Clinical and Experimental Toxicology of Organophosphates and Carbamates

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Foreword

A knowledge of the type of chemical structures which possess anticholinesterase actions began with the determination by Stedman and Barger in 1925 of the structure of the carbamate, physostigmine, isolated from the *Calabar* bean and with the synthesis of organophosphorus compounds by Lange and Schrader in the 1930s. Although the latter compounds first became known as chemical warfare agents the synthesis of many modifications of the basic completely substituted oxyacids of phosphorus has lead to compounds effective and safe to use as pesticides. Although the lock and key principle for drugs was known long before the structure of these anticholinesterases was established, toxicological research on their mode of action was one of the earliest to establish the initiating reaction in precise chemical terms and to link this with the many derangements produced in whole animals, including humans.

The acute and selective toxicity of many of the anticholinesterases can be very high (e.g. μg/kg) and is the result of inhibition of the enzymic hydrolysis of acetylcholine. The enzyme responsible is acylated by both the organophosphorus compounds and the carbamates in a reaction in which they take the place of the normal substrate, acetylcholine. The phosphylated and carbamylated acetylcholinesterases are stable, unlike the acylated intermediates formed in the hydrolysis of normal substrates. The reaction sequence for inhibition of esterases is now known to be general; its elucidation laid the theoretical groundwork for the development of many biologically active molecules through their chemical affinity for and reactivity with the catalytic centre of enzymes or receptors. It is difficult to remember the days when it was not always appreciated that the mechanisms of enzymes followed the normal laws of chemistry.

Along with an increasing use of anticholinesterases, the firm chemical basis for their action has allowed many practical problems to be solved, e.g. biomonitoring techniques in human and other species after exposure and the development of rational therapeutic agents for poisoning by them.

The number of possible chemical structures around the phosphorus atom or derivatives of carbamic acid is almost limitless. It is not surprising that it has been found that some structures cause other forms of selective toxicity, e.g. delayed neuropathy and lung damage. Similar studies in depth of the mechanisms of these forms of toxic reactions has established how to avoid these undesirable chronic toxicities during the development of new pesticides. Thus the anticholinesterases are classic examples of the value of basic research for the solution of practical problems; progress can be slow but the dividends are great.

In 1963 a textbook appeared with the title *Cholinesterases and Anticholinesterases*, with chapters by many experts and the whole edited by G.B. Koelle. This has been the source book on the subject for over 25 years. However, with the increasing use of the anticholinesterases during this period much research has been published. Organophosphorus compounds and/or the carbamates have been used as pesticides, in industry, as therapeutic agents and as tools for the study and elucidation of complex

physiological systems (as recommended by Claude Bernard in 1875). The literature is vast and the time is ripe for another textbook which embraces these new developments. A look at the Contents page of the present volume indicates its enormous scope; it provides a reference book with a sound appreciation of past research and a look ahead to the future.

Professor W.N. Aldridge
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Preface

The organophosphates and carbamates have important, sometimes unique, applications for the benefit of mankind. Principal uses in this respect are in agriculture, general commerce, therapeutic medicine and as insecticides in domestic and public health applications. Additionally, and perhaps to the detriment of mankind, the organophosphates are of potential usefulness in chemical warfare operations. In most circumstances, when organophosphates and carbamates are handled correctly and with appropriate protective, precautionary and guidance measures, they may be used safely. However, because of their wide usage, potential for misuse and often high biological activity, the likelihood for adverse effects developing by accident or intent is high. Also, the possibility for environmental contamination, with severe ecological consequences, is well appreciated. Therefore, a considerable wealth of experience and source of information has developed about the biological activities of organophosphates and carbamates, their general and mammalian toxicology, ecotoxicology, human and veterinary poisoning, management of poisoning, prophylaxis and prevention against intoxication and industrial hygiene. Although the central biological activity that underlies the mechanistic basis for the use of carbamates and organophosphates, namely inhibition of the cholinesterase group of enzymes, also mediates some of their toxicity, the potential for a wide spectrum of differing toxicity by other mechanisms is well appreciated. The intentions of this volume are to present both a review of the more important aspects of the basic and applied toxicology of anticholinesterase organophosphates and carbamates, and also to allow the reader the benefit of unpublished information and experience from a number of experts in this area. To allow viewpoints as globally representative as possible from East and West, contributors have been drawn from 17 countries. We are particularly pleased and grateful to have as one of the authors Dr George B. Koelle, who 27 years ago edited *Cholinesterase and Anticholinesterase Agents,* for long widely recognized as the leading text in this field.

In view of the varied, extensive and global uses of organophosphates and carbamates, it is hoped that this volume will be of value to a wide spectrum of professionals and organizations. Particularly, we believe that this text will be a reference source to biochemists, pharmacologists, general physicians, general and clinical toxicologists, emergency room and occupational health physicians, poison control centres, pesticide scientists, ecotoxicologists, industrial hygienists, and forensic toxicologists and pathologists. Additionally, the book should be useful to those reading for undergraduate and postgraduate degrees in subjects with a toxicological facet, or for those working towards various toxicology certifying examinations.

We are grateful to Dr W.N. Aldridge, OBE, for writing a Foreword to this volume. His name is universally synonymous with anticholinesterase toxicology. Finally we are acutely aware of the tolerance and unfailing help provided by Ms Sue Deeley, Managing Editor and Ms Cathie Staves of Butterworth-Heinemann.

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July 1991

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Abbreviations used in the text

ACh:	acetylcholine
AChE:	acetylcholinesterase
antiChE:	anticholinesterase
BCh:	butyrylcholine
BChE:	butyrylcholinesterase
CB:	carbamate
ChE:	cholinesterase
CNS:	central nervous system
ECG (EKG):	electrocardiogram
EEG:	electroencephalogram
EMG:	electromyogram
LC_{50}:	median lethal concentration
LD_{50}:	median lethal dose
im:	intramuscular
ip:	intraperitoneal
iv:	intravenous
NM:	neuromuscular
OP:	organophosphate
PAM:	pralidoxime
2-PAM:	pralidoxime chloride
pc:	percutaneous
PNS:	peripheral nervous system
po:	peroral
P2S:	pralidoxime mesylate
PsChE:	pseudocholinesterase
RBC:	red blood cell
sc:	subcutaneous
TMB4:	trimedoxime

1

Overview of the biological and clinical aspects of organophosphates and carbamates

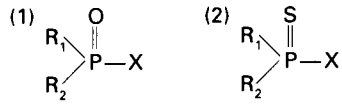
Bryan Ballantyne and Timothy C. Marrs

This overview presents introductory information on OPs and CBs for readers unfamiliar with this area.

Chemistry

OPs

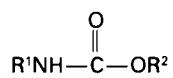
The OPs are usually esters, amides or thiol derivatives of phosphoric acid, having the following general structural formulae:



(1) is a phosphate and (2) a phosphorothioate. The P=O form is sometimes referred to as the oxon. Various substitutions are possible. R_1 and R_2 are usually alkyl or aryl groups, which may be both bonded directly, or through –O– or –S– (phosphates), or R_1 may be directly bonded and R_2 bonded through –O– or –S– (phosphonates). In phosphoroamidates, C is linked to P through an –NH group. X may be one of a wide range of substituted or branched aliphatic, aromatic, or heterocyclic groups, linked to P through a labile group (usually –O– or –S–). Detailed physicochemical properties are to be found in WHO [69]. The largest volume of OPs are used as pesticides.

CBs

CBs are N-substituted esters of carbamic acid, having the general structural formula:



R^1 is a methyl, aromatic or benzimidazole group, and R^2 an aromatic or aliphatic group. Physicochemical properties have been reviewed in WHO [70].

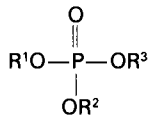
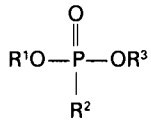
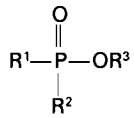
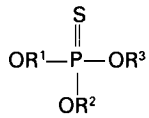
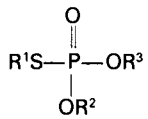
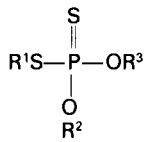
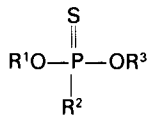
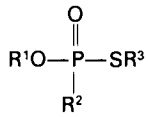
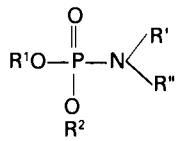
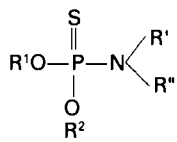
They are used extensively as pesticides. Medical uses include some tranquilizers, and the treatment of myasthenia gravis, glaucoma, anticholinergic poisoning, and paroxysmal atrial tachycardia.

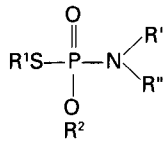
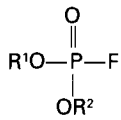
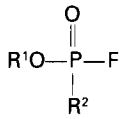
Nomenclature of antiChEs

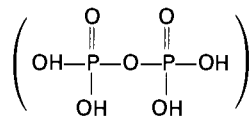
OP compounds

In general, except for pesticides, nerve agents with common names or code letters and drugs, systematic chemical names are used for chemicals in this book. British spellings are used. However, any book on antiChEs is likely to be bedeviled by differences in nomenclature of OPs. A major problem is that caused by the terms phosphorothionate, phosphorothiolate and phosphorothioate and the analogous phosphonates. The term phosphorothioate is used by the IPCS [36] and is similar to the International Standards Organization [37–39] description of all this type of OP as esters of phosphorothioic acid. The continued use phosphorothionate and phosphorothiolate, is doubtless because these terms represent a convenient way of distinguishing between P=S phosphorothioates (phosphorothionates) and those where the alkyl substituent is linked to phosphorus via sulphur (phosphorothiolates). The terms phosphonothionate and phosphonothiolate have similar advantages. However, the apparent ambiguity in the use of the name phosphorothioate can be avoided by

Table 1.1 Organophosphorus compounds

Type[a]	*Outline of structure*	*Other name*[b]	*Examples*
Phosphate			Dichlorvos Chlorfenvinphos TEPP[c]
Phosphonate			Trichlorfon
Phosphinate			Glufosinate
Phosphorothioate		Phosphorothionate	Diazinon Parathion Bromophos
Phosphorothioate (S-substituted)		Phosphorothiolate[d]	Demeton-S-methyl Omethoate Profenofos
Phosphorodithioate			Malathion Prothoate Dimethoate
Phosphonothioate		Phosphonothionate	Leptophos
Phosphonothioate (S-substituted)		Phosphonothiolate[d]	VX
Phosphoramidate			Fenamiphos
Phosphorothioamidate			Isofenphos

Phosphorothioamidate (S-substituted)			Methamidophos
Phosphorofluoridate		Fluorophosphate	DFP
Phosphonofluoridate			Sarin Soman

[a]Nomenclature used in IPCS Environmental Health Criteria 63[36]
[b]These names have the advantage that they distinguish between the =S phosphorothioates and the S-alkyl phosphorothioates. In this book, phosphorothionate and -iolate is used to distinguish these two types of compound, but not in the names of individual compounds
[c]TEPP is a derivative of pyrophosphoric acid 
[d]It is sometimes convenient to distinguish between phosphorothioates where the substituent attached to the phosphorus atom through sulphur is the leaving group from those where it is not (*see* Ch. 6)

specifying whether alkyl groups are attached to phosphorus via oxygen or sulphur, e.g. parathion is described as O,O-diethyl O-(4-nitrophenyl)phosphorothioate. With parathion a rapid calculation would result in the conclusion that the phosphorus configuration is a P=O one. With the phosphorodithioates such specification was always necessary because a description of an OP as a dialkylphorphorodithioate could mean an S,S-dialkylphosphorodithioate or an O,S-dialkylphosphorodithioate, the latter with a P=S bond and the former with a P=O bond.

In this book, the phosphorothioate and analogous nomenclature is preferred, except when there is a need repeatedly to contrast P=S phosphorothioates and S-alkyl phosphorothioates. Sometimes there is a need to distinguish between phosphorothioates where the S-substituent bond is between a sulphur atom and the leaving group and where it is between a sulphur atom and an alkyl group that is not the leaving group. There is no recognized way in which that can be done. Maxwell and Lenz (*see* Ch.6) contrast alkoxy OPs and use the term mercapto for the sulphur-containing analogues in this context.

There are arguments both for and against the use of the terms phosphorothionate and phosphorothiolate as against phosphorothioate. The most compelling argument against the earlier nomenclature is that the coexistance of two sets of names for compounds will result in confusion. Similarly, DFP is called diisopropyl phosphorofluoridate, despite the lack of any advantage of this name over diisopropylfluorophosphate.

OP pesticides

Wherever possible, the English language version of ISO common names are used for pesticides [13–16,37,39]. If such do not exist, names given in Annex 3 of the International Standards Organization lists are used [38]: these are common names approved by various national standards organizations. Where no ISO name is available, nor is there a listing in under ISO Annex 3 listings [38], chemical names are used, except where a proprietary name is so widely used that there is no likely ambiguity; in such cases the name is denoted by an initial capital.

OP nerve agents

These are generally called by their common names, tabun, sarin and soman. Code letter

names are used where no common name exists (GE,VX).

Drugs

There are few OP drugs: those mentioned in the text are given their British Pharmacopoeia non-proprietary name and the United States Pharmacopoeia name, if different (e.g. ecothiopate, echothiophate) [11,64]. Proprietary names for drugs are capitalized.

CBs

The nomenclature of CBs presents fewer difficulties than OPs. With pesticides, wherever possible, the English language version of International Standards Organization Common names are used for pesticides [37,39]. If such do not exist, names given in Annex 3 of the International Standards Organization lists are used. If the substance appears in no approved list, the systematic chemical name or a capitalized proprietary name is used. CB drugs are given their British Pharmacopoeia non-proprietary name and the United States Pharmacopoeia name, if different [11,64].

Drugs used in the treatment of OP poisoning

Drugs, in general, are given their British Pharmacopoeia and the United States Pharmacopoeia names [11,64]. Names for the oximes are abbreviated: PAM denotes pralidoxime in general, 2-PAM is the abbreviation used for the chloride salt of this drug and PAM-methylsulphate is used for the methylsulphate of pralidoxime. P2S is used to denote pralidoxime methanesulphonate (mesylate). Bispyridinium oximes lacking approved names are given the alphanumeric codes.

Ecological aspects

OPs pose health hazards to humans, domestic and wild animals, birds, and aquatic organisms. Resulting from use-pattern there is direct contamination of soil, and of water from 'run-off'. Contamination occurs from industrial effluent and waste sites.

Regulations exist to control the levels of antiChEs in foodstuffs. Exposure of the general population is by the food chain and also through contaminated ground water. Also, cases of heavy accidental contamination of foodstuffs have occurred.

Bioaccumulation of CBs is known to occur in foodstuffs. Persistence in soil is determined by a variety of environmental conditions, including pH. Groundwater is a known route of exposure to CBs through drinking water [51].

ChEs and cholinergic function

Inhibition of the ChE enzymes is relevant to both the mechanistic basis for the use of antiChE OPs and CBs, and explains several manifestations of intoxication, particularly in the early stages of poisoning.

ChEs

The ChEs (choline ester hydrolases) belong to the class of hydrolases acting on ester bonds, and specifically to a subclass of carboxylic ester hydrolases.

Acetylcholine hydrolase (Enzyme Commission 3.1.1.7), also known as acetylcholine esterase and true ChE, has maximum substrate affinity for acetyl choline and is inhibited by substances such as 1,5-bis(4-trimethylammonium phenyl) pentane-3-one di-iodide.

Acylcholine acyl hydrolase (Enzyme Commission 3.1.1.8), also referred to as butyrylChE and pseudoChE, has maximum substrate affinity for butylcholine, and is inhibited by N,N-di-isopropylphosphorodiamidic fluoride.

ChEs are widely distributed in neuronal and non-neuronal cells [30,60]. Examples of non-neuronal ChEs include reticuloendothelial cells [4,5,9], hepatocytes [7], renal tubule [27], and adipocytes [6]. Their functional significance may include lipid metabolism, detoxification processes, and monovalent cation transport.

With respect to acute antiChE poisoning, the important sites of ChE activity are the cholinergic synapse and myoneural junction (AChE), RBC (AChE), and blood plasma

(BChE). Plasma BChE, of hepatic origin [7], varies with age, sex, and steroid hormone levels [25]; other influences are malnutrition, acute infections, exercise, xanthine compounds and certain drugs (e.g. codeine and chloroquine) [45,71].

A number of variants of ChEs are known, based on electrophoretic mobility (isozymes), chromatographic characteristics, sedimentation analysis, and molecular biology [24]. Studies on abnormally sustained reactions to short-acting muscle relaxants revealed atypical plasma BChE variants. Suxamethonium has a short duration of action because of its metabolism by plasma BChE. Genetic deficiency of BChE, or atypical variants, results in suxamethonium not being rapidly hydrolyzed and a prolonged paralysis ensues. A related problem is the use of suxamethonium in the OP-poisoned patient, where OP inhibition of BChE results in prolonged paralysis [58].

Cholinergic neurons

Cholinergic neurons release acetyl choline (ACh) which stimulates ACh receptors on the cell surface mediating the response. Cholinergic receptors are classified as nicotinic (N-cholinoceptors) or muscarinic (M-cholinoceptors), on the basis of their response to the corresponding alkaloids. N-cholinoceptors mediate effects of ACh at the skeletal muscle myoneural junction, autonomic ganglia, and within the CNS. M-cholinoceptors are found at smooth muscle myoneural junctions, and exocrine and endocrine secretory systems. M-cholinoceptors have been divided into three pharmacologically identifiable types, and five molecular forms have been described. Goyal [31] suggests the functional significance of cholinoceptors is as follows. At cholinergic synapses, the postsynaptic N-cholinoceptors mediate fast postexcitatory potentials, the postsynaptic M-cholinoceptors mediate slow excitatory postsynaptic potentials, and the M_2-cholinoceptors mediate inhibiting postsynaptic potentials. Presynaptic M_2-cholinoceptors inhibit the release of ACh from the presynaptic nerve ending. At the myoneural junction, postjunctional M_3-cholinoceptors mediate the excitation of smooth muscle. Prejunctional M_3-cholinoceptors provide feedback inhibition of ACh release.

The interaction of OPs with receptor subtypes may be complex, and explains certain features seen after chronic OP poisoning. For example, Katz and Marquis [42] suggested that blockade of M_2-and M_3-cholinoceptors on the presynaptic neuron in the CNS, by paraoxon, may interfere with negative feedback inhibition of ACh release and facilitate the development of behavioural and motor deficits. There is evidence that the toxicity of OP antiChEs also includes a reversible interaction with N- and M-cholinoceptors [3]. In the heart, the high affinity of cardiac M_2-cholinoceptors for OPs suggests that cardiac functions may be vulnerable to OP antiChE intoxication [61].

Under normal physiological conditions, ACh at the postjunctional cholinoceptor is rapidly hydrolyzed by AChE, which abolishes its activity at the postjunctional membrane. The anionic position of the ACh molecule binds to an anionic site on the AChE molecule, and then combines with an ester site on the enzyme, followed by hydrolysis. Rapid reactivation of AChE then occurs. AChE has the capacity to hydrolyze 3×10^5 ACh molecules per molecule of enzyme; equivalent to a turnover of 150 μs. Cholinergic synapses have been reviewed by Dun and Perlman [22], Whittaker [68] and Edelman *et al.* [23].

Toxicology

OPs and CBs have been studied extensively with respect to their antiChE and neurotoxic effects, but other aspects of specific organ and long-term effects have been investigated. They are readily absorbed across the respiratory and gastrointestinal mucosa. Lipophilic OPs are readily absorbed through the skin.

Many OPs require metabolic activation, mainly by mixed function oxidases, hydrolases, and transferases. Phosphorothioates are toxicologically inert until activated by conversion of the P=S to an oxon (P=O), which results in a latent period between exposure and cholinergic signs. Some lipophilic OPs are not rapidly excreted, resulting in a recurrence of clinical features after apparent initial recovery. Hepatic aliesterases offer protection by their ability to sequester hepatically generated species [18]. Most CBs are direct ChE inhibitors.

Pathophysiology and consequences of antiChE toxicity

ChE inhibition by OPs involves several stages. First, the formation of a Michaelis enzyme-subtrate complex, followed by phosphorylation of the enzyme, in which a serine residue is phosphorylated with loss of the leaving group. Spontaneous reactivation of inhibited ChE may occur, whose rate depends on factors such as species, tissue, OP attached groups, and the presence of nucleophiles which catalyze the reactivation process. The rate of reactivation is a major determinant of the rate of resolution of intoxication. In some cases a process of 'ageing' occurs, in which there is cleavage of an R–O–P bond with the loss of R and the formation of a charged monosubstituted phosphoric acid residue still attached to the enzyme protein. It is time-dependant (hence ageing), and the aged enzyme is no longer responsive to nucleophilic reactivating agents. Some quaternary OPs interact with both the esteratic and anionic subsites in the active centre of the ChE molecule to produce a stable complex, contributing to high toxicity. If the alkyl groups in the phosphorylated enzyme are methyl or ethyl, spontaneous reactivation takes hours. Secondary or tertiary alkyl groups further enhance the stability of the phosphorylated enzyme, and the return of ChE activity depends on the synthesis of new enzyme [63].

CBs produce carbamylation of the ester site of the ChE enzyme, preventing AChE from hydrolyzing ACh. Because CBs do not possess a phosphate group, the complex is unstable and spontaneous reactivation of ChE occurs. The cholinergic effects induced by CBs are therefore of shorter duration than with OPs.

Inhibition of AChE by OPs and CBs produces effects which are primarily the result of accumulation of ACh. This results in activation of M-cholinoceptors and N-cholinoceptors, but with quick densitization of the latter. Effects may be grouped as muscarinic, nicotonic, and in the CNS (Table 1.2). As CBs penetrate the blood-brain barrier less effectively than OPs, the CNS component is comparatively small with CBs.

Table 1.2 Effects of anticholinesterase agents

Muscarinic	Lacrimation
	Rhinorrhoea
	Hypersalivation
	Bronchorrhoea
	Bronchoconstriction
	Hyperhydrosis
	Miosis
	Decreased visual acuity
	Urination
	Defaecation
	Bradycardia
	Hypotension
Nicotinic	Mydriasis
	Tachycardia
	Hypertension
	Skeletal muscle twitching
	Skeletal muscle fasciculations
	Skeletal muscle weakness
	Skeletal muscle paralysis
	Pallor
CNS	Ataxia
	Convulsions
	Respiratory failure
	Coma

Neurotoxicity

Peripheral neuropathy

A well documented delayed complication of acute OP poisoning is peripheral neuropathy. Histopathologically the lesion involves axonal degeneration of the larger diameter and longer fibres. The initiation of the pathogenesis is ascribed to covalent binding to, and modification of the structure of, neuropathy target esterase (NTE), a membrane-bound protein whose physiological function is uncertain [40]. The potential for OPs to cause peripheral neuropathy can be studied by histological examination of nervous tissue from animals (*see* Ch.11), or by assay for NTE inhibition.

Neurobehavioural

In view of the implication of the cholinergic system in learning and memory, it is not unexpected that altered behavioural patterns have been seen. These include deficits of learned behaviour [17,28,29,56]. Behavioural changes may be long lasting [32].

Potentiation

Carboxylesterases catalytically detoxify OPs by hydrolysis of carboxylic acid ester bonds

and carboxyl-amide bonds. Inhibition of carboxylesterases may therefore enhance the toxicity of certain OPs, e.g. with malathion the acute peroral LD_{50} is lowered from 10 g/kg to 0.1 g/kg by carboxylesterase inhibitors [69]. Thus OPs which inhibit carboxylesterases may potentiate the acute toxicity of other OP esters that are metabolically detoxified by decarboxylation. Also, carboxylesterase inhibiting impurities in an OP may enhance the toxicity of the major antiChE component, e.g. malathion and phenthoate [1,53].

Other toxicity

Depending on chemical structure, there may be a potential for toxicity other than that from inhibition of ChEs and NTE.

Carcinogenicity

Interest in carcinogenic potential of antiChEs arises from the widespread long-term exposure to them in domestic and occupational environments, and their occurrence as residues in foodstuffs. In many countries, mandatory regulations exist for testing and limitation of exposure to antiChEs. Efforts have been directed to using genotoxic effects as predictors of carcinogenicity of AntiChEs.

Developmental and reproductive toxicology

Reproductive and developmental toxicity studies have been conducted for reasons similar to those for carcinogenicity. One area of concern, and probable future research and regulation, is that of developmental neurotoxicity from fetal and/or early postnatal exposure.

Cardiotoxicity

Adverse effects on the heart have been studied both morphologically and functionally; including cardiomyopathy [62] and arrhythmias [55].

Nephrotoxicity

Renal injury of clinical significance is uncommon with antiChEs, although some show experimental evidence of renal tubular toxicity. Impurities in antiChE OPs may cause kidney injury [43].

Myotoxicity

Direct skeletal muscle fibre necrosis may be produced by OPs, possibly via calcium influx [12].

Immunotoxicology

The influence of antiChEs on the immune response has recently become an area of concern. Modulation by both enhancement and suppression of immune function have been described. Immunosuppression by long-term low level exposure is an area of concern.

Human poisoning

The presentation and progression of acute antiChE poisoning may be conveniently described as follows:

(1) Acute cholinergic crisis (OPs and CBs)
(2) Intermediate syndrome (OPs)
(3) Peripheral neuropathy (OPs)
(4) Other specific organ injury and dysfunction (OPs and CBs)

In any case, the sequence and nature of the clinical features will vary. Major determinants include chemistry of the antiChE (and its metabolism), exposure dosage, and route of exposure.

Cholinergic crisis

Depending on the factors mentioned above, signs and symptoms appear within a few minutes to a few hours of exposure. Typical clinical manifestations are:
Muscarinic effects: Predominant features are hyperhidrosis, hypersalivation, excess lachrimation, miosis, intestinal cramps, vomiting, diarrhoea, urinary and faecal incontinence, bronchorrhoea and bronchoconstriction.
Nicotinic effects: These include muscle cramps, fasciculation, weakness, paralysis and pallor.

Effects on the cardiovascular system are determined by the relative predominance of

M-cholinoceptor (bradycardia, hypotension) *versus* N-cholinoceptor (tachycardia, hypertension) activity. Typically there is transient sinus tachycardia followed by sinus bradycardia, A-V block, ST and T-wave abnormalities, and various ventricular arrhythmias.

CNS features: These include anxiety, restlessness, dizziness, confusion, ataxia, convulsions, respiratory and circulatory depression, and loss of consciousness. Individual cases may present with rare features, e.g. choreoathetosis [41].

Respiratory failure, hypoxaemia, and cyanosis, result from bronchoconstriction, laryngospasm, bronchorrhoea, respiratory muscle paralysis, and central depression of respiration. Diaphragmatic paralysis has been described [54]. Death is usually ascribed to asphyxia, but cardiac failure may also be involved.

Major differences in clinical features of the cholinergic phase between OPs and CBs are: (1) because of rapid spontaneous reactivation of the carbamylated enzyme, the cholinergic phase is shorter with CBs, and (2) as CBs do not readily penetrate the blood-brain barrier, the CNS component of the cholinergic phase is usually not as marked as with OPs.

Intermediate syndrome

This syndrome, seen with OP poisoning appears after the cholinergic phase but before the expected time of delayed peripheral neuropathy. It was specifically identified by Senanayake and Karalliedde [59], but Wadia *et al.* [67] presented cases of the same syndrome. The intermediate syndrome consists of weakness or paralysis of proximal limb muscles, neck flexors, motor cranial nerves, and respiratory muscles. If respiratory insufficiency is managed, recovery occurs within 4 to 18 days. Neurophysiological studies suggest a postsynaptic dysfunction and a myopathic lesion.

Delayed peripheral neuropathy

Delayed-onset peripheral neuropathy after OP poisoning is well documented. Two major outbreaks have been recorded, both caused by triorthocresyl phosphate. In the USA during 1930–1931 there were 16 000 cases of neuropathy after adulteration of rum ('ginger-jake paralysis'). In Morocco during 1959, adulteration of vegetable oil produced neuropathy in 11 000 persons.

The delay to clinical signs ranges from 1 to several weeks. It is characterized by a distal, symmetrical and sensorimotor polyneuropathy with burning and tingling sensations, progressing to atrophy and flaccid paralysis of the lower limbs [20,47]. The upper limbs may be subsequently affected [19]. Rarely, delayed onset peripheral or CNS effects occur in the absence of a cholinergic phase [49]. Measurement of lymphocytic NTE may predict the development of OP-induced polyneuropathy [48].

Because enzyme ageing does not occur with carbamylation it is not anticipated that CBs will cause delayed-onset peripheral polyneuropathy. Only one case of delayed neuropathy after ingestion of a CB could be found [21].

Other features

(1) Longer term psychological problems have been described with OP poisoning, including depression, schizoid reactions, memory deficit, and exacerbation of pre-existing psychiatric problems [52,57].
(2) Transient non-ketotic hyperglycemia and glycosuria may occur [34,52], possibly related to elevated levels of corticosterone, catecholamines, glucagon, and decreased insulin [28].
(3) Hypokalaemia results from an intracellular shift of K^+ [66].
(4) Pancreatic injury has been described [35].
(5) Hypothermia has occurred in several cases [33,35], possibly secondary to CNS effects and the absence of shivering.

Diagnostic investigations

Various diagnostic procedures are available for confirming acute antiChE poisoning and its complications.

Direct measurement

Analytical methods for measurement of parent molecules and metabolites provide a specific identification of poisoning. However, they may not necessarily give a functional index of

toxicity. These methods are more appropriate for monitoring for occupational exposure and forensic purposes.

ChE measurements

It has generally been considered that RBC AChE provides a functional index of toxicity because of its identity with the enzyme at cholinergic synapses, whose inhibition forms the mechanistic basis for antiChE poisoning. However, plasma BChE activity is reduced more rapidly than RBC AChE activity, and some consider that measurement of BChE activity is a better index of recent exposure [2,71]. This is more relevant to exposure monitoring than diagnosis of established poisoning.

Neurophysiological monitors

Various approaches are available for the diagnosis of incipient or established peripheral neuropathy [10].

Treatment

The major elements in the management of acute antiChE poisoning are as follows.

Immediate and supportive measures

(1) Decontamination using measures not likely to contaminate the first-aider.
(2) Gastric lavage for swallowed antiChEs, with airways protection in the partially or fully unconscious patient.
(3) Oxygen, because tissue hypoxia is a significant lethal component of OP toxicity [50].
(4) Maintenance of airway, because of increased secretions and bronchospasm. Artificial ventilation may be required, but suxamethonium should be avoided [58].

Antidotal measures

Atropine

The mainstay of treatment for poisoning by OPs and CBs is atropine, a non- depolarizing competitive blocker of the action of ACh at peripheral M-cholinceptors. Rapid full atropinization is required, as indicated by mydriasis, tachycardia, flushing and dry mouth.

Oximes

Inhibited AChE may be reactivated with appropriate nucleophilic agents. The salts of N-methylpyridinium-2-aldoxime are the most commonly used. Although it has been stated that oximes do not cross the blood-brain barrier [63] there is evidence that some may do so to variable extents [8,46]. A limitation to oximes is the time-dependent ageing of the enzyme, and they become less effective with increasing time after poisoning. A combination of atropine and oximes is synergistic and, in most cases of OP poisoning, is more effective than either alone. This is because, at least in part, separate components of intoxication are treated; atropine antagonizing peripheral M-cholinoceptor cholinergic effects and some central effects, and oximes producing reactivation of N-cholinoceptor AChE.

As carbamylated AChE spontaneously and rapidly reactivates, oximes are not required in antiChE CB poisoning. With some CBs, oximes may enhance toxicity. Kurtz [44] recommends that PAM should not be used except in cases where atropine has been proved inadequate, or in mixed OP and CB poisoning.

Anticonvulsants

Benzodiazepines both relieve anxiety and counteract some CNS effects not alleviated by atropine [65]. Additionally, diazepam is useful in controlling convulsions during the cholinergic phase.

Other antidotes

Based on mechanisms of toxicity and experimental studies other agents have been proposed as useful in acute antiChE poisoning, including calcium channel blocking agents.

Prophylaxis

A considerable literature exists on the use of drugs as pretreatments for prophylaxis where exposure to OPs is a possibility. This includes the possible use of OPs as chemical warfare agents, and the need for prophylaxis in occupational situations with high risk of overexposure. Oximes have been investigated extensively for prophylaxis. Clearly, drugs used in prophylaxis must have high safety

margins, and without the risk of complications acceptable in antidotal applications.

Prevention and protection

In view of the wide usage of antiChEs the potential for poisoning is high. There is a constant need to examine preventative, precautionary and protective measures to reduce the incidence of poisoning in both domestic and occupational environments.

General measures

(1) Labels and product literature should be sufficiently precise to allow the user fully to understand potential hazards. Protective and precautionary measures, and the management of poisoning, should be clearly specified.
(2) The use of procedures to limit accidental swallowing, e.g. incorporation of markers, olfactory warning materials and emetics.
(3) Legislation should be sufficient to control the safe and effective use of antiChEs.

Occupational exposure

Programmes on safety require to be specifically developed for any given situation. Nevertheless, the following generalizations can be made. (1) Education on the hazards from antiChEs and the precautions and protective measures is necessary. (2) Collective protection includes maintenance of equipment and alarm systems, and adherence to workplace exposure guidelines. Personal protection includes good hygiene, and the use of protective clothing and equipment considered appropriate. (3) Preplacement medical examinations to determine suitability to work with OPs, including neurological, liver, and ChE examinations are needed. Periodic medical surveillance is required to detect early indications of chronic intoxication. (4) Periodic monitoring, such as blood ChE activity and/or urine metabolites, as an index of exposure is necessary.

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2

Biochemical determination of cholinesterase activity in biological fluids and tissues

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Introduction

AChE (EC 3.1.1.7) and BChE (EC 3.1.1.8) are present in various biological fluids and tissues of mammals, birds and fish. AChE is found mostly in RBC and nervous tissue, while BChE is in blood plasma and serum with brain and peripheral nervous tissue also contributing to the pool of plasma AChE [8]. Natural and specific substrate specificity is different for each esterase. Both AChE and BChE hydrolyze ACh, however, an excess of ACh inhibits AChE but not BChE [21]. The major reasons for measurement of ChE activity [17,50,115] are presented in Table 2.1.

Table 2.1 Major reasons for measuring ChE levels in humans and animals

Monitor RBC, plasma and/or serum ChE levels in humans or animals exposed to OP or CB pesticides
Characterize and detect the presence of serum ChE variants in human plasma by differential enzyme inhibition
Preanaesthesia screening to predict human susceptibility to prolonged suxamethonium-induced apnoea
Monitor human and animal plasma or serum ChE as a prognostic tool in hepatocellular disease and dysfunction
Determine cause of death at autopsy in human or animal when antiChE compounds are suspected
Assess post-mortem levels of brain AChE as diagnostic markers in investigation of the role of cholinergic system in senile dementia and in degeneration of ageing brain or to aid in the diagnosis of pesticide poisoning
Assess amniotic fluid ChE activity as a diagnostic test for open neutral tube defects in early human pregnancy (clinical and experimental)
Diagnose Hirschsprung's disease using biopsied rectal mucosa for AChE determination (clinical and experimental)
Study the role of nervous tissue ChE in experimentally-induced polyneuropathy in animals by measuring plasma AChE or biopsied tissue
Study the use of human blood and CSF ChE activities as diagnostic markers in various types of dementia especially senile dementia of Alzheimer type (clinical and experimental)
Monitor prognosis in cancer, kwashiorkor and renal diseases using plasma ChE
Determine brain ChE in experimental neuropharmacology, neurotoxicology, neurophysiology and neurobehavioural studies
Diagnose a new myasthenic syndrome in man characterized by endplate ChE deficiency [34]
Monitor adverse effects of reversible ChE inhibitors commonly used in the diagnosis and treatment of myasthenia gravis in human and dog
Study the diagnostic value of plasma AChE in human and animals for differentiating neuronal degenerative disorders from primary muscle degenerative disease (experimental)

How to preserve sample quality

Sample collection and storage

Sample collection and storage (time and temperature) are critical to the catalytic stability of ChE and thus influence the quality and interpretation of results of the assay. Recommended procedures for the collection and storage of biological fluids (blood, CSF and semen) and tissues are: (1) avoid sample contamination by acid or alkali, (2) collect and store sample in glass rather than plastic vessels to avoid contamination by leachates from plastic, (3) refrigerate samples immediately because ChE catalytic activity is temperature dependent, (4) separate fluid and cellular components of blood, CSF and semen, and (5) determine enzyme activity as soon as possible. If enzyme activity is not determined immediately, samples can be stored for several days at 4°C. For

longer periods, samples should be kept at –20°C or lower. Tissue (e.g. brain) should be refrigerated immediately or frozen, although it is best to homogenize tissue at pH 7.6–8.0 using a sonicator or non-metallic homogenizer before storage. Samples requiring transport should be placed in a precooled, insulated container with enough dry ice (–76°C) to ensure that the samples arrive frozen. If it is impossible to transport material frozen, samples should be kept sterile and stored at the coolest ambient temperature possible during shipment providing transit is <24 h.

There is no consensus regarding time and temperature storage for ChE stability. Human serum has been stored at room temperature (20°C) for 50 h, at 4°C for 9 days, or at –20°C for 1 year without significant loss of ChE activity [54]; weekly freezing and thawing of human serum for 9 weeks reportedly had no affect on ChE activity[54]. Human CSF can be handled like serum; its AChE activity is not affected by low-temperature storage for up to 50 days [63], nor by repeated freezing and thawing [25]. However, Johnston and Huff [64] observed a 30% decrease in activity following a single freezing and thawing of plasma. At room temperature plasma slowly coagulates and ChE accumulates in the coagulum [14]. Any apparent loss of activity can be restored by thorough mixing or making a fivefold dilution of the plasma before storage to prevent coagulation [14]. Avoid haemolysis, especially if ACh or thiocholine is used as the substrate. Human plasma ChE can be collected for forensic purposes up to 72 h after death without significant decreases in ChE activity [115]. Human plasma is stable for several weeks at 0°–5°C [122], while samples stored at 5°C for 4 months retained 80% of their original activity [14].

Freezing whole blood or storage at refrigeration temperature is to be avoided because appreciable loss of catalytic activity occurs in the fractions separated after storage [64]. For RBC AChE, heparin is the anticoagulant of choice and the separated blood fractions are stable at 0–5°C [64].

ChE stability shows species variation [69,76,82,98], but in general RBC and plasma samples from dog, pig, cat, rat, cattle and chicken can be stored at 4°C for 24 h and at –17°C for 2 weeks without excessive loss of ChE activity. Of these species, plasma BChE of cattle, chicken and rat and the RBC AChE of rat, cat and chicken are the least stable under storage conditions [76]. Equine plasma and RBC ChE are stable for 8 weeks at either 4°C or –17°C [98]. ChE of haemolyzed RBCs of cattle and swine is stable for 90 days at –20°C and for at least 540 days when lyophilized and stored at 4°C [69]. The loss of activity caused by the single thawing (37°C) of frozen (–20°C) haemolyzed RBC samples is avoided by storing the cells intact [69,76]. Surprisingly, both equine plasma and RBC ChE are stable after repeated freeze-thaw cycles [98].

Brain ChE of domestic animals (cattle, swine and sheep) is stable at room temperature (25°C) for 4 days, at 4°C for 7 days, –22°C for > 3 months, but activity is decreased within 24 h at 37°C [82]. It can be inferred that brain sampling from dead animals is possible for up to 3 days at ambient temperature or below 25°C.

Avian brain ChE activity is preserved for up to 5 weeks when stored in dry ice (–76°C). However, deterioration occurs with storage at –18°C or –22°C [124]. Whole blood is advisable in the cat as AChE is in platelets, not in RBCs [76].

Problems causing erroneous results

Important factors contributing to erroneous ChE results are: (1) traces of alkali or acid at puncture site, in reagents or on glassware, particularly with methods based on the formation of acids, (2) contamination of blood samples by extraneous antiChEs, a problem when collecting finger-prick samples from workers handling pesticides. This can be largely prevented by immediate sample dilution or by using venipuncture, (3) denaturation of ChE by elevated temperatures and/or prolonged storage, (4) reactivation of inhibited enzyme by the presence of an oxime in blood of treated human or animal patients, (5) poor choice of substrate for enzyme hydrolysis; the choice of substrate depends not only on the purpose of the study and on the method but also on the animal species involved. Investigators have shown the importance of substrate specificity of serum and brain [5,61]; (6) use of wrong buffer and/or assay temperature[116]; and (7) inappropriate method of killing the animal, e.g. death by microwave irradiation completely

destroys ChE activity. On theoretical grounds CNS drugs with antiChE activity such as urethane (ethylcarbamate), chloroform, chloral hydrate, phenothiazine tranquilizers and thiopentone should be avoided. Acceptable procedures include killing small laboratory animals and fish by decapitation, pentobarbitone injections for laboratory animals and CO_2 asphyxiation for birds.

Analytical methods

Many methods have been proposed for detecting ChE activity in biological tissues and fluids; all involve hydrolysis of various choline ester substrates by AChE or BChE. The rate of substrate disappearance or reaction product formation is used as a measure of ChE catalytic activity. In general, methods measuring reaction product formation are more accurate than methods measuring substrate disappearance. The choice of method depends primarily on the reason for the analysis, the facilities and expertise available, the species involved, and the size and nature of the samples. Excellent reviews have addressed the technical aspects of electrometry, titrimetry, visible region and UV absorption spectrophotometry and gasometry [9,21,73,78,102,118,122].

In general the most commonly used manual method is the electrometric method of Michel [77]. The method of Ellman *et al.* [33] is the spectrophotometric procedure of choice and is the basis for a number of automated methods. As a rule, the automated methods are more reproducible and precise, less time consuming, and require less technical skills than the manual methods. The method of Dietz *et al.* [28], based on the Ellman reaction, is the preferred method for identification of human serum ChE variants.

Fluorimetric methods

In general, fluorescence techniques are several fold more sensitive than absorption spectrophotometric methods. Enzyme assays can occur at high dilution so that the generation of minute products of hydrolysis preclude inhibition by product formation. Interference by extraneous materials may be a disadvantage. As substrates may not be specific for ChE [48,49,93], a comparative assessment should be made for each method. Fluorimetric methods are potentially useful for determination of low levels of ChE activity.

Guilbault and Kramer [48] proposed the use of indoxyl acetate or resorufin butyrate as fluorogenic substrates for ChEs. They are hydrolyzed at pH 6.5 to stable fluorescent products. Indoxyl acetate is hydrolyzed by indoxyl, which in turn is oxidized to indigo white; both are highly fluorescent compounds only at acidic pH. The rate of change in fluorescence is measured (λ_{ex}=395 nm, λ_{em}=470 nm). The rate of substrate hydrolysis by BChE is three times that of AChE, and hence BChE can not be measured in the presence of AChE without the use of an appropriate AChE inhibitor. When resorufin butyrate is replaced by indoxyl acetate as the substrate, the rate of change in fluorescence owing to the production of resorufin is measured (λ_{ex}=540−570 nm, λ_{em}=580 nm). AChE has little effect on resorufin butyrate. Employing the methods described, as little as 0.0003 units/ml of horse serum BChE is detectable and as little as 2.5 x 10^{-8} mol substrate is required.

Another method with potential for the routine measurement of serum ChE is based on the enzymatic hydrolysis of the fluorogenic N-methyl-indoxyl ester of butyrate or acetate to a stable fluorescent product [49]. The rate of increase in fluorescence (λ_{ex}=430 nm, λ_{em}=501 nm) is measured at pH 7.5 and is correlated with ChE activity. N-methyl-indoxyl acetate is hydrolyzed by both AChE and BChE. However, the butyrate ester is not hydrolyzed by AChE. This allows serum BChE to be measured in the presence of AChE.

Prince [93] proposed measuring the rate of formation of the fluorescent 1-methyl-7-hydroxyquinolinium iodide (λ_{ex}=406 nm, λ_{em}=505 nm) hydrolysis product produced by ChE from the substrate 1-methyl-7-acetoxyquinolinium iodide for determination of small quantities of ChE in muscle or nerve fibres. This simple technique is not specific for ChE and has no advantages over the methods of Guilbault and Kramer [48] and Guilbault *et al.* [49].

A semi-automated method described by Sharman and Cooper [99] for measuring AChE activity in small volumes of CSF and tissue extracts using ACh as substrate is a simple and promising method for both AChE and BChE.

ACh is hydrolyzed to choline which undergoes catalytic oxidation by choline oxidase, the hydrogen peroxide formed is reacted with homovanillic acid in the presence of peroxidase. Formation of the fluorescent product is measured (λ_{ex}=312 nm, λ_{em}=430 nm) at 37°C in 0.1 M sodium phosphate buffer (pH 7.5) over an incubation time of 30 min. The utility of this method extends to the determination of enzyme kinetics of ChE.

Parvari *et al.* [89] converted the spectrophotometric method of Ellman to a microfluorimetric assay for measuring ChE activity in biological tissue with very low and uneven enzyme distribution. The assay is based on reacting thiocholine produced from ChE hydrolysis of acetylthiocholine, with the fluorogenic maleimide, N-(4-(7-diethylamino-4-methyl-coumarin-3-yl)phenyl) maleimide (CPM). CPM reacts selectively with thiol groups and the fluorescence is measured (λ_{ex}=390 nm, λ_{em}=473 nm). The precision and specificity of this assay, which is also suitable for kinetic studies has been examined using μg quantities of rat brain tissue as the source of ChE [89]. Little concerning the efficacy of these potentially useful fluorimetric methods for measuring biological fluids and tissue ChE activity has been published.

Electrometric methods

Modifications of the electrochemical method of Michel ([77] are still widely used in laboratories which perform blood (RBC, plasma, serum and whole blood) ChE assays with regularity. This method, a macro technique for RBC or plasma ChE activity, is based on enzymatic hydrolysis of ACh to produce acetic acid, which dissociates liberating H^+ thus decreasing the pH of the reaction mixture. Electrometric determination of the change in pH from 8.1 over a definite period of time (e.g. 1 h) at a specific temperature (e.g. 25°C) represents the enzyme activity (pH/h). In addition to a pH meter the only other equipment needed for this assay is a water bath. If an anticoagulant is required, heparin is used to avoid enzymatic inactivation by removal of Mg^{2+} and Ca^{2+} ions present in the blood [9,122]. The rate of the enzyme-catalyzed reaction is temperature dependent and is thus important in determining the ChE activity. A 1°C change in incubation temperature results in a 5.5% and 3.0% change in plasma and RBC ChE activity, respectively [32]. The method is unsuitable for enzyme kinetic studies as pH is measured after a fixed time interval rather than at different times. Another minor drawback of the method is the limited shelf-life of the phosphate buffer [79]. The pH of the buffer is critical as the buffer is designed so that during the assay the decrease in enzyme activity with pH over the range 8.1–6.0 is compensated for by a decrease in the buffer capacity and a straight-line relationship is maintained between fall in pH and time. The Michel method is better suited for monitoring ChE activity following OP exposure than for CB exposure because CB-induced enzyme inhibition may be significantly reversed during the long incubation period.

A modification described by Johnson and Whitehead [62] is suitable for detecting and estimating reversible ChE inhibition. It requires only 3–4 min, involves little dilution of the serum or plasma sample and only brief contact of sample with the ACh substrate. The Michel method, however, is unaffected by colour (e.g. haem) and is as sensitive and reproducible as pH-Stat, Hestrim colour reaction and Acholest methods in measuring small variations in blood ChE activity [9,90,122]. The many micromodifications of the Michel method, reviewed by Witter [122], allow the use of two or three drops of blood collected and separated into cells and plasma in capillary tubes. Witter *et al.* [123] found the day-to-day change of initial pH of buffer and red cells or plasma is negligible, thus requiring only the final pH reading and allowing twice the number of samples to be analysed.

Barenghi *et al.* [11] used differential pH measurement (Delpas CL, Kontron AG, Analytical Division, Zurich, Switzerland) to determine RBC AChE and plasma ChE activity. A differential amplifier connected to a capillary glass electrode measures the pH change relative to a second reference capillary glass electrode. The procedure is simple (no pretreatment of sample and automatic correction for sample blank) and allows RBC ChE activity to be determined in 2 min with high reproducibility. Plasma and RBC samples analysed by this method and the Ellman *et al.* [33] procedure correlated well.

Table 2.2 Modification of Michel's method to measure blood ChE activities in different species

Species	*Esterase*	*Modifications and advantages*	*References*
Dog	EChE[a]	Higher concentration of washed RBC; useful in animals with low ChE activity	Frawly and Fuyat [38]
Horse, goat	WBChE[b]	Substituting whole blood for washed RBC; faster than Michel's method	Palmar *et al.* [86]
Pig	EChE	Doubling volume of RBC and ACh; useful in animals with low ChE activity	Moncol and Battle [81]
Goat, horse, pig, dog, cat, rabbit, guinea pig	EChE	Increasing RBC volume eightfold; various incubation times; useful in animals with low ChE activity	Callahan and Kruckenberg [18]
Guinea pig, goat, cattle, rabbit, cat, horse, rat, sheep, dog, mouse	WBChE EChE PChE[c]	Doubling volume of RBC, tripling volume of plasma; useful in animals with low ChE activity	Kruckenberg and Vestweber [67]
Sheep, goat, cattle, pig, horse, dog	EChE PChE	Increasing sample volume, incubation temperature increased to 37°C, time of incubation reduced; sensitive, high pH reading, short incubation time, both macro and semimicro methods could be used, non-washed RBC may be used	Silvestri [103]
Sheep	EChE PChE	Increasing sample volume, adapting volumes of reagents and samples for convenience (modified from Silvestri [103]; faster than Michel's method, useful in animals with low ChE activity, adapted for sheep PChE determination	Mohammad and St. Omer [79]

[a]RBC ChE
[b]Whole blood ChE
[c]Plasma ChE

The Michel method was originally designed for the analysis of human blood ChEs. As domestic and laboratory animals, and birds, have lower blood ChE activity than humans [79,80,119], various modifications of Michel's method are used for veterinary purposes to enhance the products of the enzymatic reaction and thus the pH (Table 2.2). From experience, however, we recommend the procedure described by Silvestri [103] and modified by Mohammad and St Omer [79].

Radiometric methods

Radiometric assays for AChE activity are very sensitive and allow analysis of μg quantities of blood or tissue. Winteringham and Disney [120,121] reported a microdetermination of AChE activity in which [^{14}C]-labelled ACh is incubated with ChE and the unhydrolyzed [^{14}C]ACh is measured following removal of the volatile [^{14}C]acetate by vacuum desiccation or air drying. When compared with an electrometric method [29], for measuring inhibition of OP and CB insecticides, both methods were similar for inhibition of OP. However, the radiometric method indicates much higher levels of inhibition by the CBs than the electrometric method, suggesting substrate and dilution effects. A different approach by Reed *et al.* [96] uses [^{14}C]ACh as the substrate for ChE but production of [^{14}C] acetate is followed to determine the ChE activity. The unhydrolyzed [^{14}C]ACh is removed by binding it to an ion exchange resin and the [^{14}C]acetate in the acidified supernatant is measured. Potter [92] used selective extraction with toluene : isoamyl alcohol (5 : 1) to isolate the labelled acetate for counting. McCaman *et al.* [70] precipitated the unhydrolyzed substrate with ammonium reineckate, which compared with Potter's [92] extraction procedure, was more quantitative. Siakotos *et al.* [100] have shortened the method by adding Amberlite CG-120 resin suspended in dioxane to the enzymatic preparation and the substrate is absorbed and centrifuged. The supernatent is counted directly for labelled acetate. Fonnum [37] quantitatively removed the labelled ACh with ketonic sodium tetraphenylboron. With this approach there is less contamination of the labelled acetate (0.2–0.3%) with labelled ACh ketonic sodium tetraphenylboron treatment than from

the previous methods where 1% contamination remains after reinecke precipitation [70]. The method of Potter [92] also has relatively low contamination levels of 0.2–0.4%. All three methods have a similar range of sensitivity. A very simple approach is to incubate [^{3}H]ACh with the enzyme preparation in a scintillation vial and the reaction product [^{3}H]acetate is extracted into a toluene-based scintillator and counted directly in the reaction vial [60]. Radiometric assays are very sensitive and allow multiple sample analysis using small sample size.

Visible-region spectrophotometric methods

Because of its relative simplicity and sensitivity, the kinetic spectrophotometric method of Ellman *et al.* [33] is commonly used in research and diagnostic laboratories to measure ChE activity in whole blood, plasma, serum, RBC and various tissue homogenates of humans [8,20,44,75,101,104,106,111,117], mammals [39, 46,76,82,85,113], birds [26,124] and fish [61]. The original method uses acetylthiocholine as substrate, which in the presence of 5,5-dithiobis-2-nitrobenzoic acid at pH 8 and at 25°C, undergoes enzymatic hydrolysis to thiocholine and acetic acid. The thiocholine rapidly reacts with the dithionitrobenzoate to form the yellow anion of 5-thio-2-nitrobenzoate. The rate reaction is followed at 421 nm in a recording spectrophotometer. ChE activity in 10 µl of blood or 25 µl of other tissue homogenate can be determined. Units are reported as the hydrolysis of 0.01 mg of ACh bromide per min per 3 ml of 9.2×10^{-3} M ACh bromide. Enzyme hydrolysis has been followed at wavelengths varying from 405 to 450 nm [23,28,33,43, 44,47,72,82,101]. The activity of BChE can be inhibited with quinidine sulphate, thus allowing only AChE to be measured [33]. Haemolysis and turbidity of cells interfere with the measurement of thiocholine and under optimal conditions each sample should provide its own blank.

For the estimation of RBC ChE, George and Abernethy [44] have made use of propionylthiocholine as substrate and the detergent benzethonium chloride to stop the enzyme action. The spectral interference of haem is overcome by reading the absorbance of 5-thio-2-nitrobenzoate at 440 nm because peak absorbance is shifted from 410 to 435 nm and the Soret absorption band of haemoglobin is shifted from 410 to 405 nm. MacQueen *et al.* [72] inhibited the enzyme reaction with quinidine sulphate but read the absorbance of RBC activity at 450 nm. Chow and Eslam [20] modified Ellman's method for AChE by doubling the substrate concentration and using haemolyzed RBCs. Both investigators observed that at pH 8 both barbitone and phosphate buffers gave optimal enzyme activity, but Tris and borate buffers were inhibitory. The method of Garry and Routh [43] for assaying plasma ChE is an endpoint modification of the kinetic Ellman's method. The enzyme activity is measured after incubation for 3 min at 37°C at pH 7.4. ChE activity is expressed as µmol of sulphydryl groups produced in 3 min per ml of plasma or serum. The low levels of RBC AChE in domestic animals preclude the use of the micro method of Gary and Routh [43] as the larger amounts of haemolyzate (relative to human) required would interfere with the spectrophotometric reading.

For the determination of human serum ChE and its genetic variants by differential inhibition with dibucaine [23,28,40–42], suxamethonium [40] or sodium fluoride [23,40–42], investigators have used modifications of the Ellman method in which the substrate was acetylthiocholine [40,41], propionylthiocholine [28,40,41] or butyrylthiocholine [23,40,41]. Suxamethonium is as reliable as dibucaine in differentiating 'usual' [E^u, E^u,] and atypical [E^a, E^a,] forms of the enzyme [40]. All three thiocholine ester substrates with dibucaine as inhibitor can distinguish 'usual' from 'atypical' enzyme [23,28,40]. However, the method of Dietz *et al.* [28], in which propionylthiocholine is the substrate, is reportedly preferred by the American Association for Clinical Chemistry for identification of these ChE variants [35]. Such a selection has been criticized by Brown *et al.* [17] and Whittaker *et al.* [116]. Garry [40] demonstrated that sodium fluoride, when used as a differential inhibitor, gives conflicting results depending on whether Tris or phosphate buffer is used.

Accurate automated methods based on the principle of the Ellman's method have been developed and used to determine plasma and RBC ChE in humans [40,41,56,68] and various

animal species [76,113]. A recent adaptation of Ellman's method to a programmable photometer (Compur M 2000 photometer) for the determination of ChE activity in human whole blood gives good accuracy and precision [114]. Field-type spectrophotometric methods for the measurement of ChEs in whole blood, plasma and RBCs, using the principle of Ellman's method have been developed [16,74] and evaluated in workers at risk of OP poisoning [15,74]. The methods are simple, cost effective and quite precise with good reproducibility.

Recently, Testyler and Gourmelon [107] described the use of a photometric system which utilized the principle of Ellman's method, and allowed the spectrophotometric assay of ChE in the brain tissue of live anaesthetized rats. The system utilizes a miniaturized optical probe consisting of a multibarrel micropipette for reagent injections and optical fibres for light absorption measurements. This new and unique technology has potential as a research tool for *in vivo* studies in neuropharmacology, neurotoxicology and neurobehavioural effects.

Augustinsson *et al.* [10] described an improvement of Ellman's method which allowed the use of whole blood for the determination of plasma and RBC ChE. The modifications involved the use of propionylthiocholine as substrate, 4,4'-dithiodipyridine as the chromogenic disulphide and (10-(-diethylaminopropionyl)phenothiazine as a selective inhibitor of plasma ChE. The absorption of the reaction product 4-thiopyridone is measured at 324 nm, to avoid interference by haemoglobin.

A plethora of new methods have been proposed for detecting ChE genetic variants, but there are insufficient data on which to judge their effectiveness when used in other laboratories. Okabe *et al.* [84] have determined serum ChE activity by estimating the choline derived from hydrolysis of benzoylcholine and using choline-oxidase to quantitatively generate H_2O_2 which is coupled with 4-aminoantipyrine and phenol by horseradish peroxidase to form a red quinone chromogen having an absorption maximum at 500 nm. The procedure which appears adaptable to automation, is relatively simple, precise and accurate [87]. The procedure of Okabe *et al.* [84] was found by Panteghini and Bonora [87] clearly to differentiate the genetic variants of BChE by dibucaine inhibition; the results obtained from usual and atypical forms of human sera showed excellent correlation (r=0.973−0.984) with those obtained with the reference methods of Dietz *et al.* [28], Das and Liddell [23] and Garry and Routh [43]. Abernethy *et al.* [1] recommended a precisely timed incubation at a temperature of 25°C when using the Okabe *et al.* [84] method for the differentiation of variants of BChE by differential inhibition with dibucaine and fluoride. A recent modification of the method of Okabe *et al.* [84] involved replacing phenol with 2-hydroxy-3-5-dichlorobenzenesulphonate to provide about a 4.4-fold increase in sensitivity [6]. The red chromogen generated by this technique is measured at pH 7.8 at 510 nm. Ratnaike *et al.* [95] automated the method modification described by Artiss [6]. Interference by bilirubin and haemoglobin in the described peroxidase coupled reactions is minimal and non-deleterious [6,84,91].

Van Hooidonk *et al.* [110] devised a spectrophotometric procedure using 1-[2-thiazolylazo]-2-acetoxybenzene derivatives as substrate for the assay of ChE. The coloured alcoholic products of hydrolysis absorb at long wavelengths (485–585 nm) where interference from protein absorption is excluded.

McComb *et al.* [71] devised a procedure for differentiating the usual, intermediate, and atypical serum ChE phenotypes by determining the inhibition of the hydrolysis of o-nitrophenylbutyrate by suxamethonium. Suxamethonium number is defined as the percentage of inhibition of the formation of the yellow o-nitrophenolate anion.

Hestrin's [51] method has been applied to the analysis of ChE activity of RBCs, plasma and whole blood and is based on the determination of the rate of disappearance of ACh. The reaction of ACh with hydroxylamine forms hydroxamic acid, which reacts with ferric chloride in an acid medium to form a red-purple complex, the intensity of which is read at 540 nm. The various modifications of the original Hestrin method have been reviewed [9,119,122]. The method of de la Huerga *et al.* [24] is a popular modification. The method is not as accurate as the manometric, titrimetric or Ellman methods.

Ultraviolet spectrophotometric methods

Kalow and Lindsay [66] measured human serum ChE activity by following the disappearance of the substrate benzoylcholine by ultraviolet (UV) absorbance at 240 nm. Kalow and Genest [65] used the same approach in combination with the inhibitor, dibucaine, to differentiate serum ChE variants by determining the percentage inhibition by dibucaine (dibucaine number,DN). The UV rate reaction method of Kalow and Genest [65] is still used for the differentiation of serum ChE variants based on dibucaine inhibition. The rate of benzoylcholine hydrolysis by serum is measured at 240 nm. The method of Dietz *et al.* [28] is now the preferred method. UV absorbance has been used to measure the 2,2'- or 4,4'-dithiodipyridine derivative of released thiocholine from acetyl- or butyrylthiocholine [108] or the decrease of NADPH at 340 nm when p-hydroxybenzoylcholine is used as the substrate with p-hydroxybenzoate hydroxylase as the coupling enzyme [7,55]. Hsiao *et al.* [53] described the determination of serum ChE activity using p-hydroxybenzoylcholine as a substrate and p-hydroxybenzoate hydroxylase as the coupling enzyme. The substrate is hydroxylated to 3,4-dihydroxybenzoic acid with the simultaneous decrease of NADPH, which is measured kinetically at 340 nm. This UV procedure is commercially available as CHE Auto UV assay (Fujirebio Inc., Tokyo, Japan). It has been automated for the Cobas-Bio Centrifugal Analyser for the analysis of serum ChE phenotypes by dibucaine inhibition [88] and was found to be precise, and the results obtained from normal and pathological sera showed good correlation with the methods of Dietz *et al.* [28] and Das and Liddell [23].

Gasometric methods

The gasometric method measures the rate of acid produced from the hydrolysis of ACh by estimating manometrically the CO_2 released in a bicarbonate acid buffer. Although the technique is accurate and reliable, technical complications make it primarily a research assay. The utility and drawbacks of this technique have been reviewed [3,66,109,112,119].

Titrimetric methods

In this procedure the acetic acid formed during hydrolysis of ACh is determined by titration with standard alkali at constant pH using either a pH indicator or a potentiometer. The automated pH-stat [83] which uses electronic means to maintain constant pH of the reaction mixture, is perhaps one of the best and most precise techniques for assaying ChE and studying kinetics. Activity is expressed as μmoles of ACh hydrolyzed per min per ml of sample. Aldrich *et al.* [4] described a micromodification of the pH stat method in which a weaker titrant is used to allow the use of samples with low enzyme activity. The utility of this method is limited by high equipment cost and technical complexity. Thus, the Michel and Ellman methods are preferred in laboratories with limited demand for ChE testing.

Tintometry and other methods using pH indicators

When pH indicators such as phenolphthalein [13], bromothymol blue [36,45,112], phenol red [19,56,97] and m-nitrophenol [94] are incorporated into the enzyme-substrate reaction mixture, changes in pH from acid production can be measured visually [36,45,112] or by colorimetry [13,19,45,56,97,105]. Most procedures utilizing pH indicators were devised for serum ChE [13,56,94,97] or plasma ChE [112]; only a few measure whole blood ChE [36,45] or RBC ChE [36]. In one technique the sensitivity of the colour reagent phenolphthalein was reduced by 75% to permit determination of dibucaine and fluoride numbers [13]. Colorimetric methods using phenol red [105] and phenolphthalein [13] have been automated. A problem with colorimetric indicators include spectral shifts which occur with bromothymol blue and phenol red, and limited sensitivity owing to small colour changes with m-nitrophenol.

Currently, selected indicator methods are routinely used only in large-scale screening tests of pesticide workers for ChE activity [21,109]. A number of field kits and test papers devised for rapid screening of a large number of samples for ChE activity are available. The best field pH-indicator method is the tintometric method [30] in which fingerstick

whole blood from persons exposed to OP compounds and from non-exposed persons is incubated with bromothymol blue and ACh without the use of a buffer. The time required for the reaction mixture with normal ChE to match one in a series of eight coloured glass standards marked 100% activity is noted. Exposed samples are incubated for an identical time as the control. The colour of each exposed sample is compared with a series of coloured-glass standards. Miller and Shah [78] field tested a commercial tintometric kit (Tintometer Ltd) by monitoring ChE activity of workers exposed to OP compounds and found good correlation with the Michel procedure. The ChE activity was expressed as a percentage of activity in normal blood. An improved Acholest method by Wang and Henschel [112] utilizes filter paper impregnated with ACh and bromothymol blue. The test-paper, on contact with serum or plasma, changes colour reflecting acid hydrolysis of the ACh. The endpoint is the time required for the test-paper to turn to the shade of yellow of the control paper. As a field test the Acholest method is not as fast and convenient as the tintometric method [109]. Good correlation was reported with the Acholest and the Michel methods [112]. The Acholest is inadequate as a screening test for ChE variants [27].

'Acholest' test paper is commercially available (Österreichische Stickstoff Werke A. G. and Fougera and Co.). Gerarde *et al.* [45] described the use of a commercially available Unopette system (Becton Dickinson Company, Rutherford, NJ) for visual determination of whole blood ChE. In this system 20 µl of whole blood reacts with ACh and bromothymol blue in a closed system designed to exclude atmospheric CO_2 contamination. After 20 minutes of incubation at 37°C the colour of the reaction-mixture in the Unopetter reservoir is compared with a colour chart. Results are expressed in terms of zero, 25, 50, 75, 100% of normal ChE activity.

Miscellaneous methods

Gas-liquid chromatography has been used to measure the rate of production of acetic acid from ACh [2] and 2,2-dimethyl-1-butanol when 3,3-dimethylbutyl acetate is used as the substrate [21]. Chemiluminescence has been used to measure ACh concentration [57–59] and was adapted by Birman [12] to measure AChE activity. Homolka [52] proposed using butyrylthiocholine as the substrate and measured the released thiocholine electrochemically with a dropping mercury electrode.

Conclusion

Several methods for measuring both AChE and BChE activity have been reviewed. A commonly used manual method, especially in veterinary laboratories assaying small and variable volumes of blood ChE, is the electrometric method of Michel [77]. The method of Ellman *et al.* [33] is the spectrophotometric procedure of choice and is the basis for a number of automated methods. As a rule, the automated methods are more reproducible, precise, less time consuming, and require less technical skills than the manual methods. The method of Dietz *et al.* [28], which is based on the Ellman reaction, is the preferred method for identification of human serum ChE variants. In Third World countries, the tintometric and field-type Ellman spectrophotometric methods are preferred for monitoring occupational hazards of exposure to insecticides.

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3

Histochemical localization of cholinesterase in anticholinesterase poisoning

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Introduction

Histochemistry and more recently immunocytochemistry have made a major contribution to the understanding of cholinergic mechanisms: in particular they have proved useful in studies of central nervous cholinergic pathways and of the peripheral nervous system, including the motor endplate. As techniques, they have, in many situations, an advantage over a biochemical approach, enabling considerably more precise localization of ChEs. Although, in addition to sensitivity and specificity which have to be considered when biochemical approaches are used, with histochemistry it is necessary to consider the possibility of artefact. Moreover, the localization is achieved at the expense of quantitation which in most histochemical methods is not usually possible except to an inexact degree.

Glick [17,18] found that ChEs hydrolyzed acetylthiocholine, as well as choline esters of lauric, palmitic, stearic and myristic acids. These observations gave rise to early methods for the histochemical localization of ChEs, as well as subsequent modifications, many of which are still in use. The first method that was published, that of Gomori [19], used the long chain fatty acid esters as substrates, while the indirect method of Koelle and Friedenwald [23] and direct method of Karnovsky and Roots [22] made use of acetylthiocholine or butyrylthiocholine for localization of AChE and BChE respectively. These methods have since given rise to many modifications and have been adapted to ultrastructural studies.

Histochemical methods

Histochemical methods for ChEs fall into two groups: those that employ substrates that confer intrinsic specificity upon the method and those that do not. The former group includes those methods that rely on the hydrolysis of acetylthiocholine and butyrylthiocholine such as the methods of Koelle and Friedenwald [23] and Karnovsky and Roots [22]. Methods that do not use thiocholine esters as substrates lack specificity so that inhibitors must be used.

Thiocholine ester methods

AChE and BChE will hydrolyze the appropriate acylthiocholine ester, acetylthiocholine and butyrylthiocholine. Histochemical techniques exploiting these reactions have a certain intrinsic specificity not possessed by other ChE methods.

Methods using acetylthiocholine or butyrylthiocholine as substrates can be divided into two types. The first type are indirect methods such as that of Koelle and Friedenwald [23], in which the reaction product of thiocholine ester hydrolysis has to be visualized by a second reaction. The second type is a single stage procedure, where a second step is not needed, as typified in the method of Karnovsky and Roots [22]. The distinction between the two is blurred by the omission of the second stage of the Koelle and Friedenwald method in some ultrastructural studies and by modifications to the Karnovsky and Roots method which have turned it into a two-stage procedure.

Method of Koelle and Friedenwald and subsequent developments

Koelle and Friedenwald [23] used acetylthiocholine iodide as substrate, having found that AChE and BChE hydrolyzed this choline ester more quickly than they hydrolyzed acetylcholine. The method is carried out on fresh frozen sections and the thiocholine liberated is precipitated as the white complex cuprous thiocholine iodide [38]. It is necessary to saturate the medium with copper to bring about immediate precipitation. The method is described as indirect because the initial precipitate is not easily visible, therefore a second stage is required for visualization. Ammonium sulphide solution is used to convert the white precipitate to dark brown deposits, mainly copper sulphide. It appears that iodide, cyanide or thiocyanate ions are required for the first reaction [6,43], all three ions forming insoluble salts with cuprous copper. The method will not work if chloride is substituted for iodide, the chloride of copper thiocholine being soluble in water [44]. Furthermore, although iodide was specified in the original description, and is almost always used, better localization was achieved by Brzin and Pucihar [6] with acetylthiocholine cyanide and thiocyanate, when these compounds were substituted in the method, and conversion of the primary precipitate was omitted, for cytochemistry. Selectivity was conferred on the method by incubation of sections with a low concentration of DFP and the use of butyrylthiocholine, to detect AChE and BChE respectively. Although unfixed frozen sections were used in the original method, previous fixation of tissues with, for example, cold neutral 10% formalin was carried out by Couteaux [7,8] before performance of a variant of the Koelle technique. The use of formol-fixed tissue was also described by Lewis [28]. Fixation is necessary for the optimum preservation of structural detail but at the same time it has to be realized that some loss of activity is probably always involved [7]. Because the degree of inactivation may vary with tissue and species, it would seem wise to use both fixed and unfixed tissues to obtain detail without missing low degrees of ChE activity. In some cases fixation by perfusion has been used. Thus Barron and Hoover [2], before applying the indirect thiocholine method to the rat pituitary gland, perfused fixative (phosphate buffered picric acid-2% paraformaldehyde or 4% paraformaldehyde in phosphate buffer) via the ascending aorta. As well as the use of prior fixation, Lewis [28] also discussed a number of other modifications, mostly in processing. Furthermore, the use of sodium sulphide to replace ammonium sulphide was advocated. Numerous other modifications have been described because optimal conditions for the reaction, when used for localization, depend on the species and tissue and importantly the magnitude of the enzyme activity present. Other modifications have been designed to increase the sensitivity of the method, including the use of prolonged incubation [40], as well as the employment of a semipermeable membrane, as was described by Hüther and Luppa [21]. In the latter method, the intention was to prevent loss of enzyme activity. Too intense staining tends to result in diffusion artefacts, so that such methods are not indicated in tissues of high ChE activity.

Method of Karnovsky and Roots

More frequently used nowadays than the method of Koelle and Friedenwald [23] is the direct thiocholine method of Karnovsky and Roots [22] and its modifications. It has the advantage that the colour is produced directly at the site of enzyme activity which makes it easier to estimate the optimum time of incubation, as colour development can be seen as it proceeds. Also the precipitate is more finely granular. Additionally, artefacts which are liable to appear in the two step procedure are less likely in the Karnovsky and Roots technique [30]. The method is less satisfactory for electron microscopy. In the unmodified version [22], block-fixation of tissues overnight in 10% formalin containing 1% calcium chloride was used but unfixed cryostat sections are also suitable. The basis for the reaction has been thought to be as follows: acetylthiocholine or butyrylthiocholine iodide is hydrolyzed and the resulting thiocholine iodide reduces ferricyanide to ferrocyanide, which combines with copper (Cu^{2+}) to form copper ferrocyanide, which is brown (Hatchett's brown). It is probable that events at the site of ChE activity are

more complicated, since X-ray energy microanalysis of the reaction products has shown that sulphur, iodine, iron and copper are all present [42]. In the original unmodified Karnovsky and Roots method, cupric ferrocyanide and cuprous ferrocyanide together with the precipitate produced by the technique of Koelle and Friedenwald [23], cuprous thiocholine iodide, are produced [42,43]. By increasing the concentration of the ferricyanide and decreasing that of copper (cupric) ion, it is possible to slow down the Koelle reaction: this reaction can be completely eliminated by substituting acetylthiocholine chloride for the iodide, because cuprous thiocholine chloride is soluble [44]. In the same modification of the direct method, it was reported that lowering of the pH of the reaction from 6 to 5 reduced artefactual nuclear staining. Since the original description of the direct thiocholine method a number of other modifications have been introduced; thus acetylthiocholine salts, other than the iodide, have been used to prevent secondary precipitation and Tsuji [43] recommended the use of the perchlorate. Raineri [34] used, in addition to the usual substrates, acetyl-β-methylthiocholine, and applied the method to 80% ethanol, acetone or acetone-2% glutaraldehyde-fixed tissues.

Later thiocholine methods

A considerably modified thiocholine-ferrocyanide method was described by Erankö *et al.* [13], in which lead complexed with tris acetate buffer trapped the ferrocyanide ion. In this method, a faint yellowish white precipitate is formed, which is more easily visualized as lead sulphide, after treatment with ammonium sulphide. A related method, also employing lead, was described by Gautron [15]. This method uses diacetyl sulphide (thiodiacetyl, the monosulphide analogue of the thiolacetic acid histochemical method) as a complexing agent for lead: acetylthiocholine perchlorate is hydrolyzed by ChE and the resultant sulphydryl groups are reacted with lead thiodiacetyl to form lead sulphide. The author claimed that diffusion artefact was reduced compared with the original method and that the precipitate was adequately visible, both at the light and electron microscopic level. This method was also used by Stephens *et al.* [39]. Lukáš and Fiolová [31] added agar to the incubation mixture and used a semipermeable membrane between the section and the medium. The latter feature was designed to prevent leakage of enzyme activity, enabling easier detection of low levels of ChEs. Other methods of increasing the sensitivity of the Karnovsky and Roots reaction have often involved converting the method into a two stage procedure, with a second, amplification step. For example, Tago *et al.* [41] followed the Karnovsky and Roots incubation with a mixture containing diaminobenzidine and hydrogen peroxide. The products of the first stage cause the hydrogen peroxide to oxidize the diaminobenzidine. A fine precipitate is produced and the addition of nickel or other metals results in good contrast images. This method was stated by the authors to be considerably more sensitive than previous histochemical methods. The Karnovsky and Roots procedure has been combined with silver impregnation to allow staining of myelinated and unmyelinated axons, as well as other uses. A variant of the Karnovsky and Roots [22] method, using silver nitrate, was applied to studies of *Philodina* and *Rotaria* ssp. [34]. In the use of fixed or unfixed tissues, similar considerations apply as with other ChE histochemical techniques: some loss of activity is probably inevitable with fixation. Nevertheless, formalin-fixed tissue has frequently been used and it is noteworthy that formal-fixed brain homogenate did not show a substantial diminution of acetylthiocholine hydrolyzing activity, compared with homogenate of unfixed brain, in the study of Broderson *et al.* [4].

Non-thiocholine methods

The methods described below rarely present advantages over the thiocholine methods, at least for light microscopy, lacking as they do the specificity of the thiocholine methods and consequently requiring the use of inhibitors.

Methods using long chain fatty acid esters of choline

These methods use the hydrolysis of long chain fatty acid esters of choline. In the case of the

method of Gomori [19] and modifications by Denz [12] and de Almeida and Couceiro [11], myristoyl choline is the substrate. The original method utilized acetone fixation, while that of Denz was carried out on fresh frozen sections. The fundamental problem with these methods is that the higher fatty acid esters of choline are not attacked easily by AChE. Therefore, if short incubation periods are used, the activity visualized is probably not mainly that of AChE. However, Denz [12] found the method reliable when used on fresh frozen tissue and thought it was showing AChE activity. Nevertheless, methods using esters of long chain fatty acids are largely of historical interest.

Thiolacetic acid methods

The principle of these methods is the thiolacetylation of the active site of ChEs with subsequent elimination of hydrogen sulphide. The thiolacetic acid method was published by Crevier and Bélanger [9]. Koelle and Horn [26], comparing results using triple-distilled thiolacetic acid and the original impure preparation, subsequently found that the true substrate was diacetyl-disulphide, a dimer of thiolacetic acid. Lead nitrate is also used and localization is by deposition of lead sulphide. Aurous gold can be used instead of lead, as the capturing agent [25], but thiolacetic acid methods without exception suffer because this substrate can be hydrolyzed by numerous esterases other than AChE or BChE. Adequate specificity is thus difficult to obtain.

Azo dye-coupling methods

Azo-coupling methods, like thiolacetic acid methods, lack specificity for AChE: they are therefore most useful in the demonstration of non-specific esterases. By the appropriate use of certain inhibitors, some specificity may be gained [9]. Carbonaphthoxycholine is a moderately specific substrate for BChE and is not hydrolyzed by AChE: its hydrolysis and subsequent azo-coupling formed the basis of a method described by Ravin *et al.* [35]. β-naphthylacetate is hydrolyzed satisfactorily both by AChE and BChE. The hydrolysis product can be coupled with an azo dye such as tetrazotized diorthoanisidine [35]. A recent use of an azo-coupling method is by Anglade *et al.* [1].

Quantitative histochemical methods

A quantitative microspectrophotometric method, based on the Karnovsky and Roots technique, was described by Wenk *et al.* [46]. A number of problems were associated with this method, when the results were compared with biochemical estimation. The difficulties probably arose from the heterogeneity of distribution of the enzyme in the tissues that were tested. A quantitative analysis of rat and human brain AChE activity was carried out by Biegon and Wolff [3]. This study utilized the method of Geneser-Jensen and Blackstad [16], with video camera-based computerized image analysis equipment.

Histochemical methods – conclusion

It is impossible to make a determinative choice of method for all circumstances because AChEs and BChEs from various sources have different properties. Moreover, methods designed for the detection of ChEs at sites of high activity are often unsuitable for use where activity is scanty and *vice versa*. Most widely used of the methods described above are the thiocholine methods. They are more specific than the other methods particularly with respect to non-specific esterases. Furthermore, the existence of two substrates allows distinction to be made between AChE and BChE. Direct thiocholine methods are, in general, easier to perform than the original Koelle technique, as the optimum incubation time is easier to attain. Probably those modifications of the Karnovsky and Roots method, which substitute acetylthiocholine chloride for the iodide, represent the best technique in terms of diffusion and sensitivity as well as ease of performance, at this time.

Inhibition tests

The thiocholine methods are probably the most satisfactory histochemical techniques for the location of ChEs. However, even the thiocholine methods, using respectively acetylthiocholine and butyrylthiocholine, are not completely specific for either AChE or BChE. Thus Koelle [24] showed that mammalian brain homogenates contained other enzymes that could hydrolyze acetyl and butyrylthiocholine. With the non-thiocholine methods the

use of inhibitors is even more important. Over the years a number of inhibitors, more or less specific for the inhibition of non-specific esterases, AChE or BChE, have been studied and some of them are discussed below; different tests give different recommended concentrations.

Physostigmine (0.01 mmol/l) can be used to inhibit both AChE and BChE; by the use of controls including physostigmine it is possible to increase the specificity of both direct and indirect thiocholine methods. Use of physostigmine enables the adoption of less specific methods, such as the azo-dye methods, to detect ChEs. Alternatives are the use of 0.01 mmol/l DFP [30], as well as paraoxon.

The degree to which it is possible to distinguish between AChE and BChE, using inhibitors has improved since the earliest histochemical methods were described. Two compounds of Burroughs Wellcome, 62C47 (1:5-bis-(4- trimethyl ammonium phenyl) pentan-3-one diiodide) 10 μmol/l or BW248C51 (1:5-bis-(4-allyl-dimethylammonium-phenyl)-pentan-)-one diiodide) 50 μmol/l can be used specifically to inhibit AChE. BChE can be inhibited by tetraisopropyl pyrophosphoramide (isoOMPA) [2,36] at a concentration of 1 μmol/l ; an alternative is ethopropazine hydrochloride [16].

In the past, less specific inhibitors were used. Thus, the partial inhibition of AChE by 0.001 mmol/l physostigmine or DFP and the total inhibition of BChE by the same two substances was used. Another method was the use of mipafox to inhibit BChE. These and numerous other inhibitors of one or other enzyme are discussed in Pearse [33].

Cytochemical methods

Many of the methods described above for use in light microscopy have been adapted for the electron microscope. Many use the hydrolysis of acetylthiocholine and its butyryl homologue. Both direct and indirect thiocholine methods have been applied to ultrastructural studies but diffusion artefact has proved to be a problem and the penetrating power of the medium into the block is poor [43], with some of the thiocholine techniques. This is particularly true of methods using ferricyanide, for example Karnovsky and Roots [22], which are generally unsatisfactory with compact material because of poor penetration.

Some of the earliest cytochemical techniques used variants of the indirect (Koelle's) method [5]. The use of ammonium sulphide, in Koelle's method, causes diffusion artefacts and if the activity of ChEs is at all appreciable, the precipitate is so electron-dense that much detail is obscured. For cytochemistry the method is therefore usually modified, considerable improvement being obtained by improving the efficiency of the capture reaction, the use of isotonic solutions and the addition of calcium [29]. Silver has been used instead of copper [5] and the simple omission of sulphide has been proposed. Another solution is to add acetylcholine perchlorate or chloride which slows down the precipitation by competition at the esteratic site of the enzyme [37,43]. Some of the problems with electron microscopic variants of the thiocholine method may be related to the method of treatment before incubation and Erankö *et al.* [14] found that prolonged fixation in formaldehyde-sucrose gave better results than glutaraldehyde, and that freezing gave poor preservation with ice crystal artefacts destroying much of the detail. Accordingly the fixed tissue was sectioned without freezing using a McIlwain tissue chopper. After the histochemical procedure of Karnovsky and Roots [22] and washing, the tissue was postfixed in osmium tetroxide. Following dehydration, tissues were embedded in Epon and sections cut. Counterstaining was with lead citrate. Lewis and Shute [29] fixed tissue in buffered glutaraldehyde, often by perfusion but found, as had Erankö [14], that cryostat sections were unacceptable for the detail required to examine sections from the CNS. They therefore cut sections 200–250 mm thick freehand from the chilled block of brain. A modification of the indirect histochemical procedure, using sodium sulphide as the second stage, was carried out and required areas of tissue were dissected out and embedded in Dalton's solution. The material was dehydrated and embedded in Araldite. Sections were stained with lead citrate. This method was applied to glutaldehyde-paraformaldehyde fixed tissue from chick embryos by Miki and Mizoguti [32]: uranyl acetate was used instead of lead citrate. An indirect ultrahistochemical technique, described by Satler *et al.* [36] and

using acetylthiocholine, was applied to rat liver fixed in ice- cold 2% glutaraldehyde in 0.01 mol/l phosphate buffer pH 7, by portal vein perfusion; in this method staining was with uranyl acetate or lead citrate. Tsuji and Fournier [45] described a variant of the Koelle technique in which the cuprous thiocholine iodide reacted with phosphomolybdic acid and osmium tetroxide to produce an electron-dense precipitate. Diffusion artefact was not seen and, as in some other methods, acetylcholine chloride was added to slow down the reaction. It was reported that 5% aqueous phosphomolybdic acid stabilized the cuprous thiocholine iodide *in situ* and thereby prevented dissolution of the precipitate when the material was postfixed with osmium tetroxide. The electron density of the material was increased, presumably by complexation of the precipitate with the phosphomolybdic acid. In a modification of the direct thiocholine method, suitable for both light and electron microscopy [20], the copper ferrocyanide deposited at the site of enzyme activity was amplified by bridging to osmium with thiohydrocarbazide or by using the ferrocyanide catalytically to bring about oxidative coupling of 3,3' diaminobenzidine to form a polymer. This is intensely coloured, and after reaction with osmium tetroxide, electron dense. The amplification procedure allows the use of lower temperatures and shorter incubation periods. The method of Gautron [15], a hybrid between the thiolacetic acid and thiocholine methods, has been used for cytochemical studies. In the hands of Stephens *et al.* [39], but not in the original ultrahistochemical method, postfixation with osmium tetroxide and staining with uranyl or lead acetate were omitted.

Methods of azo-dye type have been used for cytochemistry: thus Lehrer and Ornstein [27] described a method using α-naphthylacetate and hexazonium p-rosaniline and applied it to the study of the ultrastructure of the motor endplate.

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4

Pharmacology and toxicology of organophosphates

George B. Koelle

Historical note

The earliest synthesis of an OP, tetraethyl pyrophosphate, was reported by Phillipe de Clermont at a meeting of the French Academy of Sciences in 1854. However, there is some question about the identity or purity of his compound, for he mentioned its taste. Holmstedt [7] has pointed out that this might have precluded his surviving to present the report; he died many years later at 90 years of age. The toxicity of the OPs was first noted nearly 80 years later by Lange [15], who stated that inhalation of the vapour of dimethyl or diethyl phosphorofluoridate produced a choking sensation and dimness of vision (miosis). It was probably this comment that led Schrader [19] to synthesise a huge number of OPs in his search for insecticides, beginning at I.G. Farbenindustrie in 1936. One of the compounds resulting from this early investigation was parathion (O,O-diethyl O-(4-nitrophenyl) phosphorothioate), which is still a widely employed agricultural insecticide. Therefore the authorities changed Schrader's work from insecticides to chemical warfare agents. Continued exploration of OPs led to diisopropyl phosphorofluoridate (DFP), the first tentative nerve gas, and subsequently to the more potent G agents (tabun, sarin and soman).

What followed is now history. When Russian troops captured the major nerve gas factory at Dühernfurt, near the Polish city of Wroclaw (then Breslau, in German Silesia), it has been estimated that it contained stores of 12 000 tons of tabun (GA), 600 tons of sarin (GB) and an unknown amount of soman (GD). It is still a mystery why these vast quantities were not unleashed at the time of the Normandy invasion. The most likely explanation is that the OSS had effectively rumoured that the Allies were prepared to retaliate in kind (which we were not). The history of these events has been recorded in full in the foregoing references and by Meselson and Robinson [18] and Koelle [12].

Mechanism of action

The primary pharmacological actions of the OPs is attributed almost entirely to the inactivation of AChE; acetylcholine acetylhydrolase; EC 3.1.1.7) [11]. This results from alkylphosphorylation of a serine hydroxyl group at the esteratic site of the enzyme. ACh combines with the enzyme here and at the anionic site as the first step in its hydrolysis. The permanence of the covalent linkage is dependent on the nature of the alkylphosphate group. For example, a dimethyl phosphate group is removed by spontaneous hydrolysis in a period of hours; on the other hand, the action of a diisopropyl phosphate group is essentially irreversible. Immediately following alkylphosphorylation of the esteratic site, a process called 'ageing' begins. This is the splitting off of a single alkylphosphate group, which renders the enzyme non-reactivatable by reactivating agents such as PAM. The various molecular aggregate forms of AChE (G_1, G_2, G_4, A_4, A_8, A_{12},) do not differ significantly in their susceptibility to alkylphosphorylation by the OPs [17].

This brief account entails some reservations. First, several other esterases in addition to AChE are similarly alkylphosphorylated by

the OPs. However, with the exception of the still unidentified enzyme or system whose inactivation is responsible for the production of OP-induced delayed neurotoxicity (OPIDN), discussed later, it is unlikely that this potential action results in significant pharmacological effects. Most of the other esterases are much less sensitive to the OPs and no significant effects have been attributed to their inactivation. As an exception to this generalization, butyrylcholinesterase (BChE; acylcholine acylhydrolase; E.C. 3.1.1.8) is more sensitive to most OPs than is AChE. However, its physiological function is unknown; with a few exceptions, no effects can be detected following its near total inactivation at most sites. Not surprisingly, the OPs have also been shown to produce varying degrees of alkylphosphorylation of non-enzymatic sites (i.e. receptors or ion gates). It is questionable whether this action contributes significantly to their major pharmacological or toxicological effects [6].

Cholinergic nerves

Cholinergic nerve fibres are characterized by their specific content of four factors: (1) acetylcholine (ACh), (2) choline acetyltransferase (ChAT), (3) the sodium-dependent high-affinity choline uptake (SDHACU) system, and (4) acetylcholinesterase (AChE). The first three are confined to the presynaptic cholinergic nerve terminals; AChE is present at both pre- and postsynaptic sites [4]. At all sites of cholinergic transmission AChE is present in considerable excess over normal physiological requirements. This proportion varies from two- or threefold at the smooth muscle of the gut to 10–20-fold at the motor endplates (MEPs) of skeletal muscle and in the CNS. Consequently, from 50% to 95% of AChE at these sites must be inhibited or inactivated before pharmacological effects become apparent. The picture is complicated by the fact that AChE occurs as internal (reserve) and external (functional) fractions. The former presumably represents newly synthesized AChE within the granular endoplasmic reticulum, and the latter enzyme that has passed on to the neuronal membranes (axonal or perikaryonal) with its active sites oriented externally [11,14]. Inhibition of only the external portion is associated with the production of pharmacological effects [16].

There are four classes of cholinergic nerve fibres. These and their corresponding postsynaptic sites and receptors, muscarinic and nicotinic are shown in Table 4.1.

In autonomic ganglia, physiological transmission is mediated essentially by N receptors; the role of M receptors and their associated potential changes remain to be defined [20]. In the CNS, N receptors predominate in the spinal cord and M receptors in the brain.

Pharmacology

The pharmacological actions of the OPs are the result of accumulation of endogeneous ACh at sites of cholinergic transmission, and its action at the corresponding postsynaptic receptor sites. It is remarkable that the receptors are still classified essentially as proposed by Dale over 70 years ago, although numerous subtypes have now been identified. It is also notable that Dale's distinction between muscarimc (M) and nicotinic (N) receptors, based on the peripheral actions of ACh, still held after cholinergic transmission had been demonstrated in the CNS.

At muscarinic (M) receptors of smooth muscle (class 1), over a wide range of concentrations ACh characteristically produces either

Table 4.1 Four classes of cholinergic nerve fibres and their postsynaptic sites and receptors

Class	*Cholinergic fibres*	*Postsynaptic sites*	*Receptors*
1	Postganglionic parasympathetic	Autonomic effectors	Muscarinic
2	Preganglionic sympathetic and parasympathetic	Sympathetic and parasympathetic ganglion cells	Nicotinic Muscarinic
3	Somatic motor	MEP of skeletal muscle	Nicotinic
4	CNS	Central neurons	Nicotinic Muscarinic

excitation (e.g. bronchioles) or inhibition (e.g. arterioles). On the other hand, at nicotinic (N) receptors of skeletal muscle (class 3), ACh produces excitation at low doses and block or paralysis at high doses. These same characteristics apparently apply to the M and N receptors of autonomic ganglia (class 2) and the CNS (class 4).

Following inhibition or inactivation of critical proportions of AChE (as noted earlier) at the four classes of cholinergic fibres, the following occur: (1) effects equivalent to activation of postganglionic parasympathetic fibres, including miosis, ciliary spasm, bradycardia, vasodilatation, bronchospasm, increased tone and motility of the gastrointestinal tract and urinary bladder, salivation, increased tracheobronchial secretion, lacrymation, and sweating; (2) stimulation followed by paralysis of sympathetic and parasympathetic ganglia, (3) skeletal muscle, and (4) various centres of the CNS.

Toxicology

The toxicology of the OPs is of two distinct types: cholinomimetic and OP-induced delayed neurotoxicity (OPIDN). The cholinomimetic toxicity is an extension of the pharmacological effects described earlier. It can occur acutely, in minutes or even seconds, or chronically over the course of weeks. All pharmacological effects may develop to an exaggerated degree. However, with lethal exposure by any route compromise of the respiratory system is the primary cause of death. In terms of the cholinergic nerve fibres, contributing effects include (class 1) bronchoconstriction and increased tracheobronchial secretion, (class 3) peripheral paralysis of the diaphragm and accessory respiratory muscles, and (class 4) paralysis of the respiratory centre. Which of these effects is primarily the cause of death varies with species. In cats and dogs bronchoconstriction (class 1) is predominant, in rabbits peripheral respiratory paralysis (class 3), and in the monkey paralysis of the respiratory centre (class 4) [5]. A progressive fall in blood pressure following a lethal dose of an OP suggests that the vasomotor centre is a critical site. However, if adequate respiratory exchange is established the vasodepressor effect is usually reversed.

The diagnosis of cholinominetic OP poisoning is made on the basis of physical signs, blood levels of AChE and BChE, and history. Treatment is highly specific and effective. It includes establishment at an adequate airway, atropine, artificial respiration, and administration of an AChE-reactivator such as PAM.

OP-induced delayed neurotoxicity (OPIDN) appears to be unrelated to the antiChE actions of this class of compounds [1,21]. It is characterized by the sequential development of axonal degeneration, demyelination and flaccid paralysis after a latent period of approximately 2 weeks following exposure. Triorthocresyl phosphate (TOCP), as an adulterant of beverages and cooking oil, has been responsible for outbreaks of OPIDN affecting several thousand persons; its antiChE potency is extremely low.

Several theories have been proposed to account for the production of OPIDN. The most widely considered at present is that it is the result of inactivation of neurotoxic esterase (NTE). This enzyme has been defined empirically by Johnson [8,10], as the phenylvalerate-hydrolyzing esterase that is resistant to 40 mM paraoxon but inhibited by paraoxon plus 50 mM mipafox (N,N' diisopropylphosphorodiamidic fluoride). While this concept is valuable in predicting the potential of OPs for producing OPIDN, it has failed to explain several observations. When hens, which are particularly susceptible to OPIDN, are treated with certain OPs, the level of NTE in the CNS returns to nearly normal during the 2-week latent period before the development of OPIDN. Certain compounds that cause near-total inactivation of NTE do not produce OPIDN [9]. Only OPs that undergo 'ageing' (splitting-off of an alkoxy group) cause OPIDN. These and other findings have led to some modified or alternative proposals: (1) alkylphosphorylation of NTE is only the first step that culminates in the production of OPIDN, (2) alkylation of some other enzyme or receptor, with properties similar to those of NTE, is the critical action, or (3) NTE and an essential protein kinase may be combined in a single macromolecule [21].

Hen brain has been shown to contain 11 phenylvalerate-hydrolyzing isoenzymes and primate brain eight [2,3]. A recent histochemical survey indicated that distribution of NTE in the neuronal cytoplasm of the hen brain does not differ from that of the other isoenzymes

[13]. The critical step in the production of OPIDN remains to be established (*see* Ch.10).

Therapeutic applications

Shortly after the discovery of the original nerve gas, DFP, during World War II, and the demonstration of its mechanism of action, it and subsequent OPs went on clinical trials in the conditions that had been treated with the short-acting CB antiChEs, physostigmine and neostigmine. They included atony of the gastrointestinal tract and urinary bladder, myasthenia gravis, and glaucoma [11]. The prolonged action of OPs held promise of marked therapeutic advantage, particularly in the latter two conditions. However, results were disappointing [11]. In atony of smooth muscle, DFP had no advantage over the older drugs and its use entailed a higher degree of hazard. In myasthenia gravis the dangers attendant upon cholinergic crisis, resulting from overdosage, were much more severe than with the standard drugs, and several fatalities occurred. In addition, the high lipid-solubility of DFP allowed its passage across the blood-brain barrier, with the production of marked side-effects on the CNS. With glaucoma, where the drugs are instilled locally and hence systemic effects are minimized, DFP and another OP, ecothiopate (echothiophate), were employed advantageously for several years. However, it was discovered that prolonged administration of long-acting antiChEs causes the development of irreversible lenticular opacities. The OPs are now used in glaucoma only in special circumstances. More recently, trials of the OPs in the treatment of Alzheimer's disease have generally been disappointing.

On the positive side, the OPs are widely and effectively employed as agricultural insecticides throughout the world. Malathion (O,O-dimethyl S-(1,2 dicarbethyoxyethyl phosphorodithioate))and certain other OPs are considerably safer than parathion but are more expensive to manufacture.

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5

Pharmacology and toxicology of carbamates

Alvito P. Alvares

Introduction

ACh is an important neurotransmitter in both the PNS and CNS. It is secreted at the postganglionic parasympathetic nerve endings and at some sympathetic nerve endings; at sympathetic and parasympathetic ganglia, at the adrenal medulla; at motor endplates at the NM junction of skeletal muscles; and at certain synapses in the CNS. ACh is rapidly catabolized by AChE, an enzyme found in the CNS, the NM junction, and RBCs. This enzyme primarily hydrolyzes ACh and has very poor affinity towards BCh. Serum BChE is found primarily in serum and liver and hydrolyzes BCh at a much faster rate than ACh. AChE, in contrast to BChE, is a membrane-bound enzyme, and is localized in close proximity to the sites of action of ACh.

The pharmacological and toxicological properties of antiChEs are primarily the result of inhibition of AChE. The active subsites of the enzyme may be either an esteratic group or an anionic site. ACh binds to the enzyme surface through its quaternary nitrogen and the carbonyl carbon. The positively charged quaternary nitrogen binds to the anionic site, while the carbonyl carbon binds to a positively charged nucleophilic group at the esteratic site. The hydrolysis of ACh occurs in at least three steps. Initially, the enzyme forms a high-energy complex with the substrate. The enzyme-substrate complex results in an acetylated enzyme and the release of choline. The acetylated enzyme then reacts with water to form acetic acid, thus releasing the free enzyme. Current information suggests that the anionic site contains at least one carboxyl group, possibly from glutamate, and the esteratic site involves a histidine residue adjacent to serine. The overall reaction is written as follows:

$$E + ACh \leftrightarrow E.ACh \rightarrow E.O\text{–}\underset{\underset{O}{\|}}{C}\text{–}CH_3 \rightarrow E + CH_3COOH$$

$$+ \text{choline}$$

ChE inhibitors may prevent the hydrolysis of ACh by several different mechanisms. They may prevent the interaction with the enzyme by interacting (1) at both the anionic and esteratic sites (e.g. physostigmine and neostigmine); (2) at the anionic site forming a reversible enzyme-inhibitor complex (e.g. edrophonium); (3) at the esteratic site forming a stable acylated enzyme (e.g. the OP inhibitors). Physostigmine, edrophonium and neostigmine are clinically useful ChE inhibitors. The OP compounds produce more prolonged inactivation of AChE. Examples of slowly reversible inhibitors include parathion, malathion, diazinon, and dichlorvos. OP inhibitors react at the esteratic subsite phosphorylating the enzyme. The resultant complex may be extremely stable; significant regeneration of the active enzyme is not observed. The return of AChE activity will, therefore, depend on the synthesis of new enzyme. Because of the extremely potent, long-lasting effects of the OPs, they have been used primarily as insecticides and chemical warfare agents. Only the 'reversible' antiChE agents will be considered in this review. Of these, the CBs of general therapeutic or toxicological interest will be discussed.

The first recognized antiChE agent, obtained from the Calabar bean, was physostigmine (also called eserine). The main alkaloid was obtained in its pure form in 1864 by Jobst and Hesse; they called it physostigmine. In 1877 Laquer first used the drug therapeutically for the treatment of glaucoma. The property of physostigmine to produce miosis and other symptoms was attributed to the urethane group of the drug. This observation

prompted the preparation of a number of substituted urethanes, the best known being neostigmine, which was synthesized in 1931 by Aeschliman and Reinert. It was initially used for its stimulant action on the intestinal tract. Subsequently, it was found to be effective in the treatment of myasthenia gravis.

The use of CBs as insecticides occurred in the 1950s with their development as insect repellents. One of these compounds, 5,5-dimethyl dihydroresorcinol dimethylCB, showed promising insecticidal activity. As a consequence, a considerable number of CB analogues were synthesized and tested. The naphthyl CBs were found to have a high degree of selective toxicity against insects and to have potent antiChE activity. Among those used as insecticides are the CBs, carbaryl and propoxur. Carbaryl is one of the most widely used broad-spectrum insecticides. Its remarkable success is attributable to its low acute and chronic toxicity to mammals, and its environmental degradability. Propoxur is widely used for household pest control and for residual spray in malarial eradication programmes. The structures of CBs which are of clinical and toxicological interest are shown in Figure 5.1.

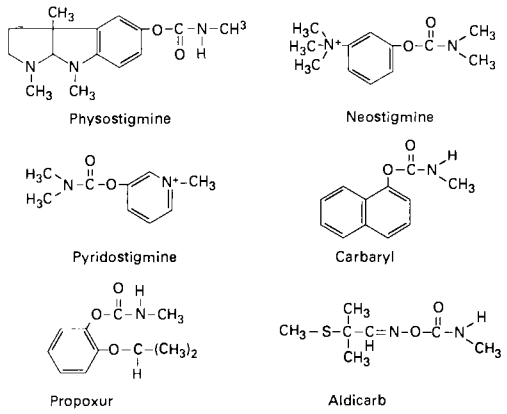

Figure 5.1 Examples of 'reversible' carbamate inhibitors of AChE

The structures of the OP and CB insecticides are diverse. Their structural features are not confined solely to their inhibitory effects on AChE. Other factors which confer their insecticidal features may involve penetration, stability, cost, and the insect or pest over which control is sought. In spite of their diverse features and uses, the basic mode of action is the same for all these compounds. They bind to the active sites of AChE. A scheme for the inhibitor interaction with AChE is shown in Figure 5.2. The CBs are alternative substrates for the esterase. They are hydrolyzed by the enzyme much more slowly than ACh. In contrast to the acetylated enzyme, the carbamylated ChE is much more stable. The inhibitory effects of the CBs is of the order of approximately 30 min to a few hours, whereas the hydrolysis of the natural ACh substrate takes place in less than a second.

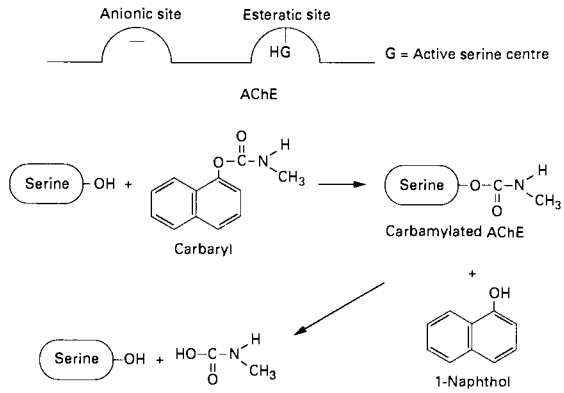

Figure 5.2 Reaction of carbaryl insecticide with AChE. Adapted from Murphy [18]

Pharmacological action of CB drugs

Absorption of the quaternary CBs, such as neostigmine, from skin or after oral administration, is poor. Physostigmine, in contrast, is well absorbed from all sites and can be used topically in the eye. The CB drugs can be metabolized by non-specific esterases as well as by ChE. However, their half-life is determined by the stability of the inhibitor-enzyme complex rather than by metabolism or excretion.

The pharmacological actions of the CB drugs are from an increase in the concentration of ACh in the vicinity of the cholinoceptors, because of their inhibitory actions on the ACh metabolizing enzyme, AChE. The main action of the CB antiChEs of therapeutic importance is concerned with the effector organs: eye, intestine and NM junction.

Physostigmine is used principally as a miotic; it is used to treat narrow-angle glaucoma and to reverse the mydriasis caused by atropine. Physostigmine is also the antidote of choice to treat poisoning with belladona alkaloids.

The action of the CB drugs, such as neostigmine, on smooth muscle and parasympathetic ganglion cells in the intestinal tract results in increased intestinal tone and motility. In the urinary system, the agents increase peristaltic activity in the ureters and increase atony of the bladder. Neostigmine is the most widely used of the ChE inhibitors for these applications. Another prominent action of the ChE inhibitors is on the skeletal muscle NM junction. Low, therapeutic concentrations of these inhibitors moderately prolong and intensify the actions of physiologically released ACh. The CB inhibitors, in addition, have an additional direct nicotinic agonist effect at the NM junction. These actions may contribute to the effectiveness of the CB drugs on the treatment of myasthenia gravis. Pyridostigmine and neostigmine are the major antiChE agents used in myasthenia gravis therapy.

Toxicological action of CB insecticides

CBs form a large class of pesticides that include two major types: (1) antiChE insecticides (compounds such as carbaryl, aldicarb, methomyl, carbofuran) used extensively as miticides and broad-spectrum insecticides, and structurally analogous to the therapeutic CBs physostigmine and neostigmine; and (2) thiocarbamate compounds, such as thiram, maneb, and zineb, widely used as fungicides, and metham and pebulate, used as herbicides. After OPs, CBs are the second largest class of insecticides in terms of worldwide usage. For the past 30 years the most widely used CB insecticide has been the broad-spectrum compound, carbaryl. CB insecticides vary in their toxicological potency from the highly toxic aldicarb and carbofuran to the minimally toxic fenethacarb and carbaryl. CB insecticides, such as aldicarb, that are more closely analogous to ACh are several-fold more potent than carbaryl, and also tend to have low insecticidal selectivity.

Aldicarb is used primarily by the agricultural industry; home and garden use is not permitted. It is biodegraded to its oxidative sulphoxide and sulphone metabolites. These appear to be the major metabolites formed *in vivo*. The primary mechanism of its toxic action is ChE inhibition; however, the carbamylation process that produces the antiChE action is quickly reversible. It is readily absorbed through the skin and intestine; it is also rapidly metabolized and excreted in the urine within 24 h. In cases of accidental poisoning, the cholinergic symptoms generally subside within 6 h with no side-effects or complications (*see* review by Risher *et al.* [20]). Some recent studies have indicated that CB compounds may present serious problems to the health of the developing fetus in experimental animals. Cambon *et al.* [1,2] demonstrated inhibition of AChE in both fetal and maternal tissues of pregnant rats after oral intake of a number of CB insecticides, including carbaryl and aldicarb.

Because carbaryl has a relatively simple structure, it is a model compound for toxicological and metabolic studies of the CB group of insecticides. Carbaryl is a widely used insecticide in agriculture and in urban gardens. It is also effective in controlling pests in buildings and on a wide range of indoor plants. The mode of action of carbaryl is similar to the other reversible ChE inhibitors. Signs and symptoms of poisoning are typically cholinergic, with lacrimation, salivation, miosis, convulsions, and death. As with other CBs, atropine sulphate is the recommended antidote for poisoning by this class of insecticides. Administration of pralidoxime (2-PAM), generally used in treatment of OP poisoning, is not recommended in the treatment of CB poisoning. There have been reports that 2-PAM aggravates the toxicity of carbaryl [3].

The known pathways of carbaryl metabolism are depicted in Figure 5.3. Hydrolysis of carbaryl to naphthol is a major pathway. The insecticide also undergoes oxidative metabolism by enzymes localized in the endoplasmic reticulum to form 4-hydroxycarbaryl, 5-hydroxycarbaryl, 5,6-dihydro-5,6,-dihydroxycarbaryl, and 1-naphthyl-N-hydroxymethylCB [14]. Most animals excrete between 65–75% of the radioactivity derived from [1-naphthyl-^{14}C] carbaryl in the urine within 24 h of dosing

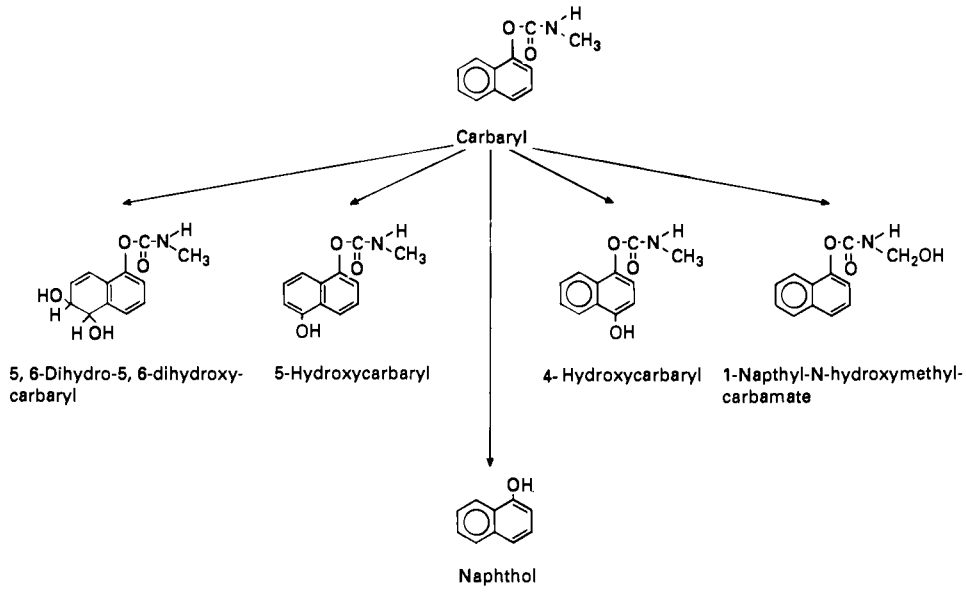

Figure 5.3 Major pathways of carbaryl metabolism. From Knight *et al.* [13]

with carbaryl. The main quantitative difference between the various species studied by Knaak *et al.* [10,11] was the extent to which carbaryl was cleaved to 1-naphthol. The reaction was minimal in monkey and pig, but occurred extensively in rat, sheep, and man. In insects, hydrolysis of the CB is a very minor pathway. Using human liver tissue explants, Chin *et al.* [5] showed than the anionic metabolites of carbaryl lay in the order of liver > lung > kidney > placenta. Hepatic tissues was shown to hydrolyze, hydroxylate and conjugate carbaryl. The oxidative metabolites are excreted in the urine as glucuronides and sulphates.

In the case of N-methylCBs, such as carbaryl and propoxur (Figure 5.1), the N-hydroxyl methyl derivatives appear stable enough to be isolated from microsomal incubation mixtures, however, this derivative appears to be a minor pathway in CB metabolism. Note that the hydrolysis of the CBs invariably results in loss of their antiChE activity, but oxidation products formed by microsomal enzymes can retain some antiChE activity and may not always be less toxic than the parent compound. Oonnithan and Casida [19] showed that the 4-hydroxy metabolite of propoxur is three-fold more potent than the parent compound.

Propoxur, a widely used CB insecticide, is hydroxylated to a 5-hydroxy metabolite. None of the 4-hydroxy isomer has been found. Propoxur, like carbaryl, also undergoes hydrolytic reactions [21]. In humans about 30% of propoxur is excreted as 2-isopropoxyphenol [7].

The hydrolytic reactions which CBs undergo are catalysed by carboxylesterases. The products are devoid of antiChE inhibition. After cell fractionation by differential centifugation the carboxylesterase activity of hepatic and extrahepatic tissues is found predominantly in the microsomal fraction. Occasionally, the bulk of liver carboxylesterases is found in the soluble fraction. This is probably from autolysis, which readily solubilizes the membrane-bound carboxylesterases [8].

The oxidative reactions are carried out by the cytochrome P450 family of enzymes localized in the endoplasmic reticulum of the liver and extrahepatic tissues. The initial step in the oxidative process consists of the substrate binding to the oxidized form of cytochrome P450 to give a type I or a type II spectral change. Type I substrates exhibit an absorbance spectra with a maximum at 385–390 nm and a minimum at approximately 420 nm. Those substrates which bind to liver microsomes and exhibit a peak at 420–435 nm

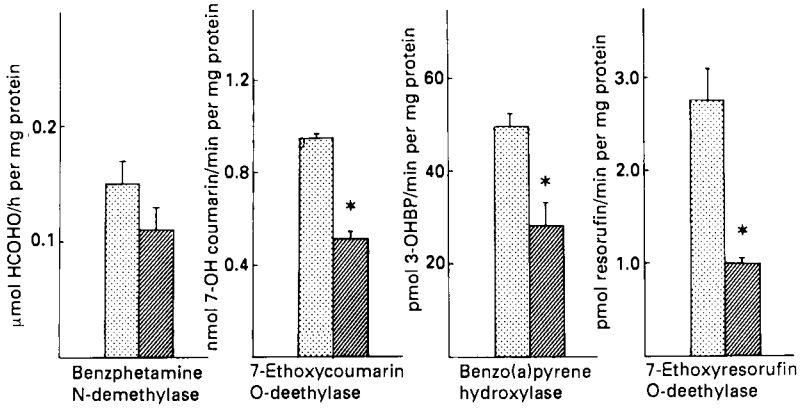

Figure 5.4 Effect of *in vitro* addition of carbaryl (■) on the metabolism of benzphetamine, 7-ethoxycoumarin, benzo (a) pyrene, and 7-ethoxyresorufin by rat liver microsomes. Asterisk represents value significantly different ($P < 0.05$) from the respective value obtained in absence of carbaryl (▨). Data from Knight *et al.* [13]

and a trough at 390–410 nm are termed type II substrates. Carbaryl, when added to hepatic microsomes from untreated rats, gives a weak, type I spectrum [13]. Knight *et al.* [13] also showed that using microsomes from untreated rats, carbaryl caused marked inhibition of cytochrome P450-dependent oxidations of the model substrates benzphetamine, 7-ethoxycoumarin, benzo(a)pyrene and 7-ethoxyresorufin (Figure 5.4). Benzo (a)pyrene and 7-ethoxyresorufin are substrates metabolized preferentially by cytochrome P448, whereas benzphetamine is preferentially metabolized by cytochrome P450; 7-ethoxycoumarin is metabolized by cytochromes P450 and P448 in rat liver.

Inhibition of drug metabolism by exogenous substances, such as carbaryl, may result in exaggerated and prolonged response to drugs with an increased risk of toxicity. Conversely, inhibition of carbaryl oxidative metabolism will result in prolonged antiChE action by the insecticide. Indeed, piperonyl butoxide, an inhibitor of cytochrome P450-mediated xenobiotic metabolism, has been used to prolong the insecticidal properties of various insecticides. Mechanistic studies have shown that such interactions involving cytochrome P450-mediated oxidations include substrate competition or functional impairment of the P450 isozymes. *In vitro* studies using liver microsomal enzyme preparations have shown that competitive inhibition kinetics are observed with many drugs and other xenobiotics that are metabolized by the oxidative enzymes.

It is now well established that a variety of environmental agents can induce oxidative as well as hepatic microsomal carboxylesterases. The enhanced rate of drug biotransformation is often associated with a faster termination of drug action. More recently, however, extensive data have shown that, in some instances, induction of cytochrome P450 can result in the formation of electrophilic metabolites of xenobiotics, which are biologically more active and are often responsible for the acute or chronic toxicity associated with certain foreign compounds. Inducers of hepatic cytochrome P450 have been characterized to a much greater extent than those of carboxylesterases. One group, of which barbiturates and the insecticide DDT are prototypes, enhances the metabolism of a large variety of substrates and induces cytochrome P450. A second group of chemicals, such as the polycyclic hydrocarbons present in cigarette smoke and charcoal-broiled meat, induces the synthesis of cytochrome P448 which differs in spectral, catalytic, electrophoretic, and immunological properties from cytochrome P450 present in livers from untreated or phenobarbitone-treated rats [6].

Induction of hepatic microsomal carboxylesterases has been reported by pretreatment of rats with xenobiotics such as phenobarbitone [22] and DDT [23], but not by the polycyclic aromatic hydrocarbon 3-methylcholanthrene [8]. Hosokawa *et al.* [9] have purified three isozymes of carboxylesterase from rat liver microsomes and have shown

that they differ considerably from each other in their inducibility by phenobarbitone, and in their substrate specificities.

Hydrolytic and oxidative metabolites of CBs are excreted as glucuronides and sulphates. Conjugation with glucuronic acid catalyzed by microsomal UDP-glucuronyltransferase is quantitatively the most important phase II reaction of xenobiotic metabolism. Glucuronyl transferase activity towards 1-naphthol is detectable in the liver, kidneys, intestines and other tissues. Similar to the cytochromes P450, glucuronyl transferases consist of a family of closely related enzymes with differing substrate specificity and inducibility. The glucuronidation of 1-naphthol, a metabolite of carbaryl, is induced primarily by the polycyclic hydrocarbon class of inducers, and not by the barbiturate class of inducing substances [16]. Sulphate conjugation is another important phase II reaction which the oxidative metabolites of carbaryl undergo. This reaction is catalysed by more than one cytosolic sulphotransferase. One isozyme is specifically termed as a phenol transferase. Recent studies [12] have shown that pretreatment of rats with phenobarbitone caused significant increase in sulphate conjugates of carbaryl in urine, but phenobarbitone failed to induce increased excretion of glucuronide conjugates of carbaryl. The relative extent to which sulphation and glucuronidation of CBs occur will depend on the substrate, species, and dosage of CBs. Both conjugative pathways are saturable.

Various insecticides can affect the microsomal oxidative enzymes. In general, chlorinated hydrocarbons such as DDT and chlordane are effective inducers, while OP insecticides, such as parathion, are inhibitors of the mono-oxygenases. The *in vivo* effects of CBs on these microsomal oxidative enzymes are not well-characterized. Studies by Lechner and Abdel-Rahman [15] have shown that carbaryl, administered subacutely, resulted in no inductive properties when liver microsomes were assayed for aminopyrine demethylase and aniline hydroxylase. In other studies, Madhukar and Matsumura [17] showed that in rats administered carbaryl orally, the insecticide moderately induced several N-demethylation reactions. More recent studies [13] have shown that subacute treatment of rats with carbaryl administered by gavage or ip daily for 4 days had no effect on cytochrome P450 content or on the metabolism of ethylmorphine or the carcinogen, benzo(a)pyrene. The lack of effect of daily administration of carbaryl may result from preferential degradation by esterase present in the blood and liver. Studies in rabbits show that plasma was more than twice as active in the conversion of carbaryl to naphthol, than liver, kidney, spleen and other tissues [4].

In recent years, the use of CB and OP insecticides in combination has become quite popular. Treatment of rats with a combination of carbaryl and malathion has been shown to result in a fivefold increase in the urinary excretion of malaoxon when compared with rats administered malathion alone [15]. Malathion is metabolized through the cytochrome P450 system to malaoxon. Changes in the bioavailability of a pesticide used in combination with other pesticides are of toxicological importance in humans occupationally exposed to these combinations. Furthermore, a large number of drugs are metabolized by the cytochrome P450 system. Humans handling pesticides on a routine basis, who concomittantly ingest these drugs, may demonstrate differences with the pharmacological and toxicological action of the drugs, when compared to the general population who ingest such drugs but are not occupationally exposed to these insecticides.

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6

Structure-activity relationships and anticholinesterase activity

Donald M. Maxwell and David E. Lenz

Introduction

Investigation of the relationship between antiChE activity and chemical structure has been a productive research area in pharmacology and toxicology as well as pesticide development. Inhibition of AChE has been reported for diverse chemical structures. Although many biochemical and neurophysiological effects have been attributed to CBs and OPs [2] the inhibition of AChE in the nervous system is the primary cause of the pharmacological and toxicological effects of these compounds [27,37,47].

This chapter presents a structure-activity analysis for the effects of antiChEs on in vivo toxicity and *in vitro* AChE inhibition, with incorporation of the kinetic parameters that are available from mammalian systems, in accord with our view that these data are more relevant to the human situation.

Kinetics of AChE inhibition

The inhibition of AChE by CBs and OPs is produced by the formation of a covalent bond between these inhibitors and a specific serine at the active site of AChE. The kinetic descriptions of the reactions of inhibitors, as well as substrates (i.e. ACh) with AChE are analogous. Wilson *et al.* [48,49] described this process using the following scheme:

$$E + AX \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EAX \underset{X}{\overset{k_2}{\rightarrow}} EA \xrightarrow{k_3} E + A$$

where E is the AChE enzyme; AX is a substrate or inhibitor; EAX is a reversible complex; and k_{+1}, k_{-1}, k_2, and k_3 are all reaction rate constants. If AX is acetylcholine, A is an acetyl group and X is choline. If AX is an inhibitor, A is the part of the inhibitor that forms a covalent bond with the enzyme and X is the leaving group. This scheme is usually simplified by combining rate constants. The affinity with which the substrate or inhibitor binds to AChE to form a reversible complex is described by a dissociation constant, K_D, defined by $K_D=k_{-1}/k_{+1}$. The smaller K_D is, the greater the affinity. For inhibitors, k_3 is small enough to be ignored in brief experiments. K_D and k_2 can be measured independently by methods developed by Main and Iverson [36]. However, K_D and k_2 have not been measured for many inhibitors. For most inhibitors the bimolecular rate constant, k_i, as shown in the following simplified scheme, is used to describe the reaction of inhibitors with AChE:

$$E + AX \xrightarrow[k_i]{} EA + X$$

The relationship between this simplified scheme and the one previously described was provided by Main and Iverson [36] who demonstrated that k_i is equal to k_2/K_D. A less rigorous expression of inhibitor potency is I_{50}, which is the concentration of inhibitor that inhibits 50% of AChE in a given incubation time (t). Aldridge [4] demonstrated that $k_i = 1n\ 2/I_{50}t$ which allowed the estimation of bimolecular rate constants from I_{50} values.

Structure of AChE

The active site of AChE consists of several subsites, each of which plays an important role in

Table 6.1. Structures of common organophosphorus compounds

Name	*Use*	*Structure*[a]			
		A_1	A_2	X	O,S
Parathion-methyl	Insecticide	MeO	MeO	OPh–4–NO_2	S
Terbufos	Insecticide	EtO	EtO	SCH_2SCMe_3	S
Fonofos	Insecticide	EtO	Et	SPh	S
Phorate	Insecticide	EtO	EtO	SCH_2SEt	S
Parathion	Insecticide	EtO	EtO	OPh–4–NO_2	S
Chlorpyrifos	Insecticide	EtO	EtO	O–2–Pyrid–3,4,6–Cl_3	S
EPN	Insecticide	EtO	Ph	OPh–4–NO_2	S
Malathion	Insecticide	EtO	EtO	$SCH(CO_2Et)CH_2CO_2Et$	S
Diazinon	Insecticide	EtO	EtO	O–4–Pyrim–6–Me–2–Pr^i	S
Dichlorvos	Insecticide	MeO	MeO	$OCH{=}CCl_2$	O
Isophenphos	Insecticide	EtO	Pr^iHN	OPh–2–CO_2Pr^i	S
DFP	Research	Pr^iO	Pr^iO	F	O
Paraoxon	Research	EtO	EtO	OPh–4–NO_2	O
Echothiopate*	Drug	EtO	EtO	$SC_2H_4N^+Me_3$	O
Sarin	Warfare	Me	Pr^iO	F	O
Tabun	Warfare	EtO	Me_2N	CN	O
VX	Warfare	Me	EtO	$SC_2H_4NPr^i_2$	O
Soman	Warfare	Me	$Bu^tCH(Me)O$	F	O

Abbreviations for structures are Ph (phenyl), Me (methyl), MeO (methoxy), Et (ethyl), EtO (ethoxy), Pr^i (iso-propyl), Bu^t (tert-butyl), pyrid (pyridinyl), and pyrim (pyrimidinyl).
*Echothiophate (USP)

defining the activity of this enzyme with substrates or inhibitors. As noted above, the active site of AChE contains a specific serine residue whose properties are uniquely modified by the effects of neighbouring amino acid residues. The hydroxyl group of this serine can be phosphorylated, carbamylated or acetylated depending on whether it reacts with an OP, CB or ACh. The active site serine, which forms covalent bonds with either inhibitors or substrates, is also known as the esteratic site because it forms an acetyl ester with ACh, the natural substrate of AChE. A second portion of the active site is known as the anionic site in recognition of possible ionic bonds that the active site can form with the positively charged part of the natural substrate (i.e. the choline of ACh) or charged inhibitors. Finally, the active site of AChE also has hydrophobic regions that can form hydrophobic bonds with various aliphatic or aromatic groups of inhibitors or substrates.

OP compounds

The structural diversity of some common OP antiChEs is shown in Table 6.1. As previously described, X is the leaving group, while A_1 and A_2 are groups that remain with the phosphorylating part of the inhibitor that covalently bonds to AChE. All OPs contain a pentavalent phosphorus atom with a double bond to either oxygen (P=O) or sulphur (P=S). Insecticides usually contain a P=S rather than a P=O moiety because it reduces their mammalian toxicity. The OPs that are used in research, medicine or warfare contain a P=O, which increases their reactivity with AChE as well as their toxicity. The leaving groups are diverse with structures ranging from a single halogen atom to a complex substituted aryl ring.

Leaving groups have the common property that the bond between phosphorus (P) and the leaving group (X) is the most labile of the four bonds attached to phosphorus. The lability of the P–X bond is an important determinant in the reactivity of OP inhibitors with AChE. This chemical lability can be measured by the rate of hydrolysis in alkaline aqueous solution [4], the wavelength of P–X bond stretching [17], or the acidity of HX, the acid formed by the hydrolysis of the P–X bond [28]. Regardless of the choice for measuring the

strength of the P–X bond, a labile P–X bond is essential for an inhibitor or substrate to form a covalent bond with AChE.

Electronic effects

The lability of the P–X bond in OPs can be influenced by a variety of electronic effects. Some of these electronic effects result from the electronegativity of the other atoms or groups attached to phosphorus. Electronegativity is an estimate of the ability of an atom to attract electrons in comparison to other atoms in a molecule [15,33]. The effect of a less electronegative atom on an AChE inhibitor is illustrated by a comparison of organophosphothioate and organophosphate analogues, which contain either P=S or P=O moieties, respectively. Organophosphates are consistently better inhibitors of AChE than P=S organophosphothioates [3,7]. Because both oxygen and sulphur are more electronegative than phosphorus, they tend to shift the electron density around the phosphorus toward oxygen or sulphur, thereby weakening the bonds between phosphorus and A_1, A_2 and X. Because oxygen is more electronegative than sulphur, P=O analogues exert a greater electron-withdrawing effect and produce weaker P–X bonds than P=S analogues. The greater lability of the P–X bond in P=O analogues in comparison to P=S analogues is reflected in their consistently greater reactivity with AChE. The difference in the effects of oxygen and sulphur on P–X lability are insufficient to explain the entire difference between the AChE reactivities of paraoxon and parathion because the difference in their rates of alkaline hydrolysis is tenfold, while the difference in their AChE reactivities is 9170-fold [22]. Because sulphur is a less effective participant in hydrogen bonding than oxygen [44], it has been suggested that the P=O in paraoxon forms hydrogen bonds with the AChE active site which the P=S of parathion does not [15].

The effects of electronegativity differences can also be observed in OPs containing halogen or nitrogen atoms. A-groups containing halogens, which are electron-withdrawing groups, can affect AChE reactivity by increasing P–X lability. For example, Lieske *et al.* [32] have reported that substitution of a chlorine

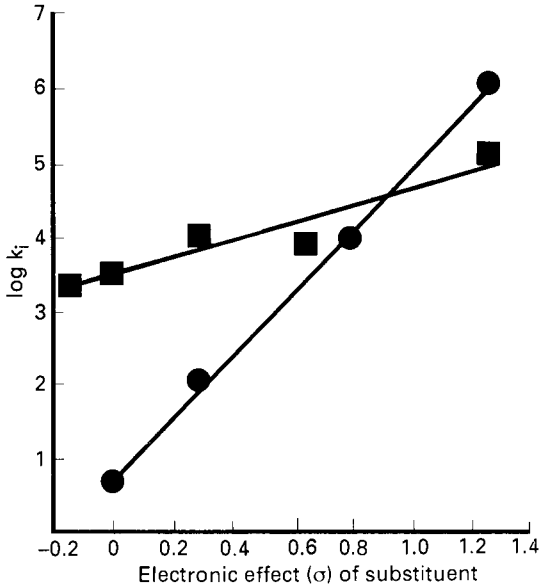


Figure 6.1 Effect of electronic parameter (σ) of aryl leaving group substituent (X) on AChE reactivity (k_i) of organophosphorus inhibitors. Substituents are presented in order of increasing σ for $(EtO)_2P(O)OPhX$ (●) where X=H, 4-Cl, 3-NO_2 or 4-NO_2 and EtO(PrS)P(O)OPhX (■) where X=4-Me, H, 4-Cl, 4-COOMe, or 4-NO_2. Data for k_i from Aldridge [4] and Hart and O'Brien [20]. Data for σ from Hansch and Leo [19].

atom for a hydrogen in the methyl substituent of a methylphenylphosphinate increased its AChE k_i by tenfold. In contrast, A-groups containing nitrogen are generally less reactive as inhibitors of AChE than their oxygen analogues. The amido groups of phosphoramidate inhibitors are less electron-withdrawing than similar alkoxy groups and not as effective at weakening the P–X bond. This results in an 11- to 78-fold decrease in AChE reactivity [18].

Electronic effects are also observed when electron-withdrawing substituent are present on aryl leaving groups of OPs. The effect of substituents on aryl rings usually are evaluated by use of the electronic parameter σ which is an estimate of the ability of a substituent to withdraw electrons (+σ) or donate electrons (−σ) to an aryl ring. Electron-withdrawing groups such as nitro or halide substituents invariably produce increases in the k_i of OPs by weakening the P–X bond, while electron-donating groups produce no effect (Figure 6.1). However, the maximal effect of the same

electron-withdrawing substituents on aryl leaving groups varies considerably between compounds with different A-groups. As shown in Figure 6.1, a para-nitro substituent on a phenyl leaving group increases the k_i of O-ethyl S-propyl phosphorothioate by 10^2-fold while the same leaving group increases the k_i of diethyl phosphate by 10^6-fold.

The position of the electron-withdrawing group on the aryl ring has a large influence on the degree of this electronic effect. OPs containing phenyl leaving groups with para-nitro substituents are more effective inhibitors of AChE than those with ortho- or meta-nitrophenyl leaving groups [3,42]. These observations correlate with the estimates of electron-withdrawal (σ) for ortho-, meta-, and para-substituents compiled by Hansch and Leo [19].

Oxygen-containing alcohol leaving groups are much less reactive with AChE than their sulphur-containing thiol analogues [9,10]. Except for a few organophosphates for which k_i are uncommonly small, the organophosphates are generally two to six times less reactive with AChE than the analogous S-alkyl organophosphorothioates.

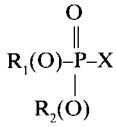
The difference between the effects of alkyl (R) and alkoxy (RO) groups on AChE reactivity are not amenable to explanation by electronegativity arguments even though oxygen is more electronegative than carbon. The order of P–X alkaline hydrolysis rates of these compounds is phosphinates > phosphonates > phosphates [15] while the order of AChE reactivity is phosphonates > phosphates > phosphinates (Table 6.2). Therefore, the greater AChE reactivity of phosphonates cannot be explained by P–X lability, and is probably related to the specificity requirements of the enzyme.

Hydrophobicity

Hydrophobicity is an estimate of the attraction of a chemical compound or group to non-aqueous environments such as the non-polar regions of AChE. Hydrophobicity parameters (π) are derived from partition coefficients between an organic solvent and an aqueous solvent. Increasing the length of the n-alkyl moiety of A-groups for either symmetrical dialkylphosphinates or dialkyl phosphates produces an increase in their reactivities with AChE (Table 6.2). These increases in k_i have been attributed to hydrophobicity since this parameter (π) increases as the chain length of n-alkyl groups increases, while electronic parameters are virtually unaffected by the length of n-alkyl groups.

The influence of hydrophobic groups on AChE reactivity is dependent on the symmetry of the resulting OP compound. As the data shown in Table 6.3 suggest, asymmetric OPs are better inhibitors than their symmetrical analogues. For example, the k_i of ethylphenylphosphinate, an asymmetric compound, is more than 100 times greater than the k_i for either of its symmetrical analogues, diethylphosphinate and diphenylphosphinate.

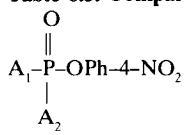
Table 6.2. Comparison of organophosphinates, -phosphonates, and -phosphates[a]



			AChE k_i (l mol^{-1} min^{-1})		
			phosphinate	phosphonate	phosphate
R_1	R_2	X	R_1, R_2	R_1O, R_2	R_1O, R_2O
CH_3	CH_3	OPh–4–NO_2	3.1×10^{4}[b]	8.2×10^{5}	1.2×10^{5}
C_2H_5	C_2H_5	OPh–4–NO_2	3.5×10^{3}	1.8×10^{6}	4.5×10^{5}
		F	—	3.1×10^{6}	2.1×10^{5}
		$SC_2H_4NEt_2$	—	4.1×10^{7}	3.3×10^{6}
C_3H_7	C_3H_7	OPh–4–NO_2	1.8×10^{4}	—	8.5×10^{5}
C_4H_9	C_4H_9	OPh–4–NO_2	6.6×10^{4}	—	1.3×10^{6}
C_5H_{11}	C_5H_{11}	OPh–4–NO_2	7.9×10^{4}	—	1.8×10^{6}

[a]From Ooms [42] except where noted otherwise
[b]From Lieske, unpublished data

Table 6.3. Comparison of symmetrical and asymmetrical organophosphinates



A_1	A_2	*AChE* k_i *(l mol^{-1} min^{-1})*	*Reference*
Me	Me	3.1×10^4	Lieske (unpublished data)
Et	Et	3.5×10^3	Ooms [42]
Ph	Ph	1.9×10^3	Lieske (unpublished data)
Me	Ph	4.6×10^5	Lieske *et al.* [30]
Et	Ph	4.2×10^5	Lieske *et al.* [30]

A similar situation occurs with methylphenylphosphinate. Observations such as these have led to the suggestion that two different hydrophobic binding sites exist for A-groups [25].

For alkylphosphonates containing a methyl A-group, the AChE reactivity increases as the n-alkyl chain of the other A-group increases up to a maximum where n=4 (Figure 6.2), which has suggested an n-butyl hydrophobic

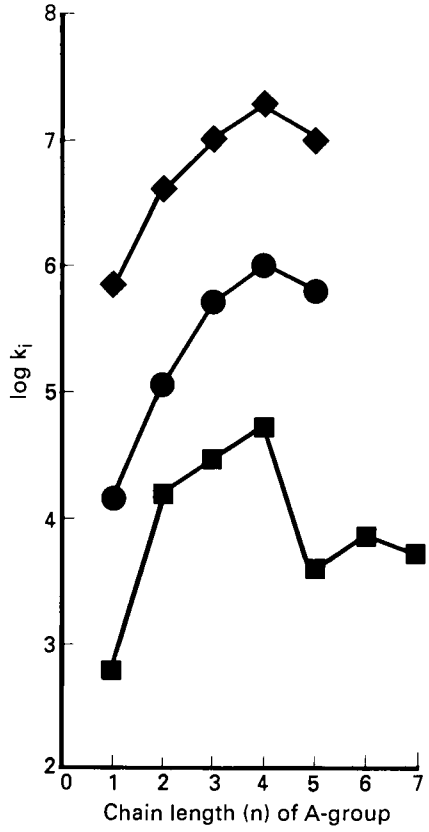


Figure 6.2 Effect of chain length (n) of n-alkyl A-group on AChE reactivity (k_i) of organophosphorus inhibitor for $Me(C_nH_{2n+1}O)P(O)OPh\text{-}4\text{-}NO_2$ (◆), $Me(C_nH_{2n+1}S)P(O)OPh\text{-}4\text{-}NO_2$ (●), and $Me(C_nH_{2n+1}O)P(O)SC_6H_{11}$ (■). Data from Ooms and Boter [43] and Kabachnik *et al.* [26].

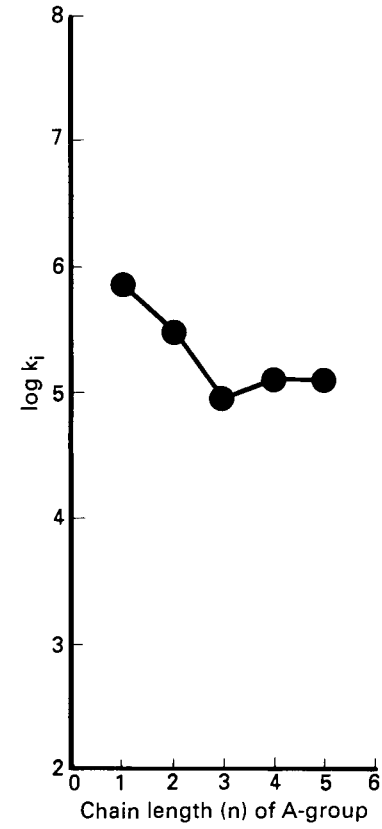


Figure 6.3 Effect of chain length (n) of n-alkyl A-group on AChE reactivity (k_i) of organophosphorus inhibitor for $C_nH_{2n+1}(Pr^iO)P(O)OPh\text{-}4\text{-}NO_2$) (●). Data from Ooms [42].

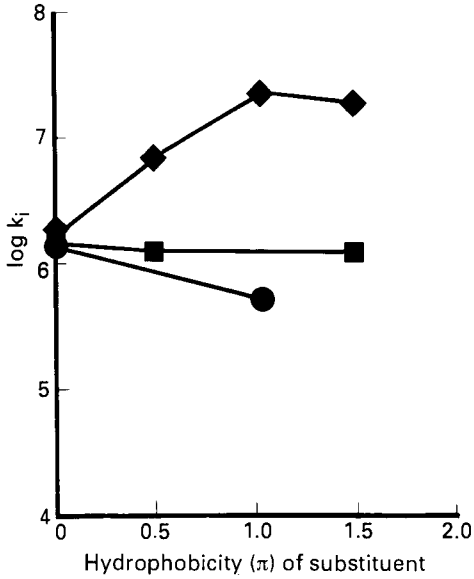


Figure 6.4 Effect of hydrophobicity (π) of aryl A-group substituent (R) on AChE reactivity (k_i) of organophosphorus inhibitors. Substituents are presented in order of increasing π for Et(PhR)P(O)OPh-4-NO_2 with meta-R (◆), para-R (■), and ortho-R (●) where R=H, Me, Et or Pr^i. Data for k_i from Adams [1]. Data for π from Hansch and Leo [19].

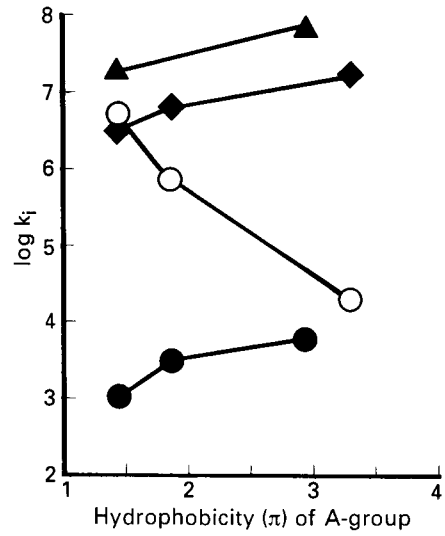


Figure 6.5 Effect of hydrophobicity (π) of bulky A-group (R) on AChE reactivity (k_i) of organophosphorus inhibitors. Substituents are presented in order of increasing π for Me(OR)P(O)OPh-4-NO_2 (○), Me(OR)P(O)$SC_2H_4NPr^i_2$ (▲), Me(OR)P(O)F (◆), and Me(OR)P(O)SC_3H_7 (●) where R=Et, Pr^i, cyclo-C_5H_9, or $CH(Me)Bu^t$. Data for k_i from Ooms [42], Kabachnik *et al.* [26], and Harvey *et al.* [21]. Data for π from Hansch and Leo [19].

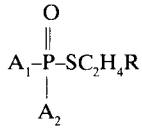
binding site. If one of the A-groups is large (e.g. Pr^iO), the maximal AChE reactivity is achieved where n=1 (Figure 6.3), suggesting a methyl hydrophobic binding site. These observations are believed to be related to the steric restrictions that the AChE hydrophobic binding sites for A-groups impose on the length of the n-alkyl groups that they can bind. One hydrophobic binding site for A-groups is limited to methyl groups and the other hydrophobic site is limited to butyl or shorter n-alkyl groups. An n-octyl hydrophobic binding site has been suggested for leaving groups of OPs, because the AChE reactivity increases for n-alkyl leaving groups to a maximum where n=8–9 depending on the particular OP [26].

Although the methyl, n-butyl, and n-octyl hydrophobic binding sites have steric restrictions on the length of the hydrophobic group that can be accommodated, they can bind branched alkyl, or substituted aryl groups of varying widths [26]. Aryl and cyclo-alkyl A-groups and leaving groups are found in some of the most effective AChE inhibitors [1,7]. Alkyl substituents in the meta-position of phenyl A-groups are effective at increasing AChE reactivity as their hydrophobicity values increase up to maximum where π=1.0 (Figure 6.4). In contrast, alkyl substituents in the ortho- or para-position are ineffective. In most cases any hydrocarbon grouping (i.e. n-alkane, branched alkane, cyclo-alkane, aryl) that increases the hydrophobicity of an A-group or leaving group without exceeding the steric restrictions of the hydrophobic binding sites on AChE will increase the reactivity of an OP with AChE.

Steric effects

In addition to the previously described steric limitations imposed on the structure of OPs by the size of AChE hydrophobic binding sites, there are other types of steric effects encountered in the interaction of organophosphorus compounds with AChE. For example, OPs containing two isopropoxy groups are 40-fold less reactive with AChE than compounds containing two n-propoxy groups, and OP containing two sec-butoxy groups are 27-fold less reactive with AChE than their n-butoxy

Table 6.4. Comparison of charged and uncharged leaving groups



ACLE k_i(l mol^{-1} min^{-1})						
A_1	A_2	R	*Uncharged*	*Partially*	*Charged*	*Reference*
Me	EtO	CH_2Me	1.2×10^3	—	—	From Kabachnik *et al.* [22]
		$CHMe_2$	1.3×10^2	—	—	
		CMe_3	1.6×10^3	—	—	
		SMe	4.0×10^4	—	—	
		S^+Me_2	—	—	5.5×10^7	
Me	Pr^iO	$CHMe_2$	7.1×10^2	—	—	From Boter *et al.* [8]
		NMe_2	—	1.0×10^7	—	
		N^+Me_3	—	—	5.3×10^7	
		SMe	1.5×10^4	—	—	
		S^+Me_2	—	—	3.7×10^7	

analogues [42]. Compounds containing the bulky para-nitrophenyl leaving group exhibit decreased AChE reactivity in response to the effects of increasing the hydrophobicity of an A-group, while compounds containing less bulky leaving groups (i.e. $SC_2H_4NPr^i_2$, F, SPr^n) exhibit increased AChE reactivity (Figure 6.5). This probably occurs because highly hydrophobic A-groups are also bulky and may exhibit steric hindrance with the bulky para-nitrophenyl leaving group. In general, AChE reactivity is reduced by steric hindrance if two or more of the four groups attached to phosphorus are bulky.

Ionic bonds

Charged groups influence the AChE reactivity of OP inhibitors whether they are leaving groups or the A-groups of inhibitors [25]. The effects of positively charged leaving groups are of importance because ACh, the normal substrate for AChE, has a positively charged leaving group, choline ($OC_2H_4N^+Me_3$). Investigations by Tammelin [46] revealed that OPs containing quaternary choline-like A-groups were about 10^3 times more reactive with AChE than their tertiary counterparts. Subsequent analyses with sulphur, nitrogen and carbon analogues of leaving groups have compared the AChE reactivities of uncharged, partially charged, and charged choline-like leaving groups (Table 6.4). The k_i values of the charged sulphonium and ammonium analogues of methyl isopropylphosphonate are nearly equal suggesting the charge effect is independent of the atomic composition of the charged moiety.

Stereochemistry

The presence of a centre of asymmetry at the phosphorus atom of an OP gives rise to a pair of stereoisomers. Several OPs with centres of chirality at the phosphorus atom have an additional chiral carbon atom as well, which gives rise to two pairs of diastereoisomers, or four stereoisomers. As a consequence of these asymmetric centres in OPs, the resultant stereoisomers often have widely different reactivities with AChE [13]. If an inhibitor contains a chiral phosphorus atom that exhibits P(−) optical rotation, the k_i of that stereoisomer is as much as 1.8×10^4 times greater than the k_i for the comparable stereoisomer exhibiting P(+) optical rotation (Table 6.5). The stereospecificity associated with the carbon chiral centre of an OP that contains both carbon and phosphorus centres of chirality is much less than for the phosphorus chiral centre. The differences in k_i between the carbon stereoisomers is sixfold or less for OPs (Table 6.5), although larger differences (i.e. 40-fold) have been observed when the chiral carbon is found in the leaving group of the OP [8].

Toxicity

The relationship between the toxicity of OPs and their structures has been investigated by

Table 6.5. Effect of chirality on AChE reactivity

Compound	*Isomer* C	P	*AChE* k_i ($l\ mol^{-1}\ min^{-1}$)	k_i *ratio*
O ‖ Et–P[a]–SC_2H_4SEt | $OC^aH(Me)Et$	+	+	6.3×10^2	1.0
	−	+	1.5×10^3	2.4
	−	−	5.5×10^4	87
	+	−	6.5×10^4	103
O ‖ Me–P[b]–F | $OC^bH(Me)CMe_3$	+	+	$<1\times10^4$	1
	−	+	$<1\times10^4$	1
	−	−	2.7×10^7	2700
	+	−	1.8×10^8	18000

[a]Chiral centre [b]Wustner and Fukuto [50], Benschop *et al.* [6]

several approaches. One has been to examine the relationship of the *in vivo* toxicity (LD_{50}) of these compounds and their *in vitro* ability to inhibit AChE (k_i or I_{50}). Early work by Heath [22] indicated a 'rough correlation' between AChE I_{50} and LD_{50} for a heterogeneous group of 23 OPs. However, Cosic *et al.* [11] concluded that k_i for AChE inhibition correlated with the LD_{50} for 5 phosphonyloximes but not for 5 phosphonylfluoridates. Lieske *et al.* [26] also reported a lack of correlation between k_i for AChE and LD_{50} for 8 organophosphinates, and Ellin [16] concluded in a review that there was no correlation between AChE k_i and LD_{50} for a variety of OPs. This lack of correlation between an *in vitro* pharmacodynamic effect and its *in vivo* manifestation may result from problems in experimental design.

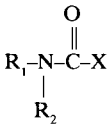
Becker *et al.* [5] demonstrated the importance of evaluating both AChE inhibition and agent metabolism in multiple regression analysis of the LD_{50} for 12 phenyl alkylphosphonates in rabbits. Only 48% of the variation in LD_{50} could be explained by variation in I_{50} alone while 86% of the variation in LD_{50} of these phosphonates could be explained if both the variation in I_{50} and rate of plasma hydrolysis were used in the regression analysis. Mager [35] analysed the relationship of LD_{50} with I_{50} and hydrophobicity. Using multiple regression analysis, Mager observed a correlation of r=0.996 for LD_{50} *versus* I_{50} and hydrophobicity for a heterogeneous group of 42 OPs.

Another method has been to examine the direct correlation of the chemical properties of OPs with *in vivo* toxicity, without concern for the underlying toxic mechanism. In an analysis of 12 analogues of parathion-methyl, Mundy *et al.* [38] found a correlation (r=0.94) between LD_{50} and hydrophobicity. Taft's steric parameter (E_s), and an electronic parameter (F). Mager [34] also succesfully to correlate LD_{50} with hydrophobicity using multicategorial regression analysis.

Carbamates

The structures of some common CBs are shown in Table 6.6: X is the leaving group, while R_1 and R_2 are groups which remain with the carbamylating (R_1R_2NCO) moiety of the inhibitor that forms a covalent bond with AChE. Although the leaving groups of carbamates are diverse, the R-groups are methyl groups or hydrogen atoms for most carbamate

Table 6.6. Structures of common carbamates



Name	Use	Structure R_1	R_2	X
Carbaryl	Insecticide	Me	H	O–1–Naphthyl
Aldicarb	Insecticide	Me	H	$ON{=}CHC(SMe)Me_2$
Propoxur	Insecticide	Me	H	$OPh{-}2{-}OPr^i$
Methomyl	Insecticide	Me	H	$ON{=}C(SMe)Me$
Methiocarb	Insecticide	Me	H	$OPh{-}4{-}SMe{-}3,5{-}Me_2$
Carbofuran	Insecticide	Me	H	$O{-}7{-}Benzofuran{-}2,3{-}H_2{-}2,2{-}Me_2$
Neostigmine	Drug	Me	Me	$OPh{-}3{-}N^+Me_3$
Pyridostigmine	Drug	Me	Me	O–3–Pyrid–I–Me
Physostigmine	Drug	Me	H	Eseroline
Carbachol	Research	H	H	$OC_2H_4N^+Me_3$

inhibitors. The effects of these small changes in R-groups on the lability of the C–X bond are striking. The alkaline hydrolysis of N-methylcarbamates is 10^5 to 10^6 times greater than analogous N,N-dimethyl carbamates [41]. These large differences in chemical lability are not reflected in their k_i values for reactivity with AChE, which differ only by a factor of two to three.

O'Brien *et al.* [41] found that alkaline hydrolysis rates were highly correlated (r=0.87) with AChE reactivity for N,N-dimethylcarbamates, but these same parameters were poorly correlated (r=0.10) for N-methyl carbamates. This lack of correlation may be explained by Dittert and Higuchi [14] who concluded that the alkaline hydrolysis of CBs occurs by two different mechanisms depending on the presence or absence of hydrogen atoms on the carbamyl nitrogen. They proposed that the mechanism of alkaline hydrolysis for N,N-dimethylcarbamates (Me_2NCOX), which contain no carbamyl hydrogens, was by hydroxyl ion attack on the carbonyl carbon, a mechanism analogous to the attack of an active site serine on a carbamate. In contrast, the alkaline hydrolysis of N-methylcarbamates (MeHNCOX) and unsubstituted carbamates (H_2NCOX), which contain carbamyl hydrogens, occurred by deprotonation of the carbamyl nitrogen by hydroxyl ion, a mechanism that is probably not involved in the reaction of AChE with CBs. Therefore, the alkaline hydrolysis rates of N-methyl carbamates significantly overestimate the lability of their C–X bonds in relation to their reactivity with AChE.

Electronic effects

Leaving groups of CBs behave differently from leaving groups of OPs with regard to the effects of electron-withdrawing or electron-donating substituents. For OPs with aryl leaving groups, electron-withdrawing substituents (e.g. NO_2 and halogens), on phenyl leaving groups increased k_i as much as 10^6-fold (Figure 6.1). For CBs the same electron-withdrawing substituents increased k_i less than 100-fold (Figure 6.6). In addition, substituents that are normally considered electron-donating groups (e.g. alkyl, alkoxy) also produce increases in k_i values. Indeed, comparison of substituents producing electronic effects (σ) of equal magnitude but opposite direction revealed that the increases in k_i caused by electron-donating substituents were as large as the increases in k_i values caused by electron-withdrawing substituents [39].

The increase in AChE reactivity caused by electron-donating substituents on phenyl leaving groups has been correlated with the ability of these CBs to form 'charge transfer complexes' with the active site of AChE [23]. However, a more general explanation for increases in k_i from both electron-donating and electron-withdrawing effects has been proposed by Nishioka *et al.* [39]. They suggested that the ability of both electron-donating and electron-withdrawing substituents to increase the k_i of CBs was indicative of a dual reaction mechanism between CBs and AChE. Nishioka and co-workers proposed a 'biphasic electronic mechanism' leading to a common tetrahedral intermediate by either nucleophilic attack or carbonyl oxygen protonation.

Hydrophobicity

In addition to their electronic effects, many substituents on aryl leaving groups can

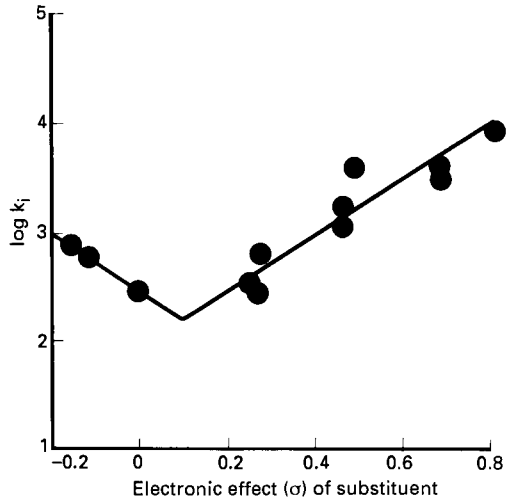


Figure 6.6 Effect of electronic parameter (σ) of aryl leaving group substituent (X) on AChE reactivity (k_i) of N-methyl-phenylcarbamates. Substituents are presented in order of increasing σ for Me(H)NC(O)Ph-4-X where X=MeO, Me, H, Br, Cl, I, COEt, CH_2COOH, CHO, SO_2Me, CN, or NO_2. Data from Nishioka *et al.* [39]

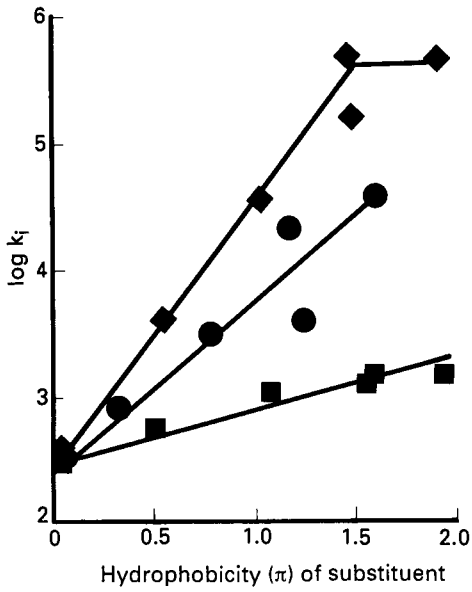


Figure 6.7 Effect of hydrophobicity (π) of aryl leaving group substituent (R) on AChE reactivity (k_i) of N-methylphenylcarbamates. Substituents are presented in order of increasing π for Me(H)NC(O)OPhR with meta-R (◆), ortho-R (●) and para-R (■), where R=H, Me, Et, Pr^n, Pr^i, or Bu^t. Data from Nishioka *et al.* (1977).

produce increases in k_i owing to their hydrophobic properties. For example, a series of meta-alkyl phenyl analogues of N-methylphenylcarbamates have a 1600-fold range of k_i values, but the electron-donating abilities of these groups are virtually identical. In contrast, the hydrophobicity (π) of these groups is variable and has a linear relationship to the log k_i of these analogues (Figure 6.7). The position of the hydrophobic alkyl substituent on the phenyl ring has a strong influence on this hydrophobic effect. Substituents in the meta-position are much more effective than the same substituent in the ortho- position or para-position (Figure 6.7). The maximal k_i for meta-alkyl substituents on the phenyl leaving group of the N-methylphenylcarbamates is reached with the iso-propyl substituent.

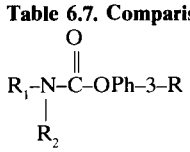
Steric effects

In addition to the limitation on the size of the substituents on aryl leaving groups, there are also steric limitations on the carbamyl portion of carbamate inhibitors of AChE. A variety of N-ethyl, N,N-diethyl, N- benzyl, and N-phenyl carbamates were tested by Kolbezen *et al.* [29] and Wilson *et al.* [49], but these compounds showed little or no carbamylation of AChE.

Ionic bonds

As with OP inhibitors, charged groups influence the reactivity of CBs for AChE. A comparison of the k_i for charged trimethylammonium (N^+Me_3) substituents *versus* their uncharged tertiary-butyl (CMe_3) analogues indicate that charged carbamates are 10^2 to 10^3 times more reactive with AChE (Table 6.7), which is similar to the ratio of AChE reactiv-

Table 6.7. Comparison of charged and uncharged carbamates



			AChE reaction constants			
R_1	R_2	R	K_D(M)	k_2(min^{-1})	k_i ($l\ mol^{-1}\ min^{-1}$)	Reference
H	Me	H	2.4×10^{-2}	8.1	2.6×10^{2}	a
H	Me	$CHMe_2$	1.6×10^{-4}	76.2	4.8×10^{5}	a
H	Me	CMe_2	5.5×10^{-5}	21.3	3.7×10^{5}	a
H	Me	N^+Me_3	—	—	2.8×10^{7}	b
Me	Me	H	1.7×10^{-2}	0.4	2.4×10^{1}	a
Me	Me	CMe_2	2.8×10^{-4}	0.7	2.4×10^{3}	c
Me	Me	CMe_3	2.0×10^{-4}	0.5	2.4×10^{3}	a
Me	Me	N^+Me_3	3.3×10^{-6}	6.1	1.9×10^{6}	c

[a]From Iverson and Main [24]
[b]From Reiner and Aldridge [45]
[c]From Nishioka *et al.* [40]

ities (k_i) for the same charged *versus* uncharged substituents with OPs (Table 6.4).

Stereochemistry

The carbonyl carbon of a CB is not asymmetric because it has only three groups (O, R_1R_2N, and X) attached to it, in comparison to the four groups (A_1, A_2, O and X) attached to the potentially asymmetric phosphorus of OPs. Chirality is only a factor in the inhibitory activity of CBs with regard to asymmetric carbons which may be found in the leaving groups of CBs. Physostigmine and physovenine are CBs that contain such asymmetric carbons in their heterocyclic leaving groups. The C(–) form of physostigmine and physovenine are about 20 times more inhibitory than the C(+) form [12].

Toxicity

The direct toxic effects of CBs on cholinergic receptors [2], in addition to their inhibition of AChE, has hindered attempts to establish close correlations between AChE reactivity and the toxicity of CBs. While studies of the structure-activity relationship of CBs and insect toxicity are numerous, mammalian toxicity of CBs has been examined only when estimating human safety. As a result of the small data base for mammalian toxicity of CBs, evaluations of the structure-activity relationship between CBs and mammalian toxicity have been qualitative [40] rather than quantitative.

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Neurobehavioural toxicology of anticholinesterases

Glenville D. D'Mello

Introduction

Scope and rationale

'Why consider the influence of antiChEs on animal behaviour'? A substantial literature exists describing in detail the physiological, neurological and behavioural sequelae of exposure to antiChEs in humans [54, 59, 74, 120,125,135]. The experimental validity of these data have been questioned [26,103]; they are anecdotal and dosage information is inadequate for evaluation. The reliability of clinical reports of adverse and toxic effects in humans has also been questioned [43,138]. Few controlled studies of antiChE effects on behaviour in humans have been reported. Such studies are constrained by ethical considerations.

Data suggesting that drugs which modify cholinergic transmission in the CNS have profound effects on behaviour have been more than adequately reviewed [8–10, 26, 33, 68, 95, 118]. Other published works have been concerned with the detailed examination of theories proposed to explain the role of central cholinergic systems in behaviour [114,130]. In extracting information from existing discussions and reviewing new data, no attempt has been made to evaluate further the validity of such theories.

No previous review of the behavioural toxicology of antiChEs can claim to be representative of the vast range of studies published to date. No single test has been shown to be representative of behaviour, antiChEs are not a homogenous group of chemicals and the concept of 'behavioural toxicity' has yet to be defined adequately. Previous reviews have avoided these difficulties by focusing attention on a few specific issues [8,10,68].

The influence of antiChEs on learning and memory has been discussed previously [8,10,22,33,62,67,89,95,118,121,130]. It may seem unnecessary to review this aspect of antiChE action again. However, a number of factors suggest otherwise. The majority of previous discussions predate 1980. The recent emergence of Alzheimer's disease as a priority area of research has renewed interest in antiChEs [65] and new data are available. The research before 1975 was restricted to a narrow range of species, antiChEs and test methods. Furthermore, early research focused on two issues: the neurochemical correlates of antiChE actions [113] and the evaluation of conflicting theories of antiChE action on learning and memory [132]. A clear description of the influence of antiChEs on learning and memory has not emerged.

Validity of pooling data

One objective of a review is to pool data from disparate sources enabling general trends to be perceived more readily. Several arguments militate against pooling. Although all antiChEs react covalently with AChE in essentially the same manner as ACh [125], many physicochemical differences exist [27, 54, 69, 136] which suggest the group is not homogenous. It seems unlikely therefore that the profiles of biochemical changes and thus the profiles of behavioural toxicity following adminstration of different antiChEs will be identical. It is recognized that significant species differences in susceptibility to the toxic effects of antiChEs exist [23,38,39,135]. Strain [29,102] and sex [92] have been identified as sources of variability. The effective pooling of data from different studies requires a high degree of methodological concordance across

studies. In behavioural toxicological research, this has rarely been achieved.

Three possible methods exist whereby different studies might be tentatively compared. Reliance on dose administered is clearly inadequate given the myriad of factors known to influence the biological potency of antiChEs [18,116,135]. RBC, plasma and brain ChE activities have been used as independent measures of the degree of antiChE intoxication [36,47,66,109]. However, antiChEs differ widely in their affinities for different ChEs and blood ChE activity is not a reliable index of either AChE activity in nervous tissue or a measure of the pharmacological actions of antiChEs [59,136]. Virtually no behavioural toxicological studies have provided data on AChE activity in brain [10]. A further potential method whereby the effects of different antiChEs, described in different studies, might be compared is to express dose administered as a fraction of the dose estimated to produce 50% mortality (i.e. the LD_{50}). This approach has been utilized on a within-study basis to estimate relative behavioural toxicological potency of different antiChEs [106,109,137], and to compare data across different studies [11,75,107]. However, it is generally accepted that the LD_{50} value cannot be regarded as a biological constant [139].

It is concluded therefore, that no good method is available to compare the behavioural toxicological potency of antiChEs within and between studies. Furthermore, the magnitude and to some extent the nature of the errors associated with the comparative methods described have not been assessed. The selection of one method in preference to another must therefore be an arbitrary decision. In this chapter, doses of antiChEs are, wherever possible, expressed as a fraction of their acute LD_{50} values; where such data have been omitted, relevant average data from other studies have been used instead. Acute LD_{50} data for physostigmine in primates are not available and therefore doses have been expressed in the normal manner. The comparison of studies involving repeated dosing has been achieved by incorporation of a temporal factor.

The analysis of behavioural function

A very simple definition of 'behaviour' is the dynamic interaction of an organism with its environment. To accomplish this an organism must first sense the properties of its environment, transform these into meaningful messages and then respond appropriately. No single test has been developed or recommended for the assessment of behaviour. Instead, a vast array of tests is available to assess sensory, cognitive, motor and affective functions [86,126]. In this chapter, the behavioral toxicity of antiChEs related to these four basic functions is described.

The concept of behavioural toxicity

WHO has defined drug toxicity as, 'Unwanted actions of the substance on organs or tissues of the body sufficient to impair their function or cause cell death' [134]. An alternative definition has been provided by Dewar [34] '... deviations from the "normal" functioning and structure of the nervous system ...'. Evans and Weiss [40] have suggested that a distinction

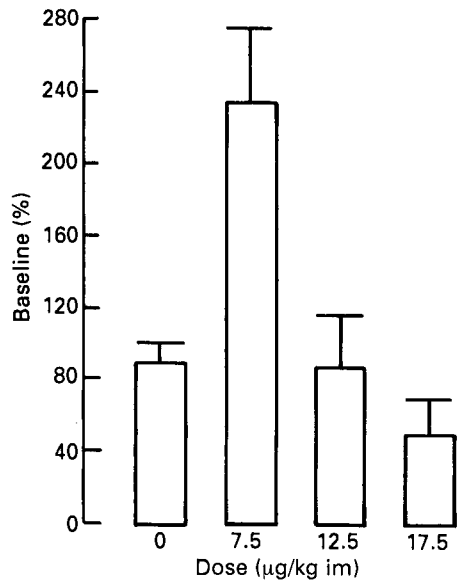

Figure 7.1 Influence of sarin on whole-body locomotion in marmosets (n = 4). Movements in the rostro-caudal plane of each animal were detected by a mercury tilt-switch device mounted in a back-pack and carried by each animal. Locomotion was monitored within each animal's home cage (dimensions 750 × 480 × 600 mm) immediately after im administration of sarin and for 15 min. Data shown represent total number of movements (mean + s.e.) expressed as a percentage of total movements on the preceding day after administration of saline. Reproduced from D'Mello and Scott, unpublished data

should be made between a 'change' and a 'toxic effect' (i.e. a true impairment of some function). Burt [21] has stated that, 'Any behavioural effect could be considered deleterious'.

Another problem concerns the relationship between the dose of a chemical and its effect on behaviour. It is generally accepted that the severity of a toxic effect is related directly to the dose administered and that somewhere on the dose-effect continuum a threshold dose exists below which no signs are detected. With behaviour, not all dose-effect functions are linear or sigmoidal in nature (Figure 7.1).

Influence of antiChEs on sensory function

Physostigmine, pyridostigmine, paraoxon, carbaryl, propoxur, TEPP, DFP, sarin, soman and VX have been shown to modify, generally suppress, the performance of tasks involving the perception of either visual [45, 47, 49, 55, 60,61,81,85,127,137] or auditory [46,48,100] cues at doses of ≥ 0.1 LD_{50}. Physostigmine was

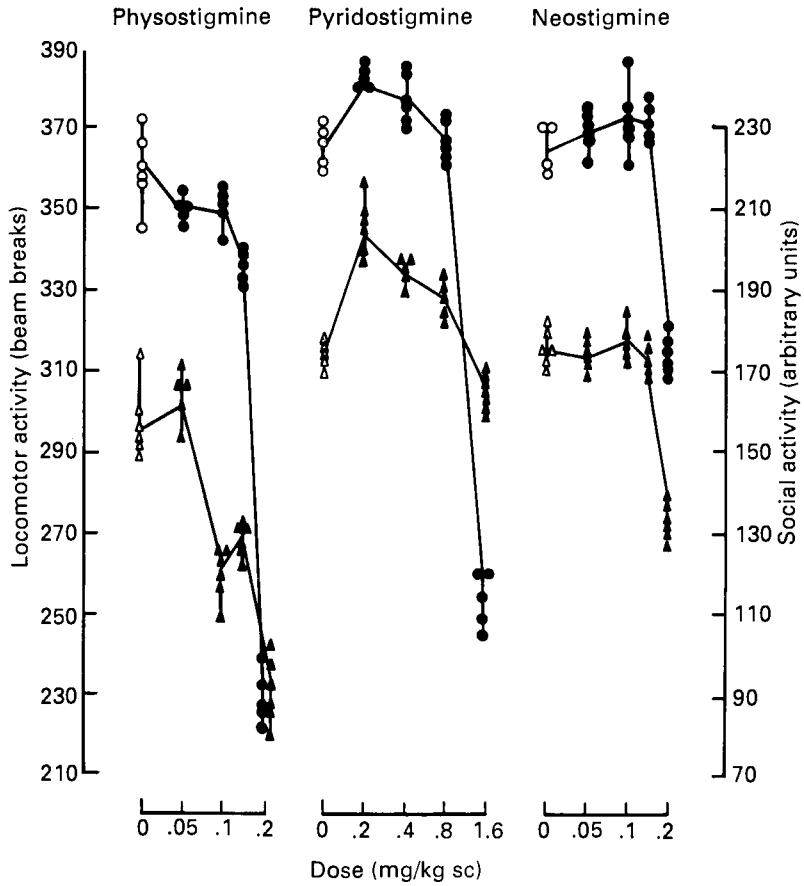

Figure 7.2 Influence of CB antiChEs on whole-body locomotion and social activity in rats. Pairs of male rats were placed in a familiar observation cage equipped with an array of photocell beam detectors and observed under conditions of subdued lighting. Locomotor activity (○,●) was assessed by counting the number of photocell-beam breaks. Social activity (△,▲), i.e. sniffing, following, walking over, crawling under and grooming of one animal by another, was recorded by a trained observer. Data shown represent total social activity, expressed in arbitrary units, recorded for individual animals. Locomotor and social activities were monitored 15 min after sc administration of drugs and for 10 min. Reproduced from Stringer and D'Mello, unpublished data

shown to improve the recognition of three-dimensional 'trial unique' objects by rhesus monkeys [2]. However, these data do not demonstrate specific disruptions of visual or auditory functions as other possible actions of the drug on memory, attention or other mechanisms cannot be excluded.

Parathion, dichlorvos, DFP, sarin and soman have been shown to produce analgesia at doses ranging from 0.3 to 0.9 LD_{50} depending on the species, antiChE and test used [28, 53, 55, 106, 107, 109, 115, 119]. Physostigmine has been shown to produce analgesia at doses in the range of 0.2–0.3 LD_{50} [30,57,79].

AntiChEs can reduce social activity in rats (Figure 7.2) although such an effect may be secondary to a depression of gross whole-body locomotion (Figure 7.3).

Physostigmine, DFP, paraoxon and sarin have been shown to modify (increase or decrease) drinking [1,52,96,117] and feeding [9,52,78] depending on antiChE dose, chronicity of administration and deprivation state of the animals. Interpretation of these data is not simple. Methods used were not ideal [14] and the influence of changes in locomotor activity is not clear [36,96].

Physostigmine has been shown to modify the perception of an electrical stimulus delivered intracerebrally [37].

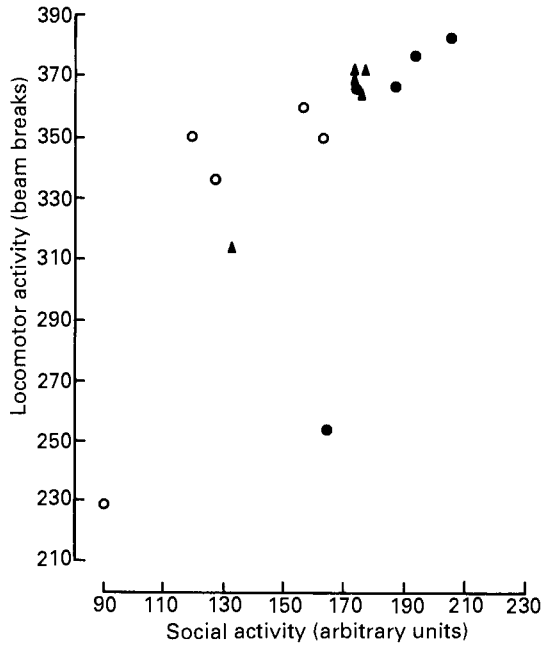

Figure 7.3 Correlation between the effects of three CB antiChEs on social and locomotor activities in male rats, assessed as described in legend to Figure 7.2. Data shown represent the mean scores for either five or six animals. Outliers show effects of the highest doses of physostigmine (○), pyridostigmine (●) tested. (▲), neostigmine. Reproduced from D'Mello and Stringer, unpublished data

Influence of antiChEs on cognitive function

Physostigmine, DFP, tabun and soman had no effect on habituation learning [91, 106, 107, 115, 124].

Physostigmine (0.005–0.78 LD_{50}) and parathion (0.3 LD_{50}) have been shown to modify (generally increase) response latency in passive avoidance tasks [15,19,35,56,101] depending on whether the antiChE was administered before or after the learning trial. Whether such changes in response latency actually reflect a modification of memory is uncertain. For example, the reduced mobility of animals may confound interpretation [137]. The repeated administration of soman at a rate of 0.2 LD_{50} per day for 3 days was shown to have no effect on response latency [115]. Physostigmine (0.03–0.1 LD_{50}) and soman (0.3–0.6 LD_{50}) and DFP (0.1 LD_{50} per day for 10 days) have been shown to reduce the rate at which rats learn an active avoidance response [46,105,110,111,131]. In this latter study the majority of animals learned to escape shock suggesting that both perception of the shock and ability to execute the avoidance response were unimpaired. Physostigmine (0.02 LD_{50}) has been shown to facilitate passive avoidance responding but interfere with active avoidance responding [111]. Such data have been used to support the view, expressed by many authors [132] that central muscarinic systems play an important role in response inhibition.

Increased rates of spatial learning in rats administered physostigmine (0.01–0.03 LD_{50}) have also been reported [31,32]. Learning rate was decreased following a higher dose (0.05 LD_{50}) of physostigmine suggesting that the shape of the physostigmine dose-response function in these tasks is essentially biphasic. The learning of complex maze tasks by rats [98] was not affected by soman (0.5 LD_{50}) but

a significant increase in the variability of responding was observed and two animals continued to retrace their paths through parts of the maze unnecessarily. Acquisition of a water maze task and its reversal was impaired by DFP (0.4 LD_{50} per day for 11 days) but only if mice were trained before treatment [127].

Heise and Hudson [61] demonstrated the effects of physostigmine (0.2–0.6 LD_{50}), carbaryl (0.03–0.07 LD_{50}) and propoxur (0.1–0.5 LD_{50}) on the performance of tasks that assessed either working or reference memory were qualitatively similar. As the dose of each agent was increased trial initiations decreased. However, accuracy of performance remained high until the number of initiations decreased to 25% of control levels. The accuracy of a single alternation lever press task during the repeated administration of DFP (0.12 LD_{50} per day for 20 days) was impaired, but only over the first 7 days of treatment [131]. A large, not consistently significant, increase in the number of unnecessary responses was also observed in DFP treated animals.

Physostigmine (0.025 LD_{50} per day for 15 days) and soman (0.8–0.9 LD_{50}) have been reported to either increase or decrease the rate of extinction of various responses in rats [8,26,82].

The effects of carbaryl (0.02–0.07 LD_{50}), propoxur (0.1–0.5 LD_{50}) and physostigmine (0.08–0.6 LD_{50}) on the performance by rats of a delayed non- matching to sample task (DMTS) were essentially similar. Only small (presumably non-significant) effects on accuracy were observed at low doses [60]. At higher doses, responding was simply suppressed. As a supplementary experiment failed to reveal any specific changes in sensitivity to the stimuli presented, the authors concluded that no effects on working memory could be demonstrated. A similar conclusion has been reached by others [81] using a similar task with rhesus monkeys and following administration of physostigmine at doses (0.025–0.08 mg/kg) that induced changes in other non-memory related aspects of performance. The working memory of baboons trained on a delayed matching to sample (DMTS) task involving unspecified visual stimuli and maintained by food reward seemed surprisingly resistant to the effects of soman [47]. The performance of only two of six monkeys were affected at doses of 0.6–0.7 LD_{50}. The results were confirmed in a subsequent study [45] with the same monkeys retested 4 months later during a sub-chronic dosing schedule (soman 0.04 LD_{50} per day for 28 days). In a unique variation of the DMTS procedure [2] the sample stimuli presented to rhesus monkeys were novel on each trial. Significant improvements in performance were observed following administration of physostigmine at all doses between 0.32 and 32 μg/kg. At 56 μg/kg, performance accuracy was reduced to about 60% of baseline level. The authors concluded that although physostigmine improved performance, an exclusive action of the drug on working memory could not be claimed as effects on attention or other processes could not be excluded.

Physostigmine (0.005–0.5 LD_{50}) had no consistent effects on learning by rats of a four response chain [63]. A similar pattern of results was found in cynomologus monkeys following administration of physostigmine at doses of 0.025–0.075 mg/kg [94] or carbaryl at doses of 1–10 mg/kg [4]. The acute toxicity of carbaryl in large monkeys may be remarkably low.

Administration of soman either acutely (0.1 LD_{50}) or subchronically (0.2 LD_{50} per day for 39 days) had no influence on the performance by rats of a lever press task requiring the perception of time [64,115]. However, soman administered at a rate of 0.009 LD_{50} per day for 105 days decreased responding early in the interval; a result that could be interpreted as an improvement in time perception. In another study rats surviving a high dose of soman (0.8–0.9 LD_{50}) responded at a much higher frequency than controls and thus earned fewer rewards [82]. Physostigmine (0.2 LD_{50}) disrupted performance of a similar task, but only if the penalty for inaccurate responding was footshock [71]. When the penalty was delayed access to a food reward, a higher dose of physostigmine (0.07 LD_{50}) was required before performance was impaired.

Influence of antiChEs on motor function

Soman (0.6–0.9 LD_{50}) has been shown to induce a variety of spontaneous behaviours,

i.e. tremor, convulsions, hind-limb abduction, chewing and immobility in rats [16,20,75,99]. A similar profile of behaviours is observed following repeated administration of either soman or DFP at dosage rates of 0.25–0.5 LD_{50} per day [48,78]. No indication of tremor was observed in rhesus monkeys following administration of physostigmine (0.025–0.1 mg/kg) [97]. Teeth chattering and yawning has been reported in rats but only following administration of physostigmine at a dose of 0.05–0.4 LD_{50} [128,129].

The incidence in rats of catalepsy, rigidity and various simple reflexes was assessed by Rondeau *et al.* (1981). Forelimb extension and traction reflexes could not be detected in 83% of rats during the repeated administration of fenitrothion [108] at dosage rates of 0.04–0.08 LD_{50}) per day for 40 days. Forelimb grip strength in rats was reduced by 0.6 LD_{50} soman [105].

In general, antiChEs suppress whole body locomotion [19, 20, 36, 53, 55, 58, 73, 75, 96, 106, 107, 109, 112]. However, while carbamates suppress activity at doses in the range 0.025–0.03 LD_{50}, OP antiChEs do not suppress activity until the dose administered is at least 0.4 LD_{50}. The reason may be that, at low doses, OP antiChEs increase activity (Figure 7.1). Thus, several studies have demonstrated clear increases in activity after sarin [36,75] and soman [98,105] and repeated dosing with fenitrothion [108] at doses in the range 0.3–0.8 LD_{50}. Although soman has been shown to suppress activity at a dose of 0.03 LD_{50} [137] this result was queried by the investigators and has yet to be replicated. Repeated administration of OP antiChEs suppress activity at doses within the range 0.04–0.2 LD_{50} per day [108,115].

Very high doses of antiChEs (0.5–0.97 LD_{50}) are required to disrupt motor coordination in rats [58,75,107,109,112,137]. Sarin (0.3 LD_{50}) has been shown to disrupt the performance of a visually-guided reaching response in marmosets [36]. However, the pattern of behavioural change observed did not suggest a specific effect of sarin on coordination. Lower doses (0.02–0.1 LD_{50}) are effective in disrupting coordination if administered repeatedly, e.g. once per day for 5–40 days [11,13,108].

The chronic administration of pyridostigmine (producing a 40–70% carbamylation) had no effect on endurance in rats [12,25]. Physostigmine (0.25 LD_{50}) however, reduced by 25% the time to 'exhaustion' in rats [83].

The motivation of rats and baboons to work for food rewards was decreased by physostigmine, carbaryl and propoxur at doses of 0.04–0.7 LD_{50} respectively [45,47,61]. The occurence of lapses in responding or deficits in attention were suggested as possible explanations. The work motivation of rhesus monkeys trained to exert a specified force on a lever was not affected by physostigmine until the dose administered was at least 0.1 mg/kg [97].

The response time of rats and guinea pigs was increased by sarin, soman and VX at doses in the range 0.6–0.7 LD_{50} [85]. Repeated administration of soman (0.12 LD_{50} per day for 3 days) increased the time taken by rats to depress a lever to gain access to water reward [115]. A high dose of soman (0.7 LD_{50}) was required to increase the response time of baboons and then only 50% of monkeys were affected [47].

Soman (0.1 LD_{50}) reduced the response rate of rats by 50% on both fixed ratio and fixed interval schedules [17,64]. Complete suppression of responding in all animals is observed at doses of approximately 0.4 LD_{50}. This dose-effect relationship seems consistent with data for physostigmine [51,96] and carbaryl [5]. Rats trained to respond at a slow rate and surviving a high dose of soman (0.8–0.9 LD_{50}) emitted 100% more responses per reward than controls [82]. Worthy of note is the high degree of variability observed between animals, e.g. of four rats administered carbaryl (0.025 LD_{50}) the degree of response suppression ranged from 10% to 70% of baseline rates [5]. In rats administered soman (0.6 LD_{50}), the time to onset of effects ranged from 2 to 30 min [16]. The responding of some rats administered soman (0.2 LD_{50}) was completely suppressed while the performance of others was relatively unaltered [64].

Non-consequential maze running activity in rats surviving a high dose of soman (0.8–0.9 LD_{50}), persisted for up to 4 months [98]. Non-consequential responding by baboons trained on a DMTS task has also been reported 2–4 weeks after administration of 0.6–0.7 LD_{50} soman [47]. In most monkeys, such unnecessary responses returned to baseline levels within 1–10 weeks. However, one monkey continued to emit such responses until 16 weeks.

Influence of antiChEs on affective function

Karczmar and Scudder [70] showed that physostigmine at doses of 0.025–0.125 LD_{50}, increased isolation-induced aggression in mice. Charpentier [24] reported that physostigmine (0.78 LD_{50}) increased the length of attacks in mice. Also, Allikmets [3] has reported that sc administration of physostigmine (0.15 mg/kg) lengthens and intensifies ACh-induced aggression in cats. The relative paucity of information in this area may be due to the idea, yet to be validated, that peripherally administered cholinomimetics do not usually induce or increase aggression because they simultaneously activate cholinergic systems of behavioural control [3].

More recent work has been largely anecdotal in nature. For example, of nine rats administered soman at a dosage rate of 0.25 LD_{50} per day, two of five survivors exhibited 'aggression' 13–18 days into the repeated dosing schedule [48]. However, the intensity and nature of the aggression was not described. In another study [82], all rats surviving a high dose of soman (0.8–0.9 LD_{50}) were hyperactive to handling, a response that persisted for 6 months. Rats surviving doses of soman (0.5–0.8 LD_{50}) exhibited an increased reactivity that was highly correlated with an increase in locomotor activity [98]. Mollenauer [88] showed that physostigmine (0.125 LD_{50}) increases the incidence of 'freezing' and the time spent either exploring the centre of the arena or feeding in rats, but only when a 'fear- inducing' stimulus was present. Locomotor activity was not altered. The authors concluded that physostigmine may have intensified the 'fear' experienced by the rats.

Discussion

Methodological considerations

The majority of published reports on the behavioural toxicology of antiChEs in animal species are descriptive in nature. The simple demonstration of a change in a single index of behavioural function has been an acceptable objective. Although this approach has provided much information and has resulted in the quantification of observable toxicity, attempts to understand the nature of changes observed are rare. The comparison of antiChE effects across a range of different tests is essential if understanding is to be achieved. Although many recent studies have utilized several test methods, the rationale seems to be a search for the most sensitive behavioural assay.

Although the most likely exposure to antiChEs for humans is ingestion, inhalation or percutaneous absorption [54,135] the majority of behavioural toxicological studies have utilized ip, sc or im routes. The precise influence of route of administration on behavioural toxicity of antiChEs is not known although both quantitative and qualitative differences in toxicity might be anticipated, e.g. various local and peripheral effects on visual and respiratory systems are observed following inhalation but not after administration by other routes.

The range of antiChEs tested has been restricted primarily to physostigmine, DFP and soman. Whether these can be considered representative of antiChEs in general is not known. The range of species might be considered representative of vertebrate mammals in general. However, given that the ultimate objective of toxicological studies is the extrapolation of animal data to humans it is surprising that virtually no interspecies comparative studies have been conducted.

The data reviewed suggest that the sensitivity of different test methods may vary by a factor of up to five depending on the species and antiChE agent. The implications of such differences for assessments of the behavioural toxicity of antiChEs have not been determined. Test sophistication, e.g. automated *versus* manual measurement of behaviour, does not seem to have a major impact on the ability of test methods to detect antiChE actions.

Overview of studies cited

Few studies have been designed specifically to assess the influence of antiChEs on sensory [84] or affective functions. AntiChEs have been shown to disrupt behaviours where the perception of visual or auditory stimuli are critical. However, 'specific' actions of

antiChEs on visual or auditory system function cannot be determined from these data. The antinociceptive action of antiChEs is clear at doses of 0.2 LD_{50} and above. However, it should be noted that tests of analgesia depend on the integrity of reflexive responses and these are disrupted at similar doses.

The range of studies concerned primarily with assessment of antiChE effects on cognitive and motor functions are reasonably comprehensive. AntiChEs have been shown to improve, have no effect, or disrupt behaviours where adaptive changes are required. The absence of any consistent pattern is not surprising as many factors are involved in determining the nature and direction of change in different tests. The majority of studies assessing the effects of antiChEs on motor function have been conducted using rats. How representative these rodent studies are of vertebrate mammals in general is uncertain. Contrary to popular belief antiChEs do not exclusively suppress motor function. Increases, for example, in locomotor activity have been observed but the time of observation seems to be critical. AntiChEs have been shown to disrupt motor coordination, the voluntary initiation of motor responses and response time. However, 'specific' actions of antiChEs on these aspects of motor function have not been discovered.

Nature of antiChE-induced changes in behaviour

Tentative comparison of the behavioural toxicity of antiChEs when administered either acutely or sub-chronically suggests that the profile of effects observed does not differ qualitatively. The ease with which pharmacological tolerance may be induced differs substantially across different antiChEs. Also, there is no clear qualitative difference in the behavioural toxicity of antiChEs between rodent and primate species.

The slopes of dose-response functions for OP effects on behaviour are generally very steep. Large primates seem particularly sensitive to small increments in dose. An increase or facilitation of behaviour may be observed at low doses (e.g. 0.01–0.1 LD_{50}) and a decrease or disruption of behaviour at high doses (e.g. 0.3 LD_{50} and above). The facilitation of behaviour at low doses is observed frequently with physostigmine. Studies with OP antiChEs have rarely included doses in the lower range and thus facilitation has only been observed at early times (e.g. 0–15 min) after the administration of high doses.

An increase in the variability of responding both within and between animals seems to be a common feature of antiChE poisoning. This may have masked significant changes in behaviour as the majority of investigators utilize group measures (i.e. averaged scores) of toxicity. Although the use of 'single-subject' designs [11,47] undoubtedly increases the information content of experiments, it is uncertain whether an increase in test sensitivity is also obtained.

One striking feature of the behavioural toxicity of OP antiChEs, particularly soman, is the frequent observation of pausing during test sessions. Investigators have commented that animals seem to 'wait out' the effects of an agent. Responding may be suppressed completely and then begin abruptly at, or near, control rates. The majority of studies have concentrated assessment on the first hour after poisoning. However, persistent effects (lasting beyond 1 h) have been reported, particularly following high doses of agents. These data suggest that extended assessment of antiChE effects on behaviour should be routine rather than the exception.

Few studies have compared the behavioural effects of antiChE agents with those of drugs and chemicals from other pharmacological classes. Thus it cannot be claimed that the effects described are specific to antiChEs. Neither can it be concluded that antiChEs have no specific actions on behaviour.

Disruptions of behaviour induced by low doses of antiChEs disappear quickly (within 1–2 h) and behaviour may seem 'normal'. Many investigators have equated this apparent 'normality' with 'recovery of function'. The validity of such an interpretation has to been determined.

If the idea that any change in behaviour can be regarded as 'toxic' [21] is accepted, then it follows that CB antiChEs, and in particular physostigmine, are an order of magnitude more toxic to behaviour than are OP antiChEs.

Concluding remarks

This chapter has attempted to integrate those studies designed specifically to assess the behavioural toxicity of antiChE agents in rodent and non-human primate species. Many relevant and important areas of antiChE research, each involving the assessment of behaviour have, of necessity, been ignored. These include studies of antiChE effects in neonatal [90,122] and aged [7,44] animals, studies of the 'stimulus properties' of antiChEs [93,106,109] and the phenomenon termed 'behaviourally-augmented tolerance' [41,50]. No reference has been made to studies assessing the neurochemical correlates of antiChE-induced changes in behaviour [72,80,87], the pharmacological antagonism of antiChE effects [76,77] and studies assessing the influence of environmental stressors on antiChE effects [6,133].

During the past decade, the vast majority (the author estimates about 90%) of behavioural toxicological research relating to OP antiChEs and published in the open literature, has been supported by USA defence funding. In contrast, virtually no research in this area has been funded by environmental health research agencies. The emphasis of defence research has been descriptive in nature and thus the number of mechanistic studies has been low. Furthermore, defence research has concentrated on the assessment of soman toxicity, and soman is not representative of antiChEs in general. Also, the criteria used by the military to assess 'risk' are quite different to the criteria used by environmental health research agencies. For example, the military recognize that in any future war a significant degree of hazard is inevitable and therefore, acceptable. In contrast, the primary objective of environmental health research is to minimize, if not eliminate, such hazards.

It is clear that antiChEs possess a very broad spectrum of effects on behaviour. The reason for this might be the ubiquity of cholinergic systems in the PNS and CNS [42,104]. However, although profound changes in behaviour have been observed, 'specific' actions of antiChEs on sensory, cognitive, motor or affective functions have never been demonstrated. Thus, it must be concluded that although much research has been conducted, surprisingly little is known of the behavioural toxicology of antiChE agents.

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8

Central neurotoxicity and behavioural effects of anticholinesterases

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Introduction

Much information is available on the effects of antiChEs on the CNS and has been extensively reviewed [16,43,44,50,52,68,80,90,96].

The major features of CNS toxicity from antiChEs are well understood, although some reported effects are contradictory and often difficult to interpret. Thus conflicting results are available on long-term sequelae of acute exposure and on possible effects of chronic exposures not causing overt cholinergic toxicity. In the future some of these controversial issues may be clarified because our knowledge of the functional anatomy and biochemistry of the CNS cholinergic system is rapidly expanding.

Evaluation of CNS cholinergic toxicity is complicated by the widespread distribution of cholinergic systems in non-nervous tissues [81] and by cholinergic transmission in the PNS. It is often difficult to differentiate CNS selective effects of neurotoxicants in general [72], and of antiChEs in particular [36], because they have a strong peripheral component. Except where mentioned, this chapter deals more specifically with OPs and provides an integrated overview of CNS effects of antiChEs based on biochemical, physiological, behavioural, and clinical studies.

Cholinergic system in the CNS

The cholinergic system has four components [91]: (1) choline acetyltransferase (CAT) which catalyses the transfer of an acetyl group from acetyl coenzyme A to choline (Ch) to synthesize ACh; (2) ACh which is released by vescicle exocytosis and recycled; (3) AChE that hydrolyzes ACh to Ch and acetic acid; and (4) cholinergic receptors: CNS cholinergic receptors include M_1 and M_2 muscarinic receptors (excitatory and inhibitory) and nicotinic receptors (Motoneuron-Renshaw cell).

Immunohistochemical studies using monoclonal antibodies to CAT have visualized intrinsic cholinergic CNS neurons [51]. Also some projections of central cholinergic neurons have been identified using combined AChE histochemistry and retrograde tracing techniques [1]. Localization of cholinergic neurons in the CNS has been summarized by Fibiger and Vincent [29]. The majority of CNS cholinergic neurons have large perikarya and have been identified in several species, including humans, in the striatum (local circuit neurons), in the rostral cholinergic column of the basal forebrain (projections to the olfactory bulb, hippocampus, cerebral cortex and amygdala), in the caudal cholinergic column of the mesencephalic and pontine reticular formation (projections to the interpeduncular nucleus, tectum, hypothalamus, thalamus, basal forebrain and medial prefrontal cortex) and in the spinal cord.

The cholinergic system interacts with various CNS neurotransmitters including serotonin [75], dopamine [55], catecholamines [63], GABA [83] and neuropeptides [54]. It has also been shown that the neurogenic control of the cerebral vasculature may in part be regulated by the cholinergic neurotransmitter system [5].

ACh is found in motor neurons, in the spinal cord and in the cranial nerve motor nuclei where it acts as the fast chemical signal of NM transmission. The physiological functions of the intrinsic CNS cholinergic system is mainly modulatory in nature, and has been associated with temperature [15], cardiovascular and respiratory regulation [59], and with a number

of behavioural effects including depression [48], response to stress [30], arousal [46], sleep and dreaming [84], and memory [9].

Biochemical effects of antiChEs on CNS

Biochemistry of AChE inhibition

AChE is widely distributed within the CNS with some subcortical areas, like the nucleus caudatus and globus pallidus, being particularly rich [33]. It exists in several molecular forms [92], the soluble globular form being that present in the brain. The difference probably reflects the mode of membrane attachment rather than a different catalytic activity. Therefore variations in brain AChE inhibition after systemic dosing with OPs [86], suggest selective access of the inhibitor rather than a differental sensitivity of AChE.

OPs and CBs interact with AChE at the serine residue of its catalytic site [2]. The nature of the biochemical reactions between OP and AChE is the same as that for the enzyme and ACh. The major difference is in the rate constant of enzyme reactivation (k_3) which is fast for ACh and extremely slow for OPs (see Chapter 6). The 'ageing' reaction can be very fast (high k_4). OPs vary in their potency to inhibit AChE (different 'affinity' constants, i.e. $k_{+1} + k_{-1} + k_2$) as well as in the rates of 'ageing' and spontaneous reactivation. The latter is substantial for some inhibitors (dimethylphosphates) and almost nil for others ('irreversible' inhibitors). CB antiChEs behave similarly except for their faster k_3; i.e. 'reversible' inhibitors.

Some OPs have a chiral structure and their enantiomers have the phosphorus atom as a stereocentre; they show major differences in antiChE activity. Very limited information is available on the stereoselectivity of 'ageing' and spontaneous reactivation reactions [20].

Biochemical consequences of AChE inhibition on CNS cholinergic system

Following AChE inhibition by OPs the percentage increase in ACh and the time-course of its accumulation may vary widely from region to region of brain [87].

Two processes are responsible for the recovery of inhibited AChE; spontaneous reactivation of inhibited AChE and *de novo* synthesis of AChE [11]. Different AChE isoenzymes may recover at different rates both after single [41] and repeated exposures to inhibitors [67].

Single doses of OPs do not affect brain M-cholinoceptors [86], whereas repeated exposures reduce both their density and affinity for specific ligands [27]. This reduction in M-cholinoceptors shows regional specificity [14], reflecting either a different duration or intensity of cholinergic stimulation, or a selective access of the inhibitor. Reductions of high affinity brain nicotine binding sites have also been reported after chronic cholinergic stimulation [17]. Symptoms of excessive cholinergic stimulation are gradually reduced during chronic OP exposure, despite significant inhibition of AChE [8]. The development of this tolerance has been, in part, associated to effects on M-cholinoceptors caused by prolonged AChE inhibition and ACh stimulation [80].

Other components of the cholinergic system are not directly affected by AChE inhibition. CAT levels do not change after lethal doses of several OPs [85] and variable effects on Ch levels are reported for some OPs, suggesting a mechanism not related to AChE inhibition [32].

Other biochemical effects associated with AChE inhibition

Because of interactions of the CNS cholinergic system with other neurotransmitter systems, several effects beside AChE inhibition have been reported. All of them appear secondary to the increased ACh levels. Thus single doses of DFP increase the number of dopamine and GABA receptors shortly after exposure, whereas this increase is less marked after repeated dosing [86]. Some OPs also alter the turnover of serotonin [28]. Other effects after severe acute intoxication, e.g. on brain glucose, are secondary to seizures [74].

An important effect of antiChEs is that they increase permeability of the blood-brain barrier [44]. This facilitates the access of other chemicals to the brain [71], including oximes [31], the latter having important therapeutic implications in OP poisoning.

There are no reports of biochemical effects on the CNS from antiChEs at doses not causing AChE inhibition, except for NTE inhibition and axonal degeneration of some spinal cord tracts which are caused by OPs with relatively low anti-AChE potency.

Clinical and behavioural effects on CNS of antiChEs

Acute poisoning

A clinical discrimination of primary effects of antiChEs on the CNS is difficult, particularly in assessing their relative importance in causing death.

The clinical picture of severe anti-AChE poisoning is dominated by respiratory insufficiency, from a combination of peripheral muscarinic and nicotinic effects, and CNS toxicity [68,70]. Central toxicity seems the major cause of respiratory failure [77]. Selective effects of antiChEs on the CNS, obtained by intracerebral or intracarotid dosing, have been summarized [50]. Effects of OP poisoning on CNS functions have also been summarized [16].

The relationship between *in vivo* OP toxicity and brain AChE inhibition is influenced by many factors. In general 50–80% of nervous system AChE must be inactivated before symptoms are noted. Brain AChE activity around 10–15% of normal is associated with severe toxicity, and below 10% with respiratory failure and death. Lethal exposures in the absence of treatment have been estimated to correspond to approximately 30 to 50 times the minimal symptomatic exposure [44].

Early effects are characterized by stimulation or facilitation at various sites, which are followed, at higher concentrations of antiChEs, by inhibition or paralysis [90].

Acute OP poisoning causes various neurological signs in humans [95]. The time between exposure and onset of symptoms varies with the compound, route and degree of exposure: it is within a few minutes after massive ingestion, but may be delayed to 5 days [64]. CNS signs depend on the severity of poisoning and include behavioural changes, sleep disturbances, slurred speech, tremors, convulsions, coma, hypothermia and respiratory and circulatory failure. Early CNS signs and symptoms correlate with substantial inhibition of blood ChEs and include anxiety, depression, emotional lability, headache, giddiness, insomnia, excessive dreaming and tremor. These effects may last for several days, when moderate symptomatology is produced [39].

EEG abnormalities can be detected at the onset of symptoms and are characterized by irregularities in rhythm, variation and increase in potential, and intermittent bursts of abnormally slow waves of elevated voltage similar to those seen in epilepsy. They persist for about a week [40] (*see* Ch.9).

Unusual CNS clinical features of acute antiChEs poisoning have been also reported, including extrapyramidal symptoms [49] and atypical ocular bobbing [42].

Recovery from cholinergic toxicity depends on factors such as the type of inhibitor, the dose and the treatment. The half-life of recovery of inhibited AChE in the nervous system is about 1 week in experimental animals, and 1% a day in RBCs in cases of human poisoning [43,70]. Usually, recovery from major cholinergic signs is almost complete within a few days, but EEG changes may persist for several weeks [40]. On occasions symptomatology may last for several weeks [97] owing to a slower than normal recovery of AChE, probably because of the slow elimination of inhibitors.

Studies based on interview, physical examination, and blood biochemistry on long-term effects of acute OP poisoning revealed no significant neuropsychiatric sequelae in a group of 114 individuals; six had severe poisoning, the others mild to moderate[89]. Conversely, using a combined clinical and neuropsychological evaluation, statistically significant changes in cognitive functions of 100 subjects with previous acute OP poisoning were detected, when compared with a matched control group [82]. In this study however, the severity of the acute episodes was unknown and it is unclear whether these small psychological changes were from brain hypoxia or other factors. Furthermore, blood levels of organochlorine pesticides in the study group were about twice those of controls. As statistical analysis failed to show any association between blood levels and the neuropsychological tests, the authors ruled out organochlorines as the causative agents.

Changes in EEG, lasting for 1 year, have been reported after single sublethal iv doses of sarin in monkeys [13]. Animals were artificially ventilated during the seizure activity. These data are difficult to assess, because of the limited number of animals, the complex statistical treatment of the electrophysiological parameters, and it is not clear whether the variability of these EEG parameters in controls was assessed over time. Parallel experiments with dieldrin produced similar alterations in the EEG spectrum. Interpretation is therefore further complicated by the same effects being produced by compounds with different chemical structures and mechanisms of toxicity.

Anecdotal reports of various sequelae of acute OP poisoning, including parkinsonism [19], have appeared.

CNS pathology after severe acute OP poisoning in humans [62], or animals [45] is non-specific, with common features of vascular damage associated to increased permeability of the vessel walls. In addition, other histopathological findings related either to the seizure activity and/or to the hypoxic status have been observed after lethal doses of nerve agents [60]. Recent evidence confirms that neuropathology is not a direct neurotoxic effect, that it is unlikely to be a result of peripherally induced hypoxia, but rather a primarily seizure-induced effect [58]. OPs vary in their potency to induce seizures [47] and this clinical manifestation is perhaps not entirely related to AChE inhibition [94], also being blocked by benzodiazepines known to act via GABAergic mechanisms [57].

Tributyl S,S,S-phosphorotrithioate (DEF), which has low anti-AChE potential, produces hypothermia in experimental animals. This is observed at doses causing AChE inhibition and is probably produced by a metabolite active on central thermogenic control processes [76].

Effects of chronic exposure

Neuropsychiatric disorders

CNS cholinergic effects caused by repeated dosing with OPs are similar to those after a single dose [96]. The question is whether chronic exposures can cause neuropsychiatric, behavioural, and electrophysiological changes without overt toxicity, whether or not related to AChE inhibition.

Schizophrenic and depressive reactions, with severe memory impairment and difficulty in concentration, were reported in 16 workers after variable exposures to OPs [34]. This study however was seriously flawed by lack of evidence of exposure [7]. In a study on 53 and 68 workers with varying degrees of unquantified exposure to OPs , there was no indication that exposure at levels insufficient to produce clinical illness had any important effect on mental alertness [26]. Other studies on volunteers showed that altered awareness correlates with substantial inhibition of whole blood AChE [12].

Studies on EEG changes in industrial workers who had repeated accidental exposures to sarin have been reported [25]. The exposures caused symptoms and significant inhibition of RBC AChE, but it is not clear if cases of severe poisoning occurred. A number of differences, derived from complex analysis of EEG spectra, were reported between exposed workers and controls. The changes, present for up to 1 year after the last exposure, included increased beta activity, increased delta and theta slowing, decreased alpha activity, and increased amounts of rapid eye movement sleep. Comparable changes have been observed in monkeys exposed to subclinical doses of sarin given once a week over 10 weeks [13]. It should be noted that controversy exists concerning the value of computerized analysis of brainwave topography [23,73] and caution is necessary in its use [3]. Similar minimal EEG disturbances were observed in an earlier study [66], which to a lesser degree reflect the most severe disturbances seen after acute exposure. Work history and exposure data were lacking, and it was stated that the workers were also exposed to chlorinated hydrocarbons. Also, it is not clear in these studies whether persistent EEG changes were accompanied by changes in psychological or behavioural parameters. The authors also raise the question of the toxicological significance of the findings [24]. In another study claiming a correlation between neuropsychological tests and EEG changes, actual exposure data to both OP and dieldrin were not reported, and the EEG changes are

different from those reported by Metcalf and Holmes [66]. Both neuropsychological and EEG data indicate a 'selective' effect on the left frontal hemisphere [53].

EEG changes without AChE inhibition were reported after 3 months of daily consumption of diets containing various fractions of the LD_{50} of six OPs [21]. However, close examination of the results shows that none of the OPs produces effects not seen in the vehicle-alone group [96].

It is concluded that neuropsychiatric disorders are unlikely to occur from repeated exposure to OPs at doses not causing overt cholinergic toxicity.

Behavioural effects

Behavioural effects of acute OP poisoning and in asymptomatic subjects during chronic exposure have been reviewed [56].

Changes during acute poisoning, lasting for several months, include impaired vigilance, information processing, and psychomotor speed and memory [12,70]. Such changes were not found during subacute low level exposure [61] in chronic asymptomatic exposures [26], and in chronic exposures with normal blood ChEs activities [79]. Therefore there is no evidence of behavioural disturbances among workers with asymptomatic exposures, a finding supported by animal studies.

Experimental animal studies demonstrated that prolonged exposure to OPs can cause typical patterns of behavioural and physiological changes followed by recovery toward pre-exposure values. The initial behavioural effects are related to decreased AChE activity and consequently to an increase of ACh levels [6]. During the later phases of acute intoxication and during subacute and chronic intoxication, behaviour can return toward normal despite low enzyme activity [10]. A number of mechanisms could be involved in the development of this tolerance [80]. An effect called 'behaviourally augmented tolerance' is based on the observation that behavioural changes during acute intoxication are often so rapid as to lead to the hypothesis of both 'behavioural' and 'physiological' processes in tolerance development [35].

These and other observations raise the question whether behavioural effects, not related to AChE inhibition, might occur at low dose levels of OPs. In this respect it has been shown that extremely small single doses of certain OPs can produce behavioural changes in experimental animals; ChE measurements were not performed [98].

There is no evidence of neurochemical correlates other than AChE inhibition with behavioural changes.

Therapeutic uses

Some drugs including antidepressants, antiparkinsonian drugs, antihistamines, antispasmodics, etc. and toxic plants including mushrooms, potato sprouts, bittersweet, etc. are known to have central and peripheral anticholinergic toxicity. Effects include delirium, anxiety, hyperactivity, hallucinations and seizures and can be rapidly reversed by physostigmine salicylate [37].

Recently a number of reports described the effects of cholinergic treatment on memory and global mental functions in Alzheimer's disease [4]. Since the demonstration that low levels of CAT are associated with the extent of degenerative changes in the CNS of Alzheimer's patients [69], and that memory is associated with the cholinergic system [22], a number of reports indicate the effectiveness of such treatment in these patients [88,93].

Conclusions

The primary molecular interactions of antiChEs are well understood as are some of their CNS biochemical, physiological, behavioural and clinical consequences. Clinical and behavioural effects seem, so far, always to be correlated with AChE inhibition. The considerable redundancy of CNS structure confers on the system a reserve capacity which is not exceeded even at relatively high inhibition of AChE, including behavioural effects [77]. When clinical cholinergic signs occur they are relatively short lasting and completely reversible, unless major brain hypoxic effects have been produced. Therefore there is no indication that repeated low dose OP exposure can cause adverse effects without AChE inhibition. Alterations of behaviour may be the earliest and most sensitive signs of CNS toxicity [72].

Recent developments in knowledge of CNS cholinergic neurons [18] will provide explanations for either the selective access or effects of antiChEs in certain brain areas. Study of regional differences in the cholinergic innervation of cerebral cortex [65] and of their functions [9] should highlight the correlation between behaviour and the cholinergic system.

Study of the interactions of the cholinergic system with other neurotransmitter systems might indicate other possible primary or secondary targets, which have been inferred on the basis of several observations [35,50].

In conclusion, the cholinergic functions of the CNS are extremely complex and not well understood, and the effects of antiChEs have been studied at different levels of biological complexity. The simplest is the interaction with AChE, the most complex is that which studies subtle behavioural changes in large populations. In between, an almost infinite variety of studies are possible.

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9

Acute and subchronic neurotoxicity and cardiotoxicity of anticholinesterases

Illés Dési and László Nagymajtényi

Introduction

As is widely known the symptoms and signs of antiChE poisoning depend on the decrease in effective AChE activity [7,8,17,39]. In the literature dealing with antiChEs, there is little material discussing functional neurotoxic effects.

Neurotoxicity

Duffy and Burchfield [5], using monkeys given a single massive dose of sarin, observed distinct EEG alterations not only 24 h after the treatment but even 1 year later, as shown by increased beta activity. Dési [3] noted behavioural alterations in maze tests and EEG changes in the complex and its component bands when rats were treated with bromophos in subchronic experiments. Alterations in beta activity and REM sleep were observed by Duffy and Burchfield [5], while long-term behavioural and psychological changes have been seen following OP exposure [19,23].

Functional disorder in the PNS, namely enhanced motor reflexes, were noted after administration of chlorothion [3]. Following administration of a single massive dose of dichlorvos, mild ataxia was observed in hens [2]. However, in the dog, dichlorvos did not alter the conduction velocity of peripheral nerves [9] and did not induce EEG alteration in rats [10]. Several studies have noted the effects of OPs on the EMG.

In addition to the studies discussed, there has been much interest in the delayed neurotoxic effects of OPs (*see* Chs 10 and 11). In this process well-defined, degenerative alterations occur in the PNS combined with morphological change [12,13,15,35]. It has been demonstrated that numerous OPs, including leptophos, isofenphos and chlorpyrifos, are capable of producing peripheral neuropathy after administration as a single large dose. OPs with a delayed neurotoxic effect appear not to be effective when given as repeated low doses. OP-induced peripheral neuropathy has been a feature of some human accidental or suicidal intoxications with certain OPs. In these cases, the clinical picture and impairment of function has been similar to that seen in animal studies [14,24,40,41].

It is generally accepted that the clinical signs and symptoms of OP poisoning are, except for peripheral neuropathy, from ChE inhibition. Nevertheless, the relationship is not completely straightforward. Thus in some instances correlation was observed between symptoms and signs and enzyme levels [18], while in other cases such correlations were not noted [36]. Moreover, variable decreases in ChE activity have been reported in intoxications produced by identical doses of the same compound: in such cases measurements have included ChE activity in plasma, RBCs and tissues, including the brain.

Experimental

Three OPs have been studied at Szeged, dimethoate, dichlorvos and parathion-methyl. These three pesticides are widely-used in Hungary and are frequent causes of human poisoning. Moreover, none produces OP-induced peripheral neuropathy, so that effects of the OPs on peripheral nerves are likely to be reversible. The concomitant use of atropine [21,32,37] (*see* Ch.51) and diazepam [6,38] in experimental poisoning has also been studied.

Poisoning by the three OPs was investigated

in rats, using three dosing regimens: (1) superacute, 2.5–3 × LD_{50} ip, (2) acute, 1.1 × LD_{50} by gavage, and (3) subchronic, $\frac{1}{50}$ LD_{50} by gavage, 5 days per week for 6 weeks. These protocols were intended to model overwhelming, less severe and chronic OP poisoning, respectively. Additionally, the effects of atropine or diazepam were studied on animals treated with OPs according to the superacute and acute protocols.

EEGs were studied using a 12 channel EEG apparatus, while the conduction velocity of the rat tail ventral nerve was also measured [29]. Additionally, the relative and absolute refractory times of the nerve were measured by the method of Anda *et al.* [1].

ChE activity was estimated by the method of Meinecke and Oettel [28] in RBC and brain (cortex and white matter), and in liver and cardiac and skeletal muscle. In addition to being measured at the same times as electrophysiological measurements, ChE was determined on decedents or at sacrifice.

In the supralethal experiments, clinical signs characteristic of severe OP poisoning were seen. They included intense salivation and fasciculation and all the treated animals died within 50–70 min. In the acute experiments, the clinical signs were considerably milder and appeared later. In the combination experiments, atropine relieved the cholinergic signs, but with diazepam, only the restlessness and spasticity were ameliorated.

After dimethoate and parathion-methyl administration in the superacute experiment, the EEG mean amplitude decreased by 20 min and this later became more marked. By the end of the experiment, there was a significant increase in mean frequency. The EEG index, because of increased activity of the fast frequency bands, decreased significantly after 40 min with all three OPs. According to the power density values of the component frequency bands, within 20 min the activity of the slow frequency bands began to diminish slightly, with a simultaneous but less intense decrease in the activity of the beta bands. Later the decrease in the activity of the slow bands was greater than the reduction of beta 1 and beta 2 activity.

In the acute experiment, the EEG mean amplitude showed a significant decrease by 24 h, which was of similar magnitude for all three OPs. The mean frequency increased most for parathion-methyl, but not to a statistically significant degree. The EEG index decreased slightly in the case of dimethoate, but the other two OPs produced a significant fall. With dimethoate, there was a similar decrease in all EEG bands, both fast and slow. However, while with dichlorvos and parathion-methyl the decrease in the activity of the slow bands was similar to that seen with dimethoate, there was a smaller decrease in the values of the fast bands.

After subchronic dosing, the mean amplitude decreased significantly by the end of the first week and remained at that level, the decrease being similar for all three OPs. The mean frequency data, however, showed definite differences: in the case of dimethoate, there was a highly significant increase by the end of the first week. At the end of the second and fourth weeks, mean frequencies were obtained similar to initial values. By the end of the treatment period, significant changes, similar to those seen at the end of the first week were evident. For dichlorvos, the increase in the mean frequency was significant at the end of the first week and was maintained, with slight fluctuation, at this level. At the end of the first week of parathion-methyl dosing, the mean frequency was still almost the same as the control value, only increasing significantly by the end of the second week. Thereafter it remained at this level. In the cases of dimethoate and dichlorvos, the EEG index showed a significant decrease by the end of the first week. The values measured during the second and fourth week showed a transient improvement, but thereafter a decrease was observed with both OPs. With parathion-methyl, the EEG index was similar to the control value at the end of the first week, but by the end of the second week it had decreased significantly. The differences in behaviour of the EEG indices were explicable by alterations in activity of the individual components. Slow band activity decreased similarly with all three OPs, so that the index alterations seen were primarily from changes in beta activity.

EEG changes observed after OPs combined with atropine were broadly similar to those obtained with the OPs alone, although there were some minor changes. Thus atropine and

diazepam modified the clinical response, without changing the EEG pattern substantially.

In the PNS, conduction velocity in the tail nerves decreased significantly with all three OPs in the supralethal studies. However, there were some differences: thus with dimethoate the decrease was more marked initially. In the acute experiment a similar and significant decrease in conduction velocity was seen with all three OPs. With subchronic administration, by the end of the first week, the three OPs had all produced significant depressions in nerve conduction velocity. The depression persisted with all three test compounds. Changes in relative and absolute refractory times showed, in the supralethal study, a similar ordering of changes to those seen in conduction velocity. Thus the refractory times increased most with dimethoate and least with dichlorvos, parathion-methyl being intermediate. In the acute studies, there was a significant increase in both the relative and absolute refractory times. This was similar for the three OPs in the case of relative refractory times, but in the case of absolute refractory time the increase was, for parathion-methyl, less than for the other two OPs. After subchronic administration, the three OPs caused increases in the refractory time but some differences were seen. With dimethoate and dichlorvos, there was temporary improvement in relative refractory time, but by the sixth week it had markedly increased. In the case of parathion-methyl, although there was, after initial deterioration, some improvement, relative refractory time was still significantly increased at the end of the 6-week experimental period.

In supralethal combination studies, the effects of the OPs on conduction and refractory times were only slightly improved with atropine. In acute studies, atropine did not appreciably change conduction velocity or refractory times. Similarly atropine was ineffective in subchronic studies, in respect of peripheral nerve measurements. Combination studies with diazepam did not involve studies of the PNS.

In the supralethal experiments, similar depressions were seen with the three OPs in each tissue examined and in the RBCs. There were, however, considerable differences between the tissues. In the acute studies, there were marked differences in enzyme inhibition between the OPs for the same tissues. For example, in the white matter there was a highly significant decrease in activity after dimethoate but not with parathion-methyl. Differential inhibition between the three OPs also occurred in the subchronic experiments. Thus cortical enzymic activity showed a highly significant decrease with dimethoate but with dichlorvos ChE was normal during the first week and then, after an abrupt period of inhibition, the enzyme activity began to rise again. There was no notable correlation between changes observed in electrophysiological measurements and ChE inhibition, whether RBC, plasma, cerebral cortical or white matter ChE was used for the comparison.

Discussion

Based on literature surveys and on our own studies, the neurotoxic effects of the OPs examined can be summarized as follows. All three OPs, at very large doses, produced definite alterations in electrophysiological measurements, in both CNS and peripheral nerves. The use of large doses of OP revealed important differences in the effects of the OPs, while ChE inhibition was similar. In acute studies, differences in effects evoked by the three OPs were present, in respect of EEG amplitude, frequency and index changes. Divergence between ChE inhibition and electrophysiological alteration was notable. From the subchronic experiments, the conclusion was that electrophysiological changes were detected both in the CNS and PNS. Moreover, ChE activity, whether in RBC, CNS or in other organs, did not correlate with electrophysiological findings. Thus determination of ChE may not be a satisfactory guide to the functional state of the nervous system. In view of this, it is particularly important in cases of continuing exposure to OPs, not only to monitor OP activity, but also to follow the function of the nervous system. Sensitive electrophysiological techniques (EEG and electroneuromyograph), which are easy to use and non-invasive, will give early indication of nervous system involvement. It is important to note that, despite its beneficial action on the clinical state, the effects of atropine on electrophysiological findings were nugatory.

Cardiac effects

Reports of cardiotoxic effects of OPs have been limited until recently to descriptions of bradycardia from increased vagal tone. Only a few references have referred to other effects such as arrhythmia [16,20,31,34] (*see* Ch.14).

Marosi *et al.* [27] concluded, after analysis of the clinical data on 461 patients presenting with acute OP poisoning at an intensive care unit, that with large doses of OP cardiotoxic effects were common. These effects could be divided into well-defined groups, but did not appear to correlate with ChE activity. Where there was a lethal outcome, death was caused by progressive cardiogenic shock or by atrioventricular block with sudden asystole. The cardiotoxic effect appeared to be dose-related and possibly depending on the type of OP. The lethal outcome could not be avoided by atropine treatment.

In animal studies, intending to model the pattern of human poisoning, four OPs, dimethoate, formothion, dichlorvos or trichlorfon, were administered to rats or guinea pigs. It was observed that the cardiotoxic effects were clearly dose-dependent and related to the chemical structure [25,26,30]. Administration of small doses of OPs only produced bradycardia induced by increased cholinergic effects. By increasing the dose above a threshold which was characteristic of each compound but always well above the LD_{50}, additional effects were observable. Dimethoate and formothion produced similar effects on the ECG, presumably related to their similar chemical structure. These effects were ST segment depression, QT interval prolongation and T-wave inversion. Dichlorvos and trichlorfon gave rise to atrioventricular block and ventricular asystole. Even with the administration of large doses of atropine (8 mg/kg), effects except for bradycardia were unaltered. No morphological alterations attributable to the cardiotoxic effects were seen using light and electron microscopy.

ChE determination during the study showed no correlation with the cardiac effects. However, a relationship was found between OP dose and toxic effect. Furthermore, it was revealed that a direct cardiotoxic effect was observable only when the concentration of OP in the heart muscle was above a certain level.

To elucidate the mechanism, we carried out an *in vitro* study on the isolated rat and guinea pig heart. Using Langendorf preparations [4] dimethoate, dichlorvos, parathion-methyl and bromophos were studied alone or in the presence of antidotes. The electrophysiology and mechanical function of the heart were investigated using computer analysis.

The two mechanisms, disorder of repolarization and of impulse generation and conduction, could be distinguished even *in vitro* and depended on the structure of the active substance. To produce effects OPs have to reach a threshold concentration in the perfusing solution, which is characteristic of a given compound. ECG and mechanical disorders appeared in parallel when the threshold concentration was surpassed. The direct toxic effect could not be prevented by continuous coadministration of atropine. Neither adrenaline nor isoprenaline (isoproteranol) was able to ameliorate the toxic effects. From previous studies, it is apparent that OPs may affect ion transport through the myocardial cell membrane, suggesting a mechanism for their direct toxic effects on the heart. In the presence of Ca^{2+} channel blockers, such as verapamil or nifedipine, the cardiotoxic effects are shortened, increasing the probability that the underlying cause is interference with ion transport.

In view of the frequency of death from cardiac toxicity, particularly in those resuscitated from overwhelming doses of OPs, it is important that the mechanisms underlying this toxic effect be elucidated. This is the approach most likely to produce effective therapy.

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10

Molecular events in delayed neuropathy: experimental aspects of neuropathy target esterase

Martin K. Johnson

Introduction

The clinical syndrome now known preferably as organophosphate-induced delayed polyneuropathy (OPIDP) or neuropathy (OPIDN) is described in Chapter 11, with the historical detail. The key features of the syndrome are: (1) the syndrome can be produced in humans and certain other species by a single dose, severe cases are virtually irrevocable with some peripheral repair capability overwhelmed by permanent damage to certain central neurons; and (2) responsible agents contain (initially or after metabolic activation) at least one labile ester /acid anhydride bond linking phosphorus to a recognizable 'leaving group' and all have activity against some esterases *in vivo*. Such compounds may or may not have significant antiChE activity. Thus the stereotype nerve agent DFP is both an antiChE and neuropathic while the neuropathic TOCP is a plasticizer-type compound with negligible activity against AChE.

The role of neuropathy target esterase (NTE, initially called 'neurotoxic' esterase) in the process of initiation of OPIDP was introduced in a short review, Johnson [48] which was subsequently elaborated [52,53]. Both latter reviews dealt with mechanism but the former discussed NTE in relation to the biological aspects of OPIDP while the latter listed the dose/OPIDP responses for over 200 OP compounds with their effects on NTE *in vitro* and *in vivo*. Davis and Richardson [28] also set NTE in the context of a complete review of OPIDP. Abou-Donia [1] restated the biology and structure-activity information for OPIDP but with little mention of NTE. The most extensive account [59] of all aspects of NTE, including its role in initiation, its biochemical properties and its interactions with chemicals *in vitro* and *in vivo* and how NTE assays relate to acute and chronic testing for OPIDP, requires updating.

This chapter presents a critical survey of the experimental studies on NTE which pertain to its status as the primary molecular target for initiation of OPIDP as shown in Figure 10.1. Fifteen brief statements of observations and interpretations were set out along with five questions by Johnson [58]. They are still valid and provide a useful framework for this survey. The statements and questions (with updated nomenclature) are presented in bold type; for convenience they are now arranged in groups and in a revised order. Headings and subheadings are sufficiently detailed that perusal of them may not only suffice to inform a casual reader, but to orientate the serious student.

The chapter also contains a brief survey of recent studies on NTE as an enzyme *per se* and of how NTE assays fit into the framework of testing for regulatory purposes.

A review of statements about NTE and OPIDP

Background to experimental studies

Statements 1–3

Some species (humans, cat, sheep, pig, and chicken) are susceptible to neuropathy after a single dose of some OP esters. Other, usually smaller, species appear resistant to varying degrees and immature animals are always less susceptible.

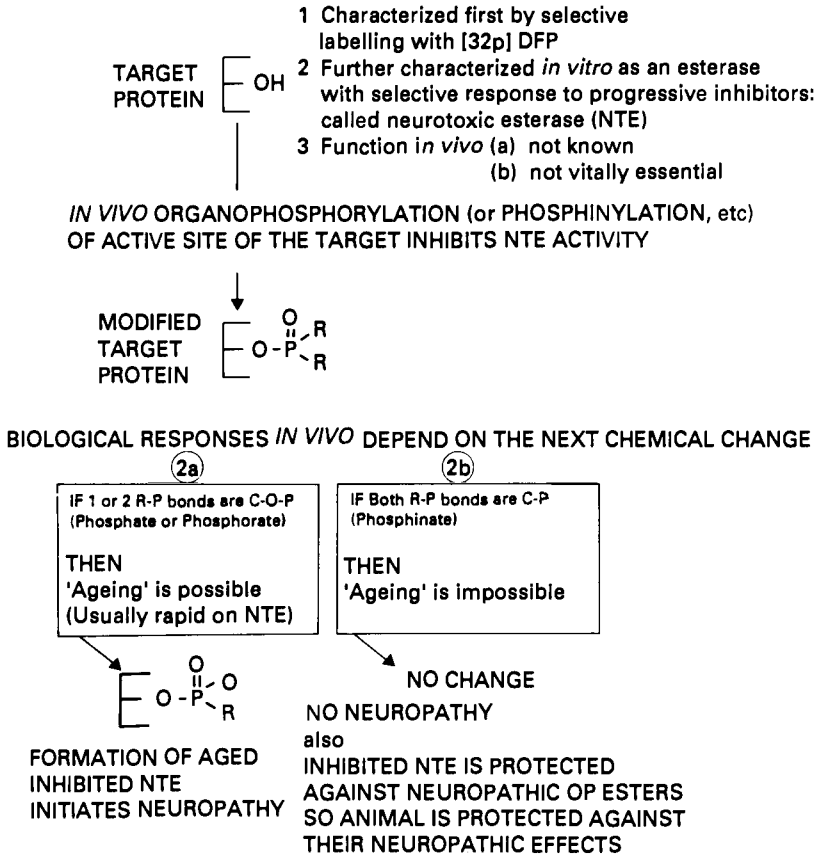

Figure 10.1 Respective consequences of alternative further chemical modifications (2a and 2b) of the OPIDP target protein after it has been modified by covalent binding of certain OP esters. The pathological effects are related to the biochemical effects. (Reproduced from Johnson [59] with permission)

The adult hen is the most uniformly susceptible test animal and has been used for biochemical studies on initiation.
Most studies used brain tissue while recognizing that lesions occur only in hindbrain, spinal cord, and peripheral nerve.

These statements need little comment. Comparative data for other neural tissues of the hen and comparison of NTE in tissues from other sensitive species including humans are covered in this chapter (pp. 94, 102). The problem of apparently insensitive species has been partially clarified and is dealt with under Question (2).

Dissection of the target for OPIDP

Statement 4

Studies *in vitro* with brain of adult hen examined total sites phosphorylated by a neuropathic OP ([^{32}P]DFP). These sites were dissected and a small subgroup was identified as the only portion having appropriate characteristics of the target with respect to attack by a variety of neuropathic and non-neuropathic OPs (Figure 10.2).

After ranging studies it was found that only 4% of labelling sites were resistant to reasonable concentrations of the non-neuropathic anti-esterase compound, paraoxon, but were susceptible to the neuropathic compounds mipafox and phenyl saligenin cyclic phosphate [46,49,62].

Target-site labelling assays in delayed neuropathy tests

The amount of paraoxon-'resistant' mipafox-'sensitive' [DFP]-labelling sites which were

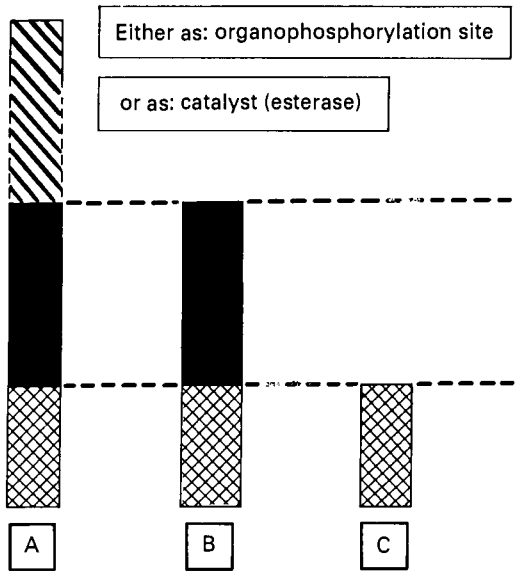

Figure 10.2 Representation (not to scale) of the effects of various preincubations on the quantities assayed in both the [^{3}H]DFP labelling assay for the neuropathy target protein or the substrate hydrolysis assay for NTE. (A) Preincubation with buffer. (B) Preincubation with paraoxon (usually 40–160 μM for 20 min at 37°C). (C) Preincubation as in (B) but with mipafox (50–100 μM) added either with paraoxon or added after removal/ dilution of paraoxon. The filled blocks calculated as B−C is a measure of NTE in both assays. However the hatched blocks measured as A−B or C are not exactly equivalent. Thus, for example, AChE is labelled by [^{3}H]DFP and is sensitive to paraoxon so that it contributes to A−B in the labelling assay but it does not significantly hydrolyze PV

found by *ex vivo* assay to have remained in autopsy samples of brain taken from dosed hens correlated well with the neuropathogenicity or otherwise of many test compounds, both antiChE and plasticizer-type OP esters; correlations included a half-way effect of single doses of two compounds which were found to be non-neuropathic at that dose but positive on three repeated daily doses [40,47].

Is the identified subgroup of labelling sites homogeneous?

In recent years the homogeneity of the above subgroup of labelling sites has been examined extensively, principally by polyacrylamide gel electrophoresis in SDS-PAGE of DFP-labelled proteins. Williams and Johnson [131] and Williams [129] examined the PAGE patterns from whole brain homogenate or from washed particulate fractions ('microsomes') after many minor variations of preincubation conditions including varying paraoxon concentration from 40 to 160 μM, mipafox from 50 to 100 μM and pH 5.2–8.0. They found labelled polypeptides covering the whole M_r range from 30 K to 200 K. Lowering the pH of the labelling medium (but not the medium for preincubation with unlabelled OPs) to 5.2 increased the specificity (B-C/B in Figure 10.2) of labelling of microsomes to >60%. The main effect was elimination of many of the sites which were insensitive to mipafox and had M_r less than 100 K without affecting the target protein; it is not known whether this was a reversible effect of pH but it correlated with what had been discovered of the stability of the catalytic activity of NTE [59]. After labelling at pH 8.0 or 5.2 and separation of labelled polypeptides by SDS-PAGE, bands of paraoxon-'resistant' mipafox-'sensitive' labelled sites with M_r around 155 K and 92 K were found. The larger proportion (80%) was in the 155 K band but, clearly, each of the bands was a candidate for identification as the labelled subunit from the neuropathy target protein, assuming only one organophosphorylation site/polypeptide chain.

Moreover, titration experiments showed that sites in both regions had indistinguishable I_{50}s for inhibition by mipafox. By five criteria the 155 K (c. 178 K on SDS gel filtration) subunit has been preferred; several of these involve consideration of the esteratic activity of NTE which is described in more detail later. The criteria are:

(1) For preincubation times of 20 min at 37°C, the I_{50} of paraoxon as an inhibitor of the catalytic activity of NTE was known to be >400 μM and changing the concentration of paraoxon from the commonly used 40 μM to 160 μM decreased activity by about 15% as expected for a first-order kinetic reaction. When the same change was applied to the labelling assay the quantity of 155 K site also decreased by 15% but the 92 K site decreased 60–70% [131].
(2) The proportions of labelled 155 K in forebrain and hindbrain equalled the proportions of the esterase-assayed NTE while the proportions of 92 K did not [131].

(3) 4-Nitrophenyl di-n-pentylphosphinate is a potent inhibitor of the catalytic activity of NTE *in vitro* [54] and a prophylactic agent in *vivo* against OPIDP by its capacity to block NTE (Figure 10.1, later). *In vitro* the 155 K but not the 92 K labelling-site was blocked by concentrations of the phosphinate which inhibited NTE [131].
(4) Similar contrasting effects on the 155 K and 92 K sites to those described in (3) were elicited when the phosphinate was injected into hens at a dose of 1.5 mg/kg sc which effectively protects them against the neuropathic effect, but not the cholinergic effect, of a challenge dose of DFP [131].
(5) After attack on the target by DFP, an intramolecular transfer of an isopropyl group from the covalently bound di-isopropyl group is an integral part of the process of initiation of OPIDP (Figure 10.1). When the DFP is labelled in its isopropyl group, the transferred isopropyl group is assayable as 'volatilizable counts' after alkali treatment of the labelled protein [129]; only the 155 K peptide carried such 'volatilizable counts' (Figure 10.3) [88].

Carrington and Abou-Donia [15] repeated the work of Williams and Johnson [131] and also varied both concentrations of DFP and time to achieve the same net CT. They came to the same conclusion concerning the identity of the 155 K (160 K by their measurement) polypeptide with the target site as preferred over a smaller amount of one with M_r = 82 K (compared with 92 K in the earlier work). However, they also reported the presence of a very small amount of a previously unreported polypeptide (M_r = 115 K) which was 'resistant' to paraoxon and 'sensitive' to mipafox and which, therefore, might be a candidate for the target site. This trace polypeptide is also reported by Thomas *et al.* [120] and by Pope and Padilla [102]. In recent experiments with bigger loadings of labelled protein onto SDS-PAGE (unpublished data) a labelled polypeptide has been detected of that M_r and which carried 'volatizable counts'; the quantity was variable depending on the conditions of processing of brain tissue. Virtually no 'volatilizable counts' are found in the 115 K region if freshly excised brain is processed rapidly with special care being given to thorough chilling of all homogenates. The proportion compared

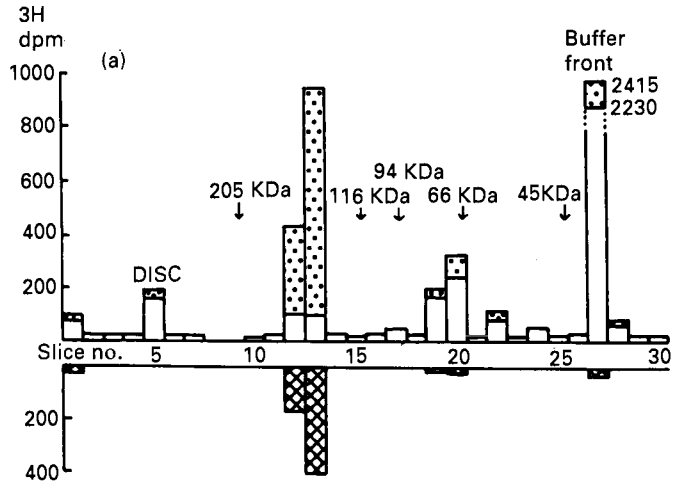

Figure 10.3 Electrophoretogram (SDS-PAGE) of the radioactivity in particles from 20 mg of hen brain labelled with [^{3}H]DFP. Above the baseline complete blocks represent quantity of labelling resistant to paraoxon (160 μM per 20 min at 37°C); each block is subdivided into labelling that is eliminated by inclusion of mipafox (100 μM) in preincubation (stippled portions) and labelling that is not (open). Below the baseline, represents the 'volatilizable counts' in each gel slice. Adapted from Meredith [86] and Meredith and Johnson [88]

with the 155 K polypeptide was about 1:3 if the brains were frozen at −20°C overnight before processing, and more if other conditions which might favour autolysis were permitted. It is presumed, therefore, that the 115 K polypeptide is an artefact. It may derive from the 155 K polypeptide because it appears to carry the full catalytic site which is involved with substrate hydrolysis and with inhibition and ageing of the inhibited esterase (*see* below and pp. 98–100). Carrington *et al.* [19] suggest that the 115 K unit might be derived from the 155 K by deglycosylation although there might be a family of partially deglycosylated intermediates. An alternative explanation is that it is formed by cleavage by an OP-insensitive protease of a single peptide bond in the 155 K polypeptide.

Radiation inactivation studies [19] led to the conclusion that the target size of hen brain NTE is approximately 105 kDa (97–129 in other species). It is difficult to relate this data to the M_r derived by SDS-Page because the target size for AChE in parallel experiments was about 53 kDa, although the considered molecular mass was 90–110 kDa. Again, glycosylation may explain the discrepancies. The main conclusion was that there was no reason to consider DFP-binding polypeptide was part of a larger protein that is responsible for the enzyme activity.

Target site in other species and tissues

The target polypeptide in rat brain has been identified by inhibitor-response criteria as having M_r of approximately 160 K [92]. The same size polypeptide has been identified using the more discriminating 'volatilizable counts' assay in brain of rat, guinea-pig, sheep and pig [88]. Also a polypeptide (M_r = 178 K on SDS gel filtration) is the principal paraoxon-'resistant' mipafox-'sensitive' DFP-labelling site in spinal cord and, slightly surprisingly, also in non-neural tissue, liver, kidney and spleen of hen and in human placenta; in each case this was the only polypeptide which yielded 'volatilizable counts' [129].

Conclusion from all labelling studies

The quantity of target site as originally measured clearly embraced several separate polypeptides. The 155–160 K polypeptide present in considerably greater proportion than others has the 'right' characteristics by many criteria. The lesser amount of 82–92 K polypeptide fails by at least two criteria. The 115 K polypeptide appears likely to be a degradation product of the 155–160 K and is virtually absent from preparations from fresh unfrozen brain. Evidence for a 61 K polypeptide as a candidate for the target seems tenuous at present. For a wide variety of compounds there was excellent correlation between biochemical assay *ex vivo* of the composite site in tissue from dosed hens and the clinical responses in pair-dosed birds. However, tests to relate the effect of dosing compounds to hens with the response of the individual labelled polypeptides assayed *ex vivo* have been few.

Statement 5

The target protein possessed catalytic (esterase) activity: *in vitro* and *ex vivo* tests.

The cyclic process of organophosphorylation and dephosphorylation of OP-sensitive esterases (Figure 10.4) is well known to be analogous to the steps of substrate hydrolysis, the principal difference is that k_3 for inhibitors is many orders less than that for substrates so that inhibition is often considered virtually irreversible (*see* Ch. 6) [3]. Although inhibition involves formation of covalent bonds, there is good evidence for the initial and reversible formation of a Michaelis complex between the OP ester and the esterase. The known high degree of specificity of OP esters at low concentrations for esterase catalytic sites led to a screening of hydrolyzable substrates which would halt the progressive labelling of the neuropathy target site by [^{32}P]DFP. Only phenyl 2-phenylacetate (PPA) was effective and in hen brain homogenate only 3–4% of the total PPA hydrolase was TEPP-'resistant', paraoxon-'resistant' and mipafox-'sensitive' (see Figure 10.2) [47]. The inhibitor responses of this portion were similar to those of the target labelling site in many *in vitro* and *ex vivo* tests and was called 'neurotoxic esterase' [47,49]. It now carries the less ambiguous title of NTE. The residual 6–7% of PPA hydrolase was relatively resistant to all three above inhibitors although not to all OP esters.

That the loss of catalytic activity of NTE as usually assayed was a measure of insult to the

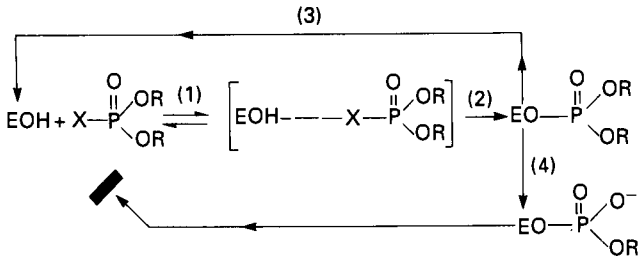

Figure 10.4 Steps in the interaction of an esterase with an OP inhibitor. (1) Formation of Michaelis complex. (2) Phosphorylation of enzyme. (3) Reactivation (spontaneous or forced by oximes or KF). (4) Ageing to a form which cannot be reactivated as in (3). In this chapter unaged and aged inhibited forms of NTE are referred to as unmodified (UI-NTE) and modified inhibited NTE (MI-NTE)

neuropathy target has been demonstrated in many tests combining clinical observations with *ex vivo* assay of NTE in tissue taken from pair-dosed hens. Provided that the necessity for ageing of inhibited NTE is respected, the NTE assay has successfully predicted both positive and negative responses elicited by more than 100 OPs and phosphonates plus the protective effects of about 20 organophosphinates, sulphonyl fluorides and CBs. Partial lists of such tests are given by Johnson [53,59]. For single doses of initiating compounds about 70–80% inhibition of NTE in hen neural tissues appears to be the threshold level to elicit clinically obvious OPIDP. The necessity for checking enzyme responses in spinal cord and sciatic nerve as well as in brain is elaborated later in this chapter.

Is NTE as usually assayed homogeneous?

The early studies with PPA as substrate did not detect any heterogeneity by simple graphical analysis of first order plots of concentration of inhibitor *versus* log (% of esteratic activity remaining) for several inhibitors [47,49].

The efficacy of phenyl n-butylCB as an inhibitor of NTE [49] led to consideration of analogous aliphatic carboxylic acid esters as potential substrates for NTE [54] and phenyl valerate (PV) was introduced as preferred substrate because of higher absolute rates obtained and improved selectivity. NTE accounts for 65–70% of the paraoxon-'resistant' PV hydrolase activity of whole hen brain homogenate. Definitive conditions for assay of NTE are laid down by Johnson [56] except that the quantity of brain tissue should be 6.0 mg (not 0.61) and that use of 2% SDS (instead of 1%) in the stop medium makes the procedure immediately applicable to homogenates of spinal cord. That PV hydrolysis is measuring the same enzyme as did PPA under the fixed differential assay conditions originally set for NTE was shown by the constant ratio of activity remaining against the two substrates during experiments in which NTE activity was titrated against mipafox concentration [54]. Also, observed inhibitory power of a number of OP esters and CBs against NTE was unchanged regardless of which ester was used as substrate.

Using a computer-aided iterative ('curve-stripping') procedure to analyse semi-log inhibition data for three inhibitors used alone or in sequence, Chemnitius and Zech [24] claimed to detect as many as eleven PV esterases in homogenates of hen brain including two esterases with the general characteristics attributed to NTE. They then attempted [22] to dissect NTE activity by improvements in technique involving:

(1) Preincubation of concentrated tissue suspensions with paraoxon followed by a substantial dilution step before a separate preincubation with mipafox: this should minimize any competitive inhibition of mipafox-sensitive sites by residual paraoxon. However, the two-step procedure does introduce the possibility of some degree of spontaneous reactivation of paraoxon-inhibited enzyme(s) during the period of incubation of the diluted mixture. The paraoxon-'sensitive' activity

is much greater than NTE, and one of the two enzymes concerned is mipafox-sensitive, so that a small percentage reactivation after removal of paraoxon may introduce a significant extra 'pseudo-NTE' into the quantity identified by sensitivity to mipafox. We have found it necessary to use benzenesulphonyl fluoride rather than paraoxon in our studies on spontaneous reactivation and ageing of inhibited NTE because of problems introduced apparently by the spontaneous reactivation of paraoxon-inhibited activity [69,70]. However, we have never quantified accurately the error which might be involved so that no definite criticisms can be made on this basis.

(2) Use of 28–31 closely-spaced concentrations of mipafox or DFP in titrations at 25°C after the paraoxon step; concentrations were also checked scrupulously. These precautions compare with the use of only 8–10 concentrations in earlier work. However, no time-course studies were reported for these or the earlier studies so that derivation of rate-constants of inhibition still depended on the assumption that first-order kinetic conditions were maintained throughout.

From these experiments Chemnitius *et al.* [22] concluded that there were indeed two isoenzymes, called NTE_A and NTE_B, in the mean proportions of 18:82 in hen brain homogenate with mean catalytic activity against PV of 160 and 724 nmoles/min per g at 25°C. However, two criticisms may be levelled at the procedures used.

First, the iterative analysis protocol is flawed in two respects: (1) the errors at opposite ends of the line in a semi-log plot are markedly different, a fact which is ignored in the iterative procedure; and (2) the error in determining rate constants iteratively is cumulative and may become substantial by the time a third component is reached. It is generally accepted by kineticists that the best way to analyse such experiments is to attempt to fit the untransformed data to models with increasing numbers of components: the goodness of fit of each model is evaluated by a residual least-squares method. When we have used this procedure we have been unable to reproduce the Chemnitius *et al.* [22] result, although using exactly their published techniques and number of data points (Johnson and Read, unpublished data). Second order rate constants for inhibition found in the different laboratories agreed at about 5×10^3 l/mol per min for mipafox and $5–10\times10^4$ l/mol per min for DFP at 37°C.

Second, when very close analysis of kinetic data is attempted (unlike the earlier studies) then consideration must be given to the possible effect of formation of Michaelis complexes between the OP inhibitor and the enzyme, as well as between the substrate and enzyme. Carrington and Abou-Donia [17] made approximate determinations of the K_m of both PV as substrate and mipafox as inhibitor and showed that the inhibitor-NTE Michaelis complex was not fully discharged by added PV after preincubation with higher concentrations of mipafox. Curve-fitting of the untransformed data as described above but with allowance also for the skewing effect on the lines of this undischarged Michaelis complex led to the conclusion that the data on paraoxon-'resistant' mipafox-'sensitive' activity was best fitted by a two-component model consisting of a single NTE ($k_a = 5.6\times10^3$ l/mol per min at 25°C) plus a residual 'non-NTE' activity.

In spite of the above criticisms a screening of many compounds has produced evidence that trace amounts of NTE activity as usually assayed is from a different enzyme [64]. When brain homogenate was preincubated *in vitro* with higher concentrations of several neuropathic or prophylactic compounds about 3–5% of activity survived treatment with $10–20 \times I_{50}$ whereas first order kinetics require that <1% of a single enzyme should survive $7 \times I_{50}$. Because of its resistance to the compounds this tiny quantity cannot be a candidate for the neuropathy target; it might be an expression of the 92 K DFP-binding protein.

Statement 6

Correlation between blockade of the phosphorylation site and inhibition of the esterase (neurotoxic esterase, NTE) was excellent. Radioassay is technically more difficult and therefore is now only used at key points of experimental advance.

The first correlation was discussed earlier, namely that only one of many hydrolyzable

compounds tested hindered DFP-labelling of the target site in a Michaelis-complex style and that ester was hydrolyzed by NTE.

Effects of inhibitors in vitro and in vivo

A good correlation was found for both parameters (26 observations) [49]. Correlation was maintained for reappearance of NTE assayed by refined versions of both procedures after inhibition *in vivo* [87], and for migration of semi-solubilized NTE activity on a sucrose density gradient [120].

Table 10.1 shows the catalytic centre activities obtained by the standard assay procedures applied to neural and non-neural tissue from a number of species. The sciatic nerve values are subject to the greatest variation because assays were near the technical limit in that study. The overall similarity of numbers for all other tissues is within a threefold range and may be taken as further evidence that the two parameters from which the catalytic centre activity is derived are truly related.

Furthermore, the rate of loss of rectivatibility of inhibited NTE (hydrolase) was identical to the rate of 'ageing' determined by direct radiochemical analysis [26].

Table 10.1 Activity and catalytic centre activity of NTE in various tissue preparations

	Catalytic activity of whole homogenate (nmol min^{-1} g^{-1} tissue)	*Reference*	*Catalytic Centre activity of particulate preparation (mol/min ($\times 10^{-5}$))*	*Reference*
Hen				
Brain	2300–2620	Johnson [59]	2.6; 1.55–1.85; 1.53; 1.95	Williams and Johnson [131]; Williams [129]; Meredith and Johnson [87]; Meredith and Johnson [88]
Spinal cord	550; 340	Johnson [59]; Williams [129]	1.18–1.42	Williams [129]
Sciatic nerve	94–120; 65–94	Johnson [59]; Caroldi and Lotti [11]; Vilanova *et al.* [125]; Rueffer-Turner *et al.*, unpublished data	0.44–1.37	Rueffer-Turner *et al.*, unpublished data
Liver	Not detected*; 250	Johnson [59]; Williams [129]	1.33–1.49	Williams [129]
Spleen	1500; 830	Johnson [59]; Williams [129]	1.30–1.51	Williams [129]
Human				
Frontal cortex	2390	Lotti and Johnson [82]		
Spinal cord	800	Lotti and Johnson [82]		
Femoral nerve	50	Lotti and Johnson [82]		
Placenta	650	Williams [129]	2.60–2.95	Williams [129]
Pig				
Brain	c. 1500; 1330	Johnson [59]; Meredith and Johnson [88]	1.95	Meredith and Johnson [88]
Sheep				
Brain	c. 1500; 1280	Johnson [59]; Meredith and Johnson [88]	1.95	Meredith and Johnson [88]
Guinea pig				
Brain	730	Meredith and Johnson [88]	1.82	Meredith and Johnson [88]
Rat				
Brain	800–1100; 580	Johnson [59]; Meredith and Johnson [88]	2.78	Meredith and Johnson [88]

Catalytic activity of whole tissue homogenates was determined according to Johnson [56]. The list refers only to species for which catalytic centre activities have been determined. For other species see references cited in sections on pp. 102, 105, 106 and by Johnson [59]. Catalytic centre activity of particulate preparations (representing c. 60–90% of whole homogenate activity) was calculated from values of NTE catalytic activity and quantity of target binding site of a common sample pretreated with paraoxon (160 μM) for 20 min at 37°C as recommended by Williams and Johnson [131]. All values except those of Williams and Johnson [58] depend on some form of the 'volatilizable counts' assay

*Not detected, overlooked in presence of very high activity of paraoxon-'sensitive' PV hydrolase

Conclusions

Characterization has been achieved according to many criteria of responses *in vitro* and *in vivo*. The analysis of [^{3}H]-binding sites has led to a more stringent definition of the target site. The esteratic activity assayed as NTE appears to be about 95% homogeneous and the remaining 5% can be discounted since it is unaffected by several neuropathic and/or prophylactic compounds. The target site and NTE appear to be identical by several criteria listed and also according to further evidence discussed in the following major section.

Molecular events at the target

Statements 7–10

Two linked events are necessary for initiation in the adult hen: (1) organophosphorylation of the target protein (more easily monitored as inhibition of NTE), and (2) conversion of the bound group to a charged form by cleavage of one residual group from phosphorus (demonstrated radiochemically for DFP but monitored usually as 'ageing' of inhibited NTE).

Ageing occurs rapidly on NTE for a range of chemical structures: the rates are dissimilar to those with cholinesterases.

Blockade of the phosphorylation site by inhibitory esters which cannot age prevents the two-step initiation and birds become resistant to the neuropathic effects of challenge doses of DFP, etc. for so long as the site is covered by a prophylactic compound.

Prophylaxis is specific for neuropathic effects with no influence on acute cholinergic effects.

Prediction was made that some CB esters structurally related to phenyl phenylacetate would inhibit NTE *in vitro* and *in vivo*. The prediction was confirmed and also the radiolabelling target site was shown to be blocked by such compounds: there was excellent correlation between the two effects [49,67]. Duration of inhibition of NTE *in vivo* by such CBs was short-lived ($t_{1/2}$ = 1–5 h). Therefore, it was predicted that these compounds would be prophylactic agents against the neuropathic effects (but not the acute cholinergic effects) of DFP: the target would be temporarily covered while circulating DFP was being disposed of. The prediction was confirmed [49,67].

Correlated inhibitory effects on the target site and on NTE were also found for another class of analogues of phenyl phenylacetate. Phenylmethanesulphonyl fluoride (PMSF) and n-butanesulphonyl fluoride were inhibitory while the more sterically cramped compound benzene-sulphonyl fluoride was ineffective. The two inhibitory sulphonyl fluorides differed from CBs in that their effects on NTE *in vivo*, lasted as long as those of DFP. Contrary to expectation, neither of the sulphonyl fluoride inhibitors of NTE was neuropathic. However, both were found to be prophylactic even if given several days before (but not after) DFP [49] while the non-inhibitory benzene analogue was neither neuropathic nor prophylactic (unpublished observation). It was concluded that completion of only reaction (1) in Figure 10.1 was an insufficient trigger for initiation of OPIDP but that NTE must be involved in the process.

This conclusion led to the design of di-n-butyl and n-pentylphosphinate esters as non-ageing inhibitors of NTE which also blocked the neuropathy target site *in vivo*. These compounds were sterically very similar to some neuropathic compounds but tests showed them to be prophylactic agents just like PMSF [51]. This finding led to the concept (depicted in Figure 10.1) of the inter-related mechanisms for prophylaxis against OPIDP and for its initiation. The latter involves a two-step process of inhibition of NTE followed by a modification of the structure of the protein (or its environment) by an ageing reaction of the type illustrated in Figure 10.4.

Direct evidence for ageing of inhibited NTE has been obtained using tritiated DFP and one other radiolabelled compound. The time-course is very rapid ($t_{1/2}$ = 3–4 min for DFP) and identical when measured either as loss of reactivatability or as the radiochemical change from a di-isopropyl phosphoryl to mono-isopropyl phosphoryl residue attached to the protein [26]. For numerous other phosphate and phosphonate esters the ageing has been demonstrated as time-dependant loss of reactivatability [27,70].

A particularly convincing proof of the necessity for the second step in the initiation of OPIDP comes from the observed effects of the

enantiomers of either EPN or of its oxon (EPNO). Both isomers of EPNO are active antiChEs *in vitro* and acutely toxic *in vivo*. The anti-NTE activities of the isomers *in vitro* are similar [95]. However, the ageing characteristics of the derived inhibited NTEs are totally different and their toxicological effects match the ageing. Thus *in vivo* the L-(–) isomers of EPN or EPNO produce inhibited NTE which ages rapidly whereas the D-(+) isomers produce an inhibited NTE which does not age in spite of the presence of a potentially cleavable

Table 10.2 Agents which have been shown to act prophylactically against OPIDP without influencing antiChE effects of the challenge agent

Prophylactic agent	*Neuropathic challenge agent*	*Remarks*	*Experimental procedure*[a]
Short-term[b]			
Phenyl phenylcarbamate	DFP		Johnson and Lauwerys [67]
Phenyl benzylcarbamate	DFP		Johnson and Lauwerys [67]
	Mipafox		Johnson [49]
Phenyl N-methyl N-benzylcarbamate	DFP	c	Johnson [49]
Phenyl n-butylcarbamate	DFP	c	Johnson [49]
Long-term[b]			
Phenylmethanesulphonyl fluoride	DFP	c	Johnson [49]; Johnson [51]; Carrington and Abou-Donia [14]
	Mipafox	Rats[c]	Johnson [49]; Veronesi and Padilla [123]
	Leptophos-oxon	c	Johnson [60]
	Diphenyl mono–2–cresyl phosphate		Johnson [49]
	TOCP	c,d	Carrington and Abou-Donia [14]; Carrington and Abou-Donia [18]
	Triphenyl phosphite	c,d,e	Carrington and Abou-Donia [18]
	Haloxon	Sheep[d]	Johnson [*see* pp. 102]
	DFP	Cat peripheral[c,f]	Baker *et al.* [5]; Drakontides and Baker [30]
		Hen peripheral[c,g]	Caroldi *et al.* [12]; Lotti *et al.* [79]
n-butane-sulphonyl fluoride	DFP	c	Johnson [49]
4-nitrophenyl di-n-butylphosphinate	DFP		Johnson [51]
4-nitrophenyl di-n-pentylphosphinate	DFP		Johnson [51]
	2,2-dichlorovinyl di-*n*-propyl phosphate		Johnson [51]
	2,2-dichlorovinyl di-n-pentyl phosphate		Johnson [51]
	Diphenyl mono-2-cresyl phosphate		Johnson [51]
	4-nitrophenyl n-pentyl n-pentylphosphonate		Johnson, unpublished data
2,2-dichlorovinyl di-n-pentylphosphinate	DFP		Johnson [51]
Phenyl di-n-pentyl-phosphinate	DFP	c	Johnson *et al.* [66]
D(+)-EPN oxon	Phenyl saligenin	c	Johnson and Read [68]
D(+)-EPN	cyclic phosphate	c	Johnson and Read [68]
Soman	DFP	c	Johnson *et al.* [72]

[a]For every agent tested at Carshalton except 4-nitrophenyl di-n-butylphosphinate and soman it was established that blockage of the DFP-labelling target site was similar to the NTE inhibition. Protection was assessed by walking behaviour in all cases and also by other means as indicated.
[b]Short-term prophylactic agents were effective provided that challenge was given within a few hours; long-term agents protected against labile challenge agents such as DFP, mipafox or phenyl saligenin cyclic phosphate given at any time up to 4–6 days later
[c]Assessed also by histopathology
[d]Effective only when challenge was not more than 1 day later: some challenge agent persisted in the body beyond 4–6 days
[e]Partial protection only: apparently related to some protection against OPIDP but none against another spinal syndrome with a different histopathological picture
[f]Protection against unilateral peripheral nerve effect of DFP into one femoral artery; confirmation also by electrophysiology
[g]Unilateral and bilateral protection by PMSF injected into sciatic artery(s) of hen against peripheral effect of systemic DFP

R-O-P bond; the L-(–) isomers induce OPIDP but the D-(+) isomers are actually prophylatic rather than neuropathic agents [68,70].

Table 10.2 lists compounds which have been shown to exert protective effects. Doses of prophylactic agents were usually those which gave high inhibition of NTE soon after dosing; in some cases lower doses were also shown to be effective. For many compounds the delay before challenge was extended and it was shown that sensitivity reappeared in parallel with reappearance of NTE after a few hours for short-term agents and up to 6 days afterwards for long-term agents. For labile challenge compounds, such as DFP, mipafox or phenyl saligenin cyclic phosphate, protection was achieved whenever available NTE was <70% of normal at the time of challenge. However, challenge compounds such as TOCP or diphenyl mono-2-cresyl phosphate or haloxon were much less rapidly cleared than DFP and the prophylaxis afforded by PMSF (shown as a 'long-term' agent in Table 10.2) was only effective if challenge with those agents was made within 1 day.

Conclusions

The demonstration of prophylaxis by the compounds listed in Table 10.2 indicates that the molecular target cannot be indifferent to these agents. This does not by itself prove NTE is that target. However:

(1) Prophylactic agents were designed from scratch on the basis of their structural similarity to NTE substrate and do, in fact, both block the labelling site and inhibit NTE.
(2) Close analogues which do not inhibit NTE do not block the target labelling-site and are not prophylactic.
(3) Duration of period for prophylaxis by both short-term and long-term agents coincides with time-courses for reappearance of active NTE *in vivo*.
(4) Neuropathic and prophylactic effects of stereoisomers of EPN and its oxon are completely correlated with stereo-specificity of ageing of the derived inhibited NTEs.

When the above correlations of NTE with prophylaxis are added to the mass of *in vitro* and *in vivo* data in the section 'Dissection of the target for OPIDP' which link the labelling site with NTE, there remains little room for doubt that the initiation target has been correctly identified.

Central and peripheral NTE responses in relation to neuropathy tests

Statement 11
Key experiments have been cross-checked comparing brain with spinal cord and peripheral nerve. No basic differences were found although in a few cases quantitative differences were seen. Inhibition in brain is never less than in the other nervous tissues and brain is a good but not totally precise monitor of the initiation event in all nervous tissue.

Apart from a few terminals in the pons, medulla and cerebellum, nerve endings in brain are not seen to degenerate in OPIDP [21,105,119]. Degeneration is largely in nerve endings of long nerves of spinal cord and peripheral nerve. Convenience dictated the use of homogenates of whole hen brain in early studies.

Comparisons of neural NTEs in vitro

Examination of DFP-binding sites and of PV hydrolases in homogenized spinal cord or sciatic nerve revealed similar patterns of response to the inhibitors paraoxon, mipafox and DFP which were used to characterize the target in brain. However, for hen and human the concentration of target in cord and nerve is lower than in brain (Table 10.1).

The catalytic centre activity of NTE in all tissues determined are very similar (Table 10.1). The sensitivity of procedures for determining M_r of subunit(s) in hen sciatic nerve is inadequate but the size in hen spinal cord and brain of other species is like that for hen brain NTE.

No differences were found in sensitivity of brain or spinal cord NTE to dichlorvos *in vitro* [57]. However, Moretto and Lotti [90] reported that NTE in homogenates of sciatic nerve seemed four times less sensitive than NTE in spinal cord and brain to the di-n-butyl analogue of dichlorvos. Few other comparative tests have been reported for compounds other than paraoxon, mipafox and DFP used to characterize the site.

Comparison of NTE responses elicited in vivo

The assumption that degrees of response to intoxicating doses would be similar in brain and spinal cord *in vivo* seems to have been vindicated [11, 68, 95, 113, 118]. However, a discrepancy was seen after doses of three related dimethyl phosphates, dimethyl phosphorofluoridate, dichlorvos and its monochlorovinyl analogue [57]. Thus assays after a dose of dichlorvos (100 mg sc) showed residual NTE activities of only 18% and 11% in brains but 70% and 47% in spinal cords taken from two dosed birds although homogenized control tissues were equally sensitive to dichlorvos *in vitro*. It was presumed that the differences *in vivo* were a function of the great lability of the test compounds in physiological media from spontaneous and enzyme-induced hydrolysis: this could lead to a lower CT for tissue less well served on first pass of blood. It is interesting that when dichlorvos was given as a 50% commercial formulation no discrepancy was found between the inhibitions in brain and spinal cord [10]; presumably the formulation adjuncts slowed the processes of metabolic disposal of dichlorvos and allowed a more uniform distribution of the compound to nervous tissue. Inhibition of NTE in spinal cord was 25–30% less than in brain after a high dose of a triaryl phosphate mixture [118].

Statement 12

Initiation of severe neuropathy requires that about 80% of the phosphorylation site in spinal cord and brain be transformed to the aged inhibited form soon after dosing.

There are few published studies in which responses in all three neural tissues have been monitored. The earliest study by Caroldi and Lotti [11] showed similar tissue responses after doses of DFP or PMSF (three birds each), but 65% inhibition in spinal cord and sciatic nerve compared with 78% in brain after one triaryl phosphate. A recent study involved 36 assayed and 51 observed hens dosed with one or other of six related phosphoramidates [71]. At effective doses, inhibition of NTE in spinal cord and sciatic nerve was 5–15% less than in brain. Unambiguous clinical effects of grade 3 (grade 8 being maximum severity) were produced in about 90% of observed birds when the inhibition of NTE in pair-dosed assayed birds was >90% in brain, > 85% in spinal cord or >75% in sciatic nerve. These values seem slightly above the threshold which I have indicated in the past based on experiences with a variety of other compounds: the reason is not apparent. However the phosphoramidates studied were of an unusual molecular structure which may have modulated the effect. Unlike the classical neuropathic phosphorodiamidate, mipafox, the single amido-group in these compounds was not N-substituted; it is possible that this feature could affect the net charge generated by an ageing reaction of inhibited NTE.

In the few cases noted above where spinal cord inhibition did lag behind inhibition of brain NTE, the clinical response correlated with the former with signs not being seen unless spinal NTE was much inhibited. The lower sensitivity of sciatic nerve than spinal cord to di-n-butyl dichlorovinyl phosphate noted above leads to threshold doses of the compound causing only spinal neuropathy without significant signs of peripheral effect: NTE responses matched the clinical effects and the full syndrome with appropriate NTE inhibition was elicited by higher doses [90].

The need to consider ageing of inhibited NTE

The protective effect of some isomeric forms of phosphonates [72,68] was not envisaged when statement 12 was written. Experience of significant rapid ageing of NTE inhibited by a variety of compounds [27] masked the possibility of a partial-only ageing when a compound is administered which consists of a racemic mixture of isomers. It seemed the assumption could be made – 'if it could age (according to molecular structure) then it would'. This still seems valid for phosphates, chiral or otherwise, as in any steric configuration one remaining bond in the molecule of inhibitor which is attached to the active site will be oriented towards the site which receives the group which is cleaved from phosphorus. There appears to be no exception. However, toxicity tests for other molecular structures should include a measure of ageing if NTE is found to be inhibited.

NTE assays for regulatory purposes

Inclusion of assays of NTE responses have been recommended to improve existing safety

evaluation tests required by law [93,94,128]. For such formal studies one suggested protocol which integrates NTE assays with clinical and histopathological tests to give more data from fewer animals has been published [61]. The position to 1982 was summarized [53,59] and recent examples which verify the predictive value of the NTE assays include pesticides [33,36,115], nerve agents [41,72,130] and non-pesticidal compounds [73,111,118].

Some of the above investigations did not use the large groups of birds as required by the regulators. However, excellent correlation of NTE assay results was obtained with clinical and histopathological data in toxicity tests on pirimiphos-methyl and on EPN performed under GLP for regulatory purposes [75] (Johnson and other, unpublished data).

Conclusions

For coarse screening of a test compound, monitoring only of brain responses is adequate (particularly if the effect in brain is very small). Further analysis of the NTE response in other tissues and of ageing may be desirable if more precise dose/responses are required in a full toxicological evaluation. Clearly if inhibition is negligible in any test then the question of ageing does not arise and a positive statement of a quantitatively *negative* response can be made. Such a valuable conclusion could not be drawn from any other test procedure however lengthy.

NTE responses in other sensitive species

Statement 13

Limited studies in sheep and cat show that NTE of these species: (1) is similar *in vitro*, (2) responds to neuropathic doses *in vivo* and (3) is blocked by protective compounds at doses which do indeed protect against neuropathic challenge.

Sheep, cats and dogs are sensitive to OPIDP induced by sufficient of a single dose of certain OP esters [52]. Data for these species is not extensive but confirms the Statement [20,35,52,59,113,117].

Statement 14

Human and hen brain NTE responses to inhibitors are similar.

Using fresh frozen autopsy tissue the I_{50} for some 20 compounds, with a wide range of structures was determined. Values for human tissue were approximately within threefold of those for hen tissue [81].

Extension to tissues other than whole brain

Dudek [31] and Dudek and Richardson [32] detected NTE activity in spleen, lymphocytes and various other hen tissues and also in human lymphocytes and platelets: similar degrees of inhibition were observed in *ex vivo* samples of hen brain and lymphocytes 24 h after doses of several compounds. A wide distribution of NTE in regions of human brain and in other tissues has been reported and rapid ageing of DFP-inhibited human NTEs has been demonstrated [82,89]. NTE activity can be preserved in stored frozen human lymphocytes according to Bertoncin *et al.* [7] although Maroni and Bleeker [85] had less success with different conditions of storage. Close agreement was found for effects of eight compounds on NTE measured *in vitro* in lymphocytes isolated from fresh human blood and in autopsy samples of human brain [87]. Additionally, in two cases of fatal human poisoning by OPs the NTE responses of lymphocytes drawn before death proved an accurate indicator of the effect on neural NTE as measured in post-mortem samples; the agents were respectively chlorpyrifos causing 50–70% inhibition [96] and omethoate causing negligible inhibition [80]. Moreover, in a patient who survived a suicide attempt involving ingestion of chlorpyrifos, detection of substantial inhibition of lymphocytic NTE during the early days of recovery from acute effects correctly predicted the onset of a delayed peripheral neuropathy [83].

Repeated monitoring of lymphocytic NTE in workers occupationally exposed to OP pesticides as suggested by Dudek and Richardson [31] has indicated in some workers significant effects which may be related to sensory defects [77,97].

Conclusions

NTE of different sensitive species appears to behave similarly towards inhibitors in so far as tests have been performed, and human inhibited NTE ages. The limited data from human

poisonings suggests that substantial rather than marginal inhibition of NTE is required to initiate OPIDP in man.

What happens after formation of MI-NTE?

Statement 15
The physiological disturbance set up by this initial change (on NTE) is unknown.

Esteratic activity of NTE is not essential to health of axons

Prolonged inhibition over 2–3 weeks by repeated doses of PMSF which forms an unageable UI-NTE exerts no apparent deleterious effect: the only effect detected is transformation of the PMSF-treated animal into one resistant to the OPIDP insult [49].

What aspect of ageing of inhibited NTE is important to initiation of OPIDP?

It seems reasonable that generation of a fixed negative charge on the residual OP group attached to protein may be deleterious to some normal physiological function of the protein or in its immediate environment. However this is not the only possibility. Thus a cross-link might be formed from phosphorus to another point in the molecule. Again it is known that cleavage of the alkyl group from phosphorus in DFP-inhibited NTE involves the rest of the protein molecule which actually traps the 'R' group with 100% efficiency [26,131]. Such an intramolecular transfer appears unique to NTE among brain enzymes [129]. A possibility was, therefore, that the site 'Z' on NTE which actually receives the 'R' group may have a normal and essential physiological function so that initiation of OPIDP follows from blockage here rather than by generation of the charged group. However, it has been shown recently that some phosphoramidate inhibitors of NTE induce OPIDP by a modification of the catalytic centre without transfer to site 'z' [71a].

Does MI-NTE block control processes involving protein kinases?

It was suggested that the negative charge on aged inhibited NTE might disrupt processes normally controlled by cAMP-modulated protein kinases, possibly by causing the system to remain in 'ON' or 'OFF' position [55]. However, no influence of ATP ± cAMP on the rate of phosphorylation of NTE by labelled DFP has been found [59]. Also, preincubation with DFP did not affect *in vitro* labelling by ATP of certain cAMP-sensitive sites in brain [4]. However the possibility that there is some effect on cGMP-sensitive sites has not been excluded.

A series of reports exist on changes in Ca^{2+}/calmodulin-dependant protein phosphorylation which develop in parallel with histologically detectable changes 7–21 days after dosing with TOCP [2,100]. The changes were suggested to have arisen from changes in the activity of a protein kinase in response to physical disruption of the neuron. However, some very variable changes were reported as early as 1 day after dosing [99]. There seems scope for clarifying whether these apparent early changes are in the causal chain of events. Direct effects of neuropathic compounds added *in vitro* to these systems were not found [100]: any effect mediated via a primary attack on NTE would not have been seen as NTE was covalently blocked in these experiments by PMSF added as an anti-protease.

Initiation appears to be axonal rather than in cell bodies

In cats a mild unilateral neuropathy has been produced by intra-arterial injection of DFP [39,84]. Tracer studies with [^{3}H]DFP confirmed that label was bound to the nerve of the injected leg far more than elsewhere [43] and pretreatment of the cats with PMSF systemically blocked the neuropathic effects but not the early acute effects of DFP [5,30]. A similar study in hens by Caroldi *et al.* [12] produced a high degree of inhibition of NTE in the sciatic nerve axon in one leg with negligible inhibition in the contralateral nerve, in spinal cord and in brain: a typical unilateral peripheral neuropathy developed thereafter. Also PMSF applied bilaterally via sciatic arteries protected against the usual peripheral flaccid paralysis effects of systemic DFP while leaving the hens with a distinct spinal and cerebellar syndrome [79]. The birds were severely ataxic but leg movements could be performed and a simple leg retraction reflex

was preserved which is not the case in the fully developed OPIDP syndrome. Thus the central and peripheral effects in OPIDP can be elicited separately and the target in the latter case appears to be axonal NTE as the cell bodies received negligible dose.

Effects which precede clinical signs

Electrophysiological measurements on the sciatic nerves of hens 24 h after a neuropathic dose of di-n-butyl 2,2-dichlorovinyl phosphate indicated a significant increase in the threshold of excitability which did not occur if the birds were pretreated with a prophylactic phosphinate [109]. Also 4 days after initiation the responses of the biventor cervicis muscle to either indirect stimulus through the nerve or direct application of ACh indicate that muscle and nerve are no longer in a normal functional relationship [34]. The latter observation implied that a partial 'biochemical transection' of the nerve had occurred and that further development of a Wallerian-type degeneration was to be expected. However, unlike physical transection, it may be that not every fibre in an axon ceases to function after moderate doses of the agent so that a range of clinical severity can be expected.

There was an early and progressive deficit of retrograde axonal transport of iodinated tetanus toxin from the gastrocnemius muscle of hens which had been dosed previously with the very neuropathic di-n-butyl 2,2-dichlorovinyl phosphate [91]. The rate was reduced to almost 30% by 7 days after dosing with intermediate effects on days 2–5 although not all the intermediate values were statistically significant owing to some variation in control values. It cannot be said whether these changes were causative or only early expressions of axon degeneration. However they are certainly directly related to OPIDP as they were: (i) maximal before onset of clinical or histopathological indications of OPIDP, (ii) dose-dependant and related to greater than 70% inhibition of NTE in the nerve, (iii) dissociated from the trivial anti-AChE effect of this compound because it could not be produced by a dose of paraoxon of equal antiChE potency, and (iv) totally prevented by predosing the hens with PMSF.

The observed decrements in fast retrograde transport stand in marked contrast to the reported absence of significant abnormalities in anterograde axonal transport of peripheral nerves in the early stages of OPIDP in cats or hens [8,20,45,101], although a defect in anterograde axonal transport in the most distal parts of the axon has yet to be excluded. Only Reichert and Abou-Donia [106] report changes in anterograde axonal transport: this was in the rat optic nerve after local injection of TOCP or some phosphonothioates with a P=S bond. It should be stressed, however, that degeneration of optic nerves is not a usual finding in the neuropathy and that TOCP and P=S phosphonothioates require metabolic activation, while the extent of activation capacity in the rat optic system is unknown. Furthermore, the rat is not the animal of choice for OPIDP studies.

Several reported observations have defective experimental design or have not been substantiated. These include phospholipid metabolism, blood copper status, striatal dopamine levels or effects in cell culture [38,40,42,74,78,103,127].

Meaningful experiments in this realm require that: (1) Investigations should focus on the early events after a single dose to a susceptible species; it is necessary to distinguish causal events from responses to the degeneration process. (2) Neuropathic agents that are directly active without needing metabolic conversion should be used. (3) Compounds with as low as possible antiChE activity should be used and control birds should be dosed with non-neuropathic agents having antiChE activity similar to that of the test compound. (4) Food intake should be kept similar (and preferably normal) in dosed, control, and normal birds. (5) Further control birds should be predosed with a protective agent such as phenylmethanesulphonyl fluoride before the neuropathic agent; this will allow irrelevant responses to be observed and discounted.

Similar rules can be drawn for *in vitro* studies with the added proviso of setting sensible limits to exposure.

Conclusions

It is encourageing that several biochemical effects have been detected during the first

week after intoxication and inhibition of NTE and before frank clinical signs. However, the controls suggested above were applied in only one case. It is difficult to devise experiments which will show whether these effects are in the causal sequence from initiation at NTE or are only early signals of the process of axon degeneration or response to injury.

A review of questions about NTE and OPIDP

Five questions were listed [58] to highlight aspects of OPIDP which had not been clarified by the molecular studies on NTE. Progress in clarifying the answers has been variable and each is discussed in turn but in a different order from that in which they were presented originally.

Question 1. Why use brain to predict events in spinal cord and peripheral nerve?

The similarity of responses of NTE in all neural tissues to inhibitors *in vitro* and *in vivo* was reviewed on pp. 100–101. There do not appear to be fundamental differences which would invalidate interpretations about mode of action. The decision on whether to examine NTE in all target tissues or only in brain in a toxicity test can, therefore, be based on the stringency required. For sighting studies or tests with negative responses, NTE assay only in brain is convenient and acceptable. Going to measurements in spinal cord and/or sciatic nerve will yield increasing amounts of quantitative data without sacrifice of extra birds and is appropriate if the purpose is to identify the exact dose likely to elicit clinical neuropathic effects.

Besides the similarity in response to inhibitors, the rates of reappearance of active NTE in the three tissues after inhibition by several compounds appeared to be similar [11]. However, after complete inhibition by phenyl di-n-pentylphosphinate *in vivo* the UI-NTE disappeared and active NTE reappeared at rates which were identical, but t½ for brain was 2.1 days while for spinal cord it was 3.6 days [87]. Both these rates were markedly faster than after inhibition by DFP which formed unreactivatable MI-NTE or after PMSF where the inhibited NTE appears to be resistant to reactivators [11,51]. Presumably spontaneous reactivation of the phosphinylated NTE as well as *de novo* synthesis contributes to the faster rates but there is no obvious reason why the rates in brain and cord should differ.

Question 2. Why the species difference?

Not all mammalian or avian species are overtly sensitive to the clinical neuropathic effect of a single high dose of OP esters which are effective in the hen. Some are sensitive to repeated doses of varying duration while clinical expression of the syndrome in rats and several other rodent species is very difficult to achieve [52,59]. Six-month old rats do show clinical ataxia (Lotti, unpublished).

Do insensitive species have less or more or different NTE than sensitive ones?

Rat and hen brain NTEs were compared *in vitro* by Novak and Padilla [92]. No significant differences were found in inhibitor-sensitivity, activity/pH relationships or size of DFP-labelled subunit.

Clinically insensitive rats and mice have somewhat less NTE than sensitive mammals such as sheep and pigs [59] but no correlation exists when comparing birds, thus bobwhites have more than hens while Japanese quail have less but both bobwhites and quail are insensitive [9].

Is species insensitivity a result of failure to generate/deliver enough proximal toxin to the target?

Although such a possibility has been mooted it only became an answerable question when target assays came into being. Soliman [113] showed that brain NTE of rats and mice given 30 daily oral doses of leptophos (60 mg/kg) was only reduced to about half of control values and no clinical OPIDP ensued. In contrast lesser doses to four sensitive species, namely chicken, Peking duck, dog and sheep, caused 71–87% inhibition and clear clinical OPIDP. These experiments support a positive answer to the question posed above. However, in old Fisher rats given repeated doses of d-n-pentyl 2,2-dichlorovinyl phosphate, inhibition of brain NTE was maintained around 80% for 12 days without clinical OPIDP developing in the weeks thereafter; similar results were

found with phenyl saligenin cyclic phosphate given to marmosets [60]. Likewise, Bursian *et al.* [9] gave up to eight times the dose of TOCP necessary to cause 80–90% inhibition of brain NTE in both bobwhites and Japanese quail without eliciting a clinical response. Therefore different toxicokinetic patterns do not alone account for differences in sensitivity.

Does inhibited NTE of insensitive species not age?

Rat brain NTE inhibited by DFP aged rapidly by the criterion of loss of reactivatibility [59] and both rat and guinea pig NTE (as well as NTE from sensitive sheep and pigs) also aged according to the radiochemical assay of volatilizable counts [88].

Axon degeneration does occur in some apparently insensitive species

The apparent anomalies noted above were partially resolved by further studies in rats. It was shown that, although no clinical signs developed, lesions typical of OPIDP were found on careful histopathological examination of spinal cord and peripheral nerve of rats given a single dose of either TOCP or mipafox [98,124]. In these experiments NTE responses correlated with dose, and inhibition in brain and spinal cord was 70–80% after the threshold doses. Furthermore, predosing with PMSF inhibited NTE and protected against OPIDP exactly as in the hen [123].

Conclusion

The rat is not the only species which appears clinically insensitive to one or more doses of neuropathic OP esters [37,52–59]. In view of the data now available for rats it seems that the apparent lower sensitivity of these species does not signify that the initial molecular events at the target are fundamentally different from those in sensitive species. Veronesi [122], who gave the first adequate report of lesions in the rat, suggested that the lack of clinical signs might be related to a greater ability to repair or adapt to peripheral nerve damage in the rat as compared with the hen.

Question 3. Why the age difference?

Johnson and Barnes [65] reported that inhibition and reappearance of NTE in chick brain was similar in time-course to that in the adult but that clinical effects only followed multiple doses involving prolonged high inhibition of NTE. The realization noted above that dosed rats may go through all the stages of inhibition up to axonal damage without clinical expression invites a more thorough histopathological examination of dosed young birds to see if the same situation occurs.

Question 4. Why the delay with concomitant reappearance of NTE between initiation and appearance of degenerating axons?

A certain degree of alkylation of some sites in DNA by one-shot carcinogens is generally considered to be a sufficient stimulus or switch to set in motion the whole process of carcinogenesis. However, tumour expression may be delayed for months and the alkylation molecules apparently disappear soon after dosing. Arguing by analogy the reappearance of active NTE before clinical expression of OPIDP may be puzzling but is no reason at all for objecting to the relevance of NTE to initiation.

Does MI-NTE persist in the nervous system?

At present no analytical method is sensitive and specific enough to trace MI-NTE *in vivo* for long periods after dosing to see if it persists. Using a reactivation procedure, di-n-pentylphosphinylated NTE (a form of UI-NTE) has been followed and shown not to persist: it disappeared from both brain and spinal cord at the same rate as fresh NTE appeared [87].

Possible involvement of axon/cell body relationships

It was noted on p. 103 that localized delivery of DFP or PMSF to distal regions of nerve could elicit appropriate responses without insulting the NTE in cell bodies and that decline in retrograde transport was a comparatively early event. It can be speculated that the delay period covers the period before 'information' normally carried to the cell body by retrograde flow begins to fail and that positive 'instructions' to degenerate are not sent out until that time, which appears to be about 1 week according to Moretto *et al.* [91]. Alternatively the failure of retrograde flow

might be construed as a failure of a signal calling for further supplies essential to the maintenance of the axon. No experiment to distinguish between these concepts has been reported.

Question 5. What is the physiological function of NTE?

Although prolonged inhibition of NTE without ageing does not initiate neuropathy or other obvious effects this does not actually mean that the esteratic activity of NTE is not part of a normal physiological process but only that such a process is not vital or rate-limiting. It may be that the system can survive long periods with only a small proportion of its esteratic capacity or that the substrate can be channelled through another metabolic route. However, no obvious naturally occurring analogues of the best NTE substrates seem to be candidates for a 'true' substrate (*see* lists in Johnson [59]) and the normal function of NTE remains obscure, but see conclusions below.

While OPIDP starts with inhibition of NTE the undesirable event appears to be some subtle change in the whole molecule as UI-NTE is converted to MI-NTE: the subsequent effects brought about by the presence of MI-NTE or of phosphoramidated NTE [71a] (see p. 103) may have no relationship to the esteratic activity. NTE is not the only esterase whose catalytic activity seems superfluous. Thus AChE has a vital function in nervous tissue but no conceivable function in RBC; BChE (PsChE) in nervous tissue has no identified function and can be inhibited *in vivo* for a considerable time without apparent effect [128].

A possible way to identify the physiological significance of NTE is to determine whether the site (Z) which is involved in ageing of UI-NTE has any function. However, attempts to attack this site *in vivo* with 4-bromophenylacetylurea or with alkylating agents related to NTE substrates have failed. Further work in this direction seems desirable.

Purification of NTE to the point where protein chemistry and immunohistochemical and molecular biological studies can be performed, is a priority in the author's laboratory. By a combination of protein purification techniques, preparations free of other DFP-labelled proteins and enriched about 650-fold compared with original brain homogenate have been obtained [110,110a]. Complete purification requires about a further 60-fold enrichment but this is not essential to the raising of antibodies for use in larger-scale affinity chromatography and for study of the intracellular location, etc.

Conclusions

Questions concerning differences between different neural tissues, species and ages seem resolved in principle if not in all details. The reason for delay in onset of neuropathy remains obscure. There is recent evidence that NTE has a vital role in the response to, or repair of, axonal damage induced by nerve crush or some chemicals [67a,90a]. It is hoped that the studies listed above will lead to understanding more about NTE in both its normal state and in its modified neuropathic state.

Other recent studies of NTE

Structure-activity studies and searches for alternative substrates or inhibitors for NTE assays

Early studies with many compounds [53,54] led to some general criteria for inhibitors having high affinity for NTE. The principal criteria are intermediate alkyl or aryl group size, with an optimum around benzyl or n-butyl, and small/flexible leaving groups (fluoride or dichlorovinyl) in the phosphorus esters. Further studies have examined phosphates, phosphonates, phosphinates, phosphoramidates, sulphonates, sulphamates and CBs with a variety of leaving groups and a range of potential reversible inhibitors [59,64]. Benzenesulphonyl fluoride has some advantages over paraoxon for eliminating non-NTE esterases but is ineffective in removing some DFP-binding proteins such as AChE which is sensitive to paraoxon. No adequate substitute for mipafox as a selective inhibitor has been found; ethyl 2,6-dichlorophenyl phenylphosphonate [107] does not appear to be as specific as originally claimed [see 64].

Several compounds have been suggested as substrates instead of PV. While 4-nitrophenyl valerate [114] may have an advantage in some kinetic studies it is rather unstable and less sensitive and specific than PV; three of 14 carboxylate esters structurally related to PV

were hydrolyzed faster in a standard NTE assay [64]. However, the proportion of 'non-NTE' to 'apparent NTE' was higher than for PV so they are unlikely to be useful except for assay of part-purified NTE.

Solubilization and purification of NTE

Several variations of detergent and ion concentration provided apparently soluble active NTE but further purification was negligible [6,25,29,44,50,102,103].

Recently the combination of molar NaCl with non-ionic detergent plus lipid has enabled a purification greater than 600-fold of [^{3}H]DFP-labelled NTE [86,110,110a]. Also there is a preliminary report of purification by affinity chromatography in a similar medium [121].

Although many organic solvents inactivate NTE, Schwab *et al.* [112] found that treatment of brain microsomes with DMSO (40% v/v in water) appeared to solubilize one-third of the 67% NTE activity surviving along with half the protein; inhibitor characteristics of the solubilized material were unchanged. Interestingly, we have failed in our laboratory to extract any [^{3}H]DFP-labelled NTE with DMSO, which suggests that the MI-NTE generated after DFP-inhibition may be in a changed environment in the membrane; this may, indeed, indicate the change which is brought about by ageing of UI-NTE and which initiates the whole chain of events leading to OPIDP.

Other basic studies of NTE

Studies of kinetics of heat-inactivation of all PV hydrolases present in buffer homogenates of hen brain, in microsomes suspended in buffer or in DMSO, and in solubilized preparations, show that biphasic reactions occurred with similar proportions and rate constants regardless of the particular enzyme concerned [108]. As the relative amounts of activity lost in the fast and the slow phases depend on temperature, it was concluded that there was an underlying heat-induced structural change in all cases rather than that each enzyme was present in multiple forms.

Kinetic studies of the inhibition of NTE by mipafox and radiation-inactivation studies have already been mentioned (Dissection of the target for OPIDP, pp. 91–98). NTE is transported relatively fast (approximately 300 mm/day) in hen sciatic nerve [16].

In preliminary studies it has shown that, in MI-NTE obtained from [^{3}H]DFP, the bond which links the isopropyl group directly to protein (i.e. not through phosphorus) is fairly labile at pH 1–2 as well as at pH 9 (Rueffer-Turner, unpublished data). This pH profile may be compatible with the group being bound to a side-chain carboxyl group in the protein. The acid-lability hinders attempts to obtain suitably labelled peptide fragments for identification.

General conclusion

The history of the elucidation of the role of NTE in initiation of OPIDP illustrates the value of testable hypothesis and of attending to apparent anomalies [62]. The previously laid foundation of understanding of OP ester interactions with esterases has led from active-site labelling to (1) competition in formation of Michaelis complexes, (2) use of inhibitors to dissect esteratic activity, (3) substrate structure-activity studies, (4) prediction of prophylactic compounds (and of ineffective analogues), (5) prediction of the necessity for ageing, (6) detection of a unique intramolecular ageing reaction and (7) stereospecific toxic or prophylactic properties within a pair of enantiomeric phosphonates. At every point the structure-activity and kinetic studies have identified the target and correlated the large polypeptide (M_r approximately 155 K in SDS) and catalytically active NTE in constant proportions with a catalytic centre activity in excess of 10^5 per min. From this a very large number of NTE-based neuropathy tests of chemicals have been successful and several apparent anomalies have been dispelled.

'Proofs' seldom progress beyond an overwhelming weight of evidence and casuistic argument can always be advanced. Carrington [13] found no evidence for anything but NTE as the target for initiation of OPIDP but wondered whether the 'true' target might be a previously undetected protein with characteristics virtually identical to those of NTE but which had been overlooked for technical reasons. The examination of homogeneity in

this chapter should dispel any lingering doubts and there appears now to be no valid reason to doubt that NTE as normally characterized is the target for a 2-stage initiation of OPIDP by neuropathic OPs and phosphonates. The process is illustrated in Figure 10.1.

Our present ignorance of the biochemical steps after initiation of OPIDP does not limit the value of NTE assays to toxicological evaluations. Isolation and purification of NTE is progressing and there is hope that subsequent immunohistochemistry and molecular biological studies of the location and function of NTE will assist the understanding of processes which maintain long axons of healthy neurons. These are proceeding with studies showing a role of NTE in responses to axonal damage or repair thereof [67a,90a].

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11

Validation of a rodent model of organophosphorus-induced delayed neuropathy

Bellina Veronesi

Introduction

Exposure to some OPs may produce a neurological response distinct from the characteristic cholinergic crisis. After a quiescent period of 2 – 3 weeks following OP exposure, sensory-motor deficits develop, affecting the distal extremities and reflecting a selective degeneration of long and large fibre tracts of the spinal cord (CNS) and peripheral (PNS) nervous system in a 'dying-back' pattern of degeneration [11,12]. Known as OP- induced delayed neuropathy (OPIDN), it has occurred in epidemic proportions in areas where the unrestricted use of pesticides is commonplace. Little is known about the pathogenesis of OPIDN. This is due, in part, to the almost exclusive use of chickens to describe the pathological and biochemical changes induced by OPs. To most investigators the chicken represents an awkward test species with a sparse biochemical, pharmacological, and neuroanatomical database. Because of its sensitivity to OPIDN, however, it is used exclusively to differentiate OPIDN-producing compounds. The delayed neuropathy is readily produced in chickens, cats and farm animals [5,46,48] but rodents are considered neurologically insensitive because of their failure to develop hindlimb paralysis after exposure to neuropathic OPs [1,4,44]. Various notions have been proposed to explain the insensitivity of the rat, including species-specific nervous tissue [11,24], differences in the rat's pharmacokinetics, and metabolic handling of OPs [2,16,20], and qualitative differences in the target enzyme [24,48,49]. This alleged rodent resistance to OPIDN has been reassessed, and rats exposed to single or multiple doses of OPs have been shown to develop neuropathology and biochemical endpoints typical of OPIDN but remain resistant to the ataxia, refuting the notion that the target tissue of OPIDN is species-specific. Our experiments have shown that pretreatment with hepatic metabolic inhibitors greatly increases rodent sensitivity to OPIDN in both CNS and PNS, suggesting that interspecies differences in the metabolism of TOCPs do play a major role in the neuropathic expression of this neuropathy. These experiments with rats have demonstrated that, as in cats, chickens and humans, NTE inhibition can be used to predict neuropathic damage and provide a biochemical marker for the rodent model.

Distribution of neuropathic damage in rat OPIDN

Triortho-cresyl phosphate (TOCP), a neuropathic OP, came into notoriety in the 1920s after being identified as the culpable agent of the 'Ginger Jake' epidemic [46]. Much data exists on TOCP neurotoxicity, and it is considered a model OPIDN-producing chemical. In the first experiment [52] Long Evans male rats (250 g) were given a biweekly high oral dose of TOCP (1160 mg/kg) or daily low (116 mg/kg) doses. High-dosed rats were pretreated with atropine sulphate (10 mg/kg) 20 min before TOCP exposure and 8 h after. With such prophylactic treatment, all rats survived. Tissue was taken for pathology at 2 weeks and at intervals of 6 weeks. Although neuropathic

damage occurred as early as 2 weeks (see later), neurological dysfunction was not observed until after 12 weeks of exposure to high doses of TOCP. Affected rats developed hindlimb splay, a noticeable 'heel-walk' and, in some instances, overt 'criss-crossing' of the hindlimbs when lifted by their tail, a dysfunction described in mercury poisoning and related to severe loss of sensory fibres [13,17]. Histopathological examination of Epoxy 1 μm sections indicated that severe spinal cord damage occurred as early as 2 weeks after a single high dose of TOCP and was localized in the upper cervical cord (C2-C5) dorsal columns (fasciculus gracilis), an area combining large diameter sensory nerve fibres [61]. In the lumbar cord, only a scattered distribution of degeneration was seen in the ventrolateral columns, which contain distal ends of various descending tracts [62]. Microscopically, this damage consisted of degenerated nerve fibres, myelin debris, severe astrocytic proliferation, hyaline bodies and giant axonal swellings (Figure 11.1) which by electron microscopy contained accumulations of fragmented smooth endoplasmic reticulum (i.e. tubulovesicular profiles) and intra-axonal vacuoles. Such axonal lesions were ultrastructurally identical to those described in humans and in experimental models of OPIDN

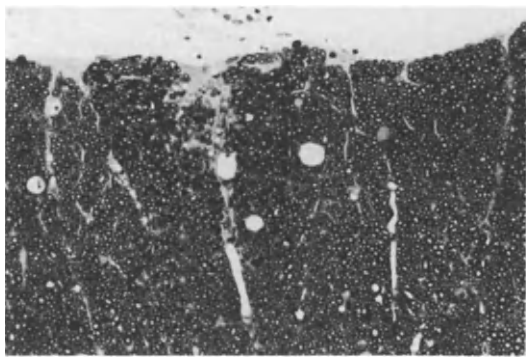

Figure 11.1 Light micrograph of the cervical cord dorsal column from TOCP-treated rat (TOCP 1160 mg/kg, 6 weeks). Note myelin ellipsoids and other evidence of axonal degeneration. Cord damage characteristically involved the large diameter ascending tract fibres of the fasciculus gracilis in the cervical region. Epoxy resin, semi-thin section. Magnification × 1200, reduced to 77% in reproduction

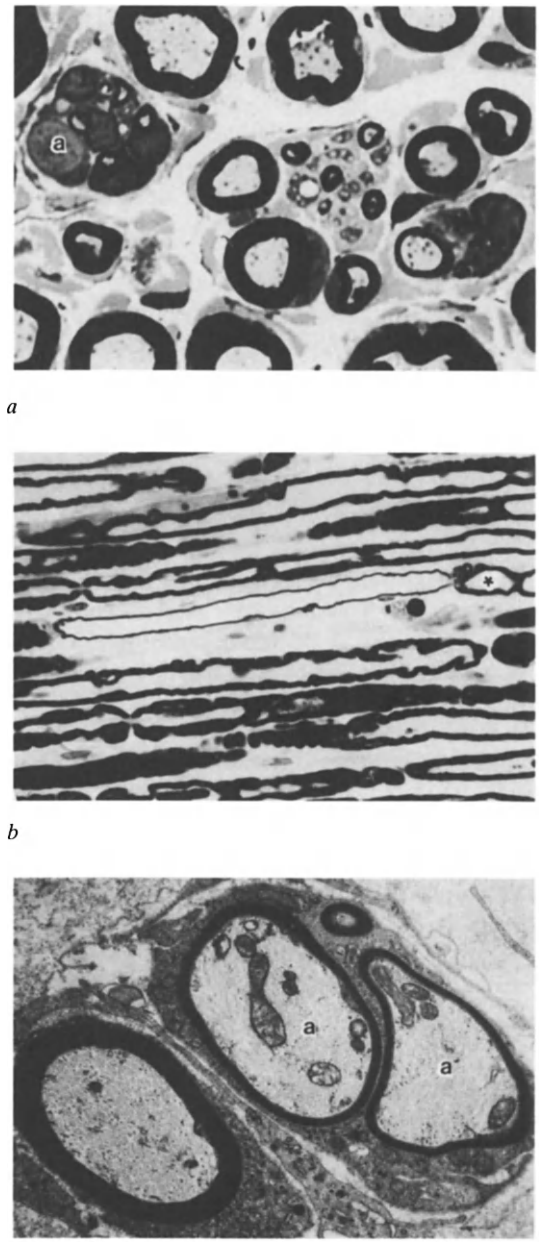

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Figure 11.2 (a) Clumps of myelinated and non-myelinated fibres housed within common Schwann-cell cytoplasm as evidence of PNS regeneration throughout early exposures. Note swollen axon (a). (b) Lengths of axons covered with disproportionately thin myelin suggested segmental remyelination. Note adjacent internode (*) with normal myelin thickness. (c) Depicts several regeneration axons (a) surrounded by thin, newly formed myelin. (a,b) Epoxy, semi-thin section. Magnification × 1500, reduced to 77% in reproduction. (c) Electron micrograph. Magnification × 18 500, reduced to 77% in reproduction

[6,7,41]. The PNS at 2 weeks exposure was largely devoid of degenerative changes; a salient feature during early (2–6 week) exposure was excessive axonal sprouting and other evidence of regeneration and remyelination (Figure 11.2). At later stages of intoxication, however, extensive degeneration was noted including giant swollen axons, collapsed myelin sheaths, myelin ellipsoids and fragmented axons. Examination of teased nerve fibre preparations showed progression of morphological changes as individual PNS nerve fibres degenerated in response to TOCP. The proximal length of the fibres appeared relatively preserved with giant axonal swellings interfaced between normal internodes and degenerated fibre lengths (Figure 11.3). This preservation of the more proximal lengths, with distal degeneration, supported a 'dying-back' classification similar to the pattern of neuropathy seen in chickens, cats and humans. A delay period of 14–21 days is considered requisite for ataxia and neuropathic damage in the hen model of OPIDN [1]. However, using contemporary ultrastructural techniques on the rat, we documented the initial outset of tubulovesicular axonal swellings in the cervical cord and vacuolated neuromuscular junctions within 48 h of exposure to acute high doses of either DFP or TOCP (Figures 11.4 and 11.5). This demonstration suggested a very rapid translation from biochemical (NTE) inhibition to neuropathic damage in the rat.

These experiments suggest that although resistant to ataxia, the rat expresses neuropathic degeneration similar to that seen in conventional test species such as the chicken. This damage commences in the distal ends of

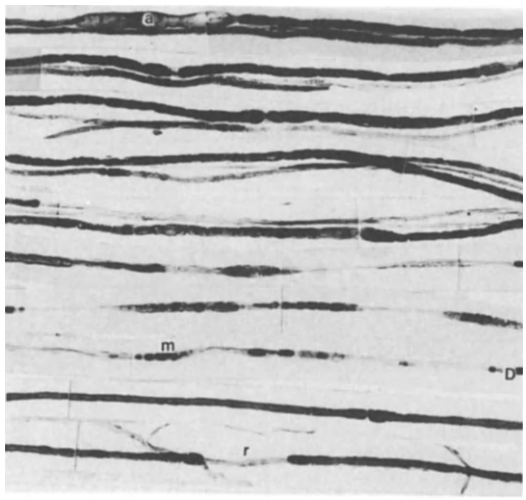

Figure 11.3 Consecutive lengths of a single PNS fibre teased from the LRLN of an animal exposed to intermittent doses of TOCP (1160 mg/kg) for 12 weeks. Changes noted in more proximal lengths of the fibre included elongated nodes of Ranvier (r), and giant axonal swellings (a). More distally (D), degenerative changes include myelin ellipsoids (m). Note remyelinated internode. Magnification × 1500, reduced to 56% in reproduction

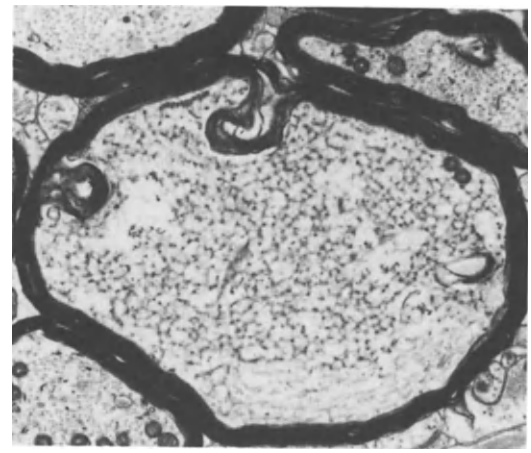

Figure 11.4 Electron micrograph of typical axonal swelling filled with tubulovesicular profiles. Rat dosed with TOCP 2360 mg/kg at 48 h. Magnification × 12 500, reduced to 77% in reproduction

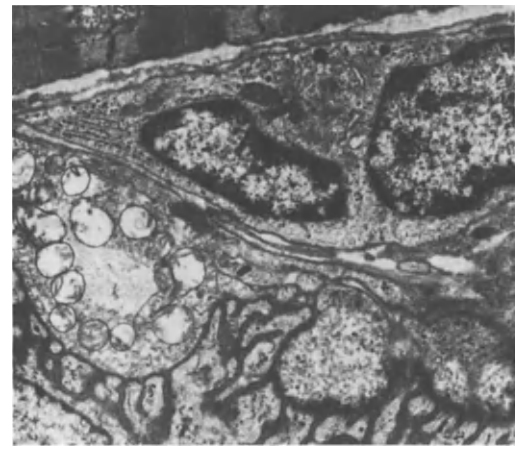

Figure 11.5 Disrupted neuromuscular junctions taken from DFP (2.0 mg/kg) treated rat at 48 h. Note disrupted mitochondria and neurofilamentous accumulation of affected nerve terminal compared with less affected axons. Muscle is relatively normal. Magnification × 16 000

larger diameter ascending and descending spinal cord tracts. In the PNS, selective vulnerability of larger diameter (tibial branches) over small diameter (plantar nerve) fibres are typical of the neuropathy in a 'dying-back' pattern [12]. Thus, early investigators incorrectly labelled the rat as insensitive to OPIDN.

Biochemical index of rat OPIDN

We investigated whether the known biochemical parameters of OPIDN also occurred in the rat. It has been demonstrated that inhibition of NTE by more than 70% shortly after OP exposure predicts subsequent pathology in chickens, cats, farm animals and humans [24,29,30,49]. Because this relationship holds for hundreds of tested compounds, NTE depression is considered to be a reliable predictor of OPIDN. To test this relationship in the rat, animals were dosed acutely with TOCP (145, 290, 580, 835, 1160, 2320 and 3480 mg/kg) and brain and spinal cord NTE levels were determined at 44 h. Two weeks later similarly treated animals were killed and examined for neuropathic damage. The cervical cord pathology was scored (0–4) depending on its severity. A damage score of 3, which described extensive degeneration of the dorsal columns, was taken to indicate definitive cord damage in the OP-treated rat. Severe spinal cord degeneration (damage score of ≥ 3) was observed in 90% of the rats dosed with ≥ 835 mg/kg TOCP, with only minimal pathology occurring at the lower doses. This dose correlated with 66% and 72% depression of NTE activity in the brain and spinal cord, respectively [39]. The relationship between NTE activity and pathology in rats was tested with mipafox. Again, severe cervical cord pathology, occurring in over 85% of the animals, was associated with depressed NTE activity of 73% in the spinal cord and 67% in the brain [57].

These studies also demonstrated that interspecies differences in the time course of NTE inhibition and recovery occur. For example, in rats dosed with 1160 mg/kg TOCP, brain NTE is depressed by only 25% after 20-h exposure, whereas in birds dosed with the equipotent dose of 1000 mg/kg TOCP, brain NTE is inhibited 90% to 95% 24 h after exposure [38,47] indicating differences in the biotransforming or partitioning of OP compounds between models. The recovery of NTE activity also differs in the rat and chicken. In birds exposed to an acute dose of 1000 mg/kg TOCP, which depresses NTE by 90%, NTE activity remains depressed 45% to 50% after 14 days exposure [38,47], whereas in rats treated with the equipotent dose of 3480 mg/kg, NTE activity returns to control values by day 14. This faster recovery of NTE activity in the rat can be explained either by less 'ageing' of the inhibited enzyme [14] or by a more rapid resynthesis of NTE [48] and suggests that the duration of NTE inhibition may contribute to species vulnerability. These studies also demonstrated a highly significant correlation in NTE inhibition between brain and target tissue (cervical spinal cord) in rats exposed to TOCP, for all dosages and all time points. This relationship has been reported in the chicken [8,23,38], and implied that in both species the brain can be used as a tissue source for the NTE assay in predicting spinal cord damage. A subsequent study examined the qualitative differences between chicken and rat brain NTE, by evaluating their sensitivities to inhibition by OPs *in vitro* [37]. These authors concluded that rat and chicken brain NTE were very similar with respect to inhibitor sensitivities, pH sensitivity and molecular weight. A noted difference was that the specific activity of hen brain NTE is approximately twice that of rat [37].

The significance of the above studies was the linkage of NTE inhibition with spinal cord damage using the rat, which raised the possibility that rodents might be viable models in the testing of putative OP neurotoxicants.

Manipulation of OPIDN in the rat

Protection against OPIDN

An experiment was designed to address the role of NTE in precipitating OPIDN in the rat [56]. Hypothetically, initiation of the delayed neuropathy in molecular terms involves two separate events: the inhibition of NTE activity owing to the binding of the OP to the active site of NTE and 'ageing' of the NTE-OP complex in which an alkyl substitution of the OP is spontaneously hydrolyzed [14,24,60]. Both extensive inhibition and 'ageing' are

necessary steps for neuropathy. Certain phosphinates, CBs and sulphonates can inhibit NTE over the critical 70%, but because they are unable to 'age' they are non-neuropathic. Exposure to such chemicals before treatment with an 'ageable' (neuropathic) OP will theoretically block the active site of the NTE and protect against subsequent neuropathy [3,8,27,30]. To differentiate between these two events, rats were exposed first to a non-neuropathic, 'non-ageing', protecting agent (phenylmethylsulphonyl fluoride; PMSF) and then to a neuropathic, 'ageable' OP (mipafox). Other animals were dosed with identical dosages of these compounds but in reversed order. Further groups were exposed to either PMSF (250 mg/kg) or to mipafox (15 mg/kg) and a time course of brain NTE inhibition and recovery was defined. A separate group of PMSF-treated rats was exposed to mipafox 4 h later when brain NTE inhibition was 89%. Conversely, another group of rats, pretreated with mipafox was dosed 4 h later with PMSF when NTE inhibition was 90%. A third group of animals was treated with PMSF and exposed to mipafox 14 days later, when NTE activity had recovered to within 10% of control values. Histopathology indicated severe critical cord damage in the following frequencies: PMSF, 0%; mipafox, 85%; PMSF-4h-mipafox, 0%; mipafox-4h-PMSF, 100%; PMSF-14 days- mipafox, 75%; controls, 0%. These data indicated that PMSF-pretreatment protected rats against mipafox-induced neurological damage but that the timing of administration and the order of presentation were critical to this protection. The demonstration that PMSF protected the rats against a challenge dose of a neuropathic OP also suggested that the initial molecular events of OPIDN are common to all tested species and underscored the critical role of the inhibited and 'aged' NTE for the neuropathic process of OPIDN.

Pharmacological manipulation

TOCP is activated by mixed function oxidases (MFO) to the neurotoxic metabolite saligenin O-tolyl-phosphate, which is subsequently detoxified by the MFO system. The effect of metabolic interference on the onset of OPIDN was examined by pretreating Long Evans rats with 50 mg/kg of the MFO inhibitor, piperonyl butoxide (PiPB), 1 h before administering TOCP (1160 mg/kg). The animals were killed after three treatments and the spinal cord, various PNS nerves and liver were examined microscopically [53]. Rats treated with PiPB in combination with TOCP showed significantly more damage in both the spinal cord and PNS than those treated with TOCP alone. PiPB was used in this experiment to modify the biotransformational balance (i.e. activation and deactivation), an important determinant in the development of TOCP-induced neuropathy [2,16]. PiPB, which inhibits MFO when given in low doses shortly before exposure, is reported to inhibit some phase II conjugations [21,31]. There is pharmacological evidence that glutathione-dependent reactions play important roles in the detoxification of various OPs [18,33,35]. The thesis of this study was that pharmacological interference with these phase II reactions could modulate the onset of TOCP neuropathy in rats by allowing the reactive intermediate (saligenin-o-tolyl cyclic phosphate) a longer serum/tissue residence time to exert deleterious neurotoxic effects. To support this, in an earlier experiment [54] Sprague Dawley rats were pretreated with PiPB for 4 days before TOCP administration. The exposure variables (multiple PiPB exposures) were designed to induce the synthesis of conjugating proteins, thereby improving the efficacy of deactivation of TOCP. In these experiments, quantitatively less CNS and PNS degeneration developed in rats treated with PiPB and TOCP compared with TOCP alone. This study demonstrated that pharmacological manipulation of metabolic pathways can affect both the onset and severity of TOCP neuropathy in rats and suggested that rodent resistance to OPIDN may partially have a hepatic basis.

Age related sensitivity in rat OPIDN

The effect of age on rodent sensitivity to OPs was examined by dosing 2- and 24-month-old Long Evans rats with low levels of TOCP (116 mg/kg) daily (5 days per week, po). Both groups were age matched with vehicle controls and examined for functional and histopathological change after 2 and 6 weeks. Functional

signs of incipient neuropathy were present by 6 weeks in TOCP-treated senescent rats and included ambulation and plantar-extension abnormalities as well as hindlimb criss-crossing. Because of the age- related morphological changes known to exist in senescent animals [15,34], tissues from old TOCP-treated animals were carefully age matched with old corn oil controls. Both groups of senescent rats showed severe degeneration of the fasciculi gracilus and cuneatus and spinocerebellar tracts by 2 weeks. The histopathology included the range of cytological abnormalities noted in early studies (axonal swellings, metachromatic cells, myelin ellipsoids and severe astrocytosis). Controls showed typical age-related changes in the PNS (swollen myelinated and demyelinated axons surrounded by overlapping rings of Schwann cell basal laminae, spherical metachromatic bodies and excessive endoneurial collagen). Because these changes were so extensive in the plantar nerves of both control and treated old animals, fascicles that were spared age-related, compression damage (tibial branches, phrenic nerve, left recurrent laryngeal nerves) were used to compare damage. In TOCP-treated senescent rats, all these PNS nerves showed a significantly higher incidence of degeneration and regeneration after 6 weeks compared with age-matched controls and young TOCP-treated animals. This experiment suggested that old rats functionally and neuropathologically are more vulnerable to OP exposure (Veronesi, unpublished results). The basis of this age-related enhancement may be hepatic since senescence diminishes the ability of the liver to detoxify.

Other studies in our laboratory examined juvenile sensitivity to OP-induced neuropathic damage and concluded that in the rat as in chicks [26], OPIDN is an age-dependent phenomenon (Veronesi, unpublished results). Long Evans rats at various ages (20, 25, 30, 35, 40 and 60 days) were dosed with TOCP (2360 mg/kg) and killed for histopathology 2–3 weeks later. Although a high variability occurred in the frequency and severity of cord damage, neuropathic sensitivity gradually increased with age (Figure 11.6). Definitive neuropathy (damage score ≥ 3) occurred only at day 30 and after. This phenomenon does not appear to relate to the maturation of the target enzyme since paraoxon-resistant activity (an

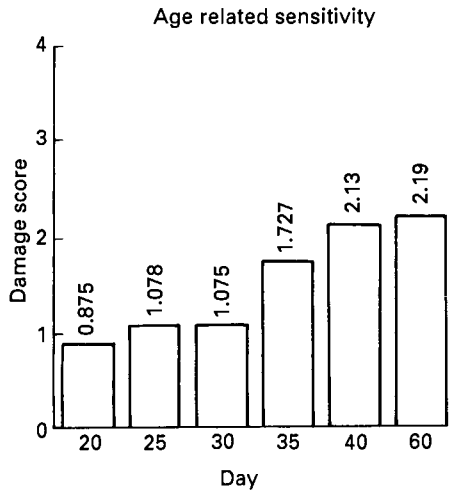

Figure 11.6 Neuropathic sensitivity of Long Evans female rats at various ages. Damage scores reflect increasing severity with age

index of NTE activity) is age- dependent and mipafox sensitivity (*in vitro*) is stable throughout the above time points (Padilla, personal communication). However, this age- related susceptibility may result from hepatic microsomal maturation since P450 activity, determined by anilase dehydrogenase activity, steadily increases throughout these time points, almost doubling in activity from 20–25 days (Lapadula, personal communication).

Triphenyl phosphite neuropathy

We recently used the rat model to evaluate the neurotoxicity of triphenyl phosphite (TPP), an aryl phosphite structurally similar to TOCP. TPP was investigated over 50 years ago and found to produce extensor rigidity in cats in contrast to TOCP which produced flaccid paralysis [44–46]. In spite of its increasing use [51], TPP had escaped experimental scrutiny until recently when it was reported that chickens exposed to TPP developed the ataxia, pathology and biochemical features of OPIDN [42,43]. We examined the neurotoxic effects of TPP in rats given two single doses (2×1.0 ml/kg, sc) and examined neuropathological and biochemical criteria, pathognomonic of the delayed neuropathy in rats. Our results did not support TPP as producing OPIDN for several reasons. Within 7 days of exposure

Figure 11.7 Cervical damage in TPP-treated rats was confined to ventrolateral and ventral columns with a distinct sparing of the dorsal columns. Semi-thin Epoxy section. Magnification × 155, reduced to 77% in reproduction

severe dysfunction occurred in TPP-treated rats, consisting of hind- and forelimb paralysis and circling behaviour, dysfunctions certainly not reported in any previous description of OPIDN in the rat. In addition, spinal cord degeneration occurred in the lateral and ventral columns (Figure 11.7) in a pattern of damage topographically different from that produced in the rat by classic OPIDN compounds such as TOCP and mipafox, which predominantly damage the dorsal columns of the cervical cord. In the TPP-treated animals, swollen axons and occasional anterior horn necrosis were seen also in the spinal cord grey matter suggesting interneuron involvement (Figure 11.8). Severe damage to the spinal roots and dorsal root ganglion neurons was also present (Figure 11.9). Examination of the brainstem indicated spinocerebellar tract degeneration and axonal swellings in the reticular formation. Lastly, both the dysfunctions and neuropathic damage occurred in conjunc-

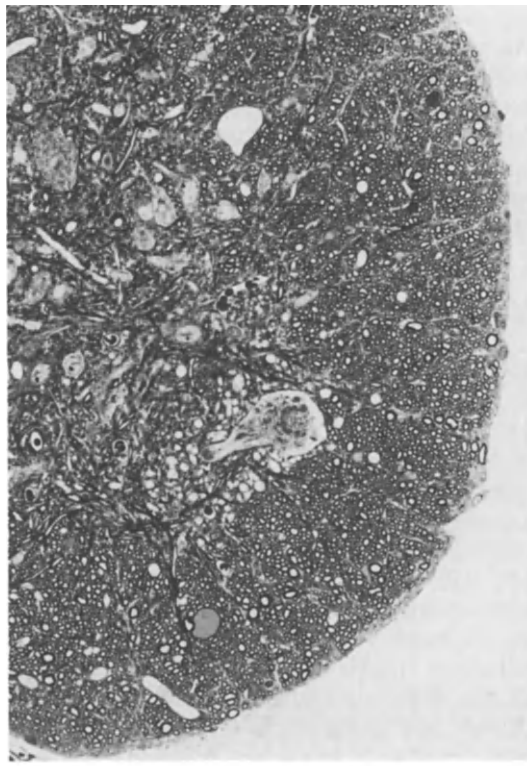

Figure 11.8 Anterior horn cell necrosis and giant axonal swellings often occurred in the lower spinal cord level of TPP-treated rats. Magnification × 1314, reduced to 93% in reproduction

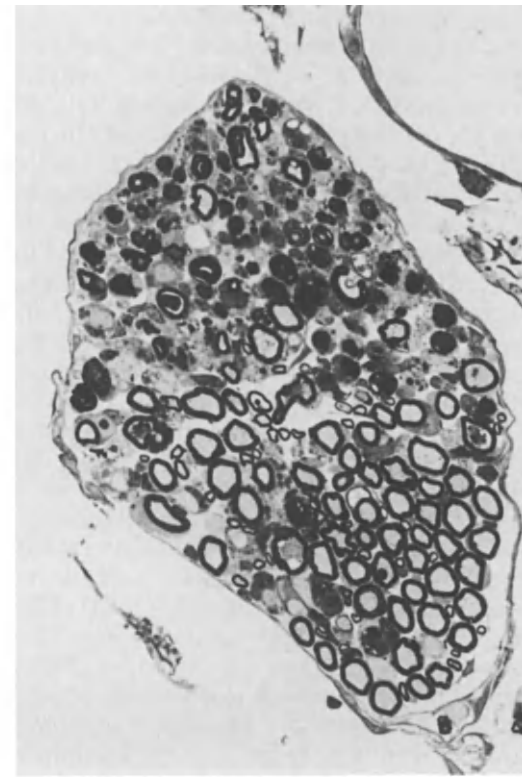

Figure 11.9 Spinal root degeneration and dorsal root ganglion necrosis (not shown) was also seen in TPP neurotoxicity. Magnification × 1700

tion with only marginal (33%) inhibition of NTE [58].

To expand on this study, rats were exposed to TOCP, TPP or a combination of the OPs at neuropathic dosages. In these rats, severe dysfunction occurred within days of exposure and neuropathic damage was noted involving the dorsal, lateral and ventral columns of the cervical cord, in addition to anterior horn cell damage (Figure 11.10). This experiment [55] showed definitively that in rats, TPP and TOCP produce two distinct patterns of neuropathy. When TPP is administered to the chicken, the common test model of OPIDN, a 'false-positive' is obtained. Hens exposed to TPP show extensive NTE inhibition (> 90%) and ataxia (7–14 days later), and display spinal cord damage similar to OPIDN [9,42,43]. More detailed histopathology, however, shows that in addition to degeneration of the spinal cord tracts, TPP-dosed chickens show anterior horn cell necrosis and brainstem pathology (Brown, personal communication), features not seen in OPIDN. To explain this curious difference in the neuropathic expression of mammals and avians, Abou-Donia and colleagues have suggested that in chickens, TPP produces both OPIDN and a neuronopathy, the latter being expressed only in mammals [9].

Mouse model of OPIDN

We have recently demonstrated light and electron microscopically that mice (CD strain) also are neuropathically sensitive to acute or multiple doses of TOCP and that inhibition of NTE can be loosely correlated with the appearance of spinal cord pathology 2–3 weeks later [59], although a much more variable response occurs morphologically and

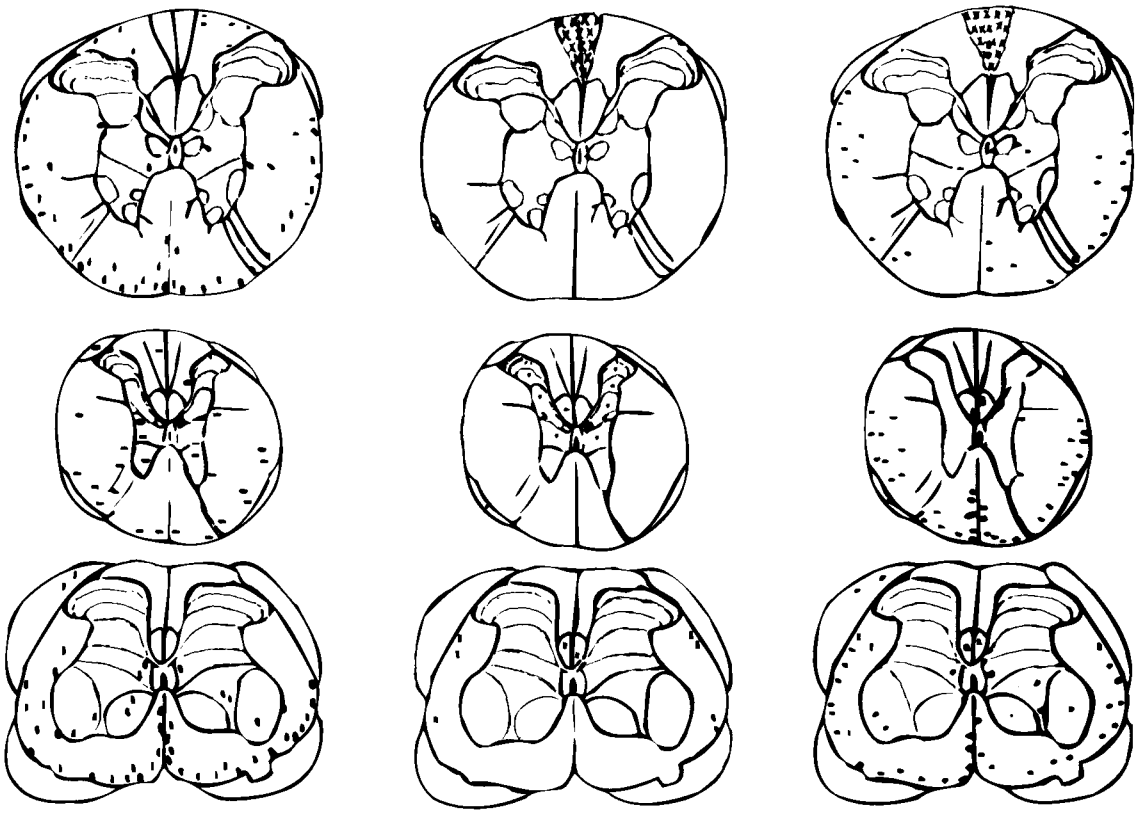

Figure 11.10 Topography of spinal cord damage (i.e. axonal swellings, myelin ellipsoids) seen in (a) TPP-treated, twice dosed rats; (b) TOCP- treated rats and (c) rats exposed to TPP (two doses) in combination with TOCP (one dose). (•••), TPP; (xxx), TOCP

biochemically. In this model, 1160–3480 mg/kg TOCP caused >70% inhibition of brain NTE but *in vitro* sensitivity to mipafox was similar to that seen in the rat and chicken (Pope, personal communication), again suggesting that hepatic influences may play a major role in interspecies variability to OPIDN.

The demonstration that mice also develop OPIDN might provide an opportunity to isolate the NTE moiety. In the mouse there are over 25 commercially available genetic variants for esterase genes and the affected chromosomes have been identified and mapped. It may be possible to arrange these variants according to their neuropathic sensitivity to OPs and to attempt to perform linkage studies to see if the NTE sensitivity segregates with the esterase variant, indicating that the NTE and esterase genes are close by if not actually linked. Molecular genetic techniques might then be applied to isolate the affected arm in kilobase lengths, identify the polyA RNAs, translate in an *in vitro* reticulocyte system, and identify the mRNA for NTE. This is obviously a herculean task reserved for molecular geneticists but one which might permit the isolation and subsequent immunocytochemical tagging of the elusive NTE moiety.

Variations in the rat and chicken models

There are several variations in the rodent model of OPIDN compared with more conventional chicken test species. The topography of spinal cord damage seen in rats differs from that seen in hens, a variability that may be associated with interspecies neuroanatomical differences underlying their modes of locomotion [4,11,36]. In OPIDN, tracts housing the largest and longest nerve fibres are the most susceptible, regardless of the species, in accord with a 'dying-back' neuropathy. In humans and cats, the most severely damaged descending tracts are the pyramidal tracts, which are absent in the bird [19,36]. In the hen, the most vulnerable tracts are scattered throughout the lateral, ventral and dorsal columns. In the rat, the most severely affected tracts appear to be the large diameter sensory fibres terminating in the dorsal columns of the upper cervical cord. In addition to topographic differences, it has been suggested that PNS degeneration precedes CNS damage in the chicken [1,11]. The rat expresses a more protracted outset of PNS degeneration, possibly owing to excessive and somewhat confounding regeneration. Ultrastructurally, the axonal lesion of OP neuropathy is common to all tested species, with excessive amounts of fragmented smooth endoplasmic reticulum (tubulovesicular profiles) [6,7,41].

The most striking difference in the response of chickens and rats to OP exposure is the retention of hindlimb function in the rat despite severe CNS and PNS histopathology. This lack of clinical signs has led to the conclusion that the rat is insensitive to OPIDN [1,4,24,25,44,45]. Early studies have reported that even after 24 weeks of daily or intermittent exposure to OPs, rats demonstrate only minor ambulation problems in spite of severe central and peripheral nerve degeneration [32]. This contrasts with the chicken, which becomes grossly ataxic after minor spinal cord pathology [6,40]. The paradox is an engaging problem neurologically whose explanation may involve differences in modes of locomotion. In the rat, the ascending tracts housing the sensory nerves are almost exclusively damaged whereas descending motor tracts, because of their smaller diameter, are relatively spared. In spite of the pathology which involves largely the sensory system in the rat, little dysfunction is seen until after lengthy and repeated exposures. It is possible that the rodent has a neuronal reserve that subserves the vulnerable sensory-motor function, 'masking' dysfunction until a certain threshold of spinal cord damage is reached. Indeed, Lapadula *et al.* [28] report that mice must be dosed for 1 year before hindlimb paralysis occurs. Species (e.g. chickens and man) that do not have a similar neuronal compensation become ataxic when fewer CNS neural elements are damaged. This hierarchy of species sensitivity to ataxia (chicken > cat > rat > mouse) is not unique to OPs, being seen in other toxicant-induced neuropathies, notably the hexacarbons [50].

Another feature which appears to play a key role in protecting the rat from functional debil-

itation is the excessive PNS regeneration seen in rodent OPIDN. Although regeneration is often seen as a response to traumatic or experimental nerve injury, the predominance of this event, which would help to re-establish muscle strength and coordination throughout the early stages of TOCP damage may explain, in part, the preservation of hindlimb function in the rat until later stages of intoxication. The low level of PNS damage in rodent OPIDN may in itself be sufficient to protect against detectable ataxia because even in the chicken, when PMSF, administered in the sciatic artery, is used to protect the hen from subsequent DFP-induced PNS damage (but not cord degeneration), only minimal ataxia occurs [8].

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12

Intermediate syndrome in anticholinesterase neurotoxicity

Nimal Senanayake and Lakshman Karalliedde

The intermediate syndrome (IMS) in OP neurotoxicity [35] sets in after the cholinergic crisis [26], but before the expected onset of the delayed polyneuropathy [32,33]. Sudden unexpected deaths and respiratory embarrassment in patients who had apparently recovered from the cholinergic crisis drew initial attention to IMS [35]. The cardinal feature is muscular weakness, affecting predominantly the neck flexors, proximal limb and respiratory muscles. Motor cranial nerve palsies are common. Unlike delayed polyneuropathy, IMS carries a risk of death because of associated respiratory depression. IMS is probably the human equivalent of some observations in animal experiments [1,13,17,30]. The pathophysiological process is different to that of both the cholinergic crisis and the delayed polyneuropathy. The clinical situation suggests a triphasic neurotoxic effect of OPs after human intoxication.

Clinical features

IMS follows a cholinergic phase treated conventionally or while on therapy. About 24–96 h after intoxication, respiratory insufficiency may draw attention to the onset of IMS. The patient is usually conscious. Muscles innervated by cranial nerves show varying degrees of weakness. The external ocular muscles are most commonly affected, producing ptosis of mild to moderate severity and defects of ocular movements. These may be accompanied by weakness of muscles of mastication, face and soft palate. Weakness is bilateral and usually symmetrical. Mild weakness of these muscles may easily be overlooked.

Weakness of neck flexion, often such that the patient cannot raise the head, is a constant feature. In the limbs, the muscle tone is usually normal but may be decreased. Testing of muscle power demonstrates a characteristic proximal weakness affecting predominantly shoulder abduction and hip flexion. Muscle weakness is symmetrical but the severity may vary. Power in the distal muscles is normal and this may give a false impression that the limbs are spared. The muscles do not fasciculate. Tendon reflexes are usually decreased or absent, and the plantar response is flexor.

There is no sensory impairment. However occasional patients show spasticity of limbs, hyperreflexia, and sometimes dystonic reactions.

Respiratory insufficiency develops over approximately 6 h, and initially the accessory muscles of respiration are used. Then there is increase in respiratory rate, sweating, restlessness and later, cyanosis. If unattended, the patient may soon become unconscious and death may follow.

The following case report highlights several important aspects of IMS. A 40-year-old man was seen at the Teaching Hospital, Peradeniya, at 01.15 hours on 7 May 1987 with a history of having ingested dimethoate 2 h earlier. On admission he was unconscious, responding only to deep pain. Pupils were pin-point and there were generalized muscle fasciculations. He was sweating, frothing and vomiting. Pulse rate was 64/min, blood pressure 90/60 mm Hg and respiratory rate 20/min. Immediate measures were taken to clear the airway. Secretions were removed by suction and a gastric lavage was carried out after endotracheal intubation. Atropine, 18 mg, and PAM 1 g were administered iv within 5 min of admission. Atropine was repeated in 2–4 mg doses to maintain mydriasis and a pulse rate over 120/min. A total of 62 mg was required in the

first 24 h. PAM was repeated in 1 g doses three times within the first 24 h and thereafter daily for 5 days. He regained consciousness in 6 h, and 24 h after admission he was rational, had dilated pupils and was free of muscle fasciculations. There were no detectable clinical deficits.

At 45 h after admission, physical examination revealed mild restriction of external ocular movements and weakness of neck flexion, shoulder abduction and hip flexion. Seven hours later he became restless, began sweating profusely and developed difficulty in breathing. Oxygen was administered by mask and he was closely observed. By 96 h after admission he was extremely restless, dyspnoeic, and cyanosed. The tidal volume was 150 ml, respiratory rate 40/min and the blood pressure 170/90 mmHg. Endotracheal intubation was carried out immediately and the patient was mechanically ventilated. Neither procedure required the use of a muscle relaxant. With assisted ventilation, the restlessness became less marked. Diazepam in 10 mg doses was administered iv to provide rest and sleep during mechanical ventilation. Tracheostomy was performed on the third day of ventilatory care. He was weaned off the ventilator after 6 days. The weakness of external ocular muscles lasted 6 days, and weakness of shoulder abduction and hip flexion lasted 16 days. The weakness of neck flexion was detectable up to 10 days after poisoning. The patient was discharged, symptom-free and with a normal physical examination, on the 20th day after admission.

Investigations

Urine analysis, biochemical and haematological profiles, and CSF show no characteristic abnormality. Sensory and motor conduction in peripheral nerves and routine electromyography of distal and proximal muscles of the limbs are normal. However, in a preliminary study [35], tetanic stimulation of the abductor pollicis brevis, 24–48 h after the onset of the IMS showed a marked fade at 20 and 50 Hz. The muscle was stimulated via the median nerve at the wrist and the muscle action potentials recorded with surface electrodes. At 50 Hz a fade of 30–75%, was observed in 5 s, and at 20 Hz a fade of 20–30 %, in 10 s. A train of four supramaximal stimuli at 2 Hz did not produce a change in the amplitude of the motor action potential. There was no post-tetanic facilitation.

Diagnosis

Diagnosis of the IMS is clinical, and should be suspected when a conscious patient, recovering from the acute cholinergic crisis, develops muscular weakness 1–4 days after OP poisoning. Respiratory insufficiency may be the functional disturbance drawing attention to the syndrome. The characteristic distribution of muscle weakness makes the diagnosis evident. The patient may be on atropine or oxime therapy but it should not negate the diagnosis.

The early onset distinguishes IMS from delayed polyneuropathy. There is an obvious contrast between the distribution of muscular weakness in the two conditions (Table 12.1). Electrophysiological findings are also totally different. In delayed polyneuropathy the nerve conduction is impaired and muscles show denervation [31,33] whereas in IMS these parameters are normal.

Table 12.1 Comparison of the intermediate syndrome and delayed neuropathy

Variable	*Intermediate syndrome*	*Delayed neuropathy*
Time of onset, after poisoning	1–4 days	2–3 weeks
Sites of weakness		
Limb muscles	Proximal	Distal
Neck muscles	+	–
Cranial nerves	+	–
Respiratory muscles	+	–
Electromyogram	Tetanic fade	Denervation
Recovery, from time of onset	4–18 days	6–12 months
OPs commonly involved	Fenthion Dimethoate Monocrotophos	Methamidophos Trichlorfon Leptophos

Reproduced from Senanayake and Karalliedde [35] with permission

Treatment

Prompt and effective management of respiratory insufficiency is the major treatment of IMS. Because respiratory difficulty may

appear suddenly in a patient recovering from the cholinergic crisis even while on conventional therapy, all patients should be observed in hospital for up to 5 days after poisoning. Should early signs of respiratory insufficiency develop, i.e. increase in respiratory rate, use of accessory muscles of respiration, decreased tidal volume, and reduced PaO_2, facilities for ventilatory care should be within reach. Oxygen therapy should be initiated. Diazepam in 10 mg doses iv may be given if the patient becomes anxious or restless. Clinical or biochemical evidence of hypoxia or respiratory failure is an indication for ventilatory support. Diazepam assists in stabilizing and calming the patient on mechanical ventilation. Several reports have indicated that benzodiazepines are helpful as antidotes against poisoning by antiChEs [14,19]. The duration of ventilatory care may vary from days to a few weeks. It is necessary to assess daily the patient's ability to adequately breathe spontaneously. Frequent blood gas analyses are of value in monitoring and in weaning patients off ventilatory care. PAM therapy initiated during the acute cholinergic phase should be continued during IMS until the patient recovers adequate respiratory function. The dose proposed is 1 g iv t.d.s. Reactivation of inhibited alkyl phosphorylated enzyme is probably the most relevant in the treatment of IMS. High doses of PAM can cause NM blockade, but is minimal at the dose advocated [38].

Atropine therapy in IMS needs further evaluation. It is virtually without effect against peripheral NM dysfunction [38]. Although most OPs and their active metabolites are eliminated within 40 h, some such as fenthion and fenitrothion, are known to persist for longer periods [24]. Thus, our patients continued on atropine to maintain mydriasis and a pulse rate over 100/min, for the same duration as that of PAM therapy.

Clinical course

There is no definite pattern in the development of neurological manifestations. Nevertheless, the regression of signs follows a characteristic pattern in survivors. Cranial nerve palsies (palatal, facial and external ocular, in that order) are usually the first to regress, followed by improvement of the respiratory difficulty and recovery of strength in the proximal limb muscles. Neck flexion is the last function to recover. In our initial series [35] of ten patients, recovery ranged from 5 to 18 days after the onset of weakness, except in one patient who subsequently developed delayed polyneuropathy. Weakness of neck flexion lasted 32 days, by which time he had weakness of the limb distal muscles from delayed polyneuropathy. Two of our initial patients died in the early phase of IMS because of delay in recognizing respiratory failure and in initiating ventilatory care. Deaths can be prevented if respiratory failure is managed promptly and adequately. In survivors, recovery from IMS is complete.

Pathogenic mechanisms

Muscular weakness following OP intoxication in animals had been known for many years. The earliest observations were by Carey [3] in 1944. Paralysis appearing within 24 h of poisoning by OPs (fenchlorphos, parathion-methyl or malathion), and lasting a few days or weeks, had been described in hens [12,43]. This early onset of muscle weakness was distinct from that of delayed polyneuropathy which developed approximately 14 days after poisoning with compounds such as triorthocresyl phosphate [12]. Ariens *et al.* [1] demonstrated that myopathic changes occurred in the diaphragm, gastrocnemius and psoas muscles of the rat following sublethal doses of OPs, such as DFP, tabun and paraoxon. At 2 h after dosing, localized eosinophilia, swelling of the sarcoplasm and loss of striations were seen in several muscle fibres. After 4–6 h, segmented leucocytes appeared, the sarcolemmal nuclei became pyknotic and the sarcoplasm broke up in floes. Approximately 12 h after dosing, a complete localized necrosis developed in the affected fibres. Phagocytosis followed by mesenchymal cell proliferation ensued and fibres with striations appeared in 3–4 days. After 10 days, the fibre unity was restored. Salpeter *et al.* [30], incubating mouse sternomastoid muscle with DFP observed necrotic changes to begin and be most extensive in the region of the endplate. The peak of necrosis was seen between 1 and 3 days. At 7 days,

signs of muscle recovery were evident. Full recovery occurred by 2–33 weeks. This myopathy was different to the delayed neuropathy which began at nerve terminals approximately 3 weeks after exposure to DFP [20,21] when muscle contractile strength was returning towards normal [17].

Onset, progression and recovery of muscle weakness in IMS corresponds closely to the sequence of myopathic changes observed in these animal experiments and is distinguishable from the muscle weakness following the delayed polyneuropathy which develops in 2–4 weeks after poisoning. The myopathic changes in experimental animals have been studied in the diaphragm and leg muscles [1,10] and external ocular muscles [2]. The diaphragm was the most severely affected in most instances [1,17], which corroborates the cardinal feature of respiratory failure in IMS.

Relapse of symptoms despite apparently adequate therapy had been observed after OP poisoning [13]. Prolonged respiratory depression in the human following fenthion poisoning was reported by Dean *et al.* [6] and Mahieu *et al.* [23]. Respiratory paralysis 6 days after intoxication with dicrotophos [27] and relapse with respiratory difficulty after malathion intoxication [11] are reported. Wadia *et al.* [39,40] reported late paralytic signs in patients after poisoning with OPs such as fenthion, malathion and fenitrothion. The basic symptomatology in most cases seems consistent with IMS.

The abnormal electromyographic responses to tetanic stimulation, of pathophysiological significance, incriminate a dysfunction at the NM junction as the mechanism for the muscle weakness of IMS. These abnormalities: fade on tetanic stimulation, absence of fade on low frequency stimulation, and absence of post-tetanic facilitation, differ from those seen in other NM disorders. Tetanic stimulation produces an enhancing response in botulism and myasthenic syndrome [22]. Fade on tetanic stimulation is seen in myasthenia gravis, but a decline in response at low frequencies of stimulation and post-tetanic facilitation are also observed [22]. In depolarization block, seen with ACh and decamethonium, both fast and twitch rates of nerve stimulation are well sustained [8]. It may be concluded that the NM junctional dysfunction in IMS is postsynaptic.

Inability to sustain tetanic stimulation has been observed in OP-dosed animals. While normal muscle is capable of sustaining tetanic stimulation at 25, 50, 100 and 200 Hz for 10 s, 2–4 h after OP intoxication, contractions were subnormal at 100 Hz and not observed at 200 Hz [1]. Similar observations were made by Salpeter *et al.* [30] who found the ability of muscle to sustain tetanus in response to nerve stimulation is eliminated when esterases at the NM junction are saturated with DFP. This ability was regained partially when <10% of DFP binding sites had recovered. There was a positive correlation between the frequency of stimulation at which the tetanic response could be maintained and the extent of AChE recovery. Tetanic responses at 100 Hz appeared indistinguishable from controls with only about 25% of normal AChE.

The mechanisms underlying the myopathic changes of OP intoxication have been investigated. Ariens *et al.* [1] suggested that the high ACh concentration at the endplate created a long-lasting depolarization resulting from a permeability change of the junctional membrane. They concluded that prolonged permeability was the cause of necrosis. Fenichel *et al.* [9] showed that excessive ACh could induce skeletal muscle necrosis even with normal ChE. The myopathy was not just the result of excessive depolarization and contraction but more likely related to a disturbance in the trophic effect mediated by ACh. Laskowski *et al.* [18] observed that OPs caused antidromic firing of motor nerve action potentials. This increased activity in the presence of accumulated ACh had a possible causal relation with the disorganization of subsynaptic fine structure. Severity of the myopathy appeared to depend on a critical degree and duration of AChE inhibition which triggered the neurally mediated events, including increased neurotransmitter release and antidromic nerve activity [42].

The depolarizing effects of paraoxon were initially thought to be from a combined depolarization of ACh receptors and extrajunctional receptors controlling Na^+ conductance channels. Paraoxon did not have an effect on K^+ conductance channels [17]. However, Salpeter *et al.* [30] noting Z band dissolution and breakdown of sarcoplasmic

reticulum argued that disturbances in Ca^{2+} flux rather than Na^{+} flux was the mediator of the detrimental effect of OPs on muscle structure. Their suggestions were based on: (1) a proteolytic enzyme specific for Z bands [28] is activated by elevated levels of Ca^{2+} ions [29]; (2) membrane depolarization causes Ca^{2+} ion influx, and when depolarization is produced by agonist receptor interaction, Ca^{2+} influx is greatest in the region of the endplate [25]; (3) Ca^{2+} is released from the ACh receptor itself as a result of ACh binding, adding to Ca^{2+} in the region of the endplate; and (4) sarcoplasmic reticulum is a major site for Ca^{2+} binding. Dettbarn [7] demonstrated that atropine and gentamicin, in concentrations not blocking NM transmission, alleviates the necrotic action of OPs by interacting with the presynaptic Ca^{2+} uptake mechanism.

Several factors influence the myopathic reaction to OPs in the experimental animal. These, if applied to the human situation, could prove relevant in the prevention and treatment of IMS. Increased muscle activity as produced by phrenic nerve stimulation aggravated, whereas inactivity as produced by previous denervation prevented, the myopathic change [1,9]. Hemicholinium and tetradotoxin had an inhibitory effect, while alpha bungarotoxin and curare totally prevented the myopathic reaction [9,17,30]. Pretreatment with atropine or gentamicin was shown to have a significant antimyotoxic effect [7].

Clinically, the most important observation is that PAM minimizes or prevents the myopathy [1,17]. The time of PAM dosing appears critical. Ariens *et al.* [1] showed that PAM administered within 2 h of intoxication prevented myopathic change. However, Laskowski *et al.* [17] found that PAM, to be fully effective, had to be administered within 10 min of intoxication and that a delay of 2 h decreased effectiveness.

Agents commonly encountered in cases of OP intoxication admitted to our institution are dimethoate, methamidophos, malathion, monocrotophos and fenthion, in that order of frequency [15,16]. Agents responsible for IMS in the initial series [35] were fenthion in four cases, monocrotophos in two, dimethoate in two and methamidophos in one. It is likely that the chemistry of the OP is a determinant in the development of IMS. In the experimental animal, the severity of myopathy varied with the OP [7] and there was a relationship between chemical structure and muscle group and the muscle type affected [17].

Some of our patients with IMS had additional features such as transient hyperreflexia and dystonia, suggesting involvement of the pyramidal and the extrapyramidal systems. The significance of these findings is not clear. We feel they are incidental and do not necessarily influence the symptomatology or clinical course of IMS.

Conclusions

Pesticide poisoning is an important cause of morbidity and mortality and a serious public health problem particularly in developing agricultural countries [5,41]. OPs rank high among pesticides causing human health hazards [34,36]. The description IMS has features of substantial medical and public health importance [4]. Among the diverse health hazards varying from cholinergic crisis to delayed peripheral neuropathy and chronic effects, IMS presents a life-threatening, yet treatable, consequence of OP intoxication.

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Cardiac effects of anticholinesterase agents

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Introduction

The cardiac pharmacology of the antiChEs has been largely attributed to the actions of ACh on the heart. The cardiac pharmacology of ACh and other cholinergic agents has been extensively reviewed elsewhere [40,60]. By illustrating the cardiac actions of ACh, these reviews provide a framework for a greater understanding of the cardiac actions of the antiChEs.

Considering that the toxicity of ACh can result in cardiac slowing, arrhythmias, and then cardiac death, it is not surprising that agents that increase ACh levels by ChE inhibition at the cardiac site will also produce arrhythmias followed by cardiac death. It is recognized that ChE inhibitors affect peripheral nerves that impinge on the myocardium, CNS centres that control rhythmicity of the heart, and changes in lung function that may produce pulmonary insufficiency. Excess ACh at these sites can contribute or may, under certain circumstances, be the primary reason that cardiac toxicity is initiated.

Cardiac biochemistry

The cardiac effects of antiChEs are manifested in the accumulation of ACh at synaptic sites [28]. This excess ACh interacts with muscarinic M_2 myocardial receptors [34]. AF-DX 116, a selective M_2 muscarinic antagonist, was found to reverse the negative inotropic effects of ACh and soman [99]. AChE compounds may act as long-acting ACh. Studies in the heart have not yet determined whether the presence of excess, prolonged ACh concentration or the inhibition of the enzyme itself is responsible for some of the serious toxicity observed.

Both sympathetic cholinergic discharge and ACh itself elicit changes in oxygen uptake in muscle [1]. Tomov *et al.* [100] believed that increased synthesis, in part, could account for the increase in ACh. Biosynthesis of ACh does not seem to be a factor [88] in the pharmacological responses of cholinergic agonists on atria and ventricles. It is possible that the biochemical consequences of cholinergic overload and/or AChE inhibition itself may play a role in the pathogenesis of OP toxicity.

A correlation was found between signs of toxicity and several biochemical changes in rats poisoned by soman [47]. As the severity of effects increased, there was greater inhibition of blood ChE and elevation of plasma glucose levels. When soman is administered to cats, ACh levels increase differentially, tissue respiration decreases, and cytochrome oxidase activity decreases in several tissues (e.g. cerebral cortex, medulla oblongata, spinal cord, liver, and muscle) [100]. Cardiac muscle could be differentially affected because its metabolism is primarily aerobic. Metabolism, however, is not uniform throughout the heart, which suggests that biochemical changes may not be equivalent in all cardiac structures (e.g. subendocardial myocardium, Purkinje fibres, atria). Holmstedt [43] also observed that oxygen consumption is significantly decreased following tabun administration in the unanaesthetized rabbit.

CPK activity increased in the blood after soman intoxication. These levels could be diminished by suppressing the convulsions with a combination of an oxime, benactyzine, and atropine [92]. As isoenzymes of CPK were not measured, it is unclear if cardiac enzymes are changed. It was proposed that skeletal muscle CPK accounts for the change, but further work needs to be performed to verify this hypothesis.

The antiChE dimethoate was found to induce serious ECG disturbances (QT lengthening, ST depression, and T-wave inversion) and cardiac failure in the guinea pig. Data indicated that the effects on the myocardium were independent of the antiChE action [70]. Similar effects were shown with dichlorvos and trichlorfon [72]. It has been proposed that phospholipase A_2 may play a role in muscarinic regulation [41]. Analysis of this enzyme may provide another method to examine the function of antiChEs on cardiac systems. Non-esterified fatty acid plasma concentrations increased in humans [51,52], dogs [87], and rats [85] that displayed arrhythmias following OP administration.

In studies performed in rats cAMP, cGMP, ATP, and CP levels were unchanged following the rhythmic disturbances caused by VX administration. However, compounds that stimulate muscarinic receptors increase cGMP levels in intact myocardium [106]. As a result, Robineau [85] concluded that OP-induced arrhythmias may result from mechanisms different from muscarinic stimulation. Corbier and Robineau [18] have suggested from electrophysiological studies that the cardiac arrhythmias caused by VX may result from Na^+/K^+ ATPase inhibition. The biochemical differences between dog and rat may be explained by species differences in the cardiac sodium pump between the two animals [2]. This proposal is consistent with the observation that oximes will antagonize the action of ouabain on Na^+/K^+ ATPase inhibition [8]. Although it has been suggested that ChE and Na^+/K^+ ATPase share some common properties, the pharmacological effects of OPs and digitalis-like compounds that are thought to act on these enzymes are not usually considered to be identical.

Following insecticide overdose in humans, hypernatraemia and hyperkalaemia have been observed. Humans gradually recover between day 2 and 5 after such an exposure [1]. It is unclear whether this change in ion concentration is from inhibition of a Na^+ pump [18]. Further studies need to be performed to determine the cause of the NA^+/K^+ abnormality. Similar changes in Na^+ and K^+ were not observed in rats receiving convulsive doses of soman [17]. However, the Na^+ pump of the rat heart has different properties for ligands than other species [2].

Cardiac electrophysiology

Muscarinic modulation of cardiac rate by cholinergic agents is thought to occur from Ca^{2+} currents [32], K^+ currents [5,80], or by an incompletely defined hyperpolarizing (I_f) current [26]. Because the effect of the hyperpolarizing current (I_f) is observed at much lower ACh concentrations than seen with Ca^{2+} or K^+ currents, it is believed that this current is responsible for the initial mechanism of action for cholinergic agonists. Changes in the cardiac electrophysiology following administration of OPs have been reported in animals [79,81] and in humans [16, 61, 73, 74, 89, 94, 103, 104]. In dogs poisoned with either '1 or 2 LD_{100}' soman, Kotev *et al.* [55,56] found sinus bradyarrhythmia initially. Eventually complete atrioventricular blockade took place. Ventricular extrasystoles that were 'polytonal' in nature were also observed. These arrhythmias may not be different from the torsade arrhythmias [50] described in humans by Ludomirsky *et al.* [67] or those observed in dogs [49]. Administration of VX in rabbits revealed multiple events. First, a complete disappearance of the P-wave followed by the appearance of ventricular automatism was observed. This automatism was evident after the effect of the poison was shown in the initial ECG changes. Second, it was found that centres of ventricular automatism are characterized by very depressed frequencies. Third, it was shown that changes in the primary centre of ventricular automatism are a characteristic phenomenon (e.g. specific for a particular OP) [81]. This suggests that there are specific cholinergic sites in the ventricle which could be responsible for normal ventricular rhythm.

During convulsive periods, additional waves are observed. Their presence may be caused by hypoxia or anoxia [38]. The time of exposure to a given OP may influence the cardiac rhythm [76,77]. It has been noted that the heart will continue to beat after respiration has ceased; however, the changes in rhythm which may lead to serious cardiac arrhythmias were not analysed in these studies [21]. Anoxia produced by respiratory failure certainly could exacerbate the cardiac difficulties. However, in these same historical studies, the data on TEPP show that blood pressure changes occur before

respiratory changes. This suggests that cardiac pathophysiology may influence pulmonary indices. The administration of VS [O-ethyl S-(2-diisopropylaminoethyl) ethylphosphonothioate] or VM [O-ethyl S-(2-diethylaminoethyl) ethylphosphonothioate] changed ECG patterns in dogs that were pretreated with atropine: these dogs were also receiving artificial respiration. The T wave was often abolished or inverted. If the animals became anoxic, the T wave became enlarged [98]. Analysis of this collected data strongly suggest that ChE inhibitors can exert a ventricular rhythm change that is not dependent on anoxia. Anoxia can further impose ventricular rhythm disturbances that may complicate interpretation of an ECG pattern.

Alternatively, cholinergic agonists may possess antiarrhythmic effects under certain conditions. The administration of carbachol following the application of a 50 Hz alternating current could increase the threshold of arrhythmias and asystole in isolated guinea pig atria and papillary muscles [12]. These results may suggest that factors such as ventricular rate and ionic milieu could influence the propensity of OPs to generate an arrhythmogenic condition. These same factors may account for the many observations that arrhythmias are not found in all subjects after they receive an arrhythmogenic dose of a compound. Further studies with OPs need to be performed to determine if this antiarrhythmic property is present for specific antiChE compounds.

Direct effects of antiChEs

A direct pathological lesion on the atrium has been described in the heart following acute poisoning with sarin [111] or with parathion, trichlorfon, or malathion [10,91]. Administration of Guzathion, usually a mixture of azinphos-methyl and -ethyl, also produces similar lesions [6]. Chhabra *et al.* [16] found that fatty infiltration, dilation of blood vessels, and haemorrhage of tissue occurred following malathion intoxication in humans. This lesion may be the result of peripheral increases in vascular resistance which are observed after sarin [110] or ACh is administered [112]. McDonough *et al.* [69] showed that the combination of atropine and 2-PAM blocked the soman-induced cardiac lesions, but not the accompanying brain lesions. They suggested that the cardiac lesions may be secondary to the brain lesions or develop independently, perhaps owing to sympathetic overstimulation. Similar lesions with ACh at lower doses have not been cited in the literature presumably because of its rapid hydrolysis.

It may be important to note that all AChE inhibitors may not demonstrate the same cardiac effects. Thus, notably DFP did not elicit a pressor response [90]. Sulphur-containing OPs showed a slower onset of hypotensive signs compared with their oxygen analogues; however, the same sulphur compounds appear to have a greater effect in reducing heart rate before cardiac arrest [4]. After tabun was administered in the dog, a drop in arterial pressure accompanied by bradycardia was observed [39]. Following sarin or TEPP administration in the dog, there is a vasodilator effect which may be masked by vasoconstriction in the limbs and splanchnic area. This results from an increase in sympathetic vasoconstrictor tone [20]. Although earlier studies suggested that only blood vessels of certain size were mainly affected by cholinergic agonists, it may be that all blood vessels are affected by ACh [19].

However, additional mechanisms have been suggested for this vasodilation. Banjac and Drewes [7] have proposed that the mechanism of OP-induced vasodilation is not completely related to the action of muscarinic receptors. They propose that the vasodilation is, at least in part, caused by EDRF (endothelium-derived relaxing factor). De Neef *et al.* [22–25] concluded that the vascular effects of ChE inhibitors are, in part, the result of their action on central muscarinic receptors [107]. The accumulation of ACh then induces a decrease in blood pressure.

Several studies have been performed utilizing a wide variety of cardiac preparations to characterize the toxicology of antiChEs. There is a lack of agreement in this area. Administration of the antiChE drugs TEPP, DFP, sarin or soman did not produce significant effects in the isolated cat papillary muscle even at the high concentrations employed [71]. Nevertheless, it has been reported that OPs have an effect in the isolated heart preparation [43,58].

VX, VE (O-ethyl S-(2-dimethylaminoethyl) methylphosphonothioate), and VS producd a dose-dependent bradycardia and negative inotropy in the isolated rabbit heart [59]. Similarly, VX was found to produce arrhythmias in the isolated dog heart [102]. Although atropine did not increase ChE, it did increase contractile force and heart rate in the same VX-treated heart. However, DuBois found that administration of parathion or its oxygen analogue showed little sensitivity in the isolated rabbit heart but induced cardiovascular effects characteristic of AV block in the *in vivo* preparation [27]. Further studies are needed to clarify the source of the cardiac actions of the ChE inhibitors. The controversy might be resolved if Purkinje tissues and/or endothelium were suggested to be an important site for the action of these compounds. In isolated papillary muscles or in certain isolated (e.g. initially hypoxic) heart preparations, the endothelium or Purkinje tissue may be damaged. This may prevent the release of factors such as EDRF, which in turn could affect heart function.

OP effects of vagal activity

Holmstedt [43] reviewed the relative ability of OP compounds to lower blood pressure. This capacity is thought to be correlated with their effect of inhibiting ChE. The vagal effects of OPs are potentiated by the addition of exogenous ACh. The bradycardia may lead to cardiac arrest. Ventricular escape after 30–60 s (20–30 beats/min) or death may ensue. Demonstrations in anaesthetized dogs have shown that muscarinic blockade alone is not equivalent to vagal blockade. It is suggested that increases in vagal activity (e.g. caused by administration of OPs) need to be antagonized with both atropine and a nicotinic agent so they are blocked completely [14]. Anaesthesia changes the circulatory and respiratory effects of sarin. In unanaesthetized dogs, there was usually a decrease in pulse frequency, an increase in arterial carbon dioxide tension, and a fall in arterial blood pH before changes in respiratory rate or volume were evident [30]. This suggests that an initial cardiovascular change could, through effects on carbon dioxide receptors or blood pH, trigger respiratory changes. Previously, it has been assumed that respiratory changes occurred before cardiovascular changes.

ChE inhibitors may exert their cardiovascular (i.e. blood pressure) effects, in part, by acting on cardioinhibitory vasomotor and respiratory centres. The role of these centres is questioned by different results. Polet and De Schaepdryver [82] suggest no direct involvement; however, studies by Preston and Heath [83,84] indicated that the central vasomotor system was at least partially responsible for OP-induced hypotension.

Atrial effects of antiChEs

OP-induced bradycardia and negative inotropy have been shown to occur in isolated atrial preparations [9,13], in whole animal preparations [44], and in clinical situations [29]. Similar changes occur with other ChE inhibitors such as eserine [15]. The atrial bradycardia, which is accompanied by a decrease in oxygen consumption, occurs before blood pressure changes are noticed [44]. The heart rate and blood pressure effects may be bimodal; they depend upon several factors such as the species examined, the dose and type of ChE employed, and whether the animal was apneic or not [46].

Tachycardia, as well as bradycardia, has been described in humans following OP poisoning by a mixture of Cosan (elemental sulphur), zineb and demeton-S-methyl [42], sarin or soman [93], and parathion, trichlorfon or malathion [91]. The cause of this unexpected effect is not known, but it has been suggested that it may result from the co-intoxication of other chemicals such as fungicides (e.g. zineb). Another more probable cause is the release of endogenous catecholamines. When conditions exist for catecholamine release, tachycardia may predominate over the expected bradycardia. This phenomenon could lead to clinical confusion in an emergency situation. If trained to recognize OP intoxication using bradycardia as a sign, the appearance of tachycardia clouds the diagnosis. Following administration of sarin or TEPP, for example, there may not be a stoichiometric relationship between blood pressure and heart rate [113]. All the factors, including the

appearance of cardiac arrest [91], are not completely understood.

In atria, both AChE and BChE function to modulate the chronotropic effect of ACh [95]. It is suggested that OPs decrease atrial rates by inhibiting both AChE and BChE activity. It is also suggested that PsChE may be more important for the decreased rate induced by ACh and that true ChE may be more important for the amplitude of atrial contractions caused by ACh [13]. This leads to speculation that OPs would preferentially effect one enzyme more than the other, and may display characteristic pharmacology. This suggestion needs to be studied in greater detail.

AntiChEs and the ventricular system

In addition to the atrial effects of cholinergic agents, it is known that cholinergic stimulation of the ventricles occurs [40,115]. In the rat, administration of soman produced a sinus bradycardia, reduced the height of the P wave, prolonged the P-Q interval, prolonged the QRS complex, and caused AV block [105]. In adult baboons receiving soman, cardiac arrhythmias occurred in all animals and were occasionally the first sign of intoxication. Premature atrial or ventricular contractions, first-, second-, or third-degree AV block, and ventricular tachycardia or fibrillation were observed. The severity of arrhythmia was dose-related. All animals receiving 1.25 LD_{50} showed major arrhythmias [3]. Hassler *et al.* [36]found complex arrhythmias in dogs treated with soman. In rats, Robineau [86] observed several ventricular premature complexes after administration of VX. In rhesus monkeys, however, insignificant arrhythmias were observed when intramuscular soman injection was followed by treatment with atropine, 2-PAM, and pyridostigmine [37].

In humans, apparent ventricular arrhythmias induced by antiChEs have been treated with atropine, oximes, cardiac glycosides, diuretics, and anti-adrenergic drugs [58]. Analysis of 73 cases in the acute period of poisoning with parathion, trichlorfon or malathion, revealed an increase in the electric systole appeared to be correlated with blood ChE inhibition. Changes in electric systole were proposed to be characteristic for the cardiac intoxication of OP agents [68]. Frequent intracardiac, atrioventricular, and intraventricular conductivity disorders were observed in 1305 persons, aged 18–54 years [96]. It was suggested that these conductivity disorders were a result of dystrophic myocardium. It is of interest that one of these same authors found that changes in catecholamine metabolism may be responsible for the cardiac intoxication seen with the chlorinated terpene pesticides [97].

It has been shown that DFP could produce ventricular premature complexes, 'torsade de pointes' in humans ([67]. Similar effects have been observed following VX administration in the dog [87], and they possibly occur in sarin intoxication in baboons [45]. Recent observations indicate that cholinergic autonomic innervation of the ventricle [115] may provide a site for these ventricular arrhythmias to occur in response to OPs.

Therapy for antiChEs

There is a lack of agreement on how to treat the cardiac arrhythmias and cardiac failure resulting from antiChE intoxication. Factors including type of compound and route used, preparation and species examined, and dose and the period after intoxication, appear to influence the results.

Several papers have described that cardiotoxic effects of certain ChE inhibitors can be reversed by cholinolytics such as atropine [20] or oximes like 2-PAM [105]. However, Bethe *et al.* [9] found that cardiovascular effects were non-specific because they were reversed by wash-out in isolated heart preparations but not abolished by atropine or 2-PAM. Larson and Brown [59] found that, in the isolated heart, atropine did not appear to antagonize all the effects of V agents and 2-PAM did not offer complete antidotal action. They concluded that a portion of the toxic effect was not from the cholinergic actions of the chemical. In other studies in the isolated atria, bradycardia is not reversed by wash-out but is abolished by atropine or 2-PAM [9]. In rat, 2-PAM did not reduce the initial hypertension produced by soman [105]. 2-PAM increased blood pressure after administration of ACh in dogs. This effect may not be due to

serum ChE [65]. In several clinical reports atropine has been shown to be effective against insecticide overdose, but 2-PAM treatment shows an unsatisfactory response. The lack of effect observed with 2- PAM may be from the time delay at which it was administered in relation to the initial OP toxicity.

When atropine is used in the treatment of OP poisoning, it can cause excessive tachycardia or ventricular fibrillation. To prevent this intoxication, propranolol has been used clinically when atropine was administered as a treatment for insecticide overdose [101]. Oberst *et al.* [75,77] found that atropine was not effective against sarin intoxication in the dog if the blood pressure had fallen. This observation may reflect the possibility that cardiac OP effects are not solely muscarinic actions (e.g. nicotinic) and are not treatable by atropine. In the dog model, parathion-induced ChE inhibition was followed by atropine treatment during the preterminal period. This procedure resulted in either ventricular fibrillation or spontaneous resuscitation and survival. However, death usually occurred after the use of atropine during anoxia without previous precipitation of ventricular fibrillation [31,66]. Atropine produced ventricular fibrillation in the dog following VX, sarin, or soman administration when anoxic cyanosis ensued [57,114]. Ventricular fibrillation is also seen following iv injection of TEPP in the dog when there is a 4–5 min delay in atropine or caramiphen [109]. This type of fibrillation was not observed in rabbits, cats, or monkeys. However, cardiac arrest was seen in monkeys. A combined treatment of atropine and clonazepam showed less cardiovascular change than when atropine was administered alone [62–64].

A series of pharmaceutical preparations which are referred to as 'nemicol' have been described by Kotev *et al.* [49,54–56,81,100]. The preparations composed of a mixture of an oxime (TMB-4), atropine and benactyzine (TAB) appeared to reverse the OP-induced ventricular arrhythmias back to a single sinus rhythm.

Although pretreatment with atropine may prevent bradycardia [113], the use of atropine and oximes may not be the treatment of choice for OP-induced 'torsade de pointes' ventricular arrhythmias. Atropine prevents the atrial bradyarrhythmia but it does not completely antagonize the ventricular arrhythmia. The use of isoprenaline (isoproterenol) to produce overdrive suppression or cardiac pacing has been suggested [67]. Use of cardiac pacemakers developed for emergency room application [116] may provide a practical treatment for acute OP intoxication. De Neef *et al.* [25] found that the pressor effects of paraoxon were not influenced by atropine methonitrate or mecamylamine. These results suggest a role for CNS muscarinic receptors in OP toxicity. However, it was found that when dexetimide, phentolamine, or prazocin was combined with yohimbine, the pressor effect was reduced or prevented. The usefulness of these antiadrenergic drugs suggests that postganglionic sympathetic activity may play a role in the effect of antiChE inhibitors. Mixtures of atropine and a substituted catecholamine such as Sympatol (2-N-methylamino l-p-hydroxyphenyl ethanol) have been reported to provide a reduction in mortality in tabun or sarin poisoning [108]. The administration of phenoxybenzamine to soman-poisoned dogs following atropine and 2- PAM treatment increased the time for recovery of blood pressure to normal values [35]. Similarly, Ojewole [78], found that phenoxybenzamine could antagonize ACh effects in isolated, paced guinea pig left atria.

Gucciardi and Scorsone [33] found a combination of atropine, chlorpromazine, and ACTH was useful in reversing Malatox 20 (50% malathion and 20% parathion)-induced arrhythmias that were manifested as AV dissociation in a guinea pig model. The role of ACTH needs to be evaluated further. The capacity of antidotes to reverse OP cardiotoxicity varies with the animal species [48]. Selecting a species that closely resembles humans would be important for the development of a successful antidote for this group of poisons.

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14

Effects of organophosphates on the heart

J.M. Pimentel and R.B. Carrington da Costa

Introduction

The clinical picture produced by acute OP pesticide poisoning is classically divided into muscarinic, nicotinic and CNS effects. The symptoms and signs may appear in different combinations according to severity of poisoning and route of absorption, and dose and type of compound. In spite of reports on ECG changes, the magnitude of muscarinic symptoms, of which the most important is acute respiratory failure often associated in the more severe cases with CNS disturbances, has monopolized monitoring as well as attitudes to therapy.

Cardiac arrhythmias and OPs

Cardiac arrhythmias related to OP poisoning have been described [9– 11,13,15]. Fazekas and Kiss [7] reported a case of congestive cardiomyopathy caused by long-term OP exposure. More recently Ludomirsky [13] related the ECG changes to the delayed sudden death after patients appeared to recover from the more dramatic respiratory and CNS symptoms. OP poisonings with a similar course have been observed in the intensive care unit (ICU) at Coimbra [6].

Treatment of such patients in intensive care units with continuous ECG monitoring has confirmed that the presence of those alterations has been underestimated.

Types of ECG effects

In spite of the different percentages mentioned in the literature, probably related to differences in severity of poisoning and/or OP type, changes observed can be divided into: (1) arrhythmias (ventricular and supraventricular), (2) disturbances of conduction (A-V and intraventricular), and (3) repolarization abnormalities.

However, the most frequent are arrhythmias and alterations of ventricular repolarization. In a sample of 40 patients (77.5% male, mean age 49.5±18 years) treated in our Unit [6], the study of 244 ECGs showed that a total of 55% of patients had arrhythmias. Of these, 25% showed supraventricular arrhythmia, atrial extrasystoles, flutter or fibrillation, and 15% ventricular arrhythmia (extrasystoles, bigeminal rhythm, ventricular tachycardia and pleomorphic ventricular tachycardia 'Torsade de Pointes') and 15% mixed arrhythmias. Disturbance of conduction was observed in 25% of patients, 10% of which being atrioventricular. Abnormalities of repolarization were seen in 52.5% of patients.

In the first 3 days after admission 82.5% of the ECGs showed a prolonged QTc interval. This is a frequent change [11,13,15] and is indicative of cardiac toxicity, with an increased risk of serious arrhythmias [13,14].

Cause of arrhythmias

The administration of large doses of atropine associated, in early stages of OP poisoning with transient episodes of hypoxia, acidosis and electrolyte imbalance (hypokalaemia) [8], and immediately corrected on admission to the ICU, has been implicated in such ECG changes. They may occur early but may appear as late as 7 days after poisoning. Kiss and Fazekas [9] reported cases where arrhythmias occurred between the third and 15th day in a high percentage of cases and were not related to electrolyte imbalance or therapeutic use of atropine or obidoxime. It is therefore difficult

to implicate atropine in the causation of arrhythmias occurring in the later stages of poisoning.

Continued and unequal intense parasympathetic and sympathetic activity may account for some of these arrhythmias [13]. However, in a proportion of cases, it is likely that OP pesticides (mainly in commercial formulations) are directly toxic to cardiac cells.

Morphological observations

Limaye [12] described a 'toxic myocarditis' which he found in 76 necropsies, and in 1982 Kiss and Fazekas [11] described focal myocardial damage with pericapillary haemorrage, micronecrosis and patchy fibrosis at autopsies of victims of OP poisoning.

Recent published papers [1–3,5] suggested that insecticides may exert some of their toxic actions by modifying basic cell membrane mechanisms. OPs such as parathion and azinphos-ethyl have an immediate effect of stimulating Ca^{2+} uptake and ATP hydrolysis promoted by the Ca^{2+} pump system of the sarcoplasmic reticulum. The same authors suggested that compounds such as parathion would preferentially accumulate in highly functional membranes or organelles such as mitochondria and microsomes. The myocardial cell is highly energetic and its electrical properties may make the heart particularly susceptible to alterations in cellular ionic balance, which can affect automaticity and conductivity. On the other hand the reaction to injury is usually seen either as changes in cell or tissue function or as changes in cell structure [4].

The morphological changes described led us to study the myocardial ultrastructural changes immediately after death, and results are discussed below.

Clinical studies

Ten patients (aged between 19 and 70 years) with severe poisoning with OP pesticides (nine with azinphos-ethyl and one with dimethoate) all presenting ECG changes were studied. They died between the third and 17th day after admission. Three samples of the left ventricle (2×2 mm), were taken 5–15 min after death and fixed with 1% glutaraldehyde +4% formaldehyde in phosphate buffer (pH 7.3). They were postfixed in 1% osmium tetroxide buffered with cacodylate (pH 7.3) and stained with uranyl acetate and lead citrate for electron microscopic studies.

All patients showed myocardial ultrastructural morphological changes (Figs 14.1–14.4) of different degrees of severity. The lesions observed were as follows: (1) areas of partial or extensive lysis of myofibrils; (2) mitochondria which exhibited decreased electron density, and swollen forms, fragmentation or lysis of cristae; (3) nuclei appeared to show irregularity in shape and various degrees of disorganization of chromatin; and (4) Z band abnormalities of various degrees.

Experimental studies

To amplify these data an experimental study with male Golden hamsters (average weight 100 g) was carried out. The chosen OP was azinphos-ethyl (AzE) because 50.8% of our patients took that compound. The route of administration of the test material was ip and the animals were killed on the fifth day under ether anaesthesia. The hamsters were randomly allocated into four groups: group 1, control (n=8); group 2, 0.8 mg AzE daily, for 4 days (n=10); group 3, 1.6 mg AzE (0.25 mg atropine on the day 1 and 0.8 mg daily for 3 days (n=10); and group 4, 9.25 mg atropine on day 1 (n=10). The same techniques of fixing and staining were used throughout. All hamsters injected with AzE (Figs 14.5 and 14.6) showed myocardial morphological changes, however they were more severe in group 3 but less severe than those observed in the human cases. Groups 1 and 4 were normal.

The explanation for the milder changes observed in hamsters could be that human suicide cases took proportionally higher doses than those which were given to hamsters; it is possible that short periods of ischaemia and/or hypoxia before admission to the ICU and the composition of solvents may worsen the myocardial lesions. However, we believe that OPs act directly on the heart; it is noteworthy that group 4 in the animal study, the atropine-only group, showed no histopathological abnormalities in the heart. This could explain both the ECG changes and the cases of sudden death after a good clinical outcome.

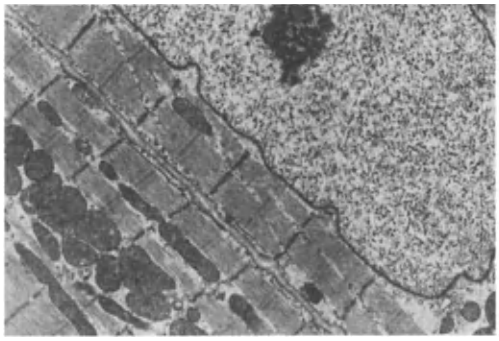

Figure 14.1 Normal human myocardium. Magnification 6600

Figure 14.2 J.L., male, 19 years old, azinphos-ethyl poisoning, died 3 days after admission. Magnification 9800

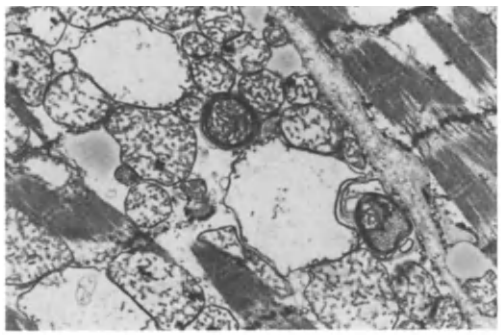

Figure 14.3 M.D.M., male, 60 years old, azinphos-ethyl poisoning, died 39 days after admission. Magnification 8300

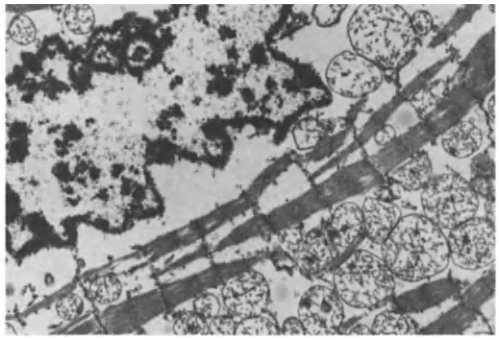

Figure 14.4 J.L., male, 19 years old, azinphos-ethyl poisoning, died 3 days after admission. Magnification 5000

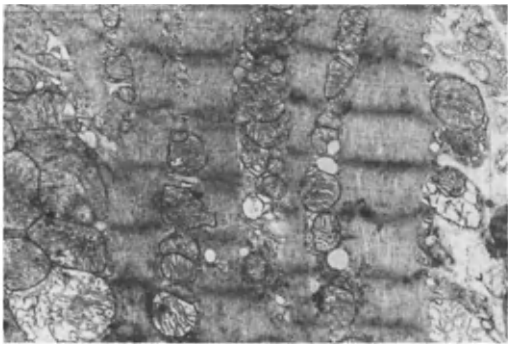

Figure 14.5 Myocardium of a Golden hamster (group 2). Magnification 6600

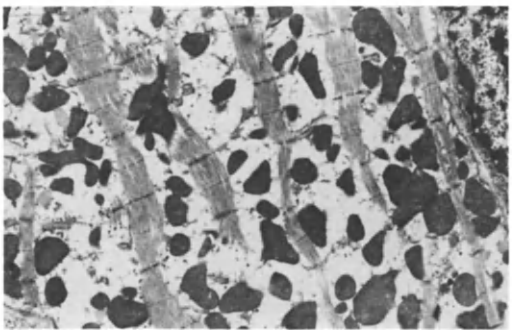

Figure 14.6 Myocardium of a Golden hamster (group 3). Magnification 5000

Acknowledgments

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15

Effects of anticholinesterases on airway smooth muscle

Michael Adler, David H. Moore and Margaret G. Filbert

Introduction

Exposure to ChE inhibitors leads to accumulation of ACh at both N- and M- cholinoceptors. The signs of exposure vary with the agent, dose and route of administration and generally include constriction of the airways and increases in tracheobronchial secretion [13]. These effects are especially pronounced following inhalation of antiChE vapours or aerosols and usually precede the onset of generalized systemic effects [14].

The airways are especially susceptible to airborne mediators because of their continuous interactions with the external environment. This vulnerability was best described by Ziment [15] in the introduction to his chapter on bronchospasm, 'The airways of the human lung are endowed with a spiral covering of involuntary muscle, which probably causes more harm than benefit. It is unfortunate that these muscle fibres undergo inappropriately severe constriction in reaction to various types of irritation...'. Among the factors responsible for the sensitivity of airway smooth muscle to antiChEs are the dominance of excitatory cholinergic innervation [12] coupled with a relative resistance to muscarinic receptor desensitization and muscle fatigue.

This chapter will focus on the mechanisms underlying the antiChE-induced constriction of isolated canine tracheal smooth muscle. Canine trachea was selected as a model because of its morphological and pharmacological similarities to human trachea. Most experiments described in this chapter were performed with the potent, irreversible, OP ChE inhibitor, soman; qualitatively similar results were also obtained with sarin, paraoxon, physostigmine, neostigmine and pyridostigmine. [The term ChE used here designates the combined activities of acetylcholinesterase (EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8).] This convention was adopted because in canine trachealis butyrylcholinesterase constitutes an appreciable fraction of the total ChE activity and appears to coregulate ACh lifetime [2].]

Characterization of antiChE responses

Low concentration effects

For all ChE inhibitors examined, exposure to low concentrations caused enhanced contractions to electric field stimulation (EFS) or exogenously applied ACh, while higher concentrations caused sustained contractures. At 10^{-9} M, the lowest concentration of soman that had a consistent effect on muscle tension, the inhibitor increased the amplitude and prolonged the half-relaxation time (HRT) of contractions elicited by EFS (Figure 15.1) and shifted the ACh concentration-response curve to the left. The effect on EFS responses was evident within 2 min of soman addition and, by 30 min of exposure, the amplitude and HRT were increased to 152 (18.9%) and 220 (34%) of control, respectively (mean (s.e.m.); n=8).

The ability of soman to prolong the decay of EFS-elicited contractions was markedly dependent on frequency of stimulation (Figure 15.2). Between 3 and 60 Hz, the HRT of soman-treated muscles increased exponentially with frequency, undergoing a 2.4-fold change over this interval. The frequency-dependence reflects the increasing difficulty in

hydrolyzing ACh by the residual ChE as the release rates are augmented.

Muscles bathed in control Tyrode's solution showed no increase in the decay of EFS contractions elicited at frequencies between 0.3 and 10 Hz and only slight increases at 30 Hz and above. This suggests that under control

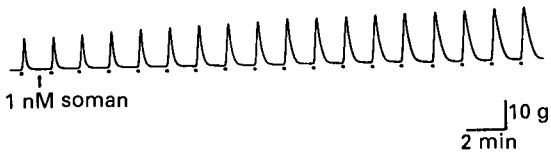

Figure 15.1 Effect of soman on contractions elicited by 10 Hz EFS pulses. Stimulations (.) were for 20 s at intervals of 1.6 min. Note the gradual increase in the amplitude and decay as ChE is inhibited. The ChE activity corresponding to the time of the last trace was 68 (6%) of control

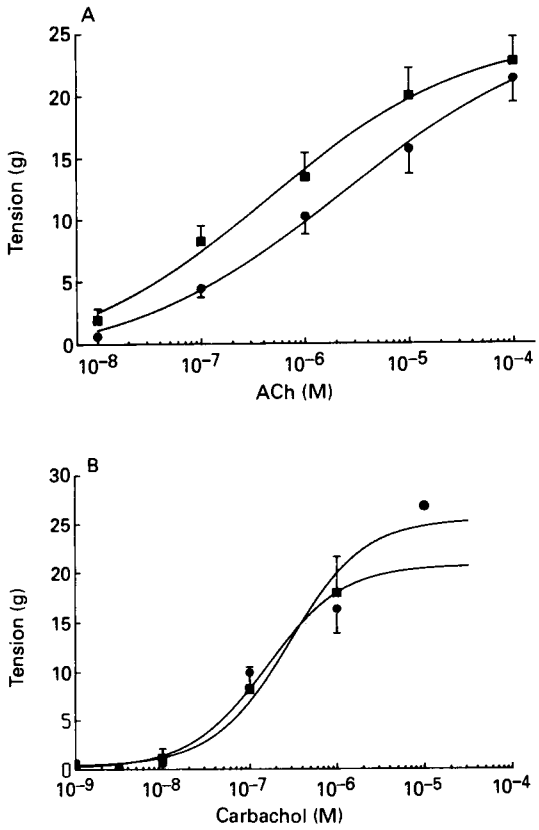

Figure 15.3 Concentration-response curves for contractions elicited by ACh (A) or carbachol (B). The symbols represent the mean values from 8–16 strips under control conditions (●) and after exposure to 10^{-9} M soman (■)

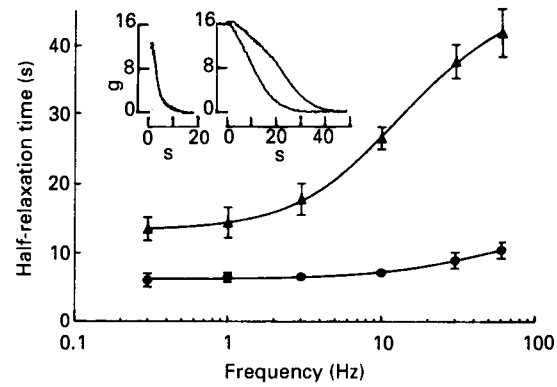

Figure 15.2 Effect of stimulation frequency on the half-relaxation time of EFS-elicited contractions. The symbols represent the mean values obtained from 8–12 strips under control conditions (●) and 43 min after exposure to 10^{-9} M soman (▲). The inset shows superimposed decays at 3 and 10 Hz under control conditions (left) and in the presence of soman (right). In each case, the amplitude was scaled to the higher frequency

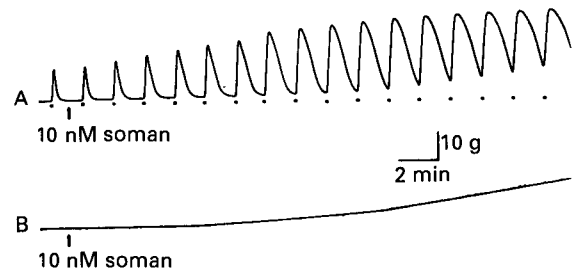

Figure 15.4 (A) EFS-elicited contractions before and during exposure to 10^{-8} M soman. EFS trains (.) were evoked every 1.6 min for 20 s at 10 Hz. (B) Spontaneous increase in baseline tension in a non-stimulated strip exposed to 10^{-8} M soman

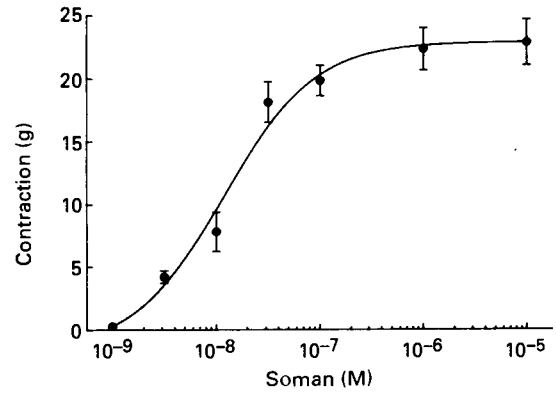

Figure 15.5 Concentration-response curve from trachealis strips exposed for 30 min to soman. The symbols represent the mean contractures recorded from 11–23 muscle strips

conditions the levels of ChE are sufficient to prevent ACh accumulation even during sustained high release.

Figure 15.3 shows the effect of soman on contractions elicited by bath- applied ACh (Figure 15.3A) or carbachol (Figure 15.3B). Under control conditions, ACh produced steady contractions with an EC_{50} of 2.3 μM (n=16). In the presence of 10^{-9} M soman, the ACh concentration-response curve shifted significantly to the left such that the EC_{50} was reduced to 0.44 μM. No significant increase was observed in the sensitivity of tracheal smooth muscle to the non-hydrolyzable agonist, carbachol (Figure 15.3B). These results suggest that the shift in the ACh concentration-response curve did not arise from soman-induced changes in receptor affinity but from impairment of transmitter hydrolysis. Raising the soman concentration to 10^{-8} M led to a more pronounced increase in the response to EFS pulses and to the appearance of a sustained contracture (Figure 15.4A). This contracture developed with a latency >10min., occurred with or without periodic stimulation (Figure 15.4B) and was always preceded by enhancement in the EFS tension. If allowed sufficient time for equilibration (approximately 90 min), muscles exposed to 10^{-8} M soman generated contractures of 16.4 (1.7) g. This represents nearly 80% of maximal tension observed with soman concentrations $\geq 10^{-6}$ M (Figure 15.5). For comparison, tensions obtained in the presence of 10^{-5} M ACh or 30 Hz EFS trains in control muscles were 19.7 (1.9) and 20.4 (1.1) g, respectively (n=18).

For all antiChEs examined, contractures were detected when ChE activities were reduced by $\geq$ 52% (Figure 15.6). Further inhibition of ChE activities led to an approximately linear increase in the contracture amplitudes. The finding that contracture depends on degree of ChE inhibition and not on the nature of the inhibitor suggests that it is mediated by ACh accumulation.

Direct effects of the OP inhibitors were not observed in the concentration range studied; direct effects of the CBs were observed but these occurred in concentrations in excess of those required for complete ChE inhibition ($\geq 3 \times 10^{-5}$ M). Furthermore, the direct effects of the CBs were always inhibitory.

Unlike agonist-induced contractions, reversed readily by washout, maximal soman-induced contractures generally showed little or no recovery after multiple washes with control Tyrode's solution (Figure 15.7). Aas *et al.* [1] reported a similar finding in guinea pig bronchial smooth muscle. With submaximal contractures, washout generally resulted in partial relaxation followed by re-contraction.

The situation was different during prolonged wash. Relaxation of soman-induced contractures occurred slowly with a half-time of 4.6 (0.5) h (n=4) beginning after 8.7 (0.4) h of sustained tension. No reversal of the abnor-

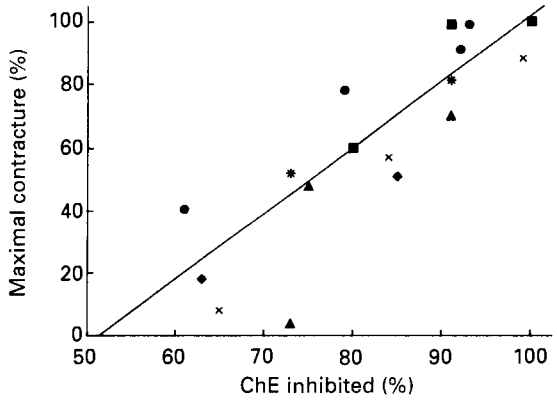

Figure 15.6 Contractures plotted as a function of ChE inhibition for three OP and three CB ChE inhibitors. Each symbol represents a single strip on which both tension and ChE activity was determined. The inhibitors studied were: ◆, soman; *, sarin; X, paraoxon; ●, physostigmine; ▲, neostigmine; ■, pyridostigmine

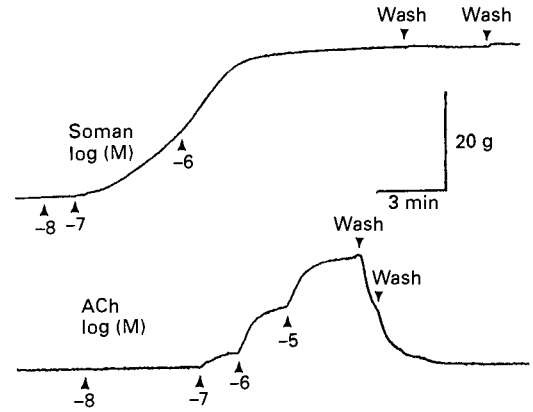

Figure 15.7 Comparison of tensions generated by soman (upper) and ACh (lower). Additions are denoted by arrows. Note the absence of relaxation after washout of unreacted soman and the prompt relaxation following washout of ACh

mal decays of EFS-induced contractions or recovery of enzymatic activity was observed. The relaxation of baseline tension may result from reduction in resting ACh release, alterations of muscarinic receptor function or muscle fatigue.

Soman-induced contractures were not altered by the ganglionic blocker hexamethonium (C6) or the sodium channel blocker tetrodotoxin (TTX) (Figure 15.8). These results suggest that contractures are independent of ganglionic transmission and nerve impulse generation. The contractures are, however, dependent on endogenous ACh release. This was demonstrated by Adler *et al.* [3], who showed that soman-induced contractures were significantly depressed after ACh stores were depleted by hemicholinium treatment.

For all concentrations of soman that produce complete inhibition of ChE, although the magnitude of the resulting contractures do not differ, their latency and rise time show a marked concentration-dependence (Figure 15.9). Over the concentration range 10^{-8}–10^{-5} M, the latency before onset of tension was reduced from 13.8 min to 24 s and the 95% rise time decreased from 33.1 min to 5.6 min. The latency appears to reflect the time required to inhibit a critical fraction of ChE. Inhibition of this critical fraction allows accumulation of basally

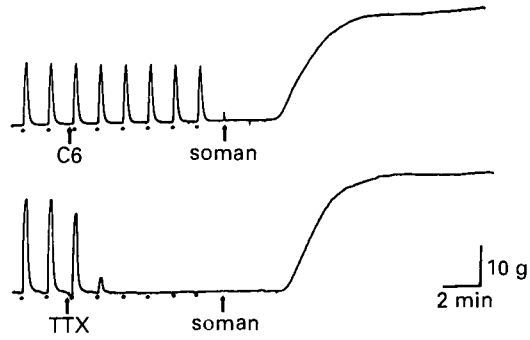

Figure 15.8 Traces illustrating lack of dependence of soman-induced contractures on ganglionic transmission or nerve impulse generation. The first arrow denotes addition of 10^{-5} M of the ganglionic blocker, hexamethonium (C6) or 2×10^{-7} M of the sodium channel blocker, tetrodotoxin (TTX); the second arrow denotes addition of 10^{-6} M soman. EFS responses were elicited every 1.5 min as indicated by (.)

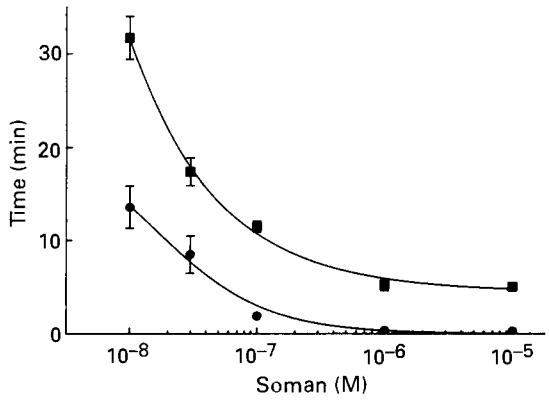

Figure 15.9 Concentration-response curves for alterations in the latency (●) and 95% rise-time (■) of soman-induced contractures. The symbols represent mean of 11–23 muscle strips

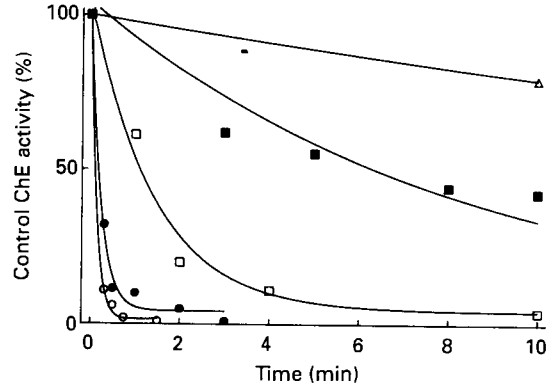

Figure 15.10 Kinetics of inhibition of ChE by soman. Tension and ChE activities were determined on the same strips in the presence of soman concentrations of 10^{-9} M (△), 10^{-8} M (■), 10^{-7} M (□), 10^{-6} M (●) and 10^{-5} M (○)

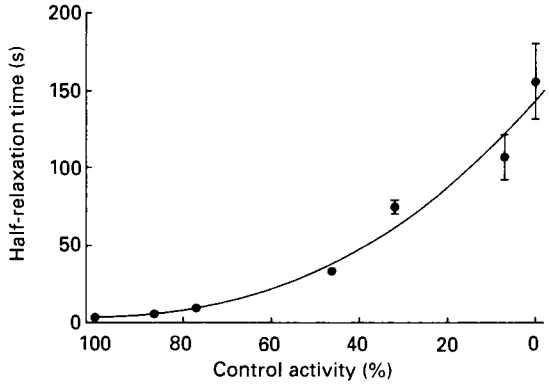

Figure 15.11 Prolongation in the half-relaxation time of EFS-elicited contractions in the presence of physostigmine (10^{-8}–10^{-5} M). The data were obtained by using 3-s trains with 30 min equilibrations between additions. The last four responses were superimposed on contractures

released ACh that presumably underlies the development of contractures.

Comparison of the concentration-latency relationship with the kinetics of soman inhibition (Figure 15.10) reveals that this critical fraction is 58.2 (3.4) % of the control ChE activity.

Rise time appears to reflect the combined processes of ChE inhibition and ACh accumulation. With soman concentrations between 10^{-8} and 10^{-7} M, the rise time is determined primarily by enzyme inhibition, but with soman concentrations $\geq 10^{-6}$ M, the rise time is nearly constant and reflects the time required for ACh accumulation to reach steady state. From rise time data obtained in the presence of 10^{-6} M soman, ACh accumulation reaches steady state approximately 6 min after transmitter hydrolysis is abolished.

The time course for ACh elimination was assessed from the the decay of contractions produced by brief (1–3 s) electrical pulses in the presence of increasing concentrations of physostigmine. Physostigmine was selected because it equilibrated rapidly and the brief trains were chosen to avoid complications from activating the non-adrenergic/non-cholinergic inhibitory pathway [5]. In Figure 15.11, before ChE inhibition, a brief EFS train produced a contraction with a HRT of 4.3 (0.56) s (n=9). In the presence of increasing concentrations of physostigmine, the HRTs became progressively longer; when ChE was completely inhibited by exposure to 10^{-5} M physostigmine, the contraction elicited by a 3-s train decayed with a HRT of 156.0 (24.5) s.

Although tension is many steps removed from the transmitter receptor interaction, its decay nevertheless establishes an upper limit for the lifetime of neurally released ACh. Thus the persistence of ACh when ChE is active cannot exceed 4.3 s and is likely to be much shorter [8]. The decay is determined primarily by the relaxation of the contractile proteins. The prolonged decays in the presence of ChE inhibitors, on the other hand, exceed the time required for passive relaxation and thus appear to be an accurate reflection of the time required for the clearance of ACh when hydrolysis is impaired. The graded prolongations shown in Figure 15.11 arise from the decreasing contribution of enzymatic hydrolysis and increasing contribution of diffusion to transmitter efflux. The 156 s determined for the HRT when ChE is completely blocked represents the half-time of transmitter diffusion in the absence of hydrolysis; this process is over 5-orders of magnitude greater than that expected for free diffusion of ACh in the absence of receptor interactions [6].

The enormous slowing in the elimination of ACh observed in the presence of ChE inhibitors in tracheal smooth muscle is analogous to that reported at vertebrate NM junction. For the latter, the antiChE effects have been attributed to hindered or buffered diffusion of ACh as a result of multiple transmitter-receptor interactions [9]. In skeletal muscle, the factors responsible for multiple interactions are the high density of ACh receptors at the motor endplate in conjunction with diffusional constraints imposed by the close apposition of the pre- and post-junctional membranes [11].

In tracheal smooth muscle, the receptor density of 21 sites/μm^2 [4] is low relative to skeletal muscle but the spare receptor population is considerable [7]. Therefore the slowing of ACh efflux is suggested to arise from interactions with the large number of spare muscarinic receptors that exist in this tissue. Geometric constraints to ACh diffusion do not appear to be relevant in this tissue because the transmitter release sites are separated by large distances (1–1.5 μm) from the postsynaptic membrane [12].

Evidence consistent with the hypothesis that spare receptors account for the slow removal of ACh is provided in Figure 15.12. The record (in A) is a contraction elicited by a 10-s EFS train in the presence of 10^{-8} M soman and is superimposed on a 12.5 g contracture. The EFS response underwent a striking prolongation lasting nearly 4 min. The presence of a plateau before the slow relaxation of this contraction suggests that ACh is saturating during the early portion of the decay.

Addition of 10^{-7} M atropine (B) led to 40% shortening in the overall decay of the EFS response within 15 min, in addition to the expected reduction in its amplitude. The shortening arises presumably from the blockade of a portion of the muscarinic receptor population. Receptor blockade is expected to curtail the number of transmitter-receptor interactions thereby permitting the removal of ACh

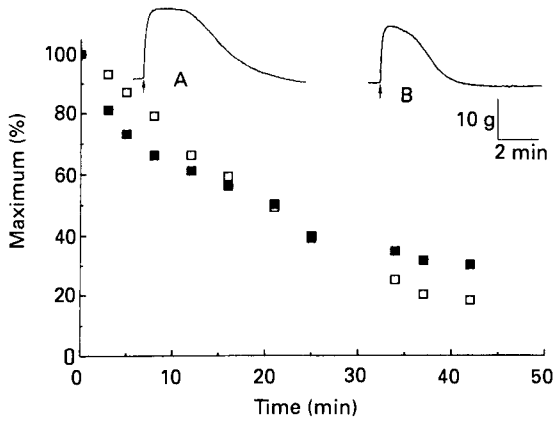

Figure 15.12 Effect of atropine on the amplitude (□) and half-relaxation time (■) of soman-prolonged EFS contractions. The insets show a contraction in the presence of 10^{-8} M soman (A) and 15 min after addition of 10^{-7} M atropine (B)

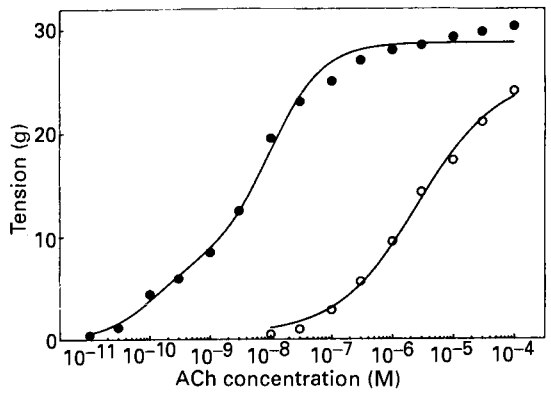

Figure 15.13 Concentration-response curves for ACh-induced contractions before (○) and after (●) complete ChE inhibition by exposure to 10^{-7} M soman

to approach its free diffusion limit. Atropine can restore EFS responses to within a factor of 2 of their pre-ChE inhibited value.

In contrast to the relatively small shift in the ACh concentration-response curve observed with 10^{-9} M soman (Figure 15.3), a much more pronounced shift is obtained after complete ChE inhibition (Figure 15.13). The control response was recorded after depletion of endogenous ACh by incubating strips in hemicholinium and stimulating with 10 s EFS trains every 1.5 min for 6.5 h. The EC_{50} calculated under these conditions was 2.6 μM, a value similar to that obtained on non-depleted strips. After exposure to 10^{-7} M soman for 30 min to inhibit ChE completely, responses to ACh were markedly enhanced. Tension could be elicited by ACh concentrations as low as 3 × 10^{-11} M. The curve in ChE-inhibited preparations appeared to have at least two components, with an inflection at approximately 3 × 10^{-9} M ACh. Based on the differences between ACh concentration-response curves before and after soman exposure, ChE inhibition appears to unmask the presence of a high affinity subpopulation of M-cholinoceptors. Whether this is indeed the case or a reflection of the increased residence time of ACh is not clear.

From Figure 15.13, it is apparent that in ChE-blocked preparations, the concentration of ACh that approximates a soman induced contracture is 10^{-8} M. This suggests that in non-depleted muscles, the concentration of ACh that accumulates from resting release is also 10^{-8} M. It is of interest that Katz and Miledi [10] proposed a similar value for the synaptic ACh concentration of skeletal muscle during ChE blockade. In skeletal muscle, however, 10^{-8} M ACh produces less than 1 mV of depolarization while in airway smooth muscle, the same ACh concentration generates large sustained contractures sufficient to occlude the airways.

Acknowledgements

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16

Pulmonary toxicity of anticholinesterases

David Dinsdale

Introduction

The lungs provide an important route for the entry of many xenobiotics into the body and antiChEs are no exception. Pulmonary reactions to the administration of these compounds, by inhalation or by other (systemic) routes, include bronchoconstriction with tightening of the chest, wheezing respiration and increased secretion of fluid into the airways. Symptoms of human exposure to vapours, aerosols or fine particles of antiChEs have been reported at doses far lower than those required to elicit similar responses during oral or dermal exposure to the same compound [17]. The inhalation of doses sufficient to produce cholinergic symptoms does not, however, produce changes in basal lung resistance in experimental animals, except when provoked by an aerosol of ACh [42]. Inhaled antiChEs may thus have a systemic action rather than a local effect on the lung and so pulmonary function tests are probably less sensitive, than plasma ChE levels, as indicators of inhalation exposure. These results suggest that inhaled toxins may exert an essentially systemic effect on the lung and other routes of administration may thus be satisfactory for studying pulmonary toxicity.

Most pulmonary effects of antiChEs result from parasympathomimetic, muscarinic effects which account for increased glandular secretion throughout the respiratory tract and, in bronchial tissue, for the bronchoconstriction. Nicotinic effects on the thoracic musculature account for the laboured respiration whereas effects on the CNS, particularly at high doses, result in depressed respiration.

Exposure to antiChEs

Therapeutic agents

The use of compounds such as ambenonium and derivatives of physostigmine or neostigmine, which inhibit AChE reversibly, usually present very little danger of toxicity. Large, intentional overdosage of physostigmine salycylate has, however, resulted in severe pulmonary oedema [11].

Irreversible inhibitors of AChE are also used therapeutically, notably demecarium bromide and the OP compounds dyflos and ecothiopate (echothiophate). These inhibitors obviate the repeated dosing required for their reversible counterparts, but the risks of poisoning are correspondingly greater. Systemic toxicity can result from inhalation of the vapour from dyflos and so it is only used as a dilute solution in arachis oil.

Pesticides

The main cause of human exposure to toxic doses of antiChEs is pesticide usage and abuse. The OP pesticides are the most widely distributed antiChEs and their action is less readily reversible than that of carbamates.

The toxic effects of the vapour of OPs were first reported after the synthesis and incidental inhalation of the more volatile diethyl and dimethyl phosphorofluoridates [32]. This work was initiated to develop new forms of insecticide but its relevance to neural transmission and to chemical warfare was studied intensively during World War II. The induction of debilitating effects at low doses is clearly a

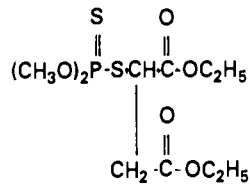
major disadvantage in a pesticide and the end of hostilities resulted in a renewed search for compounds of low mammalian toxicity. The most successful of these compounds were the phosphorothioates [24], characterized by the presence of a P–S–alkyl moiety and/or a P=S group. The most notable phosphorothioate pesticides are parathion and malathion (Figure 16.1).

The problem of accumulation of pesticides in the environment and the development of resistance by pests encouraged the continued development of other agents, such as the CB pesticides. These derivatives of carbamic acid also exhibit a high level of antiChE activity but retain the reversibility of the carbamic acid esters such as physostigmine and neostigmine. Most have a low dermal toxicity to mammals and no specific effects on the lung have been reported.

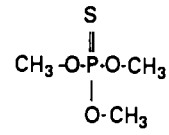
OP compounds

Metabolism and toxicity

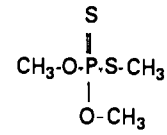
The mechanism of toxicity of the major OP pesticides has been studied extensively. Exposure of animals to many of the P=S phosphorothioates, e.g. parathion or malathion, results in the inhibition of AChE activity but the purified compounds lack this activity *in vitro* [20]. AntiChE activity results from oxidative desulphuration of the pesticide, probably



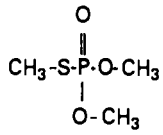
O, O-dimethyl phosphorodithoate of diethyl mercaptosuccinate
Malathion



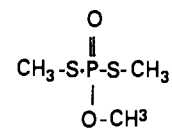
O, O, O-trimethyl phosphorothioate
OOOMe (S)



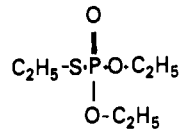
O, O, S-trimethyl phosphorodithioate
OOSMe (S)



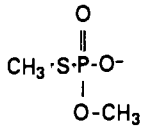
O, O, S-trimethyl phosphorothioate
OOSMe (O)



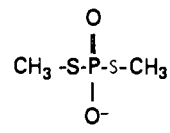
O, S, S-trimethyl phosphorodithioate
OSSMe (O)



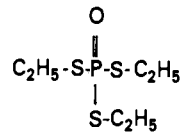
O, O, S-triethyl phosphorothioate
OOSEt (O)



O, S-dimethyl phosphorothioate
OSMe O⁻



S, S-dimethyl phosphorodithioate
SSMe O-



S, S, S-triethyl phosphorotrithioate
SSSEt (O)

Figure 16.1 Chemical structures of the OP compounds discussed in this chapter, together with the abbreviations used in the text

via a sulphine (S-oxide) intermediate, to the corresponding triesters or oxons (for review *see* De Matteis [12]). The oxons are potent inhibitors of ChE activity. They are formed *in vivo* by the microsomal mixed function oxidases, particularly in the liver, but there is increasing evidence of similar activity in other tissues, including the lung. The cytochrome P-450-dependent monooxygenase system also metabolizes P=S phosphorothioates to produce reactive metabolites which may inactivate the P-450 itself [12]. In cells in which there is a particularly high level of monooxygenase activity, or where this activity has been induced, these metabolites may also result in damage and hydropic degeneration [46]. Cellular injury has not been reported in the lung after administration of P=S phosphorothioates but microsomes from the lungs of rats treated with these compounds show a marked depletion of P-450 activity [50]. The 'suicidal metabolism' of this type of phosphorothioate by P-450 enzymes is much more marked in lung than in liver and so there is a selective inhibition of pulmonary P-450 activity. Inhibition of this activity may protect the lungs from injury by compounds which are converted by P-450 enzymes, into toxic metabolites [53].

The P-450 enzymes may also convert the pesticide, and its oxon, to non-toxic metabolites but the major mechanism of detoxification involves esterase activity. The relatively high level of carboxylesterase in mammalian liver, in comparison with that in insects, accounts for the low mammalian toxicity of many of these pesticides, e.g. malathion.

Potentiation of toxicity

Malathion has a particularly low mammalian toxicity. The poisoning of about 2500 spraymen in a Pakistani malaria control programme was thus of major concern. This outbreak of poisoning was largely attributed to the presence of contaminants in the formulation although poor work practices also contributed to the problem [6]. Two formulations of the pesticide were particularly closely associated with the development of symptoms and these were found to contain several degradation products of malathion. One of these contaminants, isomalathion, correlated particularly well with toxicity but other degradation products present in these formulations were also implicated as possible synergists [3,35]. The LD_{50} of pure, recrystallized malathion is quoted as 10–12 g/kg body-weight [3] but samples from batches implicated in this incident were toxic at a dose of 0.15 g/kg, indicating an isomalathion content of up to 10%.

The potentiation of malathion toxicity by other contaminants, in addition to the isomalathion, was subsequently confirmed and the investigation of technical formulations of malathion, particularly after storage under inappropriate conditions, indicated the presence of many such impurities [36]. Impurities containing phosphorus, especially the S-acid diesters of phosphoric acid, were found to be particularly important as they increase the rate of isomerization of malathion into isomalathion *in vitro* [54]. These diesters also become methylated *in vitro* to form trialkylphosphorothioates.

Trialkylphosphorothioates

The trialkylphosphorothioates (Figure 16.1) are potential contaminants formed during the synthesis or storage of P=S phosphorothioate pesticides. They may exacerbate the antiChE activity of these pesticides, indeed OSS trimethyl phosphorodithioate (OSSMeO) is two to three times more effective than isomalathion as a potentiator of malathion toxicity. Trialkylphosphorothioates are also antiChEs in their own right [4,10]. The severity of this effect may be dose-limiting, for some of these compounds, but many cause fatal lung damage at doses which induce only minor cholinergic effects.

Lung injury

The oral LD_{50} of OSSMeO to rats was found to be 26 mg/kg body weight but deaths occurred 3–4 days after dosing, long after the minor cholinergic effects had resolved [3,49,51]. The terminal stages of poisoning were characterized by laboured breathing and a 2–3-fold increase in lung weight. The oral administration of a lethal dose of either OSSMeO or OOSEtO was found to result in selective injury to the Type 1 pneumocytes. This damage occurred within 12 h and became more severe after 24 h. It was

followed by a proliferative response, by the Type 2 cells, and an influx of monocytes/ macrophages [15,52] which resulted in consolidation of the lungs (Figure 16.2). The effects of the injury, a common response to toxic insult in the lung, were exacerbated by the development of pronounced alveolar oedema. The extent of the oedema was clearly apparent in samples fixed by vascular perfusion and the appearance of the fluid suggested a high protein content (Figure 16.3). The high density of this fluid together with the influx of monocytes/ macrophages, involved in the phagocytosis of cell debris, probably accounts for the increase in dry weight observed during the process of lung enlargement [51]. Despite the severity of this oedema no signs of capillary damage were found but subtle changes, such as opening of the intercellular junctions, [43], could not be ruled out. Studies on the toxicity of O,O,S-trimethylphosphorothioate (OOSMeO), have also shown the lungs to be the main target organ (Figure 16.4) although some changes have been reported in other tissues. Morphological studies, on the effects of this compound were initially confined to surface changes in the bronchiolar epithelium [27] but more recent studies have reported injury to Type 1 pneumocytes [13,18,21]. Durham *et al.*'s [18] study also reported signs of endothelial damage.

A study of arterial PO_2 and PCO_2 in germ-free rats dosed with OSSMeO, has confirmed respiratory failure as the cause of death and also shown that it was not caused, or exacerbated, by bacterial superinfection [40].

The significance of early injury to Type 1 cells has also been investigated by studies of putrescine uptake in lung slices from rats dosed with OSSMeO. The active uptake of this diamine is a characteristic of both Type 1 and Type 2 pneumocytes [47]. The depressed accumulation of [^{3}H]putrescine, within a few hours of dosing with OSSMeO, probably results from injury to the Type 1 cells [39]. A reduction in uptake is even more marked by the end of the first day and at 4–6 days it is reduced to 50–60% of that observed in controls. This further reduction, at later time-points, may reflect the dedifferentiation of Type 2 cells during their proliferative phase.

A recent investigation of OSSMeO toxicity [29] has confirmed the injury to Type 1 cells in another strain of rat and also in mice. This

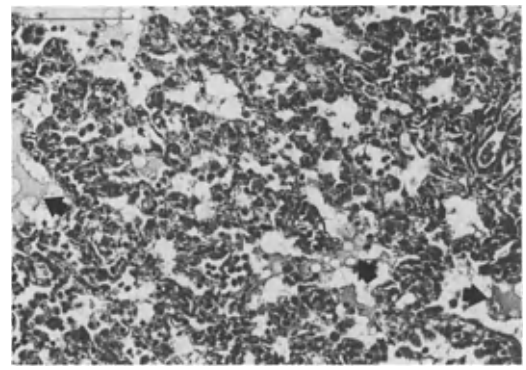


Figure 16.2 Lung tissue from a rat 3 days after 25 mg/kg OSSMeO, showing marked consolidation with thickening of the alveolar walls and oedematous fluid (arrows) in many of the airspaces. Scale bar: 100 μm

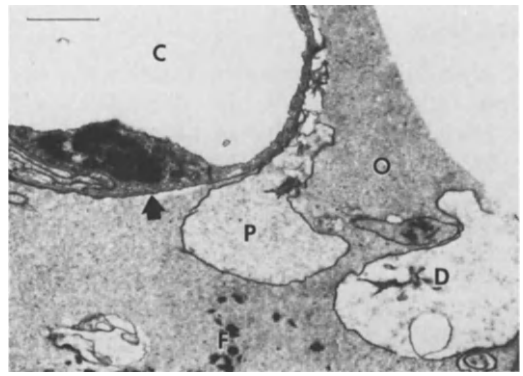


Figure 16.3 Alveolus of a rat 2 days after 25 mg/kg OOSEtO showing damage to Type 1 pneumocyte (P) and exposure of the basement membrane (arrow). The lung was fixed by vascular perfusion to retain the oedematous fluid (O) which occupies most of the airspace and contains both fibrin (F) and cell debris (D). The capillary (C) is lined by an intact endothelium. Scale bar: 1 μm

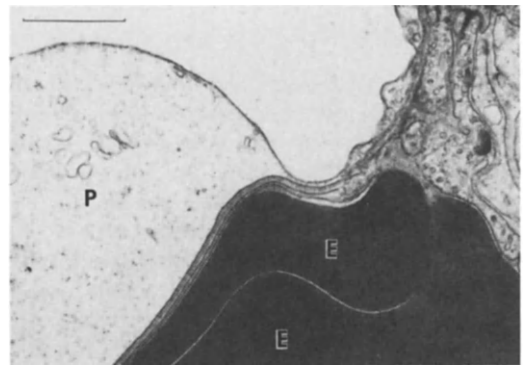


Figure 16.4 Alveolar wall of a rat 24 h after 60 mg/kg OOSMeO showing a swollen Type 1 pneumocyte (P). Fixative was instilled via the trachea and so RBCs (E) are present in the capillary. Scale bar: 1 μm

study has also shown, by the incorporation of tritiated thymidine, that the proliferation of Type 2 cells is followed by an even more widespread division of cells in the interstitium. No morphological signs of lung fibrosis have been reported after OSSMeO but a significant increase in total lung hydroxyproline was found, in mice, 21 days after treatment [30]. This increase was not enhanced by subsequent treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) or exposure to 70% oxygen for 7 days. The absence of this enhancement, together with the susceptibility of both rats and mice to OSSMeO distinguishes this compound from many other lung-damaging agents.

Distribution

The distribution of trialkylphosphorothioates following administration has been followed by the gas chromatography of blood and other tissues [5]. The compounds OSSMeO, OOSMeO and OOSEtO, on which most of the morphological studies have been based, mix rapidly and uniformly with the body water. They are rapidly eliminated from the circulation with half-lives of <1 h. There is no evidence for any selective accumulation of any of these compounds, in any particular organ although those of higher molecular weight are more lipophilic and so tend to accumulate in the brain. Low aqueous solubility may account for the low toxicity of some of these compounds, e.g. SSSEtO, as only a small fraction of the dose enters the circulation.

Toxicity

Comparisons between compounds indicate that the toxicity of the molecule decreases as the number of carbon atoms increases. This is the converse of the effect on the antiChE activity of the compound [4] and underlines the independence of these two properties. The major criterion for toxicity is the characteristic presence of at least one P−S−alkyl moiety and further such groups do little to enhance this toxicity.

A close correlation has been found between the degree of cellular injury and the increase in lung weight within 3–4 days of administration [1]. The growth-rate of adolescent rats, however, may be altered by administration of some of these compounds and so final lung-weights are best compared with body-weight at the time of dosing [50].

The reaction of AChE, and probably some other esterases, with these OPs [10] must account for the removal of part of any dose of a trialkylphosphorothioate from the circulation. These compounds are also detoxified by several hepatic glutathione S-transferases to produce methyl-S glutathione and the O-diester [2]. The major mechanism of detoxification, however, appears to involve the cytochrome P-450 system.

Metabolic activation

The intrabronchial instillation of OSSMeO directly into the lung does not result in a significant increase in the toxicity of this compound [52] and so the lesions after systemic administration, probably result from the formation of toxic metabolites. These metabolites may be formed within the lung or elsewhere in the animal.

The metabolism of OSSMeO, by slices of lung and liver, results in the formation of two OP diesters (Figure 16.1), O,S-dimethyl phosphorothioate ($OSMeO^-$) and S,S-dimethyl phosphorodithioate ($SSMeO^-$). The production of $SSMeO^-$, by cytosolic glutathione-S-transferase, predominates in the liver whereas $OSMeO^-$ is the major diester formed in the lung [38]. In metabolic studies, in which OSSMeO was labelled on one of the CH_3S-groups, the formation of $OSMeO^-$ and the binding of label was shown to be, at least partly, a P-450-dependent process [37].

The role of cytochrome P-450 enzymes

Protection

The affinity of the P=S group for lung P-450 is clearly very great as compounds which include both this moiety and the P−S−alkyl group do not result in lung injury. At least one such compound, OOSMeS, also protects against lung injury by trialkylphosphorothioates. Protection against OOSMeO toxicity, by the simultaneous administration of OOOMeS, was

reported by Umetsu *et al.* [49] and this was later attributed to inhibition of at least some of the cleavage of the CH_3S-moiety from the OOSMeO molecule [25]. Many compounds which incorporate the P=S moiety cause a marked decrease in lung microsomal 7-ethoxycoumarin O-deethylase (ECOD) activity and yet have a minimal effect on this activity in liver microsomes [50]. Similar observations have been made, independently, using OOOMeS to protect against OOSMeO toxicity [22].

The ECOD activity of microsomes is an indicator of the overall activity of several isozymes of P-450 in a tissue [55]. Some substituted benzenes (e.g. p-xylene and pseudocumene), but not benzene itself, also inhibit lung ECOD activity [44] without significant effect on the level of similar activity in the liver. This inhibition is clearly not the result of the liberation of an activated sulphur from the molecule but these compounds, like the P=S phosphorothioates, do protect against the lung injury caused by trialkylphosphorothioates. In addition to inhibiting pulmonary P-450, p-xylene also inhibits NADPH cytochrome reductase activity in lung microsomes [41] but has little effect on lung, or liver, glutathione levels [8]. Both the substituted benzenes and the trialkylphosphorothioates also protect against lung injury caused by ipomeanol [53]. This lung toxin has previously been shown to be activated by pulmonary P-450 metabolism [7].

Pretreatment of rats with the P=S phosphorothioates, OOSMeS or bromophos, or with the substituted benzene, p-xylene, inhibits the protein binding of labelled OSSMeO and also the production of $OSMeO^-$ by lung slices [37]. These compounds do not have a similar effect on the metabolism of liver slices and so their protective action probably results from the selective inhibition of the activity of one or more pulmonary isozymes of P-450. The formation of toxic metabolites in the lung may thus be inhibited while the elimination of the parent compound continues in the liver.

Inhibition of P-450 activity in both the lung and the liver, by previous administration of piperonyl butoxide, also protects against lung toxicity by some trialkylphosphorothioates [52]. These treatments, like the more specific inhibitors, probably reduce the formation of toxic metabolites in the lung until the circulating levels of the parent compound have been reduced by other mechanisms.

Surprisingly, the induction of cytochrome P-450 enzymes, with phenobarbitone, also decreases the toxicity of the trialkylphosphorothioates [52] even though protection against lung injury, by the inhibition of these enzymes, has been described above. Protection, in this case, may result from an increased rate of clearance of the parent compound, by the liver, from the plasma rather than an effect on pulmonary metabolism [1].

This complex and apparently inconsistent response, to inducers and inhibitors of P-450 activity, probably results from differences between the lung and other tissues in their complement of the relevant isozymes.

Isozymes of cytochrome P-450

The isozymes of pulmonary P-450 have been characterized, most thoroughly in the rabbit. This is mostly because of the large size and high P-450 content of rabbit lung. Two P-450 isozymes, rabbit 2 and rabbit 5, together constitute about 80% of this content; both are strongly induced by phenobarbitone. A third isozyme, rabbit 6, normally makes up <5% of the total lung content but it is induced by several aromatic hydrocarbons [48].

Rat lung, like that of humans, has a lower content of P-450 isozymes per unit weight than the rabbit and these enzymes are not induced, significantly, by phenobarbitone [33]. The activity of at least one isozyme, P-450b in rat liver or isozyme 2 in rabbit lung, may be assayed by the O-dealkylation of pentoxyresorufin [34]. Much of the activity of rat lung microsomes against pentoxyresorufin is inhibited by compounds affording protection against trialkylphosphorothioate-induced lung injury but similar inhibition does not occur with rat liver microsomes [53]. The importance of an isozyme equivalent to rabbit 5, probably P-450e [9], has yet to be established in rat lung [45]. In the rat lung P-450c is equivalent to rabbit 6, it is present at a very low concentration but is strongly induced by polycyclic aromatic hydrocarbons, e.g. 3-methylcholanthrene [31] and β-naphthoflavone. The activity of this enzyme may be assayed by the O-deethylation of 7-ethoxyresorufin, it is not

inhibited during protection by either the P=S compounds or the aromatic hydrocarbons [53]. Thus P-450b, rather than P-450c, is the most probable activator of trialkylphosphorothioates in rat lung but the possible role of P-450e has yet to be determined.

Bronchiolar effects of trialkylphosphorothioates

Superficial changes

In addition to the injury to Type 1 alveolar cells the administration of some trialkylphosphorothioates also results in changes in the bronchiolar epithelium. A lethal dose of OOSMeO results in bizarre distortion of the Clara cells (Figure 16.5) within 5 h [13] and similar changes may be found 24 h after a sublethal dose. Three days after this sublethal dose of OOSMeO, which results in minimal cholinergic symptoms, the non-ciliated epithelial (Clara) cells appear hypertrophied but less numerous than in control animals [27]. The development of these alterations in the Clara cells has been correlated with changes in the lactate dehydrogenase activity of bronchiolar lavage fluid. These changes have been confirmed, repeatedly, with sublethal doses of OOSMeO but similar changes do not accompany the administration of comparable, or even lethal doses of OSSMeO. The Clara cells of rats are much more sensitive to OOSMeO than to equitoxic doses of OSSMeO but injury to these cells is probably not a critical factor in the delayed deaths caused by trialkylphosphorothioates [13].

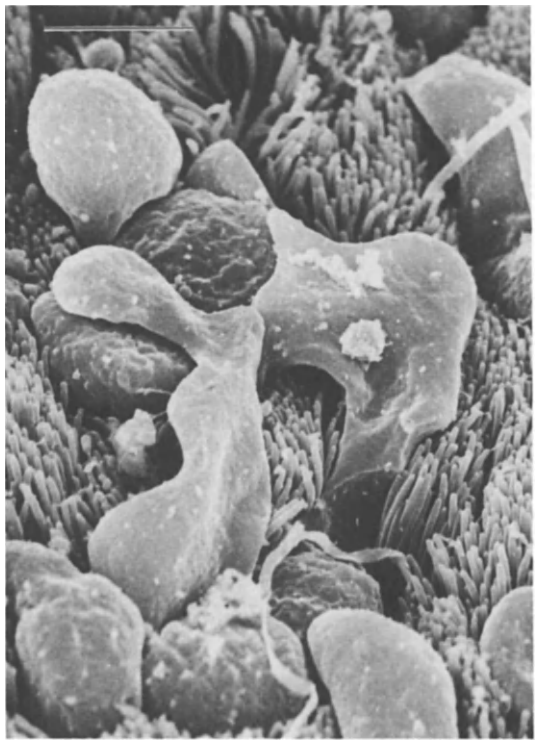


Figure 16.5 Bronchiolar epithelium of a rat 5 h after 60 mg/kg OOSMeO showing distorted Clara cells surrounded by ciliated cells of normal appearance. Scale bar: 5 μm

Modification of response

The changes induced in Clara cells may not result in the mortality associated with trialkylphosphorothioate administration but they have proved to be of value in the investigation of the metabolism of these compounds. Pretreatment of rats with phenobarbitone, which induces liver but not lung P-450, protected against the changes in Clara cells [23], thus providing further evidence for the elimination of these compounds by P-450 enzymes. Protection was also produced by pretreatment with small doses of OOSMeO itself but no changes were detected in pulmonary or hepatic monooxygenase activity [26]. A sublethal dose of OOSMeO stimulated the proliferation of Clara cells [21]. This proliferation may have been a response to the injury or to the loss of some of these cells but clusters of Clara cells in mitosis have also been found following the administration of the LD_{50} dose of OSSMeO. This dose of OSSMeO did not result in detectable signs of bronchiolar cell injury [16] but caused the loss of the characteristic granules from the apical cytoplasm of the Clara cells. The almost complete absence of these granules, within 1 h, was followed 6 days later by a large overcompensation before control levels were regained [16]. The rapid loss of granules was provisionally attributed to the antiChE activity of the compound but a later study established that the continuous administration of atropine did not provide any protection against degranulation [13]. The loss of granules was found to be a very sensitive

indicator of exposure to as little as 10% of the lethal dose of trialkylphosphorothioates [13].

Clara cell secretion

A totally different change occurs in the morphology of Clara cell granules after the administration of P=S trialkylphosphorothioates. Large, electron-lucent granules were found in the Clara cells of rats given high doses of p-xylene [1] and similar granules have been found after the administration of other inhibitors of lung P-450 enzymes including the P=S phosphorothioates [14]. These granules may constitute accumulations of material normally metabolised by the P-450 enzymes of the Clara cell. The nature of this material has not been established but preliminary studies suggest that it may be a cholesterol ester [14].

Conclusions

AntiChE activity *per se* does not impart particular lung-damaging properties to a compound even though cholinergic death often results from respiratory insufficiency.

One particular group of compounds, the P=O trialkylphosphorothioates, are potential impurities in various OP pesticides and may potentiate the cholinergic toxicity of the parent pesticide, by inhibiting its detoxification.

These compounds also damage specific cell-types within the rat lung. The specificity of this injury may provide a valuable understanding of the metabolism and enzyme complement of each individual cell-type.

The results of experimentation with the trialkylphosphorothioates suggest that the parent compound is activated to form a toxic metabolite, which probably results from oxidation of the S-alkyl moiety but has yet to be identified. The rate of this activation is particularly rapid in the lung where it overwhelms all mechanisms of detoxification and results in injury to the Type 1 cells. Activation involves at least one isozyme of P-450, most probably P-450b. This isoenzyme has been demonstrated by immunocytochemistry in Clara cells and Type 2 cells [31]. Compatible enzymic activity has been found in freshly isolated preparations of these cells [33] but the presence of P-450b, or any other P-450 isozyme, has yet to be demonstrated in Type 1 pneumocytes. These cells present particularly great technical difficulties for immunocytochemistry or isolation and so this situation does not, necessarily, reflect the relative activity of the Type 1 pneumocyte.

The selective injury of Type 1 pneumocytes must depend on many factors in addition to the absolute activity of a particular endogenous enzyme. The duration and level of exposure of these cells to circulating levels of the trialkylphosphorothioate may be controlled by the metabolic activity of other cells both in the lung and elsewhere in the body. Toxic metabolites may be formed within the target cell itself or they may be translocated from a different type of cell. All the evidence available so-far indicates that toxic metabolites are formed within the lung itself, either in the Type 1 pneumocyte or in an adjacent cell-type. The latter option is consistent with evidence for particularly high levels of P-450 enzymes within Type 2 and Clara cells but it does imply that these cells are much less sensitive than Type 1 pneumocytes to the metabolites.

The development of injury must depend on the effects of the toxic metabolite overwhelming the detoxification and repair mechanisms of the cell. The nature of these mechanisms, in the case of trialkylphosphorothioate injury, is not known but they would appear to deplete pulmonary reserves of glutathione [28]. These mechanisms involve the activity of GSH-S transferase and epoxide hydrolase [2]. Type 1 cells may be particularly deficient in their ability to repair cell damage as they are sensitive to a wide range of apparently unrelated toxins. The possibility should not, however, be discounted that the selective injury of these cells results from a particular facility to metabolize trialkylphosphorothioates [38].

The selective injury to lung cells and the potentiation of OP toxicity, by trialkylphosphorothioates, is well established and precautions are taken to minimize the levels of these contaminants in commercial pesticides. The effect of phosphorothioate compounds on P-450 activity is, however, less well-known and the possible effects of these compounds on the lungs of exposed personnel, especially spraymen, have not been evaluated. If the pulmonary P-450 activity of these individuals

is depressed then consequent changes in the detoxification/activation of xenobiotics should be investigated as a matter of urgency.

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17

Anticholinesterase-induced myonecrosis

Wolf-D. Dettbarn

The classical actions of ChE inhibitors have been studied over a long period [28]. One characteristic of antiChE toxicity is muscle hyperactivity induced by inhibition of AChE at the neuromuscular (NM) junction [23] which ultimately leads to muscle fibre breakdown [37]. All CBs and OPs cause myonecrosis when given in symptom producing dosage. The objective of this chapter is to review studies on the myotoxicity of CBs and OPs. These more recent *in vivo* mammalian studies generally follow a previous review [29].

Overall objectives

AChE at the NM junction is essential in the removal of ACh from the synaptic cleft. Inhibition of AChE profoundly modifies NM transmission, as seen in twitch potentiation, fasciculation, muscular weakness and acute focal necrosis of the muscle fibres. The objective of this chapter is to describe the mechanisms whereby antiChEs induce muscle fibre breakdown. We will describe: (1) morphological changes of nerve and muscle as a direct consequence of AChE inhibition and the repair of these lesions, (2) factors that modify the toxicity such as tolerance development and false targets such as carboxylesterases (CarbE), (3) prevention and treatment, and (4) discuss mechanisms that may underly the myonecrosis.

Background

ACh interaction with the ACh receptor (AChR) at the postsynaptic membrane causes a non-specific increase in permeability which gives rise to an endplate potential (EPP). When the EPP is large enough it elicits a muscle potential that in turn triggers muscle fibre contraction.

Inhibition of AChE, a constituent of the membranes and basal lamina at the synapse, profoundly modifies NM transmission, shown by electrophysiological analyses, the response of the muscle to nerve stimulation, and by observations on muscular activity in the intact animal in the absence of applied nerve stimulation [23].

AChE inhibitors have a number of characteristic actions at the NM junction. They produce spontaneous fasciculations of muscle fibres and, with large doses, muscle weakness or complete NM block. In the muscle stimulated through its nerve, the effects vary with the frequency of stimulation; at low frequencies, the twitch tension is potentiated by repetitive firing of the muscle fibres; at high rates of stimulation, the muscle is unable to maintain a tetanic contraction [23].

The primary cause of death from antiChEs is respiratory failure, generally arising from central impairment of respiration, combined with bronchoconstriction, bronchosecretion and failure of NM transmission. The relative importance of these factors varies with the species of animal and the lipid:aqueous phase distribution of antiChEs.

AChE inhibition and muscle necrosis

Morphological changes induced by irreversible AChE inhibitors

Light microscopic changes

A single injection of an antiChE at a non-lethal dose causing fasciculations produces a myopathy in skeletal muscles such as the rat

diaphragm, soleus (SOL), extensor digitorum longus (EDL), sternomastoid, gastrocnemius and tibialis anterior muscles. The earliest lesions are focal areas of abnormality close to the subjunctional region of the muscle fibre [30]. The trichrome-stained mitochondria, usually identified by LDH and NADH reactions, are disrupted by clumping of highly reactive material. These focal changes progress to a generalized breakdown of fibre architecture, characterized by a loss of staining quality followed by phagocytosis. Longitudinal sections indicate that in early stages the focal necrosis affects only a small segment of fibre length. During later stages progressively greater lengths of muscle fibres are affected [35,43].

Within 1–2 h of an injection, the earliest light microscopic changes are characterized by localized eosinophilia, swelling of the sarcoplasm and loss of striations in several muscle fibres. Approximately 12 h after the sc injection, a complete but localized necrosis develops in affected fibres [17,19–21].

No lesions are found in endplate-free regions of the muscle. Serial cross-sections 10 μm thick, through the muscles at intervals of 25 μm indicate that the number of lesions found per muscle is highest in the areas with the greatest density of endplates (Table 17.1) [35]. In all cases, the lesions start at or in close proximity to the endplate and extend 10–200 μm through the fibre, depending on the time that elapses between injection and termination (1–24 h). The longer the delay between injection and termination, the greater the spatial extent of the lesions. When corrections for total number of fibres are made, the diaphragm muscle has the highest number of lesions per 1000 muscle fibres, followed by SOL and EDL muscles. The EDL, a fast twitch muscle, has the greatest proportion of type II fibres, using mainly glycolysis for its energy metabolism, while the diaphragm and SOL have the highest percentage of type I fibres, utilizing mainly oxidative phosphorylation. Seven days following the injections few lesions remain and AChE activity recovers to more than 50% of control [17,19–21]. Coinciding with the appearance of myonecrosis there is a significant increase in the blood level of creatine kinase indicating destruction of muscle membrane [10].

Table 17.1 Frequency of end-plates and necrotic muscle fibres after a single dose of DFP (1.5 mg/kg, *sc*) as evaluated by serial cross sections from soleus muscle

Distance of sections (μm)	*No. of end-plates*	*No. of necrotic fibres*
250–500	0±0	0±0
500–1000	1±1	3±1
1000–1500	58±14	129[a]±38
1500–2000	147±14	326[a]±41
2000–2500	165±22	296[a]±30

*The midbelly area of each of seven muscles was serially cross-sectioned at 10 μm thickness. Every twenty-fifth section was microscopically examined for the presence of end-plates and necrotic fibres. The numbers of end-plates and necrotic fibres in this table are the cumulative values for sections examined at the distances listed. Values for end-plates and necrotic fibres are given as the means ± SD/1000 muscle fibres.
[a]Significant difference between untreated and inhibitor treated muscle at $P<0.001$ level.

Ultrastructural changes

Motor nerve terminals show varying degrees of change within 30 min to 2 h after the injection of antiChE. Some nerve terminals appear relatively normal with the exception of swollen mitochondria. More severely affected nerve terminals display myelin figures, membrane enclosures and an increase in the number of large coated vesicles. More obvious changes are seen in the subsynaptic area and the surrounding muscle fibre, such as vesicular structures in the primary and secondary subsynaptic cleft. Occasionally, some of these are seen in the sarcoplasm. Many of the cleft vesicles are similar in density and size to synaptic vesicles but with considerable variations in diameter. The severity of lesions in the subsynaptic folds varies even within the same muscle. Normal subsynaptic clefts with few cleft vesicles are seen side by side with subsynaptic clefts with many cleft vesicles and a widening of the cleft itself [30,33,39].

The antiChE, in addition to the changes seen in the region of the endplate, causes changes in the muscle fibre itself. Supercontraction of subjunctional sarcomeres is always present and muscle fibres surrounding the motor endplate show a disruption of cytoarchitectural organization. Initially, the first changes are in the mitochondria which show swelling leading to lysis of the central cristae. Myelin figures beneath the endplate are frequently observed while the region more distal is less affected. The nucleoli of the muscle cell nucleus are enlarged and move to

Table 17.2 Activity of AChE and number of necrotic fibres in rat skeletal muscles found 60 min after a single sublethal injection of various AChE inhibitors (antiChE)

AntiChE	*SOL*		*EDL*		*Diaphragm*	
	AChE[a] *(%)*	*Lesions*[b]	*AChE*[a] *(%)*	*Lesions*	*AChE*[a] *(%)*	*Lesions*[b]
Tabun	10	9±2	32	0	10	6±2
Soman	13	7±3	53	0	24	4±2
Sarin	22	12±4	83	0	24	21±6
VX	8	16±3	17	0	12	10±4

Number of lesions are given per 1000 muscle fibres in cross section of muscle. Ten muscles were used for each group. Values of AChE activity are expressed as percent of control. Data are given as the means ± SD. Significant difference between inhibitor treated and untreated muscle at, [a]$P<0.001$, [b]$P<0.05$ level.

Table 17.3 Activity of AChE and number of necrotic fibres of rat skeletal muscles from 24 h after a single sublethal injection of various antiChEs

AntiChE	*SOL*		*EDL*		*Diaphragm*	
	AChE[a] *(%)*	*Lesions*[a]	*AChE*[a] *(%)*	*Lesions*[b]	*AChE*[a] *(%)*	*Lesions*[a]
Tabun	7	66±14	23	33±5	7	302±32
Soman	18	48±9	30	1±1	7	260±48
Sarin	19	127±20	43	69±27	13	424±78
VX	23	75±17	38	69±13	22	257±47

Number of lesions are given per 1000 muscle fibres in cross section of muscle. Ten muscles were used for each group. Values of AChE activity are expressed as percent of control. Data are given as the means ± SD. Significant difference between inhibitor treated and untreated muscle at, [a]$P<0.001$, [b]$P<0.05$ level.

the periphery of the nucleus. There is an increase in the number of sarcoplasmic ribosomes with subsequent dilation of the sarcoplasmic reticulum and loss of striation of the myofibrils, followed by total destruction of the myofilaments and fragmentation of Z bands.

Reversible AChE inhibition and myopathies

In acute experiments, prostigmine and pyridostigmine, as well as physostigmine in concentrations between 0.1–0.8 mg/kg cause muscle fibre necrosis, not unlike that seen with the irreversible antiChEs. The number of necrotic fibres rises with increasing inhibitor concentration.

Exposure to physostigmine at symptom-free concentrations causes no detectable alteration [38] while a symptom-producing dose (0.8 LD_{50} or 0.6 mg/kg) causes a characteristic lesion, namely concentric hemispheres surrounding the endplate region with each successive zone toward the endplate exhibiting increased damage to sarcomeres, mitochondria and sarcoplasmic reticulum. Starting from the unaffected non-junctional cytoplasm and terminating at the junctional folds, this apparent continuum of mitochondrial changes associated with severe endplate depolarization includes the following sequence: 'granular' mitochondria, 'blistered' mitochondria, 'frothy' mitochondria, 'swollen' mitochondria, 'exploded' mitochondria. Supercontraction of the subjunctional sarcomeres and the associated formation of exploded, swollen, frothy and blistered subjunctional mitochondria were thus confirmed as the most obvious markers for the characteristic toxic lesion of the junction produced by this antiChE agent [38]. Pyridostigmine, neostigmine and physostigmine, within 24 h, caused acute focal necrosis, leukocytic infiltration and marked changes at the motor endplate, similar to those seen with irreversible antiChEs [11,26,27,43].

Recovery of the antiChE induced changes are very rapid. Within 7 days only a few fibres showed any abnormality [35,38].

Selective susceptibility of muscles

The muscle least sensitive to the effects of antiChEs is the fast twitch EDL. This is demonstrated by the total number of necrotic muscle fibres as well as the slower rate of AChE inhibition in this muscle when compared with SOL and diaphragm (Tables 17.2 and 17.3). The reason for this reduced sensitivity of AChE activity in EDL to the antiChE is not well understood and may be

found in: pharmacokinetic variables influencing delivery of specific inhibitors, differences in location and accessibility of AChE to the agent, variations of ACh release owing to changes in firing patterns, and selective distributions of enzymes hydrolyzing or binding OPs in serum and muscle. In addition, there may be variations among these muscles in their ability to act as depots for nerve agents. Thus the EDL may act as a better storage facility from which there is a slow antiChE release. The faster the rate at which AChE activity is reduced to a critical level, the faster the onset of symptoms, i.e. fasciculations, and the greater the number of necrotic fibres. Differences in central and peripheral neural actions between the OPs are another reason for changes in muscle response [33,34].

The differences in muscle sensitivity to a given antiChE may be related to muscle use patterns which would favour the more active soleus and diaphragm muscles over the fast twitch EDL [38]. While this is one possibility, the observation that AChE in EDL, and especially the functionally important 16S form at the endplate, are the least inhibited during treatment with a given inhibitor argues against this (see later). Unless one postulates that interaction between inhibitor and enzyme is controlled by muscle activity, this explanation may account only in part for the low sensitivity of the EDL.

Distinction between central and peripheral effects of AChE inhibitors

There are differences in the pattern of motor symptoms in animals treated with various antiChEs. Soman produces complex posturing movements and tremors affecting virtually the entire body, while DFP produces extensive high frequency fasciculations. In addition to the typical cholinergic symptoms, muscle fibre necrosis is more frequent at symptom-producing doses of DFP than of soman. This suggests that the antiChEs differ in their propensity to produce central or peripheral effects and that peripheral effects are more important for muscle necrosis [34].

Activity recorded from acutely denervated muscle can be only of peripheral origin, while activity of non-denervated muscle is a combination of peripherally and centrally generated discharges. Soman and DFP produced very different amounts of necrotic fibres and patterns of muscle electrical activity. Soman produced mainly centrally generated motor unit activity with little peripheral muscle fibre discharge and few lesions. In contrast, DFP produced more peripherally generated activity and a greater number of necrotic fibres (Table 17.4). There are at least four possible explanations for these differences in electrophysiological effects: (1) non-specific effects of the inhibitors on muscle or CNS independent of

Table 17.4 Effects of soman and DFP on electrical activity (EMG) and AChE activity of the normal and acutely denervated lateral gastrocnemius

Inhibitor	*Denervated*	*Innervated*	*Difference*	*AChE (% Control)*
Control	0.29 ± 0.34	30.5 ± 22.6[a]	30.2 ± 23.8	58.4 ± 5.4 (100%)
Soman	3.33 ± 10.7	66.3 ± 20.1[a,b]	62.9 ± 33.6[b]	26.2 ± 8.9[a] (45%)
DFP	12.6 ± 4.4[c]	21.6 ± 16.1	8.98 ± 18.3[c]	3.6 ± 3.6[a] (6%)

Values are discharges per second ± SD of single units. There were 18 animals in each treatment group. The response from the denervated side represents activity of peripheral origin and the activity of the nondenervated side (of the same animals) represents combined centrally and peripherally generated activity. The calculated 'difference' is an indicator of central activity. Note that there was a very low discharge frequency in denervated muscle before administration of inhibitor. This was due to irritation of the muscle by the electrode with movement of the animal (i.e., insertional activity) and not due to spontaneous activity at rest.

[a]Significant difference between the normal and denervated side at $P<0.01$ level by ANOVA.

[b]Significant difference between the normal and denervated side at $P<0.01$ level. All other comparisons were not significant ($P>0.05$).

[c]Difference between inhibitor-treated and untreated activity at $P<0.05$ level.

AChE activities for innervated muscle are expressed as μmol acetylthiocholine hydrolyzed/gram tissue wet weight/hour ± SD for five animals in each group.

changes in AChE activity, (2) differing affinities of central and peripheral forms of AChE for each inhibitor, (3) varying diffusion rates across the blood-brain barrier which may select the degree of central actions of the individual agents, favouring soman, or (4) possible degradation of DFP before it reaches the CNS in sufficient concentrations to produce central effects. Inhibition of BChE with the specific inhibitor iso-OMPA does not produce symptoms or muscle lesions. The myonecrosis is caused by inhibition of AChE [43].

The AChE data suggest that the reason for reduced peripheral effects of soman compared with DFP is less complete inhibition of AChE at the NM junction by soman. This is supported by the previous finding that sciatic nerve AChE is inhibited less by soman than are brain and muscle AChE [8].

Interaction of antiChEs with different molecular forms of AChE

AChE is a polymorphic enzyme that occurs in a number of molecular forms which are equivalent in their catalytic activity but differ in their molecular parameters and interactions [32]. These forms may be classified as globular (4S, 6S and 10S) and asymmetric (12S and 16S). In rat SOL, the majority of total enzyme activity is contributed by the 12S and 16S forms, whereas the 4S and 10S forms predominate in the diaphragm, and 4S in the EDL [12,35]. In all muscles, the 16S form is found in high concentration at the endplates, and it is thought that this form is involved in NM transmission and that its inhibition causes the symptoms seen as fasciculations and muscle necrosis [8,9]. The effects of antiChEs on total AChE activity (as shown in Tables 17.2 and 17.3). are also reflected in this action on the activity of the individual forms. All forms in SOL and diaphragm muscles are critically inhibited within 1 h. The molecular forms of EDL showed a much slower rate of inhibition, similar to those described for total muscle homogenate (Figure 17.1). Only DFP and VX rapidly inhibited all the molecular forms in this muscle, while tabun, sarin and soman caused much slower rates of inhibition of the individual forms, especially of the critical 16S form of the NM junction. This again is reflected in the much lower number of lesions seen in this muscle when compared with SOL and diaphragm [17,19–22]

Changes in energy metabolites during nerve agent toxicity

There is a clear relationship between OP-induced muscle lesions and changes in the high-energy phosphate compounds (PC) [14]. Both DFP and soman significantly reduce PC in EDL, SOL, and hemidiaphragm, coinciding with the reported appearance of muscle necrosis. Both of the AChE inhibitors significantly reduced the ATP content and elevated the level of AMP in all the muscles. The quantitative effects of DFP and soman on high-energy phosphate compounds are slightly variable.

The observed decrease in PC may be caused by the increased demand for maintaining ATP levels, since muscle fasciculations utilized greater quantities of ATP. A decreased synthesis of PC cannot be ruled out until effects on the synthesis have been established. A complicating factor in the interpretation of our data is that only a small percentage of the muscle fibres in a given muscle (at the most, 20% of the total fibre count) develop fasciculations and necrosis. Therefore, the changes of high-energy compounds reported here reflect only average changes from muscle fibres with very low, moderate or unchanged levels of these compounds. It may very well be that PC and ATP are critically reduced in muscle fibres that develop necrosis, while others may develop only small reductions in PC and ATP and therefore, no necrosis develops.

Modification of antiChE toxicity

Tolerance development to DFP

Repeated non-lethal injections of DFP in concentrations that induce fasciculations and other signs of cholinergic hyperactivity only slightly increase the number of lesions beyond that seen after the first 24-h period. Furthermore, the lesions begin to disappear after the third day even when the DFP is applied for more than 7 days. Still lower doses of DFP when administered repeatedly produce symptoms similar to those seen after a single

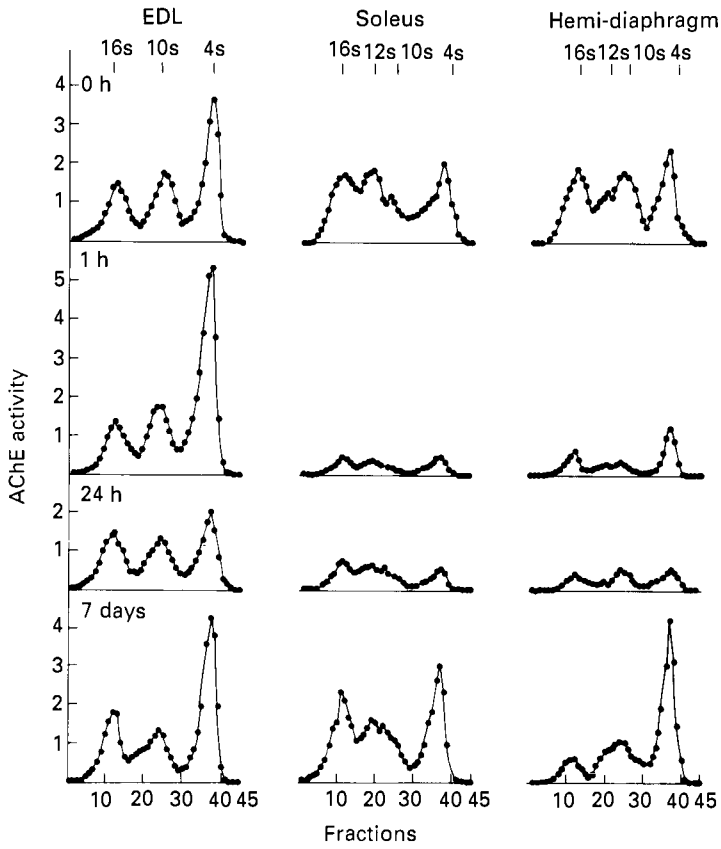

Figure 17.1 Representative profiles of activity of AChE molecular forms in EDL, soleus, and hemidiaphragm muscles from rats following an acute sublethal injection of sarin (100 μg/kg, sc). Profiles at the top of each column are from untreated muscles. Subsequent profiles are of activity of AChE molecular forms 1 h, 24 h and 7 days, respectively, after sarin treatment. The AChE activity scale is in arbitrary units based on μmol substrate hydrolyzed per min by the enzyme activity in each fraction. The sedimentation values of the AChE molecular forms are shown above the profiles and were determined by added sedimentation standards, β-galactosidase (16.0S), catalase (11.1S) and alkaline phosphatase (6.1S)

acute toxic dose on the third day. Further injections do not produce any increase in the severity or duration of symptoms and the animals become symptom- and lesion-free throughout the remainder of the injection period (28 days). The highest number of muscle lesions is seen after the third injection, at a time when the drug causes the first symptoms. Further injections do not cause any additional lesions.

Continuous injections of DFP up to 14 days do not produce additional inhibition of AChE activity after the third injection. Instead prolonged application of DFP does not sustain the level of enzyme inhibition and recovery of AChE activity from <15% to 75% of control is seen. Simultaneously, a significant decrease in binding sites of the nicotinic AChR, with no change in affinity constant is observed [19].

These experiments demonstrate adaptation of rats to repeated exposure to low doses of DFP and survival of a cumulative concentration of severalfold the acute LD_{50}. One reason for this adaptive process is found in the recovery of AChE from a significant inhibition to levels of activity that were closer to controls despite a continuous dosage of DFP that originally had produced a significant reduction in

AChE activity. All muscles tested showed a similar behaviour. This is in contrast to that seen in brain AChE of the same animals. Brain AChE does not recover during continuous dosage with DFP but remains at a very low level of activity. An additional mechanism of adaptation possibly related to reduction in symptoms or myonecrosis can be seen in the reduction of binding sites or nicotinic AChR in the muscle tested.

Tolerance is defined as loss of fasciculation and susceptibility to the necrotic effects of DFP, as well as the survival after a cumulative dose of DFP severalfold the acute LD_{50}. In all the muscles tested AChE activity approaches the normal values during the course of tolerance development. Several mechanisms may be considered for this phenomenon, such as (1) spontaneous reactivation, (2) decrease in sensitivity of the active site of the enzyme, or (3) increase in active sites owing to rapid *de novo* synthesis of an AChE with reduced sensitivity to DFP. Experiments support an increased *de novo* synthesis of AChE as one of the mechanisms that make muscle less susceptible to the toxicity of DFP [13]. The return of the lower molecular weight form activities (4S and 10S) in muscle before that of the higher molecular weight forms (12S and/or 16S) after 5 days of treatment and the increased level of the 4S activity after 14 days of treatment support the hypothesis that increased *de novo* synthesis of AChE has occurred. Whether the active site of this new enzyme has a reduced sensitivity to inhibition by DFP remains to be seen [17–19].

Other possibilities such as induction of greater synthesis of enzymes that hydrolyze and thus detoxify DFP ('DFPase'), do not occur during prolonged administration of DFP or soman [8,42]. An alternative suggestion is an increase in non-specific binding sites for DFP. These possibilities remain to be investigated.

Animals pretreated with an inhibitor of protein synthesis such as cycloheximide (0.5 mg/kg, sc) 30 min before the daily DFP injection developed signs of toxicity after the second DFP injection and none of the animals survived the fourth injection. Daily pretreatment with cycloheximide prevents the development of DFP tolerance and the recovery of AChE and CarbE activity [15]

The role of carboxylesterase (CarbE) and BChEs

Additional mechanisms that may contribute to general tolerance are the availability of other serine-active site enzymes such as CarbE and BChE. Binding to and inhibition of these enzymes reduce the free concentration of antiChE otherwise available to interact with AChE. In addition, the existence of enzymes that hydrolyze certain OPs may facilitate mechanisms that lead to adaptation [3].

Mipafox and iso-OMPA, two specific inhibitors of BChE, are effective inhibitors or CarbE and of the 'Mazur-type' DFPase [24,25, Hoskin, unpublished observations]. These inhibitors reduce the number of serine-active sites of non-AChE enzymes, and prevent the hydrolysis and binding of DFP and soman, thereby increasing the availability of DFP for AChE and thus abolishing natural tolerance.

Rats injected with low concentrations of soman (25 μg/kg) or DFP (0.5 mg/kg), showed no signs of toxicity. Pretreatment with iso-OMPA (1–3 mg/kg) or mipafox (0.05 mg/kg) 1 h before administration of antiChEs caused severe signs of hypercholinergic activity, similar to that seen with an acute sign-producing non-lethal dose of 100 μg/kg soman or 1.5 mg/kg DFP. Within 1 h iso-OMPA or mipafox alone significantly reduced the activity of CarbE and BChE in all tissues studied. The combined treatment of iso-OMPA and soman reduced CarbE activity in liver (0%) and produced significantly greater effects than iso-OMPA or soman alone on AChE and BChE in all the brain and skeletal muscles tested. The number of necrotic lesions found in skeletal muscles was many times higher with the combined treatment than was seen with soman (25 μg/kg) or DFP (0.5 mg/kg) alone, and was equal to that seen with an acute toxicity sign-producing dose of soman (100 μg/kg) or DFP (1.5 mg/kg) (Table 17.5) [16,17,20]. Neither iso-OMPA nor mipafox cause toxicity signs or muscle necrosis when given alone in the concentration used.

Both iso-OMPA and mipafox pretreatment completely abolished the tolerance development to DFP, as no animal survived more than 5 days of combined treatment. The observed adaptation to DFP toxicity appears to result from recovery of CarbE, BChE and AChE

Table 17.5 Number of necrotic fibres/1000 muscle fibres of rat hemi-diaphragm after iso-OMPA, DFP, and soman

	Treatment of animals							
	Control	*DFP 1.5 mg/kg*	iso-*OMPA 3 mg/kg*	*DFP 0.5 mg/kg*	iso-*OMPA 3 mg/kg + DFP 0.5 mg/kg*	*Soman 100 μg/kg*	*Soman 25 μg/kg*	iso-*OMPA 1 mg/kg + Soman 25 μg/kg*
Number of lesions	0±0	308[a]±58	0±0	0±0	170[a]±25	260[a]±44	0±0	257[a]±5

Lesions were counted 24 h after the drug administration.
[a]Statistical significance between control and treated rats ($P<0.001$).

activity as well as decreased nicotinic binding sites at the NM junction, as previously reported [19].

To rule out an accelerated DFP hydrolysis through the induction of an enzyme, DFPase activity was measured following DFP administration. At that time there was no increase in DFPase activity over that in untreated animals (Hoskin, unpublished observations). Rapid enzymatic hydrolysis of DFP or soman appears not to be involved as a mechanism leading to tolerance [8,16,17,19,41,42].

These experiments confirm the contribution of CarbE to detoxification and to the development of tolerance seen with DFP and other antiChEs. The time for tolerance to develop may differ among different physiological systems. It may be shorter for receptor adaptations than for other functions [17].

Prevention of the myopathy

Protection against the necrotic action has been achieved previously by reactivation of the phosphorylated AChE with 2-PAM, denervation or complete blockade of postsynaptic receptor sites by d-tubocurarine [30]. While the two latter protective mechanisms are highly effective, they are only of theoretical interest, as both measures lead to total paralysis. Nevertheless, they support the requirement of a viable nerve terminal with an unimpaired release of ACh and available AChR for the development of the myopathy. Reduction of ACh release from nerve terminals may be another mechanism for prevention or treatment of OP intoxication. Thus by lowering the amount of ACh accumulation at the NM junction, signs of toxicity such as antidromic nerve activity and muscle fasciculations can be prevented or reduced. Soman or DFP do not cause backfiring, fasciculations or lesions when release of ACh from the nerve terminal is prevented [4,8,9,36].

Drugs having presynaptic actions by reducing ACh release

Studies have established that agents such as 2-PAM, atropine, d- tubocurarine or gentamicin prevent OP-induced fasciculations, antidromic firing and NM necrosis. Botulinum toxin, when given 48 h before the nerve agents and applied locally to the nerve terminal, prevents spontaneous nerve firing and fasciculations as well as necrosis [4,5,8,9,36].

It is likely that d-tubocurarine in the low dose (40 μg/kg) has an effect on the presynaptic nerve terminal, since subparalyzing doses prevented fasciculations without interfering with normal NM transmission [2]. Atropine in concentrations that cause slight sedating effects also reduces fasciculations and the lesions. Both compounds also reduce the MEPP frequency when it has been increased by AChE inhibitors. Whether d-tubocurarine and atropine also have postsynaptic channel effects leading to the reduction of lesions cannot be ruled out [2].

Other observations indicate that creatine phosphate (CP) may increase the energy supply to the frequently contracting muscle and thus preserve its structure by stimulating the Ca^{2+} pump of the sarcoplasmic reticulum (SR).

Rats pretreated with CP developed a notable increase in their overall activity so that they walked about, dug in their bedding and resisted handling with more than usual vigour. AChE inhibition produced some fasciculations, but they were less than those in controls.

Also, the increased vigour of these animals was sustained throughout the experiment. Lesions were reduced significantly [4].

If Ca^{2+} is a factor in the development of the myopathy, three approaches may modify the onset of lesions. The first would be to reduce Ca^{2+} available to muscle. This would require that total body stores of Ca^{2+} be reduced to a critical level, below which muscle contractility and other cell functions would be compromised. A second mechanism would reduce transport of Ca^{2+} from SR to myofibrils. This is the action of dantrolene, which has not been studied yet for this purpose. The last would be to enhance reuptake of Ca^{2+} by SR. In fact, the experiments presented earlier with CP suggest that the latter mechanism may be effective in preventing muscle lesions caused by antiChEs. Thus the most probable explanation for the efficacy of CP in preventing muscle lesions caused by excess of ACh is in providing the energy for the Ca^{2+} pump and thereby the resequestering of Ca^{2+} in the SR [4].

Drugs acting centrally

Selective effects of diazepam, phenytoin and ketamine can prevent of nerve agent-induced muscle toxicity.

The list of drugs effective in the treatment of NM signs of nerve agent toxicity include: (1) oximes which reactivate the phosphorylated AChE, (2) subparalyzing as well as paralyzing doses of d-tubocurarine, (3) atropine, and (4) creatine phosphate. All but creatine phosphate reduce fasciculations, and all attenuate muscle necrosis following administration of nerve agents. The various mechanisms accounting for this effect have been discussed previously. One hypothesis is that excess activation of muscle fibres owing to sustained interaction between ACh and AChR, regardless of whether it arises indirectly from the CNS or directly from the PNS stimulation, results in muscle lesions. Reducing motor activity alone, regardless of origin of excitation, may reduce NM toxicity. Recently described electrophysiological experiments have distinguished muscle activity arising in the CNS and/or arising in the PNS. These experiments also demonstrate that soman has its predominant action through CNS stimulation, while DFP has prominent PNS as well as CNS activation [34].

Diazepam, a potent anticonvulsant and muscle relaxant, reduces the nerve agent-induced convulsions and tremors but does not affect fasciculations. High doses of diazepam (20 mg/kg), at which rats become comatose, markedly reduce the fasciculations and the number of necrotic muscle fibres. This response to diazepam may be from its effect on the central motor system, affecting motor tone and thus reducing cholinergic output [4].

Therapeutic agents may attenuate the muscle activity generated at the endplate and peripheral nerve or reduce motor unit activation owing to CNS effects of the nerve agents. Phenytoin, an anticonvulsant without sedative effects, and ketamine, a general anaesthetic, proved effective as pretreatment agents against soman and DFP. DFP was selected because it causes both peripheral and CNS symptoms of toxicity, while soman was chosen because it primarily causes CNS symptoms. Ketamine caused a significant decrease in discharges of CNS origin, while atropine methyl nitrate and phenytoin had no effect. For muscle fibre discharges of peripheral origin, both drugs produced a significant drop in muscle fibre discharges, but phenytoin showed slightly more efficacy than the others [6].

The induced muscle hyperactivity arises from actions on the CNS and on the peripheral nerve in varying proportions for different nerve agents. Treatment for the muscle toxicity may be accomplished by administering drugs with distinctive pharmacological actions at target sites in the CNS and PNS where nerve agents exert their selective effects. By attenuating the effects at either CNS or PNS sites with selective antagonists, the NM toxicity can be reduced in a more specific manner, avoiding unwanted site- effects.

Myopathy in human muscles

Although clinical symptoms from antiChE poisoning of humans are well described, there have been only a few reported cases of myopathic alterations from OP insecticide exposure. Necrotic lesions similar to those found in rat muscle are seen in human skeletal muscle of workers after exposure to spray containing malathion and diazinon or

parathion. In all these cases, the muscle necrosis was established by biopsy or autopsy. In humans, the incidence of acute muscle necrosis is probably greater than reported, as would be the case if more attention were focused on muscle at autopsy. Whether this necrosis contributes to the symptoms of muscle weakness in acute poisoning remains to be investigated.

The histological picture from these case studies is comparable to pathological alterations in rat skeletal muscle after acute exposure to OP inhibitors of AChE such as parathion, paraoxon, DFP and soman [1,7,44]. More recently, a reversible paralysis of proximal limb, neck and respiratory muscles was described following an acute toxic phase of antiChE poisoning involving fenthion, monocrotophos, dimethoate, and methamidophos. The paralysis appeared after an acute cholinergic crisis but before the expected onset of delayed neuropathy [40]. It is likely that the NM dysfunction described in these patients is related to the NM necrosis.

Comments and conclusions

The onset of damage to muscle fibres from antiChEs is probably determined by a number of factors, such as the availability of energy supplies in the form of glycogen, CP and ATP. DFP or soman administration significantly decreased the concentration of PC in all three skeletal muscles. The highest level of PC was found in the fast-twitch EDL muscle followed by the slow-twitch muscles such as soleus and hemidiaphragm. The hemidiaphragm was the one most severely affected. CP was maximally reduced at 6 h by about 40% with DFP and by about 50% with soman. Full recovery was seen within 72 h. The fast-twitch muscle, EDL, has the highest concentration of ATP. A significant decrease (P <0.01) of ATP concentration was observed in the hemidiaphragm. Within 1 h ATP was reduced by 25% and remained depressed up to 24 h (23–30%). The ATP level in soleus and EDL showed a reduction during the 3–6-h period. DFP and soman significantly reduced the ATP/ADP ratio and ATP/AMP ratio in skeletal muscles as a result of corresponding increases in ADP and AMP values [14]. The time course of the necrosis as reported earlier correlates well with the reduced levels of PC, the reduction of which may have been the result of an increased demand for energy and a low rate of ADP phosphorylation caused by an increased level of sarcoplasmic Ca^{2+}. During OP or agonist-induced twitch potentiation and prolonged fasciculations an increased influx of Ca^{2+} is observed in the region of the endplate [31,39]. This increased sarcoplasmic Ca^{2+} during the prolonged muscle activity may cause a reduction in PC and ATP needed for the sequestration of sarcoplasmic Ca^{2+} into the SR. Some of the first changes seen following OP-induced muscle activity are supercontraction, vacuolization and destruction of muscle mitochondria [30]. The rapid and large reductions in PC levels in contrast to the small changes in ATP in fasciculating muscle indicate that an immediate equilibrium does not exist between these two high-energy phosphate metabolites.

The observed decrease in PC may be related to the increased demand for maintaining ATP levels because muscle fasciculations utilize greater quantities of ATP. A decreased synthesis of PC cannot be ruled out until effects on the synthesis have been studied in more detail. A complicating factor for the interpretation of our data is the fact that only a small percentage of the muscle fibres in a given muscle (at the most 15% of the total fibre count) develop fasciculations and necrosis. Therefore, the observed changes of high-energy phosphate compounds reflect only average changes from muscle fibres with very low, moderate or unchanged levels of these compounds. It may well be that PC and ATP are critically reduced in muscle fibres that develop necrosis, while others may only develop small reductions in PC and ATP, and therefore do not develop necrosis. The technical difficulties for a quick isolation of the necrotic fibres from muscles has so far prevented separate analysis.

Still puzzling is the observation that only a limited number of fibres in a given muscle undergo necrosis. In no muscle did more than 15% of the fibres show a fully developed necrosis, while ultrastructural changes were apparent at almost all endplates. The lesioned fibres probably belong to the same motor unit(s), but this remains to be determined. It

can be assumed, however, that more than 15% of the fibres in the muscle must have been recruited during the hyperactivity period. While fibre death could result from hypoxia or relative ischaemia of the fibres during the sustained activity, exercise-induced ischaemia or hypoxia would be expected to affect a larger population of fibres. It is possible that all of the fibres recruited during the fasciculation did sustain reversible injury such as ultrastructural changes at nerve terminal and endplate and that the necrotic fibre population may represent fibres with the lowest CP and ATP supply. Moreover, some authors suggest that calcium-activated proteases are activated by the increased influx of calcium, resulting in myofibre destruction and Z-band disappearance [31,39]. According to this proposal, the proteolytic alterations are essentially irreversible. If the proposed preoteolytic digestion of substantial numbers of subjunctional sarcomeres were to occur, a substantial period would be required for gene reactivation and for the synthesis of the requisite amounts of actin, myosin, troponin, actinins, tropomyosin and other myofibrillar proteins [38]. However, the rapid repair of damage suggests the reassembly of pre-existing components rather than *de novo* synthesis following widespread proteolysis [38]. It seems relevant to point out that sustained NM activity even in the absence of antiChE can produce many of the changes observed following antiChE toxicity, the exception being that prolonged high frequency stimulation does not cause the supercontraction of the subjunctional sarcomeres which is always present in antiChE-induced myopathy [38]. High frequency stimulation, however, potentiates the toxicity of low ineffective dosages of antiChE precipitating the typical morphological changes.

The supercontraction seen with antiChE indicates the presence of prolonged continuous depolarization of the endplate and supports the suggestion that the morphological changes seen occur only when antiChE induces sustained NM activity [36].

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Ophthalmic toxicology of anticholinesterases

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Introduction

Much has been learned about the ophthalmic toxicity of antiChEs from testing in animals and humans, from accidental poisonings, and with their uses in treatment of glaucoma, accommodative strabismus and myasthenia gravis. Best known effects include miosis, induction of pupillary cysts, enhancement of accommodation, formation of cataracts and reduction of intraocular pressure.

Distribution of ChEs in ocular tissues

The distribution of ChEs in ocular tissues of various species is varied. Assays have been published for ChE in eye tissues of humans [42, 68], monkeys [101], rabbits [68, 101, 113, 114, 162], cats [69,101] and beagle dogs [73]. Humans are notable for having entirely BChE in the lens, monkeys for having almost entirely AChE in the ciliary muscle [Erickson-Lamy *et al.*, unpublished] but much less AChE in the cornea than rabbits and cats. Rabbits have high AChE activity in the corneal epithelium. ChE activity in the cornea and lens bears no relationship to the effects of antiChEs on these tissues.

Penetration and distribution of antiChEs

Penetration and distribution from the anterior surface was examined in rabbits by Laties [101] utilizing autoradiography of [^{3}H]DFP and by measuring inhibition of tissue ChE by ecothiopate* iodide eyedrops. Binding of DFP by corneal epithelium and inhibition of corneal ChE by ecothiopate is striking, but of unknown consequence. From the cornea, antiChEs diffuse across the anterior chamber to the iris, to the pupillary surface of the lens and to the aqueous outflow system. Even after iris ChE has become saturated by inhibitor, the lens still has some enzyme activity behind the iris. AntiChEs also enter the eye by passing through the sclera, entering the ciliary body, and spreading toward the crests of the ciliary processes, but even after complete inhibition of ChE at the roots of the processes some activity still exists at the crests of the processes, near the equator of the lens [101].

*Echothiophate (USP)

Cornea and conjunctiva

Clinical examination after application of antiChE eyedrops reveals only conjunctival hyperaemia; the cornea usually shows no abnormality. Exceptionally, rabbits repeatedly exposed to 1% Hektion have corneal ulceration and perforation [142] and repeated exposure to fenitrothion produces changes suggestive of conical cornea [92]. Also, exceptionally, two patients treated for glaucoma for several years by twice daily application of ecothiopate iodide eyedrops developed pseudopemphigoid in the treated eyes only [128]. Also a case was reported of bilateral tear duct stenosis after use of ecothiopate iodide eyedrops twice a day for 13 years [166]. Excessive tearing from lacrimal gland stimulation occurs in antiChE poisoning [48].

In the corneas of cattle, von Brücke *et al.* [159] demonstrated large amounts of ACh in the corneal epithelium, and van Alphen [156] showed an abundance of choline acetyltransferase. Both were present in all mammalian

species tested by Mindel and Mittag [115,116], except cats. Most investigators suggest that most ACh in the cornea is in the epithelium [54,60,116,143]; its function is unknown.

ChE is present in the epithelium, the stroma [129] and the endothelium [101,129]. That in the epithelium has been demonstrated by Howard *et al.* [77] who showed the epithelial activity to be AChE while that in the stroma is BChE. Rabbit corneal ChE activity is proportional to the contents of ACh and choline acetyl transferase [76], but Wilson and McKean [164] found rabbit corneal ChE to be disproportionately low. In cats corneal ChE activity has been reported to be less than in rabbits, and very low in monkeys [101,129].

Various antiChEs administered experimentally or therapeutically abolish corneal and conjunctival ChE activity [101] but cause no evident alterations in the corneal epithelium [54]; no M-cholinoceptors have been found in the corneal epithelium [127].

Denervation of the limbus causes a 50% fall in ChE activity of the corneal epithelium [129]. Postganglionic severing of the first branch of the trigeminal nerve has a similar effect, but preganglionic severing does not [159].

While the functional significance of the high levels of ACh, choline acetylase, and ChE activity in the corneal epithelium and endothelium of some species remains unknown, it has been suggested that they may have a role in ion movements across epithelium and endothelium or corneal sensation [53,129]. However, corneal touch or pain stimuli are not enhanced by physostigmine, nor blocked by atropine [156].

Cultured corneal epithelium from rabbits and humans has been reported to show cytotoxicity after addition of extremely high dilutions of antiChE eyedrops, but the significance is unclear [99].

Iris

AntiChEs usually produce miosis, but sometimes pupil dilation. Certain concentrations of antiChEs in contact with the eye may cause extreme miosis, but affect accommodation only moderately; e.g. volunteers exposed to sarin vapour had marked miosis but only 2.3 D of induced accommodation, compared with a potential maximum of 7–10 D [120].

In systemic antiChE poisoning the pupils may become extremely small, but paradoxically in some cases of severe poisoning the pupils are dilated [44]. Leuzinger *et al.* [105], reporting a survey of 536 cases of OP poisoning, observed in severe cases that, 'often the miosis of the pupils changes to mydriasis, making proper diagnosis difficult'.

In some animals a decrease in miosis is noted on repeated administration of antiChE eyedrops. In rabbits [11], cats and rabbits [29], monkeys [27] and guinea pigs [16], repeated application of antiChE miotic eyedrops resulted in decrease in the degree of miosis induced, and also a decrease in miosis from carbachol and pilocarpine eyedrops. However, in most cases the maximal pupillary response to bright light was unaltered even after several weeks of antiChE eyedrop treatment [27,71]. Bito [26] showed that neither the sympathetic nervous system nor sympathetic neurotransmitters had a role. Histologically the irides of guinea pigs show no atrophy or vascular damage [16].

Iris ChEs are located on cell membranes, between cells and intracellularly [95,101,102]. Two major pools are present; one is functional enzyme, while the other much larger pool is of unknown function. The pool responsible for hydrolysis of neuronally released ACh is located on cell surfaces [70,75,110]; the other pool is intracellular. Studies with ecothiopate and DFP on cat iris and ciliary muscle confirms that extracellular surface ChE regulates response to cholinergic agents, and is specifically involved in potentiation of contractility [68–71,118]. Ecothiopate iodide selectively inhibits AChE whereas DFP more selectively inhibits BChE [70]. Ecothiopate shifts the dose-response curves of pupillary constriction by AChE or methacholine, but not by carbachol, which is reversible by 2-PAM [70,71].

Miosis in humans is commonly associated with a feeling that the surroundings are dim, or that the illumination has been reduced [5,155]. This is related to the decrease in pupillary area [5,144]. Dark adaptation has little effect on strong miosis from antiChEs [5]. However, if parasympathetic innervation is abolished by ciliary ganglionectomy, then physostigmine [8] or DFP [104] fail to constrict the pupil.

Miosis increases the depth of focus, but in the miotic treatment of accommodative strabismus the pupils usually are not sufficiently constricted to have an important role [135].

When miosis is maintained for weeks or months by daily antiChE eyedrops there is, according to Romano and Jackson [136], Wilkie *et al.*, [163] and Drance [45] a gradual shallowing of the anterior chamber. In rare instances, angle-closure glaucoma results. In tests on the influence of 0.125% ecothiopate iodide eyedrops on the axial depth of the anterior chamber in patients, in the first 8 h a change was barely detectable, but with daily administration there was gradual shallowing during at least 8 weeks [163]. The mean decrease of axial depth was 0.2 mm at 1 week, and 0.44 mm at 8 weeks. None of these patients developed glaucoma. When administration of ecothiopate iodide was discontinued, axial depth gradually returned toward normal over 6 weeks.

Angle-closure glaucoma is most likely to be precipitated in eyes having a shallow anterior chamber and narrow angle which can be accentuated to the point of angle-closure by miosis and forward movement of the lens. Jones and Watson [86] and François and Verbraeken [55,56] described rare cases in which angle-closure glaucoma was induced in eyes of young persons despite previous near-normal anatomy. Glaucoma in these cases was caused by the iris closing the angle of the anterior chamber and obstructing outflow of aqueous humour; however, contraction of the ciliary muscle must also have played an important role, loosening the zonules of the lens and allowing the lens and iris to come forward. Treatment with anticholinergic drugs was effective in these cases, presumably owing to tightening of the lens zonules resulting from ciliary muscle paralysis.

Iritis is an infrequent complication in humans from contact with antiChEs, and usually is associated with conjunctival hyperaemia. One case has been described from a spray of bromophos [39]. AntiChE-induced iritis in some cases responds well to anticholinergic treatment [5], but in other cases response is slow despite treatment with mydriatics, corticosteroids and 2- PAM [24].

Development of cysts of the pupillary border of the iris in humans is a common complication of repeated application of antiChE to the eye. Attempts to reproduce the cysts in rabbits and guinea pigs have been unsuccessful [145]. Pupillary cysts were originally described after use of physostigmine [157], but they have been observed with most antiChE eyedrops. Characteristically, 1–12 brown cysts, 0.1–1 mm in diameter are seen along or behind the pupillary edge of the iris. They may develop in 1–40 weeks [1]. If miotic eyedrops are discontinued, in 2–40 weeks the cysts change to shrunken brown tags, and slowly disappear [1]. In adults, the cysts usually develop only in response to strong miosis, but in children they commonly develop in association with moderate miosis [34–36,74]. Rarely, the pupillary cysts interfere with vision, particularly in association with extreme miosis [58,147]. Simultaneous use of phenylephrine eyedrops can enlarge the pupil slightly, and reduce the tendency to form cysts [2,34–36,65]. The ciliary processes have been reported rarely to have cysts in association with pupillary cysts from miotics [97].

Histologically, Christensen *et al.* [37] found proliferation of iris pigment epithelium instead of cysts, but in another case Straub and Conrads [145] found that two posterior epithelial layers of the iris were separated in some places, forming fluid-filled cysts. The latter case fits better a suggested mechanism that miosis pinches the iris pigment epithelium against the lens, then retention of fluid between the layers produces cysts [1,147]. Consistently, in the absence of the lens, cysts are not produced by miotic eyedrops [147].

Depth perception may be unreliable after unequal eye contact with an antiChE, producing unequal miosis [155].

Ciliary body

AntiChEs can affect accommodation and aqueous outflow through the ciliary body. The innervation of the ciliary muscle is mainly parasympathetic. Contraction of the muscle in accommodation is potentiated by antiChEs. Ciliary ganglionectomy or postganglionic ciliary neurectomy in cynomolgus monkeys causes denervation of the ciliary muscle and affects the accommodative mechanism [49]. Resting refraction is the same as before denervation,

but the normal accommodative response to topical physostigmine is lost. Response to pilocarpine persists. With reinnervation, which occurs in about 6 months, there is recovery of accommodative responsiveness to both physostigmine and pilocarpine, reaching normal even when the activities of choline acetyl transferase and AChE in the ciliary muscle have reached only about 30% of normal [Erickson-Lamy *et al.*, unpublished].

AntiChEs applied to the eye can enhance the accommodative capability of the eye. In humans the near point of accommodation may move in 10 cm, and the amount of minus lens that can be overcome may be increased by more than 4 diopters at distance, yet when the effort to accommodate is relaxed, the focus of the eye, and the visual acuity at distance, may rapidly return to normal [5,155]. The rate and completeness of recovery appears to be anti-ChE-dose-dependent. With submaximal dosage one can easily demonstrate an abnormal slowness in relaxation of accommodation. With maximal dosage some accommodative myopia may persist even when no effort at accommodation is being made. If an effective dose of an antiChE is applied to the eye, distant objects come into focus appreciable slower.

Aching discomfort in the eye or forehead is commonly noted on looking at a near object after application of an antiChE because of enhanced contraction of the ciliary muscle; this discomfort fades away when gaze shifts to the distance.

The fact that antiChEs can potentiate accommodation and induce a temporary functional myopia has led some investigators, especially in Japan, to speculate if recurrent exposure to antiChEs might induce chronic myopia. No convincing evidence has been produced, although there have been some intriguing observations. In an extensive retrospective study, Tamura and Mitsui [150] reported on a correlation between the incidence of myopia in 40 000 school-age children and the use of OP insecticides in the Tokushima Prefecture between 1957–1973; peak incidences of myopia coincided with peak use of OPs. In another study, refractive errors occurred in 88% of 71 children (4–16 years old) from rural areas of the Saku district who visited Asam Hospital compared with a 2% incidence of myopia in an age-matched control group of patients in Tokyo University Hospital [83]. It is not clear that exposure of the children of the Saku region to OPs was of sufficient magnitude and duration to cause significant sustained accommodation. Furthermore, myopia has not been demonstrated to be a side- effect in children who have been chronically treated with antiChEs for esotropia [9,10]. However, recent experimental models of myopia have suggested that cholinergic nerves may play a role in the development of myopia. Abnormal eye growth (axial myopia) is restricted to childhood and apparently can be stimulated by manipulation of the visual environment. One hypothesis is that axial myopia is caused by excessive accommodation. Young [167] found that restriction of the visual space of rhesus monkeys led to the development of axial myopia, which was reversed in part by adminstration of atropine. Similarly, axial myopia induced by suturing the lids in tree shrews [111] and in rhesus (but not *M. acctiodes*) monkeys [133] was prevented by atropine.

Other findings in laboratory animals support the possibility that an antiChE-induced myopia can occur in young animals and may be mediated by pathological changes in the ciliary muscle. Beagle dogs treated daily with oral disulfoton (5–20 mg per day) or fenitrothion (10–20 mg per day) for 2 years developed myopia and corneal astigmatism [85,153]. Widespread destruction of the ciliary muscle fibres was present, whereas, axial length of the treated globes was not significantly different from that in control eyes. AntiChE-induced changes in the ciliary muscle also occur in the subhuman primate eye. Daily topical treatment with clinically-relevant doses of ecothiopate iodide to young adult cynomolgus monkey eyes resulted in mitochondrial swelling and thickening of the basement membrane of the ciliary muscle after 2 months [108]. However, although, muscle degeneration became progressively more severe after 6 months of treatment, no sign of myopia was evident.

Unrelated to induction of myopia, but of interest in respect to persistent changes in the ciliary muscle after exposure to antiChEs, 2–6 months of administration of ecothiopate eye-drops in monkeys resulted in a subsensitivity

of the accommodative mechanism in response to parenteral pilocarpine, which was associated with alteration in M-cholinoceptors, and which persisted for several months [50,89]. Interestingly, the accommodative response recovered in spite of widespread damage to the ciliary muscle.

Enhancement of accommodation by antiChE eyedrops provides a useful treatment for accommodative esotropia in children. In accommodative esotropia a relative weakness of accommodation and disproportionately strong convergence produces 'crossed eyes', with danger of emblyopia in one of the eyes. AntiChE eyedrops can enhance accommodation so that focusing corresponds to the amount of convergence, helping to straighten the eyes.

Another function of the ciliary muscle that can be potentiated by antiChEs is control of resistance to outflow from the anterior chamber. In glaucoma pressure is elevated because of excessive resistance to flow through the aqueous outflow system. Contraction of the ciliary muscle reduces the resistance to outflow and antiChE eyedrops potentiate this action [24,46]. Agents utilized in treating glaucoma include demecarium bromide, ecothiopate iodide, DFP, neostigmine, paraoxon, physostigmine and TEPP.

The mechanism by which contraction of the ciliary muscle reduces resistance to outflow of aqueous humour has not been defined. Severing the attachment of the ciliary muscle to the scleral spur prevents the change in resistance to aqueous outflow which normally occurs in response to parasympathetic innervation or to the action of cholinergic or antiChE agents [90]. In excised eyes, with the attachment to the scleral spur intact, mechanically pulling on the ciliary muscle reversibly reduces resistance to aqueous outflow. This can be prevented by detaching the ciliary muscle, suggesting that this resistance is subject to physical modulation, and that potentiation of contraction of ciliary muscle is important in the treatment of glaucoma by means of antiChEs [62]. With continuing treatment there may be decrease in effectiveness [61]. Lütjen-Drecoll and Kaufman [108,109] have described microscopic structural alterations which may account for a decrease in effectiveness.

Lens

AntiChE eyedrops used in treating glaucoma can cause changes in the transparency of the crystalline lens, leading in some patients to decrease in vision. Early suspicions that this might be so were expressed by Kreibig [98], Muller *et al.* [122] and Harrison [72] from observing instances of development of opacities of the lens in patients using antiChE eyedrops. The first systematic study was by Axelsson and Holmberg [19], followed by a series of related reports by Axelsson [12–18], de Roetth [40–42], Shaffer and Hetherington [140,141], Cinotti and Patti [38], Thoft [152], Abraham and Teller [3], Drance [45], Levene [106], Morton *et al.* [119] and Nordmann and Gerhard [123,124]. Most agree that careful slit-lamp examination of the lens after several months of daily administration of antiChE eyedrops reveals anterior and posterior subcapsular vacuoles or small opacities in about half of the patients studied. Axelsson [15] has provided good evidence that glaucoma itself does not produce cataracts. The length of treatment and observation and the incidence of lens changes vary with investigators, but it is generally accepted that antiChE drugs, including ecothiopate iodide, demecarium bromide, paraoxon and DFP produce a much higher incidence of anterior and posterior subcapsular changes than do pilocarpine or carbachol eyedrops, or than occurs in untreated controls. Not all observers have been wholly in agreement [3,38,152].

Eyes treated with pilocarpine before antiChEs are somewhat protected from the effects of antiChEs on the lens [106, 123, 124, 140, 141]. The reason is unknown.

Also unexplained is the fact that some adult glaucoma patients are highly resistant to the adverse effect of antiChE eyedrops on the lens and, despite daily use of drops of maximum concentration for years, maintain normal visual acuity and normal transparency of their lenses [41,42,45,141,142].

Several investigators have described the lens changes produced by antiChE eyedrops as typically consisting of minute anterior subcapsular vacuoles in groups, very small anterior subcapsular woolly or mossy opacities in aggregates, often associated with nuclear sclerosis, and posterior subcapsular small

vacuoles or opacities. The anterior changes are easily seen but usually do not interfere with vision, whereas the posterior changes are more difficult to see, and they often reduce visual acuity. In some patients cataract extraction has been necessary [14,45,119,151].

Age influences susceptibility to induction of lens changes by antiChE eyedrops. Susceptibility is highest in the elderly, whereas children receiving drops for treatment of accommodative strabismus rarely show lens changes [20, 21,35,72,131,165].

AntiChE drugs given orally in the treatment of myasthenia gravis do not produce lens changes and miosis usually does not develop; therefore the dose reaching the eyes must be less than from the eyedrops [107].

Acute transitory lens opacification in guinea pigs from intracarotid injection of antiChEs was reported by Diamant [43], but only in near moribund animals; coma, cessation of blinking and evaporation of water from the eyes caused this opacification. Axelsson [16] showed that ecothiopate or paraoxon eyedrops could be applied daily to guinea pigs eyes without producing cataracts. Also, in rabbits daily application of 0.25% ecothiopate iodide eyedrops for 3 months to 1 year failed to produce cataracts [30,52,66,67], yet Laties [101] showed that when ecothiopate iodide or DFP eyedrops were applied to rabbits eyes these agents reached the iris, the anterior surface of the lens and the ciliary body. The equatorial portion of the lens was protected, still showing some ChE activity. In human and rabbit eyes, de Roetth [42] showed that ChE in the pupillary portion of the lens could be inhibited by antiChE drops applied to the eye, and Bito *et al.* [28] found that there was a decrease in the number of epithelial cells in mitosis in this region.

In cultured rabbit lens, Michon and Kinoshita [113,114] determined that concentrations of antiChEs sufficient to inhibit all the lens ChE failed to produce changes in transparency, but that a thousand times this concentration caused anterior, posterior and equatorial vacuoles to appear, with an increase in permeability of the lens, an increase in water and Na^+ content, and a decrease in K^+. Ecothiopate could produce these changes without altering lens metabolism, but demecarium bromide caused anaerobic metabolism to predominate [114]. Muller *et al.* [122] reported that in pig, rabbit and human lenses, paraoxon caused a 50% inhibition of oxygen consumption. In rabbit eyes that had been chronically exposed to ecothiopate iodide eyedrops without producing visible changes in the lenses, Härkönen and Tarkkanen [66,67] found a decrease in ATP and lactate, but no change in glycogen, glucose or glucose-6-phosphate. Firth *et al.* [52] found no change in lens glutathione. In cultured calf lenses, Klethi [93] found that ecothiopate iodide, even at 0.06–0.5% produced no lens opacities, but did alter lens nucleotides and ^{32}P incorporation.

Kaufman and Bárány [89] and Kaufman *et al.* [87] showed that daily administration of 0.25% ecothiopate eyedrops to monkeys, produced anterior and posterior subcapsular opacities in 2.5–14 weeks, the anterior opacities reaching a maximum at 3–4 months, and the posterior after 1.5–3 months. Kaufman and Bárány [89] and Kaufman *et al.* [87,88] found that if the iris were removed beforehand more opacities resulted, but if eyedrops containing atropine in addition to ecothiopate iodide were used, the development of anterior and posterior opacities was delayed, and the number reduced. Experiments have been reported by Albrecht and Bárány [4] and Kaufman *et al.* [91] in which accommodation was eliminated by disinserting the ciliary muscle from the scleral spur before daily administration of ecothiopate eyedrops. Eyes so treated developed anterior and posterior opacities, the same as eyes with normal accommodation. This is a different result from what might have been expected from the experiments in which Kaufman *et al.* [88] found interference with formation of opacities when atropine was added to antiChE eyedrops.

In monkeys given daily ecothiopate eyedrops, Albrecht and Bárány [4] and Philipson *et al.* [130] observed that besides subcapsular opacities there was swelling of the anterior cortex of the lens. It is reminiscent of the increase in permeability of cultured rabbit lenses described by Michon and Kinoshita [114].

Aqueous humour

Although antiChEs reduce intraocular pressure in both normal and glaucomatous

eyes a paradoxical transitory increase in intraocular pressure may occur in some patients owing to a breakdown of the blood-aqueous barrier.

This initial pressure increase can be extreme in rabbits. Thus, application of physostigmine, neostigmine or DFP to rabbits' eyes initially causes hyperaemia of the iris, a rise in intraocular pressure, and an increase in capillary permeability, allowing entry of proteins sufficient to produce a strong flare in the aqueous humour [22,160]. This may be a prostaglandin-mediated reaction.

Rabbits can be made tolerant to antiChEs by repeated application, and then respond more like primate eyes, with a reduction of intraocular pressure and no inflammatory signs [22,100,160].

In humans an acute iritic or iridocyclitic reaction to antiChE eyedrops occasionally occurs reminiscent of the initial reaction seen regularly in rabbits [5,24].

Retina and optic nerve

A review of Alpar [6] lists case reports in which retinal detachment occurred after initiation of treatment of glaucoma with miotics, especially antiChE miotics. A cause-effect relationship between treatment with strong miotic and retinal detachment has not been established. Lemcke and Pischel [103] reported an increase in suspected miotic-induced cases of retinal detachment after the introduction of modern ChE inhibitors to glaucoma therapy. Becker and Shaffer [25] noted that patients with open angle glaucoma may be predisposed to peripheral retinal degeneration. The mechanism underlying a putative ChE inhibitor-induced retinal tear is thought to involve an intense contraction of the ciliary muscle producing retinal tears [6]. Lemcke and Pischel [103] reported holes or horseshoe- shaped tears usually in the periphery of the retina, sometimes surrounded by pigment changes, suggestive of pre-existing chorioretinal adhesion. Findings by Freilich and Seelenfreund [57] among 20 patients ranged from focal detachments to total detachments. Total detachments were caused by small horseshoe-shaped tears as well as small round breaks at the posterior vitreous base. Finally, the results of a survey of 91 retinal surgeons, along with examination of data obtained from the *National Registry of Drug-Induced Ocular Side-Effects* strongly suggested the possibility of antiChE-induced retinal detachment in patients with pre-existing retinal pathology [23].

No mention is made of retinal detachment in reports describing the sequelae of poisoning with OPs. Similarly, retinal detachment has not been noted in studies in experimental animals, including those involving long-term (e.g. up to 6 months) daily administration of antiChEs to monkey eyes [50,89]. Therefore, it is likely that antiChE-induced retinal detachment occurs only in eyes in which retinal pathology pre-exists.

Delayed peripheral neurotoxicity from OP esters is a well known clinical entity. In the case of esters lacking antiChE activity, ocular involvement has been rare [63]. DFP and mipafox have caused delayed neurotoxicity involving axonal degeneration with secondary demyelination, without clinically evident involvement of the eye [49,94]. Several studies in humans as well as experimental animals suggest that exposure to antiChEs may lead to acute alterations in the electrical properties of the retina. Alpern and Jampel [7] reported that topical application of 1% physostigmine resulted in a decrease in the critical flicker frequency in human subjects, which, on the basis of pharmacological experiments, was hypothesized to be a cholinergic mechanism. Gazzard and Thomas [59] analysed the threshold luminance of the central visual fields of human subjects after exposure to sarin vapour. Results indicated that sarin raises the visual threshold, influencing cone more than rod function.

Other studies have demonstrated sarin-induced elevation of the absolute scotopic threshold in human subjects [137,138]. Several studies have documented ERG changes in experimental animals including rat [79,80], mouse [31,32] and cat [149,158]. Carricaburu and co-workers [33] found that high doses of mevinphos and malathion disturb the electrical properties of the retina by direct action on the photoreceptor cells (increased lag time and reduced amplitude of the a wave), and cause possible damage to the bipolar and/or ganglion neurons (reduction in the b wave amplitude).

In cats, chlorfenviphos (1–16 mg/kg ip) increased the amplitude of the a wave, and at doses above 4 mg/kg also increased the amplitude of the b wave [149]. On the other hand, the evoked responses recorded from electrodes placed in the lateral geniculate body and the optic tectum were decreased in amplitude, while responses in the visual cortex were variable.

Most of these effects were inhibited by atropine, glutathione or PAM implicating the accumulation of ACh in the cholinergic projection network of the CNS. Similarly, Von Bredow *et al.* [158] reported that sarin (10 mg/cat ip) resulted in an increase in the b wave of the cat ERG which could be completely reversed by atropine. After daily im injections of fenthion (0.5 mg/kg) for 4 days, increased amplitude and decreased latency and peak times of the a and b waves of the rat ERG is observed. At high dosages (over 25 mg/kg) latency and peak times are increased and amplitudes of the a and b waves decreased [78].

In a second study, Imai [79] demonstrated that ERG changes could be induced in rats after single im injections of 5, 25 and 50 mg/kg of fenthion. The ERG in the low dosage group revealed an increment in the amplitude of the a and b waves which persisted up to 10 days after injection. After day 10 the latency and peak times were lengthened, and did not return to normal until 40 days after injection. In the mid-dosage group the a and b wave amplitudes increased, and by the fourth day, the latency and peak times lengthened, after which, the a and b wave amplitudes decreased. The a and b amplitudes recovered by 60 days after injection; but the peak time never recovered. In the high dosage group, the a and b wave amplitudes were decreased and latency and peak times increased, and no recovery of the amplitudes was noted.

Revzin [134] reported on the effect of mevinphos on flash-evoked slow potentials in the visual tectum or the nucleus rotundus of urethane-anaesthesized animals. They concluded that, 'exposure to organophosphate pesticides can cause substantial visual dysfunctions over a period of time with little or no warning from the usual perceptual signs that dangerous functional changes are occurring'.

Findings of retinal pathology in persons exposed to OP insecticides have also been reported, primarily in the Japanese literature. Kogure and Imai [96] reported the findings of Japanese ophthalmologists in individuals classified as having Saku disease including an initial reddening of the optic nerve accompanied by mild swelling. Later, the optic nerve became pale in the temporal aspect and eventually atrophic. Optic atrophy was accompanied by the finding of a reduced visual acuity. Examination of 56 school children from Saku City in Japan revealed a 65% incidence of optic neuritis and/or retinochoroidal atrophy [84]. Another study reported an incidence of 9.81% optic neuritis in a population of 164 farmers chronically exposed to insecticides, including OPs [82]. Similar findings have been reported in Egypt after severe poisoning with leptophos [112].

Other studies have implicated OPs in retinal pigmentary degeneration. Retinal pigmentary degeneration has been reported in two patients after documented severe exposure to OP insecticides [126]. Mistra *et al.* [117] also reported on the high incidence (22% of a sample population of 64) of macular degeneration among workers engaged in the spraying of OP insecticides in India. The results of fluorescein angiography suggested that the macular lesion was the result of a defect in the pigment epithelium. However, no information was provided with regard to whether the workers were also exposed to the antimalarial drug chloroquine, which is known for its toxicity to the retinal pigment epithelium.

Experimental pigmentary degeneration has been induced by OPs in laboratory animals. Biweekly sc fenthion to pigmented and albino rats over 1 year resulted in diminution of the ERG amplitude followed by retinal degeneration and eventual loss of the ERG. Pathological changes included marked degeneration of the posterior retinal pole involving the diminution of all cell layers while the peripheral retina was minimally affected and the optic nerve appeared to be normal. Biochemical changes in pigmented rats included a loss of pigment from the retina coinciding with reduced ERG amplitude and preceding histological changes [81]. OP-induced degeneration of the optic nerve and the pigment epithelium also has been demonstrated in beagle dogs [154]. In contrast, long-term antiChE treatment in beagle dogs

resulted in normal ocular findings despite lowering of serum and RBC ChE [125].

While the above studies have provided evidence for antiChE-induced retinopathy and optic atrophy in both humans and experimental animals, others have reported no or mild transient ocular effects. A follow-up study of over 232 individuals having a documented history of acute OP insecticide poisoning revealed only miosis, blurring of vision for a day or two, and eyelid twitching [148]. Examination of 63 workers in manufacture, application or transport of OP insecticides for 5 years or more revealed consistent abnormal ocular findings limited to conjunctival hyperaemia, photophobia, excessive lacrimation and a subjective burning or itching of the eyes [132].

Furthermore, toxic retinopathy has not been noted in the thousands of individuals who received topical antiChE treatment for glaucoma or esotropia. While it could be argued that retinal damage might not be differentiated from the disease process itself in glaucoma, this is not the case in esotropia. Extensive reviews of the literature presented no evidence for retinal pathology in children treated for accommodative esotropia [9,10]. Collectively, the weight of evidence suggests that the effect of sustained inhibition of ChE *per se* is limited to acute alterations of the electrical properties of the retina with no long-term pathology. However, several OPs have other pharmacological activity, e.g. some OPs are capable of inhibiting esterases other than ChE [146].

A spontaneous retinal degeneration is known to occur in the *rd* mouse, owing to increased turnover of retinal cGMP phosphodiesterase [51]. Phosphodiesterase inhibitors produce changes in the ERG of the isolated perfused cat eye which are similar to ERG abnormalities found in individuals with hereditary retinitis pigmentosa [139]. Collectively, these studies suggest the theoretical possibility that OP-induced retinal degeneration could result if substantial inhibition of cGMP phosphodiesterase occurred in addition to ChE inhibition.

Extra-ocular muscles

These muscles have the highest concentration of AChE in the body [73,121]. AntiChE eyedrops used in treating glaucoma commonly cause twitching of the eyelids for a few minutes shortly after application. Systemic poisoning can also produce this effect. Intramuscular injections of TEPP in patients with myasthenia gravis have been observed by Grob and Harvey [64] to produce transient 'jerking movements of the eyes' and 'tremor of the eyelids'. This presumably is a nicotinic action of ACh. Pilocarpine eyedrops, which do not have nicotinic actions, do not produce twitching.

Extra-ocular muscle effects have been reported in neuropathies from antiChE poisoning. Suicidal ingestion of OPs has been reported to cause bilateral sixth or third nerve paralyses in eight of 200 patients, and bilateral facial paralysis in 12 of 200 patients [161].

In beagle dogs, after daily administration of disulfoton for 2 years, some myelinated nerve bundles to extra-ocular muscles showed demyelination, but only mild axonal disturbance [120]. Within the muscle fibres swollen mitochondria were intermingled with normal mitochondria, suggesting a developing neuromyopathy.

Saku disease

Several reports appeared in the 1960s and 1970s in the Japanese literature describing a syndrome characterized by reduced visual acuity, narrowing of the visual field, myopia, astigmatism, congestion or atrophy of the optic nerve, disturbance in balance, abnormal retinal function and lowered blood ChE. This syndrome (Saku disease) was described by Ishikawa after examination of clinical records of affected patients from Japan's highly agricultural Saku region of the Nagano Prefecture, where large amounts of parathion and malathion were used [83]. The myopia associated with the Saku syndrome was of particular interest, in view of the widespread incidence of severe myopia which occurs in the Oriental population.

However, as noted by Plestina and Piukovíc-Plestina [132] in their extensive review of the Japanese literature, in most of the reports involving suspected OP-induced ocular pathology, 'the connection between the aetiology of eye impairment and effects of antiChE is

speculative'. The available literature does not exclude the possibility of a greater susceptibility of the Oriental population to antiChE-induced ocular pathology. However, the absence of significant pathological findings in other studies [132,148] suggest a cautious interpretation of the largely unsubstantiated findings concerning Saku disease.

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19

Nephrotoxicity of anticholinesterases

Gregory Paul Wedin

Introduction

Nephrotoxicity is not recognized as a clinical feature of antiChE poisoning. A limited number of case reports and experimental data, however, suggest that antiChEs may have nephrotoxic effects. The effect of antiChEs on the human kidney has not been carefully or thoroughly evaluated. As a result, there is a lack of information in the literature to elucidate recent case reports or characterize the potential nephrotoxic effects of these agents.

Studies suggests that both renal circulation and electrolyte excretion may be partially under cholinergic control, which indicates that antiChEs could disrupt normal renal function. This, coupled with biochemical and histopathological changes consistent with nephrotoxicity after the administration of antiChEs, support a contention that a nephrotoxic action may exist in humans.

Kidney function and nephrotoxicity

The kidney has important metabolic and regulatory functions to maintain body homeostasis that make it particularly susceptible to the toxic effects of chemicals. The human kidneys are exposed to toxicants to a greater degree than other organs, as they receive a disproportionately large blood supply (25% of cardiac output). The renal concentrating mechanisms can lead to higher concentrations of a substance in the tubules or interstitium than in blood. Specific transport mechanisms for secretion and reabsorption can result in a toxicant accumulating in tubular cells. The lipophilicity of antiChEs may allow their penetration through plasma membranes with direct access to the intracellular space and organelles.

The kidney is capable of oxidation, reduction, conjugation and enzymatic metabolism. Although many of these reactions detoxify substrates, some metabolites or conjugates may be more toxic than the parent compound. Covalent binding of reactive metabolites to tissue macromolecules accounts for the toxicity of many chemicals. It has been suggested that O,O,S-trimethyl phosphorothioate (OOS-Me), an impurity in some antiChEs, is metabolically activated to reactive metabolites that bind to tissue macromolecules, including those in the kidney [22]. It is probable that binding of malathion to tissue macromolecules is dependent on mixed function oxidase activity [30].

Toxicants may cause direct tubular or glomerular injury, causing impaired secretory and reabsorptive function or glomerular permeability. Prolonged vasoconstriction, either as a direct effect or compensatory mechanism, may lead to renal ischaemic damage.

The high metabolic capacity and oxygen consumption of the kidney makes it more susceptable to extrarenal effects such as hypotention or altered neural or humoral activity. Nephrotoxic insults can occur, therefore, secondary to complications of antiChE poisoning such as hypotension or dehydration. Other complications such as muscle fasciculations and seizures can lead to rhabdomyolysis associated with myoglobinuria and acute renal failure. Such effects could also potentiate or unmask an otherwise clinically insignificant nephrotoxic potential of the antiChE itself.

The renal cortex is most susceptible to toxic injury because it receives the greatest blood supply. The proximal tubule is most commonly affected. Resultant toxicity ranges from minor changes in cellular morphology and transport capabilities to tubular necrosis.

Evaluation of nephrotoxic effects

Biochemical and histopathological studies can used to evaluate and monitor patients after exposure to a known or suspected nephrotoxin. Urinalysis parameters proven most reliable indicies of nephrotoxicity include proteinuria, reduced specific gravity, cylinduria, renal tubular celluria, oliguria or polyuria, and haematuria [35]. Glycosuria, pyuria and crystalluria are other indices, and sediment such as renal tubular cells and casts are most significant.

Excess protein generally represents increased glomerular permeability. Increased urinary glucose suggests nephrotoxicity only when accompanied by protein. Glycosuria without hyperglycaemia is not usually from increased filtration but from impaired proximal tubular reabsorption. A change in urine osmolarity or impaired concentrating ability of the kidney is a very early change that indicates renal medullary function may be impaired. Renal tubular damage is associated with increased urinary excretion of Na^+. Other solutes such as K^+, phosphate, and H^+ as well as urea nitrogen and creatinine accumulate in plasma when renal function is impaired.

Other indices of kidney function include inulin clearance, to measure glomerular filtration (GFR), and renal extraction of para-aminohippurate ion (PAH) to measure renal plasma flow (RPF). AntiChEs may be added to isolated tissues to specifically evaluate direct toxic effects on the tissue by microscopic examination or measurement of organic ion transport.

Evidence of nephrotoxic injury by light or electron microscopy includes loss of brush borders of proximal tubular cells, enlarged lysosomes, vacuolization, and dilation of the endoplasmiç reticulum.

Parasympathetic control of renal function

It is known that the kidney contains adrenergic nerve fibres, but the presence of cholinergic nerve fibres remains in question [19]. AChE-containing nerve fibres have been demonstrated in the kidneys of various mammalian species [5,7,32]. Nerves that contain a high concentration of AChE would be expected to be cholinergic nerves [32]. An assessment of the nephrotoxicity of antiChEs, therefore, must take into account the effects of ACh on renal function.

The corresponding distribution of AChE-containing fibres with adrenergic nerve fibres has led to a suggestion that ACh may play a role in adrenergic neurotransmission by releasing noradrenaline [7]. The presence of AChE-containing fibres after adrenergic denervation suggests that the kidney contains two types of nerve fibres [32].

The effect of ACh on adrenergic transmission to the renal vasculature has been specifically evaluated. Takeuchi *et al.* [39] studied renal vascular response to stimulation of the greater splanchnic nerve under various conditions in an attempt to demonstrate a cholinergic component. Neither the antiChE neostigmine nor hemicholinium bromide, a drug interfering with the production of ACh, had a consistent effect on the renal vasoconstrictor response to nerve stimulation. Also, a vasodilator response was not elicited when the vasoconstrictor response to nerve stimulation was abolished with reserpine and guanethidine. ACh infused directly into the renal artery, however, did produce a vasodilator response that was reversed by atropine. It was concluded that antiChE fibres in the kidney are separate from adrenergic fibres.

In contrast, McGiff *et al.* [31] demonstrated that ACh injected into the renal artery at high doses (1000 μg) produced vasoconstriction, whereas vasodilation resulted at low doses (1–10 μg). This vasoconstrictor response was enhanced by co-administration of atropine and physostigmine in that vasoconstriction was produced at all doses studied, except 1 μg. Reserpine, which depletes stores of catecholamines, caused ACh to produce only vasodilation. The use of autonomic blocking drugs either reversed or reduced the vasoconstrictor response to ACh. Physostigmine enhanced the vasoconstrictor response to nerve stimulation, whereas atropine diminished the vasoconstrictor response. Hemicholium bromide blocked the renal vasoconstrictor response to repetitive nerve stimulation, at which point ACh was probably depleted. It was concluded that the vasoconstrictor effect of ACh was probably caused by release of catecholamines.

When paraoxon was infused to the point of systemic toxicity it produced marked bilateral renal effects, including vasoconstriction. This resulted in decreased effective RPF and GFR [46].

Based on these studies, the kidney may be innervated by both adrenergic and cholinergic components. The adrenergic pathway may be controlled in part by cholinergic stimulation, which may be dose dependent. It is difficult, therefore, reliably to predict the effect that antiChE agents might have on the renal vasculature. The apparent existence of cholinergic mediated adrenergic transmission within the kidneys, however, suggests a potential for deleterious effects of renal function in the presence of excess ACh.

Increased renal adrenergic tone could contribute to acute tubular necrosis secondary to increased renal vascular resistance, activation of the renin-angiotensin system or possibly increased transmembrane transport of Ca^{2+} in renal tubular cells [19]. Both increased vascular resistance and activation of the renin-angiotensin system may lead to renal failure secondary to a direct ischaemic insult. Increased intracellular Ca^{2+} is associated with lethal cell injury, but the precise mechanism has not been established [45].

AntiChE effects on renal excretory function

Further evidence for cholinergic control of renal function is demonstrated by the effects of ACh and antiChEs on renal excretory function. These findings suggest a cholinergic control of cellular cation transport systems [29]. Urinalysis findings provide evidence for antiChE-induced nephrotoxicity.

The kidney contains antiChE-sensitive ChEs [8]. Electron microscopic radiographs have identified radiolabelled DFP localized primarily over the cytoplasm of proximal tubule cells, particularly with the infolds of the basal plasma membrane and small amounts either in or on the mitochondria and microvilli. There was also heavy labelling of kidney tubule cell nuclei.

ChE activity has been associated with Na^+ transport in various tissues [6,23]. ChE activity has also been shown to be prominent in the thick ascending limbs of the loop of Henle in the rat kidney [17]. This suggests that ChE may be involved in Na^+ transport associated with the counter current mechanism for concentrating urine in the renal medulla.

Data indicate that ACh directly affects renal excretory function. When ACh is infused into the renal artery it produces a significant increase in water excretion, K^+, Na^+, Cl^-, Ca^{2+} and phosphate [29,34,40]. It also increases RPF but produces variable changes in GFR [34,40]. Urine osmolarity is decreased by ACh [40]. These effects on renal function are blocked by atropine.

It appears that ACh increases both efferent and afferent arteriolar vasodilation, as GFR remained stable or was variably affected [40]. The fact that Na^+ excretion is increased despite unaltered GFR indicates that ACh inhibits tubular Na^+ reabsorption. Increased Ca^{2+} and phosphate excretion suggests a proximal tubular effect [29]. It has also been postulated that these effects are secondary to increased renal medullary blood flow, which would reduce the medullary osmotic gradient [34].

Physostigmine, DFP, neostigmine methylsulphate, and paraoxon do not produce similar effects on renal function, even when administered to the point of systemic toxicity [29,40,46]. Atropine, administered alone, also failed to demonstrate any effects. These results suggest that either there is a lack of endogenous ACh or that renal ChE is resistant to these inhibitors or that antiChEs could not reach renal cell ChE [29]. It is suggested that this lack of effect may be from antiChE effects on two opposing systems; enhanced ACh at postganglionic receptors and enhanced release of noradrenaline to adrenergic sites [46]. Sympathetic stimulation produces Na^+ and water retention whereas parasympathetic stimulation causes increased water and Na^+ excretion.

Another approach to assess the effects of chemicals on renal function is the use of isolated renal tissue. The active secretion of the organic anion, p-aminohippurate (PAH), and the organic cation, tetraethylammonium (TEA), is reduced by nephrotoxins. These two distinct processes may be evaluated *in vitro* by using the renal slice technique. Reduced uptake of these substances *in vitro* corresponds to impaired proximal tubular secretion *in vivo*.

Baggett *et al.* [4] studied renal cortex slices from rats pretreated with DFP (*in vivo* study) and the addition of DFP to fresh cortex slices (*in vitro* study) and found that PAH accumulation was decreased. Only the *in vitro* study demonstrated inhibition of TEA transport. These results suggest that DFP may have a selective action on renal organic ion transport. This selectivity may also relate to intrarenal distribution of DFP rather than a selective action of DFP on a specific transport system. Na^+ and K^+ content, inulin space and total renal water were not altered. This indicates that normal tissue function was maintained [9].

Further evidence that the effects of OPs on Na^+ excretion is from direct effects on tubular membranes is provided by low dose parathion studies in the dog [18]. Intravenous parathion (1.5 μg/kg) significantly increased urinary Na^+ excretion, which was completely blocked by atropine; K^+ excretion was significantly decreased. Values for RPF, GFR and urine output either did not vary significantly or were slightly decreased. AChE activity in both RBCs and renal tissue was not changed. Therefore, the effect on Na^+ excretion is probably a result of decreased tubular reabsorption secondary to changes in tubular membranes and is unrelated to altered enzyme activity. The authors propose that the effect of atropine was from its ability to block endogenous ACh.

In unanaesthetized animals the effect of DFP (2, 3 or 4 mg/kg) on renal function was evaluated [10]. Urinary excretion of Na^+, glucose, blood and protein were increased. Urine flow increased significantly, and was accompanied by a significant decrease in osmolarity. In anaesthetized animals neither blood flow nor GFR showed consistent changes.

Increased urine output, regardless of changes in GFR or blood flow also suggests a direct tubular effect of DFP [10]. That renal ChE remained decreased when renal function parameters had normalized, substantiates the belief that the effects of DFP are unrelated to its antiChE activity, but rather a direct effect of this reactive compound. The concomitant increase in the excretion of glucose, protein and blood suggests the occurrence of acute renal damage. Increased excretion of protein, glucose, blood and concomitant oliguria also occurred in rats administered parathion [12]. This was associated with renal histopathological changes.

OOS-Me is an impurity of malathion that potentiates the toxicity of malathion and produces toxicity to rats when administered alone. A dose dependent hyperaminoaciduria has been demonstrated in rats treated with 10–60 mg/kg of OOS-Me [24]. At higher doses (40–60 mg/kg) urine volume was decreased. The blood urea nitrogen to plasma creatinine ratio was used to distinguish between prerenal, renal or postrenal OOS-Me damage. In most cases this ratio was <15 : 1 indicating renal tubular damage. Such damage may account for the increased excretion of amino acids.

To evaluate further the nephrotoxic effects of OOS-Me, an analysis of urinary proteins was performed [25]. Urinary amino acids, glucose and specific gravity also were measured. There was a substantial increase in urinary amino acids, no change in total protein but the types of protein were changed; most notably, a reduction of $gamma_1$-globulin. Urinary glucose was increased and the specific gravity was only 1.01 in the presence of oliguria. These findings provide additional evidence that OOS-Me causes proximal tubule damage. The authors also suggest that OOS-Me may have effects on both the cellular and humoral immune systems. This is based on the observed loss of $gamma_1$-globulin coupled with their previous findings of lymphocytopaenia and signs of immunodeficiency in rats treated with OOS-Me.

Histopathologic evaluations of nephrotoxicity

Histopathological studies have been performed to examine changes of the kidney when exposed *in vivo* or *in vitro* to antiChEs. Although this does not provide much insight into possible mechanisms of antiChE nephrotoxicity, it gives valuable information about sites and types of injury.

Several studies to assess the mammalian toxicity of carbaryl demonstrate diffuse cloudy swelling of proximal tubule cells [13]. The distribution of such swelling was more diffuse in animals receiving higher doses. Cloudy swelling of renal tubules was also demonstrated

in rats dying after oral parathion [12]. The kidneys also had capillary-venous congestion and there was fatty degeneration or necrosis of the tubules. There was no significant change in kidneys of surviving animals. Chlorpyrifos also causes cloudy swelling of convoluted tubules [33].

Further evidence of a nephrotoxic action of carbaryl was the deposition of fine fat droplets in the epithelial cells of proximal tubules [13]. A similar deposition of fat occurred with triorthocresyl phosphate. Fatty degeneration of kidneys has also been reported with soman and paraoxon [20,21].

Degenerative changes of renal tubular epithelium from the chronic administration of phosdrin to dogs and rats have been described [15]. Oxydemeton-methyl has also been shown in poultry to cause coagulative necrosis in tubular epithelial cells and hyperplasia of endothelial cells leading to increased cellularity in the glomeruli [14]. Fenthion did not cause such degenerative changes. Both acute and chronic administration of oxydemeton-methyl and fenthion slightly increased the total lipid content of the kidneys but decreased phospholipid. The ratio of phospholipids to cholesterol in the kidney was decreased by oxydemeton-methyl. This effect predisposes cell membranes to degeneration, which may explain the relative degenerative effects of the two OPs [14].

Acute administration of phosmet produced renal vascular dystrophy and degeneration of proximal tubule cells within 60 min [41]. Kidneys revealed signs of parenchymal and vacuolar dystrophy. Lysosomes accumulated in enlarged intracrystal spaces of mitochondria. Endothelial destruction of small arteries and of the basal lamina in capillary loops was also described. Evaluation after 24 h revealed parenchymal dystrophy in the cells of proximal tubules. The cytoplasm of tubular cells contained numerous lysosomes and large vacuoles. Dense granules were evident in podocyte pedecelles and cells of the juxtaglomerular apparatus.

Granular dystrophy in human and rat kidneys after intoxication with trichlorfon and malathion has been reported [11]. Vacuolization of podiocytes and destruction of tubular epithelial mitochondria occurs accompanied by increased lysosomal activity after administration of PEMA [16]. Vacuolation of proximal tubule cells of monkey and rat kidneys has been described with the administration of carbaryl [38]. The parenchymal dystrophy and vacuoles may explain the increased excretion of blood, glucose and protein in urine [41].

The glomeruli have also been implicated in antiChE nephrotoxicity. Although the renal parenchyma of rats was normal with the administration of phosphamidon, occasional foci of glomerulonephritis was reported [36]. Twice weekly administration of parathion caused proliferation and fibrosis of reticulin fibre of the basal membrane of Bowman's capsule and tubules [43]. This was most evident in rats receiving 8 mg/kg and surviving for 200 days.

Another method used to assess nephrotoxicity is measurement of renal enzyme activity. Impaired alkaline phosphatase activity indicates that transphosphorylation reactions may be adversely affected [37]. Increased acid phosphatase activity may be associated with cell disintegration and prenecrotic changes in renal tissue [37].

The administration of malathion, phosalone or phenthoate to rats, significantly inhibited alkaline phosphatase activity [37]. A significant increase in acid phosphatase was produced by malathion and phosalone, and a highly significant increase resulted from phenthoate administration. The administration of malathion with differing dietary levels of protein produced variable changes in alkaline phosphatase activity [42]. Alkaline phosphatase was increased in the 5% and 20% dietary protein groups but decreased in the group receiving 10% protein. In a study using single doses of carbaryl or daily doses for 7 days, acid phosphatase activity was significantly increased in rat kidneys, but alkaline phosphatase activity was unaffected [27]. The administration of malathion has also been reported to cause increased acid phosphatase activity [26]. These alterations in renal enzyme activity suggest a potential nephrotoxic action for antiChEs.

Evidence of nephrotoxicity in humans

Reports of human cases of accidental or intentional exposure to antiChEs often only report

dramatic and clinically significant toxicity, and subtle effects may be disregarded or overlooked.

One controlled study on humans suggests that impairment of renal function may occur without other objective or subjective findings [47]. Two groups of five men ingested either a placebo capsule or capsules containing 0.06 mg/kg carbaryl on a daily basis. Two other groups of six men ingested either placebo or 0.13 mg/kg of carbaryl daily.

Neither plasma nor RBC ChE activities were significantly affected. No signs or symptoms attributable to the antiChE were detected. Haematology, blood chemistry and urinalysis revealed no significant effects. The only variable affected by carbaryl was the ratio of urinary concentration of amino acid nitrogen to that of creatinine, in the group receiving the higher dose. This data indicates a slight impairment of the reabsorptive capacity of the proximal tubule.

Renal function evaluated in 30 children who received trichlorfon for the treatment of schistosomiasis [1] did not show any nephrotoxic effects.

Only two reports of renal involvement with antiChE poisoning are reported in the English language literature. The first involved a 65-year-old man who developed acute renal insufficiency and massive proteinuria 4 weeks after he began heavy use of malathion, with intermittent dermal and inhalation exposure [3]. He presented with gross peripheral oedema. Pertinent initial laboratory data included: serum creatinine of 380 μmol/l (5.0 mg/dl), serum urea nitrogen 18.0 mmol/l (50 mg/dl). Urinalysis revealed 4+ protein, four to five white blood cells and three to four RBCs per high power field and occasional granular casts. Renal biopsy evaluation by immunofluorescence revealed sparse deposits of IgG in glomeruli. Electron microscopic evaluation demonstrated uncovered, segmental, epimembranous electron-dense deposits and diffuse effacement of the foot processes. His renal function gradually improved spontaneously. The authors suggest that the presence of membranous glomerulopathy and low C_3 level, 110 (reference range 115–328), indicates that malathion caused an immune complex nephropathy. They propose that an OP could invoke an immune response, with the insecticide serving as antigen or that the toxicity of the insecticide unmasked antigens. This is consistent with the findings of Keadtisuke and Fukuto [25] who suggest possible involvement of the immune system with the nephrotoxic effects of OOS-Me.

The other report describes a 26-year-old man who ingested approximately 8 ounces of an unknown concentration solution of diazinon in a suicide attempt [44]. He developed significant muscarinic effects that were relieved by atropine and 2-PAM. His urine output shortly after admission averaged only 22 ml/h, and was dark and cloudy. Urinalysis on the second hospital day revealed trace protein, trace blood, moderate amorphous crystals and a specific gravity of 1.029. Urine output increased with iv fluids. The crystalluria gradually resolved by the ninth hospital day. The composition of the crystals was not identified. AntiChEs can increase Ca^{2+} excretion, which may have formed the crystals. It is unlikely that diazinon reached high enough concentrations in the urine to form crystals [2]. Diazinon, or its metabolites may have been partially responsible for the dark colour of the urine. It is possible that significant dehydration may have precipitated this reaction.

In a multihospital study of OP poisoning, renal impairment was reported in seven of 53 patients studied [28]. Renal dysfunction was associated with urinary sediment and decreased GFR (personal communication). Seizures and coma occurred in 32 patients. Cardiac arrhythmias occurred in 22 patients. The incidence of hypotension was not reported. A prerenal aetiology is likely to be responsible for the renal impairment in many of these cases.

Conclusion

Clinically significant nephrotoxicity is an uncommon manifestation of antiChE toxicity. It is possible that subtle nephrotoxic injury goes undetected. Clinical manifestations of antiChE poisoning such as hypotension, dehydration, and seizures can lead to nephrotoxicity, and could precipitate or unmask direct toxic effects.

Experimental data indicates that ACh, which accumulates in the presence of

antiChEs, and antiChEs themselves, can significantly alter renal function. This may result from alterations in neural, humoral, and metabolic activity. Experimental data also indicates that antiChEs may have a direct nephrotoxic effect on renal tubules.

Further work is needed to explore the potential nephrotoxicity of antiChEs in humans. Patients need to be more closely and carefully evaluated for evidence of nephrotoxic injury.

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Immunotoxicology of anticholinesterase agents

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Esterases and immune responses

Inhibition of esterases may affect the immune system either by inhibiting esterases in effector cells or via alterations in neurotransmitters, which regulate autonomic nervous system connections. Becker and Hansen [2] suggested that activatible esterases may degranulate mast cells, releasing histamine, and may be involved in the process of chemotaxis by rabbit polymorphonuclear cells. Lymphocyte activation and subsequent division after antigen challenge are prerequisites for many immunological responses. There is evidence that serine esterases are activated after surface Ig cross-linking by anti-Ig. DFP inhibits the anti-Ig-induced activation of mouse B cells [1]. Additional evidence of a direct role of esterases in immune responses is found in the cytotoxic and natural killer cell-mediated cell lysis. The target cell lysis is associated with proteins stored in cytoplasmic granules of the effector cells, which are released on target cell recognition. Many of these proteins have been identified as serine esterases [23]. T cell killing is inhibited by DFP and other serine esterase inhibitors [17].

Inhibition of ChEs may affect the immune response. The autonomic nervous system directly innervates thymus, spleen, lymph node, bone marrow and other lymphoid tissues [15,16]. Williams *et al.* [64] showed that pharmacological manipulation of post-ganglionic noradrenergic nerve fibres alters immune function. Increased levels of cyclic nucleotidase, which respond to neurotransmitters, may also alter the immune response [9]. Cholinergic stimulation of target tissues caused an accumulation of cyclic guanosine 3'5'-monophosphate (cGMP). Cyclic nucleotides influence lymphocyte activation and proliferation [56].

The possibility that OPs can be designed as selective immunosuppressors for therapeutic purposes has been considered [3]. It was theorized that highly specific, highly active, relatively stable and non-toxic chemicals can be prepared. Specificity against the first component of the complement system was selectively increased by adding a terminal amino group to the *p*-nitrophenyl ethyl pentyl phosphate. It is sometimes possible to separate the antiChE activity from the protease inhibitor activity; however, this approach has had limited success.

In mice injected with 4 mg/kg of DFP, antigen processing and presentation by macrophages for immune processes requiring the cooperation of T cells were primarily affected [53]. The response to macrophage-independent B cell antigens was not altered. DFP also interfered with the generation of memory cells, which are responsible for clonal expansion when cells encounter a subsequent challenge to the antigen. AntiChEs may act directly on the cells possessing ACh receptors, which are located on lymphocytes. However, antiChEs may also have an indirect effect via corticosteroid production.

ChE inhibitors as potential alkylating agents

OP pesticides may possess potential phosphorylating or alkylating properties. As various carcinogenic alkylating agents are immunosuppressive agents [32], phosphorylating agents may have an immunotoxic effect via this mechanism. Hilgetag and Teichmann [22]

hypothesized that dimethyl phosphates and other phosphorotriesters are good alkylating agents. Kimbrough and Gaines [28] reported that the alkylating agents tris(1-methyl-1-aziridinyl)phosphine oxide (metepa), tris(1-aziridinyl)phosphine oxide (tepa), and 2,2,4,4,6,6-hexahydro-2,2,4,4,6,6-hexakis(1-aziridinyl)-1,3,5,2,4,6-triazine phosphorine (apholate) induced malformations in newborn rats, as did other OPs such as parathion, dichlorvos, and diazinon, but generally required maternally toxic doses. Little emphasis has been placed on the relationship between phosphorylating properties of OPs and their immunotoxic effects.

The phosphorylating properties were investigated to study the mechanism of neurotoxic OPs [24,25]. A mono-substituted phosphoric acid residue was formed on proteins at specific neurotoxic and other non-specific sites. A phosphorylation of factors involved in the expression of immune responses thus warrants consideration.

Ethyl CB (urethane) is another carcinogenic alkylating agent with antiesterase properties. Carcinogenic doses of urethane produced severe myelotoxicity and a markedly depressed natural killer cell activity but had less effects on other parameters of immune function like cellular and humoral immunity, and macrophage function [33]. A selective alkylation of various processes of the immune function by antiesterase agents is thus possible.

Immunomodulation by antiChE pesticides

OPs

A number of studies suggest that antiChEs are immunosuppressive. It is difficult to compare data from different OPs because of the many variations in their chemical configuration.

Street and Sharma [55] fed rabbits low doses of parathion-methyl and reported atrophy of the thymus cortex and fewer antibody forming cells in the lymph node; splenic morphology was considerably altered. Delayed hypersensitivity response was not altered in a consistent manner (Table 20.1). There was no significant influence on circulating antibody levels against sheep RBC. Casale *et al.* [8] reported that single high doses of malathion, parathion and dichlorvos reduced primary IgM response to SRBC at levels producing cholinergic symptoms. The OPs had no effect when given in multiple lower

Table 20.1 Influence of dietary exposure to antiChE pesticides in rabbits[a]

Chemical	*Dose mg/kg per day*	*Skin reactivity to tuberculin[b]*		*Lymph node fluorescence[c]*	*Germinal centres in spleen[d]*	*Thymus atrophy[e]*
		10 days	*24 days*			
Parathion-methyl	0	1.86±0.59	1.57±0.40	25±1[f]	13±3	0.6±0.2
	0.036	2.14±0.55	1.29±0.28	15±1[f]	11±2	1.1±0.2
	0.162	1.43±0.43	0.57±0.30[f]	17±3[f]	12±3	1.3±0.5[f]
	0.519	1.57±0.53	1.71±0.36	13±2[f]	8±3[f]	1.9±0.5[f]
	1.479	1.57±0.65	0.57±0.37[f]	14±2[f]	9±3[f]	1.8±0.6
Carbaryl	0	2.57±1.05	1.28±0.52	20±2	14±2	0.8±0.3
	0.23	3.16±0.47	0.16±0.07[f]	15±2[f]	13±2	0.6±0.2
	1.08	2.33±0.49	1.00±0.43	18±2	13±1	1.1±0.4
	2.30	2.66±0.56	1.66±0.53	19±4	12±3	2.2±0.5[f]
	8.38	2.28±0.64	1.00±0.51	21±4	10±3	2.2±0.3[f]
Carbofuran	0	2.14±0.34	2.00±0.51	19±2	14±3	1.2±0.4
	0.03	0.67±0.33[f]	0.83±0.65	15±2	10±2[f]	1.4±0.4
	0.16	0.71±0.42[f]	1.00±0.49	15±2	8±2[f]	1.6±0.4
	0.49	0.71±0.42[f]	0.29±0.18	12±2[f]	9±3	1.8±0.2
	1.05	1.00±0.41[f]	2.00±0.50[f]	14±3[f]	7±2[f]	1.9±0.4

[a]Male white New Zealand rabbits were given various chemicals in feed for 8 weeks. Several parameters of immune system were evaluated
[b]Reaction scored on a scale of 0–4 (0 = no reaction, 4 = weal > 15 mm). Animals were challenged with Freund's adjuvant on day 33 of pesticide treatment and tuberculin injected intradermally on 10 and 24 days after the antigenic challenge. The reaction was scored 24 h after the tuberculin injection
[c]Number of antibody producing cells in a defined microscopic field. Popliteal lymph node sections were stained with fluorescein-labelled goat anti-rabbit globulin and examined under an ultraviolet miscroscope
[d]Number of well-defined germinal centres in a 4 mm field. Hematoxylin and eosin stained sections were evaluated in a random-blind fashion
[e]Degree of thymus atrophy scored as 0–4 (0 = no change, 4 = extensive degeneration of cortical area). Hematoxylin and eosin stained sections were evaluated in a random-blind fashion
[f]Statistically significant from respective control value, $P<0.05$

doses. The cholinomimetic agent, arecoline, reduced IgM antibody response only when given in a form which would sustain prolonged cholinergic poisoning.

OPs are also immunosuppressive on haematopoietic stem cells. Mice given 4 mg/kg of parathion for 14 days had alterations in bone marrow-derived haematopoietic stem cell colony formation up to 2 weeks without any cholinergic symptoms [20]. Results were similar with human bone marrow cells when paraoxon or malaoxon were added to *in vitro* cultures. Granulocyte-macrophage colony (CFU-GM) formation was suppressed in a dose-dependent fashion [21]. Other OPs such as dimethoate [60], fenitrothion, fenthion, diazinon and EPN [38], dichlorvos [8,11] were also immunosuppressive.

Several impurities often present in OPs may potentiate OP toxicity. For example, O,O,S-trimethyl phosphorothioate (OOS-TMP) and O,S,S-trimethyl phosphorothioate (OSS-TMP) [34,61], of many impurities in malathion, are potent ChE inhibitors; they inhibit immune responses at doses which produce no cholinergic symptoms. Various effects of OOS-TMP and OSS-TMP are summarized in Table 20.2.

Devens *et al.* [12] reported a significant reduction in generation of cytotoxic lymphocytes against P815 tumour cells and reduced antibody response to SRBC in C57BL/6 mice after a single acute dose of OOS-TMP (10 mg/kg). This dose also produced a transient reduction in the number of thymic lymphocytes on day 3 and day 5 after dosing. A dose of 10 mg/kg OOS-TMP increased non-specific esterase activity of splenic macrophages in cell separation/reconstitution experiments. Macrophages from OOS-TMP treated mice reduced the number of antibody forming cells when mixed with normal T and B cells [45]. Furthermore, macrophages from OOS-TMP treated animals suppressed the proliferation of tumour cells and the supernatant from 24-h

Table 20.2 Influence of antiChE impurities in malathion on immune responses

Chemical and dose[a]	*Tests employed and effects*[b]	*Reference*
O,O,S-trimethyl phosphorothioate (OOS-TMP)		
0.5 mg/kg per day for 14 days, oral	Generation of anti-SRBC antibody secreting cells increased. IL-2 production increased, proliferative responses to mitogens increased. Cytotoxic T cell response unchanged	Rodgers *et al.* [45]
5 mg/kg per day for 14 days, oral	CTL and specific antibody responses unchanged, other above parameters still elevated	Rodgers *et al.* [45]
10 mg/kg, single dose, oral	Increase in size of macrophages and increased esterase activity	Rodgers *et al.* [46]
10 mg/kg, single oral	Decreased antigen presentation by macrophages, increased phagocytic activity and IL-1 production	Rodgers *et al.* [47]
Incubation of splenic cells *in vitro*	Cytotoxicity by mature killer T cells inhibited at 75 μg/ml	Rodgers *et al.* [44]
Incubation of splenic cells *in vitro* with and without preincubation of OOS-TMP with glutathione	Glutathione conjugated chemical inhibited CTL and haemolytic plaque-forming-cells. Direct inhibition of macrophages, and T and B cells	Thomas and Imamura [58]
1–40 mg/kg, single oral dose	Reversible suppression of CTL and anti-SRBC plaques	Rodgers *et al.* [48]
10 mg/kg, oral	Generation of suppressor macrophages induced	Rodgers *et al.* [49]
10 or 20 mg/kg, oral	Increased phagocytic capability and respiratory burst activity of splenic and peritoneal macrophages	Rodgers and Ellefson [42]
O,S,S-trimethyl phosphorodithioate (OSS-TMP)		
In vitro, 25–1000 μM	Human CTL and NK cell activity inhibited	Rodgers *et al.* [43]
20–80 mg/kg, oral	CTL response generation elevated at lower doses, suppressed at higher doses. Mitogenic response of splenic cells elevated	Rodgers *et al.* [50]
2 mg/kg, oral	CTL and IL-2 elevated. Mitogenic responses not influenced	Rodgers *et al.* [51]
5 mg/kg, oral	CTL and antibody responses suppressed, other immune parameters unchanged	Rodgers *et al.* [51]

[a]Experiments in C57Bl/6 mice or derived cells, except where indicated otherwise
[b]CTL, cytotoxic T lymphocytes; IL-2, interleukin-2; NK, natural killer

cultures of spleen cells inhibited the proliferative response to mitogen and antibody response to SRBC [48]. This dose also increased the phagocytic activity and IL-1 production by macrophages but reduced the ability of macrophages to present antigen [47]. The same group [44] also studied the effects of OOS-TMP *in vitro* that had been preincubated with an activating system of rat liver postmitochondrial supernatant. Preincubated OOS-TMP blocked the generation of cytotoxic T lymphocyte (CTL) response but failed to inhibit mature cytotoxic T cells. Thus, OOS-TMP appears to act at the early stage of CTL activation or proliferation. Further studies by Thomas and Imamura [58] suggest that the immunosuppression by OOS-TMP is via a glutathione-mediated process that involves the functional inhibition of responder lymphocytes and macrophages.

Rodgers *et al.* [43,50] studied the effect of O,S,S-trimethyl phosphorodithioate (OSS-TMP) on the *in vivo* primary and *in vitro* secondary cellular and humoral immune response. Single non-toxic doses of 20 or 40 mg/kg elevated primary immunological responsiveness but only the higher dose elevated the secondary humoral response. Rodgers *et al.* [43] reported that *in vitro* OSS-TMP blocked the effector phase of the cytolytic reaction that is mediated by murine and human cytotoxic T-lymphocytes.

Casale *et al.* [7] investigated the influence of several antiChEs on the human complement system, which requires serine esterases. An *in vitro* assay system involved the incubation of diluted human sera with 0.5–3 mM of different chemicals. Carbaryl, carbofuran, dichlorvos and DFP inhibited the lysis of antibody-sensitized SRBC in a dose-dependent manner. Carbaryl was more potent than DFP, carbofuran or dichlorvos. The antiChE properties were not related to their ability to inhibit the complement system; paraoxon is a potent antiChE, whereas carbaryl is the least potent enzyme inhibitor of the group. The maximum inhibition of lysis in any serum by the highest concentration of carbaryl was <45%.

CBs

Street and Sharma [55] fed rabbits diets containing carbaryl or carbofuran, which reduced the numbers of activated lymphocytes in lymph nodes, decreased the number of splenic germinal centres and produced atrophy of the thymic cortex (Table 20.1). Olson *et al.* [40] reported a decrease in plaque forming cells (PFC) to SRBC in mice given aldicarb in drinking water. However, lower doses (1 ppb in water) had a greater effect than a higher dose (1 ppm). In contrast, Thomas and Ratajczak [59] reported that aldicarb did not affect various parameters of immunological responsiveness in mice exposed to various concentrations (0.1–1000 ppb) in drinking water for 34 days. A decreased T_4:T_8 ratio was reported in 23 subjects who consumed groundwater contaminated with aldicarb (<61 ppb); the decreased T_4:T_8 ratio was the result of a significant increase in the number of T_8 cells [18].

Peppy *et al.* [41] showed that carbaryl reduced the phagocytic activity of hepatic and splenic macrophages, based on colloidal carbon clearance following iv carbaryl. They suggested that phagocytic activity was impaired owing to inhibition of cell-bound serine esterases. Maroussem *et al.* [35] later showed that resident peritoneal macrophages stimulated with zymogen in the presence of carbaryl had reduced respiratory burst, altered phospholipid profile, and decreased prostaglandin levels.

Immunologic effects of plasticizer TOTP

Some OPs cause delayed neurotoxicity by inhibition of NTE [31], and alterations in immunological function have been implicated [39]. Watanabe and Sharma [62] tested the latter hypothesis by administering tri-*o*-tolyl phosphate (TOTP), a contaminant of tricresyl phosphate (TOCP), to chickens. TOTP consistently increased diffuse lymphatic tissue in the liver and spleen and increased total plasma protein levels. However, the transfer of plasma and leucocytes from atoxic chickens to normal chickens failed to passively transfer the neurotoxicity. Also, immunosuppressive therapy failed to protect chickens consistently from TOTP-induced paralysis [63]. Foil *et al.* [19] also reported a failure to produce ataxic syndrome by the passive transfer of serum

and/or lymphocytes from atoxic donors without subsequent TOTP treatment. However, T-cell mediated graft *versus* host and phytohaemagglutinin wattle responses were significantly depressed in TOTP-treated chickens. In a repeated gavage study with mice, neither TOTP nor its isomer, tri-*m*-tolyl phosphate, consistently affected immune parameters including lymphocyte blastogenesis, splenic anti-SRBC plaques, or delayed hypersensitivity [4].

Allergic sensitization by OPs and CBs

Ercegovich [14] recommended investigation because of lack of evidence linking incidental exposure to allergic sensitization. Street [54] reviewed the alteration in immune responses to a number of pesticides including allergic sensitization to OPs. Of the many OPs which alter the immune response, only malathion showed a positive patch test but the frequency of positive tests was low in people frequently handling malathion. Cushman and Street [10] produced IgE antibody in BALB/c mice to a metabolite of malathion conjugated to keyhole limpet haemocyanin. However, epicutaneous malathion did not elicit a delayed-type hypersensitivity response up to 1 month later. Matsushita and Aoyama [36] reported sensitization and some cross-reaction with OPs. Subjects from an area where several pesticides were used who tested positive to benomyl also reacted to diazinon. Similar results were obtained in a guinea pig maximization test. In a study of OP-induced contact dermatitis in 202 patients in Japan, Matsushita *et al.* [37] attributed the reactions to mainly dioxabenzafos, fenitrothion, leptophos, cyanophos, diazinon and malathion. The areas affected by dermatitis were fingers (62.4%), face (39.6%), forearm (31.6%) and neck (29.7%). One quarter (25.2%) of the cases with dermatitis had symptoms associated with acute OP poisoning.

Isocyanates as ChE inhibitors and their sensitization potential

Isocyanates have antiChE properties, and cause respiratory sensitization and asthma. Butcher [6] suggested two mechanisms for isocyanate-induced sensitization: one involving cellular adrenergic receptors, which alter cAMP levels necessary for maintaining bronchial tone, and an immunologically-mediated process, which involves IgE antibodies.

Brown *et al.* [5] studied isocyanates and ChE inhibition. Hexamethylene diisocyanate, hexylisocyanate and 2,6-toluene diisocyanate were potent inhibitors of ChE activity of purified human serum. A higher molar ratio was required to inhibit 5% enzyme by 2,4-toluene diisocyanate (2,4-TDI), phenyl isocyanate or o-tolyl isocyanate.

Tolyl-specific IgE antibodies have been reported in humans sensitized to toluene-diisocyanate (TDI) [27]. Furthermore, Karol [26] showed that guinea pigs inhaling TDI for 3 h/day for 5 days developed antibodies against TDI. TDI concentrations > 0.36 ppm caused a dose-dependent increase in the titre of TDI-specific antibodies and also in an increasing percentage of animals producing such antibodies. Liss *et al.* [30] found positive serum samples for total antibody and IgG and IgE specific for methylene diphenyl diisocyanate-human serum albumin (MDI-HSA) conjugate in workers exposed to this chemical in a steel foundry.

Koschier *et al.* [29] studied the sensitizing potential of 2,4-TDI in guinea pigs given an intradermal injection followed by a challenging dose 5 days later. The severity of sensitization was directly related to the initial induction and challenge dose. Tanaka *et al.* [57] induced contact dermatitis (MDI) by 1% methylene biphenyl diisocyanate in C57Bl/6 mice. Contact dermatitis in syngeneic mice was transferred by lymphocytes from MDI-sensitized animals. The affected cells were probably T-cells.

Dwivedi *et al.* [13] showed a significant reduction in delayed-type hypersensitivity response and reduced phagocytosis by peritoneal and alveolar macrophages 7 days after rats received a single acute dose of methyl isocyanate. Reduced phagocytosis by peritoneal and alveolar macrophages was evident 2 weeks after a single exposure [52]. Also, mortality was 100% in rats following endotoxin treatment compared with 20% in control animals.

Future perspectives and conclusions

AntiChEs have potent effects on various processes of the immune response. It has not been established whether these are mediated directly via inhibition of ChEs or other esterases that mediate the immune reactions. Esterase activation may have a role at various steps of complement cascade, release of histamine or other bioactive substances from cells, and chemotaxis of sensitized lymphocytes. It is not known if the antiChE property can be used for therapeutic modulation of the immune system. The cytotoxic effect of many potent antiChE chemicals may explain a number of *in vitro* effects observed; esterases also have lysosomal-induced cellular effects.

Much of the available information on immunotoxic evaluation of antiChEs involves pesticides. However, exposure to low levels of pesticides does not appear to adversely affect the immune system. The effects observed are not always dose-related and often occur at exposures not producing systemic toxicity. Street [54] summarized the effects of various OPs and CBs on the immune system. Allergic responses and various degrees of autoimmune diseases have been reported in workers occupationally exposed to pesticides; however, this effect may be mediated by alteration of proteins rather than ChEs. Modulation of immune responses, particularly interference with host-defense mechanisms, has been experimentally induced for a variety of antiChE pesticides.

Various antiChEs are biologically potent chemicals which inhibit ChEs via covalent interaction at the serine residue of the active site. Even though enzyme inhibition may be reversible, there is the potential that other proteins may be modified and that several related enzymes may be inhibited. In many cases it can not be determined if the immunomodulatory effects are the result of inhibition of esterases or to related mechanisms.

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21

Immunotoxic effects of anticholinesterases

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Overview of immunotoxicology

Function of the immune system

The immune system confers resistance of the host to infection by bacteria, viruses and parasites; functions in the rejection of allografts; and may eliminate spontaneously occurring tumours [63].

Proper function of the immune system is exquisitely sensitive to disruptions in physiological homoeostasis. Immunotoxicology deals with the effects of xenobiotics on this system. The immune response is highly regulated and several different mechanisms may eliminate an antigen [59]. Therefore, a toxicant can affect one facet of defence against infection without altering the ability of the host to survive challenge by an infectious agent. However, it is known that certain chemicals can influence the susceptibility of a host to infection, and that this may be mediated through the immune system.

Generation of an immune response

A simplified version of cell population in the immune system is shown in Figure 21.1. The generation of an immune response results in the formation of effector cells; either cytotoxic T lymphocytes or antibody secreting plasma cells [41,64]. The humoral response, which protects against bacterial and viral infections, is mediated by the collaboration of the macrophage, the helper T lymphocyte and the

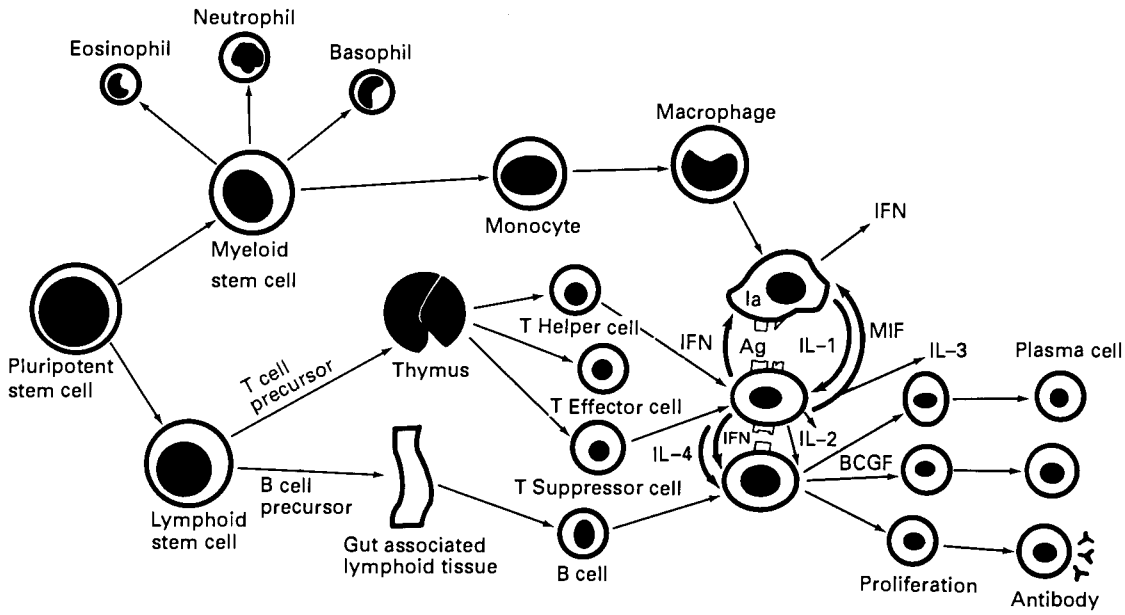

Figure 21.1 Schematic representation of the cellular components of the immune response

B lymphocyte [32,42,58,80,82]. Protein factors (often part of the interleukin series) are thought to be released from all three cell types and may provide signals for lymphocyte and macrophage differentiation. The effector cell, the plasma cell, is a terminally differentiated B cell that secretes an antibody monospecific for the antigen which initially stimulated the humoral immune response [31,35,95]. These antibodies can cause clearance of antigen by three mechanisms. (1) Opsinization, i.e. coating of the antigen with antibody, results in increased phagocytosis of the antigen by macrophages and polymorphonuclear cells [7,97,126], owing to interaction between the Fc region and Fc receptors on phagocytic cells. (2) Antibodies can cause agglutination of the antigen, thereby increasing the chance of antigen phagocytosis. (3) Antibody coating of an antigen can initiate fixation of complement which results in cell lysis displaying the antigen [47]. Complement fixation also leads to the formation of molecules which are chemotactic for macrophages and polymorphonuclear cells.

The cell-mediated immune response system eliminates virally infected host cells and mediates the rejection of transformed (neoplastic) cells and tissue allografts [70]. Cell-mediated responses result from the interaction between macrophages, helper T cells and killer T lymphocytes. This interaction ultimately produces clones of antigen-specific cytotoxic T lymphocytes [13,14]. The response elicited leads to destruction of antigen through direct contact between the cytotoxic T lymphocyte and the antigen bearing cells, and subsequent delivery of a unilateral lethal hit to the cell carrying the antigenic determinant [77].

Non-specific immunity

Mononuclear phagocytes can be effector cells in the mediation of non-specific immunity. They contribute to and regulate the generation of an immune response through several processes including presentation of antigen on the surface in conjunction with the protein of the Class II major histocompatibility locus (Ia), the release of interleukins 1 and 6 and the release of prostaglandins [12, 18, 54, 83, 92, 125, 134]. Macrophages are important in protecting the host against tumours and intracellular parasites [52]. To kill these antigens, the mononuclear phagocytes must go through a series of well-defined stages in response to separate signals to the final stage called 'activation' [2]. The function of the macrophage is multifaceted; it can remove antigen or tissue debris through phagocytosis, enhanced by opsonization. In addition, the products of the respiratory burst system, i.e. toxic oxygen radicals and tumour necrosis factor can contribute to the cytolytic activity of macrophages [21]. Macrophages also regulate the function of other systems such as inflammatory and coagulation systems [1].

Consequences of immune modulation

Routine toxicological evaluations which examine haematological parameters and the weights of spleen and thymus will allow a determination of gross effects on the immune system. However, functional assessment of the immune system is not generally investigated in routine toxicological studies. Immune suppression can increase the incidence of infection; additionally pathological lesions are associated with inappropriate function of the immune system.

Immunostimulation

A number of diseases involve hyperactivity of the immune system, e.g. diabetes and penicillin-induced haemolytic anaemia. Some forms of diabetes may be caused by viral infection of islet cells triggering an immune response leading to islet cell destruction [11, 55]. Penicillin-induced haemolytic anaemia results from penicillin binding to RBCs [28], causing a change in cell shape, a subsequent immune response, and RBC lysis. Autoimmune disease results from a breakdown of self tolerance during embryogenesis and the subsequent production of an immune response to self antigens [10,86], e.g. myasthenia gravis antibodies against ACh receptors, and systemic lupus erythematosus results from B cell hyperactivity against self antigens [44,119]. Allergies, often termed hypersensitivity, are also the result of immunostimulation. Through the interaction of IgE antibodies bound to mast cells with its antigen, mediators such as histamine, serotonin and slow reacting substance are released and cause the symptoms of an allergic reaction [56].

Immunosuppression

Immune deficiency may result from genetic abnormalities (i.e. a deficiency in adenosine deaminase leading to severe combined immunodeficiency), congenital malformations, surgical accidents, pregnancy, malnutrition, stress, disease (e.g. HIV-III virus can lead to acquired immune deficiency syndrome) and exposure to immunosuppressive agents [20, 21, 38, 49, 50, 60,81,94]. Immune deficiency can also occur in patients with autoimmune disease; e.g. in systemic lupus erythematosus, suppression of complement levels and leucocyte function have been noted [51]. Impaired host defenses can result in severe and recurrent infections with opportunistic microorganisms. As noted earlier, immune system may prevent or limit tumour growth, and a higher incidence of tumours may follow immune suppression, e.g. allograft recipients receiving immunosuppressive agents have a significant increase in the incidence of neoplasms [90,91].

Suggested assays for assessment of immunotoxicity

Several groups have proposed a series of tests which should be used to assess immune function following exposure to a toxicant. Vos [127] suggests that the initial assessment should be done during a 90-day study, without the use of additional animals. He suggests gross examination of thymus, spleen and lymph node, and determination of weight, viable cell numbers, and pathology of these organs. In addition, serum globulin levels and peripheral blood lymphocyte and monocyte counts can be determined. These parameters will indicate if the chemical has any gross effects on the immune system. Although these are appropriate parameters to examine, the results should be carefully interpreted; e.g. a reduction in splenic and thymic weight can occur without compromise of the immune system. Also, compounds can profoundly affect the immune response without altering any of the screening parameters described earlier. Therefore, functional parameters should be examined before deciding if a chemical is immunotoxic. Functional studies suggested by Vos [128] include: (1) studies of cell mediated immunity: resistance to *Listeria monocytogenes* infection, rejection of allografts, delayed type hypersensitivity, graft versus host reaction, and mitogenic response; (2) studies of humoral immunity: mitogenic response, antibody synthesis in response to sheep RBC (SRBC) or lipopolysaccharide (LPS); and (3) studies of macrophage function: resistance to *Listeria monocytogenes*, carbon clearance and phagocytosis. These assays were chosen because they can be performed on the rat, the animal used for most toxicological studies (rather than the mouse, used for most immunological studies), and without much additional equipment and expense.

Koller and Exon [66] have developed a system of several immune functional studies that can be performed on one rat without interference between the antigens administered [34], and involving an assessment of each major arm of the immune system. The *in vivo* humoral response to the antigen, keyhole limpet haemocyanin, is performed by measuring serum levels of antibody to this antigen by enzyme-linked immunosorbent assay. The cell-mediated immune response is assessed by determining the ability of the rat to generate a delayed type hypersensitivity reaction to bovine serum albumin. Macrophage function is assessed *in vitro* by measuring the phagocytic activity. In addition, the secretion of interleukin 1 and 2 and prostaglandin E is assessed following *in vitro* culture of cells obtained from the rat. Lastly, the cytotoxic activity of natural killer cells found in the spleen is measured. Therefore, seven separate assays can be performed on a single animal in this system.

Many laboratories advocate a tiered approach based on examining either the most complex or sensitive aspects of immune responsivity first. Bick [9] suggests a series of functional studies following *in vivo* immunization with antigen in the first tier. These include: (1) the generation of a humoral immune response, (2) popliteal lymph node proliferation, and (3) delayed type cutaneous hypersensitivity and circulating serum antibody levels. If an effect on the immune response is found, the cause can be determined by investigating the aspect of the immune response found to be modulated. In the second tier, immunological responses of intermediate complexity are proposed. These include: (1)

the determination of basal immunoglobulin levels, (2) mitogenic stimulation of T and B lymphocytes, and (3) enumeration of thioglycollate-elicited peritoneal cells. In the third tier, assays of the function of a single immune cell type are proposed; e.g. these would include (1) determination of the phagocytic activity of peritoneal cells, and (2) the quantitation of cytostatic and cytotoxic activity of peritoneal exudate cells.

In a recent proposal, a multilaboratory effort was suggested to validate a tiering system [74]. In this system, both the cellular and humoral immune responses along with immunopathology (haematology, weight and histology of immune organs) are on the first tier. Assessment of host resistance to infection and growth of syngeneic tumours, together with quantitation of splenic B and T cell numbers, measurement of the secondary immune response, cytotoxic T cell response, and macrophage function are in the second tier.

Immunotoxicity of OP compounds

Because large populations can potentially be contaminated by OP and CB pesticides, studies of the effects of such compounds at non-cholinergic levels on the immune system should be of value in safety evaluations.

Effects on humans based on epidemiology

Some epidemiology studies have indicated that OPs may have an effect on the human immune system. Thus, exposure to OPs has been shown to cause allergic reactions (3–4 month-exposure), a decrease in rosette forming T cells and increase in B cells, a decrease in leucocyte phagocytic activity, and an increased susceptibility to colds and subjective health complaints [6, 51, 61, 62, 75, 118, 132]. One study has shown a decrease in monocyte esterase activity in workers occupationally exposed to an OP compound [68]. Occupational exposure to OP pesticides decreased neutrophil chemotaxis and adhesion, but increased nitroblue tetrazolium-dye reduction by neutrophils [51]. In these studies there may be a suggestion of immune modulation, but the extent and mechanism of the effects are difficult to ascertain.

Parathion

This has been the most extensively studied OP. Wiltrout *et al.* [130] showed that subacute administration of parathion blocked the generation of a humoral immune response. In addition, Dandliker *et al.* [24] demonstrated that parathion was able to suppress both humoral and cellular immunity. Alternatively, one study showed a decrease in lymphoid organ weight with no change in the humoral immune response following repeated exposure to parathion. Others showed that parathion suppressed the humoral immune response following acute, subacute and *in vitro* exposure [4,17,30]. Peroral dosing of parathion to mice with a cytomegalovirus infection elevated mortality [96]. The proliferative response of human lymphocytes to mitogens was suppressed following *in vitro* exposure to paraoxon [129]. Paraoxon and two structurally related coumpounds inhibited the production of interleukin 2 by rat splenocytes following *in vitro* exposure [93]. Also, *in vitro* exposure of splenocytes to parathion and parathion-methyl blocked the generation of a cell-mediated immune response [112]. Parathion-methyl increased the virulence of *Salmonella typhimurium* infection in rabbits [36,37]. Alternatively, in another study, parathion-methyl administered orally over 4-weeks did not affect the generation of humoral and cellular immune responses in rabbits [25]. *In vitro* exposure of human peripheral blood leucocytes to parathion-methyl did not affect the proliferative response to mitogen, but decreased the chemotactic response [69]. These studies are difficult to correlate owing to differences in route of exposure, immune parameters measured and the species studied. In general, however, parathion has been shown to be immunosuppressive in several systems, but the mechanism is unknown.

Malathion

There is relatively little information on the effects of malathion on the immune system. Repeated exposure to malathion results in allergic responses in man, guinea pigs and mice [23,79]. Administration of low doses of malathion for prolonged periods results in a decrease in the humoral immune responses.

For example, low doses of malathion given 5–6 weeks to rabbits significantly lowered the serum antibody titres generated in response to *Salmonella typhi* vaccination [25]. In addition, a single cholinergic dose of malathion suppressed the generation of a humoral immune response, whereas multiple low doses did not [17]. Alternatively, non-cholinergic doses of malathion elevated the generation of a humoral immune response and proliferative responses to mitogens. Acute or subacute administration of malathion did not affect the generation of a CTL response to allogeneic tumour [113]. *In vivo* administration of non-cholinergic doses of malathion stimulated macrophage function as measured by respiratory burst activity and phagocytosis [101]. *In vitro* exposure of human peripheral blood mononuclear cells suppressed the proliferative response to several mitogens [69]. However, malathion, which had undergone *in vitro* metabolism, elevated the respiratory burst activity of human mononuclear cells. *In vitro* exposure of murine splenocytes to malathion also blocked the generation of CTL responses to allogeneic tumour and proliferative responses to mitogens [102,104,112]. These studies show that malathion can both suppress or enhance immune function depending on route, magnitude and frequency of administration.

Effects of impurities in OP pesticides

The effects on the immune system of impurities in OP pesticides (malathion, acephate and fenitrothion), O,O,S-trimethyl phosphorothioate (OOS-TMP), O,S,S-trimethyl phosphorodithioate (OSS-TMP), O,O-dimethyl, S-ethyl phosphorothioate (OO-Me -S-Et) and O,O,O-trimethyl phosphorothioate (OOO-TMP), have been studied. Extensive studies have been conducted with OOS-TMP. Following acute non-toxic doses of OOS-TMP, it blocked the generation of both cell-mediated and humoral immunity following *in vivo* or *in vitro* exposure to antigen [27,104,110]. However, OOS-TMP did not significantly affect the proliferative response of splenocytes to mitogens, but did elevate interleukin 2 production [110]. Recent studies showed that OOO-TMP elevated humoral and cell-mediated immune responses and protected against OOS-TMP-induced immune suppression when coadministered with OOS-TMP [101]. In addition, exposure to low levels of OOS-TMP protected against an immunosuppressive dose of OOS-TMP.

Subchronic (14 day) exposure to OOS-TMP increased the humoral and cell-mediated immune responses, mitogenic responses and interleukin 2 production [107]. The suppression of immune function following acute administration of OOS-TMP was dose and time-dependent [110], and macrophages were shown to be most affected by OOS-TMP [108]. Macrophages from OOS-TMP-treated mice were shown to (1) be larger in size [108], (2) have increased non-specific esterase activity [108], (3) be less effective at antigen presentation [109], (4) have increased phagocytic activity [109], (5) secrete increased levels of interleukin 1 [109], (6) have decreased Ia and F4/80 expression [99,109], (7) release suppressive factors [111], (8) have increased respiratory burst activity [100] and (9) secrete increased levels of neutral proteases, plasminogen activator, elastase and collagenase [103]. These effects were transient becoming similar to controls within 7 days (at which time immune function was similar to control) [100,103]. OOS-TMP also caused thymic atrophy [27]. Recent investigations showed a reduction in the number of cells expressing T cell markers in the thymus [98]. Studies are ongoing to determine the identity of the mouse thymus cells following acute administration of OOS-TMP. In summary, acute *in vivo* administration of OOS-TMP was immunosuppressive, but stimulated macrophage function; repeated exposures stimulated cellular and humoral immune responses.

In vivo exposure to OSS-TMP enhanced or suppressed the generation of cell-mediated or humoral immune responses at non-toxic or toxic (assessed by suppression of plasma BChE) doses, respectively, following *in vivo* or *in vitro* stimulation with antigen [114,115]. *In vivo* exposure to OSS-TMP also elevated proliferative responses to mitogens, but suppressed interleukin 2 production [115]. Fourteen-day exposure to OSS-TMP elevated or suppressed, depending on the dose, the generation of immune responses [116]. Further studies showed that OSS-TMP altered both T and B lymphocyte function [121]. *In vitro*

exposure to OSS-TMP enhanced or suppressed immune function, depending on OSS-TMP concentration and the *in vitro* metabolism system used [115,121]. OSS-TMP also inhibited the cytolytic function of cloned murine and human cytotoxic T lymphocytes, but only if present during the time when the cell to be lysed was recognized (as measured by conjugation) by the cytotoxic T cell [105]. OSS-TMP was immunostimulatory at non-cholinergic doses and immunosuppressive at cholinergic doses. *In vitro* exposure to OSS-TMP suppressed humoral and cell-mediated immune function.

Finally, one study showed that OO-Me-S-Et, a synthetic analogue of the impurities described earlier, blocked cell-mediated and humoral immune responses through impairment of lymphocyte function [122]. These studies show that the impurities found in technical malathion can modulate (suppress or enhance) immune function. The cell type affected, the length of the effect, and the immune parameter modulated varied from compound to compound.

Other OP pesticides

The effects of many OPs have been determined on one facet of immune function. Carbophenothion and crufomate suppressed the proliferation of human lymphocytes in response to mitogen [87]. Acute administration of dichlorvos slightly decreased splenic weight of mice, but did not affect the generation of a humoral immune response [17]. In addition, chronic, low level exposure to dichlorvos suppressed the generation of serum antibodies following vaccination of rabbits with *Salmonella typhi* and the generation of cell-mediated immunity following a tuberculin vaccination [25,26]. *In vitro* treatment of rabbit PMN by diisopropylethyl phosphate or triisopropyl phosphate reduced locomotion of the leucocytes [131]. Oral, acute administration of DFP to guinea pigs enhanced the serum complement and haemolysin activity and the generation of a humoral immune response, but suppressed lysozyme activity. Alternatively, repeated administration of DFP suppressed complement, haemolysin and lysozyme activities, and suppressed the generation of a humoral immune response [71]. Intraperitoneal injection of dimethoate reduced the thymic and splenic weight of treated mice and blocked the generation of a humoral immune response [124]. Administration of fenchlorphos to chickens for 3–8 weeks increased the weight of the bursa of Fabricus [117]. In addition, *in vitro* exposure of murine splenocytes to fenthion blocked their ability to generate a cell-mediated immune response [112]. Alternatively, topical administration of fenthion to newborn mice with encephalomyocarditis virus infection did not alter mortality [22]. On the other hand, leptophos administered orally for 12 weeks did not affect the generation of the humoral immune response in the mouse [65]. Demeton-O-methyl decreased the generation of a humoral immune response in the rat when a single high dose was administered [84]. Monocrotophos, given ip once a week for 6 weeks modulated several haematological parameters, including an increase in clotting time, white blood cell count, splenic cellularity and the percentage of large lymphocytes, neutrophils and basophils. Haematological parameters reduced were the percentage of small lymphocytes, the number of RBCs, the platelet count, and bone marrow activity [45].

Crufomate and carbophenothion *in vitro*, had no effect on the proliferation of human lymphocytes, but crufomate suppressed the chemotactic response of peripheral leukocytes [69]. *In vitro* exposure of human basophils to soman led to an IgE-independent release of histamine [78]. Esa *et al.* [33] showed that *in vitro* exposure of human mononuclear cells to triphenyl phosphine oxide and tetra-o-cresyl piperazinyl diphosphoramidate caused suppression of antigen specific proliferation. In addition, treatment of human monocytes with triphenyl phosphine oxide, tetra-o-cresyl piperazinyl diphosphoamidate, triphenyl phosphate and triphenyl thiophosphate significantly inhibited their ability to present antigen to immune T cells. Exposure of mice to tris (2,3-dichloropropyl) phosphate decreased the proliferative response of splenocytes to mitogens and increased the incidence of tumour formation from injection of tumour cells, but did not alter splenic or thymic weight, haematological parameters, delayed type hypersensitivity response to antigen, serum immunoglobulin levels, humoral responses to T cell dependent and independent antigens

and mortality following *Listeria monocytogenes* infection [72]. Triphenyl phosphate caused an allergic reaction and suppressed the immune system by subchronic administration [15,53].

Most of these studies indicate that various OPs are capable of suppressing some aspects of immune function.

Effects of CBs on immune function

Carbaryl increased the serum level of IgG1 and IgG2b without affecting the other immunoglobulins following oral exposure for 1 month [3]. The humoral immune response of rabbits to antigen was unchanged following oral carbaryl or carbofuran for 4 weeks [120]; while carbaryl did not affect cellular immunity, carbofuran significantly suppressed cellular immunity. Carbaryl suppressed a humoral immune response at very high doses [130]. In addition, *in vitro* exposure of splenocytes to carbaryl blocked their ability to generate a humoral immune response [112]. Carbaryl and carbofuran suppressed the expression of complement activity in human serum when added to the assay [16]. Carbaryl and some of its metabolites inhibited the proliferation of the interleukin 2-dependent T cell clone, CTLL-2 [5]. However, carbofuran did not affect the generation of a delayed type hypersensitivity response *in vivo* or a cell mediated immune response *in vitro* [37,112]. Carbofuran, given ip over 6 weeks modulated several haematological parameters. These were: increased clotting time, peripheral white blood cell count, splenic cellularity, and the percentage of large lymphocytes, neutrophils and basophils; and reduced percentage of small lymphocytes, the RBCs and platelet count, and bone marrow activity [45].

Carbofuran and aminocarb decreased humoral immune responses to neutral and pathogenic antigens and increased the cytolysis of macrophages by virus [40]. Aminocarb did not decrease the resistance of mice to *Salmonella typhimurium* and mouse hepatitis virus 3 [8]. Aminocarb suppressed the generation of a serum antibody titre to mouse hepatitis virus 3, but when given orally enhanced the humoral immune response to a non-pathogenic antigen [39]. Aminocarb did not affect the generation of a cell-mediated immune response or the ability of macrophages to process antigen [40]. Olson *et al.* [85] showed that low levels of aldicarb decreased the humoral immune response, but Thomas *et al.* [123] showed that low levels of aldicarb did not affect the generation of cellular and humoral immune response or the resistance of the host to infection or tumour challenge. Ethyl carbamate inhibited humoral immune responses to T cell dependent and independent antigens [46,73,76,88,89]. However, initial studies suggested that ethyl CB did not affect or only slightly affected cell-mediated immunity [29, 67,73,88]. In addition, ethyl CB did not affect the resistance of mice to encephalomyocarditis virus infection, but increased the incidence of virally-induced leukaemia [19]. Exposure for 14 days to ethyl CB reduced splenic and thymic weight and increased splenic myelopoiesis. Macrophage phagocytic and bacterial functions were unaffected, but the release of cytostatic factors from macrophages was elevated. Alternatively, bone marrow myelopoeitic and splenic natural killer activity were suppressed [45,73]. On the other hand, 14-day exposure to methyl CB did not affect splenic or thymic weight, cell-mediated or humoral immune responses, mitogenic responses, macrophage function, bone marrow function or natural killer activity [73]. Carbaryl given to quail for 5 days lowered their resistance to the protozoan parasite, *Histomonas meleagrides* [133]. Pirimicarb induced immune-mediated haemolytic anaemia in dogs [57].

These studies indicate that carbamates can suppress immune function, and modulate humoral immune responses, macrophage function and haematological parameters. Also, carbamates lowered the resistance of the exposed animal to parasitic, bacterial and viral infection.

Summary

Studies show that antiChEs at non-toxic, non-cholinergic doses can modulate the generation of immune responses. Suppression of immune function can increase host susceptibility to viral, bacterial and parasitic infections and increase the incidence of tumours, and possibly lengthen the course of an infection. Conversely, enhancement of the immune

system can lead to exacerbation of autoimmune disease or non-specific tissue damage (e.g. elevated pulmonary leucocyte function may lead to pulmonary fibrosis), and general malaise or lethargy. Perturbation of normal homoeostasis of the immune system may lead to deleterious side-effects that may not be observed until the facet of the immune system that was affected is required to defend the host from a foreign antigen.

Immunotoxicological studies of foreign substances may provide insight into the basic biochemical mechanisms of immune function. For example, the requirement for an event at an early postrecognition step that can be inhibited by an OP (probably an esterase) in the cytolysis of a tumour target by a cytotoxic T lymphocyte [105]. The use of various chemicals may allow the dissection of the complex and specific interactions between cells of the immune system, and help in understanding the immune response by elucidation of the sites of action of these chemicals. In addition, such studies may allow the development of immunomodulatory drugs for the treatment of immune dysfunction diseases.

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22

Genotoxic and carcinogenic potential of anticholinesterases

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Introduction

OP pesticides are used in USA agriculture in quantities exceeding 100 million pounds per year. The impact of such pesticides and related chemicals on the health of agricultural workers and consumers is still largely unknown. Continued attention must be focused on pesticides because of the large amounts of these chemicals in use, the extent of field worker exposure and the entry of pesticides into the food chain.

This chapter reviews 24 OPs for genotoxic or carcinogenic potential. These compounds are either antiChEs or closely related agents, and 20 or more have been used as insecticides. Limited data exist regarding the potential human carcinogenic or mutagenic effects of OPs. Eight of these agents have been evaluated for carcinogenic risk to humans by the International Agency for Research on Cancer (IARC) and nine have been evaluated for evidence of carcinogenicity in animals by the US EPA Gene-Tox programme. Genotoxic and carcinogenic effects are of special concern because of the generally irreversible nature of the processes and the long latency period associated with their manifestation. In contrast to the limited data on carcinogenicity 19 of 24 agents have been evaluated for genotoxicity in ten or more distinct short-term tests.

Methods

Genotoxicity in this chapter is presented in terms of genetic activity profiles, developed by Garrett *et al.* [4] to represent in a standardized format the qualitative and quantitative data from a large number of short-term tests. The purposes of this methodology are twofold: (1) to facilitate visual interpretation of genetic toxicity data from a wide variety of test systems, and (2) to enable qualitative and quantitative comparisons to be made for several chemicals tested using the same or similar bioassays.

Bioassays used in the evaluation of a given chemical are represented by profile lines displayed along the x-axis. Each profile line is labelled with a three-letter code word representing the bioassay system. Conventions for the naming of test systems and additional details are described by Waters *et al.* [11]. The sequence of presentation of test systems is entirely flexible, but has been ordered by convention in either a phylogenetic sequence (i.e. prokaryotes, lower eukaryotes, plants, insects, mammals *in vitro*, and mammals *in vivo*) or by genetic or related endpoint (i.e. DNA damage, gene mutation, sister chromatid exchange, micronuclei, chromosomal aberration, aneuploidy and cell transformation). The endpoint sequence is used in this chapter to emphasize the types of genetic or related effects represented for a selected chemical. The definition and sequence of test system code words by genetic endpoint is shown in Appendix 1. Within each category of endpoint the sequence is arranged in phylogenetic order.

The length of a profile line is a function of the dose(s) tested in a given bioassay. The longer the profile lines above the horizontal baseline, the lower the dose required for a positive response, and the longer the line below the baseline, the higher the dose applied yielding a negative response. For positive

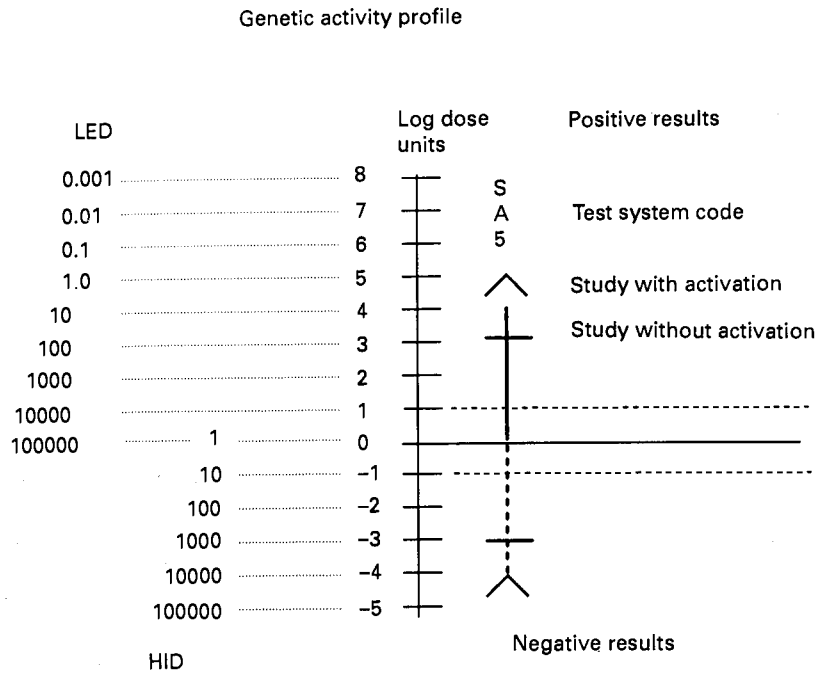


Figure 22.1 Schematic representation of the Genetic Activity Profile. Dose information in the form of either the lowest effective dose (LED) or the highest ineffective dose (HID) is converted to the logarithmic dose unit (LDU) according to the scale shown beside the y-axis. Dosage is μg/ml or mg/kg body-weight per day. Individual studies are then plotted using the symbols (-) for those not using an exogenous activation system or (^) for those that do employ an exogenous activation. The majority response (positive or negative) is indicated by the solid vertical bar drawn from the origin to the average log dose value. Conflicting data are plotted and are indicated by the dashed line drawn from the origin through all conflicting data points. If an equal number of positives and negatives exist as depicted in this model, the majority is represented as positive.

results, the lowest effective dose (LED) is recorded, for negative results, the highest ineffective dose (HID) is recorded. Figure 22.1 illustrates the dose range and log dose unit (LDU) equivalent values plotted on the y-axis. A genetic activity profile thus represents a partial spectrum of the genetic and related effects induced by a given chemical.

To determine patterns or similarities in genetic activity profiles, the data for each possible pair of chemicals can be examined by computer over the entire series of tests to find common test results. The statistically significant matches for pairs of chemicals are determined by calculating, from the binomial distribution, the probability (P) that the number of concordant tests results would occur by chance. The P value is the primary function for evaluating the significance of a qualitative match between a pair of chemicals.

The agreement in the relative magnitudes of common profile lines is determined by calculating a dose-related function (DRF). The DRF has been defined by Equation 22.1.

$$DRF = \frac{1}{n} \sum_{i} C - |LDU_{i_a} - LDU_{i_b}| \qquad (22.1)$$

where n is the total number of common tests, C is a constant (assigned a value of 3.0), LDU_{ij} represents the response or logarithmic dose unit as previously defined [4] for each short-term test i and for chemicals j_a and j_b. A DRF can be computed for common concordant test results only and is termed the DRF_c. Thus, the two dose-related functions, DRF and DRF_c, provide important additional information on the quantitative agreement between common and common concordant tests, respectively, for a pair of genetic activity profiles.

The *P* value and the DRF_c value may be used as screening tools to obtain matched profiles at preselected levels of significance and quantitative agreement. By specifying a DRF_c and *P* value, patterns of similar genetic activity can be identified from the total number of possible profile matches. Conventionally, *P* values of <0.05 to 0.01 have been used to select significant matches. The DRF_c routinely has been set at a minimum level of 1.5–2.0 for an initial screen of a data base. A DRF of 3.0 indicates perfect quantitative agreement; a

Table 22.1 Data listing for malathion 121-75-5

Test code[a]	*End point*[b]	*Results No act.*[c]	*Act.*	*Dose (LED or HID)*	*EPA reference number*[d]	*EMIC number*[d]	*Short citation*[e]
1 ECB	D	(+)	0	100.0000	2188	30811	Griffin and Hill 161, 1978
2 ECD	D	–	0	1000.0000	589	31446	Simmon, Mitchell and Jorgenson 1, 1977
3 BSD	D	?	?	300.0000	2881	37136	Shiau, Huff, Wells *et al.* 169, 1980
4 BSD	D	–	0	1000.0000	589	31446	Simmon, Mitchell and Jorgenson 1, 1977
5 SA0	G	–	–	5.0000	2317	48199	Wildeman and Nazar 437, 1982
6 SA0	G	0	+	0.0000	1477	44405	Ishidate, Sofuni and Yoshikawa 95, 1981
7 SA0	G	–	–	500.0000	589	31446	Simmon, Mitchell and Jorgenson 1, 1977
8 SA5	G	+	–	12.5000	2881	37136	Shiau, Huff, Wells *et al.* 169, 1980
9 SA5	G	+	0	10.0000	4156	45812	Shigaeva and Savitskaya 64, 1981
10 SA5	G	–	–	500.0000	589	31446	Simmon, Mitchell and Jorgenson 1, 1977
11 SA7	G	–	–	500.0000	589	31446	Simmon, Mitchell and Jorgenson 1, 1977
12 SA8	G	+	0	10.0000	4156	45812	Shigaeva and Savitskaya 64, 1981
13 SA8	G	–	–	500.0000	589	31446	Simmon, Mitchell and Jorgenson 1, 1977
14 SA9	G	–	–	5.0000	2317	48199	Wildeman and Nazar 437, 1982
15 ECK	G	–	0	66000.0000	4148	16302	Mohn 7, 1973
16 ECW	G	–	–	500.0000	589	31446	Simmon, Mitchell and Jorgenson 1, 1977
17 EC2	G	–	0	1000.0000	2330	14190	Ashwood-Smith, Trevino and Ring 418, 1972
18 BSM	G	+	–	12.5000	2881	37136	Shiau, Huff, Wells *et al.* 169, 1980
19 SCH	R	–	–	50000.0000	589	31446	Simmon, Mitchell and Jorgenson 1, 1977
20 SZF	G	–	–	66000.0000	4091	50095	Gilot-Delhalle, Colizzi and Moutschen 139, 1983
21 ACC	C	+	0	80.0000	4229	35081	Mishra and Sinha 716, 1979
22 HSC	C	+	0	250.0000	4058	27097	Singh, Singh, Singh *et al.* 688, 1977
23 HSC	C	+	0	1230.0000	4064	36028	Singh, Singh, Singh *et al.* 127, 1979
24 TSC	C	+	0	20.000	4235	51607	Ma, Anderson and Harris 127, 1983
25 DMX	G	–	0	0.5000	692	31499	Valencia 70 pp, 1977
26 SIC	S	+	0	99.0000	3350	45122	Nishio and Uyeki 939, 1981
27 SIC	S	0	+	40.0000	1358	48211	Chen, Sirianni, Huang *et al.* 621, 1982
28 SIC	S	+	0	40.0000	4087	37908	Chen, Hsueh, Sirianni *et al.* 307, 1981
29 SIC	S	(+)	+	40.3000	10100	69082	Galloway, Armstrong, Reuben *et al.* 1, 1987
30 SIA	S	+	0	130.0000	1959	43143	Sasaki, Sugimura, Yoshida *et al.* 574, 1980
31 MIA	M	–	0	160.0000	1959	43143	Sasaki, Sugimura, Yoshida *et al.* 574, 1980
32 CIC	C	+	0	76.0000	1477	44405	Ishidate, Sofuni and Yoshikawa 95, 1981
33 CIC	C	–	+	303.0000	10100	69082	Galloway, Armstrong, Reuben *et al.* 1, 1987
34 SHF	S	(+)	0	64.0000	1959	43143	Sasaki, Sugimura and Yoshida 574, 1980
35 SHF	S	+	0	40.0000	4228	31666	Nicholas, Vienne and Van den Berghe 167, 1979
36 SIH	S	+	–	0.2000	4092	45945	Sobti, Krishan and Pfaffenberger 89, 1982
37 CHL	C	+	0	10.0000	4231	40211	Walter, Czajkowska and Lipecka 375, 1980
38 CIH	C	–	0	100.0000	4224	14546	Huang 36, 1973
39 MVM	M	+	0	120.0000	4234	48363	Dulout, Olivero, Von Guradze *et al.* 413, 1982
40 CBA	C	+	0	460.0000	4236	52074	Dulout, Pastori and Olivero 163, 1983
41 CBA	C	–	0	100.0000	4099	23007	Kurinnyi 1534, 1975
42 CVA	C	(+)	0	18.4000	4101	25229	Bulsiewicz, Rozewicka, Januscewska *et al.* 361, 1976
43 DLM	C	–	0	5000.0000	589	31446	Simmon, Mitchell and Jorgenson 1, 1977
44 CLH	C	(+)	0	0.0000	4100	17984	Van Bao, Szabo, Ruzicska *et al.* 33, 1974

[a]See Appendix 1 for test code definition
[b]Endpoints are divided into DNA damage (D), gene mutation (G), etc
[c]Results are given with and without metabolic activition (ACT)
[d]EPA reference numbers and Environmental Mutagen Information Centre (EMIC) numbers are given for each publication
[e]Complete citation from authors or by EMIC number

Table 22.2 Qualitative test results for OP chemicals

DNA damage				RECB	BF	Gene mutation									SCE			MN	Chromosomal aberrations										A	T		
ESEEEBB	DUDUU		YSSA	BHH	SSSSSSS	EEEEB		SSSAN	S	H	D	GGGG	VSSSSSSS			TMMMM	AHTVP	D	CCCCCCCCCCCCC	D	M	C	NDA	TTT		
CACCRSR	IRIHH		ECCN	FMM	AAAAAAA	CCCCS		CZZNC	A	S	M	C995	FIIIHHIV			SIVVV	CSSFL	M	IIIHHIBLCGGOV	L	H	L	CMI	BCC		
BDDLDDD	APHFT		3GHG	AAM	F05789S	KW2RM		RFRFF	L	M	X	LHOT	SCTAFLHA			IAMRA	CCCCC	H	CTAFLHAACCGEA	M	T	H	NNA	MMS		
					+++	++	+			+		+	+		+++	+	+	++	+	++		+++++		++++	++	+	+	+	-	+	$	ThioTEPA
									+	+	+		+	+			+	+									+					Triethylenephosphoramide
	++		-++		++	+++--++								+	+	++				+	++		-+						+	+	$	Tris(2,3-dibromopropyl)PO4
PO	0		+	PP+	+	+	+---+	+			+		-		P	P	+		N	0												Trichlorfon
	-	-	+	+		+	++-+-	+	+	-			+			+		+	+		++		+		++-+	-	-		-		$	Dimethoate
+	-			-			-+-+-	---	+	-			-			+	++	+	-+		+++			+	+-+		+	-	+			$	Malathion
+	-	--			+-		++-+-	+-		-	-			+			+			+		+			+			-	+			$	Parathion-methyl
+	+			+		-	+	++			+		-			+	--		-		+			-								Dichlorvos
N-	+		+	P	+		++---	+		P			-		P	P	+		N														Demeton
PO	0		+	P	+		----	-		P		0	-		P	P		+	N	+					N								Monocrotophos
-			-	+	-		- - -	-		-					+	-			-												*	Crotoxyphos
++	+		-	-			- - -	-																							*	Chlorpyrifos
0	0		+	N	-		-----	-				-	-		P	P	0		N			+											Disulfoton
0	0		-	P	+		-----	-				-	-		P	P			N	0								-					Acephate
-			-	+	-		- - -	-							+	-									-						*	Azinphos-methyl
0	0		-	N	-		----	-				0	0				+	+				+											Parathion
0	0		-	N	-		----	-				0	-					+		+		+			N								Phorate
0	0		-	N	-		----	-				-	0				+	-															Fenthion
0	0		-		-		-----	-				-	-				0																Fensulfothion
-	-				-		-----	---							-	-	+					-					-				$	Diazinon
0	0		-	N	-		-----	-				-					-																Ethion
			-	-			- - -	-																							*	Fonofos
			-	-			- - -	-					-																		*	Aspon

Definitions: RECB – recombination; BF – body fluids or host-mediated assay; SCE – sister chromatid exchange; MN – micronucleus test; A – aneuploidy; T – cell transformation; S – chemicals with test results abstracted by the US. EPA; * SRI data; data for other chemicals were obtained from the Gene-Tox Program; + positive test result; – negative test result; O _ equivocal; N (negative) and P (positive) represent supplementation of Gene-Tox chemicals with data from SRI. Definitions of test codes (e.g. ECB, SAD, etc.) are given in Appendix 1 except for YE3, S. cerevisiae D3 enhanced mitotic recombination; cyclophosphamide was omitted from this table (see Figure 22.2).

DRF value of 2.0 corresponds to an average dose difference of one logarithmic unit.

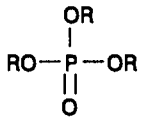
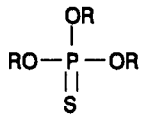
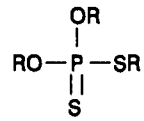
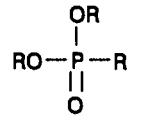
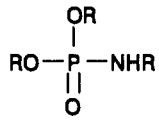
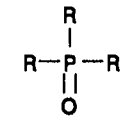
To construct genetic activity profiles for individual chemicals, the published literature was reviewed by the US EPA in Research Triangle Park, North Carolina. Listings of data and references were assembled for each chemical and abstracted, then keyed into the computer and verified. These genetic activity profile data are termed 'EPA data'. Typical data for malathion are shown in Table 22.1.

Descriptions of the procurement of pesticides, purity and qualitative aspects of the pesticide data for some of the agents here have been presented by Waters *et al.* [10]. Genetic bioassays for some of the chemicals were performed under contract with the EPA by SRI International, Menlo Park, CA. and the corresponding *Drosophila* assays were performed by WARF Institute, Inc., Madison, WI. Genetic activity profiles are available for SRI and WARF tested chemicals; however, only data derived by SRI and WARF are included in the profiles for these chemicals. These data are termed 'SRI data'.

Data that were abstracted and reviewed by the US EPA Gene-Tox programme are termed 'Gene-Tox data'. The Gene-Tox data are not currently available in a genetic activity profile format. However, Gene-Tox data were compared with EPA data by conversion of the respective original data files into a two-dimensional test data matrix. Thus, the genetic activity profile becomes a one-dimensional qualitative activity profile with +, – or 0 results (Table 22.2; Figure 22.1).

It should be noted that differences exist in test codes and in the code definitions between EPA [11] and Gene-Tox [9]. Gene-Tox originally coded all *Salmonella* tester stains using a single code, SAL. From Gene-Tox updates on *Salmonella* data individual tester strain data have been included in this report. Gene-Tox used a single code for each endpoint of *in vitro* mammalian systems for chromosome aberrations or sister chromatid exchange. EPA data uses specific codes for each endpoint for an animal species (mouse, rat and hamster).

Table 22.3 General OP classes

Chemical type	*Structure*
Phosphate	
Phosphorothioate	
Phosphorodithioate	
Phosphonate	
Phosphoramidate	
Phosphine	

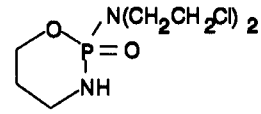
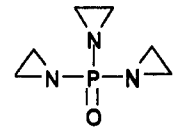
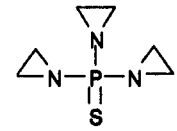
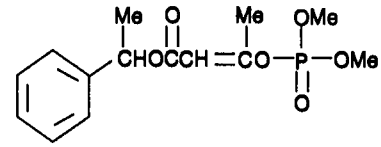
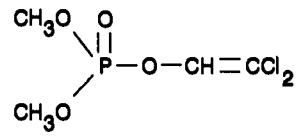
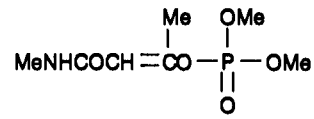
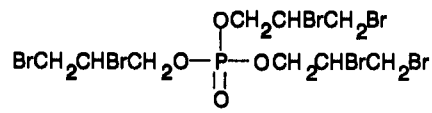
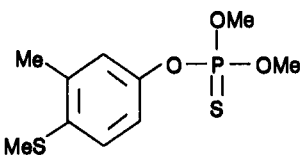
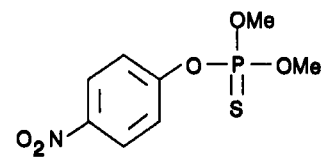
Results

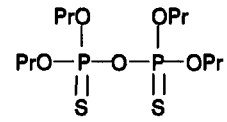
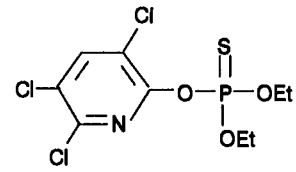
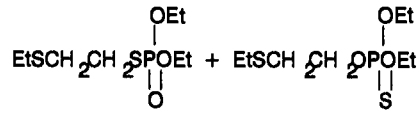
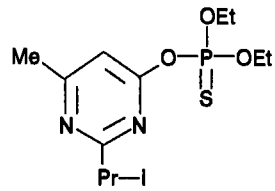
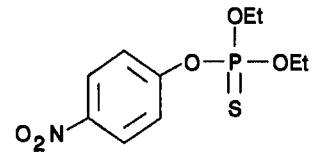
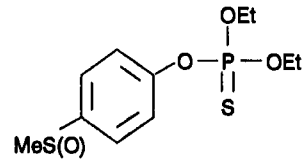
The 24 OPs described in this chapter can be subdivided into three categories based on the qualitative results. The first category of chemicals displays largely positive results; the next category gave a mixture of positive and negative results in a number of tests. The third category contains pesticides which were largely negative in tests in which they have been evaluated.

An additional way of categorizing these 24 chemicals is according to chemical structure and reaction mechanisms. Twenty-three of 24 agents can be classified as either phosphates, phosphorothioates, phosphorodithioates, phosphonates, phosphoramidates or phosphines. The basic structures of the chemicals according to this classification are shown in Table 22.3. The first five of these OP classes are antiChE.

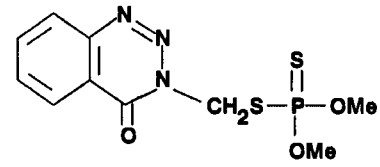
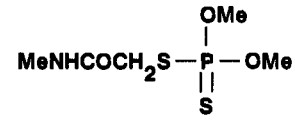
Structures and CAS numbers for each individual chemical discussed in this chapter are shown in Table 22.4. The majority of the

Table 22.4 Chemical structure and CAS number for OP compounds

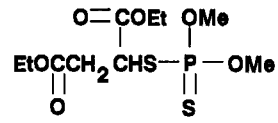
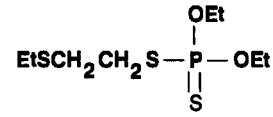
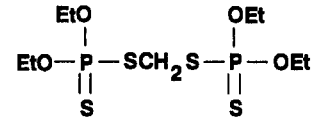
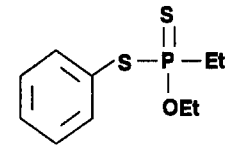
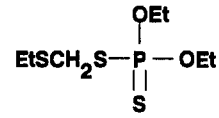
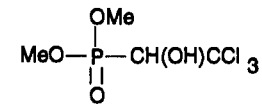
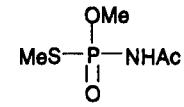
Chemical name	*CAS number*	*Structure*
Cyclophosphamide	50-18-0	
Aziridines		
Triethylenephosphoramide	545-55-1	
Thiotepa	52-24-4	
Phosphates		
Crotoxyphos	7700-17-6	
Dichlorvos	62-73-7	
Monocrotophos	6923-22-4	
Tris(2,3-dibromopropyl)-phosphate	126-72-7	
Phosphorothioates		
Fenthion	55-38-9	
Parathion-methyl	298-00-0	

Aspon	3244-90-4	
Chlorpyrifos	2921-88-2	
Demeton	8065-48-3	
Diazinon	333-41-5	
Parathion	56-38-2	
Fensulfothion	115-90-2	

Phosphorodithioates

Azinphos-methyl	86-50-0	
Dimethoate	60-51-5	

continued

Malathion	121-75-5	
Disulfoton	298-04-4	
Ethion	563-12-2	
Fonofos	944-22-9	
Phorate	298-02-2	
Phosphonate		
Trichlorfon	52-68-6	
Phosphoramidate		
Acephate	30560-19-1	

antiChEs are either phosphates, phosphorothioates, or phosphorodithioates. The thio-containing compounds must be metabolized to their respective oxygen analogues for neurotoxic action [7]. Several additional subdivisions of the OPs can then be formed based on the presence of either a methyl ester or ethyl or higher alkyl ester (Table 22.5).

OPs with mainly positive results

Category I contains five chemicals: cyclophosphamide, triethylenephosphoramide (TEPA), triethylenethiophosphoramide (thio-TEPA), tris (2, 3-dibromopropyl) phosphate, and trichlorfon (Table 22.2; *see* Figure 22.2 for cyclophosphamide). Dimethoate might also be

Table 22.5 Subdivisions of OP chemicals

Methyl ester	*Ethyl or higher esters*
Phosphorothioates	
Fenthion	Aspon[b]
Parathion-methyl[a]	Chloropyrifos[b]
	Demeton
	Diazinon[a]
	Fensulfothion
	Parathion
Phosphorodithioates	
Azinphos-methyl[b]	Disulfoton
Dimethoate[a]	Ethion
Malathion[a]	Fonofos[b]
	Phorate
Phosphates	
Crotoxyphos[b]	Tris[a]
Dichlorvos	
Monocrotophos	
Phosphonate	
Trichlorfon	
Phosphoramidate	
Acephate	

[a]Chemicals with genotoxicity data abstracted by EPA
[b]SRI data
[c]Data for other chemicals obtained from Gene-Tox programme

included in this category, however the compound produced several negative as well as a number of conflicting test results. Thus, it was included in Category II. Cyclophosphamide, TEPA and trichlorfon have been used as insecticides. Cyclophosphamide, TEPA and thio-TEPA have been used as alkylating antineoplastic agents. TEPA has been used in dyeing, grease proofing and flame proofing of textiles and other chemical processes.

The genetic activity profile for cyclophosphamide is shown in Figure 22.2. This chemical is nearly uniformly positive for all genetic endpoints in all phylogenetic categories. The compound contains highly reactive chloroethyl functional groups and is metabolized to an alkylating intermediate [6]. Patients treated with this agent show chromosomal aberrations and sister chromatid exchanges in peripheral lymphocytes and bone marrow cells. Thio-TEPA (Table 22.2) is uniformly positive in all genetic endpoints studied except for one assay of aneuploidy (AIA). The agent displays greater potency in mammalian than in non-mammalian test systems. In addition to

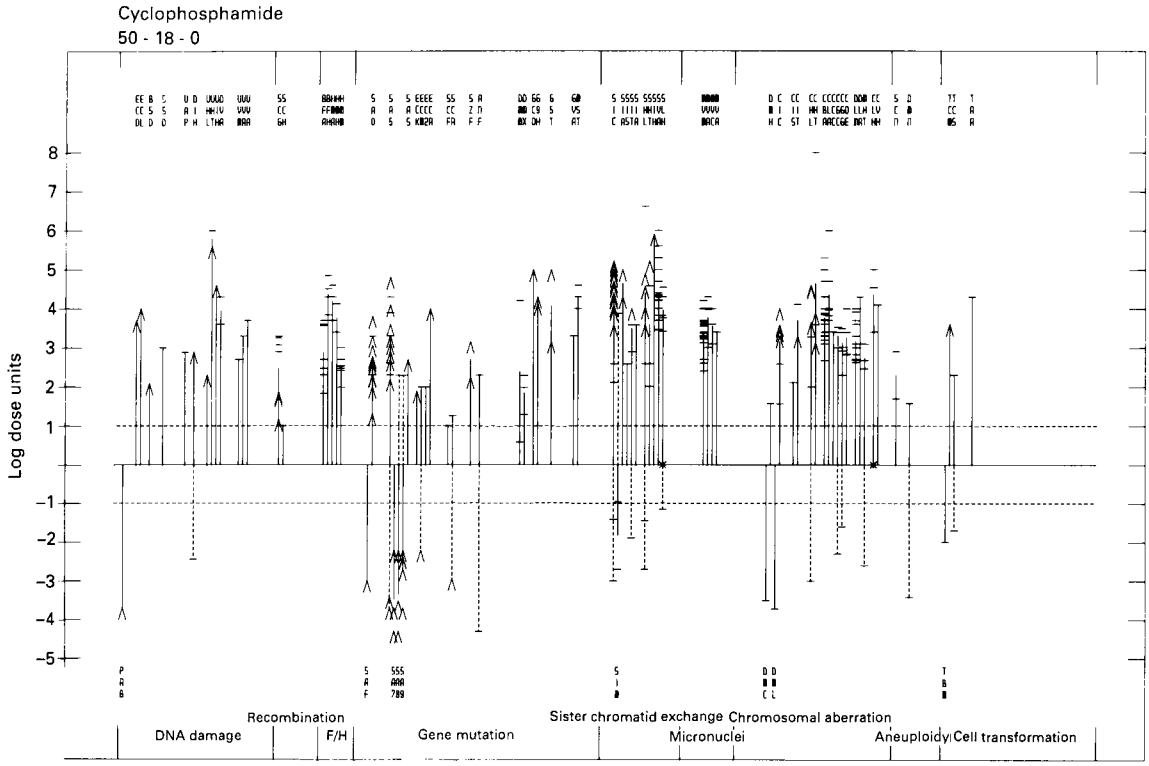


Figure 22.2 Activity profile for cyclophosphamide

causing mutation in a variety of test systems thio-TEPA caused chromosomal aberrations, sister chromatid exchange and micronuclei. An increased frequency of chromosomal aberrations was observed in one study of cancer patients receiving thio-TEPA [6]. Short-term *in vivo* tests are extremely sensitive to thio-TEPA, and the LED for this compound was approximately 0.01 mg/kg (10 ppb) in one study of sister chromatid exchange.

Tris(2,3-dibromopropyl) phosphate produced genetic effects in prokaryotes, mammals and human cells *in vitro*. No data were available on the genetic effects of this compound after direct human exposure. Gene-Tox results for TEPA were uniformly positive (Table 22.2). The genetic activity profile (SRI data) for the phosphonate insectide, trichlorfon, is in substantial agreement with data found in Table 22.2 obtained from the Gene-Tox programme. Trichlorfon causes gene mutation (SA0, ECW) and DNA damage (SAD, UHF) in prokaryotes and gene mutation (G5T, SCR), DNA damage (YE3, SCH) and chromosomal effects (SIC) in eukaryotes.

OPs with mixed positive and negative results

The pesticides comprising category II include: (1) the phosphates containing methyl esters, monocrotophos, crotoxyphos, and dichlorvos; (2) the phosphoramidate, acephate; (3) the phosphorothioate with a methyl ester, parathion-methyl; and (4) the phosphorodithioates with methyl esters, malathion and dimethoate, and azinphos-methyl. Two compounds with ethyl esters, demeton and disulfoton, also belong to this category.

The genetic activity profiles for crotoxyphos and monocrotophos showed that crotoxyphos is weakly positive for recombination (SCG) and positive for gene mutation (G5T) in eukaryotes. Monocrotophos was positive for gene mutation, DNA damage, and chromosomal effects in eukaryotes. A similar compound, dichlorvos, is positive in a variety of cell systems including DNA damage, gene mutation in *Salmonella*, SCE and chromosome aberrations (Table 22.2).

Although acephate is positive in a number of test systems, the dose is 10 g/kg or greater for three of the test systems and thus of questionable significance. The SRI data shows that the chemical produces unscheduled DNA synthesis in human cells (UHF), sister chromatid exchange (SIC), and gene mutation in eukaroytes (G5T) at lower doses. The Gene-Tox data are essentially negative for acephate although not all tests can be directly compared with the SRI data. The compound is the only phosphoramidate with short-term test data reviewed in this chapter. Acephate is considered a weak antiChE.

In general, phosphorothioates and phosphorodithioates containing methyl esters are more active in short-term tests than their corresponding ethyl or higher esters. An example is parathion-methyl, a widely used phosphorothioate containing a methyl ester. The profile showed essentially equal number of positive and negative test results (Table 22.2). Data for four test systems (SA0, SA5, SA8, and CBA) are conflicting. The chemical produces micronuclei in animal cells *in vivo* (MVA) at a dose <1 mg/kg, and produces SCEs in human cells *in vitro* at slightly higher doses. The agent also produces chromosomal aberrations in animal bone marrow cells *in vivo* (CBA) and gene mutation (SA0, SA5, SA8) and DNA damage (ECB) in prokaryotes. The *S. typhimurium* TA1538 test is positive suggesting a frame shift mutation but a conflicting negative result was also reported.

Malathion and dimethoate are phosphorodithioates with methyl esters which are used extensively in agriculture. Malathion is registered for aerial application to cereal crops, soybeans, and a variety of vegetables. Dimethoate is used on ornamental plants, vegetables, and citrus fruits. A variety of prokaryotic and eukaryotic systems are sensitive to these compounds. Both malathion and dimethoate produce sister chromatid exchange in human cells (SIH) and chromosomal aberrations in human lymphocytes *in vitro*. SCE in human cells is a sensitive test for either compound at a dose of approximately 1 mg/kg (1 ppm). It is noteworthy that a tolerance limit of 135 ppm is set for grass and green hay grazed or harvested on the day of application of malathion. Also, the generally applied tolerance limit for malathion residues is about 8 ppm [3].

Two other pesticides containing thio groups and methyl esters are fenthion and azinphos-methyl. Azinphos-methyl is positive for enhanced mitotic recombination in *S.*

cerevisiae (YE3, SCG) and for forward mutation in mouse lymphoma cells (G5T). Fenthion was evaluated only in an initial test battery. The compound which is subject to hydrolysis proved positive only for SCE in human lymphocytes *in vitro* (Table 22.2), and is more appropriately placed in Category III (largely negative results).

Phosphorothioates or phosphorodithioates containing ethyl or higher esters were often inactive in short-term tests. Demeton and disulfoton were the primary exceptions to this rule. Possible reaction mechanisms which may explain this apparent disparity are discussed later. Demeton displays rather extensive genotoxic activity, causing gene mutation in prokayotes (SA0, ECW) and eukaryotes (G5T, SCR), SCE (SIC), and primary DNA damage or recombination in prokaryotes (BSD) and eukaryotes (YE3, SCH, and UHF).

Disulfoton, one of the positive phosphorodithioates with ethyl or higher esters elicited gene mutation, primary DNA damage and SCE. Interestingly, disulfoton and the structurally similar compound phorate (which was negative in the initial SRI test battery) resemble the frequently positive agents distinguished by unbranched side chains without ring structures (e.g. demeton). Phorate has not been tested in two (G5T and SIC) of the three SRI assays for which disulfoton was positive. Because of its similar chemical structure disulfoton may exert its effects by a reaction mechanism like that of demeton.

OPs with mainly negative results

Category III consists of the phosphorothioate and phosphorodithioate pesticides containing ethyl and higher esters. These compounds include: aspon, chlorpyrifos, diazinon, ethion, fensulfothion, fonofos, parathion and phorate. Four agents, O,O,O',O'-tetrapropyl dithiopyrophosphate, ethion, fensulfothion, and fonofos are negative in all reported tests. Three of the remaining four chemicals, diazinon, parathion and phorate, were negative in the SRI data. Diazinon has been reported positive for SCE in human cells.

Table 22.6 Comparisons of genetic activity profiles for selected pairs of chemicals

Chemical	*Common*	*agreement (%+)*		*P*	*DRF(C)*
Cyclophosphamide					
Tris(2,3-dibromopropyl)PO_4	25	22	90	0.000	1.98
Thiotepa	27	26	100	0.000	1.94
Dimethoate	21	16	87	0.013	1.93
Dimethoate					
Parathion-methyl	12	12	66	0.000	2.35
Malathion	16	13	61	0.011	1.94
Thiotepa	13	11	100	0.011	1.84
Cyclophosphamide	21	16	87	0.013	1.93
Tris(2,3-dibromopropyl)PO_4	12	9	77	0.073	1.92
Malathion					
Parathion-methyl	16	13	46	0.011	2.30
Dimethoate	16	13	61	0.011	1.94
Parathion-methyl					
Dimethoate	12	12	66	0.000	2.35
Malathion	16	13	46	0.011	2.30
Thiotepa					
Cyclophosphamide	27	26	100	0.000	1.94
Tris(2,3-dibromopropyl)PO_4	13	13	100	0.000	1.50
Dimethoate	13	11	100	0.011	1.84
Tris(2,3-dibromopropyl)PO_4					
Cyclophosphamide	25	22	90	0.000	1.98
Thiotepa	13	13	100	0.000	1.50
Dimethoate	12	9	77	0.073	1.92

[a]This table shows the number of common tests between selected pairs of chemicals
[b]Concordant or agreeing results as well as the percentage of concordant results that are positive are shown
[c]*P* value is the probability that concordant results would occur by chance
[d]DRF is defined in Equation 1

Computer analysis of genetic activity profiles

The previous results have shown that the 24 OPs reviewed here can be divided into three main categories based on the strength of the positive data. Several classes of chemicals defined by chemical structure fit one of these three categories. An additional way of categorizing these agents is to examine the data for all common bioassays conducted for each pair of chemicals. Both the qualitative and quantitative agreement (matches) between chemicals can then be determined by computer. The binomial distribution is used to calculate the probability (P) that concordant test results would occur by chance.

Table 22.6 shows the agreement of a number of EPA chemicals with other OPs. Data with P values <0.05 were considered significant. Two groups of pesticides are found by this procedure: the first group contains three chemicals from Category I, cyclophosphamide, thio-TEPA, and tris(2,3-dibromopropyl)PO_4. The second group is formed by dimethoate, malathion, parathion-methyl. It should be noted that each member of both groups matches each other member of the group with a high level of significance. The P value is often 0.01 or less. Dimethoate also matches two chemicals from the first group, cyclophosphamide and thio-TEPA (P <0.02).

A measure of agreement of the magnitudes of common profile lines is given by the dose-related function (DRF). DRF values for the two groups of chemicals ranged from 1.50 to 2.35. A value of 2.0 corresponds to an average dose difference of one logarithmic dose unit.

The chemical matches determined by computer can be visualized by plotting combined genetic activity profiles. Test results for parathion-methyl and dimethoate agree throughout the endpoint spectrum (Figure 22.3). Both compounds are positive in higher eukaryotes but neither causes germinal cell mutation in mice (DLM). The quantitative agreement in test results for the two compounds is excellent.

Dimethoate and thio-TEPA although not partners in a group match in a number of common positive bioassays. In all positive bioassays thio-TEPA is more active on the logarithmic scale. This match may be fortuitous owing to the large number of positive tests for thio-TEPA (Table 22.2).

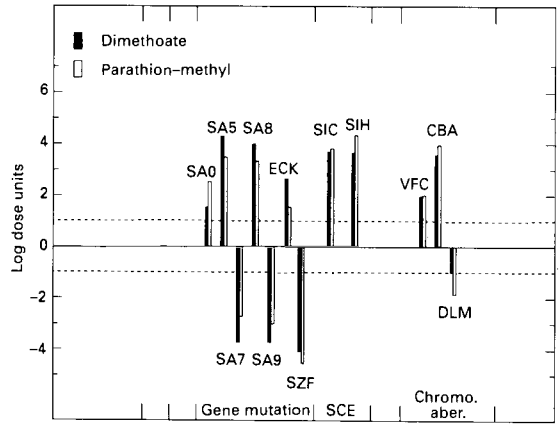


Figure 22.3 Test results for parathion-methyl and dimethoate

Table 22.7 Overall evaluation of carcinogenicity for OP agents

Chemical	*Classification IARC Human*	*Animal*	*Overall*	*Gene-Tox Animal*
Cyclophosphamide	S	S	1	SP
Thio TEPA	I	S	2A	SP
Tris(2,3-dibromopropyl)PO_4	I	S	2A	SP
TEPA	ND	I	3	I
Trichlorfon	ND	I	3	
Dimethoate				SN
Malathion	ND	I	3	SN
Parathion-methyl	ND	ESL	3	SN
Dichlorvos	ND	I	3	E
Diazinon				SN

[a]IARC codes: ESL, evidence suggesting lack of carcinogenicity; I, inadequate evidence; L, limited evidence; ND, no adequate data; S, sufficient evidence; 1, carcinogenic to humans; 2A, probably carcinogenic; 2B, possibly carcinogenic; 3, not classifiable as to carcinogenicity to humans [6]
[b]Gene-Tox codes: SN, sufficient negative; SP, sufficient positive animal carcinogen; E, equivocal; I, inadequate [8]

Carcinogenic potential of OPs

Ten of the 24 agents reviewed here for genotoxicity have been previously evaluated by panels of experts for evidence of carcinogenicity in animals or humans. Eight chemicals have been reviewed by IARC [6] and nine of the chemicals have been reviewed for and included in the Gene-Tox carcinogen data base [8]. Chemicals included in the Gene-Tox carcinogen data base included chemicals previously evaluated by IARC, selected chemicals bioassayed by the National Toxicology Programme (NTP) and the National Cancer Institute (NCI), as well as data available from the published literature.

The overall evaluation by IARC or Gene-Tox of the carcinogenicity of ten OPs is shown in Table 22.7. The chemicals are arranged from the highest to the lowest evidence of causing cancer in animals or humans. Sufficient evidence was found that three of the chemicals, cyclophosphamide, thioTEPA, and tris(2,3,-dibromopropyl)PO_4 cause cancer in animals. Only one of these three, cyclophosphamide, is considered to have sufficient evidence for a positive response in humans, although both thio-TEPA and tris(2,3,dibromopropyl)PO_4 were ranked as category 2A chemicals, i.e. they are probable carcinogens in humans.

Four compounds, TEPA, dichlorvos, malathion and trichlorfon were ranked by IARC as having inadequate evidence for being animal carcinogens. Malathion was considered a sufficient negative in the Gene-Tox carcinogen report.

Three other compounds, dimethoate, diazinon, and parathion-methyl were considered sufficiently negative as animal carcinogens by Gene-Tox. The results with parathion-methyl were consistent with the IARC evaluation.

IARC considers cyclophosphamide carcinogenic and tris(2,3-dibromopropyl)PO_4 probably carcinogenic to humans. Of the 25 common assays only three (SAF, SA9, TBM) do not agree. Tris(2,3-dibromopropyl)PO_4 and dimethoate have similar combined profiles. Dimethoate is considered to have sufficient evidence of a negative response in animal cancer studies. Of the 12 common tests, three (URP, SA8, SA9) disagree between these two chemicals. One of the *Salmonella* tests, SA8, is positive for dimethoate but negative for tris(2,3-dibromopropyl)PO_4. Also in several cases dimethoate produces positive responses at lower doses than does tris(2,3- dibromopropyl)PO_4.

Discussion

Many OPs that inhibit AChE are strong neurotoxins and are also capable of producing a variety of genotoxic effects. Genetic activity profiles have been used to represent the quantitative information (LED or HID tested) in a two-dimensional configuration that facilitates both chemical and test comparisons. We have reported previously on several agents discussed here; however the previous test results were from a single laboratory [5].

Three main categories of results were distinguished among the 24 compounds evaluated. Five Category I pesticides were active in most of the *in vitro* and *in vivo* assays. Eleven Category II pesticides displayed mixed positive and negative results. Category III consisted of eight pesticides which gave largely negative results. We have evaluated and classified these pesticides using three different techniques: (1) with respect to their overall genetic activity by inspection of the genetic activity profiles, (2) on the basis of their chemical structures, and (3) by means of computerized matching of genetic activity profiles.

Matching of profiles was based on the probability (P) that the number of concordant results would occur by chance. Thus, similarities in the profiles of genetic effects displayed by specific pairs of compounds can be determined.

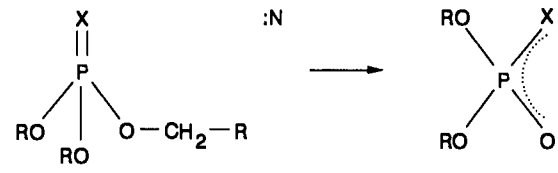
The groups of chemicals formed by analysis of pesticide performance in a number of common bioassays were correlated with chemical structure and activity. Although pesticides within a group did not display identical profiles of genetic activity, components of the profiles matched other chemicals within the group. The major reactions of pentavalent phosphorous esters are phosphorylation and alkylation reactions. Although the phosphorylation reaction is responsible for the inhibition of the AChE and produces corresponding neurotoxicity, certain biological activities of the phosphorus esters such as

mutation appear to be from the alkylation properties [2]. The general reaction mechanism is shown in Equation 22.2 where N is a nucleophile [1].

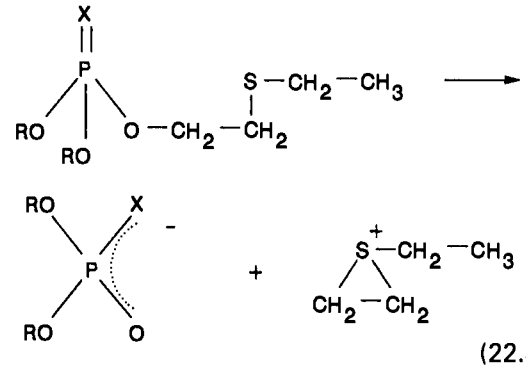
The heterocyclic bases of nucleic acids may serve as nucleophilic agents for such a reaction, and alkylation may take place on nitrogen atoms possessing high electron density. The alkylation reaction is more likely with the methyl ester group than ethyl and higher alkyl esters groups, and phosphate esters are more reactive than the phosphorothioate esters [1]. Thus, the reactivity of dimethoate, malathion, parathion-methyl, monocrotophos, and trichlorfon may be explained by methylation reactions of the phosphate esters. As demeton is an ethyl ester, this pesticide might act by a different mechanism. The 2-alkylthioethanols form cyclic ethylenesulfonium ions which are highly active alkylating agents (Equation 22.3). This mechanism may explain the genetic activity of demeton [1]. A very similar mechanism may explain the activity of phorate (Equation 22.4). Alkylation reactions may explain the majority of genotoxic effects observed for antiChEs. Wooder and Wright [12] showed that OP pesticides alkylate DNA. In addition to the phosphates, cyclophosphamide and the aziridines are powerful alkylating agents; the latter compounds exert clinically useful cytotoxicity by forming covalent linkages with nucleophilic centres in DNA and RNA. Genotoxic effects may include miscoding of DNA, imidazole ring cleavage or depurination with DNA scission. Bifunctional or multifunctional compounds such as cyclophosphamide and thio-TEPA may cause cross-linkage of two adjacent nucleic acid chains.

The evidence that some of the agents evaluated in this report are carcinogens is not conclusive. However, three compounds, cyclophosphamide, thio-TEPA, and tris(2,3- dibromopropyl)PO_4 produce mainly positive results in short-term tests and are considered animal carcinogens by both IARC and the Gene-Tox programme.

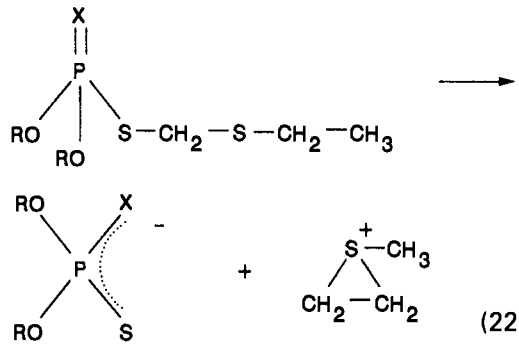
There is substantial agreement in short-term tests for thio-TEPA and dimethoate. Thio-TEPA is considered a probable human carcinogen by IARC. In contrast, dimethoate is considered to have sufficient evidence of a negative response in animal cancer studies.



(22.2)



(22.3)



(22.4)

Concordant results were obtained in the combined genetic activity profile for tris(2,3-dibromopropyl)PO_4 and dimethoate. Thus dimethoate although negative in animal cancer tests produces a profile of genetic activity similar to those of tris(2,3-dibromopropyl)PO_4 and thio-TEPA. This apparent disparity suggests a need for continued study of the potential long-term carcinogenic effect of OPs.

In general, genetic activity profiles provide a useful adjunct in the overall evaluation of genotoxicity. Structure-activity correlations based on genetic activity profiles representing a variety of test systems may provide useful information relative to possible mechanisms of genetic toxicity. As more information is gained, comparisons of preliminary test results for new OP chemicals with existing data for

known genotoxicants should aid in the evaluation of potential health hazards.

This document has been reviewed in accordance with US EPA policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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Appendix 1

DNA damage

PRB Prophage induction or SOS test, DNA damage or repair
ECB *Escherichia coli* (or *E. coli* DNA), strand breaks, cross-links or repair
SAD *Salmonella typhimurium,* DNA repair-deficient strains, differential toxicity
ECD *E. coli* pol A/W3110-P3478, differential toxicity (spot test)
ECL *E. coli* pol A/W3110-P3478, differential toxicity (liquid suspension test)
ECU *E. coli* unscheduled DNA synthesis
ERD *E. coli* recombinant strains, differential toxicity
BSD *Bacillus subtilis* recombinant strains, differential toxicity
BRD Other DNA repair-deficient bacteria, differential toxicity
SSB *Saccharomyces* species, DNA breaks, cross-links or related damage
SSD *S. cerevisiae,* DNA repair-deficient strains, differential toxicity
SZD *S. pombe,* DNA repair-deficient strains, differential toxicity
PLU Plants, unscheduled DNA synthesis
DIA DNA strand breaks, cross-links or related damage, animal cells *in vitro*
RIA DNA repair exclusive of UDS, animal cells *in vitro*
URP Unscheduled DNA synthesis, rat primary hepatocytes
UIA Unscheduled DNA synthesis, other animal cells *in vitro*
DIH DNA strand breaks, cross-links or related damage, human cells *in vitro*
RIH DNA repair exclusive of UDS, human cells *in vitro*
UHF Unscheduled DNA synthesis, human fibroblasts *in vitro*
UHL Unscheduled DNA synthesis, human lymphocytes *in vitro*
UHT Unscheduled DNA synthesis, transformed human cells *in vitro*
UIH Unscheduled DNA synthesis, other human cells *in vitro*
DVA DNA strand breaks, cross-links or related damage, animals *in vivo*

DNA repair exclusive of UDS, animal cells *in vivo*
UPR Unscheduled DNA synthesis, rat hepatocytes *in vivo*
UVC Unscheduled DNA synthesis, hamster cells *in vivo*
UVM Unscheduled DNA synthesis, mouse cells *in vivo*
UVR Unscheduled DNA synthesis, rat cells (not hepatocytes) *in vivo*
UVA Unscheduled DNA synthesis, other animal cells *in vivo*
DVH DNA strand breaks, cross-links or related damage, humans *in vivo*
UBH Unscheduled DNA synthesis, human bone-marrow cells *in vivo*
UVH Unscheduled DNA synthesis, other human cells *in vivo*

Recombination

SCG *Saccharomyces cerevisiae,* gene conversion
SCH *S. cerevisiae,* homozygosis by recombination or gene conversion
SZG *Schizosaccharomyces pombe,* gene conversion
ANG *Aspergillus nidulans,* genetic crossing-over
DMG *Drosophila melanogaster,* genetic crossing-over or recombination

Body fluid and host mediated assays

BFA Body fluids from animals, microbial mutagenicity
BFH Body fluids from humans, microbial mutagenicity
HMA Host mediated assay, animal cells in animal hosts
HMH Host mediated assay, human cells in animal hosts
HMM Host mediated assay, microbial cells in animal hosts

Gene mutation

BPF Bacteriophage, forward mutation
BPR Bacteriophage, reverse mutation
SAF *Salmonella typhimurium,* forward mutation
SA0 *S. typhimurium* TA100, reverse mutation
SA2 *S. typhimurium* TA102, reverse mutation
SA3 *S. typhimurium* TA1530, reverse mutation
SA4 *S. typhimurium* TA104, reverse mutation
SA5 *S. typhimurium* TA1535, reverse mutation
SA7 *S. typhimurium* TA1537, reverse mutation
SA8 *S. typhimurium* TA1538, reverse mutation
SA9 *S. typhimurium* TA98, reverse mutation
SAS *S. typhimurium* (other miscellaneous strains), reverse mutation
ECF *Escherichia coli* (excluding strain K12), forward mutation
ECK *E. coli* K12, forward or reverse mutation
ECW *E. coli* WP2 uvrA, reverse mutation
EC2 *E. coli* WP2, reverse mutation
ECR *E. coli* (other miscellaneous strains), reverse mutation
BSM *Bacillus subtilis* multi-gene test
KPF *Klebsiella pneumonia,* forward mutation
MAF *Micrococcus aureus,* forward mutation
SCF *Saccharomyces cerevisiae,* forward mutation
SCR *S. cerevisiae,* reverse mutation
SGR *Streptomyces griseoflavus,* reverse mutation
STF *Strep. coelicolor,* forward mutation
STR *Strep. coelicolor,* reverse mutation
SZF *Schizosaccharomyces pombe,* forward mutation
SZR *Schizosaccharomyces pombe,* reverse mutation
ANF *Aspergillus nidulans,* forward mutation
ANR *A. nidulans,* reverse mutation
NCF *Neurospora crassa,* forward mutation
NCR *Neurospora crassa,* reverse mutation
SAL *S. typhimurium* combined data, reverse mutation
ASM *Arabidopsis* species, mutation
HSM *Hordeum* species, mutation
TSM *Tradescantia* species, mutation
PLM Plants (other), mutation
DMM *Drosophila melanogaster,* somatic mutation (and recombination)
DMX *Drosophila melanogaster,* sex-linked recessive lethal mutation
GCL Gene mutation, Chinese hamster lung cells (excluding V79) *in vitro*
GCO Gene mutation, Chinese hamster ovary cells *in vitro*
G9H Gene mutation, Chinese hamster lung V-79 cells *in vitro*, HPRT

G9O Gene mutation, Chinese hamster lung V-79 cells *in vitro*, ouabain
GML Gene mutation, mouse lymphoma cells (excluding L5178Y) *in vitro*
G5T Gene mutation, mouse L5178Y cells *in vitro*, TK locus
G51 Gene mutation, mouse L5178Y cells *in vitro*, all other loci
GIA Gene mutation, other animal cells *in vitro*
GIH Gene mutation, human cells *in vitro*
GVA Gene mutation, animal cells *in vivo*
MST Mouse spot test
SLP Mouse specific locus test, postspermatogonia
SLO Mouse specific locus test, other stages

Sister chromatid exchanges

VFS *Vicia faba,* sister chromatid exchange
PLS Plants (other), sister chromatid exchange
SIC Sister chromatid exchange, Chinese hamster cells *in vitro*
SIM Sister chromatid exchange, mouse cells *in vitro*
SIR Sister chromatid exchange, rat cells *in vitro*
SIS Sister chromatid exchange, Syrian hamster cells *in vitro*
SIT Sister chromatid exchange, transformed cells *in vitro*
SIA Sister chromatid exchange, other animal cells *in vitro*
SHF Sister chromatid exchange, human fibroblasts *in vitro*
SHL Sister chromatid exchange, human lymphocytes *in vitro*
SHT Sister chromatid exchange, transformed human cells *in vitro*
SIH Sister chromatid exchange, other human cells *in vitro*
SVA Sister chromatid exchange, animal cells *in vivo*
SLH Sister chromatid exchange, human lymphocytes *in vivo*
SVH Sister chromatid exchange, other human cells *in vivo*

Micronucleus test

TSI *Tradescantia* species, micronuclei
PLI Plants (other), micronuclei
MIA Micronucleus test, animal cells *in vitro*
MIH Micronucleus test, human cells *in vitro*
MVM Micronucleus test, mice *in vivo*
MVR Micronucleus test, rats *in vivo*
MVC Micronucleus test, hamsters *in vivo*
MVA Micronucleus test, other animals *in vivo*
MVH Micronucleus test, human cells *in vivo*

Chromosomal aberrations

PSC *Paramecium* species, chromosomal aberrations
ACC *Allium cepa,* chromosomal aberrations
HSC *Hordeum* species, chromosomal aberrations
TSC *Tradescantia* species, chromosomal aberrations
VFC *Vicia faba,* chromosomal aberrations
PLC Plants (other), chromosomal aberrations
DMC *Drosophila melanogaster,* chromosomal aberrations
DMH *Drosophila melanogaster,* heritable translocation test
DML *Drosophila melanogaster,* dominant lethal test
CIC Chromosomal aberrations, Chinese hamster cells *in vitro*
CIM Chromosomal aberrations, mouse cells *in vitro*
CIR Chromosomal aberrations, rat cells *in vitro*
CIS Chromosomal aberrations, Syrian hamster cells *in vitro*
CIT Chromosomal aberrations, transformed cells *in vitro*
CIA Chromosomal aberrations, other animal cells *in vitro*
CHF Chromosomal aberrations, human fibroblasts *in vitro*
CHL Chromosomal aberrations, human lymphocytes *in vitro*
CHT Chromosomal aberrations, transformed human cells *in vitro*
CIH Chromosomal aberrations, other human cells *in vitro*
CBA Chromosomal aberrations, animal bone marrow cells *in vivo*
CLA Chromosomal aberrations, animal leucocytes *in vivo*
CCC Chromosomal aberrations, spermatocytes treated and observed
CGC Chromosomal aberrations, spermatogonia treated, spermatocytes observed
CGG Chromosomal aberrations, spermatogonia treated and observed

COE Chromosomal aberrations, oocytes or embryos treated *in vivo*
CVA Chromosomal aberrations, other animal cells treated *in vivo*
DLM Dominant lethal test, mice
DLR Dominant lethal test, rats
MHT Mouse heritable translocation test
CBH Chromosomal aberrations, human bone marrow cells *in vivo*
CLH Chromosomal aberrations, human lymphocytes *in vivo*
CVH Chromosomal aberrations, other human cells *in vivo*

Aneuploidy

SCN *Saccharomyces cerevisiae,* aneuploidy
ANN *Aspergillus nidulans,* aneuploidy
NCN *Neurospora crassa,* aneuploidy
DMN *Drosophila melanogaster,* aneuploidy
AIA Aneuploidy, animal cells *in vitro*
AIH Aneuploidy, human cells *in vitro*
AVA Aneuploidy, animal cells *in vivo*
AVH Aneuploidy, human cells *in vivo*

Cell transformation

TBM Cell transformation, BALB/C3T3 mouse cells
TCM Cell transformation, C3H10T1/2 mouse cells
TCS Cell transformation, Syrian hamster embryo cells, clonal assay
TFS Cell transformation, Syrian hamster embryo cells, focus assay
TPM Cell transformation, mouse prostate cells
TCL Cell transformation, other established cell lines
TRR Cell transformation, RLV/Fischer rat embryo cells
T7R Cell transformation, SA7/rat cells
T7S Cell transformation, SA7/Syrian hamster embryo cells
TEV Cell transformation, other viral enhancement systems
TIH Cell transformation, human cells *in vitro*
TVI Cell transformation, treat *in vivo*, score *in vitro*

23

Development and reproductive toxicity of anticholinesterases

Rochelle W. Tyl

Introduction

Toxicity to the reproductive system can occur from the time of formation (beginning *in utero*) through the time of reproductive function (i.e. generative and/or degenerative changes [14]); for the male this vulnerability includes spermatogenesis, accessory sex organ structure and function and intromission; for the female the time extends through ovulation, fertilization, implantation, organogenesis, fetogenesis, parturition and lactation. The toxicity can be directly on the reproductive system or indirectly, mediated by other systems which impact on the reproductive process, such as the CNS, endocrine and immune systems or by effects on the general well being of the adult. Developmental toxicity encompasses effects on gametogenesis, fertilization, the embryo, the fetus, the neonate, and the postnatal individual through adulthood; effects include death of the conceptus, frank malformations, developmental delays and functional deficits [154a]. Study designs to detect these end points are discussed elsewhere [139]. Anticholinesterases have been shown to affect essentially all of the processes listed above under certain assay conditions in various test animal species (for reviews see [38], [98a], [107a], and [145]).

Carbamates

Carbaryl

Of the CB antiChEs relatively few have been evaluated for developmental or reproductive toxicity. Only carbaryl has been evaluated in avian and fish embryos. Injection of carbaryl at 10–1000 μg per egg caused malformed duck and chick embryos [75]; Eto *et al.* [58] reported malformations after *in ovo* exposure in chick embryos. Injection of 0.0008% LD_{50} in chick embryos resulted in 100% mortality [38]. However, Tos-Luty *et al.* [138] injected carbaryl into chick eggs at 1.95–6.75 mg per embryo, and did not observe any histological changes in surviving embryos. Khmelevskii and Stephano [79] fed carbaryl to hens and cocks at levels which reduced blood ChE with no overt clinical signs. This exposure resulted in 0.02–0.06 ppm residues in adult tissues and eggs, but did not affect chick development. Lillie [85] administered carbaryl in the diet to chickens at doses up to 500 ppm which resulted in growth depression in the adult but did not affect egg production, fertility or hatchability of the eggs and no abnormalities were observed in embryos or offspring.

Fertile white Leghorn eggs were injected with 10 mg carbaryl and eggs were then incubated for 5 (stages 24–27) or 12 (stages 38–39) days. Embryos were examined by serial section for effects on the developing gonads. After 5 days of incubation, primordial germ cells were localized in the gonad with no significant difference in the number. After 12 days of incubation, no effects were observed on survival, testicular or ovarian morphology, or sex ratio. It was concluded that carbaryl does not affect normal migration of primordial germ cells or normal gonadal differentiation in birds [134].

Malformations were observed after carbaryl exposure in Medaka fish [126], in Killifish [154] and in yellowtail [6].

In mammals, aldicarb was evaluated for ChE inhibition, in Sprague-Dawley rat dams and fetuses after a single gavage dose of 0 to 0.1 mg/kg on gd 18.[28]On killing at 1, 5 and

24 h after administration, ChE was assayed in maternal and fetal blood, liver and brain. At 1 h after dosing, ChE activity was significantly reduced at 0.001 mg/kg for maternal liver (but not blood or brain), and for fetal blood, liver and brain. At 0.01 mg/kg, ChE levels were significantly reduced at 1 h in the maternal liver (but not blood or brain) and in fetal blood, liver and brain; at 5 h after dosing, levels were significantly reduced for all tissues except maternal brain; at 24 h, levels were still significantly reduced for all tissues except maternal brain and liver. At 0.1 mg/kg, all maternal and fetal tissues exhibited significantly reduced ChE at 1 and 5 h after dosing; at 24 h, maternal and fetal blood and fetal liver values were still significantly reduced [28]. The degree, onset and duration of the inhibition were dose-related. Maternal tissues were more affected than fetal tissues. These data are consistent with aldicarb and/or metabolite(s) crossing the placenta to affect the fetal enzymes.

Benomyl was administered by gavage to male Sprague-Dawley rats during prepubertal, pubertal and postpubertal development at 0–1000 mg/kg per day for 5 or 10 consecutive days. Animals exposed before puberty had no significant treatment-related effects. Animals exposed to $\geqslant 250$ mg/kg during puberty or after puberty exhibited one or more of the following : decreased testicular or epididymal weights, decreased epididymal and/or vas deferens sperm counts, and/or testicular lesions. Diffuse hypospermatocytogenesis was observed in 20% of the pubertal and 40% of the postpubertal animals; 10% of the prepubertal animals and 0% of the controls exhibition this lesion [32].

Carbaryl has been extensively tested for developmental and/or reproductive toxicity in at least ten mammalian species.

Rats

Harlan-Wistar rats given carbaryl in the diet at 0–500 mg/kg per day for various gestational intervals showed reduced maternal body-weights at 100 and 500 mg/kg per day; postnatal pup survival was significantly reduced at 500 mg/kg per day. No treatment-related malformations were reported [151]. Gavage of carbaryl at 0.02% LD_{50} to rats on gd 9, 11 and 13 caused decreased fetal body-weights, but no malformations were seen [53]. Sprague-Dawley rats given carbaryl in the diet at 0, 4000 or 7000 ppm (0, 300 and 525 mg/kg per day) on gd 6-15 showed no maternal toxicity and no effects on fetal survival or development [70]. Weil *et al.* [152] gave carbaryl by gavage or by dietary inclusion in CFE rats over three generations and multiple litters. The dietary doses ranged from 0–200 mg/kg per day; gavage doses ranged from 0–100 mg/kg per day. The only effect in the dietary study was an increase in the median number of days from initial pairing to birth of the first litter of the F1a to F2a generation at 200 mg/kg per day. By gavage, maternal toxicity occurred at 100 mg/kg per day (mortality, reduced weight gain, and clinical signs of ChE inhibition). Also at this dose there was reduced fertility (number of F1b litters and decreased live litter size in the F2a and F2b generation). No teratogenicity was observed in the F3b fetuses at any dose or route tested.

Rats were given carbaryl at 0, 1, 10 or 100 mg/kg per day or malathion (both formulation grade) at 1 or 50 mg/kg per day or both (at 1+1 or 50+50 mg/kg per day) by gavage daily for 90 days before and throughout gestation. Dams were killed on gd 20 and the fetuses were examined. Maternal weight gain during gestation was reduced at 100 mg/kg per day carbaryl. In the combination dosing regimen, maternal weight gain was further reduced at both doses. The total number of implantations and the number of live fetuses per litter were decreased at 100 mg/kg per day carbaryl; placental weights were reduced in both of the combination dose groups. No treatment-related increases in malformations were observed [87].

Carbaryl administered to pregnant rats late in gestation by a single gavage dose resulted in reduced ChE activity in fetal tissue [28].

Carbaryl crosses the rodent placenta slowly and the amount is small: gavage of 10 mg [^{14}C]methyl carbaryl to rats on gd 18 resulted in placental transfer of the radiolabel and persistence in the fetus; approximately 0.3% of the administered dose was localized in the fetuses 96 h later. Accumulation was noted in fetal eyes, CNS and liver [45–47]. Pregnant rats exhale a higher proportion of radioactivity as CO_2 from [^{14}C]carbonyl-labelled carbaryl

and excrete a lower proportion of radioactivity in the urine from ring-labelled carbaryl than do non-pregnant rats [83]. This suggests that pregnant rats metabolize carbaryl to a greater extent than do non-pregnant rats [130].

Weil and Carpenter [150] administered carbaryl in the diet to CFE rats at 0, 2.5 or 10 mg/kg per day for three generations, two litters per generation. No parental, reproductive or developmental toxicity was observed. Collins *et al.* [36] administered carbaryl in the diet to Osborne-Mendel rats at 0–10 000 ppm, also for three generations, two litters per generation. Maternal toxicity occurred at 10 000 ppm (reduced weight gain). The fertility index was significantly reduced in the second generation at 10 000 ppm with no F2b litters produced. Prenatal mortality and reduced survival from birth to postnatal day 4 were observed at 5000 and 10 000 ppm; reduced survival from postnatal day 4 to weaning was observed at 10 000 ppm. Body-weights of the pups at weaning (postnatal day 21) were reduced in all carbaryl-exposed groups.

The Russian literature on carbaryl is almost exclusively in rats. Rybakova [112] gave carbaryl by gavage to white rats at 7, 14 or 70 mg/kg per day for 12 months. Body-weights were reduced at 70 mg/kg per day. The 'motility period' of spermatozoa was significantly reduced at 14 and 70 mg/kg per day after 6 months and in all groups at 12 months. Histopathological changes in the seminiferous tubules were reported at 70 mg/kg per day. Even at 7 mg/kg per day histopathological changes in the interstitial tissue, parenchyma and spermatogenic epithelium were seen.

Vashakidze [142] administered carbaryl by gavage to male and female white rats at 2–50 mg/kg per day for 6 months or at 100 mg/kg per day for 1 month. One-third at each dose were fed a high protein (22%) synthetic diet, a low protein (9%) synthetic diet, or a standard rat feed with 15–18% protein. Progressively more profound weight loss was observed at 50 and 100 mg/kg per day. Decreased resistance of sperm to hypertonic saline was observed at 5–100 mg/kg per day. Sperm mobility time was reduced at 5 and 15 mg/kg per day and sperm 'longevity' (in nutrient medium) was reduced at 50 and 100 mg/kg per day. The high protein (22%) diet prevented the changes in sperm function. Only the males given 100 mg/kg per day had histopathological changes in the testes. Vashakidze [143] also gave carbaryl by gavage daily to male white rats for 6 months at 0.3–10 mg/kg per day. General toxicity was observed at 4–10 mg/kg per day. Reduced testes weight with histological evidence of damage, especially to spermatids and spermatogonia, was observed at 2–4 mg/kg per day, with no effects at 0.3–0.5 mg/kg per day. When treated males were mated to untreated females, preimplantation loss was observed at 2–4 mg/kg per day. The author concluded that the specific effects on reproduction were observed at much lower doses than those causing general toxicity.

Shtenberg and Ozhovan [122] administered carbaryl to five generations of rats at 2 or 5 mg/kg per day, and evaluated reproductive function for the second to the fifth generations. Sperm activity was reduced at 5 mg/kg per day starting with the fourth generation. Other functional parameters for sperm, such as resistance to hypertonic saline, mobility time and longevity were affected at both doses in various generations. Alterations in oestrus cycle were observed in females at both doses.

Vashakidze [144] gave carbaryl to male and female rats by gavage at 1–50 mg/kg per day for 1 month. Histological lesions and functional changes of the testes were reported for all doses. Treated males were mated to untreated females, resulting in reductions in total and live embryos per litter. The number of 'underdeveloped' and dead embryos per litter was increased at all doses. In treated females, dioestrus was prolonged at 5–20 mg/kg per day, oestrus was prolonged at 10 and 20 mg/kg per day and metoestrus was prolonged at 20 mg/kg per day. The number of ovarian corpora lutea per mated dam was decreased for all doses above 5 mg/kg per day, again with no dose-response pattern.

Mice

Mice were administered carbaryl in the diet at 0, 67 or 200 ppm (0, 10 and 30 mg/kg per day) on gd 6–18. There were no adverse effects observed in the dams, fetuses or neonates [17].

Carbaryl given to mice by sc injection in DMSO at 25–464 mg/kg per day on gd 6–14 caused reduced maternal weight gain and

reduced fetal body-weights per litter in several strains at 100–464 mg/kg per day. Fetal malformations were increased only in the BL6 strain at 100 mg/kg per day with a small number of dams. Two subsequent replicates did not result in increased malformations in this mouse strain. No developmental toxicity was observed in any strain at 25 mg/kg per day [82].

Murray *et al.* [99] administered carbaryl by gavage at 100 or 150 mg/kg per day or by diet at 1.166 mg/kg per day (5600 ppm) on gd 6–15. Gavage of 150 g/kg per day resulted in maternal mortality, reduced weight gain and clinical signs of ChE inhibition; at 100 mg/kg per day by gavage, one dam died. No developmental toxicity was observed in either gavage group. The dietary carbaryl group had reduced maternal weight gain during the treatment period and reduced fetal body-weight and crown-rump length. No treatment- related fetal malformations were observed.

Guthrie *et al.* [69] administered carbaryl to laboratory mouse strains selected for resistance to insecticides by single ip injection 1 week before mating. There were no treatment-related effects on reproduction or development of the offspring.

Population growth and genetic variation were evaluated in feral mice from areas treated with carbaryl at 21.74 kg/ha; no effects were observed on the parameters evaluated [65].

Carbaryl by gavage to mice at doses up to 34 mg/kg per day for 5 days, did not affect the weight of the testes or accessory sex glands or the ability to metabolize testosterone [52]. However, carbaryl has been reported to cause destruction of resting primordial ovarian follicles in mice [54].

Radiolabelled [^{14}C]carbaryl was shown to cross the murine placenta on gd 18 after a 10 mg/kg dose, albeit slowly and/or at a low proportion of the dose with radiolabel detected in pups 60 h after birth. Accumulation was noted in fetal and neonatal eyes, CNS and liver [47].

Gerbils

Carbaryl in the diet to three generations of gerbils, two litters per generation, at 0–10 000 ppm, produced treatment-related decreases in fertility indices in both matings of the second and third generation at 10 000 ppm. Statistically significant decreases in fertility indices were also observed in the first mating of the first generation at 4000 ppm, in both matings of the third generation at 2000 ppm and in the second mating of the third generation at 6000 ppm. The average litter size at birth was significantly reduced in the second generation for the first mating at 4000, 6000 and 10 000 ppm and for the second litter in this generation at 10 000 ppm; in the third generation, both matings were significantly affected at 2000 ppm but not at 4000 or 6000 ppm (at 10 000 ppm the first mating in this generation resulted in a total of six offspring from two females and the second mating was not productive). The viability index at birth exhibited significant reductions at 10 000 ppm for all three generations and 'sporadic' decreases for the other dose groups. Survival of gerbil pups to postnatal day 4 was significantly reduced for both matings in all three generations at 6000 and 10 000 ppm; at 2000 and 4000 ppm, survival was significantly reduced for both matings in the second and third generations. At 4000 ppm, the first mating in the first generation was also affected, while both matings in the first generation at 2000 ppm were unaffected [36].

Hamsters

Carbaryl given by gavage to pregnant hamsters at 125 mg/kg per day on gd 6–8 or 250 mg/kg per day on gd 7 or 8 produced maternal mortality at 250 mg/kg per day with clinical signs of toxicity in both treated groups. Fetal deaths were observed at 250 mg/kg per day, but there were no treatment-related malformations [108].

Guinea pig

Carbaryl by gavage to pregnant guinea pigs at 300 mg/kg on gd 11–20 resulted in a 38% maternal mortality rate and 17.5% fetal mortality rate. No fetal external malformations were observed, but skeletal defects (cervical vertebrae) were observed. This dose of carbaryl given on a single day of gestation, from gd 11 to gd 20, produced less maternal mortality than with multiple doses; fetal mortality

was unaffected. When data from the individual doses on gd 11–20 were pooled, the maternal mortality rate was 12.5% with fetal malformations observed only from dams receiving carbaryl on gd 12–16; the maternal mortality rate for this subgroup was 13.8%. Eight of the nine malformed fetuses in this group had vertebral malformations; one control fetus also had a cervical vertebra anomaly [108].

Weil *et al.* [152] compared dietary and gavage administration of carbaryl to Hart guinea pigs during various intervals of gestation; doses were 0–300 mg/kg per day in the diet or 0–200 mg/kg by gavage. All of the gavage doses produced clinical signs of ChE inhibition; 50% died at 200 mg/kg. Dietary administration resulted in reduced weight gain at 200 mg/kg with no deaths and no signs of ChE inhibition at any doses. The only developmental toxicity was a reduced number of viable fetuses per litter at 300 mg/kg in the diet on gd 15–19. Fetal weights were unaffected and no treatment-related malformations were observed.

Rabbits

With carbaryl in the diet at 0, 10 or 30 mg/kg per day on gd 9–16 the pregnancy rate was unexpectedly low for all doses, but no maternal or developmental toxicity was observed [120]. Robens [108] dosed rabbits by gavage at 0, 50, 100 or 200 mg/kg per day on gd 5–15 without maternal or developmental toxicity.

When 13–20 doses per group were given carbaryl by gavage at 0, 150 or 200 mg/kg per day on gd 6–18, diarrhoea was observed at 200 mg/kg per day and decreased weight gain during treatment at 150 and 200 mg/kg per day. Fetal toxicity was observed: increased resorptions at 200 mg/kg per day and decreased fetal body-weights at 150 mg/kg per day. However, the litters at 200 mg/kg per day with more resorptions had fewer live fetuses per litter and therefore fetuses would be heavier [139]. A significant increase in the incidence of fetal omphalocele occurred at 200 mg/kg per day [99].

Sheep

Carbaryl in the diet to Ramboillet sheep during mating and gestation at 0, 100 or 250 ppm produced no clinical signs of toxicity. Ventricular septal defect was observed in two of 23 lambs at 250 ppm; no malformations were observed at 0 or 100 ppm [103].

Swine

Smalley *et al.* [125] fed carbaryl to miniature sows throughout pregnancy at doses up to 30 mg/kg per day with no effects on reproduction or development, but clinical signs of acute ChE inhibition were observed. Carbaryl in the diet to miniature swine at 0–32 mg/kg per day produced a dose-related trend for reduced fertility and reduced number of piglets per litter [56].

Dogs

Smalley *et al.* [124] administered carbaryl to beagles in the diet at 0–50 mg/kg per day throughout gestation with pups followed until weaning. Malformations were observed at all but the lowest dose (3.125 mg/kg per day), including brachygnathia, umbilical hernia, gastroschisis, acaudia, polydactyly and other skeletal defects. In addition, pre- and postnatal mortality, therefore decreased litter size, and dystocia were observed in a dose-related pattern. Pre-implantation loss was increased at 12.5, 25 and 50 mg/kg per day. No pups were born alive at 50 mg/kg per day. Earl *et al.* [56] exposed beagles to carbaryl in the diet during gestation with the same results as those of Smalley *et al.* [124]. Beagles were also given carbaryl in the diet at 0–12.5 mg/kg from gd 1 until weaning of the pups [73]. Pre- and postnatal toxicity was observed at 5.0 and 12.5 mg/kg per day, decreased pup birth weight at 12.5 mg/kg per day, and decreased postnatal survival at all doses with no dose-response pattern. Malformations were observed in litters at 5.0 and 12.5 mg/kg per day [73].

Monkeys

Mature female rhesus monkeys (*Macacca mulatta*) with regular menstrual cycles were mated with proven fertile males and then administered carbaryl by gavage at 0, 2 or 20 mg/kg daily throughout gestation. The authors' concluded that carbaryl did not produce terata but was associated with a

higher rate of abortion than in the controls [55]. The results are confounded by the small number of females per group and the lack of a dose-response pattern for abortions.

Carbofuran

Carbofuran was administered in the diet to pregnant albino rats, white rabbits and beagle dogs at 50 ppm with no teratogenic effects in any species [90].

Carbofuran was evaluated for effects on maternal and fetal tissue ChE in Sprague-Dawley rats after a single gavage of 0–2.50 mg/kg on gd 18 at 30 min, 1, 5, or 24 h after dosing. ChE effects were dose-related for degree, time of onset and duration of inhibition, consistent with direct exposure of the fetuses to carbofuran and/or its metabolite(s) via placental transfer. Maternal tissues were more affected than fetal tissues.

Other carbamates

Ziram was administered to Swiss albino male mice at 350, 700 or 1050 mg/kg per day which produced a significant increase in the incidence of chromosomal aberrations in germ cells and a significant increase in the incidence of micronuclei in RBC. Reproductive function was not assessed [72].

Pirimicarb was also investigated for effects on maternal and fetal ChE activity in rats in the same dose and assay method as described above for carbofuran; the single gavage dose administered on gd 18 was 0, 2, 20 or 50–150 mg/kg. All dams died within 15 min from doses of 50–150 mg/kg; for these animals maternal brain and fetal blood and brain all exhibited significant reductions in ChE activity. For survivors, examination of blood, brain and liver ChEs showed inhibition was more pronounced in the maternal tissues than in fetal tissues and was dose-related for degree and onset and duration of enzyme inhibition effect [28].

Propoxur was evaluated in pregnant rats with no teratogenicity reported [146].

Dominant lethal assays have been performed in mice for two CB pesticides. Carbaryl, by gavage in mice at 250 or 1000 mg/kg per day, was negative [57]. In an 'add-on' to a reproductive toxicity study, male rats from the second generation, exposed to carbaryl at 25, 100 or 200 mg/kg in the diet or 3.25 or 100 mg/kg by gavage, were mated to unexposed females and also produced no dominant lethal induction [153]. Propoxur was negative in mice at 25 mg/kg by gavage [118,140] but 50 mg/kg by gavage for 5 days produced an increase in early embryonic deaths for the first 2 weeks after treatment [140]. The timing of the lesion indicates that the target cells were epididymal sperm and late spermatid stages.

Physostigmine

Physostigmine was injected into chick eggs. Paralysis and skeletal anomalies were observed in harvested embryos [132]. The different malformations observed were apparently via two different mechanisms of action [133].

Organophosphates

Pesticides

Early work on the developmental toxicity of OP pesticides focused on the avian embryo. A large number of these agents were teratogenic, and included the following agents positive in the chicken: monocrotophos [116], dicrotophos [158, 110], diazinon [34,58,76], dichlorvos [76, 110, 141], although dichlorvos was negative in earlier work at 10 mg per egg [109], parathion [76, 92, 107, 110, 141, 158], etrimfos [58], fenitrothion [104], azinphos-methyl [110,141], pirimiphos-methyl [58], malathion [66,149] and mevinphos [110].

Malformations were also produced in the duck embryo by diazinon, dichlorvos and parathion [76], in the quail embryo by monocrotophos [116], dicrotophos and parathion [93] and in the partridge embryo by azinphos-ethyl [88]. However, malathion was negative for teratogenic effects in chicks [110,141] and quail [94]. In chicks, mevinphos [141] and dioxabenzafos [58] were both negative.

Fish embryos have been evaluated for teratogenicity from OP pesticides. Malformations were induced in the yellowtail (*Seriola quinqueradiata*) from fenitrothion and trichlorfon [6], in medaka fish with malathion and parathion [126] and in killifish with parathion [154]. Malathion was negative in

killifish [154].

Many of these pesticides tested in mammals resulted in predominantly negative results for teratogenicity.

S-2-acetamidoethyl O,O-dimethyl phosphorodithioate (Amiphos) given to mice by gavage at 40 mg/kg per day on gd 1–14 resulted in no teratogenicity [71]. Dicrotophos was administered to pregnant mice on single or multiple days of gestation; brain ChE was substantially reduced in embryos 30 min after administration on gd 11 and in fetuses 30 min after administration on gd 19; however, there was no inhibition on gd 19 after administration on gd 8–16, indicating rapid and complete enzyme reactivation. No terata were reported [27]. Chlorfenvinphos, given in the feed to rats at 30, 100 or 300 ppm in a multigeneration design, produced dose-related prenatal mortality but no malformations [4]. Chloropyrifos administered to mice by gavage at 1, 10 or 25 mg/kg per day on gd 6–15 resulted in delayed ossification but no teratogenicity [41]. In rats, cyanophos was not teratogenic when administered orally at 10 mg/kg per day on gd 9–14 [159]. Diazinon at 0.125 mg/kg on gd 6, 7 and 8, or at 0.25 mg/kg on gd 7 or 8, and to rabbits at 7 or 30 mg/kg on gd 5–15, resulted in neither embryotoxicity nor teratogenicity [108]. However, diazinon given to beagle dogs by gavage at 1.2 or 5 mg/kg per day resulted in an increased incidence of stillbirths [56].

Dichlorvos did not cause developmental toxicity by gavage in mice when given over gd 6–15, even at the maximum tolerated dose of 60 mg/kg per day. Exposure by inhalation at 4 μg/1 for 7 h daily during organogenesis was not teratogenic in mice [117]. Rats given dichlorvos ip on gd 11 showed lethality at 20 mg/kg and maternal toxicity at 15 mg/kg. There was no prenatal mortality or fetal body-weight effect at 15 mg/kg, but three fetuses in one litter (of four litters exposed) had omphaloceles [80]. A gavage study in rats dosed on gd 8–15 at 25 mg/kg per day resulted in no treatment-related malformations [7]. An inhalation study of dichlorvos vapour at 0.25–6.25 μg/1 throughout gestation in rats did not produce terata even with a 90% maternal mortality rate at 6.25 μg/1 [137].

Dichlorvos given by gavage to rabbits over gd 6–16 resulted in no teratogenicity [31,147,148,151]. Vapour exposure of rabbits during organogenesis also produced negative results [117,137].

In a 2-year study in rats, with a reproductive component, dichlorvos was administered in the diet at 100 or 500 ppm. There were no effects on the number of litters or on litter size and no malformations were observed in the offspring [155,156]. Male and female pigs were given dichlorvos in the diet at 200 to 500 ppm for up to 36 months without effects on the number, survival or growth of the offspring and no malformations [37,123].

Darrow [40], evaluating the efficacy of dichlorvos-impregnated resin neck collars against biting lice in goats, reported no adverse effects on nannies or kids, and no effects on ChE.

Dichlorvos-impregnated resin plastic strips were evaluated for reproductive effects in a mouse breeding colony. With reduced plasma BChE and dichlorvos concentrations of 1.9 or 4.6 mg/m^3, there were no effects on litter frequency or litter size [33].

Administration of Cygon 4E (47% dimethoate) to rats by gavage on gd 6–15 at 3 or 6 mg/kg per day was neither embryotoxic nor teratogenic; but at 12 or 24 mg/kg per day the incidence of minor abnormalities was increased, unassociated with any embryotoxicity [78]. In cats, gavage on gd 14–22 at 3 or 6 mg/kg per day resulted in no developmental toxicity; at 12 mg/kg per day polydactyly was observed in the absence of embryotoxicity [81].

Spyker and Avery [127] exposed pregnant mice to diazinon and evaluated the offspring in a behavioural test battery. They reported no effects on general growth and development, open field activity, swimming posture or pattern, auditory startle response, visual placement or olfactory discrimination. No audiogenic seizures were observed. Neuromuscular performance was inhibited in the inclined plane test and rotorod test, while swimming activity and cling endurance were increased. Running speed performance in a maze was impaired but with no increase in frequency of errors.

Dimethoate in the drinking water to five generations of mice at 60 ppm resulted in no effects on litter size, pup weights or malformation incidence [26].

In rats, bromophos-ethyl was not teratogenic [146]. Demeton, given ip at 7–10 mg/kg per day on gd 7–12 in mice, resulted in reduced

fetal body- weight and 'mild' teratogenicity. Mice given demeton ip on gd 7–9, 9–10 or 9–11 at 10 mg/kg per day produced a slightly increased incidence of minor skeletal anomalies [25].

Fenthion given to mice at 60 ppm in drinking water for five generations produced no effects on litter size or teratogenicity [25,26]. However, a single ip injection in mice at 40 or 80 mg/kg on gd 7–12 caused reduced fetal body-weight and increased incidence of malformation, in the absence of increased prenatal mortality. Fenthion, administered orally to rats at 5 mg/kg per day on gd 1–8 or at 10 mg/kg per day on gd 7–10 was embryotoxic but not teratogenic [62].

Formothion given orally to pregnant rabbits at 6–30 mg/kg per day on gd 6–18 was neither embryotoxic nor teratogenic [81].

Malathion given to rats for two generations in the diet caused reductions in neonatal viability and growth at 240 mg/kg per day [75]. Rats given malathion orally throughout gestation at doses of 0.1–100 mg/kg per day [84] or on gd 6–15 at 50–300 mg/kg per day gave negative results [77], as did 600 or 900 mg/kg ip on gd 11 [80]. When malathion was given by gavage to rabbits at 100 mg/kg on gd 7–12, marked inhibition of ChE activity was observed in maternal and fetal blood and in fetal brain, but without effects on resorptions, fetal weight or fetal external or visceral malformations [89].

Azinphos-methyl given to rats and mice at 1.25, 2.5 or 5.0 mg/kg per day po on gd 6–15 was not teratogenic [121].

Parathion-methyl given ip to mice on gd 10 produced no effects at 20 mg/kg but an increased incidence of prenatal mortality and cleft palate was observed at 60 mg/kg [136]. Cleft palate may not represent a teratogenic response *per se* since maternal 'stress' in mice results in cleft palate in the offspring. In rats, a single ip injection on gd 12 of 5, 10 or 15 mg/kg, produced neither embryotoxicity nor teratogenicity but did cause reduced fetal body-weights at 15 mg/kg [136]. Rats given parathion-methyl at 0.1–10 mg/kg per day po produced prenatal mortality [84].

Parathion-methyl was administered orally to pregnant rats and the offspring were evaluated for behavioural deficits. No adverse effects were observed for reflex behaviours, startle response, passive avoidance or rotorod test [67]. However, behavioural and neurological dysfunctions have been reported in offspring after *in utero* exposure to parathion-methyl in rats [39,68,105].

Oxydemeton-methyl was given by gavage at 0–4.5 mg/kg per day on gd 6–15 in a three-phase study. In Phase I, dams were killed on gd 16 for ChE measurements; dose-related reductions were observed in maternal plasma (30–72%), RBC (18–56%) and brain (21–68%). Phase II females were killed on gd 20 for evaluation of fetuses; there were no significant effects on fetal brain ChE and no embryotoxic, fetotoxic or teratogenic effects, in the presence of maternal toxicity (tremors, reduced food consumption and weight gain). Phase III females were killed on day 21 postpartum, with offspring retained until day 44 postpartum. There were no effects on neonatal survival, growth or development, or in an extensive neurobehavioural testing battery [35].

Parathion given to pregnant mice caused no adverse effects on conditioned avoidance response or open field behaviour in the offspring, but there was a transient increased sensitivity to induction of audiogenic seizures [1–3]. Parathion administration to pregnant rats caused behavioural and neurological dysfunctions in the offspring at approximately 1 mg/kg per day [51,80,100,135].

Phosphamidon given to pregnant mice produced maximum embryotoxicity after administration on gd 7 or 13 (with little effect on gd 10) [22]. Phosphamidon (Dimecon) administered to male mice by gavage, ip or sc injection at 3–5 mg/kg, once daily for 5 days, produced sperm shape abnormalities, chromosomal aberrations and increased incidence of micronuclei [16].

Phosmet, at high dietary doses to rats during gestation, caused no increased incidence of malformations [128] but 1.5 mg/kg po daily throughout pregnancy or 30 mg/kg po once on gd 9 resulted in postimplantation loss and increased incidence of developmental abnormalities [91].

Intrajugular injections of crufomate into pregnant cows (8.8 g) resulted in no fetal malformations [111].

Trichlorfon has been evaluated in a number of species by various routes. In rats, 80 mg/kg

by gavage on gd 9 or 13 produced embryotoxicity and teratogenicity, but 8 mg/kg per day throughout gestation also by gavage caused no developmental toxicity [91]. Trichlorfon in rats by gavage on gd 6–15 at 175–519 mg/kg per day resulted in no teratogenicity even at maternally toxic levels [128]. At the same doses in the diet during the same period, 375 mg/kg per day resulted in minor skeletal aberrations; 432 and 519 mg/kg per day caused major external and skeletal malformations [128]. If rats were exposed to 480 mg/kg per day by gavage during organogenesis, malformations were also observed [129]. Exposure of rats to 0.1–10 mg/kg per day by gavage during the entire gestational period caused prenatal mortality so that newborn litter size was reduced [84]. Exposure of rat dams resulted in reduced AChE activity in offspring [131]. Inhalation exposure of rats to 0.005 or 0.02 mg/m^3 resulted in fetal skeletal defects; at 0.2 or 9.0 mg/m^3, histopathological changes in the placentae were observed [64]. Administration of trichlorfon to golden Syrian hamsters by gavage at 400 mg/kg three times per day on gd 7–11 resulted in malformations but not at doses of 100–300 mg/kg per day [129]. Trichlorfon was administered to pregnant guinea pigs by gavage at 100 mg/kg on gd 36–38 or gd 51–53. Offspring developed locomotor disturbances and post-mortem examination revealed significantly reduced brain weights. Histological examination of the cerebellum indicated reduction in the external granular layer and regional absence of Purkinje cells. Choline acetyltransferase and glutamate decarboxylase activities were reduced [18– 20]. When radiolabelled trichlorfon was given by gavage on gd 37 or 52 to pregnant guinea pigs, fetal uptake was observed after 30 min with greater uptake later in gestation [21].

Two mouse strains were given trichlorfon at 3–10 mg/kg ip; tooth malformations were observed in the AB Iena/Halle strain but not in the C57BL strain [61]. At 360 mg/kg ip in mice, no major fetal malformations were observed, but embryotoxicity was produced, with the AB Iena/Halle strain more sensitive than the C57BL or DBA strains to induction of embryotoxicity [114].

Also with mice, trichlorfon was administered by gavage at 300–600 mg/kg per day on gd 6–10 with no malformations produced; after dosing on gd 10–14, both 500 and 600 mg/kg per day produced malformations, predominantly cleft palate [129]. An outbreak of congenital tremor in piglets from sows treated orally during pregnancy with an antiparasitic trichlorfon compound (Neguvon) was reported in Sweden. All examined piglets exhibited marked cerebellar hypoplasia [24].

Chemical warfare agents

Sarin, formulated as Type I with a stabilizer and Type II without a stabilizer, was evaluated for developmental toxicity in both rats and rabbits. Sarin Types I and II given by gavage to rats on gd 6–15 at 0–380 μg/kg per day did not produce embryonic or fetal toxicity, nor teratogenicity, even at the top dose which produced maternal mortality (28%) [8,9]. Rabbits given sarin Type I or Type II by gavage on gd 6–19 at dose of 0–15 μg/kg per day showed no embryonic or fetal toxicity and no teratogenicity; the high dose produced significant maternal mortality [10,11].

Soman given to rats on gd 6–15 by gavage at 0–165 μg/kg per day produced maternal toxicity at 75–165 μg/kg per day with a 47% maternal mortality rate at 165 μg/kg per day. There was no embryonic or fetal toxicity, and no teratogenicity [12,13a]. Soman given to rabbits on gd 6–19 by gavage at 0–15 μg/kg per day produced maternal toxicity (mortality) at 5–15 μg/kg per day; there was no indication of embryonic or fetal toxicity and no teratogenicity [13,13a].

Tabun was given to rats by gavage at 0–300 μg/kg per day over gd 6–15. Maternal mortality was observed at 75 (3%), 150 (6%) and 300 (34%) μg/kg per day; maternal weight gain was reduced at 300 μg/kg per day. There was no embryonic or fetal toxicity observed and no teratogenicity [50]. In rabbits developmental toxicity was not observed even at maternally toxic doses [50a].

A single sc dose of DFP, 1.1 mg/kg, was administered to rats during pregnancy and brain AChE measured in dams, fetuses and placentae on gd 18–19 and in dams and neonates 1–10 days after dosing. Maternal brain levels were most severely affected, neonates were less affected and recovered quickly (within 48 h); fetuses were least affected and recovered the most rapidly (within 24 h).

Inhibition in weaning rats was intermediate between newborns and adults [23].

Other

Tricresyl phosphate was given by gavage to male Long Evans rats at 0, 100 or 200 mg/kg for 56 days and to female rats at 0, 200 or 400 mg/kg for 14 days before mating and during the 10-day mating period. Females were dosed through pregnancy and lactation until litters were weaned on postnatal day 21; at weaning, females and pups were necropsied. Sperm concentration, motility and progressive movement were decreased in males at 200 mg/kg. Abnormal sperm morphology was observed in males at 100 and 200 mg/kg. Mating was not affected but the number of live litters was reduced; litter size and pup viability were decreased at 400 mg/kg. Pup body-weights and developmental landmarks were unaffected. Histopathological changes were observed in testes and epididymides of the males and in ovaries of the females [29].

Dominant lethal assays on OPs

Several OP pesticides have been evaluated for dominant lethal effects in mice. Azinphos-methyl was given both acutely [5] and subchronically (7 weeks) [74] by ip injection with negative results. Dimethoate also given ip, produced positive results after acute administration and equivocal results after chronic dosing [63]. Dichlorvos given by acute gavage at 5–10 mg/kg or acute ip injection at 5–16 mg/kg, produced negative results [57]. A 4-week inhalation exposure to dichlorvos at 2.1–5.8 mg/l, 23 h per day, or at 30–50 mg/l, 16 h per day, also produced negative results [43,44]. In a rare study method females were exposed to dichlorvos by inhalation at 2–8 mg/l for 5, 10 or 15 days and mated to unexposed males with negative results [42]. The same authors also gave dichlorvos to male mice by gavage at 25–50 mg/kg per day for 2 weeks with negative results. Acute ip injection of dichlorvos to male mice at 10 mg/kg was also negative, as was subchronic (5 days per week for 7 weeks) intubation administration of 2 ppm [98]. Chronic dietary administration of malathion, parathion-methyl or parathion was also negative [74]. Trichlorfon, administered acutely to male mice, resulted in increased preimplantation loss in two of the seven mating weeks assayed [99]: this OP was also administered by acute ip injection to mice at 176–405 mg/kg [49,60,115], or for 5 weeks by daily ip injection at 54 mg/kg per day; both with positive results [115]. However, acute ip injection of 100 mg/kg [98] or of $3–4.5 \times 10^{-2}$ M [15] produced negative results, as did 0.5 ppm by gavage for 7 weeks, 5 days per week [98] or 5×20 mg/kg ip for 2 weeks [48].

Human exposures

Human exposure data relevant to reproductive and developmental outcomes from antiChE exposure are few and confounded by absence of identification of the agent, exposure to multiple agents and/or lack of any measure of exposure level, and apparent exposures after organogenesis is completed. In addition, case reports document exposure(s) and outcome but do not and cannot provide evidence for cause-and-effect relationships.

A woman, 5 months pregnant, was acutely exposed to demeton-methyl and required intensive care; she gave birth to a normal child who has shown normal morphophysiological and psychomotor development [30]. A review of infants and children exposed to OPs and CBs reported acute toxic symptoms, with a full recovery with therapy [160].

There is some unsubstantiated evidence associating maternal insecticide exposure, particularly OPs and human fetal wastage [113]. One report, not well documented, indicated malformations of the extremities and fetal death in offspring of 18 women acutely exposed to very high levels of parathion- methyl [102]. Maternal exposure to malathion during the 11th and 12th weeks of pregnancy was followed by the birth of a severely malformed infant who died neonatally [86]. One published report of a large study failed to show any correlation with antiChE insecticide exposure during gestation and human fetal wastage [101].

Wyrobek *et al.* [157] reported an increased incidence in sperm shape abnormalities in employees exposed to carbaryl, but there was no assessment of reproductive outcome.

Although the above studies do not present a strong case for human risk for developmental or reproductive toxicity from exposure to antiChEs, human pregnancy may represent a time of increased risk to such agents because there are declines in serum BChE activity in women during pregnancy, more profound in younger mothers [59], there are genetic deficiencies in serum ChEs, and many neuroactive drugs, such as suxemethonium (succinyl choline chloride) (used during caesarean section) and benzodiazepines, produced lowered peripheral ChE levels. Any of these pre-existing conditions superimposed on an acute exposure to environmental antiChEs may pose an especial risk during pregnancy.

Summary and conclusions

Carbaryl can be teratogenic in fish and fowl embryos. A mechanism for one category of chick malformations, micromelia and abnormal feathering, involves CB-induced inhibition of the enzyme kynurenine formamidase which interferes with NAD biosynthesis in the embryo [95–97]. Teratogenicity testing in mammals gives almost uniformly negative results for carbaryl except for terata produced in one dog study and one rabbit study. In both studies, doses producing terata were also maternally toxic. Cranmer [38] argues that the terata observed in the dog were 'most likely due to non-specific maternal toxicity rather than specific teratogenic mechanisms'. Reproductive effects are observed in both male and female animals if the doses are high enough, with the testis and ovary as possible targets. Developmental toxicity (but no teratogenicity) may accompany maternal toxicity and is usually only observed at high doses. The limited database for other CBs indicates no teratogenicity.

Carbaryl crosses the rodent placenta, albeit slowly and at low levels, with persistence in the conceptus. ChE levels in various fetal tissues are transiently reduced from *in utero* exposure, with ChE levels in dams being more affected. What impact, if any, this transient inhibition has on postnatal development is not clear; certainly general growth and development are only affected at high doses of CBs. Specific tests for neurofunctional development have not been performed in test animal offspring after *in utero* and/or postnatal exposure to CBs. This is an important avenue of future research and testing. Human CB exposure data relevant to reproductive or development toxicity are almost completely lacking. Many OPs are teratogenic in avian and fish embryos. The mechanism of teratogenesis in birds (at least for abnormal feathering and micromelia) may be related to reduced embryonic NAD levels from inhibition of kynurenine formamidase [58,97,119], as with CBs, and/or altered levels of available ACh resulting in NM blocks during development [83]. Teratogenicity testing in mammals has indicated two diametrically opposed types of results. Many studies in rats, rabbits and hamsters report negative results, although developmental toxicity was observed at maternally toxic doses. In contrast, terata have been reported from *in utero* exposure to OPs in rats, mice, hamsters, rabbits, horses and cats.

OPs cross the placenta in rodents. Embryonic and fetal ChE levels are transiently reduced at high doses *in utero* with fetuses least affected, young postnatal animals more affected, and adult animals most affected after *in utero* and/or postnatal exposure. Postnatal sequelae of *in utero* exposure apparently do not include effects on general growth and development, but may or may not include effects on neurofunctional development or behavioural deficits accompanied by histological lesions in the brain.

Human developmental or reproductive toxicity data on OPs are sparse, essentially anecdotal, and confounded by concurrent exposure to other agents.

Perhaps the most important area of future research, especially as it relates to potential human risk, is that of developmental neurotoxicity and *in utero* and/or early postnatal exposure. The antiChEs are appropriate for such evaluation since they are biologically active and their target, AChE, plays a major role in the function (and development?) of the nervous system. The CBs, by nature of their acute and transient effects on antiChEs may be less likely to affect the developing CNS, but a developing system has been considered, *a priori*, more vulnerable than the mature system and a transient effect at a critical time

period may produce permanent effects. The OPs have been shown to affect behaviour sometimes with accompanying CNS lesions. The US EPA is beginning to require developmental neurotoxicity evaluations as mandated by Test Rules and by testing guidelines [106].

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24

Agricultural and veterinary toxicology of anticholinesterases

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Insecticides in agriculture and veterinary medicine are economically necessary to control insects that reduce productivity in livestock, eat crops and carry diseases. The antiChEs are the most commonly employed; because of the magnitude of their application they are the chemicals most frequently associated with toxicity to domestic animals and wildlife. The specific antiChEs applied vary with the need, and formulations depend on the animal species involved, the conditions of use, and the unique factors that determine the desired effect and most effective application procedure [7].

AntiChE use on domestic animals

Extensive exposure to antiChEs occurs in beef cattle raised for rapid weight gain and meat production. Breeding animals are sprayed with OP or CB insecticides several times yearly. At least twice yearly, adult cattle are exposed to systemic OPs that penetrate the skin and control internal parasites. Young cattle are treated simultaneously until weaned and placed on pasture for early rapid development. After several months, the growing calves are moved to feedyards where confined rearing and feeding leads to increased parasite and insect infestations, resulting in frequent use of topical OP or CB insecticides.

Dairy cattle are a special problem as the production of milk mandates that residues be stringently avoided. Rapidly biodegradable antiChEs are limited to young cattle being raised for eventual milk production, and are used no later than 2 months before milk production begins. Nevertheless, accidents or errors in management occasionally result in residues appearing in human food products.

Sheep and goats are treated similarly to cattle but antiChE use is considerably less. OPs and CBs are used minimally in swine.

AntiChEs may be sprayed directly on horses and applied as mists in stalls, and selected OPs are dosed orally several times yearly to control internal parasites. Formulations are available for feeding tetrachorvinphos to horses continuously to prevent fly larvae hatching in faeces.

Internal ChE parasite medication, and flea and tick control agents applied externally, are extensively and frequently used for dogs. Concern about fleas induces owners to apply OPs around kennels and in the home, exposing both pets and humans. CBs, particularly carbaryl, are frequently used for cats. External parasite control is less common in cats, but often coupled with OP use in the house to reduce flea infestation. In dogs and cats, insecticide toxicity is the most common cause of poisoning, and in cattle is the second most frequently cited toxicity. Insecticides rank in the top two or three most commonly cited chemical poisonings of horses, swine, and sheep and goats. OPs are the most common insecticide intoxicators observed in domestic animals [6].

Circumstances of domestic animal antiChE exposure

In the $18 billion USA livestock industry, 0.1% loss is from pesticide use; i.e. $18 million per year [4]. Despite the lack of specific statistics, it seems certain that poisoning is responsible for a higher proportion of unintentional

mortality in domestic animals than in man. Thus, animals have a greater exposure, and have a shorter lifespan, making them less liable to causes of death such as degenerative disease and cancer. Animals will be injured from intentional application of insecticides at a greater rate than humans because treatment of animals is more common and aggressive. Efficiency of operation with uncooperative larger animals often necessitates using large scale spraying or dipping. Specific dosages and application rates are often generalized, and weak or highly susceptible individuals in a group are at high risk.

The greatest cause of domestic animal poisoning is the carelessness of individual farmers and livestockmen. Sometimes carelessness or errors in formulation, dosage calculation, or application methodology leads to poisoning of a large number of animals on a single farm. This is not unexpected with livestock, particularly cattle [6]. The poisoning of household pets is more associated with the pet owners over-zealous efforts to control insects.

Large-scale insecticide use

Consolidation of agricultural practices and the mass production of food through larger livestock animal units may lead to agricultural operations containing thousands of animals. If only a portion are treated with insecticides, several hundred may be exposed at one time. Errors in mixing, dosage calculation or selecting the correct chemical are compounded since many animals are at risk from one episode of misuse.

Large-scale agricultural operations also attempt to utilize efficiently equipment and movement of animals. Large amounts of a spray may be mixed and applied to as many animals at one time as possible. Mixing of breeds and age groups presents considerable hazard if breeds genetically sensitive to certain OPs are processed at the same time. A uniform concentration of insecticide causes a greater risk for young animals when sprayed together with large numbers of older cattle. Large-scale agricultural operations increase efficiency, but increased hazards may also result if attention to detail is overlooked.

Lay persons with no training using antiChEs

Shortly after the introduction and extensive employment of toxic OPs in agricultural practices, it became apparent that considerable training was necessary for their appropriate use. Training programmes were developed and continue to be available, but the extensive use of insecticides in agriculture still results in untrained persons utilizing them at risk.

A common fallacy is the notion that since a spray was used for several years, 'it' can continue to be used in the same way without concern. Although labels indicate specific formulations and companies usually notify distributors of chemical ingredient, formulation and usage recommendation changes, the familiarity of the lay person with using a certain insecticide often results in a lack of appreciation of the significance of these changes. The results have been devastating to humans and animals alike; but the high mortality and more stringent and enforceable regulations have resulted in a growing awareness of the potency of the current compounds. Careful instruction and training of persons mixing and applying pesticides is vital.

Carelessness

The majority of insecticide problems in domestic animals result from ignorance or mismanagement. Insecticides mistaken for mineral supplements have been mixed directly into animal feeds. Insecticides may be stored near the normal rations of animals and a lack of verifying the contents of containers results in contamination. Unlabelled containers have been a frequent cause of trouble. In most cases compound misuse has resulted from ignorance and failure to read and understand labels.

Agricultural toxicities from antiChEs

Exposure in agricultural use usually produces acute toxic effects. Clinical signs appear within hours after exposure. With rare exceptions, attending veterinarians encounter situations of several dead animals with numerous others showing clinical signs typical of massive overexposure [14].

A major difficulty is early and accurate diagnosis of the aetiology. Circumstances may direct attention to a recent chemical application or a new feed. In some instances, the history of exposure is misleading and diverts attention away from less obvious chemical exposures. Astute evaluation of clinical signs, prompt autopsy of recently dead animals, and early decisions to remove potential sources of exposure prevent additional absorption, and observation of the effects of early therapy can be highly successful in reducing losses [9]. The trial administration of a massive dose of atropine to a suspected antiChE-poisoned animal can be of diagnostic significance within 2–5 min. Triage techniques are effectively used in agricultural situations to gain the most effective therapeutic advantage for the recovery of the patient and the economic benefit to the owner.

An important and potentially vital legal action is obtaining appropriate tissue and environmental samples for toxicological assay. Definitive proof of cause is the identification of a specific pesticide and the quantitation of concentrations in the appropriate biological or environmental samples [10].

Clinical toxicities of antiChEs

OP and CB insecticides have grown in popularity for control of ectoparasites on companion animals and livestock, as agricultural pesticides for crop protection, and in homes and gardens for elimination of insect pests. Their lack of persistence in the environment and reduced residues in animal products have made them more acceptable to the public and government regulatory agencies. Systemic insecticides are highly toxic and frequently produce acute poisoning in ruminants. Representative OPs of this group are parathion, coumaphos, malathion, diazinon, dichlorvos, phorate, fenchlorphos, famphur, terbufos, chlorpyrifos, fenthion and trichlorfon. CBs including carbaryl, aldicarb, propoxur, and carbofuran are examples of this type of insecticide.

Depression of whole blood ChE activity may not always correlate with the degree of depletion of ChE in the nervous system and other tissues. Thus, an animal may, on occasion, show signs of poisoning with only a slight depression of blood ChE or may show no signs of poisoning and yet have complete inhibition of blood ChE activity. Nevertheless, in many cases, inhibition of blood ChE can generally reflect the situation at nerve endings and has diagnostic value. Plasma BChE tends to drop rapidly following exposure to an antiChE insecticide, and returns to normal faster than RBC AChE.

Signs of antiChE poisoning in animals include profuse salivation, gastrointestinal hypermotility (severe pain and abdominal cramps), diarrhoea, excessive lacrimation, sweating, dyspnoea with rales, miosis, incontinence of urine and faeces, fasciculation of the muscles of the face, eyelids and general musculature, followed by weakness and paralysis of these muscles. Stimulation of the CNS is followed by depression. Death results from bronchoconstriction, paralysis of the respiratory centre, and/or excessive accumulation of fluid in the lungs. Bradycardia and heart conduction blockade can also occur. The interval between exposure and signs of toxicity is usually short, ranging from minutes to hours. Some animals have a gradual onset of signs while others show explosive development.

The signs of CB toxicity are shorter in onset and are more exaggerated. They appear within only a few hours after exposure, and the progress of the disease is very rapid. If absorption of CBs is rapid, the onset of clinical signs may be followed by death within 1–2 h. With a non-lethal exposure, the animal may recover spontaneously within a few hours of the onset of signs. Ruminants may have longer-lasting clinical signs because absorption of CB from the large volume of the rumen may continue over a longer period of time.

While the clinical effects of antiChE toxicity are often not long lasting, ChE inhibition may be. That from OPs persists for several days to weeks, but CBs inhibit for only several hours, once absorption is complete.

Clinical effects in domestic animals

The onset of toxicity is rapid, usually 1–3 h after exposure, and often ending in death 3–24 h later. The signs include urination, increased peristalsis reflected as colic, and 'patchy' sweating, particularly of the skin of the neck, shoulders and ribcage. Salivation may be moderate to profuse, and parasympathetic stimulation produces defaecation, frequent

urination and a general sense of anxiety or uneasiness. Respiratory efforts become exaggerated and the animal may develop severe abdominal pains. A stiff-legged gait occurs as the syndrome progresses and leads to muscle tremors of the face, neck and general body muscles. The muscle hyperactivity from antiChEs do not characteristically develop into convulsions; rather, it is generally followed by muscle weakness, incoordination and ataxia, and prostration as the animal is unable to control its muscles. Respiratory dysfunction and paralysis are extreme signs of this. Bronchoconstriction and pulmonary oedema complicate respiratory efforts, and respiratory muscle weakness leads to difficult, frequent and shallow respiratory efforts. Death is from anoxia from bronchorrhoea, poor pulmonary ventilation and irregular bradycardia.

Some newer antiChEs can produce variations in this clinical syndrome. All signs may not be seen in any one animal, but several are usually present. In all instances, however, terminal muscle weakness and respiratory dysfunction are severe. Blood ChE activity levels may be useful as a diagnostic aid.

Interactions of OP and CB insecticides with other chemicals affecting the same enzyme systems are possible, and may result in additive and sometimes synergistic clinical effects. Phenothiazine derivatives, such as the promazine tranquilizers, potentiate the effects of antiChE insecticides. Suxamethonium*, carbachol, physostigmine or neostigmine are contraindicated if animals have recently been exposed to antiChE insecticides. Significant ChE inhibition persists for at least 14 days following OP exposure, and at least 30 days or more are required before blood ChE returns to normal. The effects of CBs are much shorter, but interaction with other antiChEs is still possible if exposures are within a few days.

Post-mortem lesions in antiChE poisoning are non-specific. Excessive pulmonary fluid and excessive fluid in the mouth and digestive tract are supportive, but not confirmatory. In some animals the excessive peristalsis will result in pooling of the blood in 'bands' in the small intestinal tract mucosa, and 1–7 cm wide areas of the mucosa will appear hypaeremic owing to underlying muscle contractions. The bladder may be empty from excessive urination, and liquid faeces may be present in the colon. Final confirmation of death from antiChEs depends on the detection of significant plasma, brain, liver or kidney concentrations of the suspected chemical. Excessively depressed brain, plasma or RBC ChE activity is also supportive.

*Succinylcholine (USP)

Management of antiChE toxicity in domestic animals

Because of anatomical and physiological differences between the various species of domestic animals, the therapeutic management of poisoning varies with the species affected. The use of emetics is limited to dogs, cats and pigs. The horse physiologically does not vomit, while the rumen of cattle, sheep and goats limits emetic action. The effect of adsorbents, e.g. activated charcoal, is markedly reduced in ruminant animals owing to the large volume of rumen content. Laxatives in ruminants are slow to work owing to the large volume of the rumen which must be bypassed before there is any effective intestinal or colon action [12].

Where antidotes are indicated, the size of the large domestic animal necessitates massive amounts of agent. Atropine is used in 100 ml or more quantities for each antiChE-poisoned cow. One g (one vial) of 2-PAM is only sufficient for one dose to the average horse. An OP poisoning outbreak involving several horses or cattle would quickly deplete the available stock [11]. The mechanics of managing the antidotal treatment of groups of poisoned poultry, swine or sheep is manpower intensive because of the number involved. The treatment of antiChE poisoning in domestic animals, particularly large animals, is principally controlled by the number of poisoned animals and the innovative ability of the treating veterinarian. Washing a herd of 100 or more cattle topically exposed to fatal doses of an insecticide is a large-scale operation!

General treatment

Principles of supportive therapy

The basis of supportive therapy is aimed at preventing continued absorption of the

antiChE and assisting the normal biotransformation. Digestive tract lavages and laxatives are utilized to remove unabsorbed material rapidly from the digestive tract. Fluid therapy is intended to maintain and aid kidney function and urine excretion. The maintenance of cardiac and respiratory function is vital. Prevention of further absorption of the toxic chemical may begin by telephone instructions to the owner following his call for help. If the skin is contaminated, the owner should be instructed to wash the animal with large volumes of water. Ingestion in small animals may be counteracted by promptly inducing vomiting; a teaspoon of salt in the back of the animal's mouth or, more reliably, 1–3 teaspoonfuls of hydrogen peroxide orally.

Because most animals are poisoned by mouth, further reducing absorption in the hospitalized small animal patient is accomplished by the use of emetics, gastric lavage, adsorbents and laxatives. Apomorphine (0.04 mg/kg iv) usually is effective in about 1 min; up to 5 times that dosage may be given im or sc. Xylazine (1.0 mg/kg im) is an effective emetic in the cat. Syrup of ipecac po is also a good emetic (10–30 ml) in cats and dogs. Gastric lavage is useful. Animals should be anaesthetized and a cuffed endotracheal tube inserted to prevent aspiration. The addition of 5–25 g of activated charcoal to the lavage fluid is useful. Several washes should be made, each at least 250 ml. After the last wash, a thick slurry of activated charcoal (200 ml volume) may be left in the stomach. To remove toxin from the small intestine, laxatives may be administered. Sodium sulphate or magnesium sulphate are given at the dose of 2–25 g orally as 20% solution; they do not aggravate CNS depression and are less likely to promote removal of poison from activated charcoal. Mineral oil or milk of magnesia (2–15 ml po) are excellent milder laxatives and protectants. A colonic lavage may be conducted, but extensive and thorough complete digestive tract cleansing will be effected by a through-and-through enema; it should be administered gently, but is effective and often life-saving.

Symptomatic and supportive therapy is one of the most important factors contributing to recovery. Initial efforts should be aimed at maintaining vital functions, particularly respiration and cardiovascular activity. Positive respiratory assistance is frequently all that is needed to assure sufficient time to institute therapeutic procedures and immensely increases the likelihood of recovery. If controlled respiration can be maintained, the majority of intoxicated animals will spontaneously recover. Body temperatures should be controlled at normal with blankets or heating pads or, in the case of hyperthermia, with cold baths or ice bags. If body temperatures are depressed, important detoxifying processes are also reduced. By maintaining adequate respiratory function and body temperature, cardiovascular function is also usually adequately maintained, assuring good organ perfusion and continued urinary output. Shock is thus avoided. When partial or complete anorexia occurs, as an aftermath of OP or CB toxicity, injections of B-complex vitamins are indicated.

Emergency therapy

Maintain vital functions

This includes establishment of a patent airway, artificial respiration, cardiac massage, and if needed defibrillation techniques. Following stabilization, additional therapeutic measures may be undertaken.

Induction of emesis

Syrup of ipecac is a good general emetic. The dose of ipecac for small animals is 1–2 ml/kg. It is about 50% effective initially but can be repeated in 10–15 min. Apomorphine is an effective and reliable emetic (0.04 mg/kg iv, or at least 0.08 mg/kg im or sc). It may cause respiratory depression and protracted emesis. These can be effectively controlled with narcotic antagonists given iv: naloxone, 0.04 mg/kg; levallorphan, 0.02 mg/kg; or nalorphine, 0.1 mg/kg. Apomorphine is contraindicated in patients with CNS depression.

Contraindications for induction of emesis are unconscious or severely depressed animals. If more than 2–3 h have elapsed most of the toxicant may have passed to the duodenum, but emesis may still be of benefit. Some agents weaken the gastric wall which could then rupture during forceful emesis.

Gastric lavage

This is a reliable early technique. The animal should be unconscious or under light anaesthesia. A cuffed endotracheal tube should be inserted with the distal end protruding 1 cm beyond the teeth. The head and thorax should be lowered slightly. The volume of lavage solution used for each washing is 5–10 ml/kg. An infusion and aspiration cycle of the lavage solution should be repeated several times. Activated charcoal in the solution will enhance the effectiveness.

Adsorbents

Activated charcoal is the best adsorbing agent. A slurry of charcoal in water (2–4 g/kg; 1 g charcoal in 3–5 ml water), is given by stomach tube, and 30 min later a cathartic. The universal antidote of activated charcoal, magnesium oxide, and tannic acid is ineffective because the magnesium and tannic acid decrease the adsorptive capability of the charcoal. Burned or charred toast is ineffective.

Cathartics

Sodium sulphate (1 g/kg) is slightly more efficient than magnesium sulphate. Mineral oil or vegetable oils are of value, but should be followed by a saline cathartic in 30–45 min. A colonic lavage or high enema may hasten elimination from the gastrointestinal tract. Warm water with soap makes an excellent enema, or several commercial enema preparations are available which act as osmotic agents.

Elimination of absorbed poisons

Urinary excretion may be increased by diuretics or by altering the pH of the urine. Monitoring of urinary flow is essential, and minimal urinary flow of 0.1 ml/kg per min is necessary. Diuretics of choice are mannitol (2 g/kg per h) and frusemide (furosamide; 4 mg/kg).

Respiratory support measures

A patent airway may be obtained with a cuffed endotracheal tube or a tracheostomy. A respirator is of value, but an anaesthetic machine may be used with manual compression of the bag. A mixture of 50% oxygen and 50% room air, or 100% room air, is generally adequate. Positive pressure ventilatory support is of most benefit.

Cardiovascular support

Cardiac activity can be aided by closed-chest cardiac massage for emergencies, but the administration of agents which stimulate ionotropic and chronotropic activity must also be undertaken in some instances. Calcium gluconate is infused slowly iv; useful agents are glucagon, 25–50 μg/kg iv and digoxin, 0.2–0.6 mg/kg iv.

CNS depression

The iv administration of analeptic agents, such as doxapram (5–10 ml/kg), bemegride (10–20 mg/kg), or pentylenetetrazol (6–10 mg/kg) are short-lived and CNS depression can return if animals are not monitored continuously. Analeptics can also induce convulsions. Respiratory support is of greater value in animals exhibiting CNS depression.

CNS hyperactivity

Pentobarbitone* sodium is the agent of choice, but a respiratory depressing dose may be required to alleviate signs, when respiratory support is mandatory. Inhalant anaesthetics are excellent for long-term management of CNS hyperactivity. Central-acting skeletal muscle relaxants and minor tranquilizers have also been useful for convulsant intoxicants. Methocarbamol (110 mg/kg iv), glyceryl guaiacolate (110 mg/kg iv), and diazepam (0.5–1.5 mg/kg iv or im) are effective. The animals should be placed in a quiet dark location.

Drugs and equipment needed

The emergency nature of most antiChE poisonings requires that therapy be promptly administered. Specific equipment and adequate supplies of life- supporting drugs and antidotes must be available, and in a location that is always accessible and never depleted. Clinicians and staff should always know the location and availability of these life-saving items.

*Pentobarbital (USP)

Specific treatment

Exposure of livestock is usually to large quantities of insecticide, and the onset and progression of the poisoning are rapid, and mortality high. Effective treatment depends on rapid initiation of therapy. This is often not feasible because of inaccessibility of the animals and lack of immediate availability of antidotes. When the necessary drugs are at hand, effective specific treatment regimens are available. They involve providing respiratory assistance, and the use of atropine and oximes.

Treatment for OP toxicity

A series of actions are required, beginning with removal of the animal(s) from additional exposure. This might involve removing the contaminated feed source, or washing of animals if exposure occurred by spray application. Clinical signs are then treated by atropine at a dosage of 0.5 mg/kg of body-weight, but the total dose is best stated as 'to effect', which depends on the degree of AChE inhibition; larger quantities than recommended may be needed. Atropine should be given slowly iv, and the animals monitored for response. Sufficient atropine should be administered to be antidotal without depressing the CNS or other vital signs. Relief of parasympathetic signs occurs almost immediately with atropine, but muscular tremors are usually not fully controlled even with full atropinization. Domestic animals metabolize atropine at various rates, and affected animals must be continually evaluated and repeated dosages or atropine may be needed every 2–6 h.

Specific AChE regenerating oximes include 2-PAM, obidoxime and TMB-4. The dose is approximately 20 mg/kg and is usually given sc as a 2% solution every 12 h. Oximes are especially effective if given within 12–18 h of OP exposure. This therapy is less effective if enzyme ageing has occurred and clinical signs are advanced.

Finally, removal of unabsorbed insecticide from the intestinal tract must be accomplished as soon as possible. All species should be given at least 2 g activated charcoal/kg body-weight orally. Ruminants may require 3 or more g/kg to assure adequate distribution of charcoal in the large volumes or rumen content [13]. Even with this therapy, mortality may still occur if clinical signs are advanced before treatment was begun or if the dose of OP was very large. Treatment may be required for several days to maintain stable vital functions. Low blood ChE levels may not recover for several weeks; care is needed to avoid exposing the animal to other antiChEs during that time.

Treatment for CB toxicity

The treatment scheme is generally similar to that for OPs; removal of the animals from the source, prompt and aggressive atropine treatment, and emptying of the digestive tract followed by oral administration of 2–3 g activated charcoal/kg. Activated charcoal may be repeated every 6–12 h for more persistent effectiveness. Oximes are not routinely employed in CB poisoning since they may be ineffective and in some cases may actually increase toxicity. Owing to rapid reactivation of the AChE-carbamate complex and rapid biotransformation of the CB, treatment with atropine alone is sufficient in most cases [5,13]. Maintenance of vital functions by careful monitoring and appropriate administration of atropine and charcoal are critical in managing CB poisoning.

Unique aspects of antiChE use in agricultural and veterinary medicine

Use of systemic OPs

Of special interest to agriculturists is the increasing use of OPs formulated for systemic action in combating external insects and internal parasites of livestock. These are commonly used in cattle as 'pour on' compounds that are rapidly absorbed and produce a systemic insecticidal effect. Their use depends on the formulation producing rapid systemic absorption, diffuse distribution throughout body tissues, and effective broad-spectrum insecticide properties. They have the absorbability and tissue distribution of the fat-soluble chlorinated compounds while retaining the potency and shorter duration of action of the OPs.

The systemic OPs have special problems if not applied at appropriate times, if used in overdose, or if applied to animals specifically

sensitive to their properties [14]. They must be applied during the autumn when the internal parasites are in their most vulnerable migratory patterns. If applied too early, they are less effective; if applied late, the location of the parasite when attacked by the insecticide may produce serious and often fatal complications in the host animal. Because of the rapid absorbability and distribution of these compounds, their dosage is critical and overestimation of body-weight or too zealous an application may produce acute toxicity. If used in combination with other antiChEs potentiation may occur. A special problem has developed with the use of chlorpyrifos in mature male cattle. While the biochemical basis is still unclear, the use of this systemic OP in actively breeding bulls produces a high risk of acute toxicity with unacceptable mortality.

The widespread use of the systemic OPs in variable agricultural situations involving numerous breeds and conditions of livestock has produced a greater risk than with the classical OPs. Livestockmen using systemic OPs must appreciate their specificity or run the risk of unexpected poisoning and mortality in animals.

Multiple chemical use

The use of multiple agricultural chemicals on or around individual domestic animals within a short time frame is increasing [7]. It is not uncommon for several vaccination procedures, internal parasite control, and external insecticide applications to be given to groups of livestock within a 1–2 day period. These procedures stress the animal and could result in significant toxicity if pesticides are applied simultaneously.

The use of systemic OP insecticides in combination with CB sprays induces significant risk potential. Both antiChEs produce additive effects that may over-ride safety margins employed in recommended prophylactic or therapeutic dosages. Agricultural practices may include the use of antibiotics, some of which can alter protein binding or enzyme bioavailability, further introducing factors that enhance toxicity of applied chemicals. While efficiency of agricultural operations often suggests the feasibility of multiple chemical use, the risk of adverse reactions increases exponentially with the number of compounds applied simultaneously.

Delayed OP-induced neurotoxicity

Occasional instances of delayed neurotoxicity in livestock occurs from compounds previously not suspected of inducing such effects. These often involve highly stressed or weakened animals combined with excessive or repeated exposure to the OP. Ten to 14 days after exposure, weakness in the rear quarters occurs with degeneration of the spinal nerves resulting in partial or complete paralysis. These results do not occur consistently, but genetic differences, even between individuals of the same breed, and previous exposures producing neurological sensitivity are important in contributing to OP neurotoxicity [1].

The widespread use of pesticides in domestic animals, particularly livestock, and the variable management and environmental circumstances under which animals are held, produce a range of circumstance and biological variation that cannot be duplicated in the test protocol for chemicals before their licensing and distribution. These random toxicities, particularly the more subtle neurotoxic effects occasionally associated with antiChEs, create a special population of animal exposures and clinical experiences that serve as potential sentinels for exposures to humans under similar conditions. While widely recognized in the veterinary and agricultural fields, these experiences may not be fully appreciated by public health and regulatory officials.

The following OPs have induced delayed neurotoxity in various domestic animals [1]: Cyanofenphos, 2,2 dichlorovinyl methyl phosphate, DFP, EPN, haloxon, leptophos, mipafox, tri-o-cresyl phosphate, and tri-o-tolyl phosphate.

Differences between animal species

A singularly unique factor in insecticide use in domestic animals is the large number of different animal species to which they are applied. When contrasted with the single human organism, the multiple-species of domestic animals offer numerous anatomical, physiological and biochemical species differences that impact significantly on the ultimate effects of xenobi-

otics [8]. Although the anatomical and physiological species differences appear more obvious (Table 24.1), of greater significance are the biochemical differences expressed by variations in digestive tract, circulating and liver enzymes, and variations in other processes that produce changes in the detoxification mechanisms. Even biochemical differences between individual animals of the same species and breed may be expressed clinically as individual animal variations in sensitivity to chemical exposures.

Effect of the rumen

Monogastric animals (horse, swine, dog, cat) have stomachs physiologically and biochemically similar to man, but cattle, sheep and goats have a rumen which serves as a fermentation vat for converting cellulose forage into protein precursors through the digestive actions of microorganisms [12]. The rumen has a reducing environment with a pH varying from 5.5 to 7.5 depending on diet. Ruminants on high carbohydrate rations, as in feedlots, generate considerable amounts of acid from carbohydrate breakdown. Grazing animals have an almost neutral rumen pH as the cellulose is digested by the rumen microorganisms.

AntiChEs ingested by cattle, sheep or goats immediately pass to the rumen which may significantly modify the diffusibility of the chemical. Dilution of the pesticide is invariable in the 40–60 gallons of rumen content, where reductive biochemical action on the pesticide is rapid. In most cases the result is detoxification, although in some instances activation may occur. Absorption through the rumen wall is retarded until the parent chemical or metabolite comes in contact with the mucosa.

Table 24.1 Examples of anatomical and physiological differences between species

Simple acid stomach *versus* voluminous neutral rumen
Absorptivity in various regions of the digestive tract
Digestive tract length
Bacteria, enzymes in digestive tract
Excretory ability
Excretion modes available (milk, sweat)
Volume and pH of urine
Amount of body fat
Dietary constituents
Physical activity and other stresses

Insecticides may be held in the fluid portion of the rumen material for many hours [12]. Ruminant animals do not vomit, further varying the normal physiological process expected in monogastric animal.

Metabolism of the insecticide

Biotransformation of insecticides is largely a biochemical event governed by enzymes present in the digestive tract or liver. Unique species differences exist between the various domestic animals in their normal biochemical capabilities. The special reducing environment of the rumen of cattle, sheep and goats initially has a profound reducing capability on the ingested insecticide. On passing into the small intestine, the digestive tract enzymes of the ruminant or the monogastric animal may produce oxidizing or conjugating changes.

The biotransforming processes continue after absorption through the action of liver enzymes in converting the absorbed materials to water-soluble materials easily excretable in urine. The biotransforming enzyme activities of ruminants are especially high in sulphatase activity, while pigs have an especially high glucuronidase activity. Horses and dogs have a uniform capability of effective oxidative mechanisms leading to good sulphate and glucuronic acid conjugation capability, while cats are notoriously deficient in glucoronide formation ability, with a high sensitivity to insecticides normally detoxified and excreted by that biochemical pathway [8].

Sensitivity of the animal

The species or individual sensitivity to antiChEs may be genetically dependent on the presence or absence of biodegradating enzymes, or result from enzyme induction or inhibition [8]. The use of diazinon for controlling flies around ducks on Long Island led to the death of an estimated 15 600 birds. Diazinon had been used for years around chickens in the same area without problems, but the species sensitivity of ducks resulted in massive fatalities [3]. The high inherent sensitivity of bees to carbaryl was recognized early, and appropriate label warnings and restrictions on its use for bees has prevented disasters. Dorset Down sheep are inherently

susceptible to diazinon, as was clinically observed in a flock composed of three sheep varieties; with fatalities occurring only in the Dorset [15].

Previous exposure, particularly to fat-soluble insecticides, may produce enzymatic changes that significantly impact on biotransformation. Enzyme induction may increase metabolism to produce resistance to toxicity by detoxification pathways; if the path is activation, enhanced toxicity occurs. OPs may limit the available ChE activity and produce enhanced toxicity to concurrently exposed CBs. While these enzyme-inducing and enzyme-inhibiting effects may be present only several days to a few weeks following exposure, their biochemical effects significantly alter individual animal responses to exposures and clinically produce at least some of the 'individual animal variation' between individuals.

Tissue residues

Many antiChEs accumulate in food or feed following their application to livestock or the environment. Their widescale use on pasture may result in hay or feedstuffs growing on neighbouring fields with residues or these foreign chemicals. If proper precautions in marketing the exposed cattle are not followed, the meat and by-products from such animals may contain unacceptable residues.

The ultimate challenge in agriculture is the avoidance of residues in products intended for human consumption [2]. To this end, studies of storage and excretion are carried out in the environment, feeds and domestic animals during early product development. If properly conducted and applied, such studies provide guidelines and appropriate safety margins for the use of antiChEs in the environment and food-producing animals so that residues do not produce public health concerns.

Of practical interest is the continuing monitoring of meat and dairy products intended for human consumption [16]. These food basket studies assure that the use of insecticides in agricultural practice is conducted in accord with label recommendations and sound chemical application. The occasional misuse of such chemicals is usually quickly detected. The regulatory action taken in confining the contamination and eliminating its access to human foods is a tribute to the diligence of the US Department of Agriculture Food Safety and Inspection Service. The high volume of uncontaminated quality foods available for the American public also validates the recognition by the agricultural and veterinary profession of the need for quality foods and their cooperative efforts to appropriately use insecticides in agriculture and veterinary medicine.

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Avian toxicology of anticholinesterases

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Neither OPs nor CBs tend to accumulate in tissues of vertebrates and both classes are comparatively labile in the environment [25, 84]. Therefore population-threatening reproductive effects on birds as found with persistent pesticides are not expected [138] and accordingly little research has been conducted on chronic avian antiChE toxicity [134]. However, because of their extensive usage unexpected exposure and effects on different wild avian populations are likely to occur.

Birds compared with mammals

Birds and mammals respond similarly to xenobiotics with any differences in metabolism more quantitative than qualitative [106]. This conclusion was based on evaluation of more than 300 studies involving 114 chemicals (including 19 OPs and 8 CBs) and 35 species of birds. Several quantitative enzymatic differences between birds and mammals are important to the sensitivity of birds to acute antiChE exposure. Birds have lower hepatic microsomal mono-oxygenase (HMO) and A-esterase activity than do mammals [9,150] which makes birds highly susceptible to both OPs and CBs. Several avian species were consistently more sensitive to acute CB exposure than was the laboratory rat [134]. Likewise, OPs are deactivated by HMOs, but many are first activated to a more potent antiChE analogue by the same HMO pathway [25]. Most active analogues are substrates for A-esterase hydrolysis and are rapidly detoxified in the liver and blood (e.g. diazoxon), but some (e.g. paraoxon) are not. Brealey *et al.* [9] studied 14 species of domestic and wild birds, three laboratory mammals, domestic sheep, and man, and determined plasma A-esterase activity was at least 13 times higher in all of the mammals than in the avian species. The relevance of these differences to birds comes from a study of dimethoate with ring-necked pheasants and laboratory rats [118]. The toxic oxygen analogue was rapidly formed and accumulated in pheasant, whereas the oxon was rapidly detoxified in rats, explaining the 10 times higher LD_{50} for rats than pheasant (Table 25.1).

Accurate toxicological prediction across taxonomic bounds is extremely difficult for antiChEs because of diverse metabolism of different compounds and differences between birds and mammals in the activity of detoxicating enzymes. This is evident from comparison of acute sensitivity of laboratory rats, ring-necked pheasants, and red-winged blackbirds to antiChEs of widely different mammalian toxicity (Table 25.1). When corrected for liver to body weight ratios, variously sized birds consistently have less HMO activity than mammals [150]. This probably explains why both avian species are more sensitive to CBs than rats, but not why blackbirds are five times as sensitive as pheasants (Table 25.1). However, red-winged blackbirds are deficient in HMO activity [107,150,151], which is probably why they consistently have LD_{50}s of <10 mg/kg for a variety of CBs (seven of nine compounds compared with one for rats and three for pheasants), and why CBs are good candidates for selective avian control. Red-winged blackbirds are also more sensitive than rats and ring-necked pheasants to OPs, whereas rats are more sensitive than pheasants to compounds of high mammalian toxicity (i.e. rat LD_{50} <200 mg/kg) and pheasants more sensitive than rats to compounds of lower mammalian toxicity (Table 25.1). The gross response of both avian species to OPs is similar to their response to CBs, with little correlation between avian and

Table 25.1 Single-dose peroral LD_{50}s[a] for laboratory rats, ring-necked pheasants and red-winged blackbirds and within-species toxicity ranking for antiChE pesticides of widely variable mammalian toxicity

Compound	*Rat*[b]		*Pheasant*[c]		*Blackbird*[d]	
	Rank	LD_{50}	*Rank*	LD_{50}	*Rank*	LD_{50}
CB						
Aldicarb	1	0.8	3	5.3	2	1.8
Carbofuran	2	11	1	4.1	1	0.4
Methomyl	3	17	4	15	6	10
Mexacarbate	4	37	2	4.6	6	10
Aminocarb	5	40	6	42	8	50
Methiocarb	6	70	8	270	5	4.6
Propoxur	7	83	5	20	3	3.8
Bufencarb	8	170	7	88	4	4.2
Carbaryl	9	850	9	707	9	56
OP						
Phorate	1	2.3	1	7.1	1	1.0
Disulfoton	2	6.8	2	12	2	3.2
Azinphos-methyl	3	13	7	75	5	8.5
EPN	4	36	6	53	2	3.2
Ethion	5	65	10	1297	9	45
Phosmet	6	113	9	237	6	18
Dimethoate	7	215	3	20	4	6.6
Fenitrothion	8	740	4	26	7	25
Malathion	9	1375	8	167	10	>100
Temephos	10	8600	5	35	8	42

[a] LD_{50} = mg active ingredient (technical grade) per kg of body-weight calculated to kill 50% of test population
[b] Sherman strain males, 3 months old, 50–60 per test; dosage via gavage in peanut oil at final volume of 5 μl/g of body-weight [34,35]
[c] Farm-reared 3–4-month-old males and females, 8–28 per test; dosage via gelatine capsule [76]
[d] Wild-captured pen-conditioned adults of both sexes and mixed ages, 8–28 per test; dosage via gavage in propylene glycol, final volume not reported [119,120]

mammalian rankings. It is noteworthy that the least toxic compounds to mammals are comparatively more toxic to birds. This may be explained through the same mechanism as for dimethoate [118] because all are activated through HMO metabolism. Some common OPs not requiring activation were also compared; most were highly toxic (exceptions: acephate and trichlorfon) and ring-necked pheasants were virtually always more sensitive than laboratory rats [134].

AChE activity in the brain of birds is much higher than in mammals. Westlake *et al.* [155] reported brain AChE activities for 28 avian and 11 mammalian species. The median avian activity level was three times that of mammals, and only two mammals (mole and grey squirrel) were above the lower extreme for birds. High levels of brain AChE have not proved advantageous to birds over mammals. This may be because avian brain AChE has a greater affinity to bind many antiChEs and a faster rate of phosphorylation and carbamylation. For example, *in vitro* studies of the oxygen analogues of malathion, parathion and parathion-methyl demonstrated that the concentrations of purified antiChE required to inhibit 50% of chicken brain AChE were about 15, 50, and 80%, respectively, of the concentrations required for 50% inhibition of rat brain AChE [102,152]. Conversely, rat brain AChE had the greater affinity to bind to the oxygen analogues of azinphos-ethyl and azinphos-methyl. The closely-related ring-necked pheasant was much more sensitive to malathion than the rat (LD_{50}: 167 compared with 1375 mg/kg) and reversed species relationship for azinphos-methyl (LD_{50}: 75 compared with 13 mg/kg); whereas both parathion and parathion-methyl were equitoxic to both species [34,76]. In an acute study of malathion, brain AChE activity was 80% higher in Japanese quail than in mice, and single oral dosage of 200 mg/kg did not affect mouse brain AChE activity, but 50 mg/kg inhibited quail AChE about 30%; the dosage required for 50% inhibition of AChE activity was 860 mg/kg for mice and 68 mg/kg for quail [15]. Also the reactivation of phosphorylated brain AChE may take up to five times longer for some avian species compared with mammals [87].

Other metabolic differences between birds and mammals in response to antiChE exposure are not generally noteworthy except that birds tend to be more susceptible than mammals to OP-induced delayed neurotoxicity (*see* Ch.9).

Lethal toxicology: acute oral and dietary

With few exceptions lethality is considered the primary environmental hazard of anti-ChEs to wildlife. According to Smith [134] over 50% of OPs and 90% of CBs are 'extremely toxic' (i.e. LD_{50} <40 mg/kg) to most bird species. Although antiChEs are labile in homoiothermic animals and recovery from acute poisoning is usually complete within a few hours [61,76], repeated lesser exposures may result in accumulative AChE inhibition and death after a few days [91]. Because multiple exposures are common in nature, a short-term feeding trial was devised for routine evaluation of the lethal toxicity of pesticides to birds [52].

Single-dose *versus* 5-day dietary exposure

Acute and subacute tests may result in very different toxicological relationships between antiChE pesticides (Table 25.2). Acute LD_{50}s for the first six compounds are statistically similar and all are extremely toxic to adult Japanese quail. When the same chemicals were given for 5 days in the diet, the direct AChE inhibitors monocrotophos, dicrotophos, and phosphamidon were more toxic than either EPN or parathion, latent AChE inhibitors, or mexacarbate, a direct N-methylCB AChE inhibitor. The comparative tolerance to latent AChE inhibitors by young quail may result from an immature hepatic microsomal enzyme system not efficiently performing oxidative desulphuration; but this does not explain why acutely toxic mexacarbate is well-tolerated subacutely. A possible explanation is that survivors of CB exposure maintain sufficient free AChE through spontaneous reactivation of carbamylated enzyme [8]. Freed CB may 'self-destruct' by inducing key hepatic microsomal enzymes [71,137] although CBs are not generally potent HMO inducers [80]. At the same time, carbamylated AChE is temporarily protected against freshly ingested CB which is also subject to HMO metabolism. The 60–80% of quail chicks that die from CB poisoning do so within 2–6 h after treated feed is presented; thereafter, virtually no mortality occurs at sub-LC_{50} levels [60,62]. In contrast, OPs are irreversible AChE inhibitors and are not potent inducers of

Table 25.2 Single-dose peroral toxicity versus 5-day lethal dietary toxicity for Japanese quail treated with antiChE pesticides

Compound	*Single-dose*[a]			*5-day dietary*[b]		
	Rank	LD_{50}	*(95% confidence intervals)*	*Rank*	LC_{50}	*(95% confidence intervals)*
Mexacarbate	1	3.2	(2.4–4.2)	9	605	(526–697)
Phosphamidon	2	3.6	(1.8–7.2)	3	90	(73–111)
Monocrotophos	3	3.7	(2.7–5.0)	1	2.4	(1.8–2.9)
Dicrotophos	4	4.3	(3.2–5.9)	2	37	(34–40)
EPN	5	5.2	(3.8–7.3)	7	437	(302–632)
Parathion	6	6.0	(3.4–10)	5	238	(153–373)
Fenthion	7	11	(8.4–13)	4	132	(106–169)
Chlorpyrifos	8	16	(10–24)	8	492	(351–680)
Propoxur	9	28	(no data)	12	>5000	
Landrin[c]	10	71	(33–154)	11	2037	(1629–2548)
Temephos	11	84	(61–116)	6	242	(183–322)
Oxydemeton-methyl	11	84	(61–116)	10	1256	(961–1642)

[a]Toxicity as LD_{50} = mg active ingredient (technical grade) per kg of body-weight calculated to kill 50% of test population, 8–28 non-breeding adults of both sexes were dosed per test via gelatin capsule [147]
[b]Toxicity as LC_{50} = mg active ingredient (technical grade) per kg of feed in *ad libitum* diet for 5 days (followed by untreated feed until toxic signs remissed) calculated to kill 50% of test population. Five to six groups of ten unsexed chicks (14 days old) were tested per chemical [62]
[c]Mixture

Table 25.3 Single-dose lethal peroral toxicity[a] of antiChE pesticides to seven avian species of four taxonomic orders and six families[b]

Compound	*Ring-necked pheasant* Rank	LD_{50}	*Mallard* Rank	LD_{50}	*Chukar* Rank	LD_{50}	*Rock dove* Rank	LD_{50}	*European starling* Rank	LD_{50}	*Red-winged blackbird* Rank	LD_{50}	*House sparrow* Rank	LD_{50}	*Sensitivity ratio[c] High/low* LD_{50}
Monocrotophos	1	2.8	3	4.8	2	6.5	3	2.8	2	3.3	1	1.0	1	1.6	6.5
EPN	2	3.1	8	53	4	14	5	5.9	6	7.5	5	3.2	4	13	17.1
Dicrotophos	3	3.2	2	4.2	3	10	1	2.4	1	2.7	2	1.6	2	3.0	6.2
Mexacarbate	4	4.5	1	3.0	1	5.2	6	6.5	8	32	7	10	10	50	16.7
Chlorpyrifos	5	8.4	9	76	9	61	7	27	3	5.0	9	13	6	21	15.2
Parathion	6	12	6	2.1	5	24	2	2.5	5	5.6	4	2.4	3	3.4	11.4
Fenthion	7	18	4	5.9	7	26	4	4.6	4	5.3	3	1.8	7	23	14.4
Propoxur	8	20	5	12	5	24	9	60	7	15	6	3.8	4	13	15.8
Temephos	9	32	10	79	10	270	8	50	9	>100	10	42	8	35	8.4
Landrin	10	52	7	22	8	60	10	168	9	>100	7	10	9	46	16.8
Sensitivity rank (mean)	2(3.4)		5(4.1)		7(6.0)		3(4.0)		6(4.4)		1(2.0)		3(4.0)		

[a]Toxicity as LD_{50} = mg active ingredient (technical grade) per kg of body-weight calculated to kill 50% of test population

[b]Reproduced from Tucker and Haegele [147] with starling and blackbird data from Schafer [119] and Schafer *et al.* [120]. All studies were conducted at the Denver Wildlife Research Centre, Denver, CO, by similar methods. Mallards and gallinaceous species were farm-reared 2–4-month-old males and females; rock doves and passerine species were wild-captured pen-conditioned adults of both sexes; 8–28 birds were dosed per test either by gavage in propylene glycol (starlings and blackbirds) or by gelatin capsule

[c]Sensitivity rank is based on the mean of across-species order of sensitivity to each chemical

hepatic microsomal enzymes; therefore, mortality from OP exposure usually begins on the second or third day of a subacute trial and progresses to day 5.

The sensitivity of birds to acute antiChE exposure varies widely among species, but the relative toxicity of an array of pesticides tends to be similar for any two species (Table 25.3). The extreme differences in sensitivities among seven avian species tested at the same laboratory averaged 13-fold for ten antiChE pesticides. Responses were most consistent across species to monocrotophos and dicrotophos, both highly toxic direct AChE inhibitors, and to temephos which is unusually toxic to birds compared with mammals (*see* Tables 25.1 and 25.3). The red-winged blackbird was either the most or second most sensitive species to seven of ten compounds whereas chukars were either the most or second most tolerant species to eight of the ten compounds (Table 25.3). For the remaining five species the mean sensitivity ratings only varied from 3.4 to 4.4. When the seven species were paired in all possible combinations, the acute LD_{50}s of the ten chemicals were well correlated between species in 18 of 21 comparisons (r=0.74, P <0.05 to r=0.99, P <0.01). The three exceptions (0.05 <P <0.1) were mallards versus chukar (r= 0.68), ring-necked pheasant (r=0.58), and European starling (r= 0.59). The mean correlation coefficient (and s.d.) for each test species against the other is: red-winged blackbird, 0.88 (0.10); chukar, 0.88 (0.12); rock dove or domestic pigeon, 0.87 (0.08); European starling, 0.84 (0.15); house sparrow, 0.79 (0.04); ring-necked pheasant, 0.79 (0.11); and mallard, 0.70 (0.10). These data therefore suggest, with the possible exception of mallard, any of the test species will adequately represent the acute sensitivity of birds to antiChE pesticides, but the response of one species cannot be used to predict the sensitivity of another species to a given antiChE compound.

When antiChE pesticides are tested subacutely in 5-day diets of young Japanese quail, ring-necked pheasants, and mallards the spread among LD_{50}s is substantially less than indicated above for LD_{50}s, but mallard LD_{50}s are not well correlated with either of the galliformes (Table 25.4). Although extreme LC_{50}s differ among the three species by only 3.2–6.4-fold ($\bar{x}$=4.4, s.d. 0.9) for the ten listed pesticides, mallards are statistically separable (*see* confidence intervals) from quail and pheasants in 18 of 20 comparisons and were always either most or least sensitive of the three species. LC_{50}s for pheasant and quail are well correlated (r=0.91, P <0.01) and usually separated by less than twofold with quail most sensitive. Mallards were the least sensitive species to all four direct AChE-inhibiting OPs (mono- and dicrotophos, ethoprophos and dichlorvos) and most sensitive to three of four latent AChE inhibitors and both CBs. The same order of response for the different classes of antiChE

Table 25.4 Five-day lethal dietary toxicity of antiChE pesticides to avian chicks of approximate equal vulnerability to the test protocol[a]

Compound	*Japanese quail*			*Ring-necked pheasant*			*Mallard*		
	Rank	LC_{50}	*(95% confidence intervals)*	*Rank*	LC_{50}	*(95% confidence intervals)*	*Rank*	LC_{50}	*(95% confidence intervals)*
Monocrotophos	1	2.4	(1.8–2.9)	1	3.1	(2.6–3.7)	1	10	(8–12)
Dicrotophos	2	37	(34–40)	2	44	(38–51)	5	94	(80–111)
Parathion-methyl	3	69	(61–78)	3	91	(77–107)	2	336	(269–413)
Fensulfothion	4	85	(62–116)	5	148	(119–179)	3	41	(32–55)
Ethoprophos	5	89	(72–109)	4	118	(103–134)	8	287	(215–382)
Parathion	6	238	(152–373)	6	336	(296–380)	4	76	(61–93)
Dichlorvos	7	265	(191–370)	7	568	(473–675)	9	1317	(1043–1674)
EPN	8	437	(302–632)	9	1075	(943–1230)	6	168	(125–237)
Carbofuran	9	746	(549–1014)	8	573	(492–666)	7	190	(156–230)
Methiocarb	10	1342	(1048–1719)	10	>5000		10	1071	(808–1405)

[a]Toxicity as LC_{50} = mg active ingredient (technical grade) per kg of feed in *ad libitum* diet for 5 days (followed by untreated feed until toxic signs remissed) calculated to kill 50% of test population. Five to six groups of 10–12 unsexed chicks (quail, 14 days old; pheasant, 10 days; mallard, 5 days) were tested per chemical [62,65]

also occurred for adults (Table 25.3) indicating that mallards have low endogeneous levels of detoxicating hepatic microsomal enzymes compared with galliformes (consistent with Walker [150]) but higher levels of A-esterase activity (consistent with Brealey *et al.* [9]). Low HMO activity would explain sensitivity to CBs and high A-esterase activity the tolerance to direct AChE inhibitors. The comparative sensitivity of the species to latent inhibitors is erratic and appears highly compound-specific; this would be expected as latent inhibitors must be activated and detoxified through the same metabolic pathway.

Clinical signs of acute antiChE poisoning tend to be similar across species and among compounds. The general progression and severity of signs follow a common sequence from a single dose and is dose-dependent for OPs but not for CBs [61]. Death usually occurred in 10–30 min at doses above the LD_{50} and 30 min to 6 h at lower doses, but occasionally took 12–24 h for some latent AChE inhibitors. Birds dosed with CBs do not follow such a predictable pattern, but are rapidly sedated, exhibit mild tremors and laboured breathing, and either die quickly (5–30 min) or recover with little evidence of toxicity after 1–2 h. Toxic signs associated with acute exposure of birds to antiChEs are presented in detail by Hudson *et al.* [76].

Toxic signs associated with OP subacute exposure were usually less intense than those in acute trials [62]. Onset of signs are often delayed until the second or third day of treatment and then either intensified and culminate in death or remit within 6–24 h after toxic feed is replaced with untreated feed, i.e. day 6. In contrast, mortality from CBs usually occurs in 2–6 h after treated feed is presented and survivors usually recover within a few hours. Signs of subacute CB exposure are consistent with those of acute exposure [61,62]. Response to OPs is usually marked by reduced activity, feather fluffing, and finally a state of lethargy sometimes accompanied with tremors before death [55].

Factors affecting interpretation of acute and subacute tests

Test animals must be susceptible to the conditions of the test protocol so their vulnerability can be quantified with reasonable statistical certainty. This can be met only for species that can be maintained in captivity in good health and cannot survive for 5 days without eating [70]. This requirement has been criticized on the basis that death by starvation would not accurately reflect direct toxicity and would confound interpretation of LC_{50} values [148]. But it has been shown that susceptible birds eventually eat rather than starve [60] and when fed antiChE compounds, brain AChE activity was severely inhibited [41]. Both standardization of test age and selection of model species were based on susceptibility to the parameters of the test rather than response to xenobiotic.

Table 25.5 Single-dose lethal peroral toxicity[a] of antiChE pesticides to mallards from hatch until adulthood

Compound	*LD_{50}[b] (95% confidence intervals)*			
	1.5 days	*1 week*	*1 month*	*6 months*
CB				
Carbofuran	0.4^{x} (0.3–0.5)	0.6^{xy} (0.5–0.7)	0.5 (0.4–0.6)	0.4^{y} (0.3–0.5)
Aldicarb	1.9^{wxy} (1.6–2.4)	3.6^{wz} (2.9–4.5)	6.7^{xz} (5.3–8.6)	4.4^{y} (3.5–5.6)
Propoxur	7.4^{xy} (6.0–9.1)	13^{x} (10–16)	15^{xy} (12–17)	9.6^{z} (7.6–12.1)
OP				
Parathion	1.6^{x} (1.4–2.0)	1.4^{y} (1.1–1.8)	1.6^{z} (1.4–2.0)	2.3^{xyz} (2.0–2.8)
Monocrotophos	5.9^{x} (4.7–7.3)	7.2^{y} (5.8–9.0)	5.1^{z} (4.4–5.9)	3.4^{xyz} (2.8–4.1)
Demeton	13^{x} (11–16)	15^{y} (13–18)	15^{z} (12–19)	8.2^{xyz} (6.6–10.2)
Chlorpyrifos	145^{x} (56–377)	29^{x} (19–47)	50 (32–78)	83 (44–158)

[a]Toxicity as LD_{50} = mg active ingredient (technical grade) per kg of body weight calculated to kill 50% of test population; 20 birds of both sexes were dosed per test via gelatin capsule [74]
[b]Within chemical age differences are indicated by the same superscripts ($P<0.05$)

Data for 5-day-old mallards, 10-day-old ring-necked pheasant, and 14-day-old Japanese quail (Table 25.4)are compatible with age of susceptibility to the 5-day subacute test, but the species are not necessarily of equivalent physiological or metabolic maturation [70]. The importance of seemingly trivial difference in age on LC_{50}s has been well documented. For example, between 7 and 14 days of age, LC_{50}s increased an average of 1.5-fold for three OPs and two CBs tested with Japanese quail from a single hatch [60]. This was demonstrated for 10-day-old ducklings (Table 25.4). LC_{50}s increased by 1.5–3.8-fold between 5 and 10 days of age for all but fensulfothion [65]. These changes probably resulted from several species variable age-dependent factors. For example, food intake in proportion to body-weight decreases naturally as age of chicks increases. Thus, in the above quail study, food consumption of controls was 48, 31, 24, and 19 g per 100 g at 3, 10, 17 and 24 days of age respectively. This decreasing requirement for food reduces toxic exposure. This is a highly individualistic response. In both the quail and the mallard studies, reduced feeding was the rule for all but CBs, and corresponding increases of LC_{50} occurred. Also, the efficacy of biochemical barriers, enzyme detoxifying systems, and excretion improve with maturation. This concept applies for direct AChE- inhibiting OPs and CBs, but not for latent OP anti-AChEs (Table 25.5). Mallard acute LD_{50}s decrease for parathion and chlorpyrifos between 1.5 and 7 days of age and then increase through adulthood. In contrast, LD_{50}s for monocrotophos, demeton, and all CBs tend to increase to 1 month and then decrease at 6 months to values comparable with or even below first week post- hatching. No important changes in brain AChE activity were detected between test ages. It was suggested that the comparative insensitivity to antiChE of the youngest age classes was from immaturity of the CNS [74].

Sex, reproductive condition, genetic lineage, nutritional status, and both exogenous and endogenous stress may have variable effects on the toxicity of antiChEs, but their importance is not well established for birds. For example, the LD_{50}s for rats of the antiChEs listed in Table 25.1 showed females were 1.4–4.7 times ($P<0.05$) as sensitive as males to six compounds, males about 1.5 times ($P<0.05$) as sensitive as females to two compounds, with no sex differences for the others [34,35]. In contrast to the mice, birds (Tables 25.1–25.3) were reproductively quiescent at the time of testing and the sexes were pooled for LD_{50} determinations as previous tests at the same laboratory showed non-breeders display only minimal sex-dependent differences in acute oral toxicity to pesticides [119,147]. This is supported by a controlled study with northern bobwhites [61] in which 26 technical grade and granular forms of antiChEs were tested on 16-week-old birds and no sex differences were seen in either LD_{50} or observed toxic response. Reproductive quiescence was ensured by use of a 10L:14D light regimen for 1 month preceding the test, and was verified by post-mortem inspection of gonadal development. Similar results were demonstrated in a subacute study of three OP insecticides using non-breeding adult house sparrows [55].

The importance of seasonal differences between sexes comes from a study with four species of wild-captured birds in which data variability increased and significant sex differences were detected during breeding seasons [67]. For example, blood plasma ChE activity of northern bobwhite is equivalent for the sexes throughout much of the year, but averages about 40% higher ($P < 0.01$) in males than in females during breeding, which is opposite to that found in breeding laboratory rats.

Avian research typically uses captive-reared birds from haphazardly outbred stocks or wild-captured birds of unknown origin. Reproducibility of acute toxicity tests with birds of such vague genetic lineage has not been thoroughly evaluated, but a limited study of equal-aged farm-reared northern bobwhite has been reported [63]. The acute toxicity of technical grade diazinon for young adult bobwhites showed statistically similar LD_{50}s of 13 mg/kg (95% confidence intervals: 8–21 mg/kg) to 17 mg/kg (95% confidence intervals: 11–25 mg/kg). The stocks did differ in apparent vigour and body-weight, but genetic variability from outbreeding could have obscured minor differences based on LD_{50} alone. Extraneous variables from laboratory differences in husbandry were eliminated by incubating eggs and rearing chicks of all stocks simultaneously in the same facilities.

Methods have not been developed to evaluate the suitability of a wild-captured individual or species for acute toxicity testing. Simple survival and weight maintenance for a few weeks in captivity may not reflect subtleties that may affect sensitivity to antiChE such as nutritional imbalance and stress response to confinement, isolation or crowding. Any avian species that breeds in captivity should be a reliable test model, but to maintain the necessary expensive facilities has proven impractical for many species. An example of species differences is illustrated by subacute feeding trials with birds believed adequately conditioned to captivity. When wild blue jays, house sparrows, and northern cardinals and wild and farm-reared northern bobwhites were fed OP pesticides for 5 days, blue jays were always the most sensitive species and farm bobwhites were always most tolerant [55]. Blue jays are reputed to be adaptable generalized feeders that seem to tolerate toxic pertubation of the environment [7] and are easily kept in captivity, yet they were about 1.5, 2.5, 51, and 54 times as sensitive to temephos as house sparrows, cardinals, and wild and farm bobwhites, respectively. Similar results were also obtained with bromophos and tetrachlorvinphos. Wild bobwhites had less subcutaneous and visceral fat than their farm counterparts, weighed about 25% less, and were more sensitive to all the OPs. The difference in bobwhite response is attributed primarily to consumption of more toxic feed than to differential sensitivity. Neither body-weight nor rate of feeding explain the differences in passerine sensitivity because blue jays are nearly twice as heavy and eat proportionally less than either house sparrows or cardinals.

Avian toxicity studies are routinely conducted outdoors for many wild-captured species and this may introduce seasonal variation in response to antiChE exposure other than sex-effects. Five-day subacute dietary toxicity tests of technical grade dicrotophos conducted on pen-conditioned adult common grackles during mid-May and mid-August gave LC_{50}s of 125 and 17 mg/kg of *ad libitum* diet [41]. Birds in the August study weighed about 5% less than in May, were judged to be in poorer fat and flesh although they tended to eat more and were in post-nuptial molt. The mean maximum daily temperatures during the tests were 21 and 31°C in May and August. Increased sensitivity is attributed to molting rather than ambient conditions because similar tests showed fenthion and fenitrothion were substantially more toxic in mid-August than late-July while the temperatures during these periods were similar.

Lethal toxicology: factors of exposure, formulation, and interaction

Ingestion is the most common route of antiChE exposure in birds and may be via water, seeds, foliage, invertebrates, vertebrates, and granular pesticides [42]. Although oral toxicity has been extensively studied with technical grade antiChEs via gelatin capsule, gavage, and addition to dry mash, little is

known about the toxicity of formulated pesticides complexed with natural matrices or effects of environmental stressors on antiChE toxicity. Likewise, few avian studies have been conducted on specific routes of exposure or on toxic interaction from either simultaneous or sequential exposure to antiChEs.

Routes of exposure and antiChE toxicity

Regardless of the route of entry, toxicity is primarily a function of antiChE availability for hepatic metabolism [103]. Thus, direct AChE inhibitors are more potent than latent inhibitors by percutaneous and inhalation routes. This is illustrated by acute studies with laboratory mice by Natoff [103]. However, the rate at which antiChE penetrates the skin varies widely among pesticidal formulations, site of application, and species [23,92,149]. Inhalation toxicity is minor compared with deposition of chemical on the skin [23]. The emphasis of controlled avian studies has been on oral or cutaneous toxicity tests and little is known about antiChE toxicity from combined routes of exposure.

Most antiChE pesticides are more toxic to birds orally than by cutaneous application. The acute toxicity of 19 antiChE pesticides were compared in non-breeding adult mallards by encapulated peroral dosage, by single application to skin or in the feed (Table 25.6) [75]. LD_{50}s were significantly (P <0.05) higher by the percutaneous route for 13 of 19 comparisons and the log LD_{50}s for the two methods were positively correlated (r=0.65, P <0.01). Mallard percutaneous tests were also compared with rats [34,35]. The log LD_{50}s for the two species were not well correlated (r=0.36, P > 0.10). In another avian study,

Table 25.6 Single-dose po and percutaneous LD_{50}s for adult male mallards treated with OP pesticides[a]

Compound	*Peroral*			*Percutaneous*			*Potency ratio*
	Rank	LD_{50}	*(95% confidence intervals)*	*Rank*	LC_{50}	*(95% confidence intervals)*	
Thionazin	1	1.7	(1.2–2.3)	1	7	(5–10)	4.1
Parathion	2	2.3	(1.9–2.9)	5	28	(20–40)	12
TEPP	3	3.6	(2.7–4.7)	8	64	(29–142)	18
Dicrotophos	4	4.2	(3.1–5.9)	3	14	(5–44)	3.3
Monocrotophos	5	4.8	(3.4–6.6)	6	30	(14–67)	6.2
Fenthion	6	5.9	(4.3–8.2)	7	44	(22–88)	7.5
Disulfoton	7	6.5	(3.8–11)	9	192	(96–384)	30
EPN	8	7.1	(5.2–10)	10	400	(180–890)	56
Demeton	9	7.2	(5.2–10)	4	24	(6–96)	3.3
Ethoprophos	10	13	(11–15)	2	11	(8–15)	0.8

[a]LD_{50} = mg active ingredient (technical grade) per kg of body-weight calculated to kill 50% of test population. Birds were farm-reared until full growth; 8 birds were dosed via gelatin capsule (peroral) or by application in propylene glycol or corn oil at final rate of 0.6 ml to each foot (percutaneous). The treated percutaneous area included the tarsometatarsus, phalanges, and webbing and is estimated to cover 12% of the body surface of an adult mallard. The treated area was covered for 24 h and then washed and observation continued for 14 days [75]

Table 25.7 Single-dose peroral and percutaneous LD_{50}s[a] for small passerines treated with antiChE pesticides

Compound	*House sparrow*		*Potency ratio*[b]	*Red-billed weaver*		*Potency ratio*[b]
	Peroral	*Percutaneous*		*Peroral*	*Percutaneous*	
Fensulfothion	0.3	1.0	3.3	0.2	0.4	2.0
Carbofuran	1.3	100	77	0.4	100	250
Monocrotophos	1.3	18	14	1.3	4.2	3.2
Parathion	1.3	1.8	1.4	1.8	1.8	1.0
Dicrotophos	4.2	1.8	0.4	1.3	1.3	1.0
Demeton	5.6	13	2.3	1.3	1.8	1.4
Fenthion	5.6	2.4	0.4	1.3	1.8	1.4
Coumaphos	10	75	8	3.2	7.5	2.3
Methiocarb	18	>100	>5	4.2	100	24

[a]LD_{50} = mg active ingredient (technical grade) per kg of body-weight calculated to kill 50% of test population. Wild-captured pen-conditioned adults of both sexes, 8–28 per test, were dosed via gavage (peroral) or to a cm^2 featherless skin area covering the pectoralis muscles under the wing joint (percutaneous). Gavage treatment was in propylene glycol at the rate of 2 µl/g body-weight with acetone evaporated by a current of air. Reproduced from Schafer *et al.* [121]
[b]Potency ratio is the percutaneous LD_{50} divided by peroral LD_{50}

antiChE was applied to a cm^2 area of skin of house sparrows, red-billed weavers, or quelea, and the LD_{50}s were compared with concurrent tests of peroral toxicity [121]. The acute percutaneous and oral toxicity was similar for most of the OPs tested, and the LD_{50}s well correlated (r >0.85, P <0.01) between species for each route of exposure (Table 25.7). In contrast, the CBs, carbofuran and methiocarb, were much more toxic perorally than percutaneously, indicating the skin is a substantial barrier to CB poisoning in birds. This was also evident in the mallard study in which aldicarb was 18 times more toxic orally than percutaneously [75]. Vehicle and site are important as determinants of percutaneous toxicity, thus radiolabelled carbaryl and famphur penetrated skin nearly twice as fast when applied in acetone compared with corn oil [105]. The LD_{50}s for fenthion in European starlings orally and to the breast and feet were 6.0, 9.5, and 41 mg/kg respectively [122].

Sources of exposure and antiChE toxicity

Nearly all antiChE research with birds is on acute tests of technical grade chemical via controlled dosage or dry commercial diet. Exposure to antiChE-contaminated water, arthropods, and foliage has not been thoroughly evaluated. Water is an important source of contaminant exposure in wildlife and the fate and availability of antiChE to birds depends on a wide variety of physical, chemical, and biological properties of the water and the pesticidal formulation. Water-soluble formulations usually remain available longest [25,84], and they also tend to be most toxic. The lethal toxicity of some emulsifiable antiChE concentrates are compared with their technical grade active ingredient in young Japanese quail in Table 25.8. By either single-dose or 5-day dietary exposure, technical grade parathion and Parathion 6EC are equally toxic, whereas Orthene is 3.0 and 4.6 times as toxic as its active ingredient (AI), acephate. How these studies relate to water-borne exposure is not clear because rates of feed and water consumption vary widely among avian species at different ages and seasons of the year. Smaller birds have a much higher water requirement relative to body-weight than larger birds under similar ambient conditions [116]. However, even closely related species of similar size and feeding habits vary their rates of free water consumption from about 15–40% of their body-weight per day at an ambient temperature of 25°C [5]. Aquatic birds undoubtedly contact much more water through feeding, swimming, and wading than do terrestrial passerines.

Feed consumption is also inversely related to body-weight in birds, but the ratio between rates of ingestion of feed and water varies according to feeding habits and ambient temperature [116]. For northern bobwhite, non-breeders of both sexes (mean change in weight = 199 g, s.d. = 12 g) ingested daily

Table 25.8 Single-dose lethal peroral toxicity and 5-day lethal dietary toxicity for Japanese quail chicks treated with technical grade and emulsifiable formulation of antiChE pesticide

Compound	*Technical grade*			*Emulsifiable*			*Toxicity ratio*[a]
	AI (%)	*LD or LC_{50}*	*(95% confidence intervals)*	*AI (%)*	*LD or LC_{50}*	*(95% confidence intervals)*	
Single-dose (LD_{50})[b]							
Diazinon	99	15	(13–17)	48	9	(7–11)	0.6*
Ethoprophos	95	25	(20–32)	70	17	(14–20)	0.7*
Parathion	99	29	(24–34)	79	28	(24–31)	1.0
Acephate	98	227	(194–260)	16	75	(56–99)	0.3*
Five-day dietary (LC_{50})[c]							
Ethoprophos	95	89	(72–109)	70	91	(68–122)	1.0
Diazinon	99	167	(131–212)	48	101	(81–126)	0.6*
Parathion	99	238	(152–371)	79	238	(181–312)	1.0
Acephate	98	3274	(2691–3986)	16	718	(593–868)	0.2*

[a]Division of liquid LD_{50} or LC_{50} by technical grade LD_{50} or LC_{50}. *Paired LD_{50} or LC_{50}s are statistically separable (two-tailed t-test, P<0.05)
[b]Toxicity as LD_{50} = mg active ingredient (AI) per kg of body-weight calculated to kill 50% of test population. Sixty 14-day-old unsexed chicks were dosed per test via gavage in corn oil (technical grade) or water (emulsifiable concentrate) at final volume of 5 µl/g body-weight [56]
[c]Toxicity as LC_{50} = mg active ingredient (AI) per kg of feed in *ad libitum* diet for 5 days (followed by untreated feed until toxic signs remissed) calculated to kill 50% of test population. Five groups of 12 unsexed chicks (14 days old at start) were tested per chemical [62]

averages of 15.0 g (s.d. = 1.8 g) of dry pellets and 20.3 g (s.d. = 1.6 g) of water, a difference of 35% (P <0.01, paired t test; E.F. Hill, unpublished data). Direct comparisons of antiChE toxicity in feed and water have not been reported, but subacute studies with young domestic chickens indicate substantial differences may be expected; with exposure via water most toxic. Five-day-old chicks were fed fresh chlorpyrifos (Dursban M) in water daily for 7 days and the LC_{50} estimated to be 100 mg/kg of *ad libitum* drinking water [13] compared with an LC_{50} of 800 mg/kg of feed for 10-day-old chicks fed technical grade chlorpyrifos for 7 days [130]. In a practical test of antiChE in water, fenthion (Baytex 4), was presented to black-crowned night herons for 24 h in shallow wading chambers at rates calculated to be equal to and 10 times field application rates (i.e. 112 g and 1.2 kg AI/ha [135]). The tenfold application yielded about 70 mg AI/kg just below the surface of the water at 24 h and resulted in no overt signs of toxicity or in brain AChE inhibition, but blood plasma ChE was inhibited an average of 32% (P <0.05). Field applications of fenthion at 47–100 g AI/ha over wetlands killed a variety of songbirds and waders [18,126,163]. Field mortality was associated with marked inhibition of brain AChE activity and was explained in part by the concentration of fenthion in prey insects in addition to water exposure.

Contaminated dead or struggling arthropods have proved attractive and sometimes lethal to wild birds that opportunistically increased their consumption after aerial insecticidal applications of carbaryl [17], trichlorfon [17], fenthion [18], and chlorpyrifos [94]. It is not known what effect phytometabolism has on the avian toxicity of systemic OPs. However, it has been demonstrated that systemics such as phorate and disulfoton may be sequentially metabolized to sulphoxide and sulphone degradation products, and each product may undergo oxidative desulphuration in plants [25]. In order, these degradation products have progressively more antiChE activity than the parent chemical; the oxygen analogues are most potent.

Secondary poisoning of flesh-eating birds foraging on antiChE-killed vertebrates has been documented for CB and OP insecticides including carbofuran [2], monocrotophos [97], fenthion [54], mevinphos [79], and famphur [31,53,54]. These poisonings were probably from eating unaltered antiChE in the gastrointestinal tracts of the prey [66]. The potential for secondary poisoning from aquatic vertebrates was demonstrated with tadpoles of *Rana catesbeiana* that concentrated parathion and fenthion from water in a continuous-flow dosing chamber [46]. Tadpoles exposed to as little as 1 mg/l parathion in water for 96 h were force-fed at the rate of 5% of body-weight to 14-day-old mallards. A single meal of tadpoles was lethal within 30 min, and before significant digestion of the tadpoles occurred. Because only parathion and not paraoxon was found in the tadpole tissues and toxicity was rapid, it is likely that residues concentrated in the protective slime layer and were almost immediately available on ingestion.

Pesticide formulation and antiChE toxicity

Residues of antiChE pesticides on seed grains, vegetation, and granular formulations have killed large numbers of wild birds under varied environmental circumstances [42,49]. Some of these kills were intentional poisonings [48,140,158], but most were likely to be applicator's ignorance of antiChE toxicology and fate and of avian behaviour. Few controlled studies have been conducted on the comparative toxicity of formulations or hazard associated with different application techniques. Instead, potential hazard is usually estimated by comparison of the theoretical concentration of active ingredient on a particular food item to results of standardized acute and subacute avian tests with technical grade chemical. However, many exposed species clearly eat proportionally more in relation to body-weight than either northern bobwhites or mallards and the toxicity of technical grade and formulated chemical may not be equal (*see* Table 25.8). Therefore, prediction of lethal hazard from a given antiChE application must consider the possibility that some species may receive substantially greater exposure than the model species and that formulated pesticide may be more toxic than technical grade chemical. Variability of toxicity among antiChE formulations was shown [57] when a single dose of carbofuran or diazinon was administered orally as technical grade (99% AI) alone

or in corn oil, as granular (GR, 14–15% AI), or as emulsifiable concentrate (EC, 48% AI). The rank of the forms tested from most to least toxic based on statistically separable (P <0.05) LD_{50}s was EC > GR = TG > CO for diazinon and EC > CO = TG > GR for carbofuran. The difference between the least and most toxic form was nearly threefold for diazinon (LD_{50} and 95% confidence intervals: EC, 3.7 and 3.0–4.6 mg/kg *versus* CO, 10.3 and 8.6–12.2 mg/kg) and fourfold for carbofuran (LD_{50} and 95% confidence intervals: EC, 3.6 and 2.7–4.8 mg/kg *versus* GR, 12.9 and 8.0–20.8 mg/kg).

AntiChE insecticide in seed dressing and granular formulation may be variably hazardous to birds of different foraging habits. Although free-choice feeding trials with adult ring-necked pheasants fed whole corn and pellets treated with various concentrations of flowable diazinon, chlorpyrifos, and carbofuran indicated the presence of pesticide was detected and avoided [6,141], incidents of large-scale waterfowl and passerine mortality have been documented for seed grains treated with carbofuran [30], monocrotophos [158], chlorfenvinphos [49], and carbophenothion [47,49]. Likewise, avian mortality has been associated with applications of granular formulations of antiChE insecticides such as aldicarb [49], carbofuran [29,88], diazinon [139], and disulfoton [49]. Ingestion of granulars is generally assumed to be haphazard and therefore proportional to application rates. This is supported by studies that showed varied thrushes, Oregon juncos, and California quail did not markedly alter their acceptance of coloured seeds against different backgrounds [108]. However, it is possible that certain granular configurations and colours may even be attractive to some species.

Many of the common granular OP and CB insecticides are extremely toxic to birds (i.e. acute peroral LD_{50} <20 mg/kg) [3,61]. In contrast with studies of liquid formulations which are usually more toxic than technical grade chemical (Table 25.8), granular formulations are more likely to be less toxic than technical grade chemical (Table 25.9). This relationship reduces the possibility that prediction of lethal toxicity on the basis of active ingredient will be underestimated for granulars, but proper hazard assessment requires determination of the acceptability and toxicity of individual granules to a variety of wildlife species. Most avian species that are likely to ingest raw granules do not have a well developed emetic reflex and as few as one to five granules of many common antiChE formulations could be lethal to sparrow-sized birds [61]. Granule size is critical to potential hazard because granule weight and resultant chemical dose may conservatively vary as much as threefold within a given batch of product [61].

Table 25.9 Single-dose peroral toxicity[a] of technical grade and granular formulation of antiChE pesticides to adult northern bobwhites

Compound	*Technical grade[a]*				*Granular[b]*				*Toxicity ratio[b]*
	AI (%)	*Rank*	LD_{50}	*(95% confidence intervals)*	*AI (%)*	*Rank*	LD_{50}	*(95% confidence intervals)*	
Fenamiphos	99	1	1.0	(0.7–13)	15	1	2.4	(1.2–4.6)	2.4
Fensulfothion	98	2	1.2	(1.0–1.6)	15	1	2.4	(2.0–2.9)	2.0*
Aldicarb	99	3	2.0	(1.4–2.9)	15	3	2.5	(1.6–4.0)	1.2
Parathion	98	4	6	(4–9)	10	6	13	(8–21)	2.2*
Phorate	93	5	7	(4–11)	15	9	21	(14–31)	3.0*
Diazinon	99	6	10	(7–13)	14	4	8	(6–11)	0.8
Fonophos	94	7	12	(10–14)	20	7	14	(12–17)	1.2
Carbofuran	99	7	12	(7–19)	10	5	12	(9–16)	1.0
Disulfoton	95	7	12	(7–19)	15	11	29	(24–34)	2.4*
Isofenphos	98	10	13	(10–16)	15	8	19	(15–23)	1.5*
Terbufos	99	11	15	(12–19)	15	10	26	(20–34)	1.7*
Bendiocarb	99	12	21	(17–26)	10	12	33	(24–44)	1.6*
Chlorpyrifos	99	13	32	(24–43)	15	13	108	(80–145)	3.4*

[a]Toxicity as LD_{50} = mg active ingredient (AI) per kg of body-weight calculated to kill 50% of test population; 40–50 birds of both sexes were dosed per test via gelatin capsule [61]

[b]Division of granular LD_{50} by technical grade LD_{50}. *LD_{50}s are statistically separable (two-tailed t-test, P<0.01)

This granule size disparity was also demonstrated by Balcomb *et al.* [3]. In nature, the hazard of granulars may be more a function of whether the granules are haphazardly or selectively ingested. If ingestion is haphazard, then application rate is the most critical variable; but if granules are selectively injested, then extreme caution must be used whenever granular formulations are used.

AntiChE interactions: chemical and environmental

Avian research on antiChE interaction has been limited to tests of the effects of subchronic exposure to realistic field concentrations of common pesticides and contaminants on the acute toxicity of antiChE pesticides, and to 5-day dietary subacute studies of simultaneous exposure to combinations of antiChE. In general, results of these studies are consistent with similar studies of laboratory mammals, but some differences are noteworthy. For example, pretreatment of laboratory rodents with chlorinated hydrocarbon pesticides that increase hepatic microsomal enzyme activity, reduced sensitivity to dicrotophos, parathion, and EPN [4,98,146]. In contrast, when DDE was fed to adult Japanese quail at the dietary concentrations of 5, 10, and 50 mg/kg for 12 weeks, blood plasma ChE increased with treatment while brain AChE was unaffected, but sensitivity to single-dose treatment of parathion and paraoxon increased significantly [90]. It was concluded that this synergism resulted from inhibition of paraoxonase (EC 3.1.1.2). However, when white Leghorn chicks were pretreated with a single dose of 200 mg/kg of DDT and treated 3 days later with 300 mg/kg of a 50% emulsion of malathion, the malathion LD_{50} was 468 mg/kg for pretreated chicks compared with 282 mg/kg for controls [77]. Ludke [90] pretreated Japanese quail with chlordane in the diets and found their acute sensitivity to parathion was decreased as previously indicated with laboratory mice [146].

Various forms of methylmercury inhibit hepatic microsomal enzyme activity in mammals [89] and blood plasma ChE activity in birds and mammals [19,20,51,68,69], and $HgCl_2$ inhibits brain AChE activity in birds [68,69]. Thus as anticipated, acute CB challenge was much more toxic to birds (carbofuran *versus* Japanese quail [21]) and mammals (carbaryl *versus* laboratory rats [89]) pretreated with methylmercury; but contrary to expectation, the latent AChE inhibitor, parathion, was also more toxic to Japanese quail pretreated with methylmercury [20]. If methylmercury is a potent inhibitor of hepatic microsomal enzymes in birds, then pretreatment with methylmercury should increase the toxicity of direct AChE inhibitors and decrease the toxicity of latent AChE inhibitors. Perhaps blood plasma ChE provides more protection of nervous system AChE from antiChE binding in birds than is generally believed. Plasma ChE was inhibited by an average of 14% ($P < 0.05$) in adult Japanese quail fed 4 mg of Morsodren per kg of diet for 18 weeks [20] and by 28% ($P < 0.05$) in quail fed 8 mg of CH_3HgCl per kg of diet for 9 weeks from hatching [68]. A single oral dosage of 25 mg of $HgCl_2$ or CH_3HgCl per kg of body-weight inhibited adult Japanese quail plasma ChE by averages of 29 ($P < 0.05$) and 47% ($P < 0.01$) within 24 h of treatment [69].

Studies of acute interaction between antiChEs paired with each other and with chlorinated hydrocarbons have usually proved additive in rats and mice [23,101]. When more than additive effects are detected as demonstrated for a few combinations of OPs, synergy is typically less than threefold [22]. In contrast, chlorinated hydrocarbons may antagonize the acute toxicity of latent AChE-inhibiting OPs [78]. Although carbaryl increased the lethal toxicity of malathion to rats by about 1.8-fold [78] and fenobucarb increased the toxicity of fenthion to mice about 1.6-fold [99], other combinations of CB and OP pesticides have not proved acutely interactive [23,101]. Few single-dose tests, have been conducted with birds, but the potential for interaction of antiChE pesticides was evaluated for birds by *ad libitum* dietary presentation of combinations of technical grade chemicals known for synergy in mammals. Combinations were fed for 5 days to Japanese quail and ring-necked pheasant chicks and only malathion plus EPN and malathion plus trichlorfon proved synergistic ($P < 0.05$ [83]). The maximum synergism was 3.4-fold for pheasants fed malathion plus EPN compared with about tenfold for this combination in acute tests with laboratory rats

[33]. These subacute dietary trials do not indicate that birds will be unusually susceptible to synergism from simultaneous exposure to combinations of antiChEs.

Some species of birds are highly mobile while foraging and may be exposed to several antiChE pesticides over a few hours or days. The potential hazard of sequential exposures to a combination of OP and CB compounds may depend on the order in which the pesticides are encountered. When the initial exposure is to CB, marked protection against OP may occur [38]. This brief protection depends on the carbamylation of AChE that protects against OP binding [160,161], and decarbamylation at a rate sufficient to maintain life as OP is destroyed [8]. In contrast, when the initial exposure is to OP antiChE, the inhibitor-AChE bond is much less reversible [104], and protection is not possible, and the toxicity of either OP or CB challenge will most likely be increased [99, 145]. Repeated low-grade exposures to antiChE may induce a degree of protection against acute antiChE toxicity. Adult female chickens were dosed daily with 1.4% (10 mg/kg) of the LD_{50} of cyanofenphos for 1–16 days and then a sample of hens was challenged each day with 140% (1000 mg/kg) of the LD_{50} [81]. The challenge dose only killed 20% of hens receiving pretreatment for at least 8 days compared with 77% of cyanofenphos-naive controls; protection continued for about 4 days after the final pretreatment dose. The acute LD_{50} of cyanofenphos for hens receiving eight 10 mg/kg pretreatment doses was 1170 mg/kg compared with 700 mg/kg for controls.

Extreme ambient temperature can influence the acute toxicity of antiChE pesticides to birds [111]. Adult Japanese quail were maintained at 26°C (thermoneutral) for 2 weeks and then either at 26°C or subjected to 4 or 37°C for 10 days. On day 11, birds from the thermoneutral group were dosed with parathion and either returned to 26°C or assigned to 4 or 37°C chambers for 7 days (acute), and birds from 4 and 37°C chambers were likewise dosed but continued in their respective chambers (chronic). At the higher temperature, the LD_{50} was significantly ($P < 0.05$) lower for both acute (39%) and chronic (52%) groups compared with the thermoneutral. The chronic cold birds also had a lower LD_{50} than thermoneutral (33%, $P < 0.05$) but the LD_{50} of the acute cold group was unaffected. The effect of temperature on food consumption has important environmental implications. Thus, the chronic cold birds, were not only more sensitive to parathion compared with thermoneutral birds (LD_{50}: 7.6 *versus* 11.5 mg/kg), but they also ate an average of 67% ($P < 0.05$) more food per day to maintain their body-weight. This may explain the extreme vulnerability of ducks, geese, and passerines to winter applications of antiChE-treated seed grains [136,140]. In contrast, birds exposed to elevated temperature ate about 55% less than the chilled birds, which implies a practical form of protection from antiChE poisoning. This advantage may be negated because of the chronic 37°C birds were more sensitive to parathion than the chronic 4°C birds (LD_{50}: 5.3 *versus* 7.6 mg/kg, $P < 0.05$) [111].

Sublethal toxicology and reproductive effects

Subchronic and behavioral toxicology

Anorexia, lethargy, feather fluffing (= piloerection), and muscular incoordination often occur with sublethal exposure of birds to antiChE pesticides; their intensity and duration are dose-dependent and highly variable among compounds [61,62,76]. These effects and weight losses of 10–20% from continuous antiChE exposure for up to 3 weeks, are usually tolerated by captive adult northern bobwhites and ring-necked pheasants [141–143], but may render equally affected free-living birds more susceptible to environmental stressors and less able to capture prey or avoid predation. For example, even a single exposure of about 5% of the LD_{50} of some OP and CB and antiChEs can reduce the core temperature up to 2°C in homoiothermic animals, and in a cold environment as much at 3–6°C [1,14,114]. Such hypothermic birds and mammals are consistently more sensitive to antiChE chemicals when tested at ambient temperatures of <6°C.

Young birds may be especially susceptible to antiChE interference with thermoregulation because many species are not fully homoiothermous until 1–3 weeks of age [132]. Day-old mallards were housed at either 10–18°C (unheated) or 39–41°C and fed graded concentrations of temephos in their feed for 7

days [28]. Ducklings on all treatments in unheated chambers had physiological stress as indicated by elevated plasma tri-iodothyronine, glucose, and uric acid, and died at much higher rates at corresponding treatment levels than ducklings in heated chambers. However, core temperatures were not different between the groups, and brain AChE activity of birds that died in unheated pens was inhibited less than is usually associated with death (i.e. $AChE_I$: 25–30% *versus* > 50% [91]), indicating alternative factors must have contributed to the mortality. Interaction between antiChE and ambient chilling was also demonstrated for chlorpyrifos by oral dosing of 14-day-old northern bobwhites and then exposing the 35°C-acclimatized chicks to 27.5°C for 4 h [95]. Brain AChE inhibition averaged 31, 50, and 76% at dosages of 42, 50, and 67 mg/kg, respectively; inhibition averaged about 30% in chicks dosed with 50 and 67 mg/kg and then continued at 35°C. There is evidence that parental care is reduced when the female is exposed to antiChE [10,44]. Temperatures below 15°C are common throughout the breeding season of birds in most temperate climates.

The effects of realistic antiChE exposure on the ability of birds to capture prey or avoid predation have not been thoroughly evaluated. American kestrels were given a single oral dose of 50 mg/kg of acephate either alone or in combination with low grade DDE pretreatment and their predatory vigilance and attack behaviour tested [117]. Whole brain and blood plasma ChE was inhibited about 25 and 40% at 1 day after dosing. Neither the frequency nor speed of kestrel responses to a familiar moving prey model was altered during repeated daily trials over 1 week after dosing. Attempts to study predation on insects by poisoned birds have been generally inconclusive because insect populations diminish rapidly after treatment [94]. In a study of domestic cat predation on adult northern bobwhites dosed orally with parathion-methyl, quail given 8 mg/kg were lethargic and easy victims, whereas those on 4 mg/kg were inseparable from controls and more difficult to capture [36]; acute LD_{50} of parathion-methyl is about 8 mg/kg for adult male northern bobwhites [76]. Increased predation on white-throated sparrows, Wilson's phalarope, and sharp-tailed grouse after field applications of fenitrothion, fenthion, and malathion respectively, has been reported [43].

Although the implications to survivability are not clear, certain avian behaviours may be affected by extremely low levels of exposure to antiChE. Adult male northern bobwhites were fed diets containing fenthion, and the birds' discrimination, acquisition and reversal performances were evaluated [82]. Birds fed 0.18 mg/kg monocrotophos diet made more than twice as many errors ($P < 0.05$) as did controls during reversals, but performance of the two groups was inseparable ($P > 0.05$) during acquisition. In contrast, bobwhites fed fenthion diet made only half as many errors ($P < 0.05$) as did controls during reversals; acquisition performance of fenthion-treated and control birds was not different ($P > 0.05$). Behavioural responses of birds to antiChE exposure have been reviewed by Peakall [109] and Grue and Heinz [43].

Reproductive toxicology

Effects on avian reproduction is usually tested by feeding graded concentrations of chemical at constant rates to pairs of breeders for several weeks. The overwhelming response to effect levels of antiChE is reduced food consumption leading to weight loss and marked reduction or cessation of oviposition [143]; but direct pharmacological action on avian reproduction also occurs [113]. This is clearly indicated by a pair-feeding study of northern bobwhite layers fed diets containing 100 mg of parathion per kg of feed for 10 days or matched amounts of control diet [115]. Birds on parathion lost over twice as much body-weight as their paired controls (mean change: 19 *versus* 8%, $P < 0.05$), stopped laying, and had significant ($P < 0.05$) reductions (i.e. 60%) in brain AChE activity and 30% in plasma luteinizing hormone (LH) level. The pair-fed controls had significant ($P < 0.05$) weight loss compared with controls, but neither brain AChE activity, egg production, follicular size, nor levels of plasma LH were affected. The action of antiChE on basal plasma LH was verified by oral dosing of male Japanese quail with parathion 5 mg/kg; no clinical signs or brain AChE inhibition were indicated, but circulating LH was depressed >50% ($P < 0.05$) within 4 h [112].

Monocrotophos may pose an important sublethal hazard to wild birds that has gone unnoticed because of its extreme acute toxicity to wildlife (Tables 25.2 and 25.3) [42, 97, 158]. For example, breeding northern bobwhites and chukars were fed 1.25–5 mg of monocrotophos per kg of diet for up to 112 days [124]. Chukars were generally tolerant of the treatments for the physiological and reproductive variables evaluated; whereas, bobwhites were consistently sensitive to the 5 mg/kg diet. Overall food consumption was depressed about 20% for quail on the 5 mg/kg diet and with an average weight loss of about 10% within 6 weeks; mortality occurred within 4 weeks and only one of 12 hens survived the 16 week study while 11 of 12 controls survived. Six of 12 hens survived the 1.25 mg/kg diet, but their egg production was depressed about 80% and hatchability of eggs laid was only about 50% of the control rate. No monocrotophos residues were detected in any eggs. Egg fertility was depressed for all treatments by eventual monocrotophos-induced sterility of males [123]. Based on a variety of lethal, chronic and reproductive variables, Schom *et al.* [125] developed a maximum acceptable toxicant concentration estimate for dietary monocrotophos between 25 and 100 mg/kg for domestic chickens, 5 and 25 mg/kg for chukars, and <1.25 mg/kg for northern bobwhites. Another study with northern bobwhites was conducted to determine whether monocrotophos effects on reproduction were the result of anorexia, whether reversal occurred once antiChE was withdrawn, and whether birds responded differently to decreasing concentrations of toxicant as expected in nature [143]. Concentrations from 0.1–1 mg of monocrotophos per kg of diet were given, then at 3-day intervals the basic concentration was either continued or reduced so that by the midpoint and last 3 days of the 15-day study the basic concentrations were reduced by 50 and 75%; all birds were then fed control diet for 2 weeks. All constant exposure birds were accompanied by pair-fed controls. Food consumption and egg production were negatively dose-related during treatment for both constant and decreasing concentration groups. The laying rate of pair-fed hens was reduced to the same extent as the constant group. Reproductive inhibition was not permanent, but the time to recovery was dose-related. There was no evidence of a pesticide effect on reproduction other than that exerted through pesticide-induced anorexia [143].

Systemic latent antiAChE pesticides that may undergo phytometabolism to their more toxic analogues and thereby function as direct AChE inhibitors, may be more difficult to detect when incorporated in a natural matrix, and may remain available for longer than antiChE exposed to the elements. Temephos is potentially one of these compounds, and therefore, Abate 4E was fed at 0, 1 and 10 mg of temephos per kg of diet to paired mallards from just before the onset of lay to natural incubation of eggs and rearing of the ducklings to 21 days of age [32]. The principal effects were an increase in the interval between eggs laid by hens fed temephos diets and decreased survival of ducklings to 21 days of age. Once lay began, controls laid an egg a day until the clutch was complete, nearly all eggs were fertile, hatched, and 94% of ducklings survived for 21 days. In contrast, hens fed temephos laid an egg on average every 1.4–1.5 days ($P < 0.05$ *versus* control). Although fertility and rate of hatch was virtually identical for both temephos treatments and controls, fewer than 75% ($P < 0.05$ *versus* controls) of temephos-treated ducklings survived for 1 week and only 60% ($P < 0.05$) survived 3 weeks. Blood plasma ChE activity was inhibited about 20% ($P < 0.05$) in temephos-treated ducklings at 21 days, but not in adults. Brain AChE activity was not altered in either ducklings or adults. No residues of temephos or its sulphoxide or sulphone metabolites were detected in eggs. This study supports the possibility that subtle OP pertubations of the endocrine-behavioural processes from trivial antiChE exposures may occur in nature and be easily overlooked.

Various laboratory studies with gallinacious species and mallards have been conducted on antiChEs such as azinphos-methyl [39], chlorpyrifos [37], diazinon [141,142], dicrotophos [129], dimethoate [131], malathion [96], methidathion [162], phosphamidon [50], and thiram [40].* These studies revealed little potential for either OP or CB antiChE pesticides to consistently impact on avian reproduction without also jeopardizing parental survival.

*A thiocarbamate

The most important effect of antiChE applications on avian reproduction in nature, other than killing or incapacitating the parents, is the removal of the prey base [42]. When prey is depleted, birds may abandon nests and emigrate from antiChE-treated areas, or at least have more difficulty in caring for their young. Abandonment of the first nesting attempt is especially critical to population success because subsequent attempts are usually less successful [85]. Some of the subtle effects of sublethal parental poisoning have been studied for free-living European starlings nesting in artificial nest boxes [44]. When nestlings were 10 days old, the male parent was eliminated and the female parent dosed once by gavage with 2.5 mg/kg of dicrotophos; the oral LD_{50} for adult female starlings is about 8 mg/kg [76]. The female was then monitored at intervals of 2 h for the next 3 days. The antiChE-dosed parents made fewer trips in search of food for their young ($P < 0.05$) and remained away from their nests for longer durations ($P < 0.05$) than did corn oil-dosed controls. As a consequence, nestlings of dicrotophos-dosed females lost about 9% of their body-weight ($P < 0.05$) which could have affected their post-fledgling success. The potential impact of reduced forage from antiChE application on avian reproduction seems clear; but when primary insect prey were actually depleted by 50–70% by aerial application of fenthion or trichlorfon, neither nestling growth rates nor fledgling success were affected [17,110]. Powell [110] suggests the effect on nestling success from food depletion is dependent on the relative abundance of prey insects at the time of chemical application.

Sharp-tailed grouse given a single dosage of malathion 200 mg/kg po were less effective in defending breeding territories on leks and sometimes exhibited bizarre behaviour [93]. One member per pair of incubating laughing gulls was dosed with 6 mg/kg of parathion or with corn oil alone, and incubation behaviour was observed for 10-min intervals throughout the day for 3 days [159]. The dosage was preselected to produce brain AChE inhibition of about 50% without overt toxic signs. No effects on incubation activities were detected on the day of dosage, but parents dosed with parathion spent less time incubating on day 2 ($P < 0.01$) and the morning of day 3 ($P < 0.05$). Sharing of nest duties by the parents seemed normal by the afternoon of day 3. This study was motivated by a natural event in which adult laughing gulls gathered parathion-poisoned insects in nearby cotton fields and either died leaving the chicks to starve, or returned and poisoned the chicks through presentation of parathion-contaminated insects [156]. In a study of European starlings, 15-day-old nestlings were given 5 mg/kg po of dicrotophos; 13% died within 24 h while survivors recovered by 24 h [45]. Nestlings of low body-weight were more sensitive to dicrotophos than their heavier nestmates. The surviving antiChE-dosed birds had weight loss of about 15% ($P < 0.05$) and brain AChE inhibition of 50–60% ($P < 0.05$) within 24 h, but returned to normal fledgling weight by day 18. In a follow-up study, fledgling survival was also evaluated [144]. Brain AChE activity was inhibited about 45% on day 18 (the normal age of fledgling) and neither age at fledgling, postfledgling survival, flocking behaviour, or habitat use were different from corn-oil dosed controls.

AntiChEs are not passed through the mother to the egg in significant amounts, but antiChE may be deposited on the egg from the parents' feathers or during pesticide application. To simulate topical exposure, fertile mallard eggs were either immersed for 30 s in an aqueous emulsion or a single dose of antiChE in a non-toxic oil vehicle pipetted onto the shell on day 3 of incubation [72], a critical period with respect to organogenesis in mallards. OPs were shown to be as much as 18 times more toxic when applied to the shell in oil compared with water immersion. The order of toxicity by LC_{50} for immersion was parathion > temephos > diazinon > malathion > carbaryl; for antiChE in oil the order was parathion > diazinon > malathion > carbaryl = temephos. Neither method of egg treatment produced teratogenicity nor developmental effects at realistic pesticide application rates. However, parathion applied in water or oil at five times the recommended rate of application, caused growth retardation (body-weight and crown-rump length, $P < 0.05$) and about 65% of the embryos surviving parathion in oil were abnormal [73]. Distortion of the axial skeleton was the most frequent malformation.

The avian egg is widely used in studies of embroytoxicity and teratogenicity. Two types of antiChE-induced teratogenesis have predominated in domestic chicken embryos: type I which leads to micromelia, parrot beak, and abnormal feathering, and type II, which involves defects of the axial skeleton [73]. Type I results from inhibition of kynurenine formamidase (EC 3.5.1.9), which impairs conversion of tryptophan to essential pyridine nucleotide cofactors in the yolk sac membrane and in the embryonic liver [100,127]. Type II affects the cholinergic system [86,100,127]. Hoffman and Eastin [73] demonstrated typical type I teratogenesis in mallards dosed topically with parathion, and type II, as indicated by depressed brain AChE activity, with parathion, diazinon and malathion.

Ecotoxicity and diagnosis of antiChE poisoning

The extreme acute toxicity of many OP and CB pesticides has resulted in hundreds of incidents of avian mortality in association with agriculture throughout the world [42,49,97,134]. Investigation of these incidents is most often restricted to collection of a sample of affected specimens and chemical determination of the causative agent.

Cause of death is difficult to establish in many episodes of wildlife mortality as neither OP nor CB residues tend to accumulate. Diagnosis is made by demonstrating depressed brain AChE and that a known antiChE was present in either ingesta or tissues [47,64]. A conservative threshold of about 50% depression in whole brain AChE activity has been proposed as diagnostic of death from antiChE poisoning [91], although depression of > 70% is routinely reported for birds killed experimentally with OP or CB pesticides [11, 12, 128, 153,154]. Depression of whole brain AChE activity is consistently between 80 and 95% in birds killed in the field by OPs [54, 64, 156, 157,159]. In contrast, when birds are killed in the field by CBs, whole brain AChE activity may vary from near normal to depressions of only 60–70% [30,64, E.F. Hill, unpublished data]. It has been proposed that high levels of CB exposure may kill by NM blockage before significant penetration of the CNS occurs [154]. Also, lower AChE inhibition may reflect spontaneous reactivation of carbamylated enzyme [59,64].

Brain AChE activity is also used as an indirect means of monitoring exposure of wild birds to field applications of antiChE pesticides. However, brain AChE depression in apparently healthy specimens obtained by random selection simply indicates recent exposure to antiChE and is only circumstantial evidence of the presence of active substance in a particular habitat [58]. A specimen is considered to have received recent antiChE exposure if its whole brain AChE (plus ChE) activity is below the lower normal bound for the species (i.e. mean change −2 s.d. [16]). Neither the time nor original degree of exposure can be determined from the coincidental degree of inhibition. Avian brain AChE inhibited by 50–60% returns to normal within 2–4 weeks after exposure [26,27].

Ideally, calculation of normal AChE activity is based on a random sample of specimens of the same species, general age class, and physiological status as the species of interest, and collected from a locale where exposure to an antiChE was unlikely. Sex is not an important variable in whole brain AChE activity [67].

Table 25.10 Whole brain AChE activity[a] of apparently healthy wild birds

Species	*n*	*Mean (s.d.)*	*Bounds[b]*
Brown pelican	22	10 (1.5)	7–13
Black-crowned night heron	9	14 (0.9)	12–16
Canada goose	19	13 (1.6)	9–17
Mallard	11	12 (1.3)	9–15
American wigeon	8	10 (1.2)	7–13
Red-tailed hawk	15	19 (3.2)	12–26
American kestrel	11	27 (2.8)	21–33
Ring-necked pheasant	10	14 (1.0)	12–16
Northern bobwhite	14	13 (1.2)	10–16
Sandhill crane	8	17 (1.5)	14–20
Willet	17	14 (2.5)	9–19
Ring-billed gull	9	18 (2.6)	12–24
Mourning dove	7	16 (1.0)	14–18
Common barn owl	11	20 (3.4)	13–27
Great horned owl	19	16 (2.5)	11–27
Barn swallow	19	12 (1.4)	9–15
American crow	8	20 (3.4)	13–27
Cedar waxwing	8	22 (3.1)	15–29
European starling	12	22 (2.0)	18–26
Red-winged blackbird	19	21 (3.3)	14–28

[a]AChE activity is μmol acetylthiocholine iodide hydrolyzed per min per g of whole brain tissue (wet weight) at 25°C [24]: data from Hill [58]
[b]Normal bounds are defined as 2 s.d. above and below the arithmetic mean [16]

Storage, processing, and biochemical assay should be the same for control and suspect specimens. Development of a broad species base is necessary because normal levels of whole brain AChE activity have been shown to differ as much as 50% ($P < 0.01$) among species of the same taxonomic genus, and an overall difference of about threefold was detected among 48 species of birds [58]. The whole brain AChE activity for an array of wild avian species evaluated over several years at a single laboratory by the same assay procedure and conditions is shown in Table 25.10.

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26

Ecotoxicological consequences of interactions between avian esterases and organophosphorus compounds

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Introduction

Interest in the effects of antiChE agents on birds stems from two main concerns. First, the widespread use of OP and CB compounds as pesticides presents a potential hazard to wild birds [19], which can be assessed through biochemical effects. Second, the use of birds as convenient subjects with which to explore general toxicological principles or as 'indicators' of effects on humans or other animals [24] depends on how closely their responses resemble those of mammals. Consequently, there has been substantial research into the effects on birds of OPs and other antiChEs. It is now accepted practice to interpret certain conventionally-agreed threshold levels of ChE inhibition in brain or blood as evidence of exposure to these pesticides, sublethal intoxication, or the cause of death [31,52,56]. This simple picture is misleading. There are many avian esterases, fulfilling a wide variety of biochemical and physiological roles, and the effects of OP compounds can be equally diverse.

Biochemical mechanisms: interaction of OP compounds with esterases

Esterases catalyse the hydrolysis of esters, and certain types of esterases hydrolyze OPs. Many OP insecticides are P=S phosphorothioates which are not hydrolyzed to any significant extent by esterases. However, within vertebrates they are converted to active phosphates ('oxons') by microsomal mono-oxygenases and the oxons are frequently good substrates for esterases.

General aspects

The interaction between OPs and esterases provide the basis for a useful classification of esterases into three groups [1,5]: 'A' esterases which hydrolyze OPs; 'B' esterases which are inhibited by OPs, and 'C' esterases which do not interact with them. Figure 26.1 illustrates the interaction between OPs and 'A' and 'B' esterases. For 'B' esterases the first stage in hydrolysis involves phosphorylation of the enzyme, and release of the leaving group. In some 'B' esterases phosphorylation is of a serine residue, but it is not known whether this is always the case. The next step involves release of the phosphoryl moiety and consequent reactivation of the enzyme. In 'B' esterases reactivation is generally slow, with the consequence that OPs function as inhibitors. The rate of reactivation of phosphorylated 'B' esterases depends on the nature of the esterases, the structure of the phosphoryl moiety, and the process of ageing, factors which will be discussed later. Metabolism occurs in two distinct phases. First there is a rapid release of the leaving group, associated with phosphorylation of the enzyme, followed in seconds or minutes by a very slow release, which is related to the rate of reactivation of the enzyme. In contrast, 'A' esterases cause continuous hydrolysis of OPs. It is not known whether the mechanism of hydrolysis is the same as that for 'B' esterases. A general mechanism is proposed in Figure 26.1, suggesting the formation of an adduct between the enzyme and one part of the OP. An important difference from the scheme for 'B' esterases is

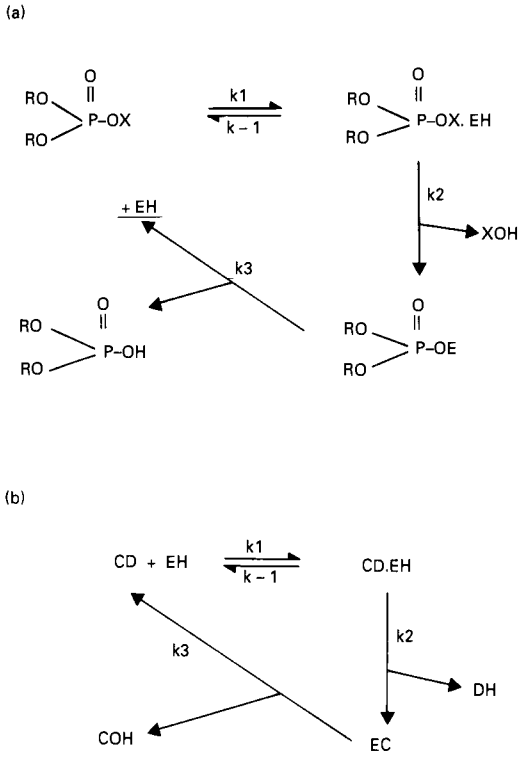

Figure 26.1 Interaction of OPs with esterases: (a) 'B' esterase. (b) Hypothetical scheme for 'A' esterase, in which C and D represent parts of the OP molecule. X indicates the leaving group

that rapid reactivation of the enzyme occurs, ie. the adduct is unstable and the rate constant k_3 is relatively large [2].

The 'B' esterases are a large and diverse group, some of which have been studied in detail. Two major subgroups are identified: (1) unspecific carboxylesterases (EC 3.1.1.1) and (2) cholinesterases (EC 3.1.1.7 and 3.1.1.8). For further discussion of classification the reader is referred to the literature [22,37,50].

Carboxylesterases

Carboxylesterases occur widely in nature, and are found in vertebrate liver, lung, blood (plasma and RBC) and brain. High concentrations are found in liver microsomes. Carboxylesterases do not apparently constitute sites of action of OPs, but they can contribute to the detoxification of them, by the mechanism shown in Figure 26.1.

ChEs

ChEs are distinguished from other B esterases by their specificity for cholinesters, a specificity that is associated with the presence of an anionic site in addition to a catalytic site. The former binds the cationic head of choline, while the latter interacts with the ester bond. The AChEs of the CNS are the principal site of action of OPs. AChE is also found in RBCs of mammals and in the blood plasma of birds. Whereas AChE has a high specificity for ACh, BChE (EC 3.1.1.8) has a wider substrate specificity and readily hydrolyzes BCh. BChE is well represented in the plasma of birds and mammals, but its physiological role is not known. The inhibition of plasma BChE provides a valuable indication of exposure to OPs.

Neuropathy target esterases

Neuropathy target esterase (NTE) (*see* Ch.10) is another 'B' type esterase of the nervous system, which has not been purified and whose physiological role is unknown. Inhibition of these esterases by certain OPs (e.g. triorthocresyl phosphate or the oxon of leptophos) can cause degeneration of myelin, and associated paralysis over a period of weeks or months after exposure. The development of delayed neuropathy depends on the ageing of the phosphorylated esterase; the transfer of the R group to a neighbouring peptide may cause the primary toxic lesion. Some birds are very sensitive to this type of poisoning, and the chicken is used in a standard test procedure for the neurotoxic effects of OPs [28,29].

Inhibition of esterases

The degree to which a 'B' esterase is inhibited following exposure to an OP is related to the values of the rate constants shown in Figure 26.1. The value of k_3 determines the rate of the slowest step, the reactivation of the enzyme, and depends on the structure of the phosphoryl moiety attached to the enzyme. In general, rates of reaction follow the order: dimethyl phosphates > diethyl phosphates > diisopropyl phosphates [2]. The rate of reactivation of a phosphorylated enzyme can be increased by reactivating agents such as 2-PAM or obidoxime chloride.

Ageing

After 'B' esterases have been phosphorylated, a process of ageing may occur. One of the organic groups (R groups in Figure 26.1) is removed to leave an ionized phosphate group. Once the ageing process has occurred, inhibition is no longer reversible, and the enzyme cannot be reactivated by 2-PAM and related oximes.

'A' esterases

'A' esterases were thought to be the same as arylesterases (EC 3.1.1.2). However, at least some are distinct from arylesterases, and are reclassified as phosphoric triester hydrolases (EC 3.1.8) [32,37,50]. 'A' esterases are found in the endoplasmic reticulum of the liver and other tissues and in the HDL fraction of serum or plasma of mammals. Little of this activity is found in birds.

Biological consequences of exposure to antiChEs

As indicated earlier, exposure to an OP pesticide leads to a variety of biochemical responses, some of which protect the animal, and some of which are detrimental. Depending on the route of exposure and the dose received, death may result from inhibition of AChE in the PNS and/or CNS. If the effect is large and sudden, paralysis of peripheral functions may occur before the compound has reached the brain; in such cases, assays of brain esterase activity would not be likely to reveal inhibition. Less extreme levels of exposure may produce a change in the quantity and distribution of enzymes, as the body's defences stimulate the release and/or synthesis of detoxifying esterases. Inhibition of esterases proceeds in opposition to this defence, and the net effect will determine whether sublethal symptoms of poisoning are seen. Behavioural debilitation is commonly observed as a reduced ability to avoid predators [9], lethargy [18] or anorexia [13]. All these may reduce the prospects of survival. If exposure occurs in the breeding season, parental care may be affected. Grue *et al.* [17] found the rates at which parent starlings *Sturnus vulgaris* fed their nestlings were reduced by > 23% after the birds were dosed with dicrotophos at a level which produced 50% inhibition of brain ChE. However, subsequent studies on the red-winged blackbird *Agelaius phoeniceus* [39] and the tree sparrow *Passer montanus* (Hart *et al.*, unpublished data) revealed no change in the feeding schedules of parent birds exposed to normal field doses of OP pesticides.

Some attention has been given to the levels of depression of esterase activity at which behavioural symptoms appear. A review by Peakall [38], and updated by Hart [20] and Grue *et al.* [14], concluded that changes in behaviour showing reduced performance are evident only at levels of exposure close to lethal intoxication. The degree of depression of brain AChE activity associated with these changes is usually substantial, but is extremely variable. However, recent studies have revealed subtle changes that are not directly detrimental, occurring at much lower exposure and with less depression of AChE (e.g. singing by male starlings; Hart, personal communication).

Dose

The dose of pesticide that a bird receives depends on many factors, including the formulation, application rate, route of exposure, and body size. Most pesticides enter birds by ingestion, although this may include preening of residues on feathers, as well as feeding. Food selection behaviour provides a means by which intake, and hence pesticide exposure, may be regulated. A hungry bird may eat nutritious treated food until adverse consequences are felt. In such cases, survival depends on the rate of feeding (i.e. how quickly the bird acquires a toxic dose relative to its size), before symptoms of poisoning are experienced. Birds such as pigeons, which rapidly fill their crops with seed when foraging in newly-sown fields, are susceptible to poisoning in this way from poorly incorporated seed treated with insecticides such as chlorfenvinphos and fonofos in the UK [7,11]. In contrast, many birds have well-developed mechanisms by which they avoid dangerous foods. This is based on inherent distaste for certain flavours, neophobia, and conditioned aversion learning [40]. It is

often thought that different chemicals act through different mechanisms; however, studies on methiocarb have revealed a complex combination of behavioural effects [12]. Some bird species require the flavour of methiocarb to be associated with illness before they learn to avoid treated food, whereas others learn slowly or not at all if there are no visual cues to accompany the flavour. Red-winged blackbirds show an increase in heart rate and respiration rate shortly after first feeding on food treated with methiocarb [48], but starlings may not avoid the food until after they have vomited (S. Yusufu, unpublished data). In some social species, birds learn aversion by observing signs of distress in others that have ingested methiocarb [34]. Although flavour aversion may protect birds against repeated ingestion of some compounds, it is not universal; for a large number of pesticides, the diet concentration that is repellent is only slightly lower than that which gives a lethal dose [42].

Because of these influences on feeding behaviour, the hazard of a pesticide depends not only on the toxic action of the compound within the body, but also on the bird's ability to detect and avoid it after first contact. The potential hazards of certain granular formulations such as carbofuran to small birds are high because a single granule may contain a lethal dose, precluding the possibility of behavioural adjustment [36]. This aspect is relevant to differences in observed poisoning between birds and mammals, because in contrast to mammals, birds rely more on vision than on their chemical senses [51] and are thus less likely than mammals to detect pesticide treatments on their food.

Variations in avian toxicity of OP and CB pesticides

Empirical assessment of acute toxicity is usually made by determining the median lethal dose in standard laboratory tests. This eliminates many of the behavioural factors mentioned earlier, and thus differences observed between species are likely to arise from biochemical mechanisms. Smith [43] has summarized LD_{50} values to birds for most OPs and CBs. From this, data for 13 bird species, and laboratory rats, to allow comparison with a standard mammal, were selected. The information covers 48 OPs and twelve CBs. Examples of the range of LD_{50} values among bird species are shown in Table 26.1 for eight OPs that provide sample sizes of at least seven species. This reveals a range of variation that is generally less than one order of magnitude between the highest and lowest bird species LD_{50}s. Differences between birds and mammals are much greater in many cases, and it appears that there is reasonable consistency among birds of very different lifestyles in their susceptibility to OP poisoning. However, the data set includes no predatory or scavenging species, which might be expected to differ in some aspects of biochemistry.

Table 26.1 Examples of the variation between bird species in LD_{50} values for OP pesticides. Data for rats are included to give a comparison with toxicity to mammals

	Chlorpyrifos	*Dicrotophos*	*Fenthion*	*Monocrotophos*	*Parathion*	*Temephos*	*Trichlorfon*	*Oxydemeton methyl*
Rat	97	16	150	8	3.6	2030	144	47
Mallard	75.6	—	5.9	4.8	1.4	79.4	36.8	53.9
Pheasant	8.4	—	17.8	—	12.4	31.5	95.9	42.4
Starling	—	2.7	5.3	3.3	5.6	>100	43.0	—
Bobwhite quail	32	—	≤4	0.9	6	—	22.4	—
Red-winged blackbird	—	—	1.8	1.0	2.4	42.2	37.0	—
Canada goose	40	2.3	12.0	1.6	—	—	—	—
Japanese quail	15.9	—	10.6	3.7	6.0	75.0	—	84.1
Rock dove	26.9	—	—	2.8	2.5	50.0	123.0	14.0
California quail	68.3	1.9	15.0	0.8	16.9	—	59.3	47.6
Chukar	60.7	9.6	25.9	6.5	24.0	—	—	113.0
House sparrow	21	3.0	22.7	1.5	3.4	35.4	—	70.8
House finch	—	2.8	10.0	8.1	—	—	—	—
Sharp-tailed grouse	—	2.3	—	—	5.7	—	—	—
Highest/lowest (birds)	9.0	5.0	14.4	10.1	17.1	>3.2	5.5	8.1

Table 26.2 Correlations between toxicity of pesticides to pairs of species

	Rat	*Mallard*	*Pheasant*	*Starling*	*Bobwhite quail*	*Red-winged blackbird*	
Rat	—	0.71 (11)	0.64 (10)	0.68 (7)	X	0.82 (9)	
Mallard	0.62 (40)	—	0.83 (10)	0.71 (7)	X	0.85 (9)	
Pheasant	0.52 (34)	0.83 (33)	—	0.21 (7)	X	0.65 (9)	Carbamate
Starling	0.52 (29)	0.62 (23)	0.40 (19)	—	X	0.92 (7)	
Bobwhite quail	0.30 (17)	0.82 (16)	0.68 (13)	0.80 (10)	—	X	
Red-winged blackbird	0.41 (26)	0.81 (21)	0.68 (17)	0.75 (26)	0.75 (10)	—	
			Organophosphorus				

[a]Values are Spearman Rank coefficients for the correspondence of LD_{50}s, with sample size (number of compounds) in brackets
[b]OP pesticides are below, and carbamates above the diagonal

The patterns were examined further by calculation of correlation coefficients between each pair of species in Table 26.1, using their LD_{50}s with respect to a range of compounds. Spearman rank correlations were used because of the non-linear scaling of the LD_{50} index and the inclusion of several extreme values. All correlations were positive, reflecting the general consistency in the ordering of toxicity of different compounds. Table 26.2 lists results for the six species giving the largest sample sizes, and covers both OPs and CBs.

This reveals three principal points of interest. First, correlations between bird species for OPs were higher than between birds and rat (overall average 0.74 *versus* 0.57, $P<0.03$, *Mann–Whitney U* test). There is no difference evident for CBs. Second, the data give no evidence for a closer similarity between species in the same order than between those that are less closely related taxonomically. Third, correlations are generally greater for CB than for OP compounds, despite the smaller sample sizes.

The most notable anomaly is the poor correspondence between the LD_{50}s of pheasant and starling (Figure 26.2). Examination of the outlying chemicals in this relationship, and in other such plots, does not provide any common pattern in the compounds which are of particularly high or low toxicity to a species. For example, in a comparison of two ecologically-

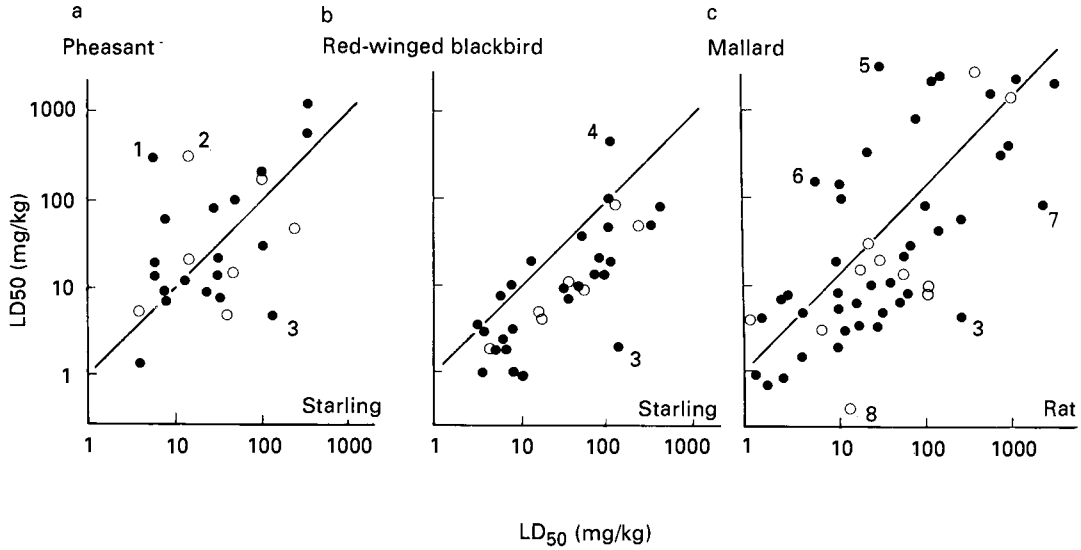

Figure 26.2 Correspondence between pairs of species in the toxicity of OP and CB pesticides. Each point represents the acute oral LD_{50} of one compound to the two species: , OP pesticide; , CB pesticides. Numbers indicate pesticides: 1, carbophenothion; 2, methiocarb; 3, diazinon; 4, sulfotep; 5, ethion; 6, azinphos-methyl; 7, temephos; 8, carbofuran. Compiled from Smith [43]

similar species within the same order, the starling is generally less sensitive than the red-winged blackbird (Figure 26.2b). The four compounds that are considerably more toxic to the red-winged blackbird (diazinon, phorate, isofenphos and monocrotophos) are of diverse structure and physical properties [44,54]. Of the compounds included, only sulfotep is noticeably more toxic to the starling. The sulfotep molecule differs from many other OP compounds included because it contains two central P=S groups available for activation by oxidative desulphuration; however, this does not explain the difference in toxicity for the two bird species.

Figure 26.2a and c also contain a number of outliers on both sides of the relationship. These do not share obvious common structural features or physical properties which indicates that the toxicity of a compound to different species is determined by a complex combination of factors. Some of these are likely to be related directly to esterase inhibition, but others probably reflect variation in other mechanisms affected indirectly by inhibition of neural ChE.

Biochemical basis of selectivity

AntiChE chemicals vary in their effects from species to species. This is a consequence of toxicokinetic and toxicodynamic differences; at one level, species may differ in the processes of uptake, distribution, storage, metabolism and excretion of a pesticide, while there may also be differences in the interaction of the toxic form of the pesticide with its site of action. Esterases can be important both because of their role in detoxification, and because AChE is the site of action of antiChEs.

Detoxification

'A' esterases have an important role in detoxifying active oxons, which sometimes are original insecticides but more often are their active metabolites. The liver receives much of the insecticide absorbed from the gut, and by virtue of its high mono-oxygenase activity, readily activates incoming P=S phosphorothioates to their oxons. The extent to which these oxons are transported in the blood to their sites of action in the nervous system depends on the effectiveness of detoxification by the esterases of the liver and blood. Most birds have no detectable 'A' esterase activity in blood [6,32]; on limited evidence, the level in the liver appears to be much lower than it is in mammals. The consequent inability of birds to rapidly and continuously hydrolyze oxons formed in the liver before they reach their sites of action in the nervous system is a major factor in determining the relatively high avian toxicity of OP insecticides such as pirimiphos-methyl and diazinon [6,49]. A similar situation exists with chlorpyrifos and pirimiphos-ethyl. All of these insecticides yield oxons which are excellent substrates for serum 'A' esterase of mammals. Consequently mammals, but not birds, carry out efficient detoxification. Within birds some differences in hepatic (microsomal) 'A' esterase (paraoxonase) have been observed, e.g. blackbird (*Turdus merula*) and barn owl (*Tyto alba*) have higher activities than the African bulbul (*Pycnonotus capensis*) or house sparrow (*Passer domesticus*) [55]. These differences may result in corresponding differences in detoxification of active oxons. By contrast with 'A' esterases, 'B' esterases are well represented in the serum/plasma of most birds. 'B' esterases are less effective in detoxifying OPs than are 'A' esterases. However, they are useful in monitoring exposure to pesticides in the field. Increases in blood levels of carboxylesterases, and inhibition of both serum carboxylesterases and ChEs can indicate responses in birds that are trapped and released for further study [46].

Species differ in their complement of serum esterases. Herbivorous and omnivorous birds tend to have a greater variety, and higher levels, of carboxylesterases than do predatory species [45]. This suggests that predatory birds may be less effective than others in detoxifying certain OPs; the same may be true of pyrethroid and CB insecticides which are hydrolyzed by esterases. The starling has a particularly high level of plasma and brain AChE [53], which might account for its lower vulnerability than other birds (Figure 26.2)

Differences in the relative amounts of the serum AChE and BChE have also been demonstrated in other groups of birds

(Hooper, personal communication). The wide range of 'B' esterases present in pheasant (*Phasianus colchicus*) tissues may have contributed to the success of this species in regions of the USA where other avian species are adversely affected by pesticides [4].

Differences in AChE activity

There may also be species differences in the target enzyme AChE, which are responsible for differences in toxicity. Haloxon, for example, is more toxic to the duck and goose than to the hen, sheep or rat [30]. Andersen *et al.* [3] reported that chicken brain AChE was inhibited by OPs, such as soman, twenty times faster than rat or mouse brain AChE. These species differences were thought to be primarily differences in the rates of phosphorylation and reactivation of brain AChE. Within a species there is often considerable inter-individual variation in the activity of the brain and serum esterases. Hill [23] reported a 1.3–1.7-fold variation in the extremes of brain AChE activity in species of wild birds. Serum BChE activity of starlings shows an apparently normal distribution with extremes differing 7.5-fold, whereas serum carboxylesterase activity of starlings exhibits a bimodal distribution with the maximum and minimum differing by tenfold (Figure 26.3). This variation in activity does not correlate with the distribution of serum esterase phenotypes of the starling shown by Martin *et al.* [33]. Patterns of individual variation are important for their influence on the total impact of birds' exposure on the population.

Temporal variations, such as diurnal, seasonal or developmental changes in the activity of serum and brain esterases may contribute to inter-individual variations and may affect susceptibility to poisoning by antiChEs. A diurnal increase in the activity of serum carboxylesterase activity has been reported in the starling [47] and in serum BChE in the buzzard (*Buteo buteo*) [10]. Seasonal and sex differences in brain AChE and serum BChE activity have been reported in a variety of species [25]. Age-related differences in sensitivity to OPs has been shown in the mallard (*Anas platyrhynchos*) [26]. Brain AChE activity in the starling has been shown to be age-related [15,16], as has serum BChE in the tree sparrow (*Passer montanus*) [21].

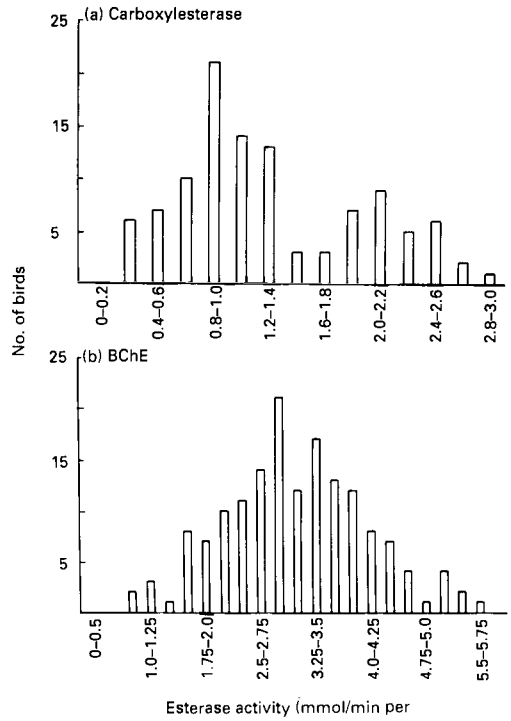

Figure 26.3 Variations in esterase activity among individual starlings *Sturnus vulgaris*. (a) Bimodal distribution of serum carboxylesterase activity. (b) Unimodal distribution of BChE activity

Within-species differences

As with species differences in susceptibility, differences in the toxicity of OPs to a single species may be determined by the rates at which oxons are hydrolyzed by esterases, and how effectively the oxons inhibit the target AChE. Because few data are available for birds generalization is difficult.

Quantitative structure-activity relationships

Studies of esterase inhibition by OPs have been carried out through derivation of quantitative structure-activity relationships. These explore the effects of small changes in molecular structure on the rate constants of biochemical processes, by measurement and modelling of a wide range of compounds within a single restricted class of molecule. Most work has involved esterases from animals other than birds, e.g. horse serum BChE and bovine RBC AChE [27], and house

fly [8]. Results have been variable and do not suggest common relationships which can be confidently extrapolated to birds. Features such as lipophilicity, steric factors, and atomic charges on the central phosphorus atom have all been found to correlate closely with stages in the esterase inhibition process in different cases.

It is also possible to seek correlations at a cruder level, by comparing molecular structure and properties with the acute LD_{50} or other measures of toxicity to a species [35]. Again, this approach has been little explored for birds, although Metcalf *et al.* [35] considered the effects of halogenated P=S phosphorothioates on delayed neurotoxicity in hens. A preliminary analysis relating structure and properties of OP pesticides listed by Smith [43] to acute oral LD_{50}s for four bird species (mallard, pheasant, starling and red-winged blackbird) suggested that molecular size and chlorination of the molecules are more closely correlated with toxicity than is their hydrophobicity (Greig-Smith *et al.*, unpublished data). However, the correlations at this level of analysis are poor, indicating that many other factors are involved in determining toxicity [41] (*see* Ch.6).

Conclusions

The role of esterases in determining the toxicity to birds of OP and other antiChEs depends both on the rates at which oxons are hydrolyzed by esterases, and on how effectively the oxons inhibit the target AChE. However, generalizations are difficult owing to lack of information available for birds. More data are required on the kinetics of metabolism of OP compounds and the inhibition of esterases *in vitro*. In this way, critical differences between species and compounds may be identified.

At present, there is a gulf between our understanding of the biochemical interactions that underlie the toxic action of antiChEs, and empirical measurement of species differences in toxicity, which reveals large unexplained variations. Also, many factors modify the inherent toxicity of pesticides, increasing or decreasing the hazard observed in the field.

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27

Toxicity of anticholinesterases to aquatic organisms

A.S. Murty and A.V. Ramani

Introduction

The history of the succession of different groups of pesticides has been well documented [6,22,49,67]. Although they are used mostly in the terrestrial environment, their transport to, or actual use in the aquatic environment and subsequent distribution, affect a much larger number of species than originally intended.

It is now established that the aquatic environment is the ultimate sink for all anthropogenic chemicals [51,80]. The aquatic environment stands second among the three planetary compartments (soil, air and water), in terms of global uniformity of composition, biotic richness, distance the pollutant is transported, and the extent of dilution of the pollutant in it. It is first in line as a sink for pollutants, for the volumes transported, and the consequent risk posed to aquatic organisms [53].

Persistence in soils and transport to the aquatic environment

The extent of transport of OPs and CBs to, and their concentration in the aquatic environment are influenced by their soil persistence and their long-range transport in the atmosphere, from which they are washed down by rain. The longer they persist in soil or the atmosphere, the greater is the chance for transport to the aquatic environment.

Persistence in the soils

Although one report notes the persistence of parathion in soils for 16 years [66], OPs and CBs are not as persistent in soil as the organochlorines (OCs). Consequently, OPs and CBs have to be applied repeatedly, resulting sometimes in their accumulation in the soil to the next crop season [45].

The persistence of a compound in nature depends on its chemical structure, water solubility, and biodegradability. The main carbon bonding influences the extent of degradability of aliphatic compounds and their derivatives [40]. Degradability of aromatic compounds depends on substituent groups. In general, the greater the water solubility of a compound (which is inversely related to its octanol-water partition coefficient; K_{ow} value), the greater is its biodegradability and the less its soil persistence. K_{ow} increases by more complex aryl substitution. High K_{ow}, poor water solubility, and resistance to hydrolysis, possessed by some OPs suggest their longer soil persistence and subsequent transport to the aquatic environment; e.g. the log K_{ow} for chlorpyrifos, dichlofenthion and leptophos are 5.11, 5.14 and 6.31, respectively, compared with 5.69 for p,p- DDE and 6.19 for p,p- DDT, two compounds with high persistence [25].

Entry into the atmosphere

During application, pesticides are carried by drift which is enhanced by aerial spraying. Hindin and Bennett [30] concluded that 5% of ethion applied by airplanes did not reach the plants, but persisted in the atmosphere for over 2 weeks. Following aerial spraying of New Brunswick forests with fenitrothion, rainwater collected in the vicinity had 77 µg/l; even 85 km away, the concentration was 0.16 µg/l [55].

Pesticides also enter the atmosphere by volatilization, and are subsequently washed down by rain. Weibel *et al.* [70] recorded the

presence of fenchlorphos in rainwater collected near Cincinnati, Ohio.

Transport to the aquatic environment

The major sources of OP and CB residues in the aquatic environment are eroded sediment and runoff from croplands. Water soluble compounds (10 mg/l or more) are carried in the runoff (Figure 27.1), whereas poorly water soluble ones (<10 mg/l) are carried adsorbed to sediment [69]. The distance of transport depends on their adsorption to soil particles, volume of runoff, extent the land is tilled (ploughed lands are eroded more than untilled lands), distance from application site, slope of the land, peak period of agricultural activity, etc.

Adsorption of residues to soil particles is a function of water solubility, size of the molecule, and soil organic matter content. In a study on six OPs and two CBs in soil and aqueous suspension of soil and sediment, Sharom *et al.* [63] reported that adsorption to soil particles was inversely, and desorption directly related to water solubility. Adsorption was positively, and leaching from adsorbed state negatively correlated with the soil organic matter. Strongly adsorbed to the soil particles, poorly water soluble residues move to the aquatic environment to the minimal extent in the runoff, but maximally with eroded particles.

Lastly, accidents occasionally carry large quantities of pesticides to the aquatic environment, e.g. when Teton Dam, USA broke on 5 June 1976 discharging nearly 30 billion m^3 of water, it washed away several thousand kg of formulated disulfoton, malathion, azinophos-methyl, demeton, phorate and other pesticides.

Residues in the aquatic environment

OP residues are less commonly reported than OCs from natural waters. Contamination of Ashley River and Charleston harbour from untreated effluent from a pesticide formulating plant (S,S,S-tributyl phosphorotrithioate) has been reported [67]. According to Dupuy and Schulze [20] contamination of coastal waters with OP residues is not uncommon. Miles and Harris [45] recorded 26, 229 and 88 ng/l of total OP residues in the water of Holland Marsh in Southern Ontario, during 1973, 1974 and 1975, respectively. Surface slicks act as concentrators of pesticide residues, especially those of moderate to high K_{ow} value. Following aerial spraying of fenitrothion, Moody *et al.* [47] recorded 701 µg/l in the surface waters, compared with a subsurface concentration of 9.5 µg/l.

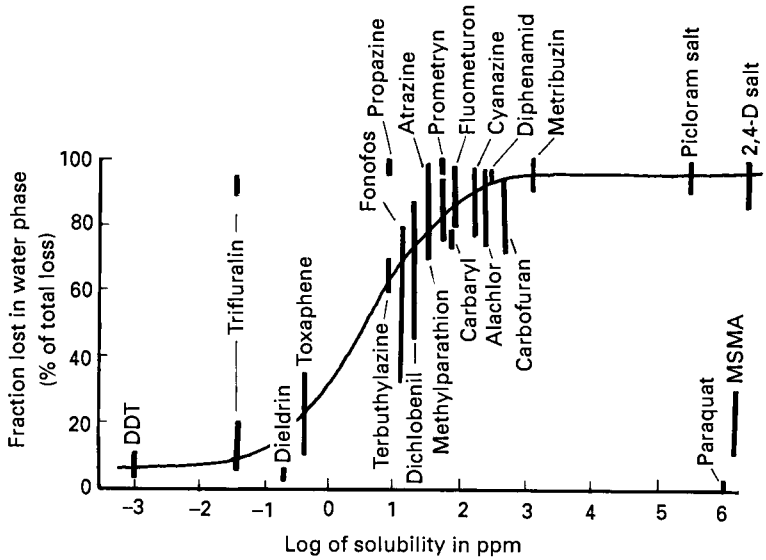

Figure 27.1 Relationship between water solubility and fraction of soil residue content lost in the runoff. Reproduced from Wauchope [69] with permission

In general, the residue levels in natural waters are as follows: closed ponds > free flowing waters > lakes > more contiguous bay-estuaries > open sea.

In a study on the fate of parathion in pond waters, high concentrations (up to 1.9 mg/kg) were reported from bottom mud [52]. Residues in water were found to persist. On the other hand [^{14}C]diazinon and parathion sprayed on a cranberry bog disappeared from water within 144 h [46]. Sharom *et al.* [63] found that in natural waters, OPs degrade chemically and microbially. Degradation of carbaryl was mostly chemical.

During 8 weeks, they found that in natural waters ethion and leptophos were more persistent and that parathion, mevinphos, diazinon, carbofuran and carbaryl were less persistent, in that order. The latter were mainly in water while the former were adsorbed to soil particles.

OP and CB residues in water and soil are rapidly degraded photolytically, chemically and microbially. Under anaerobic conditions of flooded soils, H_2S converts the amino analogues of parathion and parathion-methyl, and fenitrothion to their des-amino derivatives [2], but the antiAChE properties of the latter, and their environmental hazard remain unknown.

Bioconcentration and bioaccumulation of antiChE

Because persistence of OPs and CBs in the aquatic environment is short and their degradation is fairly rapid, their chronic toxicity to, and accumulation in, aquatic organisms is low; however, persistence in body tissues causes inhibition of AChE. The uptake of residues from water, under experimental conditions, is termed the 'Bioconcentration Factor' (BCF), which is low for OPs and CBs (Table 27.1) (There is some confusion on the use of the terms bioconcentration, bioaccumulation and biomagnification. The first two, relevant to the aquatic environment, denote uptake from water or from both water and food respectively. Biomagnification operates only in the terrestrial environment, and signifies magnification at each tropic level [49]). In general, log BCF is directly related to its log K_{ow} value, within limits. Linearity between log BCF and log K_{ow} holds only when the latter is > 2 and <6 [65]. For instance, leptophos, having one of the highest known pesticide K_{ow} values (6.31) [25] bioaccumulates only 750 times in 10 days in bluegill sunfish [42].

Because the chronic toxicity of OPs and CBs is low, and high concentrations rarely reach the aquatic environment, the concern regarding their environmental hazard seems misplaced. However, because they ultimately reach the aquatic environment and as they are potent inhibitors of AChE even at sublethal concentrations, and since long after the parent compounds have disappeared their degradation or metabolic products continue to inhibit the enzyme, an in-depth study of the effect of antiChEs on aquatic organisms is appropriate.

Fish brain AChE inhibition studies

Effect of OPs and CBs

Weiss [71] was first to investigate *in vivo* brain AChE activity of fish exposed to antiChEs,

Table 27.1 Bioconcentration of some antiChEs

Compound	*Organism*	*Exposure period (days)*	*BCF*[a]	*Reference*
Fenitrothion	Coho salmon underyearling	1	16	Bull [7]
	Killifish	10	53	Sasaki *et al.* [60]
Chlorpyrifos	Bluegills	3	1590	Macek *et al.* [43]
Parathion	Mosquitofish	34	335	Yu and Sanborn [77]
Diazinon	Topmouth gudgeon	7	152	Kanazawa [38]
	Carp	7	65	Kanazawa [38]
	Guppy	7	18	Kanazawa [38]
Thiobencarb	Fathead minnow	2.5	460	Sanders and Hunn [59]
	Bluegill	5	91	Sanders and Hunn [59]

[a]Ratio between the concentration in the body and concentration in water

analogous to OPs. He found the extent of enzyme inhibition was proportional to the concentration of the substance and extent of exposure, and suggested that fish brain AChE activity could be used to detect the presence of antiChEs in the aquatic environment. Later, he [72] studied the inhibition of brain AChE activity of largemouth bass, bluegill, golden shiner and goldfish exposed to malathion, azinphos-methyl, fenitrothion, EPN and diazinon. Brain AChE inhibition was a function of the concentration of the toxicant, period of exposure and the species of fish. In a further study with 12 OPs at concentrations expected to result in the environment following pest control measures, Weiss [73] found that the curve of brain AChE inhibition and recovery described a physiological brain ChE characteristic of the species involved. He suggested that it would be possible to demonstrate OPs in water at a concentration of 0.1 mg/l and exposure periods of 12–24 h.

This work led to other investigations on the effect of antiChEs on brain AChE levels of many fish species. Williams and Sova [76] reported that distressed and moribund Atlantic menhaden and Atlantic croakers collected from Ashley River, USA, having a history of fish kills, had reduced brain AChE activity; 8–44% in the former and 8–35% in the latter species, compared with unaffected fish. Residues of O,O-diethyl O-(2,4-dichlorophenyl) phosphorothioate and S,S,S-tributyl phosphorotrithioate were present in these waters.

Holland *et al.* [33] recorded reduced brain AChE activity in the spot and sheepshead minnows collected from areas along the Atlantic and Gulf coasts of the USA, with a history of OP contamination. Coppage *et al.* reported reduced brain AChE activity in sheepshead minnows exposed to nine OPs [12], pinfish, croakers, sheepshead minnows and pink shrimp exposed to four OPs [16] and pinfish exposed to malathion or malaoxon [10]. Similar results with other studies are summarized in Table 27.2.

Analytical methodology

Differences in the extent of AChE inhibition at death and other discrepancies partly stem from the differences in methods and in storage and handling. Earlier workers adopted the method of Hestrin [29], later workers followed the methods of Ellman *et al.* [23] or its modification.

Hogan [31] observed that AChE activity in bluegills varied directly with environmental water temperature, and suggested that such variations may be a source of considerable error in AChE estimations. Storage temperature and storage time influence enzyme estimation. Schoor and Brausch [62] reported that storage up to 3 weeks at 2-15°C or up to 4 days at 0°C did not produce loss of activity, but AChE activity steadily increased with increasing temperatures to 40°C and dropped sharply at 55°C.

Finlayson and Rudnicki [24] found that fish brain AChE was not significantly altered when homogenate was stored in 0.05 M Tris buffer (pH 7.4) up to 5 days at 7°C. Refrigerating the whole fish at 7°C for up to 5 days resulted in significantly lower AChE values; the longer the refrigeration time, the lower the value. Freezing whole fish at –5°C also yielded low values; the duration of freezing had no effect on the lowering. The initial low brain AChE following freezing was increased after 30 days of storage, possibly from loss of moisture after prolonged freezing. Homogenizing fish brain in Tris buffer shortly after death, and refrigeration of the homogenate for up to 5 days, was suggested as optimal for analysis.

Hogan [32] studied optimal substrate concentration and specific activity, using the brain tissue of cutthroat trout; ACh was hydrolyzed faster than propionyl choline, acetylβmethylcholine and butyrylcholine. He found no sex difference in brain AChE activity in bluegills. Coppage [12] obtained similar results with sheepshead minnows. Although smaller fish reacted faster, after a 2-h exposure AChE inhibition was the same in all size groups [72].

Dose-effect relationship

Low concentrations of malathion produced correspondingly less brain AChE inhibition than did higher concentrations [10]. Goodman *et al.* [27] also reported a dose-dependent inhibition of brain AChE activity in sheepshead minnows for diazinon. For the same sublethal exposure (6.5 μg/l), inhibition

Table 27.2 Summary of brain AChE inhibition caused by antiAChE compounds in representative aquatic organisms (exposure concentration in mg/l, unless stated otherwise)

Compound	*Organism*	*Exposure concentration*	*Exposure duration (h)*	*Inhibition (%)*	*Reference*
Parathion	Bluegill	0.1	7	85	Weiss [73]
	Fathead minnow	0.1	24	30	Weiss [73]
	Goldfish	0.1	18	52	Weiss [73]
	Goldenshiner	0.1	18	48	Weiss [73]
	Sheepshead minnow	10[a]	72	83	Coppage [12]
	Spot	10[a]	24	87–89	Coppage and Matthews [16]
	Pinfish	10[a]	24	88–92	Coppage and Matthews [16]
Parathion-methyl	Pink shrimp	1.3[a]	6	70–80	Schoor and Brausch [62]
Malathion	Bluegill	0.1	12	80	Weiss [73]
	Fathead minnow	0.01	24	60	Weiss [73]
	Spot	1.25	24	65–82	Coppage and Matthews [16]
	Pinfish	1	24	87–89	Coppage and Matthews [16]
	Pinfish	31–575[a]	3.5–72	8–79	Coppage *et al.* [17]
	Croaker	1	24	79–90	Coppage and Matthews [16]
	Sheepshead minnow	0.2	24	90–99	Coppage and Matthews [16]
	Carp	100	2	75	Kozlovskaya and Mayer [41]
	Pink shrimp	1	48	72–82	Coppage and Matthews [16]
Diazinon	Bluegill	0.1	6	95	Weiss [73]
	Fathead minnow	0.1	18	70	Weiss [73]
	Goldfish	0.1	18	43	Weiss [73]
	Goldenshiner	0.1	18	40	Weiss [73]
	Sheepshead minnow	6.5[a]	24	71	Goodman *et al.* [27]
Fenitrothion	Rainbow trout	2	48	75	Duangswasdi and Klaverkamp [19]
	Crab (by injection)	5–10[a]	12	57–77	Bhagyalakshmi and Ramamurthi [4]
Azinphos-methyl	Bluegill	0.01	8	65	Weiss [73]
	Fathead minnow	0.01	24	60	Weiss [73]
	Spot	20[a]	24	93–98	Coppage and Matthews [16]
	Pinfish	10[a]	24	77–84	Coppage and Matthews [16]
Acephate	Rainbow trout	2 g	24	25	Duangswasdi and Klaverkamp [19]
	Rainbow trout	400	24	38	Zinkl *et al.* [78]
Dichlorvos	Bluegill	0.1	12	40	Weiss [73]
	Fathead minnow	0.1	24	42	Weiss [73]
Fenitrothion	Bluegill	0.1	24	15	Weiss [73]
	Fathead minnow	0.1	24	10	Weiss [73]
EPN	Bluegill	0.1	12	60	Weiss [73]
	Fathead minnow	0.1	24	20	Weiss [73]
Dioxathion	Bluegill	0.1	6	95	Weiss [73]
	Fathead minnow	0.1	12	13	Weiss [73]
Coumaphos	Bluegill	0.1	18	55	Weiss [73]
	Fathead minnow	0.1	24	32	Weiss [73]
Naled	Spot	75[a]	24	82–89	Coppage and Matthews [16]
	Pinfish	75[a]	24	88	Coppage and Matthews [16]
Chlorpyrifos	Fathead minnow	0.27–2.68[a]	60 days	21–89	Jarvinen *et al.* [34]
Phorate	Sheepshead minnow	5[a]	72	83	Coppage [12]
Methamidophos	Rainbow trout	25	24	38	Zinkl *et al.* [78]
Trichlorfon	Perch	5	2	50–75	Kozlovskaya and Mayer [41]
Demeton	Bluegill	0.1	18	38	Weiss [73]
	Fathead minnow	0.1	24	13	Weiss [73]
Dieldrin	Oocytes of *Rana* sp.	2	4	22.7	de Llamas *et al.* [18]
Carbaryl	Rainbow trout	0.25	24	61–85	Zinkl *et al.* [79]
Carbofuran	*Channa*	312–462[a]	48	46–62	Bhattacharya [5]
Phenthoate	*Anabas* sp.	88–263[a]	24–48	50–72	Jash and Bhattacharya [35]

continued

Table 27.2 continued

Compound	*Organism*	*Exposure concentration*	*Exposure duration (h)*	*Inhibition (%)*	*Reference*
Natural systems					
O,O-diethyl *O*-(2,4-dichlorophenyl) phosphorothioate	Atlantic manhaden	Natural river water		17–47	Williams and Sova [76]
Effluent from pesticide plant	Catfish 96-h exposure	17–55 times dilution of of effluent		73–87	Coppage and Braidech [14]
Effluent from pesticide plant	Spot and sheepshead minnow			12–27	Holland *et al.* [33]
Carbaryl	Brook trout	24 h after aerial application at 1 lb/acre		15–34	Haines [28]

[a]Exposure concentration in μg/l

was independent of the exposure duration, being 68% and 78% on days 4 and 108, respectively. Similar results were obtained with climbing perch and carbofuran [5], with chlorpyrifos-exposed fathead minnows [34] and carbaryl-exposed rainbow trout [79].

Lethal levels of brain AChE inhibition

There is considerable controversy as to the level of inhibition of brain AChE activity that irrevocably leads to death. Weiss [71–73] considered 40–70% inhibition to be lethal. Brain AChE inhibition at 50% mortality for eight OPs is shown in Table 27.3. Gibson *et al.* [26] reported that fish with even 90% inhibition recovered completely when transferred to toxicant-free water. Coppage [12] found that inhibition with sheepshead minnows was a function of the pesticide concentration and length of exposure; at least 13% inhibition was necessary to indicate previous exposure to antiAChE compounds. He also observed that the highest inhibition caused by sublethal exposure was not as high as that caused by lethal exposure. With nine different OPs giving a 40–60% mortality rate survivors had an inhibition of 83% irrespective of the compound used. Greater inhibition indicated impending death. Coppage and Matthews [16] also found that AChE inhibition was 70–96% in fish surviving exposure to four OPs; mortality rate was 40–60%. Coppage *et al.* [17] confirmed that at this level of mortality, brain AChE activity was reduced to 20–30% of normal (Figure 27.2).

Table 27.3 Brain AChE inhibition in largemouth bass (exposure concentrations at 0.5 mg/l and mortality rate of 50%)[a]

Compound	*Period of exposure*	*Inhibition (%)*	*96-h LC_{50}[b] (μg/l)*
Azinophos-methyl	1	82–94	4.8
Diazinon	1	82–94	—
Malathion	2.6	76	285
EPN	9.5	58	380
Parathion	24	76	620
Disulfoton	24	76	620
Coumaphos	36	70	1100
Dichlorvos	48	75	—

[a]Weiss [73]
[b]Mayer and Ellersieck [44]

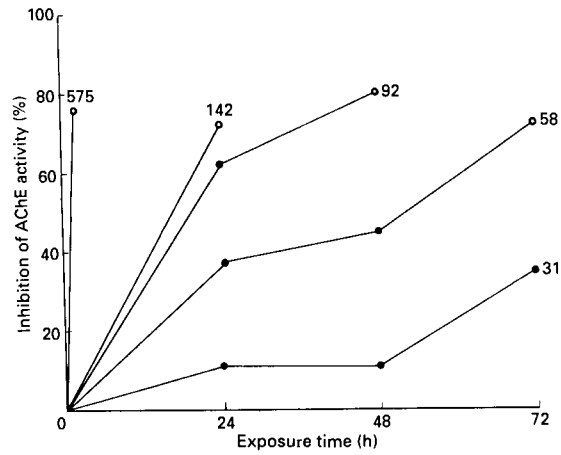

Figure 27.2 Percentage inhibition of AChE activity in the brain of pinfish exposed to lethal and sublethal concentrations of malathion. The exposure concentration (μg/l) is indicated at the end of each line. ○, 40–60% mortality rate; ●, no deaths during exposure. Adopted from Coppage *et al.* [17]

Eaton [21] noted a 30% inhibition in bluegills exposed to a 'safe concentration' of malathion for 180 days. Macek *et al.* [43] reported 80% inhibition of brain AChE in bluegills and largemouth bass exposed to chlorpyrifos at 55% and 46% mortality rate in the two species respectively. Cook *et al.* [10] reported 79% inhibition in malathion-exposed pinfish, with a 60% mortality rate. Thirujnanam and Forgash [68] recorded 56–100% inhibition in mummichogs exposed to chlorpyrifos, with a mortality rate of 20%. Coppage [12,13] concluded that AChE inhibition was similar (77–89%) regardless of the compound, species exposed and exposure period, when the mortality rate of exposed pinfish, sheepshead minnow or sailfin molly was 40–60%. Goodman *et al.* [27] found that brain AChE activity of sheepshead minnows exposed to diazinon varied with exposure concentration. Jarvinen *et al.* [34] considered that survival of chlorpyrifos-exposed fathead minnows was affected when inhibition was 80% or more. Kozlovskaya and Mayer [41] observed that perch exposed to trichlorfon died when brain AChE inhibition was 50–75%. With carp, at an 11% mortality rate there was 75% inhibition in survivors; the remaining carp died when inhibition reached 87%. They concluded that the level of enzyme activity at which death occurs, in general, depends on the physiological condition of the organism and the degree of exposure. For 48-h exposure, fish exposed to low concentrations of trichlorfon and malathion survived with enzyme activity that was fatal at higher exposure concentrations. This contrasts with the conclusions of Coppage (*op. cit.*).

For CBs, rainbow trout exposed to carbaryl had a brain AChE depression of about 85% at death, although an occasional fish showed an inhibition of only 60% [79].

It is evident that, despite a few divergent opinions, death is inevitable when AChE activity falls below 30% of normal.

Recovery period

Recovery or not of many organisms depends on the compound and the period of exposure. Weiss [71] found that surviving fish returned to normal within 2–3 weeks and that sublethal exposure made fish more susceptible to subsequent exposures. He [72] exposed largemouth bass to a slug of toxicant concentration for 1.5 h (simulating natural exposure) and then transferred the fish to clean water. The subsequent inhibition of AChE was characteristic of the individual compound. Parathion-exposed fish continued to show inhibition of brain enzyme with delayed lethality. Malathion-exposed fish recovered faster. Fish exposed to azinophos-methyl, EPN and diazinon recovered only after 2–3 weeks. Recovery was species specific; while largemouth bass exposed to fenitrothion showed a slight drop in enzyme activity, followed by a recovery period of 2 weeks, bluegills similarly exposed showed no significant inhibition. In further experiments with parathion, dioxathion and dichlorvos, among four species of fish, bluegill responded faster and recovered more slowly than the other species. It was confirmed that time to recovery was dependent on the initial inhibition, the compound and the species. Following aerial spraying of an estuarine lake with malathion, enzyme activity remained below pre-spray levels for more than 40 days in the brain of surviving spot [15]. Goodman *et al.* [27] found that AChE was depressed long after no measurable diazinon was found in exposed sheepshead minnows. In other cases, although the fish seemed to recover, periodic intermittent exposure to sublethal concentrations produced a cumulative effect [41].

Slow recovery was reported with rainbow trout exposed to acephate and methamidophos by Zinkl *et al.* [78]. They suggested that since OPs bind irreversibly with AChE, OP-exposed organisms should synthesize new enzyme to return to normal activity.

With CBs, the recovery period has been reported to be much shorter. Rainbow trout exposed to carbaryl (1 mg/l) for 24 h returned to normal and showed no AChE inhibition after 48 h [28,79]. Recovery of brain AChE activity in carbofuran-exposed fish was also fast [5,36]. It was also shown that accumulation of ACh accompanied the inhibition of brain AChE. Further, when ACh was injected into carbofuran-exposed fish, brain AChE activity was restored to normal faster than in exposed but uninjected fish. It was surmised that accumulated ACh offered protection against circulating inhibitor.

Direct and indirect inhibitors

Compounds with a P=O group are direct inhibitors of AChE, unlike those with a P=S group, which require conversion or metabolic activation. Yet, several other factors like the rate of uptake of a chemical and its movement across membranes play a more important role in determining the toxicity. This is well illustrated by the relative toxicity of acephate and fenitrothion. The former, with a P=O group is a direct inhibitor of AChE, yet its acute (*in vivo*) toxicity to rainbow trout, the extent of brain AChE inhibition *in vivo*, and its *in vitro* brain AChE inhibition are less than those of fenitrothion, an indirect inhibitor of AchE. The 24-h LC_{50} values of acephate and fenitrothion are 1880 mg/l and 1.9 mg/l, respectively [19]. *In vivo*, the AChE activity in brain, gill, heart, serum and RBC of rainbow trout showed a significant inhibition, in a shorter time, with fenitrothion than with acephate (Figure 27.3). This difference between observed and expected toxicity of a direct and indirect inhibitor is the result of the poor water (and hence greater lipid) solubility of fenitrothion and higher water solubility of acephate. This is further illustrated by the fact that fenitrooxon, the active form of fenitrothion, is at least five times more potent than acephate as an AChE inhibitor.

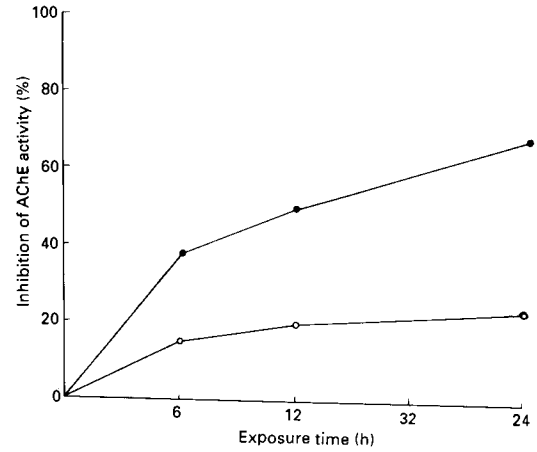

Figure 27.3 Percentage inhibition of AChE activity in the brain of rainbow trout exposed to (A) acephate (2000 mg/l) and (B) fenitrothion (2 mg/l). Adapted from Duangsawasdi and Klaverkamp [19]

In vivo and *in vitro* toxicity of antiChE

OPs and CBs act by inhibiting AChE, yet their *in vivo* toxicity is often at variance with the *in vitro* inhibition of AChE. Data in Table 27.3 reveal that the most acutely toxic compound is not the most potent inhibitor of AChE (cf. azinophos-methyl and disulfoton). Coppage [11] noted no correlation between toxicity and *in vitro* activity. While azinophos-methyl, phorate and parathion were more toxic than diazinon, the latter inhibited AChE the most. Similarly, Kanazawa [39] found no relationship between *in vivo* and *in vitro* toxicity of 12 OPs and nine CBs; *in vitro* CBs were more inhibitory.

Although a direct relationship between *in vivo* and *in vitro* toxicity does not usually exist it is occasionally reported [5], e.g. with embryos of *Bufo*, an *in vivo* concentration of 0.5×10^{-6}M dieldrin produced 36% inhibition of AChE, whereas *in vitro* 4×10^{-5}M did not decrease activity [18]. It is doubtful how such high concentrations of dieldrin could be obtained since it is equivalent to a dieldrin concentration of 16 mg/l, which is nearly two orders of magnitude higher than its water solubility. Lack of inhibition may be from precipitation of the toxicant in the enzyme assay mixture, as shown by Pocker *et al.* [56]. For a detailed discussion on the problems with hydrophobic compounds in enzyme assay studies see Murty [50]. Likewise, while parathion-methyl at 1×10^{-8} M killed 50% of the exposed pink shrimp in 96 h, an *in vivo* concentration of 0.3×10^{-3}M was needed to cause 100% inhibition [62].

While studying the additive and greater effects of piperonyl butoxide and sulphoxide on two OPs and CBs with *Lymnaea acuminata*, Singh and Agarwal [64] reported that such effects were more pronounced with *in vivo* exposure, than *in vitro*, presumably because they act on more enzyme systems in the former, whereas only AChE inhibition is examined in the latter.

Degradation product or metabolite toxicity and MFO induction

In malathion-exposed pinfish, Cook *et al.* [10] recorded a 60% mortality rate, 79% brain

AChE inhibition, no residues of malathion or malaoxon, but considerable amounts of malathion monoacid and malathion diacid in the gut. Whether the persistent enzyme inhibition was a delayed effect of the oxon or the result of the two metabolites is not clear. Although the oxygen analogues of OPs are more potent inhibitors of AChE, Schoor and Brausch [62] found paraoxon-methyl to be less toxic *in vivo* than parathion-methyl to pink shrimp, owing to the greater lipid solubility of the latter than its oxon. *In vitro*, the oxon is about 200 times more toxic and inhibitory.

Many P=S group-containing OPs require conversion to active P=O forms by microsomal NADPH-dependent mixed function oxidases (MFOs), which also detoxify them by dearylation [9]. Such an activation by liver preparations of brook trout, brown trout, pumpkinseed fish, black bullhead, winter flounder and shorthorn sculpin was demonstrated by Potter and O'Brien [58] and Murphy [48].

Increased tolerance of parathion and parathion-methyl by certain strains of mosquito fish was attributed to the high levels of liver MFOs in the resistant strains and consequent higher rate of dearylation and degradation [9]. Likewise, the resistant fish also had higher levels of AChE and carboxylesterases than the susceptible strain. Chambers [8] postulated that since carboxylesterases have a greater affinity for OPs than ChEs, the former protect the latter from irreversibly binding with OPs.

Inhibition in other tissues and other organisms, and other AChE inhibitors

Other tissues and other organisms

Although most AChE studies were undertaken with fish brain, occasionally other tissues have been studied. Duangsawasdi and Klaverkamp [19] examined ChE activity in the gill, heart, skeletal muscle, RBC and serum of rainbow trout exposed to acephate and fenitrothion. AChE inhibition in various tissues of *Tilapia* was studied by Kabeer Ahmed Sahib and Ramana Rao [37].

Occasional work with other organisms focused on the likely environmental hazard of antiChEs. Coppage and Matthews [16] reported 72–82% AChE inhibition in the ventral nerve cord of pink shrimp exposed to 1 mg/l malathion, for 48 h. Schoor and Brausch [62] investigated inhibition in the ventral nerve cord of pink shrimp exposed to parathion-methyl (MPT) and its oxon (MPO). Exposure to 1.3 µg/l MPT resulted in significant depression of AChE in the ventral nerve cord. While 1×10^{-8} M caused 40–60% mortality rate *in vivo*, a concentration of 0.3×10^{-3} M MPT was required to cause 100% inhibition of AChE *in vitro*. MPO, the active oxygen analogue of MPT, cannot be considered as the factor causing the *in vivo* toxicity (by conversion of MPT to MPO), because the acute toxicity of MPO is less than that of MPT. Greater water solubility of MPT is the cause of its lower *in vivo* toxicity.

Singh and Agarwal [64] reported on additive effects of sulphoxide and piperonyl butoxide on AChE inhibition of some OPs and CBs in the snail *Lymnaea acuminata*, and de Llamas *et al.* [18] investigated the effect of malathion and dieldrin on ChE activity of developing amphibian embryos.

Other inhibitors

As well as OPs and CBs, other substances have been implicated as AChE inhibitors. Mg^{2+}, Mn^{2+} and Ca^{2+} activated fish brain AChE, whereas, Cu^{2+}, Ni^{2+} and Zn^{2+} greatly inhibited the enzyme in bluegills, channel catfish and cutthroat trout [32]. Sastry and Sharma [61] found that mercuric chloride, at lethal and sublethal concentrations, inhibited brain AChE of murrel. Olson and Christensen [54] studied the *in vitro* AChE-inhibiting properties of 74 chemicals, using fathead minnow muscle. Eserine was the most potent inhibitor; of the 13 pesticides studied, carbaryl was the most potent inhibitor. Arsenite ion inhibition was greater than many CBs. A mixture of ten inorganic ions had the same effect as a mixture of ten pesticides.

Long-term effects of antiChEs

The absence of detectable levels of OPs or CBs in the environment cannot be taken as a lack of toxicity. Residues may disappear from water, but AChE depression at slightly below lethal levels may continue for several days or weeks. The resultant long-term effects may be

reduced ability of sustaining physical activity, searching for food, eluding predators, maintaining position in the school, or in successfully finding a mate. All or any one of these would affect survival of the species.

Weiss [71] considers that sublethal exposure to antiChEs makes fish susceptible to subsequent exposure in the early stages of recovery. Weiss and Weiss [75], reporting abnormal locomotion associated with skeletal malformations in the sheepshead minnows, hypothesized that AChE inhibition was responsible for the abnormal movement of the fry. Post and Leasure [57] reported that a 32% inhibition of AChE in brook trout accompanied a 16% decline in the 'index of activity'. Increased activity of fish without concomitant mortality soon after the spraying of a stream with temephos to control *Simulium* larvae, increased their proneness to capture [1].

Eaton [21] reported that inhibition of AChE during chronic exposure of the bluegill to malathion affected its ability to tolerate reduced oxygen tension. With fathead minnows, Jarvinen *et al.* [34] noted that at 10–40% or higher AChE inhibition, fish maturation and second generation growth were significantly reduced. At 80% or more depression, survival, growth, and embryo hatchability were significantly affected and deformities were noticed. Kozlovskaya and Mayer [41] also suggested that chronic exposure of fish to OPs may alter their growth without causing mortality.

AChE inhibition and environmental monitoring

Ever since Weiss [71] showed that the inhibition of fish brain AChE was proportional to concentration and extent of exposure, it has been suggested that this could be used to demonstrate the presence of antiChEs in the aquatic environment, and that depressed AChE in natural fish could be taken as evidence of exposure to OPs or CBs, even in the absence of identifiable residues [73]. Weiss [74] quoting Hazeltine, explained how brain AChE inhibition of bluegill sunfish was used to monitor the concentration of parathion-methyl to avoid excess doses in Clear Lake, while spraying to control larval gnat. Williams and Sova [76] also suggested that estimation of fish brain AChE inhibition, in conjunction with chemical analysis, has considerable potential for monitoring the aquatic environment for OPs. Holland *et al.* [33] and Coppage [12] also recommend the use of fish brain AChE to monitor incipient and chronic low levels of OP pollution in the estuaries. Coppage and Braidech [14] reported that carp, carpsucker and shad inhabiting downstream portions of Blue River in the USA (receiving effluent from a plant formulating OPs and CBs), showed lower brain AChE activity compared with those collected upstream. Dilutions of 1:17, 1:650 or 1:1300 of downstream river water caused 87%, 22–48% and 9–21% inhibition of fish brain AChE.

Cook *et al.* [10] demonstrated brain AChE depression in malathion- exposed fish, even in the absence of any measurable quantities of malathion or malaoxon in the tissues, and suggested that enzyme estimation is a practical way to identify natural poisoning. Goodman *et al.* [27] also considered that AChE inhibition may serve as an early indicator of OP poisoning.

Contrary to the above, Gibson *et al.* [26] noted no correlation between AChE inhibition and exposure concentration. Moribund fish exposed to 750 μg/l showed only 25% inactivation of the enzyme, whereas those that became moribund following exposure to 20 μg/l showed 50% inhibition. Antwi [3] found no significant change in brain AChE activity of four species of fish, after 6 years of intermittent aerial spraying of temephos on Volta River to control *Simulium* larvae.

Because some in organic ions are known to be common constituents of heavy metal pollution of the aquatic environment, it may be difficult to identify the actual cause of AChE depression. Any suspected aquatic environmental perturbation can at best be confirmed in a general way, by AChE inhibition studies.

One area where AChE studies would be useful is the identification of the likely presence of degradation compounds and metabolites of OPs, but only in the absence of other antiChEs. Because some of the former may persist long after the parent compounds have disappeared from the environment or from the tissues of the organisms, and as such metabolites (because of their high polarity) are

not extracted by any of the routine analytical methods for extraction of OPs and CBs from environmental matrices, only AChE inhibition may help in identifying their presence.

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28

Cutaneous absorption of anticholinesterases

R.E. Grissom and P.V. Shah

Introduction

AntiChEs are used as pesticides, to treat various diseases, and militarily as chemical warfare agents. Agricultural use of antiChEs presents a potential for skin exposure to mixers, loaders and applicators, and harvesters of process-treated crops. Studies involving such groups have shown RBC or plasma ChE inhibition, the presence of the parent compound or its metabolites in urine, or symptomatology characteristic of antiChE intoxication from skin exposure [19, 20, 21, 60, 61, 74, 75]. People who process treated crops are not exposed to the pesticides or their foliar residues in the field; however, skin exposure and absorption can occur through the hands and forearms as the produce is processed [54].

Accidental exposure and improper use of antiChE pesticides has resulted in systemic toxicities through skin exposure. For example, an individual in North Caroline, USA spilled ethoprophos and chlorpyrifos on his clothing at work. He did not change his clothing until the end of the work day, and experienced severe toxicity requiring treatment. A Japanese worker spilled mevinphos in his boots and became severely ill, requiring atropine to relieve his symptoms. The boots were washed with soap and water, but reuse resulted in severe toxicity again requiring medical attention [61]. Another worker in North Carolina who entered a tobacco field immediately after it had been sprayed with parathion became ill and died as a result of the exposure [70]. A worker in California who was intoxicated from consuming alcoholic beverages spilled parathion on his skin and subsequently died [44]. Apparently, many people do not think skin an important route of exposure.

Impurities such as O,O,S-trimethyl phosphorothioate occur in malathion, fenthion, and acephate, and O,O.S-triethyl phosphorothioate has been found in parathion [81]. Approximately 7500 field workers in a malaria control programme in Pakistan were adversely affected by malathion contaminated with isomalathion and at least five workers died [3]. Poor production procedures or inappropriate storage and use may have caused the problem. Alteration in the production of malathion and appropriate training and medical surveillance were initiated to eliminate the problem [3,80].

Topical application of medicinal antiChEs is used. Ointments and solutions such as physostigmine sulphate ophthalmic ointment, physostigmine salicylate hypothalamic solution, demecarium bromide solution, ecothiopate* iodide solution, and isoflurophate ointment are used to treat glaucoma [59, 77]. Shampoos and lotions containing carbaryl and malathion are applied on the hair and skin in order to eradicate lice and other body pests [77]. These preparations usually require a prescription from a physician and because their use is closely monitored, systemic toxicities are rare. Localized toxicities such as cataracts, blurred vision and congestive iritis, however, many occur with ophthalmic preparations [59,77].

The major route of entry of chemical warfare agents is through the skin. *In vitro* work using human skin, and *in vivo* and *in vitro* studies using animals indicates that appreciable amounts of sarin will penetrate through the skin [8,38]. Skin penetration of OPs such as VX has been investigated in human volunteers [17, 34].

Several methods are currently in use to assess skin exposure and penetration in

*Echothiophate (USP)

humans. Assessment has centred around the use of absorptive patches [20,38], foliar residues [74,75], assessment of RBC and plasma ChE [74,75], and renal excretion of antiChEs or their metabolites [20,21]. Risk assessment [27, 40] and fluorescent imaging techniques are being developed [25,26]. Evaluation of antiChEs on the body patches is supposed to indicate skin exposure. Depression of RBC and plasma ChE or excretion of antiChEs or their metabolites indicates that exposure and absorption has occurred but does not necessarily indicate total exposure or how much penetrated. Fluorescent indicators can be incorporated in the pesticide, and after application the workers are examined under UV light to assess exposure. This method has shown that workers applying malathion had skin exposure not only on bare skin but also to skin covered by both protective and non-protective clothing [25,26].

While these methods indicate that exposure or penetration has occurred, they have several limitations. They cannot be used in accurately determining skin penetration of antiChEs that are being developed or for highly toxic antiChEs. Further, they cannot adequately address the effects of variables such as temperature, perspiration, type of clothing worn, poor personal hygiene and damaged skin.

Agricultural, clinical and military use of antiChEs has created a demand to understand skin exposure and penetration. *In vivo* studies using human volunteers have been conducted on some of the less toxic antiChEs. Animals have been used in research to determine which species would be most likely to predict penetration in humans. Animals are also used in evaluating penetration of more potent antiChEs. *In vitro* systems have been developed to study penetration through skin, and both human and animal skin can be used. The relationship between *in vivo* and *in vitro* skin penetration, however, is not well understood.

In vivo percutaneous absorption of antiChE agents

Percutaneous absorption of various antiChEs has been determined to estimate quantitative risk. Methods are generally divided into three main groups, namely: *in vivo*, *in vitro*, and occupational exposure measurements [1, 2, 4, 37,50,55,56,63,76,82,84,87].

In vivo percutaneous absorption in humans

Urinary excretion analysis has been extensively utilized for measurements of skin absorption of various drugs and toxicants in humans. This method is also used in animal studies owing to its simplicity. In this method, the radioactive compound is applied to the desired area of the skin, and the appearance of radioactivity in the excreta (usually urine) is measured at various times after topical application. A tracer dose is given parenterally and radioactivity in excreta determined over the same period to correct for incomplete excretion (loss via CO_2, sweat and storage, etc.). The percent absorption is then determined as:

$$\% \text{ absorbed} = \frac{\% \text{ radioactivity in excreta (topical appliction)}}{\% \text{ radioactivity in excreta (parenteral administration)}} \times 100$$

Feldmann and Maibach [24] compared the percutaneous absorption of 12 pesticides in humans using the urinary excretion analysis technique, by applying pesticides in acetone to forearm skin. Pesticides concentration was kept at 0.62 μg/cm² (4 μg/inch²). The application sites were not covered, and subjects were asked not to wash the application sites for 24 h. Urine was collected 4-hourly on the first day, and daily for the following 4 days. Urinary excretion was corrected by a factor obtained from iv experiments. Results are shown in Table 28.1. Percutaneous absorption of carbaryl was highest compared with other pesticides. Human skin penetration for two CBs was higher than other pesticides.

Maibach and Feldmann [24] evaluated the effects of various factors such as anatomical region, concentration applied, occlusion, washing, etc. on percutaneous absorption of pesticides. Variations in percutaneous absorption have been shown in humans and animals for different anatomical regions [7,22,47,73,79,88]. Effect of anatomical region on percutaneous absorption of parathion and malathion in humans is shown in Table 28.2. The highest penetration of parathion was through scrotal skin (101.6%), which showed practically no

Table 28.1 Percutaneous absorption of pesticides in humans

Pesticide	*Absorption (%) (5 days)*[a]
Monocrotophos	14.7
Ethion	3.3
Azinphos-methyl	15.9
Malathion	8.2
Parathion	9.7
Propoxur	19.6
Carbaryl	73.9
Aldrin	7.8
Dieldrin	7.7
Lindane	9.3
2,4-D	5.8
Diquat	0.3

[a]Data corrected for incomplete urinary excretion with factors obtained in the IV experiment to calculate the percentage absorption
[b]Data from Feldman and Maibach [24]

Table 28.2 Effect of anatomical region on percutaneous absorption of parathion and malathion in humans[a]

Anatomical region	*Percentage dose absorbed (in 5 days)*[b]	
	Parathion	*Malathion*
Forearm	8.6	6.8
Palm	11.8	5.8
Foot, ball	13.5	6.8
Abdomen	18.5	9.4
Hand dorsum	21.0	12.5
Fossa cubitalis	28.4	—
Scalp	32.2	—
Jaw angle	33.9	69.9
Postauricular	34.0	—
Forehead	36.3	23.2
Ear canal	46.6	—
Axilla	64.0	28.7
Scrotum	101.0	—

[a]Data adapted from Maibach and Feldmann [46]
[b]Dose applied was 4 mg/cm^2. All data were corrected for incomplete urinary recovery from the intravenous control data

Table 28.3 Effect of occlusion on *in vivo* percutaneous absorption of pesticides in humans[a]

Pesticide	*Non-occluded (control)*[b]	*Occluded (24 h)*[b]
Monocrotophos	14.7	33.6
Azinphos-methyl	15.9	56.1
Malathion	6.8	62.8
Parathion	8.6	54.8
Dieldrin	7.7	65.5
Lindane	9.3	82.1
Propoxur	19.6	68.8
Diquat	0.4	1.4
2,4-D	5.8	14.7

[a]Data adapted from Maibach and Feldmann [46]
[b]Percentage absorption

barrier to penetration of parathion. Penetration through scrotal skin was about 12 times that of human forearm skin. A similar trend was observed for malathion. These findings confirm that the anatomical region of the body exposed plays a significant role in ultimate toxicity of a penetrant.

Increasing the area of exposure and/or increasing the concentration of the xenobiotic increases penetration [86]. Skin penetration is generally regarded as a simple diffusion process, which follows Fick's law of diffusion. It has also been postulated that a unit of skin can hold only a limited amount of chemicals, i.e. it is saturable, so that penetration cannot be increased beyond a certain point by increasing the concentration applied. Limited studies have examined the effect of concentration on skin penetration. Maibach and Feldmann [46] reported dose-related percutaneous penetration of parathion and lindane in humans. For concentrations between 4 and 2000 μg/cm^2, parathion showed an almost linear increase in penetration with increases in concentration applied, while lindane showed a decrease in percentage penetration with increasing concentration. However, the absolute amount penetrating through the skin was increased with increasing dose. A similar trend was observed for malathion penetration in humans by Serat *et al.* [65]. They reported 6.8% percutaneous absorption of malathion at 4 μg/cm^2 and 1.4% at 2000 μg/cm^2; the absolute amount of malathion was increased by 104-fold at 2000 μg/cm^2.

Occlusion of topically applied compounds increases the temperature, humidity and water content of the skin which can cause altered skin penetration [48]. Maibach and Feldmann [46] evaluated the effect of occlusion on percutaneous absorption of pesticides in humans (Table 28.3). An almost two to tenfold increase in penetration was observed due to occlusion.

Damaged or diseased skin alter normal penetration. Maibach and Feldmann [46] compared percutaneous absorption of various pesticides through normal and damaged skin of human volunteers; skin was damaged by removing the stratum corneum with cellophane tape. All pesticides showed increased penetration through the damaged skin. Monocrotophos penetration was 100% through the

damaged skin, indicating complete removal of the barrier. Penetration of azinphos-methyl was increased fourfold.

Wester *et al.* [86] found that the repeated applications did not change the percutaneous absorption of malathion from initial-day absorption.

Fredriksson [30] reported the percutaneous absorption of parathion and paraoxon, and decontamination of parathion from human skin using a disappearance measurement technique. ^{32}P-labelled parathion was distributed over about 12 cm^2 on the back of the left hand of each human volunteer, and disappearance of radioactivity measured by placing the treated hand below the detector tube. After a designated time interval, the volunteers washed their hand in the usual way. The results suggest that washing with ordinary soap and water for 30 s is not particularly effective in removing parathion from the skin surface. Wester and Maibach [84] have shown that the washing increases the percutaneous absorption of some compounds.

Hodge and Sterner [41] reported the percutaneous absorption of triorthocresyl phosphate (TOCP) in humans. [^{32}P]TOCP was rubbed onto both palms, and the urinary excretion of TOCP was observed during the first hour after application. The rapidity of penetration was striking; one subject had 13 μg TOCP per 100 ml of blood at the end of 1 h.

Exposure to parathion has been indirectly quantitated by measuring the amount of its metabolite paranitrophenol, in the urine. Funckers *et al.* [35] compared skin exposure to parathion by human volunteers at different ambient temperatures. Volunteers were exposed to 2% parathion dust at weekly or less frequent intervals at 58°, 70°, 82° and 105° F. The rate of excretion of parathion varied directly with temperature. Maximum excretion occurred 5–6 h following initiation of exposure.

In vivo percutaneous absorption in laboratory animals

In the direct method {42,91] animals are prepared by clipping hair and 24 h later the test material (usually radioactive) is applied to the skin. Generally, the back region is used. A volatile solvent such as acetone, ethanol, etc. is used for ease and uniform application of the test material. Some form of occlusive device which protects the application site is necessary to prevent oral intake and loss of material from rubbing. Perforated plastic blisters from Cathavex filters (Millipore Corp. Bedford, MA, USA) and disposable beakers (Fisher Scientific, Pittsburg, PA, USA) affixed with cyanoacrylate adhesive has been effectively used in rats [67]. Teflon rings or other suitable devices are commercially available (Crown Glass Company, Sommerville, NJ, USA). Bartek *et al.* [5] have described a method to protect the skin site in animals. Treated animals are killed at selected time intervals. Radioactivity remaining at the application site is measured by excising the area, and is assumed to be the unpenetrated dose; skin-bound radioactivity and volatility are factors that cause some uncertainty. Such experiments are usually conducted in metabolism cages, and recovery of radioactivity from urine, faeces, CO_2 and other tissues can be easily determined at each time interval permitting mass balance determinations.

Shah *et al.* [71] compared the percutaneous absorption of 14 pesticides in mice. Radioactive pesticide in 0.1 ml acetone containing 1 mg of the test material was applied to 1 cm^2 area in the back region shaved 72 h previously. Mice were placed in glass metabolism cages for collection of excreta and expired air. Treated animals were killed at selected time intervals and the application site was removed. The results are shown in Table 28.4. Percutaneous absorption of seven antiChE agents studied ranged from 25% for malathion to 85% for methomyl in 60 min, and in 8 h ranged from 67% for malathion to 95% for carbofuran.

Skinner and Kilgore [73] compared the percutaneous absorption of ^{14}C- labelled parathion through various anatomical sites in mice. Nose, scrotum, foot and tail were treated at the rate of 4 μg/cm^2 area. Excreta were collected for 7 days and corrected for incomplete excretion from iv experiments. Skin penetration of parathion in 7 days was 28%, 30.3%, 37% and 81.6% through tail, foot, scrotum and nose region of the mice, respectively.

Shah *et al.* [67] compared the percutaneous absorption of 14 pesticides applied in acetone

Table 28.4 Comparison of skin penetration of 14 pesticides in mice[a]

Pesticide	*Geometric means of percentage penetration*	
	60 min	*480 min*
Carbamate		
Carbaryl	71.7	88.5
Methomyl	84.5	88.3
Carbofuran	76.1	94.7
Organophosphate		
Parathion	31.9	85.4
Malathion	24.6	66.7
Chlorpyrifos-methyl	54.4	78.2
Chlorpyrifos	69.0	73.9
Botanical type		
Nicotine	71.5	90.7
Permethrin	79.7	88.1
Chlorinated hydrocarbons		
DDT	34.1	71.1
Hexachlorobiphenyl	55.3	66.8
4-chlorobiphenyl	84.5	97.5
Chlordecone	54.0	65.9
Dieldrin	33.7	82.6

[a]Data modified from Shah *et al.* [71]

to the previously clipped backs of young and adult rats. Percutaneous absorption in 72 h was determined at three doses of four antiChEs. Results, shown in Table 28.5, showed significant skin absorption both in young and adult rats. Percentage penetration generally decreased with increasing dose, but the absolute amount penetrating increased with increasing applied dose.

O'Brien and Dannelley [57] compared the percutaneous absorption of three antiChEs in rats. Time for 50% penetration was 5.5, 14.5 and 19 h for malathion, carbaryl and famphur, respectively.

Percutaneous absorption of malathion in guinea pigs following repeated application was evaluated by Bucks *et al.* [15] using urinary excretion analysis method. Malathion was applied at a concentration of 5 mg/cm^2 every 24 h to the same post-auricular bald area for 15 days. Comparison of percutaneous absorption of malathion between unwashed and washed application sites was also made, and absorption was found to be two to three times higher with washing compared with unwashed site. There was no significant increase in percutaneous absorption of malathion following repeated application. The authors suggest that the total penetration of malathion resulting from daily topical dosing without daily washing may be predicted from a single-dose application to the same unwashed site at an equivalent surface concentration.

Bartek and LaBudde [6] compared the percutaneous absorption of several compounds, including malathion and parathion, in New Zealand white rabbits, weaning Yorkshire and miniature Hanford swine, and squirrel monkeys. They utilized the urinary excretion method to determine percutaneous absorption. The test compounds were applied

Table 28.5 Comparison of skin penetration of pesticides in young and adult rats: effect of dose on penetration[a]

			Percentage penetration in 72 h					
	Low dose[b]		*Low dose*		*Medium dose[b]*		*High dose[b]*	
Compound	*Adult*	*Young*	*Adult*	*Young*	*Adult*	*Young*	*Adult*	*Young*
Parathion	0.0536	0.0536	82.00	81.45	70.66	57.85	—	—
Chlorpyrifos	—	—	—	—	66.33	81.53	58.70	90.05
Carbaryl	0.1536	0.1857	30.13	36.69	19.75	12.21	3.96	4.85
Carbofuran	0.0232	0.0286	83.41	24.53	8.27	9.23	5.97	3.69
Captan	0.0893	0.1071	38.24	26.74	3.72	3.78	3.65	2.64
Folpet	0.0839	0.1000	14.77	12.26	2.71	2.62	1.12	0.85
DSMA[c]	0.0893	0.1071	9.01	6.00	15.30	2.00	11.88	1.20
MSMA[c]	0.0893	0.1071	22.04	2.94	13.78	2.06	18.88	5.16
Chlordecone	0.2857	0.3357	9.20	10.17	5.96	7.23	1.03	1.93
PCB[c]	0.1089	0.1321	40.70	33.45	20.80	26.73	5.82	2.72
Nicotine	0.0143	0.0179	75.03	48.87	82.96	84.40	85.88	88.20
Permethrin	0.0161	0.0179	56.77	48.50	26.92	27.29	15.76	16.71
Dinoseb	0.2143	0.2500	86.39	77.70	90.51	81.52	93.25	82.88
Atrazine	0.2500	0.2857	7.65	9.63	4.55	6.76	2.78	3.22

[a]Data from Shah *et al.* [67]
[b]Low dosage: μmol/cm^2; medium and high doses were 0.536 and 2.679 μmol/cm^2, respectively, except parathion. Medium dose of parathion was 0.179 μmol/cm^2
[c]DMSA, disodium methanearsonate; MSMA, monosodium methanearsonate; PCB, 2,4,5,2',4',5'-Hexachlorobiphenyl

Table 28.6 Species comparison of percutaneous absorption of various antiChE agents

Compound	*Total absorption[a] (%)*				
	Rabbit[b]	*Pig[b]*	*Monkey[b]*	*Rat[c]*	*Human[d]*
Parathion	97.5	14.5	30.3	99.0	9.7
Malathion	64.6	15.5	19.3	—	8.2
Carbaryl	—	—	—	95.7	73.9

[a]Corrected for recovery following parenteral administration
[b]Data from Bartek and La Budde [6]
[c]Data from Shah and Guthrie [69]
[d]Data from Feldmann and Maibach [24]

at 4 μg/cm², and urinary excretion measured for 5 days following topical application (corrected for incomplete excretion from iv experiments). Shah and Guthrie [69] compared the percutaneous absorption of parathion and carbaryl in rats. The results of these two studies along with previously published human data [24] are shown in the Table 28.6. From these limited comparative data, it appears that rodent skin (rat and rabbit) is highly permeable. Human skin appears to be least permeable to these chemicals followed by pig and monkey skin in increasing order. A similar trend was observed with other chemicals [5,6,45,49,50,78,87].

Fredriksson [34] reported the influence of solvents and surface active agents on the barrier function of the skin towards sarin in guinea pigs; the development of toxicity was used as the method to evaluate the effect of various factors. He reported that both an increase in concentration of sarin and an increase in the area of application reduced the time required for respiratory arrest.

Fredriksson [32] measured the rate of percutaneous absorption of parathion in cats by using disappearance technique; 50 μl undiluted ^{32}P- parathion was applied over a 4.1 cm² area of skin. Disappearance of parathion was measured by using a GM tube. The rate of absorption was very slow, approximately 5 μmol/min per cm², which is close to the sensitivity limit of the method and about a 25th that of sarin. Sensitivity of detection is a major limitation in quantitation of percutaneous absorption.

Fredriksson [33] measured percutaneous absorption of paraoxon in the cat by comparing ChE activity after skin application with the activity after iv infusion. Fifty μl undiluted paraoxon was applied to the clipped left thigh over 4.1 cm² area. Under the study conditions, the rate of percutaneous absorption of paraoxon was found to be constant in a given skin. The latency period for paraoxon was found to be 12 min. Similarly, Nabb *et al.* [53] estimated that rate of skin absorption of parathion and paraoxon by comparing the ChE activity following cutaneous and iv administration in rabbits. The average rate of skin absorption was estimated to be 0.059 μg/min per cm² of skin area for parathion and 0.32 μg/min per cm² for paraoxon. This study demonstrates the utility of an indirect method, such as inhibition of target enzyme, for evaluating the percutaneous absorption of a chemical.

Moody and Franklin [52] found that in monkeys, 49% of the fenitrothion and 74% of aminocarb were absorbed from the forehead, while 21% fenitrothion and 37% aminocarb were absorbed from the ventral forearm. In rats, 84% of the fenitrothion and 88% aminocarb was absorbed from the mid-dorsal region. These results suggest rapid skin penetration of these two antiChEs in rats and monkeys, and show regional variation in percutaneous absorption in monkeys.

Hodge and Sterner [41] reported the percutaneous absorption of [^{32}P]TOCP in dogs from the abdominal surface. The urinary excretion of TOCP began in the first hour after application.

Shah and Guthrie [68] studied the percutaneous absorption of malathion, parathion and carbaryl in rabbits, and detected them in the blood in 5 min following topical application.

In vitro methods and percutaneous penetration

In vitro methods have been extensively used because of their simplicity. They offer the advantage of minimizing experimental variables and can be used in evaluating the influence of various factors on absorption. In general, *in vitro* methods are relatively inexpensive and less time consuming compared with *in vivo* methods. A major advantage is that they allow the use of human skin for absorption measurements, especially when toxicity precludes *in vivo* studies using human volunteers.

The *in vitro* methods for skin absorption measurements assume that the barrier proper-

ties of the skin reside primarily in the stratum corneum, which has been shown to be the case in many studies [10,83]. A major disadvantage of *in vitro* methods is that they do not duplicate *in vivo* situations where blood supply and metabolism may play a significant role except, perhaps, in viable tissue preparations and perfused skin flap techniques.

There are primarily two types of diffusion cells, with various modifications to meet different experimental conditions. The first has two chambers. The excised skin is placed between the chambers, and then the chambers are filled with media. The epidermal side of the chamber contains the donor medium and the dermal side is exposed to a receptor fluid without the penetrant. The donor as well as the receptor side of the two chambered diffusion cells are sometimes stirred to ensure homogeneity of the solution. The amount or rate of movement of penetrant is determined by either sampling the donor side medium or receptor side medium periodically.

The second type of diffusion cell contains only one chamber. The epidermal side of the excised skin is open to the ambient air while the dermal side is bathed by a receptor medium. These diffusion cells are divided into static [28] and flow through types [12,13]. In the static type of diffusion cells, the epidermal side of the skin is exposed to ambient air, and the dermis is in contact with the receptor fluid. The desired temperature is maintained by circulating heated water through a jacket surrounding the receptor medium. The penetrant is applied on the epidermal side usually dissolved or suspended in a volatile solvent. The receptor fluid is stirred with a magnetic stirrer to properly mix the penetrant in the receptor fluid. The movement of the penetrant through the skin is measured by sampling the receptor fluid periodically.

Bronaugh and Stewart [12,13] designed a flow-through cell for percutaneous absorption measurements. In this system, the epidermal side is also exposed to ambient air, but the receptor fluid is constantly replaced with fresh medium. The cell is mounted on a heated block to maintain desired temperature. The chemical is applied on the epidermal side, usually dissolved or suspended in a volatile solvent. Sampling is facilitated by collection of the effluent in vials in an automatic fraction collector. Similar modifications have been made with static cells to maintain a dynamic flow of the receptor fluid. These types of diffusion cells are available commercially through vendors such as Vanguard International and Crown Glass Company.

Blank *et al.* [8] studied the effects of various factors on the rate of penetration of sarin through excised human skin. The rate of penetration of sarin was independent of the amount applied, as long as the entire area was covered with a reserve amount of sarin. Penetration rate was increased by approximately twofold for a 10°C rise in temperature.

Autoradiography has been successfully employed in studying the mechanism of percutaneous penetration. Blank *et al.* [9] using an autoradiography technique suggested that the penetration of sarin through rabbit and excised human skin occurred primarily through a transepidermal pathway. Similarly, Fredriksson [31] also reported that penetration of parathion through excised skin from human, rat, rabbit and cat was through a transepidermal pathway. Parathion also penetrated into hair follicles and sebaceous glands to some extent (*see* Ch.45).

Marzulli *et al.* [51] studied the percutaneous absorption of a series of organic phosphates and phosphoric acid through excised human skin. Average maximum steady state rates of penetration ranged from 0.007 to 1.047×10^2 $\mu mol/cm^2$ per min, indicating significant skin absorption. Penetration was correlated with benzene/water partition coefficient for this series of chemicals, and related to both molecular weight and volatility of the undissociated organic phosphates.

Perfused tissue technique

In the past few years there has been great interest in viable perfused tissue techniques, especially in pigs and rats. These *in vivo* and *in vitro* techniques offer several advantages over the *in vitro* methods described earlier by allowing an investigator to maintain anatomically and physiologically intact skin preparations for both *in vivo* and *in vitro* percutaneous absorption studies and *in vitro* metabolism studies. However, the isolated perfused tissue model requires surgical techniques.

The details of perfused tissue techniques in rats and pigs have been discussed [16,43,58,62]. The isolated perfused porcine skin flap (IPPSF) technique has been described by Riviere and co-workers [62]. Briefly, weaning Yorkshire pigs are used to create the skin flaps. Following anaesthesia, a 4 × 12 cm area in the caudal abdominal and inguinal areas is demarcated. This region is perfused by the caudal superficial epigastric artery and drained by paired venae comitantes. Two flaps can be created per pig. Following incision and dissection of the subcutaneous tissue, the caudal incision is trimmed of fat, apposed and sutured forming a tubed flap. The incision in the abdominal area is also sutured. Two days following surgery, a second surgical procedure is performed to cannulate the caudal superficial epigastric artery. Then the flat is harvested and transferred to a custom-designed perfusion chamber. The pigs are allowed to heal and returned to the animal facility.

A perfusion apparatus has been specifically designed for perfusing the tubed porcine skin flap. Temperature, humidity, perfusion pressure, and pH are controlled by a computer. The perfusate flow rate through the skin is usually maintained at 1–2 ml/min. Humidity is usually maintained at 60–80%; pH is maintained at 7.4; and temperature is usually maintained at 25°C. The flap is perfused with a Krebs-Ringer bicarbonate media containing albumin, glucose, antibiotics and heparin. Dextrose is infused into the system to maintain a glucose perfusate level between 80 and 120 mg/dl. The perfusate from the venous side is automatically collected by a fraction collector or recirculated.

Xenograph techniques in rats have been shown to be successful in studying percutaneous absorption through human skin. The details of the rat-human skin system (RHSFS) have been reviewed [36,58,89,90]. Briefly, the RHSFS utilizes microvasculature techniques to create rat/human xenografted sandwich flaps supplied by the superficial epigastric artery on athymic nude rats. A human split-thickness skin graft (0.3–0.5 mm) is grafted to the skin flap created on the ventral abdomen of a rat. Following successful growth of this graft, the sandwich flap and its associated vasculature are transferred to the back of the rat through a subcutaneous tunnel. Cyclosporin is administered to prevent the rejection of the graft. Split-thickness skin from syngenetic rats can also be used for sandwich preparation in addition to human skin. This technique is labour-intensive and requires administration of cyclosporin throughout the procedure.

In vivo and *in vitro* comparisons

The majority of studies have shown that the primary barrier function of the skin resides almost entirely in the stratum corneum. This is the primary reason for expecting *in vitro* skin permeability studies to produce similar results to those observed *in vivo*. Other assumptions are that the penetration is a passive diffusion process and the dermis does not play a significant role in the barrier function of the skin. It is also assumed that the condition of excised skin for *in vitro* studies is similar to *in vivo* situations and that metabolism is not a factor.

Several studies have shown a good correlation between *in vivo* and *in vitro* skin permeability values for many chemicals and animal species. Franz [28] compared the *in vitro* percutaneous absorption of 12 organic compounds with *in vivo* results reported by Feldmann and Maibach [23]. The results (Table 28.7) showed excellent qualitative agreement between the *in vitro* and *in vivo* methods. Discrepancies were noted, however, in the quantitative aspects. Later, Franz [29] reported that the discrepancies were experimental variation; that was based on additional *in vitro* experiments to simulate *in vivo* conditions by washing skin after 24 h. *In vivo* skin absorption value of nicotinic acid was significantly lower than *in vitro* value. Franz [29] suggested that the lower *in vivo* value may be from slower urinary excretion of nicotinic acid over a 5-day collection period. Bronaugh *et al.* [14] reported identical *in vivo* and *in vitro* rat skin absorption values for benzoic acid, acetylsalicylic acid and urea. Grissom *et al.* [39] reported similar *in vivo* and *in vitro* penetrations of both hydrophilic and lipophilic pesticides in mice.

The static and flow-through diffusion cell methods have been compared by Bronaugh and Stewart [12], and the comparative skin absorption values for water, cortisone and benzoic acid through rat skin reported. They

Table 28.7 Absorption of various compounds by skin *in vivo* and *in vitro* (expressed as percentage of applied dose)[a]

Compound	*Absorption in vivo (mean ± SD)*	*Absorption in vitro*[b]	
Hippuric acid	0.2 ± 0.1	1.2 (0.8, 2.7)	1.25 ± 0.5[c]
Nicotinic acid	0.3 ± 0.1	3.3 (0.7, 8.3)	2.3 ± 0.9[c]
Thiourea	0.9 ± 0.2	3.4 (2.4, 5.5)	4.6 ± 2.3[c]
Chloramphenicol	2.0 ± 2.5	2.9 (1.0, 5.7)	
Phenol	4.4 ± 2.4	10.9 (7.7, 26)	
Urea	6.0 ± 1.9	11.1 (5.2, 29)	
Nicotinamide	11.1 ± 6.2	28.8 (16, 55)	
Acetylsalicylic acid	21.8 ± 3.1	40.5 (17, 49)	
Salicylic acid	22.8 ± 13.2	12.0 (2.3, 23)	
Benzoic acid	42.6 ± 16.5	44.9 (29, 53)	
Caffeine	47.6 ± 21.0	9.0 (5.5, 20)	24.1 ± 7.8[c]
Dinitrochlorobenzene	53.1 ± 12.4	27.5 (19, 33)	

[a]Data from Franz [28]
[b]Median (95% confidence intervals)
[c]Data from Franz [29]

Table 28.8 Comparison of *in vivo* and *in vitro* skin penetration of compounds in rats

Compound	*In vivo*[a]		*In vitro*[a]			
			Flow system		*Static system*	
	Adult	*Young*	*Adult*	*Young*	*Adult*	*Young*
Carbaryl	38.4	38.5	18.2	29.2	20.3	20.9
Carbofuran	11.8	33.5	11.2	41.1	8.8	12.0
Dinoseb	84.3	55.7	20.6	47.3	70.8	74.9
Chlordecone	7.9	9.1	1.2	1.4	2.2	2.2
Hexachlorobiphenyl	19.6	33.2	1.4	1.3	1.2	2.5

[a]Percentage penetration
[b]Data from Shah *et al.* [66]

reported similar absorption profiles and quantitative values for the two diffusion systems for three compounds. However, discrepancies in absorption values were observed for a hydrophobic compound (3-phenyl-2-propenyl-2-aminobenzoate [cinnamyl anthranilate]) between the two diffusion cell systems; the flow-through diffusion cell system gave enhanced absorption. They further reported good agreement between *in vivo* and *in vitro* (static as well as flow-through diffusion cells) skin permeation for cortisone and benzoic acid when each was applied in a petrolatum vehicle.

Shah *et al.* [66] compared penetration of five pesticides through the skin of young and adult rats using both *in vivo* and *in vitro* methods as well as static and flow-through cells (Table 28.8). They observed poor correlation between *in vivo* and *in vitro* results for highly complex pesticidal chemicals. They also reported discrepancies in the skin absorption values as determined by the two *in vitro* methods. Both *in vitro* methods underestimated skin absorption of chlordecone and hexachlorobiphenyl drastically. This may be the result of poor solubility of these chemicals in receptor fluid.

A number of factors can influence the skin absorption measurement. The influence of receptor fluid for *in vitro* skin absorption measurements has been widely documented. Bronaugh and Stewart [11] suggested that the lack of solubility of the hydrophobic penetrant into the receptor fluid for *in vitro* methods could be a contributing factor accounting for *in vivo* and *in vitro* discrepancies in skin absorption values. Bronaugh and Stewart [11] reported a maximum absorption of cinnamyl anthranilate by using 6% PEG-20 oleyl ether receptor fluid with both types of diffusion cell systems.

Preparation of the skin for use in diffusion cells can also influence absorption measurements *in vitro*. Topically applied compounds are absorbed by the microcirculation of the

papillary layer of the dermis. Full thickness skins add additional barriers to *in vitro* penetration studies. Removing part of the dermis using a dermatome reduces the thickness of these barriers but does not duplicate the *in vivo* barriers. Good correlation between *in vivo* and *in vitro* penetration has been shown when the barriers to *in vitro* penetration are similar to the ones found in *in vivo* [18,39,64].

The results obtained by different methods have to be carefully evaluated against the experimental conditions. It is also clear that the results obtained from *in vitro* studies can be influenced by a number of factors. Perhaps more comprehensive studies may improve the understanding of the relationship between *in vitro* and *in vivo* penetration. Validation of recently recommended guidelines for *in vitro* percutaneous penetration studies [72] with a number of chemicals may help in understanding the *in vitro* skin absorption process.

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29

Respiratory tract absorption of anticholinesterases

Darol E. Dodd

Inhalation exposure to antiChEs

Although, in most occupational situations, exposure to antiChEs by inhalation is not nearly as great as oral or skin exposure routes, it is toxicologically significant. CBs such as physostigmine and carbaryl are solids with negligible vapour pressures (Table 29.1). Although most OP antiChEs are liquids, several having vapour pressures high enough to produce a concentration of a few ppm in air at saturated ambient conditions (Table 29.1).

The contribution of exposure by inhalation increases when the antiChEs are intentionally dispersed as aerosols, dusts or compounds adsorbed onto inert, finely divided particulate matter. This is particularly common for agricultural insecticides. Workplace threshold limit values for airborne concentrations of antiChEs have been recommended by some health protection committees (Table 29.2). Acute inhalation toxicity testing of antiChE pesticides, in many cases, is mandated [35]. Table 29.3 lists acute inhalation LC_{50} values in rats for selected antiChEs. Repeated inhalation exposure studies with antiChE agents [7,33] are being conducted more frequently to determine possible cumulative toxicity.

There are numerous ways to prepare the antiChEs for dispersion into the air. Liquids or homogenous suspensions of partially soluble ChE inhibitors are generally diluted and aerosolized with a nebulizer or an atomizer. For solid antiChEs, formulations are prepared. Commonly manufactured formulations for spraying are dusts, flowables, sprayable powders, wettable powders, or oil fog concentrates.

Absorption of gases and vapours

Principal factors influencing the absorption of gases or vapours in the mammalian respiratory tract are the physicochemical characteristics of the agent, concentration, duration of exposure, breathing pattern, blood flow and anatomy.

Physicochemical factors influencing absorption

The concentration of the agent in the inspired air drives a diffusion process, which continues until equilibrium is attained. The diffusion rate of a vapour or gas in biological matter is a function of molecular weight and, particularly, solubility which is often determined by calculating partition or distribution coefficients based on Henry's law. Water solubility of selected antiChEs is listed in Table 29.1. Blood to gas partition coefficients may be estimated from the fractional composition of water (approximately 80%) and lipids (about 0.5%) in the blood. Agents of low blood solubility rapidly attain blood/gas equilibrium. In general, inhibition of metabolizing enzymes causes higher blood concentration for a given inspired concentration, while induction of metabolizing enzymes lowers blood concentration [*see* review, 8].

Species differences

Species differences in the uptake of inhaled gases or vapours are common and probably reflect differences in respiratory rate [5], metabolism [4] or anatomy [24]. Dosimetry modelling is most applicable for extrapolation of data from one species to another [13,21,22]. These models consider the influence of chemical reactions, diffusion, solubility, anatomical and respiratory factors [23].

Table 29.1 Physical state, vapour pressure and water solubility of selected antiChEs[a]

Class	*Physical state*	*Vapour pressure (mmHg)*	*Water solubility (%)*
Carbamates: clinically used			
Ambenonium (Cl)	Crystals	—[b]	Freely soluble
Demecarium (Br)	Powder	—	Freely soluble
Neostigmine (Br)	Crystals	—	Freely soluble
Physostigmine	Solid	—	Slightly soluble
Pyridostigmine (Br)	Crystals	—	Freely soluble
Carbamates: insecticides			
Aldicarb	Crystals	9.8×10^{-5} (25°C)	0.6 (25°C)
Aminocarb	Crystals	Non-volatile	Slightly soluble
Benomyl	Crystals	Non-volatile	0.0002 (25°C)
Carbaryl	Crystals	$<5 \times 10^{-3}$ (26°C)	0.012 (30°C)
Carbendazim	Powder	Non-volatile	0.0028 (30°C)
Carbofuran	Crystals	2.0×10^{-5} (33°C)	0.07 (25°C)
Chlorpropham	Solid	—	0.009 (25°C)
Methiocarb	Powder	1.1×10^{-4} (60°C)	0.003 (20°C)
Methomyl	Crystals	5×10^{-5} (25°C)	5.8 (25°C)
Pirimicarb	Crystals	3×10^{-5} (30°C)	0.27 (25°C)
Propoxur	Crystals	6.5×10^{-6} (20°C)	0.2 (25°C)
Thiophanate-methyl	Solid	—	Slightly soluble
Organophosphates			
DFP	Liquid	0.6 (20°C)	1.5 (25°C)
Diazinon	Liquid	1.4×10^{-4} (20°C)	0.004 (20°C)
Dichlorvos	Liquid	1.2×10^{-2} (20°C)	1
Ecothiopate*	Solid	—	Soluble
EPN	Solid	0.03 (100°C)	Slightly soluble
Ethion	Liquid	1.5×10^{-6}	Slightly soluble
Fenamiphos	Solid	7.5×10^{-7} (30°C)	0.04
Fenthion	Liquid	3×10^{-5} (20°C)	0.006
Fonofos	Liquid	2.1×10^{-4}	0.0013 (20°C)
Malathion	Liquid	4×10^{-5} (30°C)	0.014
Demeton-O-methyl	Liquid	1.85×10^{-5} (20°C)	Slightly soluble
Parathion-methyl	Solid	0.5 (20°C)	0.005
Mevinphos	Liquid	2.2×10^{-3} (20°C)	Highly soluble
Naled	Solid	2×10^{-3} (20°C)	Practically insoluble
Parathion	Liquid	3.8×10^{-5} (20°C)	0.002
Sarin	Liquid	—	Miscible
Sulfotep	Liquid	1.7×10^{-4} (20°C)	0.0025
Tabun	Liquid	—	Miscible
TEPP	Liquid	4.7×10^{-4} (30°C)	Miscible

[a]Data from The Merck Index [20], WHO [36], ACGIH [3]
[b]No data
*Echothiophate (USP)

Aerosols

An aerosol is a two-phase system and consists of finely divided condensed particulate matter suspended in a gas. The condensed particulate matter may be liquid, solid or a combination of the two and, in general, ranges in size from 0.01 μm to 100 μm in diameter. Particle size is the most significant feature determining whether an aerosol will deposit in the respiratory tract. A respirable aerosol is difficult to define because of the factors (e.g. species) influencing particle deposition. For humans, the sizes of interest range from 0.1 μm to 10 μm; larger sizes are trapped in the nose. A publication by the US EPA [34] concluded that particles between 1 μm and 3 μm (aerodynamic equivalent diameter) should be used for inhalation toxicity exposures to reproduce the proportional human deposition efficiencies in rodents.

Respiratory tract responses following inhalation of antiChEs

Human exposure to nerve gases compromises the respiratory system at several levels [16], i.e. bronchoconstriction and excessive tracheobronchial secretion, initial stimulation

Table 29.2 Workplace threshold limit values of selected antiChEs[a]

Class	*Time weighted average (mg/m³)*
Carbamates	
Benomyl	10
Carbaryl	5
Carbofuran	0.1
Methomyl	2.5
Propoxur	0.5
Organophosphates	
Azinphos-methyl	0.2
Demeton[b]	0.1
Diazinon	0.1
Dichlorvos	1
Dioxathion	0.2
EPN	0.5
Ethion	0.4
Fenamiphos	0.1
Fensulfothion	0.1
Fenthion	0.2
Fonofos	0.1
Malathion	10
Demeton-methyl[b]	0.5
Parathion-methyl	0.2
Mevinphos	0.1
Naled	3
Parathion	0.1
Sulfotep	0.2
Temephos	10
TEPP	0.05

[a]Data from ACGIH [3]
[b]Technical grade mixture

followed by paralysis of the respiratory muscles, and paralysis of the respiratory centre. These effects result in a wide variety of signs and symptoms, most notably rhinorrhoea, hyperaemia, chest tightness, wheezing and asphyxia.

There is considerable controversy regarding the mechanism of antiChE- induced bronchial constriction. The rapid absorption of compounds such as soman [1] or DFP [30] leads to widespread distribution and a systemic action on ChEs. An increase in parasympathetic activity causes contraction of the bronchial smooth muscle. Pauluhn *et al.* [26] exposed rats to different antiChE aerosols and observed an increase in lung resistance (owing to increased bronchial tone) only after ACh provocation. Plasma BChE activity was a sensitive indicator of antiChE exposure. These investigators concluded that measurement of ChE activity may be of greater clinical value than measurement of pulmonary function in victims exposed to atmospheres containing antiChEs. Furthermore, the respiratory tract effects of inhaled antiChEs may be by systemic action rather than local mediation. Histochemical preparations of bronchial tissue of rats exposed to dichlorvos [29] demonstrated a locally reduced enzyme activity in the absence of changes in enzyme activity in bronchial homogenates. Thus, following absorption the distribution of some antiChEs may be focal. Kadar *et al.* [15] concluded that the persistent binding of soman to the lung may indicate the existence of specific binding sites. This may also explain why Aas *et al.* [1] observed inhibition of ChEs in the lungs and bronchi of soman-exposed rats to the same degree as the inhibition of plasma or RBC enzymes. Irrespective of the precise mechanism of antiChE- induced bronchospasm, individuals with asthma or chronic obstructive disease may be highly susceptible to the effects of antiChEs by inhalation. Administration of ChE inhibitors to laboratory animals may serve as a model for the mechanical changes occurring in asthma [2].

Inhalation exposure systems and considerations

Many antiChE agents are potent and, in some cases, specific methodology for conducting inhalation exposure is required. For example, the generation of soman vapour by Aas *et al.* [1] was performed with a diffusion cell, and Tanaka *et al.* [33] employed a new dust generator [32] to expose rats repeatedly to a constant powder concentration of methomyl. Noteworthy reviews of the design and operation of inhalation exposure equipment are by MacFarland [18], Phalen *et al.* [27], Snellings and Dodd [31] and Cheng and Moss [6].

An early consideration is whether a nose-only (or head-only) exposure system is more appropriate than a whole-body design. Nose-only exposures eliminate the concerns of absorption through the skin or through the gut following preening. However, animal restraint is required in a nose-only design which may alter the breathing pattern during exposure [19] or cause additional stresses.

Highly volatile and/or reactive liquid test agents may have to be contained in stainless steel cylinders while metering vapour atmosphere [10]. To expose animals to nearly

Table 29.3 Acute inhalation LC_{50} values of selected antiChEs[a,b]

Class	*LC_{50} (mg/m^3)*	*Time (h)*	*LC_{50} × time ($mg\ h/m^3$)*
Carbamates			
Carbofuran	85	—[c]	—
Methomyl	300	4	1200
Propoxur	1440	1	1440
Organophosphates			
Azinphos-methyl	69	1	69
Demeton[d]	<18	1.5	<27
DFP	360	0.16	60
Diazinon	3500	4	14000
Dichlorvos	15	4	600
Dioxathion	1398	1	1398
Ethion-25% wettable powder	710–7200	1	710–7200
Fenamiphos	110–175	1	110–175
	91–100	4	364–400
Fensulfothion	113	1	113
	29.5	4	118
Fenthion	800–1200	4	3200–4800
Parathion-methyl	34	4	136
Mevinphos	128	1	128
Parathion	84	4	336
Sarin[e]	10.6	0.5	5.3
Soman[f]	21	0.32	6.7
Sulfotep	38	4	152
Tabun	304	0.16	51

[a]All values are for rats
[b]Data from ACGIH [3] and RTECS [28]
[c]No data
[d]Commercial preparation of 60% demeton-O and 40% demeton-S [9]
[e]Oberst [25]
[f]Aas *et al.* [1]

saturated vapour concentrations of pure test materials, a static exposure system can be used. However, there are drawbacks owing to loss of maintaining suitable environmental conditions and decay of the test material concentration. Differences in acute toxicity between static and dynamic exposures occur for some test materials [11].

Excellent texts describing aerosol generation and characterization of test atmosphere are those by Liu [17], Willeke [37] and Hinds [14]. A popular device for generating dust particles is the Wright dust feeder [38]. Aerosols from liquid test compounds are commonly dispersed with atomizers or nebulizers. The Laskin nebulizer [12] is well suited for single- component test materials and produces respirable particles at high concentrations. Gravimetric analysis is the most common method for determination of aerosol exposure concentrations. Particle size distribution can be assessed by various techniques including sedimentation, impaction, microscopy or velocimetry. A multistage cascade impactor is commonly used for aerosol atmosphere.

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Exposure of agricultural workers to anticholinesterases

Jack Griffith and Robert C. Duncan

Introduction

There are 3.1 million persons employed on farms in the USA; approximately 2.5 million are hired employees, 57.6% of whom work <74 days per year in farm employment. Migratory workers comprise 6.1% of the hired work force [38].

Agricultural workers are exposed to a variety of chemicals, including fertilizers, solvents, paints, fuels and pesticides. Because more than 2.29 billion pounds (approximately 1 billion kg) of active ingredient pesticides are used in the USA each year [26], pesticide exposure is one of the most common exposures for many workers.

Over 1400 active ingredients are formulated into more than 45 000 pesticide products. Before any pesticide product can be distributed, sold, offered for sale, held for sale, shipped, delivered for shipment or received, the manufacturer must register the chemical with the US EPA [42]. Data requirements for registration reflect the anticipated volume to be used, the projected use patterns and degree of potential exposure to humans and the environment.

If a pesticide is relatively harmless to humans or the environment it is considered for general use and labelled 'General Classification'. If considered potentially dangerous the product is licensed as 'Restrictive Use Pesticide'. The classification limits its use to 'certified applicators, or persons working under their supervision'. Under Sec. 23 of FIFRA [42], the EPA is charged with assisting states in the development of programmes to train and certify pesticide applicators, consistent with standards set forth in FIFRA. The Certified Applicator Programme (CAP) provides training at the state level in: (1) pesticide terminology and classification, (2) pesticide formulation and application practices, (3) use of protective clothing and equipment, (4) interpretation of pesticide labels, including safety and first aid practices, and (5) disposal and storage practices.

In 1973 EPA cancelled the use of DDT, and subsequently cancelled, suspended or restricted the use of other organochlorines. They were rapidly replaced by less persistent, but more acutely toxic OPs and CBs [13].

Monitoring worker exposure and absorption

The method of pesticide application (e.g. air and ground application), type of formulation (e.g. dilute spray, aerosol, dust), the worker's job function (e.g. applicator, mixer, loader, harvester), and the climate (rain, wind, heat and humidity) are all important in assessing a worker's exposure to and absorption of antiChEs.

Exposure

Major routes of exposure for agricultural workers are respiratory, oral and skin [22,26]. Pesticide exposure may be estimated by measuring residues on absorbent pads attached to clothing, by measuring residues on the skin, and from concentrations of residues in the ambient air.

Respiratory and oral exposure

Respiratory exposure is greatest for aerosols, intermediate for dusts and lowest for dilute

spray formulations [51]. Respiratory exposure cannot be separated completely from oral exposure because the mucosa of the upper respiratory tract may retain some residue of the inspired chemical and be swallowed. Oral exposure also occurs from contaminated hands or clothing, or ingestion of crops having pesticide residues. A worker may also 'blow out' hoses, transferring residues from equipment to the mouth [30]. Worker exposure may occur from residues drifting away from treated crops.

Dermal exposure

Skin is the primary route of exposure [29,50] and of most concern [14,28,35]. Skin exposure occurs when workers mix, load and apply pesticides, or re-enter previously sprayed fields to engage in activities involving harvesting or other functions by hand. It has proven difficult to determine the amount of pesticide residue actually reaching the skin surface through clothing and other protective materials [18,30].

Monitoring exposure

Attempts have been made to measure pesticide residues in ambient air using stationary monitoring devices, and in a worker's breathing zone by personal air samplers [5,6,49]. The intent of such monitoring is to suggest that the concentration of pesticide in the sampled air is representative of that in the worker's inhaled air. However, in field situations where pesticide residues are not of uniform size, and where they move rapidly over the treated area in variable concentrations, such estimates may be misleading.

Several investigators [25, 33, 44, 49, 53, 54] have attempted to estimate worker exposure by measuring concentrations of OP and CB residues on foliar and soil samples, and on workers clothing. Exposure has also been estimated using absorbent pads (e.g. alpha-cellulose for sprays, and pads made from thicknesses of surgical gauze and backed by filter paper for pesticide dust) attached to parts of the body or clothing [8]. It is assumed that the area covered by the pad is representative of the portion of the body being studied. However, highly absorbent knit white cotton garments that cover the entire body part being studied (e.g. gloves to estimate hand exposure, short sleeved undershirts for the upper torso) may provide a more accurate estimate.

Skin exposure may be estimated by swabbing exposed skin with surgical gauze sponges saturated with 95% ethyl alcohol [8], which are placed in a sealed jar for subsequent analysis. Durham and Wolfe found that to remove 90% or more of parathion residue from skin, the size of the back of a man's hand, several swabs were required.

Hand rinses may be used to estimate dermal exposure. Durham and Wolfe [8] emphasize the hands of the worker must be clean before entering the field. The hand is placed in a plastic container holding about 200 ml of 95% ethanol, and the fingers rubbed briskly against the thumb and palm to remove particles. Two hand rinses were found to remove 96% of parathion from one hand shortly after exposure.

Absorption

Absorption has been estimated directly from the measurement of residues or metabolites in body tissue or fluids.

Dermal absorption

Although pesticides are readily absorbed through the stratum corneum it has proved difficult to quantify absorption through the skin [28,45]. When a lipid-soluble pesticide joins with the lipid-saturated tissue of the skin, absorption is promoted [47]. Maibach and Feldmann [29] showed that damaged or diseased skin absorbs more pesticide than normal skin (e.g. parathion was absorbed 8.5-fold more through damaged than normal skin).

Concentration plays an important role in absorption. Maibach and Feldmann [29] found that as the concentration of parathion applied to human skin increased from 4 $\mu g/cm^2$ to 200 $\mu g/cm^2$, absorption increased from 0.34 $\mu g/cm^2$ to 180.0 $\mu g/cm^2$. Wester and Maibach [46] showed that by increasing the surface area of the applied dose, absorption was also increased. Thus, as Wester and Maibach [47] suggest, antiChE activity is more likely to occur when a large area of skin is exposed to high levels of the concentrate. Anatomical site also plays a role in absorption. Maibach and

Feldmann [29] applied 4 μg/cm² of parathion to the forearm, abdomen and forehead of volunteers and found that the absorbed dose ranged from 8.6% to 18.5% to 36.3%, respectively.

Protective measures to reduce dermal absorption

Although [33] dermal exposure to pesticides may be reduced up to 65% by wearing disposable protective suits and gloves, clothing is fairly standardized among fieldworkers, with cotton or cotton/polyester materials predominating [3,16]. Workers in the spray season usually wear short- sleeved shirts, while harvesters wear long-sleeved shirts, often with protective padding from elbow to wrist, and heavy leather or cotton gloves. While impermeable clothing provides the greatest protection against antiChEs [15], 100% cotton fabric reduces skin exposure more effectively than synthetic fabrics. Clothing saturated with pesticide residues may promote continuing skin exposure [13,29,48]. Such exposure enhances absorption through the skin [47] and increases the likelihood for toxicity. Wicker *et al.* [48] attributed a 50% depression in baseline plasma ChE in two workers employed as cotton scouts for 4 months to personal habits, including the failure to change work clothes daily. Clearly, workers should wash or change clothing daily to prevent an accumulation of potentially harmful residues [9–11].

Washing with soap and water following exposure might be expected to reduce the likelihood of absorbing toxic residues. However, Maibach and Feldmann [29] dosed forearm skin with parathion (4 μg/cm²) and washed the area with soap and hot water at intervals of 1 min, 15 min, 1, 4, 8 and 24 h. Absorption ranged from 8.6% of the applied dose when washed after 24 h to 2.8% when washed at 1 min. With same dosage applied to the palm of a hand and washed with rubbing alcohol, as much as 8.2% of the applied dose was absorbed within 15 min. When the washing was delayed for 4 h, 10.3% of the dose was absorbed. Fredriksson [12] showed that only 80–92% of radioactive parathion applied to the skin of volunteers could be removed by ordinary washing within 30 min after application. Workers waiting >5 h removed only 50–75%. Griffith and Duncan [15] in a study of more than 1800 citrus fieldworkers found that 56% did not wash their hands within 15 min of leaving citrus groves. Although hand washing should take place as soon as possible after using antiChEs, washing and bathing facilities are often not readily available.

Occupational hazard

Worker poisonings

Definition and symptoms

OPs and CBs are potential significant hazards to agricultural workers [1, 4, 23, 34]. Job function is clearly closely tied to poisonings because applicators, mixers and loaders are likely to have more exposure and greater absorption than harvest workers [3, 7, 22]. Wolfe *et al.* [51] found that an operator of an air blast sprayer directing spray upward into fruit trees has 12 times the exposure of a boom operator directing a comparable formulation downward into row crops. Hayes [20] reported that during air application of TEPP dust, the pesticide loader received almost three times the exposure to the concentrate than the pilot, and 4½ times as much as the flagman. Harvesting, which does not involve direct contact with the concentrate, usually produces relatively low exposures.

Davies *et al.* [4] characterized occupationally-related pesticide poisonings into two categories: applicator/mixer/loader poisonings, and picker/thinner poisonings. Applicator poisonings are usually severe, often occurring from direct contact with the concentration through accidental exposure or misuse. Picker poisonings result from contact with residues on plant foliage. Illnesses are less severe than applicator poisonings and tend to involve larger numbers of workers. Picker poisonings occur most often in warm dry climates, resulting in deposits of residue remaining on foliage for extended periods. These foliage residues, combined with dust, enhance persistence of the compound and degradation products, the oxons [21], thus posing a continual long-term threat to the safety of workers in such climates [4].

Early symptoms of poisoning include eye and skin irritation, headache, nausea, vomit-

ing, sweating, diarrhoea and abdominal pain. Later stages of more severe poisoning may show difficult and laboured breathing, loss of muscle control, convulsions and possibly death [2,31,32,37]. Symptoms of OP poisoning usually appear within a few min following ingestion, or within 12 h of skin exposure to a direct inhibitor [31]. However, symptoms may not appear for several hours with delayed inhibitors. Davies *et al.* [2] reported a dichlofenthion intoxication with severe ChE inhibition occurring from 40 to 48 h after the onset of initial minor symptoms. Almost total ChE inhibition continued for 66 days, requiring prolonged antidotal therapy. They determined that the protracted intoxication was the result of slow release of residue from adipose reservoirs.

Potentiation

Agricultural workers are frequently exposed to combinations of antiChEs. Some OPs (e.g. carbophenothion, fenthion, dioxathion) inhibit diethylsuccinase and tributyrinase (aliesterases which detoxify other OPs) at dosages lower than those inhibiting ChE. Such OPs are likely to increase the toxicity of OPs detoxified by aliesterases. Alternatively, OPs that inhibit ChEs and aliesterases at about the same rate cause additive rather than synergistic toxicity.

Several factors are necessary before potentiation occurs. First, workers must be exposed to multiple compounds. Second, compounds must be absorbed at approximately the same time and at toxic dosages.

Frequency of agricultural worker poisonings

Limited national figures are available on agricultural worker pesticide poisonings [43]. The frequency of farm worker poisonings might be expected to relate to the level of pesticide application. During a 1981 health survey of 1811 Florida citrus workers and 436 citrus growers, data were gathered on pesticide usage and field worker poisonings [17]. OPs and CBs identified in the survey and characterized by application rate, recommended application rate, and total pounds of active ingredient per acre applied yearly are shown in Table 30.1. An estimated 2 407 129 pounds active ingredient OPs and CBs are applied to Florida citrus yearly. The total estimated cost to the growers, excluding application costs, was $19 582 573

During the survey each respondent was asked: 'Are you aware of any pesticide-related incidents among your family or your fellow workers within the past 12 months'? An extensive investigation was made on each report. Physicians and hospitals were contacted and medical records reviewed. A physician's diagnosis and/or laboratory test was necessary to confirm an incident. In all, 25 incidents involving 29 people were reported by the 1811 fieldworkers interviewed. A detailed analysis is given by Griffith *et al.* [17]. Among 1200 permanent and semi-permanent fieldworkers, 11 cases were reported, with four cases subsequently confirmed. Their job categories included applicators, mixers, loaders and general combination workers. The rate of poisonings in Florida citrus for these workers is 34 per 10 000 per year. There were no confirmed poisonings among the 611 harvesters surveyed.

Safety and protection

Product labelling

Product labelling is perhaps the most important factor in the safety of agricultural fieldworkers [19,26]. A key component in registration or re- registration is the requirement that the product provide an informative label for the user, which EPA must approve before registration is completed [42]. Currently, the EPA requires that the label provide information on: the active ingredient, specific methods of handling the chemical, methods on preparation and application, guidance on storage and disposal of leftover chemical, the toxicity of the chemical, and antidotes and first aid instructions. Preparation and application information should also include permitted re-entry time.

Protective clothing and equipment

Labels may specify the use of protective clothing or equipment, either during application or before re-entry. For example, applicators to wear long-sleeved shirts and long-legged

Table 30.1 OP and CB pesticides applied to Florida citrus by pounds of active ingredient (AI)

Common name of restricted pesticide	*Application rate (lbs AI per acre)*		*Estimated total lbs AI/year*	*Estimated total costs $*
	Estimated	*Recommended*[a]		
Aldicarb[b]	6.88	5.10–10.05	394 251	4 963 620
Azinphos-methyl[b]	2.96	2.50–2.50	5 205	576 714
carbaryl[b]	2.86	Maximum 20.00	6 741	28 312
Carbophenothion[b]	2.69	3.75–3.75	387 353	3 970 368
Demeton[b]	0.52	2.50–2.50	169	1 944
Diazinon[b]	2.00	5.00–5.00	12 291	81 612
Dioxathion[b]	4.42	4.00–4.00	98 035	360 769
Ethion[b]	2.92	2.50–3.75	1 322 052	6 451 614
Fenbutatin oxide[c]	0.79	1.25–2.50	59 230	2 707 996
Malathion[b]	3.86	7.50–12.50	111 229	331 462
Methidathion[b]	0.95	1.25–2.50	10 573	108 162
Total			2 407 129	19 582 573

[a]Rates recommended on the container label or by the State of Florida
[b]Restricted use in USA
[c]Not on OP or CB

trousers, or coverall type garments, made of closely woven fabric. When handling concentrates or very toxic materials, labels may call for the use of impermeable clothing, e.g. a waterproof coat, pants or apron, and liquid-proof neoprene or natural rubber gloves. Wide-brimmed waterproof hats and boots made of natural rubber, or unlined neoprene, may also be required in applying label-restricted pesticides. Labels frequently specify the use of goggles or face shields and occasionally a respiratory device.

Re-entry standards

The EPA first proposed establishing re-entry standards for fieldworkers in 1974 [39]. Subsequently, EPA [41] addressed worker safety concerning the use of antiChE pesticides by stating in its Registration Procedures: 'Foliar residue and exposure studies will be required for products containing cholinesterase inhibiting ingredients .... Such studies shall be designed to provide data sufficient to establish satisfactory precautions to protect persons entering treated areas'. The EPA also required 'data necessary to determine required intervals between pesticide application and safe re-entry ...'.

The re-entry standard or interval has been defined as the period of time, in hours or days, following pesticide application after which a worker may legally enter a treated field to engage in normal field activity resulting in prolonged contact with foliage [25]. However, a re-entry interval established for intact residues of selected OPs may be of little value for those compounds which degrade to their more toxic oxygen analogues, such as parathion.

Three types of data are required for setting re-entry intervals: (1) dose- response data, (2) an estimate of the relationship between surface residue and total body exposure, and (3) time *versus* residue data. Using these data, it should be possible to estimate the 'dose' at the no effect or minimal risk level [52]. Initially, EPA proposed a 48 h re-entry interval for 11 OPs, and specific harvest re-entry times or preharvest intervals (PHI) for 50 crops. They subsequently reduced the proposed re- entry interval to 24 h for four of the more volatile but less toxic OPs [40]. The EPA also allowed the manufacturer to place a more restrictive re-entry interval on selected pesticides, and recognized the state's responsibility and authority to set additional restrictions. California set 48-h worker safety re-entry intervals, and 96-h harvest intervals, based on, but not limited to, toxicity, rate of persistence and degradation curves, human exposure practices, usage patterns, frequency of documented poisoning cases, rate of pesticide application, formulation, concentration or dilute application, and the possibility of potentiation [24,27]. Owing to limited data on the factors cited above, preharvest intervals were largely used to develop early re-entry intervals in California. In practice, it should be noted that California intervals frequently extend for 30

days or more. For example, when parathion or parathion-methyl is applied to citrus, intervals may be as long as 45 days after application. Where re-entry standards have been properly set and applied in California, there has been a significant reduction in systemic illness related to residues [27].

Some have questioned the wisdom of attempting to mandate nationwide re- entry standards because the occurrence of residue-related poisonings is climatically related [4,36]. However, groups representing the health interests of agricultural workers believe that national standards are necessary to ensure the safety of workers occupationally exposed to pesticides.

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31

Greenhouse exposure to anticholinesterases

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Introduction

Special features of glasshouse cultivation of plants

In recent decades, cultivation of plants under glass has spread throughout the world from the traditional areas where greenhouses were used mainly in temperate and cold climates. Formerly glasshouse use was predominantly seasonal, being largely a way of growing young vegetables before planting them out. Nowadays however, glasshouses are frequently used for permanent cultivation of vegetables, to supply the market with fresh products such as capsicums, tomatoes, cucumbers and radishes.

Cultivation of plants in glasshouses creates a more or less closed ecological system, in which most of the climatic variables such as temperature, humidity, air circulation and radiation are regulated. Therefore conditions differ considerably from those characteristic of open air market gardening or agriculture. Furthermore monoculture and close proximity of plants, the latter for economic reasons, can mean that pests cause severe and epidemic loss of plants, sometimes destroying whole crops [16]. Moreover, the ambient parameters of greenhouses such as constant high temperature and humidity, which maximize growth, also favour the reproduction of parasites. Thus culture under glass demands especially high expertise with the use of chemicals.

In glasshouses, a wide range of agricultural chemicals is used. They may include fertilizers, soil disinfectants, pesticides, crop regulators and maturity accelerators. The practice of rotation of agents may be used, to prevent the emergence of pesticide resistance. For example, rotation of insecticides (OPs, CBs, synthetic pyrthroids) can be used according to entomological demands. Consequently, in a given glasshouse, substances other than antiChEs may be used. In Hungary, use of OP and CB antiChEs, of the total pesticide used, is 25–30% [1]. Therefore the possibly injurious effects of a single substance or group of active ingredients cannot be considered, without reference to other materials [5,26,28] and in practice, impaired health may be caused simultaneously by various pesticides, with the possibility of synergism. Such adverse health effects can only be analysed by careful observation, biological monitoring, medical and laboratory examination and epidemiological surveys [4, 5, 25, 31, 34]. Additionally, occupational hygiene investigations may play an important role in defining the degree of exposure. Thus it may be possible to assess inhalation and dermal exposure by sampling from personnel, a procedure which is usually substance-specific [5,26].

In cultivation under glass, the mode of application may differ from that used in the open. In most cases mechanically-driven equipment such as containers and compressors are utilized. In these cases the operator should pull the sprayer along the row of plants backwards, so avoiding inhalation of the vapour. Other frequently-used methods include thermal aerosol hot vaporization, the so-called ultra-low volume procedure, as well as smoke cartridges. The various methods may require different individual protective equipment, including rubber boots and gloves, impregnated garments including hoods and masks or respirators. In this respect a problem arises with high summer temperatures in glasshouses, which may be up to 40°C, with a relative humidity of 80%. In such circumstances, cooler overalls of hand-woven fabric may be

Table 31.1 Dermal exposure values of sprayers, tractor drivers and plant protection workers after spraying with dimethoate[a]

		Head	*Right shoulder*	*Left shoulder*	*V-shaped neckline*	*Right elbow*	*Left elbow*	*Right thigh*	*Left thigh*	*Right shank*	*Left shank*	*Hands*	*Averages*
Sprayers[b]		0.031	0.119	0.404	—	0.243	0.844	0.455	0.762	0.609	2.380	0.548	0.580
Tractor drivers[b]		0.016	0.013	0.018	0.023	0.018	0.015	0.711	1.143	0.081	0.190	3.246	0.497
Plant protection workers	[c]	0.021	0.126	0.080	0.025	0.791	0.864	0.220	0.154	3.000	1.887	1.083	0.750
	[d]	0.031	0.091	0.123	0.012	0.186	0.236	0.209	0.098	0.082	0.057	0.150	0.116
	[e]	0.100	0.025	0.097	0.013	0.048	0.079	0.065	0.052	0.058	0.039	0.076	0.07

[a]Values expressed as μg dimethoate per 10 cm² body surface per h
[b]At the completion of spraying
[c]First day after spraying
[d]Second day after spraying
[e]Third day after spraying

worn and changed after the first contamination or not later than 2–3 days afterwards. Plant-protection work is carried out in the early morning or late afternoon in hot weather [5, 27,28].

It is important to note that, in greenhouses, the rapidly degradable pesticides, including OPs, become more persistent than in the open. Pesticides adhere to the surfaces of leaves for longer periods because the meteorological features that promote degradation, such as wind, rain and insolation, are absent or modified. Hence, for glasshouse personnel, it is important to observe a waiting time after spraying [11]. In fact the risk of exposure associated with those carrying out other tasks in the greenhouse is greater than that to persons spraying the plants (Table 31.1).

Types and effects of antiChE pesticides used in greenhouses

There are two types of antiChE pesticides used in agriculture, the OPs and the CBs. The symptomatology of acute intoxication with these compounds is well known and well described [13,24]. The chronic toxic effects, however are less well known but have been described in long-term pesticide production workers or, important in the present context, in agricultural labourers who had used pesticides regularly [9,24]. Even today there is controversy on whether food residues of pesticides can induce chronic intoxication.

The symptoms of chronic intoxication produced by OPs are headache, weakness, dizziness, impairment of memory, tiredness, restless sleep, lack of appetite and disorientation. In some cases psychological disturbance, nystagmus, tremor of the hands and other neurological manifestations, even paralysis, can occur. It has strongly to be stressed that the symptoms and signs of antiChE poisoning resemble those of alcoholism. The bibulous tendencies of farmers and farm workers may make the diagnosis of chronic OP intoxication difficult. Therefore, in the course of our investigations, the activity of the liver enzyme, γ-glutamyl transferase (GGT) has been determined.

Measuring the effects of glasshouse exposure to antiChE agents

As has been discussed earlier, it is very difficult to diagnose the subacute and chronic effects of antiChEs that are harmful to health because of confounding variables which may influence the effects of antiChEs. It is therefore beneficial to use a number of approaches to the problem. Social records and occupational hygiene data need to be recorded as does the precise activity of the worker and an epidemiological approach may be useful. With respect to the latter, there have been several proposals for standardization, notably by the World Health Organization [31]. Such proposals are very valuable, but are obviously made in the light of knowledge at the time. By considering these and other recommendations, we have attempted to elaborate a wide-ranging system, designed to cope, not only with antiChEs, but also the subacute and chronic effects of all pesticides. We have carried out a study, in which data gleaned from glasshouse workers and from agricultural spray operators working in open fields were compared. At the start of this study personal and social data were gathered on the subjects, together with

length of employment as a pesticide worker. Haematological tests carried out included haematocrit and total and differential white blood count. Clinical chemistry studies included ChE [14] and GGT [23]. Urine analysis was carried out as well as simple immunological studies of a type possible in most laboratories. The immunosuppressive effects of OPs occur relatively early [7,8,30] and thus may provide a very early means of diagnosis of mild exposure to pesticides [6] (*see* Ch.19). The results are summarized in Table 31.2. Certain immunological tests indicated abnormalities and GGT levels were elevated when ChE activity was still unchanged. It can also be seen that glasshouse workers formed the majority of those with ChE depression. Indeed of the ten greenhouse workers, ChE activity was depressed in nine; of the ten open field agricultural workers (Table 31.3), ChE activity was low in only one. In four of ten glasshouse workers there were abnormalities in more than two tests. Despite the probability that many of the changes observed in this study are caused by OPs, it must be recognized that mild changes in the humoural and cellular immune system may have other causes.

Genetic effects of OPs

The possible induction of chromosomal abnormalities may have very serious consequences not only for the agricultural worker, but also for his descendants. Therefore, as part of the study discussed earlier, regular genetic studies and tests for chromosomal abnormalities were carried out. The latter are expensive and time-consuming but, despite this, are used on all workers engaged in plant protection.

Genetic toxicological studies, including tests for chromosomal abnormalities were carried out on certain plant protection crew, working in glasshouses. It is possible to compare the data with that obtained from sprayers working in open field culture and in orchards. The sprayers were working in three counties in south-east Hungary where the crops were different and hence the degree of pesticide exposure, a factor which must be considered when comparing the data. Of the 55 workers engaged in plant protection in Csongrád county, 41 performed pesticide treatment in greenhouses. The 31 men studied in Bács county were sprayers in orchards (in the open), while there were 102 sprayers taking part who carried out ploughed field plant

Table 31.2 Summary of the changes observed in plant protection agricultural labourers working in greenhouses and in open fields

	IgA	*IgG*	*IgM*	*C_3*	*Ly number*	*T rosette*	*Gamma GT*	*ChE*
Increase in value of parameter	**C** **D4** E **H4** --- M O		**A** **B** L3	**H4** **I3** L3 S M **N** R	**D4** **H4** **G3** **I3** T X O U S	**G3**	**D4** L3 R T **N** U P	
Decrease in value of parameter			**F** **K** P	X				**A** **B** **C** **D4** E **F** **G3** **H4** **I3** **K**

(a)Bold letters, greenhouse workers; numbers, indicate more than double occurrence
(b)Workers over the dotted lines are those with decrease in ChE activity and other alterations
(c)Workers under the dotted lines are those with no decrease in ChE activity, but with other alterations

Table 31.3 Numerical abnormalities in plant protection workers[a]

Group	*Numerical abnormalities*					
	Chromosome number < 46		*Chromosome number > 46*		*Total*	
	No.	*(%)*	*No.*	*(%)*	*No.*	*(%)*
Greenhouse workers in the county of Csongrád						
After spraying with pyrethroid (Group A)	5	(2.3)	1	(0.4)	6[c]	(2.7)
After spraying with several chemicals (Group B)	8	(2.0)	2	(0.5)	10[c]	(2.5)
After an interval in spraying (Group C)	1	(0.5)	2	(0.1)	3	(1.7)
Open field workers after an interval in spraying (Group D)	8	(2.9)	2	(0.7)	10[d]	(3.7)
Total of workers in the county of Csongrád	22[d]	(2.0)	7[b]	(0.6)	29[d]	(2.7)
Controls	7	(0.6)	1	(0.9)	8	(0.7)
Open field workers in the county of Bács (Group E)	45[e]	(7.5)	6[b]	(1.0)	51[e]	(8.5)
Tractor drivers in the county of Szolnok (Group F)	49[e]	(4.4)	5	(0.4)	54[e]	(4.8)
Other plant protection workers (Group G)	28[d]	(3.0)	5	(0.5)	33[d]	(3.6)
Total of workers in the county of Szolnok	77[e]	(3.8)	10	(0.5)	87[e]	(4.3)
Controls	14	(1.2)	3	(0.2)	17	(1.4)

[a]Percentage compared with the number of cells examined in the corresponding group
[b]$0.05<P<0.1$
[c]$P<0.05$
[d]$P<0.01$
[e]$P<0.001$ compared to the number of numerical aberrations of the controls

protection. The results from the exposed persons were compared with cytogenetic data from 60 heathy blood donors matched for age and lifestyle, but who were not in known contact with pesticides.

Chromosomes were prepared from lymphocyte cultures of the peripheral blood of workers by the method of Moorhead *et al.* [15]. The evaluation included the number of cells with abnormalities, together with numerical and structural abnormalities [2,10,29]. It is possible for several abnormalities to occur in one cell, therefore the total number of abnormalities could be greater than the number of abnormal cells. The results were evaluated statistically using the Fisher's test [3].

During spraying, the workers wore protective gear including rubber boots and gloves and protective masks and hats. A rough determination of the potential of the glasshouse workers, for exposure to antiChEs, was carried out using dichlorvos [32]. In the course of workplace exposure and based on the measurement of air contamination and dermal dichlorvos exposure, the potential exposure for the worker ranged from 0.2–0.7%. This was below levels usually considered unsafe. ChE measurement [14] showed normal activity throughout the investigation.

The number of structural chromosomal abnormalities was not increased in the greenhouse workers from Csongrád County, either immediately after spraying or 1-2 months afterwards. The number of numerical abnormalities observed immediately after spraying was increased significantly compared with the controls, but this was not the case 1–2 months after spraying (Table 31.3). It may be noted that in other studies chromosomal injury related to glasshouse pesticide spraying is probably mild [18–20]. The data from open field workers, in accordance with others [21,22,33] may suggest the possibility of more important mutagenic effects.

Discussion

We have encountered no case of acute antiChE intoxication from glasshouse exposure. It has been suggested that direct skin and inhalation exposure to antiChEs is less under glass than in the open [7,11,13,17,26,28]. The probable reason is a greater awareness of the danger in greenhouse workers, together with better physical protection and occupational hygiene precautions.

Subacute and chronic antiChE poisoning in agricultural and horticultural workers is particularly difficult to diagnose because of the possibility of mixed exposures and alcoholism. To facilitate early diagnosis, the

immunological status of workers may be examined using a simple test of humoral and cellular immune activity.

Workers in glasshouses appear to be less endangered, at least from the chromosomal point of view, than orchard or open field workers.

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32

Commercial and residential poisoning with anticholinesterases

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Introduction

OP poisoning is considered a risk for farmers and migrant workers, those with suicidal intent, and, rarely, accidental community exposures. Published incidents are available only from California, where pesticide poisoning must be reported to the state health department. Annual reports are compiled and published by the Worker Health and Safety Branch, California Department of Food and Agriculture, although not presented in the peer-reviewed literature. The rate of under-reporting is estimated at 99% [8]. From 1984 to 1987 approximately 50% of the average 2500 reported incidents were occupational and 5–9% non-occupational [4,15–18]. The rest were either unrelated or not evaluated. The majority of incidents were not from antiChEs.

Sporadic reports of 'interesting' clusters and epidemics of classical antiChEs poisoning among farm workers [2], office workers [6], and innocent bystanders [5] are presented in the peer-reviewed literature, as are new forms [1,13], but no systematic collection of data exists to document frequency, source or outcome of poisoning incidents. Only one dedicated surveillance system has been established [9], although data on residential or commercial poisoning episodes are omitted.

This chapter describes two sources for estimating frequency and risk factors of anti-ChE poisoning in the USA; the Annual Reports of the Poison Control Centers (ARPCC), beginning in 1982, and the Pesticide Incident Monitoring System (PIMS), in existence from 1968 to 1980.

In 1957, a National Clearinghouse was established as a Public Health Service agency, without funding or regulatory power. In 1982, the FDA funded a pilot study to collect data systematically. All incidents are recorded on a form, automatically centrally collected, and annual reports are available in the literature [10–12,14]. Increasing numbers of centres participate each year, but under-reporting occurs. Only 65% of all poisoning incidents in specific geographic areas are reported to PCCs [14].

The EPA's PIMS was established in 1957. In 1981, funding was discontinued. Results have not been published in peer-reviewed literature, possibly because of two major weaknesses. (1) Reporting of incidents was based on knowledge of the system and an interest in reporting. (2) Incidents were generally inadequately documented. One can not determine the completeness of reporting, verify the accuracy or review the outcome.

Most documented episodes have been diagnosed because of clustering of symptoms in users with a common exposure or through surveillance of RBC AChE in those occupationally exposed. The range of normal of RBC and serum ChE is so broad that values may decrease by one-half and still be within the normal range. In several recently published incidents, prospective determinations had not been undertaken because the affected groups were not covered by mandatory surveillance provisions. Only recently have methods been described to identify poisonings retrospectively through serial observation of RBC AChE [2,6]. In the past, such episodes would not have been documented or reported.

The validity of pesticide poisonings in both reporting systems is questionable, for two specific reasons. First, none of the three avail-

able diagnostic strategies was systematically recorded: (1) therapeutic trial with atropine, (2) monitoring of urinary metabolites/excretion, and (3) temporal course of RBC ChE recovery. Second, poisoning may occur through the antiChE alone, combined with another pesticide, or through the vehicle. Each may lead to different symptomatology, requiring different strategies for documentation.

Under-reporting is certain, whose magnitude can only be estimated. PIMS was a voluntary reporting system. The PCC network has established an automated reporting system, addressing a large segment of the USA population. Nevertheless, many primary care physicians are unaware of the spectrum of symptoms induced by antiChEs and may doubt their patients who consider such disease. Finally, several typical forms of poisoning have been described; these have generally not been recognized.

Two forms, chronic dietary poisoning and asthma, were unheard of while PIMS was active. For example, Ratner *et al.* [13] reported five individuals with chronic gastrointestinal symptoms from several months to 2 years. Restriction of vegetable and fruit and serial determinations of ChE levels allowed attribution of chronic symptoms to OP poisoning. Two cases of asthma were attributed to OPs; they showed specific reactions on challenge with OPs, which could be blocked with prednisone but not atropine [1] thus excluding the anticholinergic mechanism as an aetiology.

The two systems complement each other and together allow the formation of hypotheses concerning frequency, severity and development of strategies for future prevention. The ARPCCs allow some estimate of the frequency of incidents. No specific information on individual incidents is available, except for rare fatal cases. ARPCCs are available for only 4 years (1983– 1986). Because the reports present only age, accidental compared with deliberate, and treatment they are useful primarily as descriptive epidemiology. The PIMS, on the other hand, covers more years and contains more information for examination of risk factors, allowing an analytical approach. Because it is clearly a biased reporting system, conclusions must be drawn cautiously.

Annual reports of the poison control centres

The first report for 1983 [14], describes data from 16 participating PCCs, with an effective population of 25.8 million persons, and a total of 251 012 incidents. The authors extrapolated to an estimated 2.3 million episodes per year in the USA. There were 90.0% accidental and 5.4% suicidal incidents. AntiChEs were involved in 4691 (1.9%) cases. Using the authors' extrapolation, 43 700 incidents might have occurred in the whole USA. If the authors' estimate of an under-reporting rate of 65% is accurate, approximately 67 230 incidents may have occurred.

In 1984 [12], 47 PCCs participated, covering an effective population of 99.8 million, or 42% of the USA. From a total of 730 224 episodes of poisoning, 10 521 (1.4 %) involved antiChEs. Suicidal intent was reported in 4.9%. Only 1.4% were 'accidental'; an unspecified portion was related to mislabelling. Using the same under-reporting rate, 38 500 episodes of antiChE poisoning may have occurred.

In 1985 [12] 900 513 episodes were reported in an effective population of 113.6 million, or 47.6% of the USA. This implies more than 1.9 million poisoning episodes per year, of which 1.57% (estimated 45 892) could be related to antiChEs. Suicidal intent was documented in 5.1%.

The last year for which a report is available was 1986 [11]; 57 centres participated, covering approximately 55% of the population. The reported 1 098 894 episodes suggest a total of over 2 million incidents per year. Again, 1.4% involved antiChEs. Extrapolation to the whole USA indicates approximately 28 000 incidents involving antiChEs occurred. Suicidal intent was again reported at 5.6%.

Table 32.1 shows the categories by pesticide combinations. Reports may be compared only over the 4-year period. No absolute trends are evident, primarily because an increasing number of centres collaborated each year. Each year, antiChEs were involved in 1.5% of all poisoning incidents, <0.01% of incidents involving antiChEs were fatal; the number of documented cases ranged from two to six. There is some indication that, over time, the non-fatal incidents involving antiChEs may have decreased. It is not possible to identify

Table 32.1 Annual Reports of the Poison Control Centers: incidents involving antiChEs

	Incidents	*Age group (years)*			*Treated in*	*Accidental*
	Total number (deaths)	*<6 (%)*	*6–17 (%)*	*>17 (%)*	*health facility (%)*	*(%)*
1983						
CBs	1085 (1)	63.2	18.2	4.7	20.9	88.0
OPs						
Alone	2853 (1)	54.3	24.2	5.0	27.2	85.8
With CBs	552 (0)	64.1	19.6	4.0	19.4	89.3
With chlorinated hydrocarbons	73 (0)	57.5	24.7	4.1	19.2	86.3
With other pesticides	128 (0)	71.1	19.5	2.3	21.9	89.8
1984						
CBs	3033 (1)	61.3	5.5	29.7	20.7	98.2
OPs						
Alone	5710 (9)	39.1	7.2	50.0	30.0	96.8
With CBs	1226 (0)	50.2	9.2	37.4	19.7	95.3
With chlorinated hydrocarbons	200 (1)	35.5	11.0	49.0	24.5	96.0
With other pesticides	352 (0)	56.8	5.4	34.4	23.3	97.7
1985						
CBs	4504 (0)	56.8	6.8	33.1	19.2	93.9
OPs						
Alone	7266 (0)	38.5	8.5	48.5	28.0	96.6
With CBs	1773 (0)	46.5	8.3	41.5	20.8	96.8
With chlorinated hydrocarbons	208 (0)	36.5	5.8	51.9	31.5	96.6
With other pesticides	376 (0)	47.9	8.5	37.8	33.5	95.2
1986						
CBs	4423 (2)	66.3	4.7	27.2	20.2	97.9
OPs						
Alone	8583 (4)	35.3	8.0	53.8	30.9	97.6
With CBs	2420 (0)	44.9	7.8	44.2	21.4	95.0
With chlorinated hydrocarbons	527 (0)	36.0	8.7	50.7	23.7	94.3
With other pesticides	612 (0)	46.2	6.9	44.9	27.0	97.4

risk factors for poisoning through these reports, nor are residential and office settings differentiated. Each year, approximately 90% of incidents were reported from home, 0.5–1% from schools, and 1.5–2.5% from workplaces.

The figures may not be comparable from year to year. As more PCC collaborated in the reporting system, the figures from later years are more statistically valid. Reporting behaviour may have changed; physicians may contact PCC only in severe cases. Also, 4 years is not long enough to identify secular trends in small samples.

Pesticide incident monitoring system

Materials

PIM reports were obtained from the EPA concerning incidents with CBs and OPs. Two types of reports were available: pesticide-specific reports and those by incidents.

Most pesticide-specific reports contain a synopsis, two summary tables and short summaries of the incidents ('Table 3' in the reports). The first table presents a general overview of incidents categorized according to entity and site over a particular period of years. The second table is an overview of incidents categorized by site and circumstance. The third table is a compilation of short descriptions of the incidents, which contain information on: (1) year of incident, (2) state of occurrence, (3) number of individuals and their respective ages, (4) whether the incident was documented, (5) a short description of the accident, (6) suicidal intent, and (7) outcome. Occasionally, an additional table summarizes the number of pesticide incidents involving children by site and circumstance.

In addition to pesticide-specific reports, PIMS reports were available providing summaries of incidents of one category, e.g. schools, pets or garden incidents. These generally provide only summary statistics.

Table 32.2 Pesticide Incident Monitoring System Reports: sheet 1

AntiChE	*Years covered*	*Agriculture*			*City*		
		Alone	*Combination*	*Total*	*Alone*	*Combination*	*Total*
Carbaryl	1966–1980	32	41	73	2	0	2
Chlorpyrifos	1966–1981	7	21	28	0	1	1
Malathion	1954–1980	55	56	111	3	2	5
Methomyl	1966–1981	252	210	462	—	—	—
Parathion-methyl	1966–1981	50	154	204	—	—	—
Mevinphos	1966–1980	107	155	262	—	—	—
Monocrotophos	1966–1980	20	20	40	—	—	—
Oxydemeton-methyl	1966–1981	6	32	38	2	6	8
Coumaphos	1966–1979	2	0	2	—	—	—
Dicrotophos	1966–1981	8	4	12	—	—	—
Dimethoate	1966–1978	40	12	52	—	—	—
Dioxathion	1966–1978	3	1	4	—	—	—
EPTC	1966–1979	14	4	18	—	—	—
Merphos	1966–1981	5	2	7	—	—	—
Methidathion	1966–1981	2	23	25	—	—	—
Naled	1966–1981	9	42	51	0	2	2
Carbofuran	1966–1979	48	17	65	—	—	—
Carbophenothion	1966–1980	1	3	4	0	1	1
Chloropropham	1966–1979	2	0	2	—	—	—
Coumaphos	1966–1979	2	1	3	—	—	—
DEF	1966–1981	10	12	22	—	—	—
Ferbam	1966–1979	0	6	6	—	—	—
Phorate	1966–1979	35	19	54	—	—	—
Propoxur	1966–1979	1	3	4	—	—	—
Azinphos-methyl	1966–1981	51	65	116	1	0	1
Dichlorvos	1964–1980	7	22	29	0	2	2
EPN	1966–1979	0	30	30	—	—	—
Ethion	1966–1978	7	14	21	—	—	—
Ethoprophos	1966–1979	19	10	29	—	—	—
Fensulfothion	1966–1979	9	21	30	—	—	—
Fenthion	1966–1979	4	1	5	—	—	—
Fonofos	1966–1979	7	6	13	—	—	—
Methamidophos	1966–1981	15	24	39	—	—	—
Phosalone	1966–1979	9	2	11	—	—	—
Fenchlorphos	1966–1978	1	1	2	—	—	—
Trichlorfon	1966–1981	8	17	25	—	—	—
Bendiocarb	1966–1979	—	—	—	—	—	—
Disulfoton	1966–1978	30	46	76	—	—	—
Terbufos	1966–1981	6	6	12	—	—	—
Benomyl	1966–1981	15	65	80	—	—	—
Diazinon	1966–1980	29	36	65	1	0	1
Metam-sodium	1966–1980	1	2	3	—	—	—
Oxamyl	1966–1979	4	0	4	—	—	—
Total		933	1206	2139	9	14	23

Sheet 2

	Years covered	*Commercial*			*Correctional facilities*			*Creek/pond/lake*		
		Alone	*Combination*	*Total*	*Alone*	*Combination*	*Total*	*Alone*	*Combination*	*Total*
Carbaryl	1966–1980	2	7	9	1	0	1	—	—	—
Chlorpyrifos	1966–1981	5	27	32	1	0	1	0	1	1
Malathion	1954–1980	13	22	35	0	1	1	2	0	2
Methomyl	1966–1981	1	1	2	—	—	—	—	—	—
Parathion-methyl	1966–1981	1	2	3	—	—	—	0	3	3
Mevinphos	1966–1980	1	4	5	—	—	—	—	—	—
Monocrotophos	1966–1980	0	1	1	—	—	—	—	—	—
Oxydemeton-methyl	1966–1981	1	6	7	—	—	—	—	—	—
Coumaphos	1966–1979	—	—	—	—	—	—	—	—	—
Dicrotophos	1966–1981	—	—	—	—	—	—	—	—	—
Dimethoate	1966–1988	—	—	—	—	—	—	—	—	—

Sheet 2 continued

		Commercial			*Correctional facilities*			*Creek/pond/lake*		
	Years covered	*Alone*	*Combi-nation*	*Total*	*Alone*	*Combi-nation*	*Total*	*Alone*	*Combi-nation*	*Total*
Carbaryl	1966–1980	2	7	9	1	0	1	—	—	—
Chlorpyrifos	1966–1981	5	27	32	1	0	1	0	1	1
Malathion	1954–1980	13	22	35	0	1	1	2	0	2
Methomyl	1966–1981	1	1	2	—	—	—	—	—	—
Parathion-methyl	1966–1981	1	2	3	—	—	—	0	3	3
Mevinphos	1966–1980	1	4	5	—	—	—	—	—	—
Monocrotophos	1966–1980	0	1	1	—	—	—	—	—	—
Oxydemeton-methyl	1966–1981	1	6	7	—	—	—	—	—	—
Coumaphos	1966–1979	—	—	—	—	—	—	—	—	—
Dicrotophos	1966–1981	—	—	—	—	—	—	—	—	—
Dimethoate	1966–1978	—	—	—	—	—	—	—	—	—
Dioxathion	1966–1978	—	—	—	—	—	—	—	—	—
EPTC	1966–1979	0	1	1	—	—	—	—	—	—
Merphos	1966–1981	—	—	—	—	—	—	—	—	—
Methidathion	1966–1981	0	1	1	—	—	—	—	—	—
Naled	1966–1981	1	0	1	—	—	—	—	—	—
Carbofuran	1966–1979	—	—	—	—	—	—	—	—	—
Carbophenothion	1966–1980	—	—	—	—	—	—	—	—	—
Chloropropham	1966–1979	—	—	—	—	—	—	—	—	—
Coumaphos	1966–1979	—	—	—	—	—	—	—	—	—
DEF	1966–1981	—	—	—	—	—	—	—	—	—
Ferbam	1966–1979	—	—	—	—	—	—	—	—	—
Phorate	1966–1979	—	—	—	—	—	—	—	—	—
Propoxur	1966–1979	6	11	17	—	—	—	—	—	—
Azinphos-methyl	1966–1981	0	1	1	0	1	1	0	1	1
Dichlorvos	1966–1980	3	22	25	0	1	1	—	—	—
EPN	1966–1979	—	—	—	—	—	—	—	—	—
Ethion	1966–1978	—	—	—	—	—	—	—	—	—
Ethoprophos	1966–1979	—	—	—	—	—	—	—	—	—
Fensulfothion	1966–1979	—	—	—	—	—	—	—	—	—
Fenthion	1966–1979	—	—	—	—	—	—	—	—	—
Fonofos	1966–1979	1	0	1	—	—	—	—	—	—
Methamidophos	1966–1981	—	—	—	—	—	—	—	—	—
Phosalone	1966–1979	—	—	—	—	—	—	—	—	—
Fenchlorphos	1966–1978	—	—	—	—	—	—	—	—	—
Trichlorfon	1966–1981	—	—	—	—	—	—	—	—	—
Bendiocarb	1966–1979	4	2	6	—	—	—	—	—	—
Disulfoton	1966–1978	—	—	—	—	—	—	—	—	—
Terbufos	1966–1981	—	—	—	—	—	—	—	—	—
Benomyl	1966–1981	—	—	—	—	—	—	—	—	—
Diazinon	1966–1980	15	47	62	2	1	3	—	—	—
Metam-sodium	1966–1980	2	0	2	—	—	—	—	—	—
Oxamyl	1966–1979	—	—	—	—	—	—	—	—	—
Total		56	155	211	4	4	8	2	5	7

sheet 3

		Educational facility			*Home*			*Dumping site*		
	Years covered	*Alone*	*Combi-nation*	*Total*	*Alone*	*Combi-nation*	*Total*	*Alone*	*Combi-nation*	*Total*
Carbaryl	1966–1980	0	1	1	132	75	207	—	—	—
Chlorpyrifos	1966–1981	0	5	5	72	148	220	0	1	1
Malathion	1954–1980	7	5	12	230	257	487	—	—	—
Methomyl	1966–1981	—	—	—	14	5	19	—	—	—
Parathion-methyl	1966–1981	—	—	—	7	21	28	—	—	—
Mevinphos	1966–1980	—	—	—	—	—	—	1	1	2
Monocrotophos	1966–1980	—	—	—	2	0	2	—	—	—
Oxydemeton-methyl	1966–1981	—	—	—	5	77	82	—	—	—
Coumaphos	1966–1979	—	—	—	1	0	1	—	—	—
Dicrotophos	1966–1981	—	—	—	3	0	3	—	—	—
Dimethoate	1966–1978	—	—	—	3	1	4	—	—	—

Sheet 3 continued

		Educational facility				*Home*		*Dumping site*		
	Years covered	*Alone*	*Combi-nation*	*Total*	*Alone*	*Combi-nation*	*Total*	*Alone*	*Combi-nation*	*Total*
Dioxathion	1966–1978	—	—	—	2	0	2	—	—	—
EPTC	1966–1979	—	—	—	1	0	1	—	—	—
Merphos	1966–1981	—	—	—	1	2	3	—	—	—
Methidathion	1966–1981	—	—	—	—	—	—	—	—	—
Naled	1966–1981	—	—	—	6	15	21	—	—	—
Carbofuran	1966–1979	—	—	—	3	0	3	—	—	—
Carbophenothion	1966–1980	—	—	—	2	1	3	—	—	—
Chloropropham	1966–1979	—	—	—	—	—	—	—	—	—
Coumaphos	1966–1979	—	—	—	2	0	2	—	—	—
DEF	1966–1981	1	0	1	0	2	2	—	—	—
Ferbam	1966–1979	—	—	—	0	1	1	—	—	—
Phorate	1966–1979	1	0	1	4	1	5	—	—	—
Propoxur	1966–1979	1	1	2	171	220	391	—	—	—
Azinphos-methyl	1966–1981	—	—	—	9	6	15	—	—	—
Dichlorvos	1966–1980	1	3	4	89	283	372	—	—	—
EPN	1966–1979	—	—	—	0	1	1	—	—	—
Ethion	1966–1978	—	—	—	—	—	—	—	—	—
Ethoprophos	1966–1979	—	—	—	—	—	—	—	—	—
Fensulfothion	1966–1979	1	0	1	1	0	1	—	—	—
Fenthion	1966–1979	—	—	—	2	2	4	—	—	—
Fonofos	1966–1979	—	—	—	3	0	3	—	—	—
Methamidophos	1966–1981	—	—	—	2	0	2	0	1	1
Phosalone	1966–1979	—	—	—	—	—	—	—	—	—
Fenchlorphos	1966–1978	—	—	—	3	10	13	—	—	—
Trichlorfon	1966–1981	—	—	—	5	4	9	—	—	—
Bendiocarb	1966–1979	—	—	—	1	0	1	—	—	—
Disulfoton	1966–1978	—	—	—	23	3	26	—	—	—
Terbufos	1966–1981	—	—	—	—	—	—	—	—	—
Benomyl	1966–1981	—	—	—	11	1	12	—	—	—
Diazinon	1966–1980	1	12	13	247	324	571	0	1	1
Metam-sodium	1966–1980	—	—	—	4	1	5	—	—	—
Oxamyl	1966–1979	—	—	—	—	—	—	—	—	—
Total		13	27	40	1061	1461	2522	1	4	5

Sheet 4

			Hospital			*Industrial*		*Nursery/greenhouse*		
	Years covered	*Alone*	*Combi-nation*	*Total*	*Alone*	*Combi-nation*	*Total*	*Alone*	*Combi-nation*	*Total*
Carbaryl	1966–1980	0	1	1	1	4	5	3	2	5
Chlorpyrifos	1966–1981	1	4	5	4	14	18	0	1	1
Malathion	1966–1980	0	2	2	12	13	25	15	18	33
Methomyl	1966–1980	—	—	—	19	2	21	13	6	19
Parathion-methyl	1966–1981	—	—	—	13	10	23	—	—	—
Mevinphos	1966–1980	—	—	—	43	14	57	0	3	3
Monocrotophos	1966–1980	—	—	—	1	0	1	—	—	—
Oxydemeton-methyl	1966–1981	0	1	1	1	2	3	1	14	15
Coumaphos	1966–1979	—	—	—	—	—	—	—	—	—
Dicrotophos	1966–1981	—	—	—	1	3	4	—	—	—
Dimethoate	1966–1978	—	—	—	8	5	13	6	4	10
Dioxathion	1966–1978	—	—	—	2	0	2	—	—	—
EPTC	1966–1979	—	—	—	1	0	1	—	—	—
Merphos	1966–1981	—	—	—	—	—	—	—	—	—
Methidathion	1966–1981	—	—	—	0	2	2	—	—	—
Naled	1966–1981	—	—	—	1	2	3	0	3	3
Carbofuran	1966–1979	—	—	—	9	2	11	—	—	—
Carbophenothion	1966–1980	—	—	—	1	0	1	—	—	—
Chloropropham	1966–1979	—	—	—	1	1	2	—	—	—
Coumaphos	1966–1979	—	—	—	—	—	—	—	—	—
DEF	1966–1981	—	—	—	1	0	1	—	—	—
Ferbam	1966–1979	—	—	—	—	—	—	—	—	—

Sheet 4 continued

		Hospital			*Industrial*			*Nursery/greenhouse*		
	Years covered	*Alone*	*Combination*	*Total*	*Alone*	*Combination*	*Total*	*Alone*	*Combination*	*Total*
Phorate	1966–1979	—	—	—	5	4	9	—	—	—
Propoxur	1966–1979	0	1	1	1	4	5	—	—	—
Azinphos-methyl	1966–1981	—	—	—	1	6	7	1	0	1
Dichlorvos	1966–1980	0	1	1	4	10	14	3	2	5
EPN	1966–1979	—	—	—	1	2	3	—	—	—
Ethion	1966–1978	—	—	—	—	—	—	—	—	—
Ethoprophos	1966–1979	—	—	—	0	1	1	—	—	—
Fensulfothion	1966–1979	—	—	—	1	0	1	—	—	—
Fenthion	1966–1979	—	—	—	0	1	1	—	—	—
Fonofos	1966–1979	—	—	—	—	—	—	—	—	—
Methamidophos	1966–1981	—	—	—	1	0	1	—	—	—
Phosalone	1966–1979	—	—	—	1	1	2	1	0	1
Fenchlorphos	1966–1978	—	—	—	—	—	—	—	—	—
Trichlorfon	1966–1981	—	—	—	1	1	2	—	—	—
Bendiocarb	1966–1979	—	—	—	—	—	—	—	—	—
Disulfoton	1966–1978	—	—	—	12	6	18	—	—	—
Terbufos	1966–1981	—	—	—	2	1	3	—	—	—
Benomyl	1966–1981	—	—	—	1	3	4	20	29	49
Diazinon	1966–1980	2	3	5	8	14	22	4	17	21
Metam-sodium	1966–1980	—	—	—	—	—	—	3	0	3
Oxamyl	1966–1979	—	—	—	—	—	—	—	—	—
Total		3	13	16	158	128	286	70	99	169

Sheet 5

		Park/recreational area			*Public building*			*Transportation*		
	Years covered	*Alone*	*Combination*	*Total*	*Alone*	*Combination*	*Total*	*Alone*	*Combination*	*Total*
Carbaryl	1966–1980	1	1	2	1	0	1	1	0	1
Chlorpyrifos	1966–1981	1	2	3	6	20	26	1	0	1
Malathion	1966–1980	3	5	8	6	2	8	1	1	2
Methomyl	1966–1981	—	—	—	0	1	1	3	2	5
Parathion-methyl	1966–1981	—	—	—	—	—	—	12	4	16
Mevinphos	1966–1980	—	—	—	—	—	—	2	3	5
Monocrotphos	1966–1980	—	—	—	—	—	—	0	1	1
Oxydemeton-methyl	1966–1981	0	1	1	—	—	—	—	—	—
Coumaphos	1966–1979	—	—	—	—	—	—	—	—	—
Dicrotophos	1966–1981	—	—	—	—	—	—	1	0	1
Dimethoate	1966–1978	—	—	—	—	—	—	0	1	1
Dioxathion	1966–1978	—	—	—	—	—	—	—	—	—
EPTC	1966–1979	—	—	—	—	—	—	—	—	—
Merphos	1966–1981	—	—	—	—	—	—	—	—	—
Methidathion	1966–1981	—	—	—	—	—	—	—	—	—
Naled	1966–1981	0	2	2	1	0	1	—	—	—
Carbofuran	1966–1979	—	—	—	—	—	—	1	0	1
Carbophenothion	1966–1980	—	—	—	—	—	—	—	—	—
Chloropropham	1966–1979	—	—	—	—	—	—	—	—	—
Coumaphos	1966–1979	—	—	—	—	—	—	—	—	—
DEF	1966–1981	—	—	—	—	—	—	—	—	—
Ferbam	1966–1979	—	—	—	—	—	—	—	—	—
Phorate	1966–1979	—	—	—	—	—	—	5	1	6
Propoxur	1966–1979	—	—	—	—	—	—	—	—	—
Azinphos-methyl	1966–1981	—	—	—	—	—	—	1	1	2
Dichlorvos	1966–1980	4	1	5	1	5	6	—	—	—
EPN	1966–1979	—	—	—	—	—	—	—	—	—
Ethion	1966–1978	—	—	—	—	—	—	—	—	—
Ethoprophos	1966–1979	—	—	—	—	—	—	—	—	—
Fensulfothion	1966–1979	—	—	—	—	—	—	1	0	1
Fenthion	1966–1979	—	—	—	—	—	—	—	—	—
Fonofos	1966–1979	—	—	—	—	—	—	2	0	2
Methamidophos	1966–1981	—	—	—	—	—	—	1	0	1

Sheet 5 continued

	Years covered	Park\recreational area			Public building			Transportation		
		Alone	*Combi-nation*	*Total*	*Alone*	*Combi-nation*	*Total*	*Alone*	*Combi-nation*	*Total*
Phosalone	1966–1979	—	—	—	—	—	—	—	—	—
Fenchlorphos	1966–1978	—	—	—	1	1	2	—	—	—
Trichlorfon	1966–1981	0	1	1	0	1	1	—	—	—
Bendiocarb	1966–1979	—	—	—	—	—	—	—	—	—
Disulfoton	1966–1978	—	—	—	—	—	—	6	0	6
Terbufos	1966–1981	—	—	—	—	—	—	—	—	—
Benomyl	1966–1981	2	0	2	1	0	1	—	—	—
Diazinon	1966–1980	4	2	6	1	11	12	—	—	—
Metam-sodium	1966–1980	2	0	2	—	—	—	—	—	—
Oxamyl	1966–1979	—	—	—	—	—	—	—	—	—
Total		17	15	32	18	41	59	38	14	52

Sheet 6

	Years covered	Warehouse			Unspecified			Sub-total	(%)
		Alone	*Combi-nation*	*Total*	*Alone*	*Combi-nation*	*Total*		
Carbaryl	1966–1980	0	2	2	17	10	27	337	6
Chlorpyrifos	1966–1981	0	2	2	15	31	46	391	7
Malathion	1966–1980	4	6	10	50	12	62	803	13
Methomyl	1966–1981	1	0	1	17	12	29	559	9
Parathion-methyl	1966–1981	—	—	—	15	5	20	297	5
Mevinphos	1966–1980	2	3	5	12	4	16	355	6
Monocrotophos	1966–1980	—	—	—	0	3	3	48	1
Oxydemeton-methyl	1966–1981	—	—	—	1	6	7	162	3
Coumaphos	1966–1979	—	—	—	—	—	—	3	*
Dicrotophos	1966–1981	—	—	—	1	0	1	21	*
Dimethoate	1966–1978	—	—	—	3	1	4	84	1
Dioxathion	1966–1978	—	—	—	—	—	—	8	*
EPTC	1966–1979	—	—	—	—	—	—	21	*
Merphos	1966–1981	—	—	—	1	0	1	11	*
Methidathion	1966–1981	—	—	—	1	0	1	29	*
Naled	1966–1981	—	—	—	2	4	6	90	2
Carbofuran	1966–1979	2	0	2	10	0	10	92	2
Carbophenothion	1966–1980	—	—	—	1	0	1	10	*
Chloropropham	1966–1979	—	—	—	—	—	—	4	*
Coumaphos	1966–1979	—	—	—	—	—	—	5	*
DEF	1966–1981	0	1	1	—	—	—	27	*
Ferbam	1966–1979	—	—	—	—	—	—	7	*
Phorate	1966–1979	0	2	2	3	1	4	81	1
Propoxur	1966–1979	—	—	—	12	19	31	451	8
Azinphos-methyl	1966–1981	1	0	1	5	3	8	154	3
Dichlorvos	1966–1980	0	2	2	1	27	28	494	8
EPN	1966–1979	—	—	—	—	—	—	34	1
Ethion	1966–1978	—	—	—	1	1	2	23	*
Ethoprophos	1966–1979	—	—	—	1	0	1	31	1
Fensulfothion	1966–1979	1	0	1	—	—	—	35	1
Fenthion	1966–1979	0	1	1	5	1	6	17	*
Fonofos	1966–1979	1	1	2	—	—	—	21	*
Methamidophos	1966–1981	1	0	1	2	2	4	49	1
Phosalone	1966–1979	—	—	—	—	—	—	14	*
Fenchlorphos	1966–1978	0	1	1	1	0	1	19	*
Trichlorfon	1966–1981	—	—	—	1	1	2	40	1
Bendiocarb	1966–1979	—	—	—	0	1	1	8	*
Disulfoton	1966–1978	—	—	—	1	0	1	127	2
Terbufos	1966–1981	—	—	—	1	0	1	16	*
Benomyl	1966–1981	—	—	—	4	1	5	153	3
Diazinon	1966–1980	1	1	2	36	23	59	843	14
Metam-sodium	1966–1980	—	—	—	1	0	1	16	*
Oxamyl	1966–1979	—	—	—	—	—	—	4	*
Total		14	22	36	221	168	389	5994 (total)	

DEF = SSS-tributylphosphorotrithioate
Merphos = SSS-tributylphosphorotrithioite

Methods

A summary table was constructed of all antiChE insecticides and pesticides. The pesticide with the greatest number of 'home' incidents (diazinon) was selected for further study. All episodes of poisoning by diazinon at home were coded by the criteria above: (1) years of the report, (2) number of adults and children involved, (3) suicidal intent, (4) documented or not, and (5) outcome. Also, we coded whether the incident directly involved an exterminator and whether a labelling problem was involved (usually refilling into an unlabelled container).

Data were analysed by standard statistical packages (Epistat[C] and SPSS/PC+). Because PIMS reports were often developed at different times, the denominators may vary for each calculation. χ^2 tests with Yate's correction were used to calculate statistical significance. Odd's ratios and 95% confidence intervals were calculated. Where fewer than five events of interest were expected in cells, the cells were collapsed to ensure that over 75% of the cells involved in the calculation had an 'expected value' of >5. Results are presented in the uncollapsed form for clarity.

Results

Table 32.2 shows an overview of PIMS reports. Of the total 5994 reported incidents attributed to antiChEs, 337 (6%) involved carbaryl, 391 (7%) chlorpyrifos, 843 (14%) diazinon, 494 (8%) dichlorvos, 803 (13%) malathion, 559 (9%) methomyl, 335 (6%) mevinphos, and 451 (8%) propoxur. In descending order, incidents were most prevalent in (1) home, (2) agriculture, (3) industry, (4) commercial, and (5) nurseries/greenhouses. Most incidents occurred with a chemical used in combination with another. This represents 15–28% of all reported incidents.

In all, 943 of 20 555 (4.6%) involved dichlorvos, naled, trichlorfon, tetrachlorvinphos, malathion and diazinon [7, No.179] at home or in gardens. Only two cases occurred in gardens (both malathion), the remaining 941 being home-related. Of these, 239 incidents (25.4%) involved dichlorvos, 14 (1.5%) naled, 6 (0.6%) trichlorfon, 8 (0.9%) tetrachlorvinphos, 256 (27.2%) malathion, and 418 (44.4%) diazinon.

Of 22 466 incidents, 249 (1.1%) involved domestic animals [7, No. 280]. Only 46 of 34 537 incidents (0.1%) involved schools [7, No. 281]. In 91 of 22 466 (less than 0.4%) incidents cats and dogs were involved [7, No. 385].

Table 32.3 shows causes for incidents at home and in commercial sites. Ingestion was the most frequent source of exposure at home, and inappropriate application the primary cause at work. The few cases in schools generally followed hand application of pesticides, although undocumented incidents have been reported.

Diazinon-related home incidents

Between 1966 and 1980, 843 incidents involving diazinon were reported to the PIMS, 571 (approximately 67.7%) took place in the home according to the PIMS summary. On review of the individual incidents, only 567 were identified that clearly took place at home. Table 32.4 shows the distribution of incidents by documentation. Over time, with more incidents reported, fewer were well documented (χ^2 = 28.67; P<0.0001). Incidents were less likely to be fatal over time (Table 32.5). There appeared to be a trend according to year and location of incidents. For example, most cases in 1976 occurred in Utah. Likewise, most cases

Table 32.3 Distribution of causes of poisoning from propoxur and diazinon[a]

	Propoxur		*Diazinon*	
	Home	*Commercial*	*Home*	*Commercial*
Ingestion of pesticide, food or treated material	188 (46.4)	1 (5.9)	212 (35.7)	2 (3.1)
Failure to follow label directions (improper use)	112 (27.6)	11 (64.7)	164 (27.1)	40 (61.5)
Skin/splash	13 (3.2)	3 (17.6)	—	3 (4.6)
Other	90 (22.2)	4 (23.5)	210 (35.4)	15 (23.1)
Total	405	17	594	65

[a]Numbers in parentheses are percentages

Table 32.4 Changes in reported episodes of poisoning by antiChEs over time comparing documented and non-documented incidents

Year	*Documented*	*Non-documented*	*Total*
1968–1970	7 (50.0)	7 (50.0)	14
1971–1972	9 (33.3)	18 (66.7)	25
1973	18 (31.0)	40 (69.0)	58
1974–1977	30 (15.7)	161 (84.3)	191
1978–1980	31 (11.2)	246 (88.8)	277

[a]Numbers in parentheses are percentages
[b]$\chi^2 = 31.19$; $P<0.0001$

Table 32.5 Changes in reported episodes of poisoning by antiChEs over time comparing fatal and non-fatal incidents

Year	*Fatal*	*Non-fatal*	*Total*
1968–1970	1 (7.1)	13 (92.9)	14
1971–1972	1 (3.7)	26 (96.3)	27
1973	4 (7.3)	51 (92.7)	55
1974–1977	2 (1.0)	189 (99.0)	191
1978–1980	2 (0.7)	273 (99.3)	275

[a]Numbers in parentheses are percentages
[b]$\chi^2 = 14.7$; $P<0.005$

Table 32.6 Episodes of poisoning with diazinon in children compared with adults

Characteristics	*No. of episodes*	*Odds ratio (95% confidence interval)*	*P*
Suicide	4	0.08 (0.03; 0.26)	<0.0001
Fatal outcome	6	1.03 (0.25; 4.38)	1.0000
Applied by an exterminator	97	0.24 (0.16; 0.35)	<0.0001
Labelling problem	17	1.17 (0.50; 2.81)	0.8477

Table 32.7 Episodes of poisoning with diazinon in adults: associations

Characteristics	*No. of episodes*	*Odds ratio (95% confidence interval)*	*P*
Suicide	28	9.65 (3.16; 24.20)	<0.0001
Fatal outcome	4	0.80	0.9887
Applied by an exterminator	164	5.90 (4.00; 8.71)	<0.0001
Labelling problem	11	0.82 (0.35; 1.92)	0.7770

Table 32.8 Episodes of poisoning with diazinon and fatal outcomes: risk factors

Characteristics	*No. of episodes*	*Odds ratio (95% confidence interval)*	*P*
Suicide	3	6.91 (1.49; 31.71)	0.0137
Applied by an exterminator	2	0.31 (0.05; 1.59)	0.2171
Labelling problem	3	9.02 (2.07; 42.19)	0.0034

occurring in Texas were during 1978. The reporting system therefore does contain biases which cannot be defined or reduced.

Of the diazinon incidents, 247 (45.2%) involved at least one adult, 324 (59.3%) at least one minor under the age of 18 years, and 36 (6.4%) were reported as suicides. Ten (1.8%) involved at least one fatality; 249 (44.0%) involved application by an exterminator, and 28 (4.9%) problems with labelling. Most cases were accidental and unintentional.

Adults were more likely to use pesticides in suicidal intent than children and more likely to be involved with incidents involving exterminators (Tables 32.6 and 32.7). Surprisingly, mislabelling was not a statistically significant risk factor for either adults or children.

Risk factors for fatal outcomes were examined (Table 32.8). Individuals who ingested diazinon with suicidal intent were almost seven times as likely to die as other poisoning forms. Labelling problems were associated with a ninefold risk of death.

Conclusions

Both the PIMS and the ARPCCs suggested that poisoning incidents involving antiChEs decreased over time. This must be regarded with caution because of the biases in both systems. ARPCCs involved more centres each year, so the early extrapolations are statistically unstable. As physicians have become more knowledgeable about occupational and environmental diseases, they may contact PCCs less frequently. Also, results of 4 years do not allow definition of a clear trend. The PIMS had different biases year for year. There were clear trends to less documentation, fewer

labelling difficulties, and fewer fatalities over time. Whether the latter is accurate or a chance occurrence is uncertain; it seems consistent with the ARPCCs. Increasing awareness by physicians in the 1970s may have led to increased reporting. This is consistent with substantial increases in reported incidents over time. Estimates of under-reporting are difficult to address. Kahn [8] suggested that, in California, only 1% were reported. Discher [3] similarly found rates of reporting in work-related disease in the order of 5%.

Few clear risk factors could be defined. Children were less likely to commit suicide, at least between 1968 and 1980. Nevertheless, both systems suggest that approximately 5% of incidents are suicidal gestures, and more likely to result in fatalities. Adults were more likely to suffer poisoning from application through exterminators (including the exterminators).

Only 28 incidents (3.3%) could be attributed to removing labels from containers or refilling into unlabelled containers. The emulsifiable concentrate form of diazinon resembles milk. In one incident, a young man purchased soft drinks which contained liquid diazinon, and drank some of the contents. The store owner accidentally stored the unmarked pesticide with other soft drink bottles. Labelling does not prevent pesticide poisoning, although mislabelling and label removal does markedly increase the risk of fatalities occurring.

Overall, against common assumptions, few incidents involved suicides (0.3%) or suicide attempts (5.0%). Many victims were young children. One of six common situations involved 95% of episodes: (1) inhalation of drift following spraying by a pest control operator; (2) not wearing proper safety equipment; (3) harvesting fruits/vegetables sprayed too early; (4) not storing pesticides in their original containers; ignoring label instructions or having no label instructions; (5) placing pesticides in areas accessible to young children; and (6) suicide/suicide attempts.

For points (1) to (3), either teaching or enforcement are necessary. Teaching of pesticide applicators as well as field supervisors, might prevent pesticide drift and exposure. Many applicators wear inadequate safety equipment, either because it malfunctions or it is not available. Safety in the workplace is an awareness and an enforcement issue. Only if supervisors enforce work rules unambiguously, will they be followed. Systematic review by insurance companies, with unannounced inspection of work-sites may be a useful strategy to reduce incidents. No work has been undertaken to establish appropriate re-entry times in residences. Extension of the California re-entry regulations to the rest of the USA may reduce the problem. A study of pesticide residues in residences and their relationship to symptomatology and biological indicators of exposure is needed.

Simply labelling original containers may be inadequate to prevent residential poisoning, as more severe cases are associated with lack of labels. Mislabelling, or lack of labelling, is a major risk factor for deaths in pesticide incidents. Review of labels generally suggests they are not safety oriented. They should include highly visible warnings concerning the risk of dying through removal.

No surveillance of exposed populations around pesticide application has been undertaken. Pesticide applicators are generally not subjects of medical surveillance programmes involving biological monitoring. Accurate dose-response relationships could be obtained through such programmes, and allows a more precise definition of clinically relevant decreases in RBC AChE levels.

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33

Residues of anticholinesterases in foodstuffs

K. N. Woodward

Introduction

Nature and origin of residues

The organophosphorus (OP) and carbamate (CB) insecticides are potent antiChE compounds and this accounts both for their insecticidal efficacy and for their major form of toxicity in mammals including humans. However, it should be noted that some CBs were developed as herbicides (e.g. asulam and barban) and fungicides (e.g. benomyl and carbendazim) and with some of these the antiChE activity may be weak, although other forms of toxicity may then predominate.

Source of residues

Taking the UK as an example, the antiChEs and other chemicals used in agriculture fall into two major categories: pesticides and veterinary medicines. Thus OPs and CBs used for the control of insects on crops are classified as pesticides while those used in the treatment of ectoparasitic diseases in farm and other animals are regarded as medicinal products in the UK. It follows logically that the major source of antiChE residues in edible crops are the agricultural pesticides while those in meat, offal and other animal products (fish and dairy products) arise from the use of veterinary medicines containing the products. Other countries however regard these ectoparasiticides as pesticides. In practical terms however, the end result from the point of view of residues is the same.

Acceptable residue levels

Although national regulatory requirements for authorizing the marketing of pesticides and veterinary medicines may differ, the basic underlying philosophy for setting acceptable residue levels in foods of plant and animal origin is often similar. It generally involves the assessment of a package of toxicity data, the identification of a no-observed effect level, the setting of an acceptable daily intake (ADI) and finally, the calculation of the acceptable level of the residue in the food commodity or commodities of concern.

In 1984, A Joint FAO/WHO Expert Consultation on Residues of Veterinary Drugs in Foods met in Rome. It considered the concern expressed by the Codex Alimentarius Commission about the medication of animals intended for the production of human food and it made various recommendations including one for the establishment of a Codex Committee on Residues of Veterinary Drugs in Food [22,63]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA), an already established and respected committee for assessment of food additives, first convened to consider the toxicological and residue aspects of veterinary drugs in Rome in 1987 and its first report (on chloramphenicol and steroid hormone growth promoters) was published in 1988 [25]. Similarly, the residue issues on these drugs considered by JECFA were also published [27].

It should be noted that residues may change with time from application to harvest and are also affected by other factors including food processing and cooking [6,7,56,79,88].

Analytical methods

Analytical methods for OPs specifically and for residues of veterinary drugs in general have recently been reviewed [47,71,77], and will not be discussed here.

On an international scale, residue issues are examined by the Codex Alimentarius Committees (often abbreviated to Codex) and its Commission in the Joint Food and Agricultural Organization (FAO)/World Health Organization (WHO) Food Standards Programme. The Codex Committee proposes Maximum Residue Levels (MRLs) for pesticide residues for consideration by the Codex Alimentarius Commission and in doing so it takes into account the recommendations of the FAO/WHO Joint Meeting on Pesticide Residues (JMPR). This Joint Meeting considers the toxicological and residue data on the compounds under scrutiny and makes recommendations on residue levels and other issues such as the nature of any further work required [83]. The MRL is the maximum concentration of a pesticide residue which results in a food commodity when that pesticide has been applied according to the principles of good agricultural practice [6,82]. It takes into account the toxicological findings in that the MRL must be below the limit implied by a consideration of the ADI [6] thus ensuring an adequate margin of safety. Codex ADIs and MRLs are available in published form [21,24,25].

Toxicity of residues

The toxicities of the antiChEs are discussed elsewhere in this volume and they have been widely reviewed in other publications [17, 18, 42,46,50,51,75].

The major problem with all toxicity studies lies in the interpretation of the results in terms of likely effects on humans, i.e. extrapolation to man. This process is fraught with difficulties because of (for example) species differences and the use of high doses and in-bred strains of test animal but it is even more troublesome when one is considering not the exposure to the chemical or formulation as is the case with occupational hygiene or even with food additives, but ingestion of residues. These residues may be present in plant or animal tissues not only as the parent compound, but also as metabolites and as the products of physicochemical and environmental degradation. Consequently, the toxicity of the parent compound could well be different from that of the residue-combination were it to be isolated and tested [27,65]. Nevertheless, the toxicity of residues is normally assessed on the basis of the results on the parent compound, presumably on the basis that any metabolites formed will exert their own effects and the results observed will be the sum of the effects. While this is true it ignores pharmacokinetic differences between animals and humans to an extent that might be regarded by some as being irresponsible. To attempt to overcome these difficulties the procedure known as relay-toxicology was introduced. Here, experimental animals are given the food commodity containing the residues rather than the pesticide or veterinary drug itself [28,30,80,81].

All these points considered, it has to be emphasized that poisoning incidents from pesticide and veterinary drug residues appear to be few. That is to say reports of overt toxicity in humans are uncommon. As far as the antiChEs are concerned, the major incidents have occurred after ingestion of vegetables contaminated with the carbamate aldicarb. Water melons and cucumbers treated with aldicarb have been reported to have caused illness in subjects who had ingested them [43,61]. More recently, over 300 reports of illness were made to the Health Protection Branch of Health and Welfare, Canada. The symptoms included tightening of the throat, blurred vision, eye twitching and mouth numbness accompanied by diarrhoea and vomiting. Again, aldicarb was shown to be the causative agent [49]. CBs have been implicated in other residue poisoning incidents [85]. Such incidents have raised concern elsewhere as aldicarb has been shown to be a groundwater contaminant [33,86].

Levels of residues

Arising from veterinary medicines

From the outset it is important to emphasize that in practice it is often impossible to be certain about the origin of a residue in animal tissues and products. It is generally by implication that the source is pin-pointed. A knowledge of the type of pesticide, the general use and the levels found can give clues to the origin. In the UK and Europe, the cattle

warble fly is a major economic pest [89]. The major medicinal agents in the UK for the treatment of warble fly are OPs. Similarly, OP compounds such as diazinon and chlorfenvinphos are used in sheep dips for the control of sheep scab.

That OPs are absorbed from cattle after topical application was demonstrated when groups of calves were treated with a recommended dose of 40.5 mg/kg and two other doses of 20.25 and 60.75 mg/kg of the OP famphur. The animals showed pupillary constrictions and even those given half the recommended dose had marked depressions of whole blood ChE activity for between 2 and 14 days after application [87].

Following treatment of beef steers with 0, 25, 50 or 150 mg/kg famphur, maximum residues in subcutaneous fat biopsied from the gluteal region were found 1 day later. Residues in those given 25 or 50 mg/kg were in the region of 1–3 mg/kg tissue but after 3 days residue levels were not significantly different from controls. When slaughtered on days 1, 7 and 14 after treatment with 45 mg/kg, residues were highest in muscle, omental fat and subcutaneous fat at days 1 (1.41, 1.25 and 0.4 mg/kg respectively) and 7 (0.71, 0.35 and 0.53 mg/kg respectively) and had fallen to <0.02 mg/kg in muscle, kidney, liver and perirenal fat by day 14. Levels in omental and subcutaneous fat were comparable to controls at that time point. Before treatment with approximately 23 mg/kg famphur, no residues were detected in the milk of mid-lactation Guernsey cows, but after treatment, levels of 0.197, 0.158, 0.049 and 0.031 ppm were found in the milk at the next four milkings. By the third day (morning and afternoon milkings), levels had fallen to 0.008 ppm. Most of the residues were found in the butter fat fraction [2].

After topical treatment with phosmet (60ml of a 5% solution, 6–8 mg/kg) as a warblicide, levels in the milk from dairy cows at various stages of lactation reached a peak of 0.044 mg/kg at 12 h after dosing and decayed to less than 0.02 mg/kg over the 50 h after treatment and the extent of elimination was related to milk yield [69].

A similar treatment with fenthion (4.3–6.5 mg/kg) led to a peak level (c. 0.1 mg/kg) in milk at around 10 h and falling to 0.05 mg/kg within 24 h of dosing. There was a tendency to accumulate in fat but nevertheless, the level was below 0.05 mg/kg [70]. Residues of crufomate also fell rapidly after treatment of lactating cows with 19–21 mg/kg. A peak level of 0.1 mg/kg occurred at around 10 h after topical application and then decayed to below the Codex MRL of 0.05 mg/kg by 24 h. Again there was a tendency to accumulate in the fat fraction [68].

When reindeer were given intramuscular doses of famphur (30 mg/kg) tissue levels of the parent compound were highest at the first sampling time, day 2, after treatment (0.15, 0.3 and 10 mg/kg in liver, kidney and injection site thigh muscle) as were those of the metabolite famoxon (0.03 and 0.4 mg/kg in kidney and injection site). At days 4, 5 and 6 levels had fallen markedly but there were still residues of the metabolite famoxon at the injection site (0.05 mg/kg) at day 20 [20].

Indicative of the treatment of sheep with OPs in the UK, are the levels of residues in samples of sheep fleeces. From 1984 to 1987, numbers of samples containing diazinon have risen from 15% to 38% whereas those of propetamphos have risen from 4% to 61%. Over the same period, the numbers containing gamma-HCH have fallen from 66% to 0.7% [60]. The number of samples containing dieldrin have also fallen. This situation therefore reflects the discontinued use of organochlorine compounds in sheep dips since 1985, and the increasing use of OPs as substitutes. Residues were also found in samples of meat and meat products. Samples of fat taken in 1986 were analysed and of 274, 19% contained diazinon and 2% propetamphos. In 1987 however, of 280 samples analysed 7% contained diazinon and <1% propetamphos. Of those containing diazinon, four samples exceeded the Codex Alimentarius MRL of 0.7 mg/kg fat. Samples obtained from retail outlets in the UK during 1984–1986 generally showed no or low levels of OP residues. Diazinon was not detected in samples of beef, imported lamb, pork or veal but low levels were found in UK lamb for 1984/85 (up to 1.7 mg/kg) and 1985/86 (up to 0.1 mg/kg). However, OPs were not detected in butter, milk or cheese.

Residues of OPs in Australian milk was attributed to the licking of treated areas by the animal, and the extremely low levels of chlorpyrifos, ethion and bromophos-ethyl appeared

to confirm this [64]. Various pesticides and pollutants were examined in poultry meat in Israel. The levels of these, which included diazinon and chlorpyrifos in broilers, turkeys and geese were said to be extremely low and below the USA tolerances [54].

Arising from other uses

UK

Several surveys in the UK have revealed that pesticide levels in general are low and those of OPs and CBs in particular are very low and below the Codex Alimentarius MRL values. A number of surveys have investigated pesticide residues in food commodities including those which clearly originated outside the UK, e.g. citrus fruits, bananas and rice [32, 45, 55, 57, 60,66,78]. In the 1982–1985 UK survey, levels of chlorpyrifos-methyl in imported wheat appeared to be high (0.3–1.9 mg/kg) but only six samples were positive of 139 examined and moreover all the values were below the Codex MRL of 10 mg/kg. Similarly for pirimiphos-methyl, 139 samples were investigated and 53 were positive with residue levels in the range of 0.1–9.6 mg/kg, but again even the highest was below the Codex MRL of 10 mg/kg for this OP [57]. In the 1985–1988 MAFF survey, OPs were detected in a variety of foods but here too the levels were low and generally well below the Codex MRL value where these had been assigned for the commodities in question. The exceptions were samples of malted barley. Of 136 samples of barley from England, 82 were found to have residues of OPs and of these some samples were relatively high – not detected to 1.2 mg/kg for chlorpyrifos-methyl and not detected to 4.6 mg/kg for fenitrothion; reporting limit 0.05 mg/kg. However, of these the mean residue level for fenitrothion was only 0.03 mg/kg with one other sample at 4.6 mg/kg. There are no current Codex MRLs for these two compounds in malted barley. The most similar commodities for chlorpyrifos-methyl are flour and wheat with MRLs of 2 and 10 mg/kg suggesting that the highest value found of 1.2 mg/kg should give no reason for concern. For fenitrothion the only comparable commodity is wholemeal flour with a Codex MRL of 5 mg/kg which even the single high residue value of 4.6 mg/kg is below. Moreover, the average person (65 kg) would have to consume around 140 kg malted barley/day to exceed the Codex ADI of 0.01 mg/kg body-weight [60].

Thus it would appear that for CBs and OPs at least, the residue picture in the UK over a number of years gives some grounds for reassurance.

USA and Canada

Results of residue testing from the USA suggest that in general residue levels are, or are likely to be, below Codex MRL values in individual food commodities. No significant residues of aldicarb, chlorpyrifos or methamidophos were found in alfalfa sprouts although residues were detected in the seeds from which these were grown [5]. Similarly, ethion in orange pulp and whole fruit was below the Codex MRL although this was exceeded in peel by a factor of around 3 [67]. A survey of cereals, vegetables, sugars and oils and related materials revealed that levels of malathion, diazinon and parathion were very low as were those of carbaryl, diazinon, leptophos and parathion in fruit [52]. The half-lives of carbaryl and methiocarb as dislodgable foliar residues on fruit trees were estimated at only 4.1 and 1.7 days respectively [90]. Experimental studies in the USA have indicated that the octanol-water partition coefficients may be of predictive values in estimating pesticide residue levels in milk and meat, and in substrates with relatively high fat content, whereas levels in vegetables appear to be inversely proportional to this parameter [80].

In Canada, residue levels also seem to be present at below the Codex MRL values [35,36].

Countries other than the UK, USA and Canada

Surveys in France (1967–1977), The Netherlands (1981), Sweden (1981–1984), Germany (1977) and South Africa (1971–1980), suggest that in the recent past residues of antiChEs were not a major problem on vegetables and fruits [1,8–10,13,14,16,31,33,53,84]. Trials in Spain with chlorfenvinphos, in Portugal with several pesticides, and in The Netherlands with a number of OPs suggested rapid depletion under field conditions [31,37,74].

Total diet studies

UK

Total diet studies commenced in the UK in 1966. Food offered for sale in 21 towns was purchased and analysed for various pesticide residues including those of OPs but not those of CBs. The early studies concentrated on levels of organochlorine compounds because the use of these was more prevalent at the time. For OPs in the period 1966–1967, 66 total diet samples each containing seven subsamples of cereals, meat and fish, fats, fruits and preserves, root vegetables, green vegetables and milk were examined. The only OP regularly found was malathion with 15 positive samples largely in the cereals group (0.01– 0.04 mg/kg). In 462 samples, single positive findings were made for demeton-S, disulfoton, fenchlorphos, fenitrothion and parathion (0.01–0.13 mg/kg). In the second survey, 1970–1971, malathion was again the only OP regularly found in 52 of 55 cereal samples and in samples of fruits and preserves. Carbofenthion was found in a single sample of mixed green vegetables (0.04 mg/kg). Malathion was also found in 18 of 40 cereal samples in the 1974–1975 survey but no other OP was discovered. The intakes of malathion for the 1966–1967, 1970–1971 and 1974–1975 surveys were estimated to be 11, 53 and 7 μg/ person/day, well below the current Codex ADI of 1.3 mg/kg/person/day (based on the figure of 0.02 mg/kg for a 65 kg adult) [19].

In the 1975–1977 survey, 40 of 51 cereal samples contained malathion at concentrations in the range 0.01–0.12 mg/kg. A single positive was found in oil and fats, and in fruits and preserves [55]. For the 1979–1980 survey, malathion was found in only one sample of cereals. The intake was calculated to be well below the ADI. One sample of green vegetables contained chlorpyrifos at 0.05 mg/kg [55].

The 1982–1985 survey examined 24 samples each of bread, other cereal products, oils and fats, green vegetables, other vegetables and fresh fruit. There was little evidence for the presence of OPs. One sample of 'other cereal products' contained phosalone at 0.08 mg/kg as did one sample of fresh fruit (0.38 mg/kg), and one sample of the latter also contained ethion residues (0.7 mg/kg). None of the other 141 samples were positive and intakes of those present were well below the ADI values [57].

In the latest survey, 1985–1988, 200 samples from seven food groups were investigated. The limits of detection were 0.01–0.05 mg/kg for a range of OP pesticides and only three compounds were detected. These were chlorpyrifos in 'other vegetables', malathion in 'other cereal products' both at 0.03 mg/kg and pirimiphos-methyl (see later). No residues of OPs were found in carcass meat, offal, milk and fresh fruits. Intakes of malathion, chlorpyrifos and pirimiphos-methyl were calculated to be 0.1, 0.1 and 1.8 μg/person/day all of which are far below the current Codex ADI values of 1.3, 0.65 and 0.65 mg/kg respectively, based on a 65 kg adult weight. No residues of pirimiphos- methyl had been detected in the 1982 survey of total diet but in the 1985– 1988 study, residues were found in nine of the bread group samples and in eight of the 'other cereals' group, which was thought to reflect the increasing use of this pesticide on stored wheat [60].

USA

Findings similar to those in the UK were also made in the USA [38,40]. Toddler total diets have also been the subject of investigation in the USA. Diets collected in ten American cities between 1978 and 1979 were examined. The components were drinking water, whole milk, other dairy products and dairy substitutes, meat/fish/poultry, grain cereals, potatoes, vegetables, fruit juices, oils and fats, sugars and beverages. Chlorpropham was low in potatoes and vegetables but the range extended to 0.563 mg/kg of the commodity leading to an intake of 0.4 mg/day. Unfortunately there is no Codex MRL or ADI for chlorpropham and so the significance of these findings is unclear. Levels and intakes of the other compounds of interest (chlorpyrifos, diazinon, ethion, fonofos, malathion, parathion and phosalone) were very low in the commodities investigated [39]. A similar exercise in the years 1980–1982 was conducted with samples from 13 American cities. The results were similar to those obtained in 1978–1979 with intake from carbaryl, chlorpyrifos, diazinon, ethion, fenitrothion, malathion, parathion and phosalone being low. Chlorpropham residues

in potatoes were again relatively high but intake was around half of the value in the previous study [41].

The Netherlands, France and Japan

Total diet studies conducted in these countries have not revealed any major problems related to residues of antiChEs [13,14,62].

New Zealand

A total diet study in New Zealand operated at 3-monthly intervals in the period 1974–1975 using food groups from four mail areas: Auckland, Wellington, Christchurch and Dunedin. The most common pesticide found in composite foods was malathion whilst omethoate and azinphos-methyl predominated in fruits. Of 116 samples analysed, 82 (71%) had no detectable residues. Intakes were calculated at 2.5, 2, 4, 4, 5, 1.5, 0.5 and 5 microgram/day for azinphos-methyl, diazinon, dichlorvos, ethion, mevinphos, omethoate and parathion; well below the Codex MRLs. The highest intake was that of malathion at 20 microgram/kg per day which is equal to the current Codex ADI [15].

Conclusions

The overwhelming evidence from residue and total diet studies suggests that residues of OPs and CBs are generally within the acceptable levels set by the Codex Alimentarius Commission or by individual national regulatory bodies, and this is indeed reassuring bearing in mind the toxicological properties of these substances. Data reviewed here and elsewhere [50,51,77] suggests that the compounds are rapidly broken down whether on plants or in animals, further reducing the risks to humans. Nevertheless, findings such as those discussed in this chapter should not give rise to complacency and moreover should not be taken as indicative of a reduced need for future surveillance and, where necessary, regulatory action. Not all OPs currently in use worldwide appear to have been fully investigated with respect to residues and new ones coming onto the world markets will need careful examination for their ability to leave residues. A similar requirement will arise for any new uses of existing compounds. Moreover it cannot be said that the CBs in use throughout the world have been thoroughly examined.

In recent years, the need to observe suitable harvest intervals (the time from application to a crop and harvesting) or for veterinary drugs, the respective withholding period (the time from treatment of a food-producing animal to slaughter or milk collection, for example) has been emphasized [4,12,44,58] to ensure that residue levels in food do not exceed the defined limits of national and international organizations. In addition there is a constant need for the development of simple, non-sophisticated methods of analysis so that routine monitoring can be conducted easily and cheaply [47] and where possible and practicable, for multiresidue methods of analysis [59]. More importantly is the requirement for reliable and comprehensive routine surveillance of pesticide and veterinary drug residues in food such as those currently in use in the USA and the UK [3,11,58,73] and those imposed on Member States by various European Directives [77]. Such monitoring schemes require a well constructed regulatory framework and, where shortcomings are identified, suitable scope for enforcement. Perhaps one area where progress is still required lies in the extension of the findings from animal toxicology studies to the problems of human health.

The content of this paper represents the author's views alone and in no way commits the Department of Health, UK.

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34

Organophosphorus compounds as chemical warfare agents

R.L. Maynard and Francis W. Beswick

Historical aspects of the use of OPs and chemical warfare agents

The history of the OP and other antiChE compounds has been reviewed by Holmstedt [35,36]. He has traced the development of OP compounds from Lippe De Clermont's synthesis and testing of tetraethyl pyrophosphate (TEPP) in 1854 [19], although in fact the compound had apparently been synthesized before 1854 in Murtz's laboratory, in which De Clermont was working. Another major achievement was the work of Lange [42] in incorporating the fluorine atom into OP compounds. Lange's interest in such compounds was relevant to their use as insecticides. He offered them to I.G. Farbenindustrie, 'for evaluation in pest control' where they were taken up by Bayer, the scientific leader of the company, who encouraged Gerhard Schrader to work on the compounds in 1934. Subsequently the development of OPs as chemical warfare agents involved I.G. Farbenindustrie (IGF) and echoed the involvement of this conglomerate of dye and chemical manufacturers in the large scale chemical warfare agent development of World War I.

Schrader led the programme of synthetic work at IGF and, it is claimed, by 1944 had synthesized some 2000 OP compounds; the development of OPs as chemical warfare agents can be said to have begun with him. From 1935 onwards the German Government insisted on being informed of toxic OP compounds and required that they should be investigated as potential chemical warfare agents; TEPP was included. The development of insecticidal OP compounds has been described by Schrader [70]. The history of work on OPs as chemical warfare compounds is not well documented but the following milestones can be listed.

(1) 1937: Schrader synthesized the nerve agents tabun, ethyl N,N-dimethyl phosphoramidocyanidate (also known as GA) and sarin, isopropyl methylphosphonofluoridate (also known as GB). (Sources differ regarding the data of first synthesis of GA, 23 December 1936 is quoted by several authorities. The original German code letters were G for pure tabun and GA for tabun diluted 20% with chlorobenzene.)
(2) 1938: A small pilot plant, at Münster-Lager, for production was established.
(3) 1940: Construction of a major nerve agent production plant was undertaken at Duhernfurt (now Dyhernfurth) some 40 km north of Breslau in German Silesia (now Wroclaw in Poland). The factory covered an area of 1 km^2, was heavily camouflaged and capable of producing 1000 tons of nerve agent per month.
(4) 1942: Production at Duhernfurt began in 1942 and tabun was first produced in May 1943; sarin followed in June 1944. Approximate total production figures are: tabun, 10 000–12 000 tons; sarin, 600 tons [41]. Difficulties were encountered in the production of sarin. It was discovered that quartz or silver vessels had to be used to contain the reagents.
(5) 1944: Soman (GD) synthesized by Richard Kuhn. Only laboratory quantities were synthesized in Germany during World War II.

Both sarin and tabun were filled into artillery shells and test firings performed. Tests with 250 and 500 kg bombs were also undertaken; aerial spray devices and a cluster bomb designed to

deliver agents were developed. At the end of World War II plans to deliver nerve agent by rocket were in hand. Nerve agents were seen by Germany as lethal weapons and a tactical role for them was defined. Comparatively little attention was paid to the harassing effects of low concentrations of nerve agent vapour when miosis and associated symptoms might significantly reduce a serviceman's efficiency.

The reluctance of Germany to use nerve agents during World War II has never been satisfactorily explained. Work at Cambridge, UK on fluorine- containing compounds was undertaken during the war and in 1940 dimethyl and diethyl phosphorofluoridate were synthesized. In 1941 Saunders [67] synthesized diisopropyl phosphorofluoridate (DFP), a particularly widely used OP research tool. Work on the pharmacology of DFP was undertaken in England and its capacity to inhibit horse serum ChE was demonstrated by Dixon *et al.* (unpublished). The results of these investigations were not published until after 1945. American interest in the OPs as potential chemical warfare agents lagged behind that of Germany and the UK but towards the end of World War II the Medical Division of Edgewood Arsenal became interested in them. German suspicion regarding the objectives of the work in the UK and the USA may have played a very considerable role in inhibiting the use of nerve agents during World War II.

After 1945 great effort was put into synthesizing further OPs of possible chemical warfare importance. Further G agents (GE and GF) were synthesized and in the late 1950s work which had begun with the synthesis of the insecticide Amiton, O,O-diethyl S-(2-[diethylamino]ethyl) phosphorodithioate, yielded a new series of nerve agents described as V agents. These compounds were synthesized first by Tammelin [80]. Between 1960 and 1967, 4000–5000 tons of VX were manufactured in the USA. Soviet interest in V agents was first officially admitted in 1987 when weapons alleged to contain the agent VX were shown to an international team of scientists and journalists at the Soviet Chemical Establishment at Shikhany.

It would seem reasonable to assume that any developed nation intent on an aggressive or retaliatory capacity could easily avail itself of a range of nerve agents. In 1986 a United Nations team investigating allegations of the use of chemical warfare by Iraq stated, 'the main chemical used was mustard gas (yperite), but nerve agents have probably been used on occasions' [81].

It is very difficult to list all the weapon systems which could be used to disseminate chemicals in war: some, which the authors believe could be suitable for the delivery of chemical agents, including nerve agents, are listed together with some obsolescent munitions (Table 34.1).

The practical military use of nerve agents is a matter of exploiting both their high toxicity and their physical characteristics. These properties are dealt with later but the general rule is that agents with a high volatility, i.e. likely to be encountered as a gas might be employed to produce a large number of casualties quickly

Table 34.1 Weapon systems likely to be employed for chemical agent delivery

	Sarin	*Soman*	*V-agent*
Artillery			
105 mm	Y		
122 mm	Y		
5 inch	Y		
152 mm	Y	Y	
155 mm	Y		Y
8 inch	Y		Y
203 mm		Y	
Rocket launchers			
115 mm	Y		Y
122 mm	Y		Y
220 mm	Y		
Mortars			
4.2 inch	Y		
120 mm	Y		
160 mm	Y		
240 m	Y		
Missiles			
Little John	Y		
Honest John	Y		
Frog-7		Y	Y
Lance	Y		
Sergeant			
Scud B		Y	Y
Land mines	Y		Y
Delivered from aircraft			
Bomblet dispenser	Y		
100 kg	Y	Y	Y
500 lb	Y		
250 kg	Y	Y	
750 lb	Y		
1000 lb	Y		
(Cluster)			
Spray delivery	Y	Y	Y

[a]Y, likely use

Table 34.2 Possible targets for nerve agents

Target	*Type of agent*			*Delivery system*
	Sarin	*Soman*[a]	*VX*	
Rear area targets				
Airports/airfields		Y	Y	Aircraft: bombs, cluster bombs, spray bombs, spray tanks, missiles
Seaports		Y	Y	As above
Railway junctions		Y	Y	Aircraft: bombs, cluster bombs
Headquarters and communication centres		Y	Y	Aircraft: bombs, missiles
Storage sites		Y	Y	Aircraft: bombs, cluster bombs, missiles
Troop concentrations		Y	Y	Aircraft: bombs, spray tanks
Forward areas				
Nuclear delivery weapons, other key weapons and systems	Y		Y	Multiple rocket launcher, Aircraft: bombs, rockets
Defence positions	Y		Y	Multiple rocket launchers, artillery, mortars, Aircraft: bombs, rockets
Own flanks			Y	Mines
Own defence front generally	Y		Y	Artillery, mortars, mines
to produce casualties, to harass and reduce combat efficiency	Y		Y	Multiple rocket launchers, artillery, mortars. Aircraft: bombs, rockets
To deny unwanted ground		Y	Y	Aircraft: spray, mines
Harass civilian population	Y			Aircraft: bombs, rockets, spray

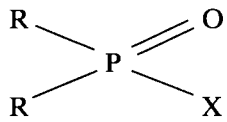
[a]Soman may be in a thickened form
[b]Y, likely use

by a direct strike on the target: an example is sarin. More persistent classes of nerve agents, e.g. soman, thickened by the addition of polyethylmethacrylate, or VX, can be laid to deny ground or make difficulties for those who have to cross it. Possible targets for nerve agents are given in Table 34.2.

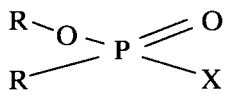
Properties of nerve agents

Physicochemical properties of nerve agents

Nerve agents are OP compounds (Table 34.3) and conform to the general formula published by Schrader [70].



Generally they are phosphonic acid derivatives:

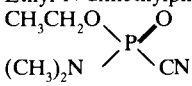
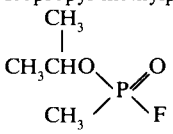
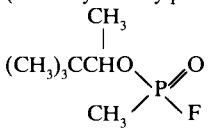
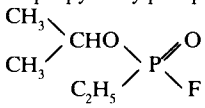
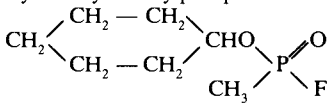
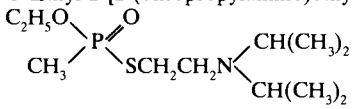
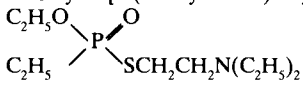
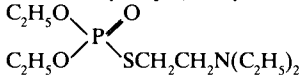
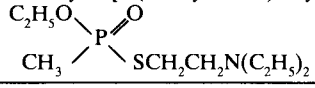


In the case of the G agents, X is a fluorine atom and the compounds are phosphonofluoridates. Tabun exceptionally is a phosphoramido cyanidate in which one R is an N-N-dimethyl group and X is a cyanide moiety. Excepting VG, the V agents are generally phosphonothioates in which one substituent group is attached to phosphorus through a sulphur atom. Selected physical properties of some of these compounds are shown in Table 34.4. All five agents in the Table are liquids at room temperature. In general terms sarin has a similar viscosity to petrol (gasoline) and VX resembles machine oil. Soman lies between them. In the pure state nerve agents are colourless and mobile liquids, although when impure they may be tinged yellow or brown. The comparative volatility of the different agents should be noted. Sarin is markedly more volatile than the others and VX is of such low volatility as to present only a low vapour hazard. Reports of the odour of nerve agents are few but a fruity smell is said to be characteristic. It is important to remember that production samples of nerve agent will not be pure and the presence of unused precursors may well contribute to the viscosity, appearance and smell of particular samples.

Toxicity of nerve agents

The progression of nerve agent toxicity, in character and speed, is not only dose-dependent but also dependent on the route of entry into the body. In the vapour phase they are

Table 34.3 Formulae of nerve agents

Abbreviation	*Common name*	*Proper name*
GA	Tabun	Ethyl N-dimethylphosphoramidocyanidate 
GB	Sarin	Isopropyl methylphosphonofluoridate 
GD	Soman	1,2,2-trimethylpropyl methylphosphonofluoridate (Pinacolyl methylphosphonofluoridate) 
GE	—	Isopropyl ethylphosphonofluoridate 
GF	—	Cyclohexyl methylphosphonofluoridate 
VX	—	O-Ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate 
VE	—	O-Ethyl S-[2-(diethylamino)ethyl] ethylphosphonothioate 
VG	—	O O-Diethyl S-[2-(diethylamino)ethyl] phosphorothioate 
VM	—	O-Ethyl S-[2-(diethylamino)ethyl] methylphosphonothioate 

absorbed rapidly across the respiratory epithelium. They are also absorbed across the cornea where by a local effect they produce miosis. As liquids they pass relatively rapidly across the skin and mucous membranes without damaging them. They may also be absorbed from the gut and, when carried into wounds, are rapidly distributed throughout the body.

The absorption of nerve agent from air taken into the respiratory tract is almost complete; thus about 90% of inhaled sarin is absorbed. The percentage absorbed remains high even at increased ventilation rates. Thus at minute volumes of 50 litres the LCt_{50} of sarin may be reduced from the often quoted figure of 100 mg min per m^3 to something of

Table 34.4 Physicochemical properties of nerve agents

		Tabun GA		*Sarin GB*		*Soman GD*		*VX*		*GF*	
Molecular weight (daltons)		162.3		140.1		182.18		267.36		180.14	
Specific gravity at 25°C		1.073		1.0887		1.022		1.0083		1.133 (20°C)	
Boiling point °C		246		147		167		300		—	
Melting point °C		–49		–56		–80		–20		–12	
Vapour pressure (VP) and volatility (Vol)[a]	°C	VP mmHg	Vol[a] mg/m³	VP mmHg	Vol mg/m³	VP mmHg	Vol mg/m³	VP mmHg	Vol mg/m³	VP mmHg	Vol mg/m³
	0	0.004	38	0.52	4279	0.044	470.9			0.006	63
	10	0.013	119.5	1.07	8494	0.11	1135.5			0.017	173
	20	0.036	319.8	2.10	16 101	0.27	2692.1	0.00044	5.85[b]	0.044	434
	25	0.07	611.3	2.9	21 862	0.40	3921.4	0.0007	10.07	0.068	659
	30	0.094	807.4	3.93	29 138	0.61	5881.4			0.104	991
	40	0.23	1912.4	7.1	60 959	—	—			0.234	2159
	50	0.56	4512.0	12.3	83 548	2.60	23516.0			0.501	4480

[a]Volatility = concentration of saturated vapour at specified temperature
Volatility calculated from PV = nRT

$$\text{Vol} = \frac{\text{VP} \times 101\,325 \times \text{MW}}{760 \times 8.3143 \times {}^{\circ}\text{A}} = \frac{\text{VP} \times \text{MW} \times 16.035}{{}^{\circ}\text{A}}$$

°A = Absolute temperature
[b]Some authorities quote values as low as 0.1–1.0 mg/m³

the order of 20 mg min per m³. This inverse relation of LCt_{50} with ventilatory state is very important and some indication of the respiratory state should be given whenever the toxicity of a nerve agent is quoted, e.g. 'the LCt_{50} of sarin for men *at rest* is 100 mg min per m³. It follows that men undertaking considerable physical activity are significantly more vulnerable to poisoning by nerve agent vapour than those at rest. Percutaneous poisoning, on the other hand, is a consequence of straight transfer and the major factors determining speed of onset and degree of intoxication are the area covered by the liquid agent and the time it is allowed to remain in contact.

A considerable range of toxicity measurements have been made in animals but although exact figures are often quoted for man, these are in fact only estimates. Some of the available data on the toxicity of nerve agents are presented in Table 34.5. These data have been collected from a wide range of sources and are only offered as sample figures, since a very large number of estimates of the lethal toxicity of nerve agents has been made.

Permissible exposure limits (threshold limit values (TLV) and time weighted averages (TWA)) have been set in the USA for sarin and VX. These are defined as the maximum time-weighted average airborne concentration expressed as mg/m³ of a chemical agent to which it is believed that essentially all members of a specific population can be exposed for a specific period without adverse effect [2]. The values are: sarin: TWA = 0.0001 mg/m³ (8 h average); TWA (pregnant woman) = 0.000003 mg/m³ (72 h average); and TWA (general population) = 0.00003 mg/m³ (72 h average). VX: TWA (workplace) = 0.00001 mg/m³ (8 h daily for an indefinite period); TWA (general population) = 0.0000003 mg/m³ (72 h average/indefinite).

Clinical aspects of nerve agent poisoning

Several excellent accounts of the effects of nerve agents on humans are available. Classical UK papers by McKee *et al.* [48,49] and Whitcher [83] and those of USA authors, Grob *et al.* [28–32] contain a wealth of information on the effects of nerve agents on individual subjects. Sidell [75] has also contributed some particularly useful papers and as noted later has prepared a survey of human exposures to nerve agents. Many of the effects of nerve agents on humans may be predicted from knowledge of the sites of action of AChE in the body. A brief but useful account is provided in the Official East German Handbook: *'Chemische Kampstoffe und Schütz von*

Table 34.5 Comparative acute toxicity of nerve agents

Species	*Route*	*Term*	*Unit*	*Tabun*	*Sarin*	*Soman*	*VX*
Man	Percutaneous	LD_{50}	mg/kg		28 [63]		
	Percutaneous	LDL_0	µg/kg				86 [84]
	Percutaneous	LDL_0	mg/kg	23 [63]		18 [63]	
	Inhalation	LDL_0	mg/m³	150 [63]		70 [63]	
	Inhalation	LD_{50}	mg/m³		70 [63]		
	Inhalation	LDL_0	µg/m³		90 [62]		
	Intravenous	TDL_0	µg/kg	14 [63]			
	Intravenous	TDL_0	µg/kg				1.5 [73]
	Oral	TDL_0	µg/kg		2 [30]		4 [73]
	Subcutaneous	LDL_0	µg/kg				30 [54]
	Intramuscular	TDL_0	µg/kg				3.2 [54]
Rat	Percutaneous	LD_{50}	mg/kg	18 [26]			
	Inhalation	LC_{50}	mg/m³/10 min	304 [26]	150 [62]		
	Intravenous	LD_{50}	µg/kg	66 [26]	39 [21]	44.5 [61]	
	Oral	LD_{50}	µg/kg	3700 [26]	550 [26]		
	Subcutaneous	LD_{50}	µg/kg	193 [38]	103 [9]	75 [5]	12 [38]
	Intramuscular	LD_{50}	µg/kg	800 [30]	108 [68]	62 [68]	
	Intraperitoneal	LD_{50}	µg/kg		218 [21]	98 [12]	
Mouse	Percutaneous	LD_{50}	mg/kg	1 [26]	1.08 [26]	7.8 [46]	
	Inhalation	LC_{50}	mg/m³/30 min	15 [26]	5 [45]	1 [45]	
	Intravenous	LD_{50}	µg/kg	150 [26]	113 [69]	35 [7]	
	Subcutaneous	LD_{50}	µg/kg	250 [50]	60 [45]	40 [45]	22 [50]
	Subcutaneous	LD_{50}	µg/kg		172 [8,51]		
	intramuscular	LD_{50}	µg/kg	440 [69]	222 [69]		
	Intraperitoneal	LD_{50}	µg/kg	604 [34]	420 [13]	393 [13]	50 [84]
	Intraperitoneal	LD_{50}	µg/kg		400 [45]		
Dog	Percutaneous	LD_{50}	mg/kg	30 [26]			
	Inhalation	LC_{50}	mg/m³/10 min	400 [26]	100 [26]		
	Intravenous	LD_{50}	µg/kg	84 [59]	19 [59]		
	Oral	LD_{50}	µg/kg	200 [45]			
	Subcutaneous	LD_{50}	µg/kg	284 [5]		12 [3]	
Monkey	Percutaneous	LD_{50}	µg/kg	9300 [26]			
	Inhalation	LC_{50}	mg/m³/10 min	250 [26]	100 [26]		
	Subcutaneous	LD_{50}	µg/kg			13 [14]	
	Intramuscular	LD_{50}	µg/kg		22.3 [20]	9.5 [44]	
Cat	Inhalation	LC_{50}	mg/m³/10 min	250 [26]	100 [59]		
	Intravenous	LD_{50}	µg/kg		22 [26]		
Rabbit	Percutaneous	LD_{50}	µg/kg	2500 [26]	925 [26]		
	Inhalation	LC_{50}	mg/m³/10 min	840 [26]	120 [26]		
	Intravenous	LD_{50}	µg/kg	63 [26]	15 [87]		
	Oral	LD_{50}	µg/kg	16300 [26]			
	Subcutaneous	LD_{50}	µg/kg	375 [16]	30 [27]	20 [3]	14 [43]
	Intraperitoneal	LD_{50}	µg/kg				66 [43]
Guinea	Percutaneous	LD_{50}	mg/kg	35 [26]			
pig	Inhalation	LC_{50}	mg/m³/2 min	393 [26]			
	Subcutaneous	LD_{50}	mg/kg	120 [27]			
	Subcutaneous	LD_{50}	µg/kg		30 [16]	24 [27]	8.4 [27]
Hamster	Subcutaneous	LD_{50}	µg/kg	245 [15]	95 [16]		
Farm	Percutaneous	LD_{50}	µg/kg	1100 [26]			
animal	Inhalation	LC_{50}	mg/m³/14 min	400 [26]			
Chickens	Subcutaneous	LD_{50}	µg/kg			50 [12]	
	Intraperitoneal	LD_{50}	µg/kg			71 [3]	
Frog	Intraperitoneal	LD_{50}	µg/kg			251 [12]	

Chemischen Kampstoffen' and in the East German manual on Military Toxicology. Equivalent Western sources [39] include the NATO Handbook [55, 56]. These official publications consider the military aspects of nerve agent poisoning.

Excellent accounts of the effects of inhibition of AChE have been provided by Koelle [41] and in other standard textbooks of pharmacology [6]. Very valuable accounts of poisoning by OP insecticides have been provided by Namba [53], Willems *et al.* [86] and Willems [85].

Table 34.6 Effects of nerve agents on target organs

Receptor	*Target organ*	*Symptoms and signs*
Peripheral muscarinic ($?M_1$)	Iris smooth muscle	Miosis
	Ciliary smooth muscle	Spasm leading to failure of accommodation, headache, pain in eyes, nausea and vomiting
	Conjunctival blood vessels	Vasodilatation: hyperaemia
	Nasal submucosal and mucosal glands and blood vessels	Rhinorrhoea and hyperaemia
	Bronchial smooth muscle	Contraction: bronchoconstriction, tightness of the chest, wheezing on expiration, dyspnoea[a]
	Bronchial mucosal and submucosal glands	Increased secretion
	Gastrointestinal tract	Anorexia, nausea, vomiting, abdominal cramps, diarrhoea, tenesmus, involuntary defaecation
	Sweat glands	Increased activity[b]
	Salivary glands	Increased activity
	Lacrimal glands	Increased activity[c]
	Heart	Bradycardia
	Urinary bladder	Frequency, involuntary micturation
Nicotinic	Skeletal muscle	Weakness, easy fatigue, fasciculation, cramps, facial paralysis
	Autonomic ganglia	Sympathetic effects may occur: pallor, tachycardia, elevation of blood pressure
CNS ? M_2 muscarinic receptors		Giddiness, insomnia, anxiety, restlessness, headache, tremor, withdrawal and depression, slowed mental recall, failure of concentration, confusion, slurred speech, ataxia, Cheyne-Stokes respiration, coma, convulsions, depression of medullary cardiac and respiratory centres[d]

[a]As reported later tightness of the chest is a very common subjective effect on exposure to nerve agent vapour. Clear evidence of bronchospasm is not available at lower level exposures
[b]Local hyperactivity of sweat glands is particularly clear after percutaneous exposure to nerve agents
[c]Of all the increases in glandular activity the response of the lacrimal glands is the most unremarkable
[d]Agents such as soman produce very marked central effects and death from respiratory failure is more likely to be the result of failure of the medullary respiratory drive than of respiratory muscles

The effects of nerve agents at different sites are shown in Table 34.6. It can be seen that the effects are manifold, affect many organs and show considerable variation depending on the route of exposure.

Clinical effects of different patterns of exposure

Vapour exposure

The eyes and respiratory system are the major sites affected by vapour exposure to nerve agents.

Low level (non-lethal) exposure

Tightness of the chest and rhinorrhoea are likely to be detected early in this type of exposure. These may appear only a few minutes after the start of the exposure. Dimming of vision, eye pain and frontal headache follow. On examination the pupils are found to be contracted and the conjunctivae hyperaemic. These local effects may last for several hours after removal of the subject from the nerve agent vapour. Headache and visual difficulties may persist for several days.

High level exposure

The clinical effects described above occur rapidly and are severe. Marked salivation and rhinorrhoea are to be expected. Respiratory problems include wheezing, dyspnoea, and expectoration of fluid occur. As the nerve agent is absorbed systemically via the lung the full range of effects listed in Table 34.6 will appear and collapse, vomiting, abdominal pain, incontinence, twitching, convulsions and respiratory failure may follow.

The validity of Haber's Law (Constant Ct = Constant effect irrespective of variations in C or t) as regards effects of nerve agent at extremes of concentration and time has not been investigated. For exposures of up to an

hour and Ct profiles of up to Ct = 15 mg min per m^3 it is felt that the law which states that equal Cts will call forth equal responses, probably holds fairly well.

Effects of skin exposure

The pattern of effects following exposure of skin to liquid nerve agent is quite different from that following exposure to vapour. It should be noted however that exposure of skin alone, i.e. exposure with respiratory tract, gastrointestinal tract and eyes protected, to nerve agent vapour produces very few effects. Exposure of the skin to liquid nerve agent is followed by sweating and fasciculation at the site of contamination, which spreads to involve whole muscle groups. These do not appear as rapidly as do the effects on the eyes seen after vapour exposure. If sufficient nerve agent is absorbed then systemic symptoms and signs as described earlier will follow.

Effects of ingestion of nerve agents

Colicky pain, nausea, vomiting and diarrhoea occur after ingestion of nerve agents in contaminated food or drinking water. Rapid absorption across the gut wall produces the symptoms and signs of systemic response already noted.

Clinical effects of nerve agent exposure in humans

Since the later 1940s both the UK and the USA, and probably other countries, have been engaged in investigating the effects of nerve agents on humans. German workers investigated the effect of nerve agents on man during World War II, under concentration camp conditions, but comparatively little information has been gleaned from these studies. The object of this section is to provide a source of unclassified material. This task has been simplified by the work of Sidell who over the years has reviewed the majority of the literature available both in the UK and the USA, classified and unclassified, relating to the effects of nerve agents on humans. Extensive studies have been carried out in humans with sarin, less extensive studies with VX and comparatively few studies with soman. Work involving the use of GA, GF and GE has only been very poorly reported (Tables 34.7 and 34.8). As well as the planned exposure of volunteers to sarin quoted earlier a number of accidents have occurred resulting in men becoming exposed to nerve agents. The most

Table 34.7 Human studies using nerve agents

Nerve agent	*Route*	*Reference*
Sarin	Inhalation	McKee and Woolcott [48]
		McKee *et al.* [49]
		Whitcher [83]
		Oberst and Koon [58]
		Harvey [33]
	Oral	Grob *et al.* [31]
		Grob and Harvey [30]
	Percutaneous	McGrath *et al.* [47]
		Adie and McPhail [1]
		Grob *et al.* [32]
		Marzulli and Williams [52]
		Freeman *et al.* [23]
Soman	Intravenous	O'Leary and Kunkel [59]
	Percutaneous	Neitlich [57]
VX	Intravenous	Kimura *et al.* [40]
		Sidell [72]
		Sidell and Groff [75]
	Oral	Sim *et al.* [78]
		Sidell and Groff [74]
	Percutaneous	Sim and Stubbs [79]
		Sim [77]

Table 34.8 Human studies of nerve agents to elucidate eye effects

Nerve agent	*Reference*
Sarin	Harvey [33]
	Grob and Harvey [30]
	Johns [37]
	Sim [76]
	Rubin and Goldberg [64–66]

Table 34.9 Accidental exposures to nerve agents

Agent	*Reference*
Sarin	Brown [11]
	Seed [71]
	Craig and Freeman [18]
	Craig and Cornblath [17]
	Gammill [24]
	Brody [10]
	Gaon and Werne [25]
	Ward *et al.* [82]
	Grob [28]
	Sidell [73]
Soman	Sidell [73]
VX	Bertino *et al.* [4]

extensive reports of accidental exposures are in the American literature and have been reported by Sidell [73] (Table 34.9).

Diagnosis of nerve agent poisoning

Cases of nerve agent poisoning displaying classic clinical effects including miosis, rhinorrhoea, salivation, fasciculation, respiratory embarrassment and CNS disturbances are comparatively straight-forward to identify. These symptoms and signs are most likely to be seen after severe vapour exposure. The effects of ingestion of nerve agent, see earlier, may be confused with a wide range of other gastrointestinal problems including various infections, e.g. typhoid and paratyphoid, encountered under military conditions.

Value of ChE estimations

It is often assumed that a knowledge of blood ChE or RBC AChE levels is essential to the diagnosis and management of nerve agent poisoning. This is not true. Despite the likely general validity of the statement 'the more severe the nerve agent poisoning the more marked will be the AChE depression', the range of depressions seen in patients with identical symptoms and signs may be very large. This point has been made most competently by Willems [85] as regards poisoning by OP insecticides. RBC AChE levels of patients were determined on admission and plotted against clinical levels of severity of intoxication using the criteria of Namba [53]. A very wide scatter of degrees of AChE inhibition in the severely affected subjects was demonstrated.

AChE determinations may be used first to confirm poisoning by an antiChE compound, and second to confirm the efficacy of oxime therapy after poisoning and to determine whether the OP-ChE complex is capable of undergoing reactivation. This can also serve to differentiate poisoning, e.g. in individuals who had not received carbamate pretreatment, reactivation on treatment with P2S would be expected in cases of sarin and VX but not in cases of tabun or soman poisoning. AChE determinations should not be used to obtain more than an extremely crude estimate of the extent of poisoning or monitor the course of recovery. Recovery of RBC AChE in the absence of oxime reactivation is very slow (see earlier), about 1% per day. These guidelines may be summarized in an aphorism: *treat the casualty not the cholinesterase depression.*

Management of nerve agent poisoning under field conditions

The management of nerve agent poisoning under field conditions presents a number of very difficult problems. Poisoning occurs suddenly and patients usually will not be seen by medical officers until some time has elapsed. Even the services of a trained first aider or medical orderly may not be instantly available to the poisoned individual. Despite this, speed of treatment remains of great importance as the interval between exposure and collapse may be short and life-saving therapy should be given as quickly as possible after poisoning. In view of this many countries have issued autoinjection devices to servicemen for self administration on recognition of the symptoms and signs of nerve agent poisoning. Such a policy carries the inherent risk that servicemen may self-administer therapy in the absence of poisoning as a result of panic. This risk is seen as justifiable given the potency and rapidity of action of nerve agents. Nearly all autoinjection devices contain a cholinolytic (atropine-like) drug and an oxime. Some countries, including the UK, supply a benzodiazepine in tablet form, diazepam 5 mg, incorporated into the safety cap on the device. The current UK autoinjection device , 'ComboPen' (Duphar) contains atropine sulphate (2 mg), P2S (500 mg) and includes a 5 mg diazepam tablet.

Self administration of a diazepam tablet under field conditions is agreed to be likely to be difficult and in the next generation of UK autoinjection devices 10 mg of a lysine conjugate of diazepam, which breaks down to diazepam and lysine in the body, will be incorporated in the injection mixture.

While few would argue with the need to include an atropine-like drug, and of such drugs atropine itself is probably a reasonable choice, the choice of oxime is far from universally agreed (*see* Ch.52). In making a choice

regarding drugs for autoinjection devices for issue to servicemen a number of factors have to be taken in to account; not all of these would be taken into account during the development of drugs and devices for civilian us. These include: efficacy; safety of components should be demonstrated to nationally accepted standards; cost; shelf life under simple and undemanding storage conditions, e.g. storage at room temperature rather than in a refrigerator; ease of access to drugs under war time conditions. This may be particularly important if only relatively few overseas sources of a particular drug are available; simplicity of use under field conditions; and capacity to function under a wide range of environmental conditions, from arctic to tropical.

These constraints on the choice of drugs for inclusion has led the UK to the combination described earlier. As circumstances will vary between countries, it is hardly surprising that different combinations have been fielded. As well as drug therapy every effort should be made to maintain respiration in nerve agent casualties by devices currently available. A bag of the AMBU type protected by a butyl rubber cover would be an excellent choice.

The adoption of CB pretreatment (*see* Ch.56) has not been universal although it is likely that a number of countries will take up the option of fielding pyridostigmine bromide tables as is done by the UK, primarily to counter soman poisoning.

The management of miosis presents a particularly difficult and important problem as it can be a very incapacitating result of exposure to nerve agents and is very difficult to reverse. Cholinolytic drops of atropine or homatropine, may be used to reverse miosis but the effects of such compounds on accommodation is seriously counterproductive. Many authorities do not advocate the use of such therapy.

Conclusions

Nerve agents are potent and effective chemical warfare agents. Defence against them rests heavily on detection systems and protective equipment. A great deal of research effort has been expended during the past 40 years in devising means of opposing the effects of such compounds and currently pretreatment with a carbamate antiChE and therapy after exposure with an atropine-like drug, an oxime and a benzodiazepine seems the best choice.

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35

Combustion toxicology of anticholinesterases

David A. Purser

Introduction

The combustion toxicology of antiChEs is a potentially serious issue involving both OP and CB pesticides, and phosphorus-based fire retardants. Fires involving materials treated with antiChEs, or in bulk pesticide stores, may release the pesticide and its thermal decomposition products, adding to the toxicity of the fire products. Some phosphorus-based fire retardants may be directly neurotoxic (triorthocresyl phosphate (TOCP)) [1,2] or toxic in other ways (tris-(2,3-dibromopropyl) phosphate (TRIS)) [10], but when phosphorus- based fire retardants are involved in fires, antiChEs may be created during the complex reaction chemistry of the fires. In 1975, it was discovered that polyurethane foams with a trimethylol propane polyol base containing phosphorus-based retardants formed a highly neurotoxic product, trimethylol propane phosphate (TMPP), when they were burned [22]. This was important politically, because it supported the belief the fires involving modern synthetic furnishing materials might be 'supertoxic' compared with those involving traditional materials, and led to demands for toxicity screening of materials. No other reports have occurred of actual fire disasters or laboratory findings involving severe or unusual toxic effects that might have resulted from exposure to antiChEs or other neurotoxic OP products. However, fire retardants, including phosphorus-based systems, often increase the toxic potency of combustion products by increasing the yields of general toxic combustion products [25], and among these products there may be some toxic products containing phosphorus. This does not necessarily mean that the toxic hazard of a fire would be increased, as fire retardants may slow the rate of fire growth, as well as reducing the risk of ignition [25].

Because combustion atmospheres contain complex mixtures of products, animal exposure, combined with chemical identification of toxic products, is the only way of determining for certain whether neurotoxicity and antiChE activity is present. The paucity of published information on both chemical and toxicological investigations is of concern considering the extreme toxicity of some phosphorus-containing compounds and the widespread use of OPs.

Decomposition conditions in fires and toxic product formation

The major cause of incapacitation and death in fires is exposure to toxic gases [24]. These consist mainly of the narcotic gases carbon monoxide (CO), hydrogen cyanide (HCN) and carbon dioxide (CO_2), affecting the nervous system, and irritants affecting the eyes and respiratory tract. The chemical composition of the combustion products and their yields in fires depend on temperature, oxygen concentration and whether the material is flaming. It is possible to identify three major types or stages of fires and the hazard situations that result from them: non-flaming/smouldering fires, early or small flaming fires and fully developed large-scale (post-flashover) fires. Non-flaming conditions may be hazardous because they provide the greatest opportunity for the formation of exotic toxic species such as TMPP by vapour phase reactions [22,29] and the most favourable conditions for the survival of CBs and OPs in the vapour phase, depending on their volatility. These substances

are likely to be largely consumed by flames, with the phosphorus oxidized to phosphorus pentoxide, although some phosphorus may remain in any char that is formed [17,27]. However, a proportion of the products may escape the flame zone, and a significant exposure to partially decomposed pyrolysis and oxidative reaction products may occur within a few minutes. With large, fully-developed fires, temperatures are high (approximately 1000°C) and complex organic substances such as OP esters or CBs are unlikely to survive such extreme conditions in significant amounts. When large fires occur in pesticide stores significant quantities of toxic species may be evolved from the periphery of the fire, where thermal decomposition conditions may be less extreme.

The validity of small-scale combustion toxicity experiments used to evaluate these phenomena depends on how well they simulate the decomposition conditions in full-scale fires, and whether the analytical and animal exposure protocols are capable of detecting toxic species and toxic effects [24]. In practice, the fire models used are often inadequate, while analytical techniques often applied do not detect OPs [29,31] and animal exposure methods may not show up neurotoxic effects, especially delayed neurotoxicity.

Combustion products of antiChEs

Smith and Ledbetter [26], who studied the combustion products of OPs, reported two large fires in the USA involving stores of parathion, one of 5 tons and the other of 42 tons, with 55 people being treated for symptoms of poisoning in the first case and three in the second. There are no known cases of death following exposure to combustion products from OPs in fires.

Because halogens, sulphur, oxygen and nitrogen are usually present in fires, with a wide variety of aliphatic and aromatic pyrolysis products, all involved in complex chemical flame reactions, there is the potential for the formation of a wide range of exotic OPs in fires. Smith and Ledbetter [26] state that OP pesticides are unstable when heated and decompose before they reach their boiling point. It is therefore likely that substantial decomposition would occur in a fire, although some evaporation from heated solutions might take place, which would be a potential source of toxicity. Three likely reactions involving OPs are decomposition, isomerization and polymerization, of which isomerization is most likely to yield toxic products. The first step in decomposition is usually loss of the electrophilic group, followed either by abstraction of hydrogen, forming dimethyl or diethyl phosphate, displacement of one of the other groups yielding methyl or ethyl phosphate, or the production of a polymer.

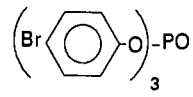
When solutions of malathion (0.1 g/ml) in xylene and kerosene were burned in a petri dish, the maximum malathion concentrations in the gaseous products were 10 $\mu g/m^3$ for xylene and 4 $\mu g/m^3$ for kerosene. For different solvents with boiling points below 150°C, most of the malathion and total phosphorus remained in the residue, while for higher boiling point solvents most of the malathion and half of the phosphorus was lost. During distillation, parathion and malathion recovery was 10% for a 150°C boiling point solvent, but while parathion recovery increased with boiling point, malathion recovery decreased, so that at 250°C parathion recovery was 15% and malathion recovery almost zero. The authors concluded that for solvents with boiling points below the pesticide decomposition temperature, the pesticide yield increased with the boiling point of the solvent, particularly as the last of the solvent boiled away, while for other solvents the yield decreased with the boiling point, since the rate of decomposition increased with temperature faster than the vapour pressure. Higher yields also occurred for higher initial concentrations. Decomposition products from malathion included dimethyl fumarate, isomers of malathion, and isomers of dimethyl phosphorodithioate.

The authors felt that virtually all the pesticide passing through a flame is destroyed, and that at the concentration found over the petri dish (10 $\mu g/m^3$) even the most toxic of the OP pesticides would not be dangerous. The malathion results were considered likely to be typical of the whole group of pesticides, as they were all easily decomposed, most at temperatures well below their boiling points. From 90% to 99% of malathion, and 85% to 98% of parathion were decomposed before

Non- reactive compounds:

Ammonium orthophosphate $(NH_4)_3PO_4$

Phosphate esters $[CH_3(CH_2)_7O]_3-PO$

Halogenated phosphate esters 

Halogenated phosphonate esters $(CH_3CH_2O)_2-\overset{\overset{O}{\|}}{P}-CH_2CH_2Br$

Reactive compounds:

Phosphonate esters $H(OCH_2CH_2)_n\ O_2-\overset{\overset{O}{\|}}{P}-CH_2OH$

Phosphorus- containing polyols

$$\begin{array}{l} CH_2OOCR \\ | \\ CHOOCR \\ | \\ CH_2OOCR \end{array}$$

where R contains phosphorus

Figure 35.1 Examples of non-reactive and reactive phosphorus-containing fire-retardants

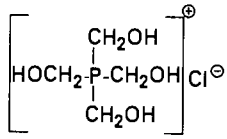
evaporation, and this would substantially reduce the amount escaping in fires. The use of a solvent with a boiling point substantially higher than the decomposition temperature of the solvent could drastically reduce the amount of pesticide which could be distilled out of a dilute solution. In general the authors suggest that the various factors reducing the concentration of pesticide in combustion products would result in their being little danger to firefighters outside a burning pesticide store, but that a serious hazard might occur inside a burning store, as pesticide concentrations as high as 10% were found in condensed vapours, and if even a 1% concentration were encountered, a fatal dose could be inhaled in a few minutes, or possibly absorbed through the skin. High concentrations of pesticides may also be liberated as pesticide-containing pool fires are extinguished.

This work provides a useful preliminary study of pesticide decomposition, but burning a few ml in a watch glass is hardly an adequate test for a pool fire of pesticides in bulk storage. It is important that larger scale fires, at least of several litres of solution, should be performed, and that the products should be dispersed into a known volume, so that realistic estimates of yield and atmospheric concentration can be made under thermal decomposition conditions representative of those known to exist in various types and stages of fire. The toxic potency of a complex mixture of products of unknown chemical identity cannot be measured without animal exposures. Acute (30- min or 4-h) exposures to decomposition products should be carried out using rodents for the detection of antiChE activity and chickens for the detection of delayed neurotoxicity [23]. Another problem is what happens when pesticides are used to treat natural materials, such as wood, which are then subsequently involved in either an accidental fire, or burned as fuel.

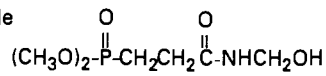
Combustion products of CBs

Neurotoxic CB residues are unlikely to occur in fires because typical CBs such as carbaryl and aldicarb are crystalline solids with low vapour pressures, so that they are unlikely to be easily volatilized. Also, the carbamic ester linkage is likely to be thermolabile, and without this there is no antiChE activity [19,21].

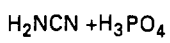
Tetrakis(hydroxymethyl)phosphonium chloride or hydroxide (THPC) and (THPOH)



N – methylol dimethyl phosphonopropionamide



Cyanamide - phosphoric acid



Tris(2,3– dibromopropyl) phosphate
(now shown to be a carcinogen, use discontinued)

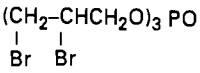


Figure 35.2 Phosphorus-containing fire-retardants used as textile finishes

Combustion products of phosphorus-based fire retardants

Phosphorus is used in many forms of fire retardants in large tonnages annually. It may be elemental (red phosphorus), inorganic (e.g. ammonium polyphosphate) or organic (e.g. phosphate esters) [20,27]. The problem is whether, when materials containing these substances are decomposed thermally, they might produce neurotoxic phosphorus esters in significant quantities in the thermal decomposition product atmosphere. In 1976 Liepins and Pearce [18] proposed that considerable toxicological problems may exist in the decomposition products of some flame retardants. This concern is not hypothetical, as demonstrated by the case of TMPP, but there is very little information on the chemistry and toxicology of the decomposition products from the majority of other phosphorus-containing fire retardants.

Major types of fire-retardants containing phosphorus

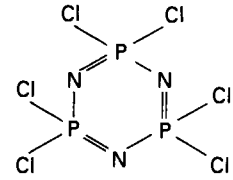
Phosphorus-containing fire retardants are non-reactive (fabric furnishes, surface coatings and fillers in resin) or reactive, combining with polymeric structure during processing. Figures 35.1–35.4 show typical examples taken from reviews [18,20,27]. These substances act in a number of different ways to impede combustion in both the sold and gas phases. The wide variety of chemical structures and reactions involved can therefore lead to a wide variety of phosphorus-containing products. In some cases the phosphorus may remain largely in the char (in intumescent coatings), in which case the formation of phosphorus-containing volatiles may be low, while in other cases (where the major reactions are in the gas phase), it is possible that a variety of potentially toxic phosphorus-containing products may be formed.

Toxicity of combustion products containing phosphorus-based fire retardants

Fire retardants reduce the efficiency of combustion, which can increase the yield of the normal toxic products. Also, the decomposing fire-retardant may itself, or in combination with other fire products, form toxic OPs. Unfortunately, acute combustion toxicity tests on rats and mice are not specifically designed to detect neurotoxicity, so that antiChE activity may be missed among the general narcotic and irritant effects of combustion product atmospheres. Also, the problem of potential delayed neurotoxicity is not covered because the young rodents used in

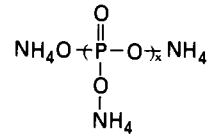
Non-intumescent coatings:

Phosphonitrilic chloride



Intumescent coatings:

Ammonium polyphosphate



Bicyclic phosphate intermediate formed with pentaerythrotol

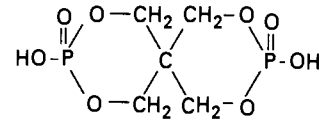


Figure 35.3 Examples of phosphorus-containing fire retardants used in coatings

Bis(2-chloroethyl) vinylphosphonate $CH_2{=}CH\text{-}\overset{\overset{O}{\|}}{P}\text{-}(OCH_2CH_2Cl)_2$

Figure 35.4 Example of a phosphorus-containing fire retardant used for copolymerization

these tests are usually unaffected, while delayed neurotoxicity does occur in humans, chickens and cats. Delayed neurotoxicity is caused by some OP flame retardants and plasticizers used in upholstery and wall coverings. A well-known example is TOCP [1]. A suitable animal model would be the hen, which develops ataxia accompanied by a distal axonopathy over a 21-day period following a single dose of TOCP [23].

Lhomme *et al.* [17] examined the effects of pyrolytic and oxidative thermal decomposition on trialkyl phosphates (trimethyl (TMP) and triethyl (TEP)) and also a tryaryl phosphate (triphenyl (TPP)). As with the OP insecticides, the trialkyl phosphates were thermally unstable, with scission of the C–O bond at 200–300°C. This yielded phosphorus pentoxide and various aliphatic products, mainly methane and ethane under pyrolysis conditions, and CO_2 with traces of aldehydes under oxidation. TPP was more thermally stable, decomposing only above 600°C, with scission of both the P–O and C–O bond. All phosphorus was recovered as phosphoric acid (resulting from hydrolysis of phosphorus pentoxide), with small amounts of red phosphorus being formed under pyrolysis. Although the authors did not analyse for OPs, which might have been present in small amounts, the work established that phosphate esters are easily destroyed by heat to release inorganic phosphorus oxides and acid. The main expected toxic hazard would therefore be from the irritant effects of inhaled phosphorus pentoxide (1 h LC_{50} of 1.217 mg/l [3]), adding to the general irritant effects of the smoke.

In these studies the OP compounds were decomposed alone. When fire retardants are added to materials the inorganic phosphate may be released to combine with other substances such as alcohols in the solid or vapour phase to form new phosphate esters, which may survive in the cooling smoke or char. An example of such a mechanism in the solid phase occurs in char formation in intumescent coatings containing ammonium polyphosphonate and pentaerythritol [27]. On heating,

ammonia and water are evolved with the formation at 250°C of a bicyclic phosphate (Figure 35.3), followed by char formation. This compound may be neurotoxic as is the caged bicyclic phosphate ester TMPP, or it might lead to the formation of TMPP in the vapour phase. However, when Wyman *et al.* [31] exposed rats to the thermal decomposition products of lubricants containing pentaerythritol and tricresyl phosphate, no signs of neurotoxicity were seen. Nevertheless, the formation of the above ester in the solid phase, and of TMPP in the vapour phase, clearly demonstrates that OP esters can be formed during the thermal decomposition of materials treated with phosphorus-based retardants. A potent lung oedemogen, phosphine (PH_3; 1 h LC_{50} 44 ppm [3]) has also been identified in thermal decomposition products from fire retardant materials [27].

Combustion toxicology studies on non-fire-retarded and fire-retarded polyurethane foams

Fire retardant (FR) additives are used in flexible polyurethane foams (FPUs), and normally give a greater yield of common toxic products when they burn [8,25], as well as any exotic products that might be formed. This is illustrated in Table 35.1, showing data from a thermoplastic polyurethane in an untreated form, and fire retarded form, decomposed using the German DIN method [25]. The non-FR sample burned cleanly, producing little CO, HCN and smoke (and therefore isocyanates), while for the FR material flaming was intermittent and inefficient, so that CO, HCN and irritant smoke yields were high, giving an approximately tenfold increase in potency from CO and HCN, and an approximate 20-fold increase in irritancy.

In a study using the NBS combustion toxicity test method two similar FPUs were tested, one containing a chlorinated phosphate so that it was cigarette and flame ignition resistant [8]. Under non-flaming conditions at 375–400°C the LC_{50} of the standard foam was 34 mg/l mass loss (i.e. when 34 mg of foam were decomposed into each litre of air) compared with 23 mg/l for the FR foaming, deaths occurring after exposure. Under flaming conditions (450°C) no deaths occurred at concentrations of up to 40 mg/l mass loss for the standard foam, while the LC_{50} of the FR foam was 27 mg/l mass loss. The increased toxicity was partly the result of a threefold increase in HCN yield, and doubled CO yield, which caused deaths during exposure, but because the majority of deaths occurred after exposure, it is likely that the main cause of death was lung irritancy from isocyanates and other pyrolysis products escaping the flame zone, or from some other factor related to the OP fire retardant, as under non-flaming conditions. These results agree with those from the DIN furnace, showing that under non-flaming and early flaming conditions, the toxic potency of FR materials can be greater than non-FR materials owing to increased yields of toxic products such as CO, HCN and isocyanates. While there may be a contribution to the toxicity from OPs, in general, the results obtained from acute experiments on flexible polyurethane foams do not suggest that OPs form the major toxic atmosphere components, although antiChE activity and delayed neurotoxicity have not been tested.

Combustion toxicology studies on non-fire-retardant and fire-retarded polyesters and cotton

The polyester material poly(ethylene terephthalate) is most frequently used in the manufacture of fibres and films. FR polyester materials were approximately twice as toxic as non-FR polyester materials when tested using the DIN tube furnace method and other methods [7]. When 100% cotton and 50/50%

Table 35.1 Composition of test atmospheres from flaming samples of thermoplastic polyurethane (600°C, 8 mg/l mass charge)

	CO (ppm)	*CO_2 (ppm)*	*HCN (ppm)*	*Smoke (OD/m)*	*Irritancy (RD_{50} mg/l)*
Normal	350	13000	11	0.07	c. 4
FR version	4200	6000	77	1.23	c. 0.2

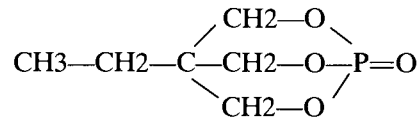
cotton/polyester fabrics were tested, some of which were treated with tetrakis(hydroxymethyl)phosphonium hydroxide (THPOH), the retardant caused a decrease in LC_{50} of from 3 to 9 times.

Kallonen *et al.* [12] tested a range of FR and non-FR fabrics using the DIN method at 500°C and 700°C, using 30-min animal exposures at 18 mg/l mass charge (18 mg of material entering the furnace per litre of diluent air). The phosphorus-containing fire retardants used were tetrakis(hydroxymethyl)phosphonium chloride and urea concentrate (Proban®, Albright and Wilson) and N-methylol-dimethyl-3-phosphonopropionamide (Pyrovatex CP®, Ciba-Geigy). From the results of this study, summarized in Table 35.2, the majority of rats survived the immediate exposure to cotton under both non-flaming and flaming conditions, in which CO levels were relatively low. However, under non-flaming conditions there was a 42% mortality rate over the following 14 days, presumably from pulmonary irritation, while under flaming conditions (where atmospheres are normally less irritant) there were no late deaths. The FR-cotton failed to flame and the yields of CO and HCN were higher than those obtained from cotton alone, which for Pyrovatex/cotton, may explain the few deaths occurring during exposure. For Proban/cotton the pattern of toxicity is less obvious. During decomposition at 500°C, four of the rats died with only 1% COHb despite a CO concentration of 3100 ppm, indicating that they died at a very early stage of the exposure for some reason, before they could take up any CO. Similarly at 700°C, 83% of rats died, with a normally sublethal average COHb concentration (44%) compared with fewer deaths at a higher COHb concentration (74%) for the Pyrovatex-treated cotton at almost identical CO and HCN concentrations. It therefore seems possible that at both decomposition temperatures some agent in the combustion products from Proban-treated cotton caused deaths during exposure in an unexplained way.

The general findings from these experiments on FR-treated fabrics is similar to those for the foams, in that FR-treatments, by reducing combustion efficiency, generally give increased yields of common toxic products and therefore some increase in toxic potency, but no clear evidence for unusual toxicity or exotic phosphorus-containing products.

Formation of extreme potency neurotoxic caged bicyclophosphorus esters in combustion products

The formation of a neurotoxic OP product in combustion products from a material treated with a phosphorus-containing fire retardant was first reported in 1975 by Petajan *et al.* [22]. The substance was found to have an extreme toxic potency and rapid action. It was identified as trimethylol propane phosphate (TMPP): 4-ethyl-1-phospha-2,6,7- trioxabicyclo (2.2.2) octane-1-oxide):



The toxic effect was discovered when rats were exposed to thermal decomposition products from a rigid polyurethane foam. Polyurethane

Table 35.2 Toxicity of the combustion products from FR and non-FR cotton[c]

Material	*Flaming (±)*	*CO (ppm)*	*CO_2 (%)*	*HCN (ppm)*	*Mortality (%)*	*COHb%*	
						Alive	*Dead*
At 500°C[b]							
Cotton	–	2400	0.85	0	42	40(12)[a]	
Cotton and Proban	–	3100	0.8	50	33	25(8)	1(4)
Cotton and Pyrovatex	–	3100	0.6	35	25	18(9)	42(3)
At 700°C							
Cotton	+	2500	1.2	5	8	41(22)	
Cotton and Proban	–	5400	1.4	50	83		44(10)
Cotton and Pyrovatex	–	5700	1.1	30	38		74(10)

[a]Number of rats used for carboxyhaemoglobin measurements in parentheses
[b]At 500°C, 12 rats were exposed on one occasion; at 700°C two experiments were carried out, each on 12 rats
[c]Data from Kallonen *et al.* [12]

Table 35.3 LD_{50}s of tricyclophosphate esters

Alkyl side group	CH_3	C_2H_5	C_3H_4 n	C_3H_4 iso	C_4H_9	$HOCH_2$
LD_{50} (mg/kg ip)	32	1.0	0.38	0.18	1.5	>500

Table 35.4 Toxicity of trimethyl propane phosphate and phosphite

	Rat LC_{50} mg/l (1-h exposure)	
	Phosphate	*Phosphite*
Male	0.037 (0.033–0.040)	0.015 (0.013–0.017)
Female	0.030 (0.027–0.034)	0.015 (0.013–0.017)

[a]Data from Kimmerle *et al.* [14]

foams consist of an isocyanate and a long chain polyol, which react together by the formation of urethane groups to form the polymer. In this case a rigid foam was formulated from a propoxylated trimethylolpropane polyol (MW 340) and polymethylene polyphenyl isocyanate. This was tested alone and with the addition of 0,0-diethyl N,N-bis(2-hydroxymethyl)aminomethylphosphonate. Exposures to products from the untreated foam caused no toxic effects, but rats exposed to foam containing 4% retardant showed focal seizures immediately after exposure which developed to grand mal seizures after 43– 70 min. Foam containing 8% fire retardant caused myoclonic jerks in the rats, progressing to status epilepticus and death. Hyperexcitability was demonstrated by the presence of very large evoked spikes in response to light flash, but there were no parasympathetic signs.

Thermal decomposition of the foam released the propoxylated trimethylol propane polyol adduct, which decomposed to form trimethylol propane. This combined with reactive phosphorus species from the retardant in the smoke to form principally TMPP. In addition to TMPP, which has an ethyl group in the 4 position, the 4-methyl homologue and other, unidentified, phosphorus-containing products were demonstrated during the combustion of the polyurethane foam [28].

TMPP is a member of a class of extremely neurotoxic compounds, the bicyclophosphorus esters (BCPEs). Their effect was first identified by Gage [11]. The structure-activity relationships of the group has been extensively characterized [4,9,14] and the mechanism of action determined [6]. The basic mechanism of action is antagonism of γ-aminobutyric acid (GABA) transmitter action by allosteric binding to GABA receptors. The convulsant effect is therefore not from potentiation of ACh through AChE inhibition as with the classic OP pesticides, but from blocking of the inhibitory control produced by GABA-mediated inhibition. The convulsive activity is not accompanied by signs of parasympathetic overstimulation or paralysis, and there is no inhibition of AChE. Barbiturates seem to be effective antidotes, as rats given 25 or 50 mg/kg pentobarbitone ip after oral LD_{50} doses of BCPEs suffered reduced convulsions and survived [14].

The magnitude of stearic effects on activity of bicyclophosphate esters is demonstrated by the range of LD_{50}s of compounds with different 4- alkyl side groups (Table 35.3) [4]. These data compare with an LD_{50} for parathion of 5.9 mg/l and 6 mg/l for DFP. The acute inhalation toxicities of the trimethylol propane phosphate and phosphite (where R is ethyl) to rats were examined by Kimmerle *et al.* [14], using a 1-h exposure followed by 14 days observation. The compounds were administered as an aerosol in a mixture of ethanol and ethylene glycol. The results are shown in Table 35.4.

Materials evolving TMPP in fires and likely toxic hazard

Woolley and Fardell [29] studied the yields of TMPP during thermal decomposition of various types of flexible (FPU) and rigid polyurethane foams (RPU). TMPP was stable at temperatures of up to 600°C in air. When six flexible and 20 rigid foams were decomposed in a tube furnace at 500°C under nitrogen, they fell into two categories with respect to TMPP yield. TMPP yields were insignificant for FPUs and some RPUs, while other RPUs containing trimethylol propane polyols (no longer manufactured) gave significant yields. The maximum TMPP yields of approximately 0.2% by mass occurred at 500°C when the foam was decomposed under non-flaming conditions in

air. Under these general decomposition conditions rat LC_{50}s for 30-min exposures to products from RPUs are between 6 and 20 mg/l mass loss, containing toxic concentrations of CO and HCN [5,15,16]. If TMPP was evolved from equivalent masses of foam at a yield of 0.2%, then the concentration would be approximately 0.012–0.06 mg/l. Because Kimmerle *et al.* [14] found a 1-h LC_{50} of 0.03–0.07 mg/l for TMPP, assuming Haber's rule, then the concentration of phosphate required for fatalities from a 30-min exposure would be 0.06–0.14 mg/l. If an RPU foam evolved TMPP at yields similar to those obtained by Woolley and Fardell, it could make a major contribution to the toxic effects of the combustion products, although additional toxic effects from CO and HCN would also be significant. Under flaming conditions CO, HCN and TMPP yields might be reduced, and evidence for this was obtained by Wright and Adams [30] in tests on FR foams based on trimethylol propane polyols. The foams contained 16% by mass of fire retardants, and the range of retardants tested included a reactive phosphonate (diethyl bis(2-hydroxyethyl)aminomethylphosphonate), a chlorinated phosphonate (bis(2-chloroethyl) (1-hydroxyethyl)phosphonate plus two similar compounds) and ammonium phosphate. Under non-flaming conditions atmospheres were lethal, but not when the sample flamed.

Formation of TMPP in heated lubricants

Polyester compounds of neopentyl polyols, including trimethylol propane esters, are in common usage as base oil stocks for hydraulic fluids, lubricants, and in particular aircraft engine lubricants, especially in US Government specification MIL-L-23699 turbo oils [13,31]. Tricresylphosphate and other phosphate esters are commonly added in the same synthetic oil blends as antiwear additives and to prevent interaction of the lubricant with engine surfaces. The formulations are principally composed of a polyol ester base stock of either trimethylol propane or pentaerythritol, or combinations or both.

TMPP or its analogues might therefore be formed when these lubricants are overheated, which may be dangerous in aircraft engines where a proportion of the air from the engines is used in cockpit or cabin ventilation. Kalman *et al.* [13] heated a lubricant in a tube furnace in a flow of air, or in a sealed tube, obtaining up to 0.7 mg/g oil from 5 min open heating at 645°C and up to 9 mg/g (38–63% of phosphorus as TMPP) after 30 min heating under sealed conditions. Wyman *et al.* [31] decomposed oils under open and sealed conditions in the same way, and then administered samples to rats by ip injection (up to 15 ml/kg bodyweight). Pyrolysis for 20 min at 500°C gave a TMPP yield of 14.3 mg/ml lubricant (approximately 50% of theoretical maximum from a 2.8% phosphorus content). Under open-air heating conditions the yields of TMPP were up to 7.7 mg/ml above 650°C. Convulsions and death occurred when animals were dosed with pyrolyzed oil shown to contain TMPP (detection limit 0.5 mg/kg). Twelve commercial lubricants were tested which contained polyol esters based either on pentaerythritol or trimethylol propane, in combination with triaryl or tricresyl phosphates. The trimethylol propane lubricants caused rapid convulsions and death, while the pentaerythritol lubricants produced no neurotoxic effects. BCPEs were not formed during normal use in aircraft engines.

As well as demonstrating the hazards associated with overheating of materials capable of forming trimethylol propane polyols in the presence of phosphates, the results with TMPP demonstrate that organic and inorganic phosphates can evolve products on heating capable of forming OP esters not present in the original material, which under appropriate conditions can account for a high percentage of the available phosphorus (50% or more). This highly toxic product was discovered only because seizures were observed in an animal combustion toxicity test, and only then was the chemical identity of the toxicant investigated and discovered. Although it is likely that in most fires involving other materials, the majority of available phosphorus is evolved as phosphorus pentoxide, it is also likely that, as with TMPP, small amounts of some phosphate esters will be formed in the smoke. Unless careful observations are made for signs of parasympathetic effects, with measurements of ChE activity, and unless special tests are performed for delayed neurotoxicity, then potentially hazardous toxic effects may not be detected.

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General considerations on workplace safety for anticholinesterases

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Introduction

The commonly used OPs and CBs, their chemical and product names and ranking according to toxicity are listed by the US EPA in a publication intended for use in recognizing and managing pesticide poisonings [18]. They are listed in descending order of toxicity: highly toxic is an LD_{50} (rat) <50 mg/kg and moderately toxic an LD_{50} > 50 mg/kg. TEPP is ranked as the most toxic OP and temephos is bottom of the moderately toxic list. Aldicarb heads the list of highly toxic CBs and carbaryl is bottom of the moderately toxic list.

In this chapter the occupational health aspects of AChEs will be discussed with particular emphasis on the types of work involved in their manufacture and use and the potential sources of exposure.

Workplace and sources of exposure

Defining the workplace with respect to antiChEs is difficult in the conventional sense. The problem arises in identifying the various groups who subsequently handle antiChE pesticides as they pass from the factory to use in agriculture and as agents in pest control. Their application is such that an indirect exposure risk may be present for the population in the vicinity of operations using pesticides.

In the manufacture of antiChE pesticides, exposure of factory workers is probably negligible as processes are largely carried out in closed reactors. Mixing and formulation may not be as easily controlled, and packaging, transporting, warehousing and distribution are likely sources of exposure to workers. Workers who repair, maintain and clean the vessels in which the products are manufactured are at constant risk of being exposed.

Occupational exposure to the chemicals from which antiChEs are manufactured is an important consideration in evaluating potential health hazards in the workplace. Potential for exposure exists in handling the raw materials which may be delivered by pipeline, rail, ship or barge, or in tank-trucks. Methyl isocyanate (MIC), used in the manufacture of aldicarb, is one of the more hazardous materials to which workers may be exposed, and its effect on the population of Bhopal is said to have been the worst industrial accident ever recorded, some 2000 lives having been lost as the result of exposure to MIC. Many interconnected production units of a single plant may be involved in pesticide production, including reaction, distillation, filtration and mixing.

Formulation of pesticides is often done in small plants, involving engineers, chemists, operators and labourers. According to Ebert *et al.* [16] 71% of all formulating establishments (presumably in the USA) employ less than 20 people and account for only 12.5% of all production; only 6% of all formulating plants employ more than 100 people, but they account for 56% of production.

Formulators dilute concentrated active ingredients using various non-pesticide materials (inerts); although many are inert as far as pesticidal activity is concerned they may be potent human toxicants. The formulating process produces a product of desired physical form for its application, e.g. dusts, powders, wettable powder, granules, pellets, emulsifiable concentrates, capsules or aerosols. Inerts used in the formulation of pesticides include

flour, silica, sulphur, lime, gypsum, talc or clays, and solvents such as diesel oil, paraffin (kerosene), xylene, petrol (gasoline), and wetting or dispersing agents. Workers require to be protected not only from the antiChE but also from the chemicals used in its formulation.

The total labour force engaged in agricultural work in the USA, as their main source of income, has been estimated at 4–5 million [13]. Some of these are at high risk for pesticide-related illness. The job categories include mixer-loader applicators (ground applicators), pilots (aerial applicators), flaggers, applicators and operators in greenhouses, field hands, tractor drivers, pickers on citrus groves, harvesters of leaf vegetables and field inspectors. Pesticide exposure of communities can result from the ingestion of residues in sprayed fruits and vegetables [48]. Children are particularly at risk from ingestion of carelessly stored materials in their homes. In a series of 37 infants and children hospitalized at the Childrens' Medical Center in Dallas during June 1975 to November 1986, with the diagnosis of OP or CB toxicity, virtually all poisonings occurred within an urban setting and, except for one child who ingested insecticide granules while in a feed store, all occurred in the home [37,52]. Risk factors related to spraying and re-entry have been reviewed [27,37,42]. Marked regional and climatic differences have been observed.

In 1974 the US EPA promulated regulations requiring specific re-entry intervals for 12 pesticides and a general re-entry interval for all agricultural pesticides prohibiting re-entry to the fields until sprays had dried or dust had settled [17]. California is the only state to require longer intervals than EPA, which may range from 5 to 30 days [37]. California's re-entry intervals, first established in 1971, varied according to the nature of the crop and the pesticide; for parathion on citrus crops the safety interval was 30 days, while on apples it was 14 days. These were not soundly based because of a lack of pertinent information on safe residue levels and poor data on decay rates in the environment. Spear [42] summarizes the important developments which advanced the understanding of the exposure and residue decay process as: (1) methods for measuring foliar residue levels and studying soil surface residues relevant to worker exposure between 1973 and 1975; (2) demonstration that residue sampling methods are good predictors of exposure; (3) findings that leaf surface residues differ in decay patterns from residues absorbed into the leaf, and agents on leaf and soil surface may be converted more rapidly to their more toxic oxygen analogues; and (4) demonstration that field workers' clothing became contaminated as a result of the dislodgement of foliar residues, which then got on to skin surfaces. The observed response was almost totally the result of exposure to the oxygen analogue.

Definitive human epidemiological data to enable the establishment of safe re-entry standards based on sound scientifically valid, dose-response calculations is absent. According to Spear [42], there are at least two problems: (1) the presence of multicomponent residues on foliage and soil surfaces needs to be better characterized; and (2) persistence of the parent compound and its oxygen analogue is affected by certain clays in the foliar dust, and by clay adjuvants in the pesticide formulation being influenced by natural elements.

The US EPA proposes to require pesticide safety training for all persons who handle agricultural pesticides or who engage in early re-entry activities [19]. All workers will have to be 'clearly and adequately' notified of all pesticide applications and resulting re-entry intervals. Warning notices will have to be posted in treated areas for some pesticides. Re-entry intervals will be established for all pesticides used on agricultural sites, based on acute toxicity and chemical class of the active ingredient(s).

Routes of exposure

Skin absorption

Skin contact and subsequent absorption is the major route of exposure in pesticide workers. The degree of absorption depends on the contact time with the skin and the presence of solvents and emulsifiers which can facilitate absorption. Other important factors include volatility, permeability of the clothing, extent of coverage of the body surface, and personal hygiene. Absorption through the skin is more efficient for the lipophilic agents. The rate of

absorption varies with skin region affected, e.g. for parathion it is much higher from scrotal skin, axillae, and skin of the head and neck than it is for the hands and arms [34]. Previously injured skin surfaces may have an increased rate of absorption. For powders, the finer the powder the more efficient the skin absorption.

Inhalation

Inhalation of dusts, vapours, mists and gases may occur. Knaak *et al.* [28] describe equipment and techniques employed in the mixing-loading and application of antiChE pesticides as used in two Californian counties in 1976. The following are important excerpts from their publication:

> In January of 1974, the California Department of Food and Agriculture adopted regulations that required the use of closed-transfer systems by employees mixing and loading liquid pesticides in Toxicity Category I and prevented the mixing and loading by employed pilots of Toxicity Categories I and II unless closed-transfer systems were used ... The regulation preventing pilots from mixing and loading went into effect in January of 1974 while the closed- transfer requirement was not put into effect for pest control operators until April 1977.
>
> Mixer-loaders diluted concentrated liquid pesticides in a mix tank with water and transferred the mixture to spray tanks mounted in or on aircraft. This was accomplished, in most cases, by hand pouring one or more liquid pesticides from their original container into a mix tank mounted on a trailer. The pesticides were mixed and diluted with water from a nurse tank. The spray was then transferred to an aircraft tank using a centrifugal pump and rubber hose. A quick disconnect device was used at the end of the hose to connect to the aircraft. The device prevented the spray mixture remaining in the hose from running out onto the ground or onto the mixer-loader after the transfer was made and the hose disconnected. In some cases, the pesticides were only partially diluted in the mix tank before being transferred to the spray tank. The final dilution was carried out in the spray tank. Pesticides in water-soluble bags, or as free powders, were added by hand to the mix tank, dissolved, diluted and transferred to the spray tank. The hand pouring of liquid pesticides makes this job a hazardous one, because concentrated pesticides are often spilled by the worker during the transfer operation.
>
> Mixer-loader applicators, in most cases, hand poured liquid pesticides from their original containers into tractor-mounted spray tanks, mixed and diluted the pesticides with water. If mix tanks were used, pumps and rubber hoses were used to transfer the pesticide mixture from the mix tank to the spray tank. Tanks were usually filled through an open lid in the top of the tank. Powders were added directly to the mix or spray tank. These workers were exposed to the spray while operating the tractor in addition to the exposure they received while hand pouring the pesticides.
>
> Flaggers assisted the pilots in the aerial application of pesticides by flagging the position where the next application was to be made ... Pilots applied pesticides with fixed and rotary wing aircraft. Pilots did not mix or load pesticides. Flaggers were exposed to spray during the application of the pesticides. Pilots were exposed to sprays or vapours which remained suspended in the air while they were spraying.

The toxicity of OPs and CBs by inhalation depends on their physical and chemical properties (*see* Ch.29). AGGIH Documentation is summarized for the 22 OPs and three CBs in Table 36.1. The AGGIH does not have TLVs for dicrotophos, dioxathion, EPN or sulfotep.

Ingestion

Ingestion is not common in the workplace but can occur in workers with poor personal hygiene, e.g. if they do not remove contaminated clothing, fail to wash their hands, or if they keep food in close proximity to antiChEs.

Ingestion of food from crops with OP residues may occur. The amount of pesticide residue on the crop at harvesting depends on weather conditions and time since the pesticide

Table 36.1 Summary of information on antiChEs abstracted from ACGIH Documentation [2]

Pesticide	*Exposure by inhalation*	*TLV (TWA_8)*
Carbaryl	The difference between a single acute dose producing typical poisoning in dogs and the dose tolerated repeatedly without ill effect is very small. Rats inhaled micronized carbaryl at a concentration of 10 mg/m³ for 7 h per day for 90 days without grossly visible injury. A human in such an atmosphere would inhale about 100 mg per day	5.00 mg/m³
Carbofuran	Inhalation toxicity is low	0.10 mg/m³
Methomyl	LC_{50} of unformulated compound (as mist) is 0.30 mg/l (4-h exposure). When the LC_{50} is divided by TLV 2.5 mg/m³ a safety factor of 120 results. (0.3 mg/l × 1000)/2.5 mg/m³	2.5 mg/m³
Chlorpyrifos	Vapour pressure insufficient to be a vapour hazard, but inhalation of particles is	0.20 mg/m³
Demeton	Similar to parathion	0.01 ppm
Demeton-methyl	—	0.50 mg/m³
Diazinon	—	0.10 mg/m³
Dichlorvos	Readily absorbed on surfaces and hydrolyzed by moisture. In animals: difficulty reaching lethal concentrations in air. A wide margin in humans between concentrations affecting plasma ChE and those causing illness	0.1 ppm
Ethion	LC_{50} 710 mg/m³ in female rats and 7200 mg/m³ for male rats per h	0.40 mg/m³
Fenamiphos	No inhalational toxicity data	0.10 mg/m³
Fensulfothion	—	0.10 mg/m³
Fenthion	Quickly absorbed in the lung	0.20 mg/m³
Fonofos	No inhalational toxicity data	0.10 mg/m³
Malathion	Low toxicity by inhalation	10.00 mg/m³
Mevinphos	Absorbed by inhalation	0.01 ppm
Naled	Not highly toxic	3.00 mg/m³
Parathion	Greater depression of ChE from inhalation than from skin absorption	0.10 mg/m³
Parathion-methyl	—	0.20 mg/m³
Sulprofos	No human data available	1.00 mg/m³
TEPP	—	0.05 mg/m³
Temephos	—	10.00 mg/m³

was applied. In very hot, dry conditions residues may be found for as long as 28 days after application. In one instance several pickers were poisoned by parathion when working in an orange grove after this length of time. This would have been considered a safe re-entry level under other circumstances. Direct poisoning of consumers of sprayed food is possible when conditions favour high residues.

The main routes of exposure to CBs are by inhalation and skin absorption. Ingestion usually results from careless storage of the product. The same possibilities for occupational exposure exist as with OPs.

The largest reported incident in the USA of illness caused by foodborne pesticide was with aldicarb-contaminated water melons in Oregon; 264 reports of illness were received and 61 definite cases were identified. Residues of aldicarb were found in the melons eaten by the defined cases (*see* Ch. 42). Another example of human illness due to aldicarb

contamination of foodstuff was reported in cucumbers grown hydroponically [19].

Medical surveillance of persons potentially exposed to AChEs

Monitoring acute effects

The US National Institute of Occupational Safety and Health (NIOSH) in its publication, *A Recommended Standard for Occupational Exposure to Carbaryl* (DHEW (NIOSH) 1977), includes the following as elements in the medical surveillance of workers:

(1) An initial or periodic occupational history.
(2) A comprehensive medical history especially regarding: frequent headaches, dizziness, chest tightness, dimness of vision, and difficulty in focusing eyes.
(3) A physical examination including the cardiorespiratory system, CNS, vision (measure intraocular tension) and kidneys.
(4) A complete urinalysis including microscopic examination.
(5) A pre-exposure baseline RBC AChE activity determination.

Measurement of whole blood AChE is the most widely adopted method for monitoring the effects of occupational exposure to OPs and CBs. Blood ChE levels vary from person to person and for the same person at different times. According to the WHO [47] the coefficient of variation for AChE activity in samples from an individual is 8–11%, and a decrease of 23% below the pre- exposure level may be considered significant. It also states that 'if the average of several pre-exposure values were available, then a decrease of 17% would be significant'. The following surveillance protocol is recommended:

(1) If AChE activity is reduced 30% or more of the pre-exposure level, repeat the test at appropriate intervals to confirm level.
(2) Depressions of 20–25% are diagnostic of exposure but not of hazard.
(3) Depressions of 30–50% are an indication for removal from further contact with pesticides until ChE levels return to normal.

The objective of a preplacement medical examination is to determine whether a job applicant is suitable for work in manufacturing, handling or using antiChEs. A clinical baseline is established for periodic monitoring. Additional special tests and procedures carried out at this time include: (1) complete blood count, including differential white cell count, RBC indices, haemoglobin concentration and haematocrit; and (2) liver function tests (including enzymes).

In determining whether applicants are suitable for employment involving exposure to antiChEs the physician must consider whether they can wear protective clothing and devices (respirators), if there is a history of allergies or asthma which could be aggravated by the ingredients of the compounds to be used, and whether the applicant's level of intelligence and understanding of the language are such that they will heed warning labels and not endanger themselves or others.

Potentially exposed workers are kept under periodic medical surveillance with the intention of detecting adverse health effects at the earliest possible stage by comparison with the baseline values established at the preplacement examination. The periodicity of the examination and ancillary tests will depend on factors such as age, sex, frequency of exposure, length of exposure and exposure levels. As a general rule, the following routine may be considered: (1) a medical history questionnaire completed annually, (2) physical examination if indicated by the response to the questionnaire, and (3) unless otherwise indicated RBC AChE is repeated at least annually.

The US EPA has proposed revising its regulations protecting workers from agricultural pesticides (40 CFR Part 156 & 170). Monitoring of blood ChE of commercial pesticide handlers only is to be done, because they are at greatest risk from the acute effects of antiChEs as they are exposed to the pesticides rather than to residues. ChE monitoring would be based on the frequency of handling Toxicity Category I or II OPs.

The US EPA proposes that both OPs and CBs be labelled so that users would be aware of possible ChE inhibition from their use. The State of California undertook an evaluation of its ChE monitoring programme and found that OPs in Toxicity Category I cause more poisoning incidents in that state than other products.

WHO [48] state that a drop of 30% in ChE

from the baseline level 'has recently been endorsed as a recommended biological threshold level for withdrawal of a worker from exposure and for implementing other preventive actions'. They indicate that this threshold has not been substantiated by existing medical or epidemiological data, and may have been influenced by the accuracy of the test methods available at the time.

Plasma BChE may be depressed in early hepatitis, in alcoholic cirrhosis and in other liver diseases as well as by various drugs. It is inhibited by OPs more rapidly and earlier than RBC AChE. However, RBC AChE is considered a better functional index of inhibition, but both plasma and RBC determinations are advisable when feasible. Baseline blood samples should be taken when the worker has not been exposed to OPs or CBs for at least 30 days. Establishing a good baseline value requires a minimum of two pre-exposure tests performed at least 3 days, but not more than 14 days, apart [11]. If they differ by as much as 20%, a third sample should be drawn and tested. The individual's baseline is obtained by averaging the pre-exposure test results. Exposure must cease when the decrease from baseline for RBC AChE and plasma BChE is 30 and 50% respectively [29]. Usually, an acute exposure will depress the plasma BChE before RBC AChE; plasma levels usually recover first.

Workers with a history of glaucoma, cardiovascular disease, hepatic disease, renal disease, CNS abnormalities and those using anticholinergic drugs should be warned that CBs or OPs could aggravate their condition.

EPA in its proposed rules [19] states that:

> Measurement of the level of the enzyme cholinesterase in the blood has been demonstrated to be a satisfactory biological index of excessive organophosphate exposure ...

and continues:

> Cholinesterase monitoring would accomplish a twofold purpose: (1) it would detect significant organophosphate pesticide exposure that would warrant worker removal from exposure, and (2) it would serve as a surveillance mechanism to identify workplace situations which require modification to minimize exposure to organophosphate pesticides.

Although CBs inhibit ChEs, the plasma and RBC activities are frequently not useful indicators because of the rapid reactivation of carbamylated ChEs. Activities return to normal within minutes or hours after CB exposure. Moreover, because toxicity may occur, labels for OPs and CBs should carry a warning about possible ChE inhibition resulting from their use.

The exposure level which should trigger ChE monitoring has not yet been adequately defined. California requires any worker exposed for 30 h in a 30-day period to receive such monitoring. This requires meticulous work records. The US EPA has proposed a day-based trigger (3 consecutive days or any 6 days in a 21-day period) because it is easier to identify such workers. US EPA rationalizes that 'this trigger excludes handlers receiving organophosphate exposure because cholinesterase levels regenerate at a rate of approximately 1% per day and are less likely to reach dangerously low levels with less frequent exposure'.

US EPA proposes that the employer be required to engage a licenced physician to supervise ChE monitoring. The physician will be required to use US EPA's ChE monitoring guidelines (or equivalent guidelines); advise when decreased ChE indicates that work practices need modification; advise the employer when the worker should be removed from further exposure, and when sufficient regeneration of ChE permits return to work. The employer will have to maintain monitoring records for at least 2 years. The EPA guidelines will cover test methods, baseline testing, frequency of testing after exposure begins, and decreases in plasma and RBC ChE levels for which work practices should be investigated and those which should be taken into consideration as an indication for worker removal from exposure.

In addition to ChE monitoring, certain OPs may be detected by measurement of their metabolites in urine (*see* Ch.39). Alkyl phosphate metabolites include dimethyl phosphate (metabolite of dimethylparathion), diethyl phosphate (parathion, disulfoton and phorate), O,S-diethyl phosphorothioate (disulfoton and phorate), and diethyl phosphate (disulfoton and phorate) [10]. Increased excretion of these alkyl phosphates may be found when OP exposure has been insufficient to lower plasma

or RBC ChE. Measurement of urinary p-nitrophenol is relatively specific for parathion exposure; approximately 8–9 h is required from first exposure to peak excretion [21]. It is also a metabolite of parathion-methyl and EPN. Concentrations in the general population are 0.01–0.03 mg/l [7]. Parathion produces no symptoms and little or no reduction in ChE as long as urinary p-nitrophenol does not rise above 2.0 mg/l [21]. A major value of urinary metabolite measurement is that it is useful to detect recent exposure.

OPs of differing toxicity may have identical metabolites in urine, but may be associated with differing RBC AChE activities. For example, parathion-methyl is about 40 times more acutely toxic than fenitrothion, and urinary metabolites will be present at much lower concentration levels when AChE is depressed 50% following exposure to parathion-methyl than following fenitrothion [47].

Urinary metabolites may also be a practical means of assessing the severity of exposure to CBs. Free and conjugated 1-naphthol are the main urinary metabolites of carbaryl [29]. In healthy unexposed subjects, urinary 1-naphthol concentration is $\leq$0.3 mg/l, and when $\geq$4.0 mg/l it represents significant exposure to carbaryl [7]. According to Lauwerys [29] insufficient human data exist for the proposal of meaningful biological monitoring methods for the majority of carbamate insecticides.

Monitoring delayed neurotoxicity

Some OPs, with a single large dose, can cause a delayed neurotoxicity of a central-peripheral distal axonopathy type with secondary demyelination [14] (*see* Ch.10]. There is always a delay of 2–4 weeks in humans between exposure and appearance of signs and symptoms. This neuropathy is from phosphorylation of NTE (*see* Ch.10). Triaryl phosphate esters, such as those used in hydraulic fluids, do not have any antiChE activity but are most commonly associated with delayed neuropathy in humans [47]. OPs reported to cause delayed neuropathy in humans include mipafox [9], leptophos [51], methamidophos [40], trichlorfon [22,26,41], trichloronat [25,49], EPN [50] and chlorpyrifos [32]. It has been shown experimentally that lymphocytes have a low level of NTE activity which in some circumstances correlates with the neurotoxic dose. The possibility of adapting this to the monitoring of exposed individuals by means of the estimation of human lymphocyte or platelet NTE activity is under investigation [8,33,35,47].

Electromyography (EMG) may be used to detect indications of exposure to OPs. It is a non-invasive technique and, when surface electrodes are used, it has been claimed to be a sensitive test, giving indications of exposure even when blood ChE activity has returned to normal [24,38]. However, specialized equipment and skills are required, the validity of published studies has not been confirmed, the reproducibility varies with many extraneous factors and the changes reported tend not to be dose-related [31,47]. EMG is considered not to be a highly sensitive measure of OP exposure [47].

Alcohol is a notorious cause of neurotoxic damage, and problem drinkers with neurobehavioural impairments may not be recognized at pre-placement medical evaluation; this could subsequently be erroneously attributed to OP exposure. Gamma glutamyl transferase (GGT), mean corpuscular volume, and the use of the CAGE questionnaire can jointly be a sensitive battery for screening problem cases [48].

Other considerations in medical monitoring

Behavioural effects

In some persons with clinical evidence of OP poisoning, behavioural and psychological effects have been recognized which are said to take 'several months' to regress [47]. However, few controlled epidemiological studies in humans have been reported. Levin and Rodnitzky [30] found that when OP exposure had been sufficient to depress plasma or RBC ChEs, some or all of the following variables might be impaired: (1) cognition: vigilance, information processing and psychomotor speed, and memory; (2) speech: both performance and perception; (3) psychic state: increased tendencies to depression, anxiety and irritability; and (4) EEG records: tendency to faster frequencies and higher voltages. They also concluded that the EEG abnormalities were positively related to the level of AChE inhibition during the initial stages of inhibition.

They also considered the evidence for the presence of less severe or latent forms of any behavioural effects in workers at risk from repeated exposure to OP, and found it to be equivocal. Savage *et al.* [39] studied 100 matched pairs of individuals with previous acute OP poisoning and non-poisoned controls, and found differences in neuropsychological tests to be much more apparent between the cases and controls. They believe that there are clear chronic neurological sequelae to acute OP poisoning, and suggest that the sequelae are 'sufficiently subtle that the clinical neurological examination, clinical EEG, and ancillary laboratory testing cannot discriminate poisoned subjects from controls'. They suggest that both neurological and neuropsychological methods are needed in a patient who has been poisoned by OPs.

WHO [47] outlines methods to be used in studying central and peripheral neurological and neurobehavioural effects for epidemiological purposes, which can be adapted for medical surveillance, if indicated.

Effects on other organs

According to the NIOSH document on carbaryl, possible effects on the reproductive system warrant consideration based on information from experimental animal studies which indicate possible effects on the developing fetus, as well as on other reproductive processes in both men and women. Female workers should be advised to minimize exposure to carbaryl during pregnancy. Nursing mothers, who may be exposed to carbaryl, should be aware of the possibility that the infant may be fed breast milk containing the compound and that precautions should be taken to avoid unnecessary exposure.

The International Programme on Chemical Safety publication on organophosphorus insecticides [47] lists the following as having been evaluated for carcinogenicity by the International Agency for Research in Cancer (IARC):

(1) Dichlorvos. EPA originally classified it as a probable human carcinogen (Category B2) based on carcinogenicity in male rats and female mice. This was reviewed by the EPA Scientific Advisory Panel (SAP) and classified as a Category C oncogen (possible human carcinogen).
(2) Dimethoate
(3) Malathion
(4) Parathion
(5) Parathion-methyl
(6) Trichlorfon

According to the American Medical Association's Council on Scientific Affairs [3], only two chemicals used previously in pesticides have been associated with cancer in humans: arsenic and vinyl chloride.

Protective measures

Although pesticides may be extremely hazardous, they can usually be used safely if proper precautions are taken in their packaging and transport, and if the agricultural user adopts safe procedures for storage, handling and application of the product and for the disposal of empty containers [23].

Engineering controls

Enclosure/segregation

Occupational health controls are designed to deal with hazards at their source, interrupt the exposure pathway, and isolate the human receiver. Some industrial hygiene controls are summarized below:

(1) Raw materials are delivered in bulk by pipeline, railroad car, barge or tanker trucks, and mechanically unloaded, thus ensuring minimal handling.
(2) Raw materials are packed in sealed containers.
(3) Weighing and dispensing of raw materials is automated where possible.
(4) Hoppers, screens, mixing equipment, milling machines and baggers should be enclosed and emissions dealt with by other engineering methods.
(5) A plant manufacturing antiChEs should not be sited in a heavily populated area. Potentially hazardous operations should be isolated to minimize exposure to employees.
(6) The use of closed-transfer systems by employees mixing and loading liquid pesticides for crop-spraying.

General control methods

Ventilation

Mechanical processing of solid materials can lead to the generation of dusts. With AChEs not only respirable dust needs to be controlled but also larger dust particles which can be swallowed and produce peroral toxic effects. The volatility of liquids has to be taken into account when designing engineering controls using exhaust ventilation. Local exhaust ventilation is the most widely practised method of control in manufacturing plants. Systems are designed to capture or contain emissions at their source before they can enter the workplace atmosphere. General ventilation systems provide and remove air from the workplace ensuring frequent 'turn over' of air, thereby keeping the concentration of airborne contaminants below hazardous levels. General ventilation is only useful in dealing with low levels of contaminants released at constant rates. On the whole this is not a suitable means of controlling exposure from AChEs.

Personal protection

Personal protection equipment is used when it is not possible to control the workplace environment by means of the above measures. However if used improperly, it does not remove the risk from exposure, e.g. the wearer may not know when a device is malfunctioning. Protective clothing should be impervious to antiChEs.

Respiratory protection devices are used in the dispensing and spraying of concentrated liquid antiChE pesticides, and when applying powders or granular formulations capable of generating dusts. However, skin exposure in these applications appears to be more important than inhalation, and therefore protective clothing is of greater importance than respirators. Working clothes should be simple in design, appropriate for prevailing climatic conditions, and provide adequate protection and freedom of movement. They should not have external cuffs or open pockets in which hazardous materials can accumulate, and sleeves should be full length. Gloves should be impermeable to the pesticide and should be carefully inspected for their integrity before use, testing them for leaks by blowing them up with air. Boots or shoes should be made of rubber or some other suitable impermeable material.

The selection of the appropriate type of respiratory protective equipment depends on the type of AChE involved, the ingredients in the mixture and their toxicity, the level of exposure anticipated, the effort entailed, and the time requirements for the job. Workers will not tolerate prolonged wearing of respirators unless they are fully informed of the risks from not protecting themselves. All respirators must fit properly, be properly maintained and cleaned and sterilized if indicated [5].

Education, instruction and training

Personal hygiene is of the utmost importance in preventing the effects of pesticides from skin contact. Workers should be instructed to wash thoroughly with soap in running water or shower after using antiChEs. Fingernails should be cleaned and short. It is essential that there is an adequate water supply in case of accidental severe skin contamination. Eating, drinking or smoking should not be permitted during work with antiChEs, and no food should be brought to the workplace or stored where it can be contaminated.

The ILO has published a monograph in the Occupational Safety and Health Series on the *Safe Use of Pesticides*; the general principles are applicable to the antiChEs.

No product should be marketed without an adequate label written in the language of the country, which gives advice on safe packaging, transport, storage and handling procedures. The dangers from antiChEs should be clearly described and First Aid measures advised. It is important that the label indicate where specialized advice and assistance in dealing with an emergency can be readily obtained. The employer and supervisors should inform workers of the hazards of individual products, describe the routes of entry into the body, and instruct them how to recognize symptoms and signs of toxicity. The supervisor must prevent unauthorized access to areas where antiChEs are stored, are being applied or have recently been applied, utilizing administrative control measures such as warning notices, locking

doors or gates, and disciplining workers who fail to abide by the job safety rules. The supervisor should ensure that unused antiChEs are returned to a secure storage area or are disposed of safely. The supervisor is responsible for inspecting all equipment and withdrawing it from use if it has any potentially hazardous defect.

An educated or well-trained worker is able to appreciate the need for and to follow the safety and health instructions given. The informed worker knows how to use safety devices and equipment correctly, understands the importance of examining the equipment before using it, and immediately reports any potentially hazardous defect to the supervisor.

Responding to emergencies

General first aid measures

In all places where antiChEs are manufactured or handled notices should be posted giving the name of the physician, nearest hospital emergency room, fire department and ambulance service. Supervisory personnel should be aware of these.

Basic steps in the event of antiChE poisoning are:

(1) Remove the victim from further exposure.
(2) Seek competent medical help.
(3) Establish a clear airway and commence artificial respiration if necessary.
(4) Induce vomiting if the patient is alert and respiration is not depressed. Exceptions to emesis include corrosive formulations and those containing hydrocarbon solvent [4]. Drowsy or semiconscious patients must have lavage, preferably with endotracheal intubation. After emesis the victim should be given a suspension of 30–50 g of activated charcoal in 3–4 ounces of water to limit absorption of residual toxicant. A sample of the vomitus or the initial gastric lavage washings should be saved for chemical analysis.

If skin is contaminated, further exposure may be avoided by removing clothing, drenching the skin with running water and cleaning the skin and hair thoroughly with soap and water as rapidly as possible. First responders should wear suitable gloves and protective clothing and avoid contact with vomitus.

The main antidote for muscarinic effects in antiChE poisoning is atropine. It is essential that tissue oxygenation be improved as much as possible before the administration of atropine to minimize the risk of ventricular fibrillation. Atropine will control excessive bronchial secretions and help maintain clear airways. It must be administered iv or im until atropinization is achieved. Medical treatment may be needed for hours, depending on the severity of the poisoning. In some very severe cases treatment may have to continue for several days. The patient must be carefully observed to ensure that symptoms do not recur as atropinization wears off.

2-PAM is required when respiratory depression, muscle weakness and twitchings are severe. When administered early 2-PAM relieves the nicotinic effects of poisoning. The use of 2-PAM in CB intoxication is controversial and considered of questionable value (*see* Ch.52). Antidotal treatment is discussed fully in Chapters 47, 49 and 52.

Coordination: medical services and poison control centres

In the United States, Federal laws (e.g. Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA); Superfund Amendments and Reauthorization Act (SARA)) require that all releases of reportable quantities (RQs) of hazardous substances into the environment be reported immediately to the National Response Center (NRC) by calling a freephone number.

The Chemical Manufacturers' Association provides a public service through its organization CHEMTREC (Chemical Transportation Emergency Center), which can provide immediate advice by telephone to onsite emergency response teams and can obtain more detailed information from the shipper of the hazardous materials involved. CHEMTREC will notify the NRC of significant incidents. It operates 24 h per day and 7 days per week, and can receive freephone calls from the USA and Canada. CHEMTREC can usually provide hazard information warnings and guidance when given the following information: name of caller and telephone number;

identification number or name of the material; nature and location of the problem; shipper or manufacturer; type of container; truck registration number or rail car number; name of the carrier; name of consignee; and local conditions (weather, accessibility, etc.).

CHEMTREC can provide the necessary communication channel between the emergency response teams and experts anywhere in the USA or Canada, enabling telephone conferences to be held if necessary.

Incidents are mainly of two kinds: (1) of frequent occurrence and involving few people per event, e.g. automobile accidents; and (2) the infrequent event involving a large number of victims in a single incident. Hazardous material spills and leaks may be the result of both types of accident. Community emergency services are more prepared to deal with the first type of accident; it is not considered to be economically feasible to have universal emergency preparedness for all types of rare occurrences [36].

In the case of a road or rail accident involving antiChEs, the emergency services personnel should adhere to the rules for approaching the scene of any accident involving any cargo as summarized in the US Department of Transportation booklet [45]:

> Approach from an upwind direction if possible.
> Move and keep people away from the scene.
> Do not walk into or touch any spilled material.
> Avoid inhaling fumes, smoke and vapours even if no hazardous substances are involved.
> Do not assume that gases or vapours are harmless because of lack of smell.

Emergency responders must not enter the area without protective clothing, and on leaving the area they should remove contaminated clothing, bathe or shower and wash their hair thoroughly and change into clean clothing. Eating, drinking and smoking in the accident area are strictly forbidden.

AntiChEs accidentally released to the environment may be toxic to varying degrees to fauna and flora as well as to humans. Every effort should be made to prevent contamination of fields and water supplies. Emergency equipment or waste generated on site must not be cleaned or disposed of in such a manner as to contaminate the water supply to the community.

The commonest emergencies with antiChEs are poisoning incidents, involving accidental ingestion or crop spraying incidents. In the case of field workers or people living nearby who are exposed, the type of pesticide is usually known and the clinical management is then simplified. Although the likelihood exists that a road or rail accident could result in the release of a large quantity of an antiChE, or a faulty manufacturing operation could similarly expose a large number of people, this has in fact not happened in the USA. Manufacturers who transport raw materials between processing plants have trained drivers who know the health hazards involved and have antidote kits with them in the event of an accident.

The most important factor in the management of an emergency situation is the establishment of communication channels between onsite emergency responders and a control centre at a medical facility. The personnel on the scene will be involved in extricating victims, triage and the commencement of treatment. Table 36.2 summarizes a system of colour-coding victims using labels (tags) based on the METTAG (Medical Emergency Triage Tag) System [6].

In the case of an incident involving antiChEs it is essential that the rescuers and medical personnel know how to categorize and recognize those who are in need of urgent attention. Symptoms will vary according to the specific product involved. For example, chlorothion, malathion or Phostex (a mixture of bis(diethoxyphosphinothioyl) disulphide with bis(diisopropoxyphosphinothioyl) disulphide) are unlikely to cause fatal poisoning, whereas EPN, parathion, disulfoton and dicrotophos can be dangerous to life [15]. According to Dreisbach and Robertson fatalities have resulted in 5- and 6-year-old children

Table 36.2 Triage categories and colour codes

Category	*Colour code*
0 Dead or near-dead	Black
1 Critical	Red
2 Serious	Yellow
3 Walking wounded	Green

from 2 mg (0.1 mg/kg) of parathion and in adults from 120 mg. They state that a single dose of carbaryl, 2.8 mg/kg, caused moderate symptoms with recovery in 2 hours. It is important that treatment be instituted promptly [15]. If improvement is seen after treatment is commenced the prognosis is good.

In the USA the CHEMTREC data bank can rapidly identify active ingredients in the products, and can provide information on first aid measures published by the manufacturer in the Material Safety Data Sheet. CHEMTREC is also able to contact and connect rescuers or emergency medical service personnel to the manufacturer's emergency response service.

Poison Control Centres are rapidly becoming part of a 'network' capable of rapidly exchanging information (*see* Chs 41 and 42). In the event of an emergency, the role of the Poison Control Centre, either as an information resource or as a coordinator of the emergency response effort, may be crucial. The most efficient medical emergency response system will utilize the services of the community EMS, the hospital emergency facilities, the manufacturers' database and the back-up support provided by the Poison Control Centres.

Decontamination and detoxification procedures

The sequence of events in the case of an accident with antiChEs is as follows.

Assessment

In the USA, and in most industrialized countries, systems have been developed for identifying materials in transport. The US Department of Transportation (DOT) has developed a system consisting of diamond-shaped signs containing a 4-digit number. These are designed to be used with the *Emergency Response Guidebook* (DOT P 5800.4) which should be in the possession of emergency services personnel at the scene of the accident. The first responder should attempt to assess the situation from a safe vantage point and try to identify the materials involved.

Decontamination

Three zones should be demarcated; i.e. the contamination area, the area of containment and the safe area. The DOT's Emergency Response Guidebook should be consulted to determine the appropriate safe distances. The DOT book may be used in conjunction with a very useful publication, *Hazardous Materials Injuries*, which is subtitled *A Handbook for Pre-Hospital Care* [43]. It contains useful advice on decontamination and the use of protective clothing.

All rescuers entering the contaminated area must wear full protective gear and appropriate respiratory protective equipment. All items brought into this zone must be regarded as contaminated. The first task is to move victims away from the accident site, remove contaminated clothing and rinse them with water as speedily as possible. Emergency life support procedures should be commenced only if lives are in danger. All water used for washing must be collected or contained in the contaminated zone.

Stutz and Janusz [43] recommend that with CBs rescuers should wear chemical-resistant, impervious clothing, boots and gloves. In the case of the OPs, they recommend that rescuers wear full protective clothing and self-contained breathing apparatus. Leather protective clothing or shoes must not be worn since pesticides can be absorbed by leather. Patient care personnel can use canister respirators, if the victim has been decontaminated; otherwise self-contained breathing apparatus may be indicated.

Triage and resuscitation

Once the victim is in the containment zone the Emergency Medical Services personnel take over. The victim is thoroughly decontaminated and moved to the clean zone. EMS personnel should decontaminate themselves before entering the clean zone where the victim is prepared for transport to the hospital. Guidelines for EMS response at a HAZMAT incident have been suggested by Plante and Walker [36]. Once the patient presents to the hospital emergency department, treatment with the specific antidote and the necessary supportive therapy should commence. The

local Poison Control Centre can provide guidance to the clinician in the management of the case. The vehicle used to transport the patient should be thoroughly decontaminated before it goes into regular service again.

In a manufacturing plant, employees should be trained to react quickly to contain the escape of harmful materials. They should immediately raise the alarm, not touch the spilled material, and only attempt to stop the leak if it can be done without risk. The plant Emergency Team should be ready to take charge of the containment operation on arrival at the scene; water sprays may be used to reduce vapours, or in the case of small liquid spills sand may be used to absorb the chemical. The contaminated area should be isolated and all entry to it stopped until it has been cleaned up and the fault corrected.

Escape procedures and routes of escape

It is essential that Emergency Services, both in-plant and in the community, are always in a state of preparedness and able to respond promptly and effectively in the event of a HAZMAT incident. It is the responsibility of management, both plant and local community, to develop an emergency action plan and to ensure that everybody knows what to do. In the United States of America Title III of the Superfund Amendments and Reauthorization Act (SARA) is also known as the Emergency Planning and Community Right-to-Know Act of 1986. The three major requirements of SARA Title III are: (1) emergency planning notification; (2) emergency release notification; and (3) reporting on chemicals and releases for community right-to-know.

Many of Title IIIs emergency planning requirements will be incorporated into the existing National Contingency Plan (NCP). The intent of item (3) above is that communities will gather and provide public access to information on hazardous materials that can affect them either through accidental releases or routine emissions. This will provide communities with an opportunity to organize their emergency response services and to decide priorities. In the case of farming communities using large quantities of pesticides and where aerial crop spraying is prevalent, an emergency response plan is essential. Knowledge of the hazards should be complemented by knowledge of how to escape and where to escape to should the need arise.

Plant shut-down procedures

Plant shut-down is a drastic step that may only be contemplated in the event of a major disaster. The consequences of shutting down certain plants may be worse than those attributable to spill or leak, and in such instances secondary containment capability must be incorporated into the plant design; e.g. concentric piping, double-walled vessels or enclosures. Should the need for plant shut-down arise it should be done with the full realization that, unless the plant design includes the necessary built-in safeguards, the release could be increased owing to lack of secondary containment, and the plant may be irreparably damaged as a result.

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Physical protection against anticholinesterases

David W. Pike

Introduction

It has been known since the 1914–1918 War that toxic chemicals could be effective in warfare to harass, maim, injure and kill the enemy and to impose a high level of additional psychological strain on men in the battlefield. During that conflict, chlorine gas was first used in 1916 and many other chemicals were then delivered in vapour or gaseous form to attack the respiratory tract of humans. Principal gases used were hydrogen cyanide, cyanogen chloride, phosgene, chloropicrin and dichloroarsine. Later in that same conflict came the first use of liquid chemicals, principally mustard gas and lewisite, filled into shells and delivered by artillery bombardment onto the target. The liquid droplets and the vapour of these chemicals damaged skin, caused blindness and also attacked the respiratory organs.

By 1917 the British 'Box Type' respirator was developed. This had a rubberized fabric facepiece fitted with a head harness to hold the unit firmly on the face in an attempt to reduce peripheral leakage between the mask and face. When the war ended there was great public and political concern over the use of toxic chemicals in warfare and this led to the Geneva Protocol in 1925 in an attempt to ban the use of chemicals in warfare. However, it did not forbid the development of protective equipment to protect servicemen and it was from such programmes between the two world wars that more advanced designs of respirators, such as the British 1924 General Service Respirator, were developed. In World War II (1939–1945) emerged the new range of military respirators in the UK (1942) and overseas, and the first respirators for protection of the civilian population were introduced. Again, the emphasis was placed on respiratory protection although the oiled silk anti-gas capes were available for protection of the body against vesicant agents.

One major focus of attention during the 1920–1945 period was on the efficacy of the respirator filter canister. Two problems were paramount. These were the adsorption of vapour and gases by charcoal and the prevention of penetration of aerosol, liquid droplets and solid particulate matter through the filter canister. The early filters were fitted with a fibrous matt of fine blue asbestos fibres blended with cotton fibres. This matt acted as a trap for aerosol and particulate matter typically in the size range 1–500 μm mass median diameter. Liquid droplets trapped on this fibrous layer evaporated into the influent airstream and the vapour given off was adsorbed on the charcoal layer. In the late 1930s, the earlier asbestos fibre particulate filters were superseded by resin impregnated wool from Merino sheep. Typically face velocities of about 5 cm/s would yield very high levels of protection. Modern high efficiency particulate filters for both military and industrial use permit the penetration of less than 0.003% of submicron particles (0.3–0.6 μm mass median diameter) of sodium chloride or dioctyl phthalate (DOP) aerosol at an airflow rate through a filter canister of 32 l/min. Such high efficiency filter canisters are widely available and provide extremely effective protection in the workplace and in military applications. The charcoals most widely used were derived from either coal or coconut shell. The carboniferous material was activated in the presence of steam or carbon dioxide in an oxygen-free atmosphere so that the enormous internal surface area of the charcoal structure

was excavated and thus made available for the adsorption of gases and vapours.

Activated charcoal is highly efficient at removing large molecules from the influent air and such molecules are held very firmly in the charcoal by van der Waals forces. A bed depth of perhaps 5 mm of charcoal would be sufficient to provide good protection against concentrations of toxic nerve agents on the battlefield. However, chemisorption is much less efficient and, as a direct consequence of this, military filter canisters have charcoal volumes of 140 ml or much greater with charcoal bed depth in the range 15–30 mm. The important point is that if the chemical being protected against is defined with respect to concentration, time of exposure and type, then a filter canister could be built to that level of protection. In practice, of course, military and industrial canisters are usually designed to meet a wide range of possible challenges in a non-specific, all embracing manner and this leads inevitably to deep charcoal beds. It should be noted that OP antiChEs are all large molecules which are very strongly adsorbed physically onto activated charcoals and it is this characteristic which permits the use of charcoal impregnated clothing by the military for protection of the body against the nerve agents.

Organophosphates which possess antiChE activity are to be found in use either as agricultural insecticides or as toxic chemicals for use in warfare. Work on such compounds began in Europe in the 1930s and many chemicals have been screened for their toxic properties. The German war gases were tabun, sarin and soman (*see* Ch. 34). The physical properties of these chemicals are very important to any consideration of protection because they range in volatility from the very high vapour pressure of sarin (2.1 mmHg at 20°C) compared with the very low vapour pressure of VX (0.0004 mmHg at 20°C). The surface tension of the nerve agents are broadly similar, in the range 2.5–3.0 $\times$ 10^{-6} Nm (25–30 dyne/cm^2) at 20°C thus making them capable of wetting surfaces very readily.

Quite apart from the physical form, vapour or droplet, of the chemicals when delivered on target to humans on the battlefield is the question of toxicity. Typical values for the toxicity of nerve agents in mice are given in Table 37.1 [5] (*see* Ch.34). Values given are for LD_{50} in mg/kg dose given sc. The data given in Table 37.1 show the close similarity in toxicity of the range of nerve agents in mice. Inhalation toxicity would be very similar. However, the percutaneous toxicity to humans of nerve agent vapour would be very high (Ct = 2000–3000 mg min per m^3, where C = concentration in mg per m^3 and t = time in min). Such Ct exposure values would be hard to sustain on the battlefield. The situation with percutaneous challenge to humans is somewhat different because once a liquid/skin contact is established there exists an opportunity for diffusion to occur across the skin barrier and into the bloodstream to bring about serious systemic poisoning. In this situation it is the low volatility V agents which exhibit the highest toxicity because the evaporation rate is much less than the G agents so that they remain longer in contact with the skin for a given mass of contamination.

Table 37.1 Toxicity (LD_{50}) of different nerve agents in mice

Agent LD_{50}	*mg/kg sc*
GA	0.40
GB	0.20
GD	0.10
GE	0.30
GF	0.15
VX	0.022
VE	0.025
VM	0.035
VS	0.035

(a) Data from Vojvodić [5]

The principal issues that arise from protection against the nerve agents are as follows. (1) Inhalation toxicity is so high that respiratory protection is essential. (2) Percutaneous toxicity is so high that protective clothing must be provided to prevent death or casualties occurring from liquid contamination. (3) The percutaneous toxicity against vapour challenge is sufficiently low that it does not require additional protection beyond that provided by available protective clothing. However, it should be noted that this argument may not be applied in a general way to all toxic chemicals; for example, mustard gas will burn the skin and cause significant casualties at exposure to much lower levels of vapour Ct and consequently is a serious hazard on the battlefield.

Consideration thus far has been directed only towards lethal effects or incapacitation from systemic poisoning by nerve agents. Direct exposure of the eyes to vapour or liquid challenge will have serious consequences. Miosis or constriction of the pupils of the eyes is also an important effect on humans on the battlefield because of the immediate effects of loss of ability to focus and to obtain satisfactory depth of field of view. Miosis occurs at very low levels of challenge (Ct 0.1 mg min per m^3 and increases in severity and military significance up to Ct = 15 mg min per m^3 and greater. A further complicating factor is that complete recovery from miosis may require at least a week.

Emphasis has been placed on the use of antiChEs of very high toxicity in warfare. However, there are many pesticides in use which possess sufficient toxicity to inhibit ChE to the point of death. Such chemicals may be used in crop spraying or as a more general agricultural pesticide perhaps stored as bulk liquid in drums. Obviously the risk of gross contamination is high and it should be borne in mind that ChE inhibition is dose-related, i.e. a high dose of low toxicity OP may be as lethal as a low dose of a high toxicity compound.

Physical protection

Respiratory protection

Modern high-efficiency respirators for military or industrial use consist of an injection or transfer moulded rubber facepiece made of polyisoprene, halogenated butyl rubber or neoprene rubber with a wall thickness of about 2–3 mm. Such materials, particularly butyl rubbers, provide good protection against penetration by OPs but ideally they should be decontaminated rapidly after contamination with liquid droplets if subsequent desorption from the rubber is to be avoided or reduced to a minimum (Table 37.2).

The seal of the respirator against the face is important. This may be accomplished by presenting a flat bearing surface of 1–2 cm between the skin and rubber facepiece but more recent designs have an inturned periphery or reflex edge face seal which conforms more readily to the facial contour. Face seal leakage of a properly fitted respirator should rarely be more than 0.1% and would normally be in the range 0.05–0.01% or less (Table 37.3). Most military respirators are available in three or four sizes whereas most industrial respirators are single size or occasionally are available in two sizes to fit the whole population.

Table 37.2 Penetration of mustard gas through and desorption from different rubber

Rubber	*Penetration time (h)*[a]	*Desorption time (days)*[b]
Viton (fluorinated elastomer)	>96	<1
Butyl	>48	1
Polyisoprene	2	4

[a]Time to the first penetration of mustard gas through a section rubber 0.020 inches thick. 1 μl drops applied; sensitivity of penetration 0.125 μg using s.d. test
[b]Mustard gas desorbed after free liquid had been removed by blotting 6 h after application
[c]Note that polyisoprene is most easily penetrated and desorption of test chemical continues for 4 days

Table 37.3 Face seal leakage of the S6 respirator

Penetration (P) of Bacillus globigii *(%)*	*Subjects having penetration in excess of stated P value (%)*
0.0001	60
0.001	40
0.01	20
0.1	5

[a]Leyland and Birmingham Rubber Company Limited
[b]The geometric mean reduction factor for face seal leakage is 0.0032%

Most military respirators are fitted with two eyepieces made of toughened glass, allyl diglycol carbonate (CR39 resin) or polycarbonate (coated with polysiloxane or similar hard coat finish to reduce damage by abrasion or scratching). The two eyepieces are normally required to enhance compatibility with optical sighting system and other weapons and equipment. Corrective lenses may be fitted to meet the specific needs of the individual. By contrast, most industrial respirators have a large visor faceplate made of polycarbonate to gain the benefits of high impact resistance and a large clear field of view in situations that do not normally impose the same constraints for compatibility with sighting systems and the like.

The facepiece is held firmly on the head by adjustable straps or a net head support which spreads the load over a large surface area of the head.

Table 37.4 Particulate penetration tests on canister NBC S6[a]

Test aerosol	*No. tested*	*Penetration median value (%)*
Dioctylphthalate (0.3 μm)	6	0.0006[b]
Sodium chloride (0.6 μm)	321	0.0001
Bacillus globigii (1 μm)	2	0.0003[b]

[a]Leyland and Birmingham Rubber Company
[b]Lower limit of detection
[c]Penetration tests were conducted at 30 l/min airflow rate

Table 37.5 Gas tests on canister NBC S6[a]

Gas	*Condition*		*Penetration time (min)*
Hydrogen cyanide	Concentration	2 g/m³	
	Flow rate	30 l/min	40
	Relative humidity	80%	
Cyanogen chloride	Concentration	2 g/m³	
	Flow rate	30 l/min	40
	Relative humidity	80%	
Sarin	Concentration	1 g/m³	230
		0.2 g/m³	1200
	Flow rate	30 l/min	
	Relative humidity	90%	

[a]Leyland and Birmingham Rubber Company

Table 37.6 Different industrial filter canisters[a]

Filter type	*Main protection against*
Gas	
A	Organic gases and vapours, e.g. solvents
B	Inorganic gases and vapours, e.g. chlorine, hydrogen sulphide, hydrogen cyanide
E	Acid gases, e.g. sulphur dioxide, hydrogen chloride
K	Ammonia
CO	Carbon monoxide
Hg	Mercury vapour
NO	Nitrous fumes including nitric oxide
Reactor	Radioactive iodine and iodomethane
Particle	
P1	Solid particles of nuisance dust
P2	Solid and liquid particles of harmful substances
P3	Solid and liquid particles of toxic and highly toxic substances

[a]Defined by DIN3181
[b]Combined filters are available which contain both particle and charcoal filter types in a common filter canister

Air is drawn through the filter canister where it passes through the pleated glass fibre particulate element (Table 37.4) and then through the bed of activated charcoal. In military canisters the charcoal is impregnated with metal salts to permit chemisorption to occur (Table 37.5). Similar treated charcoals are also used in industrial canisters but a variety of charcoal types are available to provide protection against different types of gases. The different industrial filter canister types are shown in Table 37.6. Normally the air then passes through an inlet valve which opens during inspiration but closes during the positive pressure phase of exhalation.

Table 37.7 Dynamic outlet valve leakage of S6 respirator[a]

Breathing rate (breaths/min)	*Minute volume (l)*	*Maximum flow rate (l/min)*	*Penetration (%) (s.d.)*
23	37	100	0.0017 (0.0013)

[a]Leyland and Birmingham Rubber Company
[b]Ammonia was used as the test gas with a forward wind speed of 5 km/h. The valve was tested in dry conditions. Wetting the valve by expirate will increase its efficiency

When the air is exhaled it contains carbon dioxide at 4% concentration and is normally 100% relative humidity. It is important that this hot moist exhalate passes to the outside through an outlet valve so that no opportunity exists for the build up of water or carbon dioxide within the mask. This is usually accomplished by a small orinasal cup inside the facepiece which is used to channel the exhalate to the outside via the outlet valve. Much has been said about the relative merits of low resistance to air flow passing into the facepiece through filter canister and inlet valve and subsequently out through the outlet valve. However, modern small battery-powered fan units are available which blow air through the canister and maintain a positive pressure inside the facepiece. Such devices may be continuous flow or may meet the air flow demands of the wearer. The fan powered units improve both the level of protection provided and reduce the physiological and psychological stress of wearing a respirator.

The outlet valve assembly is normally designed so that a dead space volume of air is situated on the outboard side of the valve. During exhalation air passes out through the valve under positive pressure relative to ambient. The valve has a bias to close firmly but there will be a finite time before the valve is firmly shut and during this time some leakage of ambient air could occur back into

the facepiece; this is referred to as dynamic leakage and can be measured (Table 37.7). A well-designed outlet valve assembly will have a penetration (dynamic leakage) of <0.003%. Thus total inward leakage of a respirator of good design will be <0.01%, made up of 0.003% through the canister, 0.003% through the outlet valve by dynamic leakage and 0.003% by faceseal leakage. Typical results are given in Tables 37.4–37.8 for the British S6 military respirator designed in the late 1940s and early 1950s.

Many industrial respirators are not fitted with speech transmitters and in this respect they differ from most military masks. Speech diaphragms may be made of terylene, thin gauge metal foils or plastic films such as mylar. They may be tensioned during assembly or they may be preformed before fitment. Most respirator speech devices are not very efficient but this vitally important means of communication cannot be ignored.

It should not be forgotten that there are types of respiratory protection other than the negative pressure facepiece design most commonly in use. Alternatives include closed circuit breathing apparatus where the breathable gas is recirculated through chemical scrubbers to remove carbon dioxide and water vapour. The oxygen may be available from compressed gas or may be produced chemically from superoxides. Compressed air open circuit breathing apparatus, typically worn by firemen is also available. These types of breathing apparatus are especially important where very high concentrations of toxic chemicals are likely to be encountered or where an oxygen deficient atmosphere could exist. The units are usually of the positive pressure type where an overpressure is maintained throughout the entire breathing cycle. Interestingly, the fan powered respirators form a natural niche between the lung powered negative pressure respirators and the positive pressure sets. Increasingly, fan powered systems are available in the industrial marketplace and in military applications.

Protection of the body

There are three fundamentally different approaches providing protection of the body against the OPs or indeed other toxic chemicals. First, it is possible to place a totally impervious layer between the toxic material and the skin of the wearer. Such is the case in air-fed suits used to protect workers in the nuclear power industry or by the use of heavy rubber suits of fluorinated or butyl elastomers often fitted with built-in breathing apparatus. Many plastic materials such as polytetrafluorethylene (PTFE), polyimide and polyamide films, poly vinylidene chloride or fluoride films provide excellent protection against penetration by OPs.

A second approach would be to impregnate the clothing with a chemical that would react with the compound against which protection is needed. This approach has been tried by the military but lacks performance owing to the specificity of the protection provided.

The third fundamental approach is to use an air-permeable protective clothing layer which has an adsorbent, preferably non specific such as activated charcoal, to prevent penetration of the toxic chemical.

The choice of approach adopted is determined by the nature of the problem. For example, in the military situation the need is for balance between durability of the clothing in operational use, the protection provided, and the physiological burden imposed on the wearer in a wide range of possible climatic conditions and work rates. This requirement should be contrasted with the situation of a fireman in toxic chemical spills or container drums which may soak the fireman, who is using a high pressure water hose. Clearly in such situations the firemen should be dressed in impermeable material to protect against bulk liquid (water or toxic chemical). Thus definition of the requirement is fundamental to the approach adopted in developing the right clothing for the protection required.

The history of the development of the British Mark 1, 2, 3 and 4 Nuclear, Biological and Chemical (NBC) protective suits has been described by Griffiths [4] and a brief overview of the equipment of many countries is given by Benz [1]. The best technical publication describing the British Mark 3 and 4 NBC suits was published by Ellingsen and Karlsen [2] and Gao Fang [3]. In essence, the Mark 3 suit is made of two layers. The outer layer is a 2 × 1 twill construction of nylon and modacrylic (Teklan) fibres with the outermost face rich in

nylon for durability. The Teklan fibres are inherently flame-proof. It is treated with a silicone repellent to enhance shower proofing. The innermost layer is a non-woven cross-laid or random laid with fibres of nylon 85% and viscose 15% bonded together with a synthetic rubber latex. Flame retardant and acid acceptor stabilizers are added to improve performance and the uppermost layer is treated with an oil repellent fluorochemical. The innermost surface is coated with finely ground activated charcoal particles (<50 μm diameter) to a level of coverage of 40 g/m^2.

Chemical vapour from OPs challenging the suit would be adsorbed readily into the charcoal and very effective protection achieved. Liquid droplets, expected to be less than a few mm in diameter (and possibly down to a few microns in diameter) would land on the outer fabric and spread or wick out into the capillary structure. This spreading would have two important effects. First, by increasing the surface area of contact about 30-fold, it would reduce the liquid loading per unit area substantially (30-fold). This would increase greatly the opportunity for evaporation of the toxic chemical both into the atmosphere and into the charcoal, especially when compared with a droplet repelled on the top surface of the protective layer. Ellingsen and Karlsen give graphs comparing the effects of repelled *versus* spread droplets with drop sizes of 0.5, 1.0 , 3.0 and 5.0 μl of HD (distilled mustard gas, dichlorodiethyl sulphide). These results obtained with HD remain valid for the G nerve agents since their range of vapour pressure covers that of HD. However, the V nerve agents are of such low volatility that they do not present a real clothing penetration hazard where charcoal adsorbents are used. However, they do present a serious toxic hazard on bare skin. It is for this reason, coincidentally, that wicking or spreading of agent on the outside of the suit has advantages since it removes totally the hazard of cross contamination by free liquid during such activities as suit changing, urination, defaecation drills or casualty handling.

Thus the technicalities of NBC suit design and construction are geared particularly to meet the military need for protection. The Mark 3 and 4 suits will provide > 24-h protection and are durable for up to 4 weeks in operational use. They can be folded and pressure packed for portability. The Mark 1 and 2 suits came into service use in the early 1960s and the Mark 3 followed in 1975.

Protection of the hands and feet

A number of attempts have been made by defence scientists over the past 20 years to utilize the adsorbent properties of activated charcoal impregnated into polyurethane foam or coated onto fabrics as an essential element in the development of a protective glove which is permeable to air and water vapour. These properties, which are very important to protection of the body, would be very beneficial to the military user to avoid maceration of the hands. The so-called water logging of the skin by sweat trapped inside impermeable gloves causes discomfort during prolonged use and may cause very serious damage in arctic conditions. Unfortunately, there has been little progress in producing chemically protective, air permeable gloves. This has forced the military authorities to continue with dip-coated gloves made from polychloroprene or from butyl rubber. Some attempts have also been made to use coatings of different elastomers to improve resistance to petrol, oils and lubricants with outer layers of polychloroprene or nitrile rubber while protection against OPs was provided by the butyl rubber beneath. The penetration of a nerve agent 'front' passing through thin films of rubber from direct liquid contact is such that the possibility of using thin surgical gloves made of polyethylene or natural rubber (polyisoprene) is impossible because the protection provided is too short to be of practical significance.

Interestingly, there has been some work in the UK (Pike, McDowall, personal communication), which has employed the wicking principle, described for protection of the body, to protection of the hands. Royal Air Force aircrew normally wear Cape Leather gloves during flying missions. Protection against nerve agents provided by such gloves would be minimal, perhaps a few minutes against liquid droplet challenge. However, if a thin (0.018 inch) polychloroprene glove, which by itself would provide minimal protection, is worn beneath the Cape Leather glove then the protection provided is enhanced dramatically.

The reason is that the liquid is retained in the thin leather so that only a vapour challenge is presented to the rubber. The usual opportunity for diffusion (Fick's Law) is denied and a significant protection is provided. Until approaches such as this are exploited or other avenues explored then the Services will have to tolerate protection provided by rubber impermeable materials.

Protection of the feet poses serious problems. While it is easy to design and make a butyl rubber overboot that will provide the level of protection required, there are many issues here. If an overboot is tailored to fit closely then a large number of sizes will be required. These could well be costly to make and cause serious logistic problems for replenishment and issue in time of conflict. However, they may well fit comfortably and could meet the very demanding service requirements. Any easement of the closely fitted design does relax the constraints for logistic supply but experience has shown considerable user resistance. Inevitably the user is stuck with the problem of wearing an impermeable rubber overboot in one or more sizes.

Other alternatives have been examined and include the incorporation of a chemical barrier material, rubber or plastic, into the boot itself. This approach has not been fully exploited but a solution is still a long way off. A further option would be to develop a protective sock to be worn inside the ordinary footwear. This has met with some success, one major drawback is that the wearer's normal footwear becomes contaminated and is not easily decontaminated. This issue underpins the whole concept of protection against OP antiChEs and other toxic chemicals, the basic philosophy is to use some form of protective layer which acts as a barrier to penetration, albeit for only a limited period of time, and which may then be thrown away taking the contamination with it. Thus in the UK, the primary item of chemical defence equipment, the respirator, donned as an additional item to cover bare skin and the respiratory tract, is worn in conjunction with an overgarment, overboots and overgloves, all of which are disposable and may be replaced by new equipment.

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Screening of agricultural workers for exposure to anticholinesterases

Robert C. Duncan and Jack Griffith

Introduction

RBC AChE can be distinguished from plasma BChE by difference in substrate specificity and by the use of specific inhibitors [52]. RBC AChE is membrane bound [13], and inhibited by excess levels of ACh. ACh is most effectively hydrolyzed at the maximum rate of 3×10^{-3} M solutions of RBC AChE [1]. RBC AChE will hydrolyze the following substrates in addition to ACh; acetyl-β-methylthiocholine [2]; acetylthiocholine [32]; acetyl-β- methylcholine [1,31] and propionyl choline [35]. Plasma BChE is not inhibited by excess ACh, and the most effective ACh concentration is 2×10^{-2} M [1]; it also hydrolyzes butyrylcholine [22]. RBC AChE has been reported to closely parallel the level of AChE in the central and peripheral nervous system [20,46,55] and is considered the best indicator of AChE activity at the synapse. However, it has been suggested that plasma BChE is the most sensitive measure of OP and CB exposure [6,24,38] and perhaps provides the best early warning of excessive exposure [30, 51]. Although OPs cause a virtually irreversible phosphorylation of RBC AChE requiring several weeks for recovery, carbamylation of the enzyme reverses within 24–48 h. This mitigates the effectiveness of ChE as a monitor for CBs. Plasma BChE depression reflects recent and moderate exposure to OPs and CB pesticides. A significant reduction in both the RBC and plasma enzymes would probably represent ongoing exposure, or perhaps a single large exposure.

Factors affecting ChE activity

Normal ChE values

Owing to significant inter- and intraperson variation, the results of the many studies reporting ChE values are difficult to interpret. It has been suggested that laboratory methods may account for as much as 40% of the variability in RBC AChE activity, and 24% in plasma BChE activity [54]. The method most commonly used for clinical and surveillance purposes [11] has been the electrometric method of Michel [33]. Using this electrometric method, Wolfsie and Winter [53] established a normal range for plasma BChE activity of 0.408–1.652 Δ pH per ml per h (i.e. the fall in pH produced by 0.1 ml of whole blood in 1 h multiplied by 100), and 0.554–1.252 Δ pH per ml per h for RBC. Rider *et al.* [37] also using the method of Michel, evaluated ChE levels in 800 normal subjects and determined the mean RBC AChE activity for males and females was 0.766 and 0.750 Δ pH per ml per h, respectively; ranges were 0.58–0.95 (s.d. = 0.81) for males, and 0.56 – 0.94 (s.d. = 0.82) for females. Mean plasma BChE activity for males and females was 0.95 and 0.75, respectively; ranges were 0.52–1.39 (s.d. = 0.187) for males, and 0.38–1.25 (s.d. = 0.187) for females.

Intraperson variation

Although there is less variation between serially drawn samples taken from the same individual than when samples are compared between individuals [41] a significant intraperson variation does exist. The variation is less for RBC AChE than for plasma BChE. Callaway *et al.* [8] found that the percentage standard deviation (coefficient of variation; CV) for an individual around his own personal RBC AChE was 10.7%, and 22.8% for plasma BChE. Sawitsky *et al.* [41] determined the CV for RBC AChE to be 10.2%, and for plasma BChE to be 30.2%. However,

Kilgore *et al.* [28] found that CV values for plasma BChE ranged from 3.9% to 9.1% for controls for a group of workers engaged in tree-thinning activities; the CV for RBC AChE ranged from 2.4% to 4.2%. The mean CVs for plasma BChE and RBC AChE for the entire group were 12.2% and 2.2%, respectively, with s.d.s of 0.60% and 0.27%, respectively. Davies and Rutland [14] determined the CV for plasma BChE to be 25.8%, and RBC AChE to be 15.4%. While it is clear that variation is present, it is difficult to characterize. Recently Hayes [25] suggested that in normal workers unexposed to OPs, the expected variations in RBC AChE would be in the range of 13% to 25% and for plasma BChE, from 20% to 23%.

Interperson variation

Studies to determine ChE depression among OP-exposed workers are frequently based on the comparison of group means with a control population [30,45]. From a recent review of ten studies comparing OP-exposed groups to non-exposed controls [15] it can be seen that the CV of interperson plasma BChE values range from 14.9% to 30.7% among unexposed controls for four different units of measure. There was no particular trend among measurement methods and the mean CV was 22.7%, with a s.d. of 5.7%. Data derived from Kilgore *et al.* [28] suggest that the CV for plasma BChE values over a 5-day period for a group of unexposed Mexican-American students ranged from 19% to 33%, with a s.d. of 5.9%. The mean CV was 25.9%. The RBC AChE CV for the group ranged from 5.97% to 7.5%, with a s.d. of 1.06%; the mean CV was 16.2%. These data indicate that while interperson variation is greater than intraperson variation, both variations must be considered in normal subjects used as controls.

Genetic factors

Approximately 3% of the population has a genetically determined predisposition for reduced plasma BChE owing to an atypical plasma ChE enzyme [12] (*see* Ch.48). Because the incidence of this enzyme is relatively rare, its occurrence should not add significantly to the number of 'false positives' in a screening programme. It is possible to identify persons with the atypical plasma BChE by incorporating dibucaine hydrochloride in laboratory test methods [7].

Disease factors

Characteristically, low plasma BChE activities are found in persons suffering from parenchymal liver disease, malnutrition involving deficiencies in protein or thiamine, chronic debilitating diseases, acute infections, some anaemias, myocardial infarction and dermatomyositis [12,23,47]. Plasma BChE levels are depressed in persons with low levels of serum albumin. Thus, it has been suggested that the low plasma BChE reflects impaired hepatic protein synthesis [12].

Age, sex and racial factors

RBC AChE activity decreases with age in adults [22], while plasma BChE change with age is insignificant. Gender plays no significant role in ChE activity, although plasma BChE is somewhat lower in females and fluctuates during the menstrual cycle [22]. Pregnancy significantly reduces plasma BChE during the first trimester, and during the second to seventh days postpartum [19]. Although race is not thought to be a significant factor of ChE activity, Reinhold *et al.* [36] reported lower plasma BChE levels among Black people compared with Whites of the same sex.

Analytical test procedures for ChE determination

Background

Methods are based on the ability of ChEs to hydrolyze choline ester substrates. RBC AChE acts more efficiently on ACh and is specific for acetyl-β-methylcholine. Plasma BChE hydrolyzes ACh and is specific for butyrycholine. Procedures involve either measuring the rate at which the substrate is destroyed, or the rate at which the acid (acetic or butyric) forms. Methodology has been reviewed in detail in Chapter 2, and only an essential summary is presented here.

Spectrophotometric method

Ellman *et al.* [18] developed a method using acetylthiocholine or butyrylthiocholine as substrates, and measuring the rate of the production of thiocholine by reacting with dithiobisnitrobenzoic acid (DTNB) to form a yellowish nitrobenzoate anion that can be measured spectrophotometrically. The method is suitable for whole blood, RBC and plasma ChE activity. It requires only a small sample (10 μl) of blood, and an assay time of 2 min. The rapidity of the assay permits its use for CBs. An advantage of the method is its adaptability for microdeterminations and for routine analyses that are suitable for automated procedures. A field method has been developed for the World Health Organization [50] that utilizes a portable spectrophotometer and preweighed reagents, thus permitting ChE determinations from finger prick blood samples. A single operator can perform 40 whole blood and 40 plasma assays in 1 day. Technical difficulties encountered in separating and measuring RBCs in the field (the haem in haemoglobin may interfere with the spectrophotometric measurement of thiocholine) generally limits analyses of plasma and whole blood ChE. However, Knaak *et al.* [29] developed an automated version of the method that in effect separates the RBCs from thiocholine before the reaction with DTNB occurs. Under normal working conditions, the reproducibility of the method of Ellman *et al.* [18] utilizing the modified spectrophotometer is within ±5% of the laboratory based method which utilizes standard laboratory equipment.

Electrometric method

The electrometric method of Michel [33] is the most widely used method for monitoring ChE in exposed workers. It measures change in pH, from substrate hydrolysis, as a function of time (Δ pH per ml per h). Because only one measurement is taken, this method does not permit the most accurate determination of the rate of substrate hydrolysis. ChE activity is markedly decreased with a fall in pH over a range of 8 to 6. A phosphate barbital buffer with the same pH range is used to maintain a linear relationship between the fall in pH and time during the incubation period. The method is not suitable for estimating carbamylated enzyme inhibition from spontaneous regeneration. The method of Michel requires only a pH meter and an incubation bath. A maximum of 60 samples can be analysed over a 6-h period [46]. It is not as sensitive as the Ellman method because the pH measurement is a logarithmic function of the acid concentration, rather than the measurement of the actual acid production. However, its precision (± 0.02 – 0.03 Δ pH units [52]) is adequate for field testing, and it is commonly used in ChE monitoring programmes because medical personnel are comfortable using pH units, the ready availability of equipment and because there are considerable baseline data available for comparison purposes.

Titrimetric method

The titrimetric assay was developed by Stedman *et al.* [44] and modified for use in ChE monitoring programmes by Nabb and Whitfield [34]. It involves determining the rate of acetic or butyric acid formation from hydrolysis of the substrates ACh, acetyl-β-methylcholine or butyrylcholine by titration against a standard base, usually NaOH. Using either an automatic recording potentiometer (automatic titrator), or a colour indicator (usually phenol red) the pH is held constant. However, it is difficult to match colours between the indicator standard and the assay because the indicator may combine with protein, particularly serum albumin, thus creating an apparent decrease in pH. When an automatic titrator is used, precision of the method is enhanced. Titration time ranges from 2 to 10 min, and requires 0.5–2.0 ml of blood or plasma for analysis. Although the equipment required for the assay is expensive, the method is simple. Assays require 10 min to complete, and only one sample can be run at a time. It provides an excellent means to study the kinetics of ChE reaction to either inhibitors or activators since enzyme concentrations can be maintained at levels permitting optimal activity, or they can be independently varied. During this process, a linear relationship between the concentration of ChE and the magnitude of activity is obtained that permits the reaction to be followed over a much longer time and with greater precision

and sensitivity than is possible with either the Ellman or the Michel methods.

Tintometric methods

This method, described by Edson [17], uses fingerstick samples of whole blood that are incubated with an indicator (bromothymol) and ACh. As acid is formed, colour changes occur that can be compared with coloured glass standards. CBs can be assayed if the procedure is modified to add the substrate directly to the reaction mixture immediately after the addition of the blood sample. The time required for the assay is about 20 min, and up to 100 samples can be completed in 6 h. The procedure is simple, with premeasured reagents available, has good reproducibility and correlates well with the Michel and Ellman methods [11]. Equipment is relatively inexpensive, portable and can be operated by a single technician.

Acholest method

The method, described by Sailer and Braunsteiner [40], is based on the change in pH during substrate hydrolysis being measured by the change in colour of an indicator (e.g. phenol red, bromothymol blue) after a short incubation period. A popular procedure is to use a filter paper impregnated with substrate and bromothymol blue, and dried. Plasma or serum is added to the filter paper and ChE activity evaluated by comparing colours, ranging from blue to yellow, at various time periods with colours from a chart containing standardized pH values.

ChE screening programme

Background

Screening agricultural workers by monitoring ChE values can help to select asymptomatic workers for removal from exposure before the onset of symptoms. The Commission of Chronic Illness [9] has defined screening as:

> The presumptive identification of unrecognized disease or defect by the application of tests, examinations, or other procedures which can be applied rapidly to sort out apparently well persons who probably have a disease from those who probably do not. A screening test is not intended to be a diagnostic. Persons with positive or suspicious findings should be referred to their private physicians for diagnosis and necessary treatment.

Screening model

The objective of ChE screening is to detect the effects of antiChEs before the occurrence of poisoning. To this end a 'cut-off point' must be established for the screening test, such that persons below that value are judged to be adversely exposed. The percentage of reduction from 'normal' group or individual baseline values has frequently been used as a cut-off point for determining excessive exposure.

Kahn [27] recommended that when individual RBC AChE and plasma BChE fall below 30% and 40%, respectively, a worker should be removed from exposure. To establish an individual baseline, Kahn suggested that workers should be without antiChE exposure for a minimum of 20 days before the establishment of the baseline. He recommended two ChE determinations (48–96 h apart) 1 week before the worker is exposed to antiChEs. A final pre-exposure test should be completed the afternoon before exposure is planned.

Gage [22] suggested that an individual with RBC AChE and plasma BChE respectively 20% and 30% below the 'population' average has probably been exposed to a ChE inhibitor, and that an individual dropping from 17% to 23% from his baseline mean (depending on the number of baseline values) also indicates exposure. Hayes [25] suggests that a 20% decline from plasma BChE baseline is sufficient to remove an individual from work. The California Department of Food and Agriculture has used a 40% plasma ChE inhibition as its criterion for exposure [11].

As indicated by Gage [22], ChE depression is not necessarily a toxic manifestation. Furthermore, cholinergic symptoms can appear before observable enzyme inhibition with respect to normal laboratory limits [10]. Vandekar [46] reported good agreement between the degree of ChE inhibition and the frequency of signs and symptoms. Davidsohn and

Henry [12] and Levin *et al.* [30] suggested that plasma BChE is perhaps the most sensitive indicator of excessive exposure to antiChEs.

Warnick and Carter [49] reported on 505 subjects who were moderately exposed to OPs compared with 217 closely matched controls; participants were subjects in the Community Pesticide Study [42] of the US Public Health Service. The exposed subjects had a mean plasma BChE value 6.5% below their controls (P <0.01). No effects of clinical importance were observed in the exposed workers.

Rodnitzky *et al.* [39], in an attempt to associate chronic pesticide exposure with CNS dysfunction [16], compared ChE values in a group of farmers, and OP applicators with a group of matched but unexposed controls. Although within the 'normal' range, the mean plasma BChE value for the exposed group was statistically significantly lower (11%) than the mean value for the controls. The exposed group showed no deficit in memory, signal processing, vigilance, language or proprioceptive feedback, and no cholinergic-like illness at the time of testing.

Bhatnagar *et al.* [4] compared plasma BChE values in 75 pesticide factory workers with 15 unexposed controls. The mean value for the exposed workers was 47% below the mean value of the control group (P <0.001). Effects were reported in 52% of the workers. Symptoms included loss of appetite, nausea, headache, giddiness, vertigo, fatigue, anorexia, muscular cramps, stomatitis and watering of the eyes.

Bogden *et al.* [5] compared plasma BChE in 12 migrant workers exposed to OPs with values found in two unexposed medical school personnel. Although pre-exposure data were not available and the number of exposed workers and controls was small, the authors suggested that the evidence indicated an association between health symptoms and pesticide exposure, e.g. frequent headaches, blurred vision, dizziness and muscle weakness. The mean plasma BChE for the exposed workers was reduced by 43% of the mean value of the control (P <0.010); 86% of the workers reported symptoms usually associated with OP intoxication.

Knaak *et al.* [29] compared 79 male vineyard workers complaining of cholinergic symptoms following OP exposure during re-entry with those of Sacramento Blood Bank controls. They reported that 98% of the exposed workers had cholinergic symptoms, with a mean plasma BChE value depressed 63% from the mean of their controls (P <0.001).

These reports suggest that moderate reductions (approximately 20%) in mean plasma BChE activity of an exposed group below a comparable control group can occur without the appearance of cholinergic symptoms. With more marked depressions, however, the symptoms are usually present; and for depressions of 50% or greater almost all of the exposed subjects show symptoms. On the other hand, there can be large depressions in individual values without concomitant symptomatology [11,22]. Thus, it appears that effective screening can be carried out even in the absence of individual baseline values.

Coye *et al.* [10] reporting on 31 workers exposed in a field recently sprayed with mevinphos, suggested that a monitoring/screening approach based on cholinergic symptoms and ChE values at the lower limits of normal could be applied. This combination testing approach could be used in a series or parallel fashion. In the series approach cholinergic symptoms would trigger ChE testing. A depressed ChE would trigger an enquiry about symptoms. In the parallel approach each probe would be used without regard for the other. A positive finding in either would call for caution and continued monitoring.

It may be shown that requiring both tests to be positive in the series approach always decreases sensitivity and increases specificity relative to the single tests. This would mean fewer false positives but more false negatives. In requiring only one test to be positive in contrast, parallel testing leads to increased sensitivity but a vastly decreased specificity, generating a large number of false positives.

Because enzyme inhibition and symptoms depend on the degree of antiChE exposure, the duration of exposure, the rate of exposure, the biochemical properties of the inhibitor, the exposure history of the subject and individual variation, no general statement can be made about percentage inhibition or number of symptoms to be used in the screen. Each exposure situation should be reviewed in the light of previous experience and the likelihood of serious toxic effects and screen 'cut-off

points' set accordingly. Once a screening programme is underway empirical evidence can be used to adjust initial perceptions.

Screening programme

Ware *et al.* [48] suggested that the most appropriate time for sampling a worker for ChE depression is 'immediately' after exposure. Kahn [27], perhaps reflecting 'real world' situations, would modify this approach and sample workers at the end of the work day. Sampling, however, should be conducted at the same hour each day to avoid what Kahn refers to as 'diurnal variation'. For CBs, sampling should begin as quickly as possible following exposure, with prompt analysis utilizing a rapid analytical method [18].

The drawing of blood, even by a trained phlebotomist, is an invasive procedure that is not entirely without risk. Workers may be reluctant to participate unless it is a requirement for continued employment. Thus, every care must be taken to ensure that informed consent procedures are scrupulously followed. Workers must be provided with a statement concerning procedures, associated risks and guidance for medical assistance, should illness occur. The issue of informed consent is particularly important in situations where English is not the first language. Interpreters should always be available to ensure that the worker fully understands the objectives of the screening programme.

In developing an effective employee screening programme there are several methodological considerations involved in field sampling of blood that must be well planned and executed. Beginning in the field, procedures must be standardized for collecting blood to avoid contamination by potential antiChEs in the ambient air, or on the skin of the donor. Blood samples should be collected in heparinized vacutainer tubes, placed in a test tube rack, kept at a temperature not to exceed 85° F for no more than 30–45 min, to allow for plasma separation. ChE enzymes are not stable at room temperature for long periods of time [52] consequently the tubes should be chilled with wet ice until the day's field work is concluded. On returning to field headquarters, the blood should be centrifuged at a standardized speed in a precooled centrifuge tube to prevent overheating and the subsequent loss of enzyme activity. Following centrifugation, the serum is collected in a separate tube and frozen with dry ice. RBC and plasma enzymes are stable for a relatively long period of time if kept near 0°C. The frozen tubes should be placed in a test tube rack and wrapped with foam rubber to prevent breakage during transport to the laboratory. Plasma and RBCs can be stored for several months in a frozen state [52].

Freezing is often a problem in the collecting and transportation of field samples, consequently a method utilizing the drying of whole blood on filter paper may be more appropriate in certain circumstances. Blood samples dried on filter paper can remain at room temperature for as much as 1 week while maintaining stability for a period of 3–5 months [3,26]. Whole blood samples require substrates specific for both RBC and plasma enzymes. Because only 20% of the RBC AChE can be extracted, only a method that will permit the reaction of this enzyme with substrate in the presence of paper (e.g. an electrometric procedure that will agitate the enzyme during assay) is suitable for use. About 3% and 10% of plasma and RBC activity, respectively, is lost using the dry filter paper approach [2].

The following supplies should be available for drawing blood. (1) Individually wrapped isopropyl alcohol (70%) swabs (used to thoroughly clean the skin of the donor before venipuncture). (2) Sterile disposable hypodermic needles. The needles must be non-toxic, non-pyrogenic and non-reactive to tissue. Needle size should be 20 or 21 G × 1 or 1 and 1/2 inches. (3) Pre-labelled 10 ml heparinized (red stopper) vacutainer collection tubes (size 100 × 16 mm). Interior of the tube should be sterile, with no additives. (4) Sterile band-aid plastic strips. (5) Two by two sterile gauze pads.

Additional supplies, such as tourniquets, test tube racks, needle cutters etc. are to be cleaned thoroughly with soap and water between screenings. It is absolutely necessary that detergents containing ChE inhibitors (e.g. quaternary ammonium compound) are not used to clean glassware. Contaminated needles and syringes are to be placed in a destruclip hypodermic safety device and in a container

specifically designed for contaminated products. Ammonia inhalants should be available for use in fainting episodes. Technicians hands should be washed with betadine.

Summary

The frequent screening of workers may not be practical, except in situations where large corporations employing a stable work force use trained health professionals to plan and conduct screening activities. Farmers with smaller acreages are likely to have difficulty in conducting screening activities on a regular basis owing to a transient and seasonal work force, and because costs may be prohibitive. Although there are several physiological factors that may result in diminished ChE activity, they are not consistent with active and full-time employment. Thus, depressed ChE activity can usually be accurately associated with OP and CB exposure. However, in any screening programme it is critical to limit the numbers of 'false positives', since an indication of excessive exposure may result in loss of employment. Although many employers may view screening as a mechanism to protect a worker from harm, and to provide evidence of no long-term health effects associated with employment, there is also concern that identifying workers with depressed ChE may suggest possible misuse practices in the storage, application and disposal of pesticide residues.

With all the difficulties in the use of ChE as an effective monitoring tool for antiChEs, a screening programme regardless of its effectiveness, should lead to improved work practices on the part of the employee, as well as an improved awareness on the part of an employer, that would tend to minimize the potential risk associated with exposure to antiChEs.

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Monitoring for exposure to anticholinesterase-inhibiting organophorsphorus and carbamate compounds by urine analysis

William J. Murray and Claire A. Franklin

Introduction

In view of the widespread use of OP and CB insecticides and their acute toxicity, it is evident that the potential risks to humans associated with their manufacture and use should be assessed before their registration. Also, is it evident that worker exposure should be monitored during use to prevent overexposure and illness. These scenarios represent rather different challenges. In the first, an estimate of potential risk requires accurate determination of the systemic dose that can be equated to doses used in laboratory animal toxicity tests. Through statistical analysis of this exposure estimate and the hazard assessment from toxicity studies, a risk estimate may be made [72]. The challenge is to devise methods that permit an accurate measure of exposure. The method should quantitatively determine total metabolite excretion or a recognized indicator compound. In either instance the relationship between the metabolite(s) and parent compound must be known and the kinetics of excretion defined so that duration of urine collection may be determined. The second scenario does not demand a quantitative estimate of exposure because the detection of pesticide metabolites in urine or a decrease in ChE activity may be sufficient to indicate worker exposure. In some situations random blood or urine samples taken at the end of the day or work week may be used to determine whether the worker has exceeded a specified level known to be associated with adverse health effects.

Quantitative exposure assessment

Ambient (environmental) monitoring

Because skin is the most significant exposure route for agricultural workers [30, 104, 143–145], many studies have been conducted using absorbent patches worn over and under clothing to estimate the skin contact exposure. It is presumed that pesticide will adhere to the patch as it would to skin. On this basis, the concentration of pesticide on the patch can be extrapolated to the relevant body surface area [92,139]. The data from all the patches would then be summed and used to represent the total skin contact exposure. This procedure provides a more accurate estimate of exposure than one obtained by only monitoring ambient air, but does not provide an estimate equivalent to the systemic dose necessary to conduct a risk assessment [42], primarily because pesticides are absorbed through the skin to variable extents [38]. Given the skin contact exposure value, there are two ways that it can be 'converted' to an estimate of systemic dose. One is to presume total absorption (100%), the second is to determine the percentage percutaneous penetration of the pesticide experimentally [47]. Although these approaches provide a reasonable estimate of exposure for agricultural applicators, they may be less acceptable when there are significant exposures through other routes. In such situations exposure via these other routes should be quantified to obtain a best estimate of the total systemic dose.

Biological monitoring

A useful alternative to ambient monitoring is biological monitoring [4]. It involves analysis of a chemical and its metabolites or biotransformation products in body tissues such as urine, blood, fat, hair, nails, sweat, saliva and expired air. It can also involve measurement of change in an indicator, e.g. enzyme activity. The major advantage of biological monitoring is that it provides an integrated estimate of exposure from all sources via all exposure routes. A possible limitation is that to be used effectively, the relationship between concentration of the chemical or its metabolites and a specific health effect or indicator must be known [5]. This has been established for some industrial chemicals [74]. For agricultural chemicals there is very little reliable data relating the levels of metabolite in urine with toxic effect or with the amount of material sprayed.

For meaningful data to be derived from a programme of biological monitoring it is necessary that: (1) the chemical and/or its metabolites are present in some tissue, body fluid, or excretion that can be sampled; (2) samples are representative of body burden of the chemical; (3) valid and practical methods of analysis are available; and (4) the results may be interpreted in a biologically meaningful way.

Changes in ChE levels

With pesticide workers, changes in ChE levels in blood have been used to indicate exposure to CBs and OPs. This type of biological monitoring for pesticides has been reviewed [2, 57, 85]. CB and OP insecticides inhibit a number of other esterases in addition to AChE [78]. Analytical methods have been developed to measure the degree of inhibition in blood and plasma [34,83,135]. With CBs, the carbamylated enzyme is unstable, regeneration of the enzyme is relatively rapid, and thus measurement of ChE activity may not be a reliable means of monitoring for exposure to CB insecticides [92,94,140]. However, significant interest exists in developing ChE inhibition as an index of exposure to OP insecticides [70,84,98, 103,108,138].

The measurement of ChE activity has both theoretical and practical disadvantages. Multiple blood samples are required before exposure to provide baseline ChE activity data for an individual as there is considerable variability in interindividual susceptibility of ChEs to inhibitors [51]. In addition, many factors can influence ChE activity from one period of testing to the next, whether the individual has been exposed to an OP or not [71,146]. Obtaining field worker compliance for blood sampling programmes is frequently a problem, as is storage of samples under field conditions [70]. The problem of within-subject variability is compounded by the difficulties encountered in standardizing ChE monitoring techniques among laboratories and even within a laboratory [29,116]. Spontaneous reactivation of the inhibited enzyme may be an additional source of considerable error [81]. Thus only large decreases (e.g. 30–40%) in RBC ChE activity are clinically meaningful [14,68,103].

Changes in urinary metabolite levels

An increasingly popular approach is the measurement of pesticide metabolites in urine, and many studies have been conducted in agricultural workers. However, many of these trials were not designed to estimate exposure quantitatively but were screening studies, providing evidence of worker exposure [99]. The collected data do not permit correlation of levels and toxic effects. Such screening studies, however, can be used to monitor exposure in a more general way and identify those workers who should be removed from further exposure if their urinary metabolite concentrations exceed a specified value. It is essential that the metabolism of pesticide be known and that good methods to measure urinary metabolite levels exist.

Metabolism

The metabolism of OP and CB insecticides is generally well understood in mammals, and both are rapidly metabolized and excreted, largely in urine [37,48,140,141]. Studies in rats and humans have examined the relationship between exposure levels of CB and OP compounds and urinary metabolite excretion [9,10,16,18,43–46,71]. In some instances these studies indicated that urinary metabolites, particularly OPs, could be detected without

Table 39.1 Summary of methodology used to measure alkyl phosphate metabolites in the urine of pesticide users

Compound	*Alkyl phosphates*[a]	*Reference*	
		Field studies	*Methodology*
Azinphos-methyl	DMP, DMTP	Kraus *et al.* [71]	Shafik *et al.* [121]
	DMP, DMTP	Richards *et al.* [109]	Shafik *et al.* [121]
	DMTP	Franklin *et al.* [43]	Shafik *et al.* [121]
	DMP, DMTP	Knaak *et al.* [67]	Knaak *et al.* [67]
	DMDTP, DMTP	Vasilic *et al.* [134]	Blair and Roderick [7]
Carbophenothion	DEP, DEDTP, DETP	Griffith and Duncan [49]	Lores and Bradway [76]
Chlorpyrifos	DEP, DETP	Davies *et al.* [22]	Shafik *et al.* [121]
		Hayes *et al.* [56]	Lores and Bradway [76]
		Lores *et al.* [77]	Lores and Bradway [76]
Demeton	DEP, DETP	Knaak *et al.* [67]	Knaak *et al.* [67]
Demeton-S-methyl	DMTP	Vasilic *et al.* [134]	Blair and Roderick [7]
Diazinon	DEP, DETP	Hayes *et al.* [56]	Lores and Bradway [76]
		Knaak *et al.* [67]	Knaak *et al.* [67]
	DETP	Weisskopf *et al.* [136]	Moody *et al.* [86]
Dichlorvos	DMP	Hayes *et al.* [56]	Lores and Bradway [76]
		Kraus *et al.* [70]	Lores and Bradway [76]
Dicrotophos	DMP	Lores *et al.* [77]	Lores and Bradway [76]
Dimethoate	DMP, DMDTP, DMTP	Knaak *et al.* [67]	Knaak *et al.* [67]
	DMP, DMTP	Kraus *et al.* [70]	Lores and Bradway [76]
Dioxathion	DEP, DEDTP, DETP	Griffith and Duncan [49]	Lores and Bradway [76]
Disulfoton	DEP, DEDTP, DETP	Brokopp *et al.* [13]	Lores and Bradway [76]
Ethion	DEP, DETP	Davies *et al.* [23]	Shafik and Peoples [123]
	DEDTP, DETP	Wojeck *et al.* [70]	Bradway *et al.* [11]
	DEP, DETP	Kraus *et al.* [70]	Lores and Bradway [76]
	DEP	Griffith and Duncan [49]	Lores and Bradway [76]
Fenitrothion	DMTP	Murray *et al.* [96]	Murray [95]
		Moody *et al.* [86]	Moody *et al.* [86]
Malathion	DMDTP, DMTP	Fenske [39]	Reid and Watts [106]
	DMP, DMDTP, DMTP	Knaak *et al.* [67]	Knaak *et al.* [67]
	DMP, DMDTP, DMTP	Griffith and Duncan [49]	Lores and Bradway [76]
	DMDTP	Drevenkar *et al.* [26]	Blair and Roderick [7]
Methidathion	DMDTP, DMTP	Vasilic *et al.* [134]	Blair and Roderick [7]
Mevinphos	DMP	Knaak *et al.* [67]	Knaak *et al.* [67]
		Davies *et al.* [22]	Shafik *et al.* [121]
Monocrotophos	DMP	Kummer and Van Sittert [73]	Blair and Roderick [7]
Naled	DMP	Kraus *et al.* [70]	Lores and Bradway [76]
		Knaak *et al.* [67]	Knaak *et al.* [67]
Parathion	DEP, DETP	Knaak *et al.* [67]	Knaak *et al.* [67]
		Kraus *et al.* [69]	Lores and Bradway [76]
		Morgan *et al.* [88]	Shafik *et al.* [121]
		Davies *et al.* [22]	Shafik *et al.* [121]
		Spear *et al.* [128]	Shafik *et al.* [121]
		Carman *et al.* [15]	Carman *et al.* [15]
Parathion-methyl	DMP, DMTP	Knaak *et al.* [67]	Knaak *et al.* [67]
	DMP, DMTP	Morgan *et al.* [88]	Shafik *et al.* [121]
Phorate	DEP, DEDTP, DETP	Knaak *et al.* [67]	Knaak *et al.* [67]
		Brokopp *et al.* [13]	Lores and Bradway [76]
Phosalone	DEP, DEDTP, DETP	Kraus *et al.* [70]	Lores and Bradway [76]
	DEDTP	Drevenkar *et al.* [27]	Blair and Roderick [7]
	DEDTP	Drevenkar *et al.* [26]	Blair and Roderick [7]
Phosmet	DMTP, DMDTP	Mount [90]	Bradway *et al.* [11]
Phosphamidon	DMP	Knaak *et al.* [67]	Knaak *et al.* [67]
Terbufos	DEP, DEDTP, DETP	Devine *et al.* [25]	Peterson *et al.* [102]

[a]Abbreviations given in Table 39.2

changes in ChE activity or clinically observable signs of toxicity [43,70,118]. As a result, there has been considerable interest in using urinary metabolites as an index of exposure [20, 28, 46, 49, 89, 117,118]. Monitoring urinary metabolites avoids the difficulties inherent in

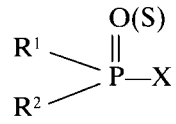
blood sampling and assessing clinical toxicity and has been shown to correlate linearly with the amount of pesticide sprayed under normal field conditions [43,46].

The purpose of the field study, whether to provide a quantitative estimate of exposure or simply to screen for evidence of exposure, determines how the samples are collected, e.g. cumulative 24-h sample or random void. If the purpose is to study exposure to a single compound under controlled conditions a method specific for metabolites of that active ingredient would be acceptable. However if the purpose is to monitor workers exposed to a variety of different actives, a method capable of determining several metabolites simultaneously is more relevant. In any case, it is essential that the analytical method used is properly validated so that the data might be considered reliable. Here we review methods that have been used, or proposed for use, by other investigators studying urinary metabolite levels of workers exposed to antiChE OP and CB insecticides as a means of monitoring exposure (Table 39.1).

Methods for analysis of urinary metabolites

OP insecticides

The chemistry and nomenclature of OP insecticides is complex, however there are excellent reviews [37,41,55,141] (*see* Chs 1 and 6). The general structure is:



There has been significant interest in developing analytical methodology for the quantitation of the principal urinary alkyl phosphates or phenolic metabolites in urine. Overall, the metabolism of OPs is such that most yield only one or more of the six common alkyl phosphates found in urine (Table 39.2) [89,137]. As many early field studies were conducted to screen workers who were potentially exposed to several different OPs, emphasis was placed on the development of methods for urinary alkyl phosphates. These procedures have also been used to provide quantitative estimates of exposure in field trials where a single active ingredient was applied. Urinary alkyl phosphate levels have been reported to correlate with the degree of exposure under controlled laboratory conditions [10,44,88,120] and in the field [43,46,142].

Table 39.2 Abbreviations of the six common alkyl phosphates found in urine and measured in field studies

Alkyl phosphate	*Abbreviation*
O,O-dimethyl phosphate	DMP
O,O-diethyl phosphate	DEP
O,O-dimethyl phosphorothioate	DMTP
O,O-diethyl phosphorothioate	DETP
O,O-dimethyl phosphorodithioate	DMDTP
O,O-diethyl phosphorodithioate	DEDTP

The urinary phenolic metabolites are for the most part compound specific and detection of these allows identification of the particular pesticide to which a person was exposed. This approach is acceptable in screening programmes where exposure is limited to a few carefully defined compounds; it has, however, presented difficulty in the development of multiphenol screening procedures [20,89]. The specificity of this approach has an advantage over alkyl phosphate analysis in the assessment of risk as it permits a quantitative estimate of exposure to a particular pesticide in a spray programme involving several active ingredients.

Phenols

Comparatively few procedures have been developed for the analysis of urinary phenolic metabolites of OPs (Table 39.1). The principal reason for this is that in most instances the phenolic metabolites are compound specific. In most occupational situations agricultural workers are potentially exposed to a range of products, so the development of a comprehensive multiphenol analytical procedure as a means of screening for exposure to OPs is an almost impossible task [89].

One approach investigated by Shafik *et al.* [124–126] was the development of a multiresidue procedure for the analysis of urinary halo and nitrophenol metabolites of a select group of compounds containing these substituents in their molecular structure. This method was validated for the analysis of a

range of urinary phenol metabolites including 2,4,5-trichlorophenol, 2,4-dichlorophenol, p-chlorothiophenol, p-nitrophenol, p-nitrocresol, 2,5 dichlorobromophenol and 3,5,6-trichloropyridinol, in rat urine following oral administration of selected OPs [10,126].

The usefulness of this approach as a general screening method for exposure to antiChE products is questionable for not only must it be validated for the specific phenolic metabolite of each product to be monitored, but urinary residues of chlorophenols or nitrophenols are not limited to metabolites of OP insecticides. Interfering residues can result from intact nitrophenolic herbicides (e.g. 2,4-dinitrophenol, dinitro-o-cresol, 2-sec-butyl-4,6-dinitrophenol), certain ubiquitous environmental contaminants such as pentachlorophenol as well as chlorinated phenol metabolites of other products such as lindane and 2,4,5-T [20]. In most instances analytical methods for phenolic urinary metabolites have been developed on a product specific basis in response to a particular need.

Significant interest has been shown in the development of analytical methods for the para-nitrophenol (PNP) metabolites of the parathion group of insecticides, as a result of their widespread use and acute toxicity. Elliot *et al.* [33] developed a colorimetric procedure, in which urine is hydrolyzed with acid, extracted and a blue colour developed with the reagent o-cresol in basic media. Cranmer [21] reported a method which employed hydrolysis and extraction techniques similar to Elliot *et al.* [33] but utilized on-column formation of the trimethylsilyl ether derivative of PNP for quantitation by electron capture gas chromatography. Application of the multiresidue method developed by Shafik *et al.* [126] to the analysis of PNP was reported to be superior to the method of Elliot *et al.* [33] in specificity and simplicity and to that of Cranmer [21] in quantitative reproducibility [88]. As a result, this method has found widespread use (Table 39.1). A procedure for confirmation of PNP in human urine as determined by this multiphenol method was reported by Kirby *et al.* [66]. A different approach to PNP analysis has been developed by Michalke [82] in which urine samples are hydrolyzed, extracted with ether, redissolved in methanol and quantitated directly by high pressure liquid chromatography with a UV detector (HPLC/UV). This technique offers significant advantages over earlier methods in that it uses only very small volumes of urine (0.5 ml compared with 3 ml), is more sensitive (5 ppb compared with 10–50 ppb) and perhaps most importantly, it involves a shorter analysis time.

Other analytical methods have been developed for specific products. A gas chromatographic procedure for the analysis of the urinary phenolic metabolite of chlorpyrifos, 3,5,6-trichloro-2-pyridinol (trimethylsilyl derivative) was reported by Nolan *et al.* [100]. Eadsforth [31] proposed a method for monitoring human exposure to chlorfenvinphos that, instead of selecting a single urinary metabolite as an indicator of exposure, degraded a mixture of metabolites to a single analysis product, 2,4-dichlorobenzoic acid which following methylation was quantitated using a gas chromatograph equipped with an electron capture detector (GC/ECD).

It is evident that analysis of urinary phenolic metabolites as a screening procedure for exposure to OPs has only limited opportunity for success. However, because of its specificity it may be that there should be increased emphasis on the development of this analytical approach as a means of determining quantitative estimates of exposure for use in risk assessment.

Alkyl phosphates

A method for the analysis of alkyl phosphates was first reported in 1968 [113] and subsequently modified for application to human blood and urine [122]. This procedure required both methylation and ethylation of each sample to obtain separation of the analytes from interfering coextractants, primarily inorganic phosphate. Sensitivity was improved with the development of a silica gel clean-up procedure which removed the majority of the inorganic phosphate [120]. In 1973 Shafik modified this method once again producing the amyl derivatives instead of the methyl or ethyl derivatives [121]. When coupled with the silica gel clean-up it was possible to determine the six principal urinary alkyl phosphate metabolites (Table 39.2) This procedure was for many years the basic method for the deter-

mination of urinary alkyl phosphates and has been widely quoted in the literature (Table 39.1). Lores and Bradway [76] reported a simplified version of this method involving precipitation of some urinary interferences with acetone, extraction of the alkyl phosphates with ion exchange resin, derivatization with diazopentane and quantitation via GC/ECD without further clean-up. In situations where the level of metabolites is too low for quantitation it is recommended that the sample be concentrated and a silica gel column clean-up included before GC analysis. Blair and Roderick [7] published a new procedure for the analysis of urinary dimethyl phosphate, which selectively removes inorganic phosphate from the urine through pretreatment with calcium hydroxide. The urine is reacidified with a cation exchange resin, treated with diazoethane and the ethyl dimethyl phosphate produced determined by GC/FPD without further clean-up. The flame photometric detector (FPD) is a phosphorus selective detector and as such avoids many of the interferences experienced with other less selective GC detectors.

Through years of use, these three methods have come to represent the accepted standard, unfortunately, there are a number of serious limitations with them. They are tedious, lack reproducible and high extraction efficiencies, and are characterized by high background interference [50,60,71,88,137]. Alkylation of alkyl phosphorothioates (e.g. DMTP, DETP) with diazoalkanes produces two isomeric products in irregular proportions resulting in a limit of detection higher than if a single product were formed [12,62,119]. In addition, diazoalkanes and their nitrosoguanidine precursors represent an unacceptable toxic and explosive hazard to the analyst [12].

Alternative methods developed have involved different approaches to the derivatization step. Churchill *et al.* [17] and Moody *et al.* [86] used on column methylation with trimethylanilinium hydroxide for the analysis of sulphur-containing alkyl phosphates by GC/FPD. Moody *et al.* [86] reported production of a single derivatized product for DMTP and minimal background interference from inorganic phosphate such that no clean-up of the initial ethyl acetate extract of urine was required. Elimination of the clean-up column and diazoalkane preparation significantly reduces sample handling and suggests that a significant increase in productivity should be attained through use of this procedure. Weisskopf *et al.* [136] adapted this method to monitor DETP in the urine of workers exposed to diazinon. They reported some difficulties, however, involving low recoveries from dilute urine (e.g. 44%) and incomplete derivatization with concentrated urine samples. For most samples, recoveries ranged from 85% to 100% at fortification levels of 8 ng DETP or higher per ml of urine; however, DETP recovered from all samples fortified at 4 ng/ml was less than 38%. In addition, this method was found to be inadequate for DEP analysis because of interferences, recovery problems and inefficient derivatization.

Virtually all of the analytical methods available for alkyl phosphate analysis considered so far involve a solvent extraction step. As a general rule the extraction of polar hydrophilic compounds such as alkyl phosphates is poor and the selectivity is low, requiring extensive clean-up of the extracts. Where it has been reported, the recovery of these compounds is frequently much less efficient than would be expected based on the published literature [60,88,136,137].

There have been a number of different approaches taken to the problem of low and erratic recoveries. Weisskopff and Seiber [137] developed a GC method suitable for analysis of the four common sulphur containing alkyl phosphate metabolites (Table 39.2). This procedure employs disposable solid phase (cyclohexyl) extraction cartridges to recover analytes from urine and selective elution to eliminate some interfering coextractants. Trimethylammonium hydroxide is used for on-column methylation. Improved separation and quantitation of the four sulphur-containing alkyl phosphates was achieved through the use of a wide bore capillary column connected to an FPD. This procedure overcomes many of the obstacles encountered in conventional approaches to urinary metabolite analysis in that it is rapid, reproducible and sensitive with a minimum detection level of 2 ppb. The principal draw back is that it is not suitable for the analysis of the non-sulphur-containing metabolites DMP or DEP. The methylation yield of DMP could not be determined because of

interference from inorganic phosphate, while the recovery of DEP averaged only 30±11%. These authors report that preliminary results indicate that these difficulties may be overcome without a significant increase in extraction or analysis time.

Bradway *et al.* [8] reported on the potential applicability of an ion-pair extractive alkylation technique to urinary alkyl phosphate analysis. The theoretical basis and potential applications of extractive alkylation have been summarized in several reviews [61, 114, 115, 127]. Ion-pair extractive alkylation has been applied successfully to analysis of trace levels of pharmaceutical compounds in complex biological matrices [32, 54, 111, 112, 129]. In 1981 Bradway *et al.* [11] outlined an extractive alkylation procedure that was under investigation in their laboratory for the analysis of the six most common urinary alkyl phosphate metabolites (Table 39.2), in which the alkyl phosphates are extracted as an ion-pair with a lipophilic quaternary ammonium cation into methylene chloride. The organic phase contains the alkylating agent pentafluorobenzyl bromide and the alkyl phosphate is derivatized *in situ*. An aliquot of this solution is injected directly for quantitation by GC/FPD. The procedure was described as having several potential advantages, but contained preliminary results only, and few details of the analytical procedure were provided. The authors concluded that extraction of DEP and DMP was incomplete and while recoveries of the remaining four alkylphosphates were adequate (unspecified) there were a number of problems. This procedure was used by Mount [90] to measure DMDTP and DMTP in urine of goats exposed to phosmet. It was concluded that urinary DMDTP was a sensitive index of phosmet exposure. However, apart from stating that some alkyl phosphates were not easily identified by the method employed, no validation data for the recovery or reproducibility of DMDTP or DMTP analysis in urine were provided. Similarly, Wojeck *et al.* [142] used this technique for the analysis of DETP and DEDTP in urine of workers exposed to ethion without comment on the effectiveness of the method of providing supportive validation data.

The extractive alkylation approach was further investigated with the development of a method for the analysis of urinary DMTP [95]. In this procedure DMTP was extracted as an ion-pair with tetrabutyl ammonium ion into methylene chloride which contained ethyl iodide as alkylating agent. Aliquots of the organic layer were passed through a silica seppac to remove excess reagents and reaction byproducts as they have been reported to interfere with GC quantitation [32,131]. The eluate was then analysed by GC/FPD and the nature of the reaction product O,O-dimethyl S-ethyl phosphorothioate confirmed by comparison to synthetic standard. Derivatized samples were stable at room temperature for several days which facilitated automation of the GC analysis. The extractive alkylation approach to urinary alkyl phosphate analysis has a number of potential advantages over earlier methods. The increased selectivity of the extraction technique results in minimal chromatographic interference and permits more rapid processing of samples. The reagents used present no significant hazard and are stable. In addition, a single derivatized product is obtained for sulphur containing alkyl phosphates (DETP and DMTP). Perhaps most importantly the reproducibility and reliability of this technique appears to be superior to traditional solvent based procedures. The main short-coming of this approach appears to be that it is not amenable to the analysis of the non-sulphur containing alkyl phosphates DEP and DMP. Further investigation in the area of different alkylating reagents and counter-ions is required.

Reid and Watts [106] described a new approach to the analysis of urinary alkyl phosphates. The problems associated with incomplete extraction are circumvented by removing water through azeotropic distillation with acetonitrile. The residue is then derivatized directly with pentafluorobenzyl bromide to form the pentafluorobenzyl esters of the alkyl phosphates. This procedure reportedly produces a single alkylation product, the thiolate esters, for each of DMTP and DETP. A GC equipped with a flame photometric detector is used for the determination step. The method was provided in two forms: a rapid screening procedure and a more rigorous quantitative procedure involving separation of the six common alkyl phosphate metabolites into thio and non-thio fractions. The reason was that alkylation of the alkyl phosphates

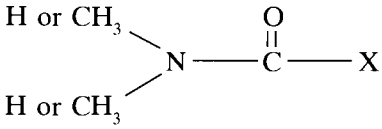
DEP and DMP proceeds in good yield only at elevated temperatures (90°C). These conditions result in degradation of 10–25 % of the sulphur-containing alkyl phosphates to their non-sulphur containing analogues DEP and DMP. The recovery of the six alkyl phosphates (Table 39.2) from urine fortified at 0.8 μg/ml was reported to be 91–102 % while minimum detectable amounts ranged from 0.04 to 0.13 μg/ml.

Peterson *et al.* [102] utilized a slightly modified version of this screening procedure for the analysis of DEP, DETP, and DEDTP in urine. This method was then applied in a field study investigating occupational exposure to terbufos [25]. As predicted by Reid and Watts [106], the recovery of DEP from the mixed standard was widely variable (e.g. 85–138%, 78–119%, and 76–120%) at all fortification levels (0.1, 0.2, 0.5 μg/ml) and for DEPT (66–106%) at the lowest fortification level 0.1 μg/ml. These values would be too variable for a quantitative estimate of absorption, but the intention was to obtain an indication of absorption of the chemical. The authors reported a validated sensitivity of 0.1 μg/ml for each alkyl phosphate analysed.

It would appear that liquid chromatography would be ideally suited to the analysis of these compounds, but work in this area has been limited by a lack of sufficiently selective and sensitive detection. Priebe and Howell [105], have proposed a method for the analysis of DEP and DMP by liquid chromatography using a post-column reaction detection system. The procedure is straightforward; sample pretreatment involves precipitation of orthophosphate and the adjustment of the pH to 4.75. Aliquots of the urine sample are injected directly on the liquid chromatograph operated in an ion-pairing separation mode. In the post-column reaction, analytes are quantitatively degraded to orthophosphate, reacted with a chromogenic reagent and the absorbance monitored at 850 nm. Recoveries for DMP and DEP were reportedly 101–102% for urine fortified at 2–11 μg/ml with detection limits of 0.3 and 0.8 μg/ml for DMP and DEP, respectively. This procedure would appear to be one of the most straightforward for the analysis of urinary alkyl phosphates, however, the requirement of a custom built post-column reaction detection system precludes its general applicability.

CBs

CB insecticides are usually esters of mono or dimethyl carbamic acid:



As with OPs, X may be one of a wide variety of groups linked to the carbonyl carbon atom via a labile ester or oxime ester bond. Complex labile groups may be attached to the alkyl group to form an inactive precursor which is metabolized to the proximal toxin *in vivo*. Most commercial CBs are phenyl N-methyl CBs, i.e. CB esters derived from phenols [19,87]. This has resulted in limited development of screening methods for urinary phenol metabolites of CB insecticides. There are two principal approaches reported in the literature, one involving the preparation of the chloracetate esters [3,124], the other formation of pentafluorobenzyl ethers [63, 64, 118]. Neither would appear to have gained widespread acceptance or use.

Drevenkar *et al.* [27] proposed a GC method for analysis of carbofuran and its urinary metabolites containing the N-methyl CB group based on a method originally proposed for crop residues [91]. This technique involves solvent extraction of the urine sample and on-column transesterification with methanol, of the N-methyl CBs to methyl-N-methyl CB. Although it is recognized that this technique monitors a significantly smaller portion of the urinary metabolite load as compared to phenol compounds, its relative simplicity and speed of analysis suggest it may, in certain instances, be useful in screening for exposure to N-methyl CB pesticides. Some further work is necessary to investigate the effectiveness of the initial extraction process and to expand the use of this procedure to other N-methyl CB compounds.

Most available analytical methodology has been developed for urinary metabolites of specific CBs, for use in controlled field studies. An example is 1-naphthol the principal urinary metabolite of carbaryl. Shafik *et al.* [124] developed a method for this metabolite based on the chloracetylating procedure of Argauer [3]. This procedure was more sensitive and

specific than the earlier colorimetric technique reported by Carpenter *et al.* [16]. This method is typical of those available in that it involves acid hydrolysis, solvent extraction, derivatization and column clean-up before GC quantitation. This procedure was used by Comer *et al.* [18] in estimating the exposure of formulating plant workers to carbaryl.

More recently methods employing HPLC have been developed. DeBerardinis and Wargin [24] reported a method for determining carbaryl and 1-naphthol in blood. This technique is more rapid than traditional GC-based procedures as samples were haemolyzed, extracted, reconstituted in mobile phase and directly injected on the HPLC. The authors stated that application of this protocol to urine was equally effective. Subsequently Keiser *et al.* [65] reported improvements in HPLC mobile phase optimizing separation of urinary phenolic metabolites of carbofuran (3-keto carbofuran phenol, carbofuran phenol), propoxur (2-isopropoxy phenol), and carbaryl (1-naphthol).

Overall, as a result of the significantly lower acute toxic hazard presented by CB insecticides and their individual urinary metabolite profiles, few methods for the analysis of urinary metabolites have been widely adapted for use as biological indices of exposure. A World Health Organization (WHO) task force on CB pesticides [140] concluded that further work be done to develop more adequate analytical methods to determine CB residues in biological materials.

Immunoassays for monitoring exposure

Although sensitive and specific, gas chromatography-based techniques generally involve time-consuming isolation and clean-up procedures, expensive analytical instruments and highly trained personnel. Such problems have motivated the search for low-cost, rapid and automated methods of analysis. Immunochemical assays appear to satisfy these criteria and while extensively used in clinical laboratories, have only in recent years been the subject of increased interest by pesticide chemists. Reviews concerning the application of immunochemical technology to pesticide residue analysis have been published [52, 53, 93,133]. Assays have been developed for OPs such as parathion [36] and paraoxon [12 ,59] in body fluids, as well as for CBs including aldicarb [93], benomyl and carbendazim on food crops and environmental samples [79,97].

These assays have advantages over traditional analytical techniques in that they require minimal sample clean-up and permit high sample through-put. A limitation however is that any one antibody will only permit detection of a very limited set of structurally similar cross-reacting residues. Thus, these techniques are best suited to screening large numbers of samples for a limited number of compounds. Recently, there has been considerable interest in developing immunoassays for trace analysis of pesticides and waste chemicals in the environment and in the urine of exposed individuals [133]. The utilization of genetic engineering for development of monoclonal antibodies should greatly enhance the possibilities for immunochemistry techniques.

Immunoassays for the urinary phenol metabolites of OPs or CBs would find ready application in field research studies estimating worker exposure or in routine monitoring programmes conducted in pesticide plants.

Use of urinary metabolite data for exposure estimation

There is considerable interest in the development of biological monitoring techniques such as urinary metabolites to allow more comprehensive assessments of the human health risk associated with occupational exposure to OP and CB insecticides [20, 75, 110]. However, development of guidelines for assessment of occupational exposure has been significantly slower than those for assessing toxicological hazards. Lack of standardization has led to a myriad of different techniques for monitoring exposure in response to individual situations.

A protocol entitled *Survey of Exposure to Organophosphorus Pesticides in Agriculture* published by the WHO in 1975 [138] was one of the first attempts to standardize exposure assessments internationally. This protocol outlined methodology for the estimation of the contact dose through the use of absorbent patches and respirators and how and when to monitor the activity of ChE in blood. This protocol was

revised in 1982 and the bias towards OPs removed as the concept of biological monitoring was expanded to include measurement of urinary metabolites [139]. This WHO protocol has been used as the basis for development of similar protocols by the US National Agricultural Chemicals Association [58,92] and the US EPA [35,107]. These protocols remain heavily oriented to the use of patches, which although recognized as imperfect, have gained favour as they are readily standardized and applied to any agricultural chemical. The fact that the method has not really been assessed for accuracy of the estimate of true exposure has been discussed by Fenske [40].

For quantitative estimates of exposure to be developed based on urinary metabolite levels a significant amount of information must be known about the chemical agent including adequate supporting data defining the absorption, metabolism, pharmacokinetics and excretion of the parent compound/metabolites in humans or an appropriate experimental animal [46,75]. In general, advice in these protocols is limited to pointing out the importance of cumulative 24-h urine samples and appropriate methods for their collection, storage and handling. The selection of an appropriate analytical method is left to the discretion of the investigator (as biological monitoring is for the most part considered to be compound specific). The WHO protocol [139] includes a list of some pesticides and the relevant metabolites in blood and urine, however, no recommendation is made regarding preferred methods of analysis.

In general much work remains to be completed before urinary metabolite levels are used routinely to provide quantitative estimates of exposure. In the meantime, they have been used successfully to determine if workers were exposed to a particular agent or class of agents, e.g. OPs and to assess the effectiveness of protective clothing or modified work practices. They may find application in the manufacturing setting or for monitoring re-entry exposure to prevent over exposure [46].

Conclusions

A number of analytical methods have been developed for urinary alkyl phosphates. The ideal method should be rapid, simple, amenable to the routine analysis of large numbers of samples, and possess the sensitivity, specificity and precision required for residue analysis. The development of a single procedure for the analysis of the six common alkyl phosphate metabolites would be highly desirable.

The methods developed by Shafik *et al.* [121], Blair and Roderick [7] and Lores and Bradway [76] are capable of measuring all six alkyl phosphate metabolites and have had widespread use. Criticisms of these procedures are that they are long, use hazardous materials, produce isomeric products for DMTP and DETP, and are prone to low and erratic recoveries in the hands of inexperienced analysts. Despite these documented shortcomings, the method of Shafik *et al.* [121] has been adequately validated [137] and successfully used in the development of exposure estimates based on urinary metabolite excretion [43,46].

The principal problem in considering the available alternative procedures is that in most instances they have not had any significant use in independent laboratories, making an assessment of their overall reproducibility and reliability difficult. The method proposed by Weisskopff and Seiber [137] would, according to their conclusions, appear to be the most rapid and reliable available. Unfortunately this procedure does not detect DMP and gives only marginal recovery of DEP, although the authors state that work is underway to address these deficiencies. The quantitative method proposed by Reid and Watts [106] is reliable, capable of quantitating all six alkyl phosphate metabolites and has been validated by Weisskopf and Seiber [137]. It is a long method for routine quantitative analysis, but the screening procedure may be worthy of further consideration in monitoring studies. The extractive alkylation approach shows promise but requires further development/study with respect to its ability to monitor non-sulphur-containing alkyl phosphates. Similarly, the application of ion-pair HPLC appears to be ideally suited to analysis of alkyl phosphate metabolites in urine as it largely avoids the principal problem steps of extraction and derivatization associated with traditional methodology.

The rapid development of immunochemistry using monoclonal antibody technology is an

exciting new area and may ultimately provide a rapid, reliable and inexpensive alternative to existing methods.

In reviewing the literature it is often difficult to determine whether some investigators have validated their methodology adequately. They either ignore this aspect of their investigation or fail to document it. This precludes a valid assessment of acceptability of the methodology or data generated. That progress may be made in developing urinary alkyl phosphate metabolites as a quantitative measure of exposure to OP insecticides it is necessary for increased attention to be given to the extent of the variability in precision and accuracy of current analytical methods. It is noted that such concerns are not limited to urinary alkyl phosphate analysis as similar problems in the quality of analyses of toxic chemicals in body fluids have been discussed previously [1]. One solution might be the development of a recommended or standard method of analysis. Some initial steps have been taken in this area under the WHO/UNEP epidemiological study on the health effects of exposure to OPs. However, there was some uncertainty as to the analytical methods to be considered for standardization [80]. Development of a standardized method of evaluation will require the establishment of an interlaboratory collaborative study to evaluate current methods and the development of an appropriate analytical quality assurance programme to maintain an acceptable level of reliability. Some of the problems associated with the development of an effective quality control programme for the analysis of organic compounds in urine were reported by Valkonen *et al.* [132] and demonstrate the need for a permanent programme of this type.

The establishment of an easy to use, reliably accurate and reproducible analytical method would greatly facilitate further investigation of the relationship between urinary alkyl phosphate levels and existing toxicology data.

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Neurophysiological monitors of anticholinesterase exposure

U.K. Misra

Introduction

Electrochemical changes produced by antiChEs can be monitored by neurophysiological techniques for use in documenting or diagnosing acute poisonings, occupational exposure and monitoring the effects of antiChE drugs. CNS effects can be monitored by EEG and evoked potential studies, and the peripheral effects by EMG and nerve conduction studies.

Neurophysiological monitoring of antiChE effects stems from the examination of patients with myasthenia gravis treated with antiChE drugs. This has revealed that the EMG response on nerve stimulation can be used to facilitate the diagnosis and control of antiChE therapy [75]. Subsequently neurophysiological observations were extended to cases of acute antiChE poisoning [94] and occupational exposure [24,37,39,42,61,73,82].

Basis of neurophysiological monitoring

The widespread distribution of cholinergic synapses in the central and peripheral nervous system make for a complexity of responses. Neurophysiological techniques have been mainly used in the evaluation of the effects of antiChEs on the NM junction, peripheral nerves and brain.

NM junction

In the presynaptic motor nerve endings are synaptic vesicles, each containing up to 10 000 ACh molecules. At rest these vesicles are randomly released into the junctional cleft causing miniature endplate potentials (MEPP) which do not produce a muscle action potential.

Action potentials propogating down the motor axon depolarize the presynaptic nerve terminal, triggering an influx of Ca^{2+}, initiating calcium- dependent release of ACh into the junctional cleft. Binding of ACh to postjunctional ACh receptors results in depolarization of muscle fibres producing an endplate potential (EPP). The depolarization of muscle fibre generates an action potential that propogates in both directions in the muscle fibre away from the endplate leading to contraction.

CNS

Recurrent excitation of spinal Renshaw neurons was found to be sensitive to nicotinic cholinergic antagonists; these cells are cholinoceptive. Although the ability of ACh to elicit neuronal discharge has been reproduced in a number of CNS cells [25], in most regions of the CNS the effects of ACh appear to be generated by a mixture of muscarinic and nicotinic receptors [48]. Prominent cholinergic circuits in cerebral, limbic and thalamic regions have been reported. They include long divergent and local circuit connections. Many actions of ACh on a number of CNS test cells are reversed by muscarinic antagonists including those in cerebrocortical and hippocampal pyramidal neurons [84].

ACh is rapidly hydrolyzed by AChE so that the life time of free ACh within the synapse (approximately 200 microseconds) is shorter than the decay of EPP or the refractory period of the muscle [19]. After inhibition of AChE the persistence of ACh at the synapse increases.

During this period ACh binds to multiple receptors resulting in the prolongation of the decay of the EPP (about three times) owing to successive stimulation of the neighbouring receptors. The quanta of ACh released by an individual nerve impulse are no longer isolated. This leads to desynchronization of endplate depolarization and development of action potentials, and therefore asynchronous excitation and fibrillation of muscle fibres is observed. Persistence of ACh in the synapse may also depolarize the axon terminal, resulting in antidromic firing of motoneurons which contributes to fasciculations [87].

Some OPs inhibit NTE and produce delayed onset peripheral neuropathy in which the pattern of denervation is related mainly to the fibre length and to some extent to fibre diameter. Cell bodies do not show primary morphological change but respond by a chromatolytic action [16] (*see* Ch.10).

Neurophysiological techniques for monitoring the effects of antiChE agents

The effects of antiChEs on the PNS can be evaluated by EMG, nerve conduction studies, NM transmission studies, single fibre electromyography and micro electrode studies. The effects on the CNS can be studied by EEG and evoked potential studies.

Techniques for studying the peripheral effects

Electromyography

Electromyography involves inserting a recording needle electrode in the muscle. Electrical activity is recorded extracellularly from the muscle fibres of a motor unit. The bioelectric signals are amplified, and displayed on an oscilloscope for visual analysis and fed to a loudspeaker for acoustic monitoring. The motor unit potentials thus recorded are summated potentials of muscle fibres in a motor unit within a few mm of the electrode. EMG signals are analysed for insertional activity, spontaneous activity, motor unit potential (MUP) analysis, recruitment pattern on mild voluntary muscle contraction and on strong contraction (interference pattern) analysis [43]. Fibrillations and positive sharp waves are recorded from denervated or partially denervated muscles or in certain myopathies, e.g. polymyositis. Fasciculation is another type of spontaneous activity which occurs in various physiological and pathological conditions. The MUPs are analysed for their duration and amplitude. In neurogenic disorders the duration of the MUP and also the amplitude may increase, whereas in myopathies both the duration and the amplitude may decrease. In both neurogenic and myopathic disorders the proportion of polyphasic MUPs may be increased. The pattern of maximum voluntary contraction may be affected in neurogenic disorders. In denervation, the number of recruitable MUPs decreases, but the muscle force is usually greater than the amount of activated motor units. In myopathies many units may be activated during a weak contraction resulting in interference pattern. In routine clinical practice the EMG is analysed qualitatively. Quantitative EMG analysis can be done manually or using a computer [78].

Nerve conduction studies

Nerve conduction studies help in assessing motor and sensory functions by recording the evoked response to stimulation of a peripheral nerve.

Motor nerve conduction

Motor nerve function is studied by stimulating the nerve and recording the compound motor action potential (CMAP) from the muscle. The standard stimulus is a supramaximal square wave pulse, thus evaluating the fastest conducting fibres. Surface or needle recording electrodes are placed on the muscle. The evoked CMAP is described by latency, amplitude, duration and configuration. The area of the negative phase of the CMAP is proportional to the number of muscle fibres depolarized; however, if the duration remains constant, then the amplitude can provide the same information. For calculation of the nerve conduction velocity, the nerve is stimulated at two sites to obtain two CMAPs [20].

F response

Nerve stimulation propogates distally (orthodromically) and proximally (antidromically) towards the anterior horn cells. By supramaximal stimulation the anterior horn cells can be activated along the axon. The recurrent discharge produces small potentials after a delay of 20–50 ms; these are the F response [54]. They provide information about conduction in the proximal segment of motor fibres, and may be a more sensitive indicator of peripheral nerve dysfunction than routine nerve conduction studies [50].

Sensory nerve conduction

Evaluation of the sensory nerve can be performed by stimulating and recording from a cutaneous nerve, recording from a cutaneous nerve after stimulating a mixed nerve, or recording from a mixed nerve after stimulating a cutaneous nerve. They are more sensitive than motor conduction in detecting early or mild disorders. Orthodromic or antidromic stimuli can be used to evoke action potentials. Both surface and needle electrodes are used for recording. A compound nerve action potential (CNAP) is measured in the same manner as a CMAP. The amplitude of CNAP provides an estimate of total number of fibres stimulated. The sensory conduction velocity is calculated as for motor conduction velocity and is generally faster [20].

In axonal neuropathies the major change is reduction in the amplitude of motor and sensory action potentials and is proportional to the severity of the disease. The latency of conduction velocity is little affected, because conduction in individual axons remains normal until axons have degenerated. Often the only finding in axonal neuropathy may be the presence of fibrillations in distal muscles, with or without low amplitude or evoked responses. Because the axonal neuropathies commonly affect the largest axons first signs are manifest initially in the lower extremities [20].

Segmental demyelinative neuropathies are associated with prolonged latencies and marked slowing of nerve conduction velocity. There is relative preservation of amplitude on distal stimulation, but a progressive reduction of amplitude and dispersion of evoked response on proximal stimulation [20].

Repetitive nerve stimulation techniques for assessment of NM transmission

In repetitive nerve stimulation, a muscle is stimulated by supramaximal stimuli through its motor nerve. A decline in the amplitude of the evoked potential provides a measure of the degree of NM block. To avoid movement-related artefacts, the extremity is firmly immobilized and the nerve is stimulated as distally as possibly. The limb should be warmed if the skin temperature is below 34°C to avoid false negative results. Artefacts are recognized by abrupt or irregular changes in the amplitude or the shape of the CMAP, whereas reliable responses show smooth and progressive alterations. Technically the distal muscles are easier and more reliable to measure, but a little less sensitive than the proximal muscles.

Generally 2–3 Hz rate of stimulation is considered most effective [23]; it is fast enough to deplete the immediately available store of ACh but slow enough to avoid superimposed facilitation from the neurosecretory mechanisms. The percentage reduction in the amplitude of CMAP is calculated for the smallest of the five responses and compared with the first in the same train. In normal persons, decrements at 2–3 Hz rate of stimulation, if present, do not exceed 5–8% [80]. The presence of reproducible decrement should be considered suspicious.

In normal muscles the size of action potentials is stable during repetitive stimulation up to 20–30 Hz [65]; both incremental and decremental responses have been reported at 50 Hz. However, assessment of amplitude changes at such a fast rate is often rendered unreliable because of movement artefacts. Most subjects can activate muscle potentials up to 50 Hz; therefore, the same effect can be achieved by voluntary effort for 10 s to 1 min [43]. A typical postactivation cycle following voluntary effort or nerve stimulation at high rate includes post-tetanic potentiation [38] lasting for 2 min, followed by post-tetanic exhaustion [23] lasting up to 15 min, after which the response returns to the original level.

Quantitative assessment of NM transmission is also possible by the use of paired stimuli. In normal muscle, the first supramaximal stimulus activates the entire group of muscle fibres. The CMAP elicited by a second stimulus

within a few ms is decreased owing to the failure of the NM junction, and the refractory period of the nerve and muscle.

In most patients with myasthenia gravis, the responses at short interstimulus intervals are normal, but in conditions with defective ACh release at very short interstimulus intervals (up to 15 ms) there is a one and a half to twofold increase in the amplitude of the second response if the first response is submaximal. The paired stimuli with longer interstimulus intervals (more than 15 ms) are effective in eliciting decremental response. The decrement begins at the interval of about 20 ms but the maximum depression occurs at the interstimulus interval of about 300–500 ms [43].

Single fibre electromyography

A special electrode having a small recording area on the side is used. Specificity of the recording is achieved by the small recording area of the recording electrode and using a high pass filter at 500 Hz. A single fibre electrode records from individual muscle fibres within 300 μm of the electrode. A single fibre potential is a biphasic potential with a rise time of the negative phase of <200 μs, duration <1 ms, and amplitude usually 5–10 mV. With the help of a trigger and signal delay unit, single fibre potentials can be displayed on a series of sweeps. Generally, potentials can be recorded from one muscle fibre in a motor unit, but pairs of potentials from the same unit in a time locked manner occur in about 35% of the electrode placements. Fibre density is the mean number of single fibre potentials recorded from motor units in at least 20 recording sites; it normally averages 1.4–1.5 [3]. Fibre density reflects the density of muscle fibres in one motor unit within the recording area. Fibre density increases in neurogenic disorders with reinnervation, and also in myopathies owing to fibre splitting and regeneration. When two or more single fibre potentials are recorded from a motor unit, the interpotential interval shows a small variation called jitter. Jitter is the result of variation in the NM transmission time in the two motor endplates involved. It is a very sensitive indicator of NM transmission abnormality. Details of the theoretical and the technical aspects of single fibre EMG are provided elsewhere [27,82].

Microelectrode methods

Membrane potentials are measured by inserting one electrode inside the muscle cell and measuring the potential difference between it and an extracellular electrode. The potential between the two electrodes is applied to an input coupler connected to a measuring device [51,55]. Membrane time constant, critical membrane depolarization, and threshold stimulating current are obtained by intracellular stimulation myography. The muscle fibre is stimulated through an intracellular electrode and the evoked changes in the membrane potentials are recorded simultaneously with the same electrode [7,52].

Electrophysiological studies for NM transmission use flow of current through ACh receptor (AChR) channels (endplate current) or endplate potential (EPP). For presynaptic studies, endplate current or EPP is used as an assay for transmitter released [22]. For postsynaptic studies, endplate current is used to measure the time course of conductance change at the endplate [86]. In classical NM preparations the nerve is stimulated with a short pulse (0.1 ms) and the resulting EPP recorded by a microelectrode in the endplate region. In these recordings miniature endplate potential is also recorded. Endplate current has been measured by a voltage clamp technique [28]. The current flowing through a single AChR has been measured. For this a micropipette, with a tip of few μm containing a bathing solution and an agonist, is pushed against the surface of muscle fibre. A low noise amplifier connected to the pipette measures the current flowing through the channel in the membrane patch under the pipette [64].

Techniques for the evaluation of central effects

Electroencephalography (EEG)

EEG waves are summated synaptic potentials generated by the pyramidal cells of the cerebral cortex, and these synaptic potentials are the responses of cortical cells to rhythmical changes from thalamic nuclei [41]. In normal conscious humans the dominant rhythm is alpha (8–13 Hz), recorded in the parieto-occipital region, and attenuated by

visual attention or mental activity. Its rhythmicity lies in a subcortical pacemaker [41]. Faster waves with a frequency of >13 Hz and amplitude of 10–20 μV, are called beta and are present in the frontal region. Theta waves have a frequency range of 4–7 Hz. Very slow waves (delta 4 Hz), spikes or other unusual patterns are absent in normal records. The stages of quantitative EEG analysis include: signal conditioning in which the signals below 1 Hz and above 50 Hz are filtered and the analogue EEG signals are converted to digital representations which are stored in a computer. This is followed by primary analysis, in which the EEG signal is broken into its constituents. Thus, the activities of alpha, beta, theta and delta range are separated and may be expressed as numerical data of the amount of energy in each frequency band, linegraph, histogram, or compressed spectral array. The next stage is summation of data (feature extraction), which can be achieved by *ad hoc* (heuristic) or formal (statistical) methods. The results thus obtained have been classified and validated [29].

Evoked potentials

Stimulus-related potentials can be evoked by a specific stimulus which can be auditory, somatosensory or visual.

Visual evoked potential

Visual evoked potential (VEP) can be recorded from the occipital cortex through scalp electrodes following retinal stimulation by a flash of light, or by reversing a black and white checker board pattern. The recording is performed by a single or an array of recording electrodes in the occipital region, 5 cm above the inion. The visual evoked potential is a major positive wave with a latency of about 100 ms. There is usually a small preceding negative wave and a subsequent negative wave. The amplitude of the response is of the order of 10 μV. In normal subjects there is a narrow range of latencies, and only a small latency difference between the two eyes [18]. Electroretinogram may be recorded concurrently with the VEP to exclude pathological conditions at the receptor level.

Somatosensory evoked potential (SEP)

Somatosensory evoked potentials (SEP) are recorded from the region of the somatosensory cortex with electrodes placed over the scalp, or from the spinal cord by electrodes placed over the spinal cord following electrical stimulation of afferent fibres in the nerves of upper or lower limbs. The pathway of these potentials is by the dorsal column medial leminiscal system. Lesions on the pathway will affect the latency, amplitude and configuration of the wave. Cortical somatosensory evoked potentials after median nerve stimulation have an early complex with a negative deflection at about 19 ms (N19) followed by a large positive wave P22 [18]. The SEP recorded by peroneal or tibial nerve stimulation provides information about conduction in the peripheral nerve and through the spinal cord.

Auditory evoked potential (AEP)

Auditory evoked potentials are recorded through scalp electrodes following click stimulus applied to the ears. Recording electrodes are placed on the mastoid bone, the reference at the vertex, and the evoked response is averaged. Click stimuli are applied at 65 db above the auditory threshold through earphones [18]. The responses obtained have small amplitude (0.5–2 μV). They are early (0–8 ms), middle (8–50 ms) and late (longer than 50 ms) [66]. The late components probably arise from the cortical region and the middle components are of unknown origin. Seven vertex positive components of the early response are recognized: waves I, II, III, IV, V, VI, VII. These are from farfield activity arising in the auditory pathway. AEP seem poorly affected even in comatose patients when the coma is due to a metabolic or a toxic cause such as uraemia, liver failure or drug overdose [83].

Event related potential (ERP)

Event related potentials are the electrical responses of the brain to external stimulus events, i.e. auditory, visual, somatosensory or electrical signals of internal events which include motor commands and broad spectrum of information processing, associative or

cognitive processes. If the subjects are required to attend, process or respond to external stimuli in a predetermined manner, like counting or button pressing, the resultant wave form will include early (sensory evoked) exogenous component, as well as a later cognitive or endogenous component. The term ERP refers to the later endogenous component associated with information processing, cognition and memory. The latency of the endogenous component is about 100 ms or more after the stimulus onset and they persist for several hundred ms.

The three most prominent ERPs are:

(1) Contingent negative variation (CNV): a slow vertex negative shift in scalp-recorded EEG that arises during signalled reaction time [97].
(2) Readiness potential (RP): a slow vertex negative shift in baseline that arises about 1 s before stereotyped movement [45].
(3) P300: a large positive wave that peaks at 300–600 ms following a stimulus that delivers relevant information to the perceiver [85].

AntiChE-induced neurophysiological changes: experimental studies

Peripheral effects

Twitch potentiation

AntiChE compounds potentiate the tension developed by the skeletal muscle in response to submaximal or maximal indirect stimulation at low frequency with both *in vivo* and *in vitro* muscle preparations [44,98]. Normally following supramaximal nerve stimulation the muscle action potentials in individual muscle fibres are synchronous and elicit a twitch. Following iv physostigmine in cats, a single indirect stimulus produced a short burst of repetitive potentials. The muscle response was similar to that at high frequency stimulation, thus explaining the phenomenon of twitch potentiation [13]. Twitch potentiation has been reported to be associated with repetitive nerve action potential [56]. Prolongation of EPP has been reported to be mainly responsible for triggering repetitive muscle action potential [26]. AntiChE compounds could produce repetitive antidromic nerve potential by an axon reflex. Following the arrival of an impulse at the nerve terminal, depolarization of the first node of Ranvier occurs at one of these terminals. This gives rise to an antidromic nerve action potential and to an invasion of the nerve terminal by the action potential; consequently a muscle action potential results. This process repeats itself [72]. Twitch potentiation occurs over a limited range of AChE inhibition [49,59]. To define the repetitive muscle activity and other NM changes, rats were exposed to chlorfenvinphos. When plasma ChE was markedly reduced, no change in CMAP to single stimulus occurred; however the animals developed a prolonged negative potential lasting up to 15 ms. Superimposed on this, repetitive activity occurred with a latency of 4.5 ms and the change was abolished by double and repetitive stimulation [57].

Fasciculations

AntiChE activity of CBs [11], OPs [59] and edrophonium-type drugs [11] produce fasciculations, which result from intermittent synchronized contraction of muscle fibres of a motor unit. Fasciculations occur with a limited range of AChE inhibition, disappearing when inhibition exceeds 95% [8]. The genesis of fasciculations following AChE inhibition is attributed to the initiation of an axon reflex which allows released ACh to have access to the first node of Ranvier and depolarize it in the absence of nerve stimulation.

Tetanic fade

Indirect stimulation of a mammalian muscle at $\geqslant$ 20 Hz for a short period, results in sustained increase in tension. Following the administration of an antiChE CB [6], OP, or edrophonium-type drugs [10], the response to such stimulation consists of rapid increase in tension followed by partial or complete relaxation, while the stimulation is maintained.

Tetanic fade is associated with marked inhibition of AChE. In an isolated nerve diaphragm preparation, with stimulation, a 50 Hz for 5 s, paraoxon produced tetanic fade when AChE inhibition was 95% [8]. The major cause of tetanic fade is depolarization block at

the postsynaptic site. Studies on the quadriceps muscle in cats with indirect stimulation at high frequencies revealed that the first effect of physostigmine was a notch in the record of muscle response, i.e. an initial increase followed by a transient fall and then further increase in muscle tension [12]. It has been observed that on nerve stimulation at high frequencies, in the presence of neostigmine or ambenonium, antidromic nerve action potentials were present in the initial period of stimulation. Low dose tubocurarine which acts postsynaptically, and does not block NM transmission, also abolished both antidromic nerve action potential and the notch in the muscle response [10].

Reversal of myasthenia gravis

Of the antiChEs, neostigmine [69] and physostigmine [96] were first reported to reverse the signs of myasthenia gravis. In doses required for therapeutic effect, various antiChEs potentiate the action of ACh at motor endplates and have a duration of action which is related to the inhibition of AChE [30]. Neostigmine was found to increase the amplitude and duration of MEPPs in myasthenic patients in the same manner as in the muscle of controls who were previously given tubocurarine. These findings are consistent with the view that antiChEs are helpful in myasthenia gravis by inhibiting AChE which intensifies the postsynaptic action of ACh, and possibly increases the quantal release of ACh [31]. However, excessive inhibition of AChE can lead to NM block by depolarization [30].

Rats treated with 1 mg/kg neostigmine for 5–7 days showed MEPP and EPP at motor endplates were reduced in amplitude. The ACh content of vesicles and the quanta of ACh in the nerve terminals released by nerve impulses were both reduced [74]. In a study on rats injected with neostigmine, 0.1 mg/kg, twice daily for 7 days, the ACh content of the diaphragm was unchanged but the quantal release of ACh on stimulation of phrenic nerve at 100 Hz was reduced by 50%. The number of cholinoceptors was also reduced [17]. These results suggest that prolonged treatment with antiChEs could be associated with progressive reduction in the effectiveness of treatment, as seen in some patients, which is usually interpreted as progression of the disease [35].

Chronic inhibition, especially by OPs, may be significant because adaptation to the inhibition of ChE may occur. Rats primed with oral neostigmine, 0.25–1 mg/ml for 4 weeks, failed to show fasciculations when injected with 40 μg/kg neostigmine, unlike controls [15]. Rats treated with a slowly increasing dose of schradan (OMPA) showed adaptation [53]. Chronic inhibition of NM AChE has been reported to precipitate a myopathy [5]. Accumulated ACh causes excessive activation of ACh receptors. The opened ionic channels allow passage of excess of Ca^{2+} which activates the protease system, which in turn attacks Z discs preferentially [68]. Neurophysiological studies on peripheral nerve conduction following OP exposure have revealed that DFP and soman initially increased sciatic nerve conduction and reduced refractoriness. However, continued exposure had a diminished effect. The results suggest that compensatory changes occur in peripheral nerves also after OP exposure [4]. In high concentration OPs block peripheral nerve conduction [102].

Central effects

Studies on the neurophysiological changes in the CNS following antiChE exposure reveal that desynchronization of EEG activity, epileptiform activity and finally quiescence of cerebral activity occurs [99]. OPs have effects on central respiratory neurons [9] and cortical activity [90]. An arousal pattern with theta rhythm is seen in the rabbit hippocampus following antiChE administration [21]. EEG, visual and auditory evoked potential studies on parathion-exposed rats revealed damping of all the EEG frequencies, reduction of amplitude, and slight prolongation of evoked potential latencies. The degree of the changes was dose-related [89].

Acute OP exposure has been reported to cause marked desynchronization of the EEG and a triad of changes consisting of increased high frequency activity, decreased low frequency activity and lowered background voltage. Higher exposure caused slowing of the EEG followed by the development of spike wave discharges which accompany

convulsions [14]. EEG abnormalities, including convulsions can be antagonized by atropine and anticonvulsants [34,62,99]. However the antagonism of OP by atropine and not by anticonvulsants has also been reported [100]. EEG changes are not specific for antiChEs, as compared with chlorinated hydrocarbons [76]. Some studies, however, have failed to demonstrate that mevinphos and physostigmine could cross the blood-brain barrier and affect the activity in the hippocampus or visual integrative neurons in the thalamus of pigeons [70,71].

The effects of OPs on the spinal cord have been reported to have contradictory results. OPs have been reported to increase monosynaptic extensor reflex, increase the firing of the multisynaptic flexor reflex, and depress the monosynaptic reflex [33]. Atropine has been reported to counteract the effect of ChE inhibitors on the spinal cord reflex, irrespective of the potentiation or depression of the reflex produced by OPs [79].

AntiChE-induced neurophysiological changes in human studies: peripheral effects

Acute poisonings

Detailed neurophysiological changes have been reported in only a few studies [40,94]. In a study on two cases of suicidal OP poisoning neither change in the amplitude of evoked muscle potential was found nor was there any repetitive muscle activity, and the results on repetitive nerve stimulation were also normal [40]. In 200 cases of OP poisoning, the neurological changes were classified into two types: type I signs were from AChE inhibition, which responded to atropine, and were present at the time of admission; type II signs appeared later, and included proximal muscle weakness, cranial nerve palsy and areflexia [95].

In a study on the neurophysiological changes in 350 cases of OP poisoning, type II paralysis was present in 49%. Motor conduction velocity and terminal motor latencies were most affected in the severely affected patients, and serially better in moderate, mild and no-deficit

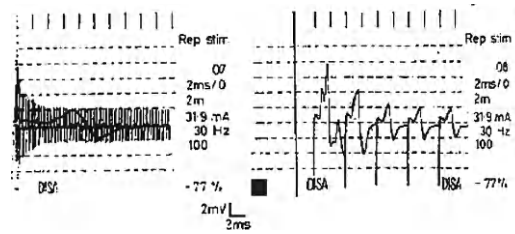

Figure 40.1 Decrement at 50 Hz in a case of OP poisoning showing moderate neurological deficit. Decrement S1–S5 is 77%. Reproduced from Wadia [94] with permission

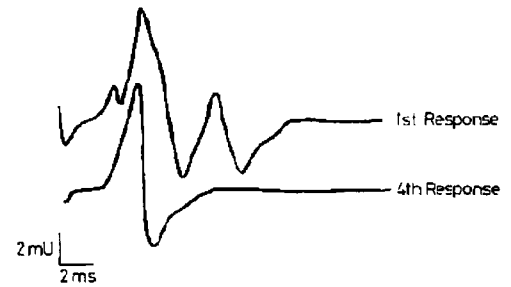

Figure 40.2 Repetitive activity in a patient with OP poisoning. The repetitive activity disappears on repeated stimuli. Reproduced from Wadia [94] with permission

Figure 40.3 Decrement shown in OP poisoning at 30 Hz; the first two responses show a small repetitive response. Reproduced from Wadia [94] with permission

group; the difference was not statistically significant. On repetitive nerve stimulation at 3 Hz a decrement was present in two cases at 10 Hz in four cases at 30 Hz in several cases, even in the absence of paralysis (Figure 40.1). Repetitive muscle activity to single stimulation was present in 60% of patients (Figure 40.2), which disappeared on stimulation within a short period (Figure 40.3). Repetitive activity was present in all patients with clinical signs. Concentric needle EMG revealed mild reduction of interference pattern. Three of these patients, exposed to dichlorvos, developed delayed neurotoxicity.

Following acute OP insecticide poisoning no

clinical evidence of peripheral neuropathy has been reported in certain studies [47]. However, in the literature there are a few reports of neuropathy from OP poisoning [63,94]. Acute infective polyneuritis-like syndrome following acute OP poisoning has also been reported [1], detailed neurophysiological studies, however, were not reported.

All OPs are antiesterase in their action, combining with varying degree of avidity to ChEs and other esterases; thus they produce cholinergic symptoms. However antiChE effects and OP-induced peripheral neuropathy appear to be independent [2]. Pathologically, myelin and axons show maximum changes which start in the periphery and affect the distal parts of the largest fibres, which show centripetal dying back. The long tracts of the spinal cord also show similar changes characteristic of a dying back process [16].

The neurophysiological correlates of delayed neurotoxicity have been evaluated in a few studies. In three patients, 3–5 days after accidental trichlorfon poisoning distal sensorimotor neuropathy occurred with paraesthesia in the feet, distal muscle weakness, gait difficulty, hypotonia, loss of ankle reflexes, brisk knee jerks and pyramidal signs. Clinical, electrophysiological studies and nerve biopsy revealed dying back neuropathy. The neurophysiological features included marked denervation of lower limbs and partial denervation of upper limb muscles. Motor conduction velocity and distal latency were normal, but the peroneal nerve was unexcitable. Sensory conduction was normal except in one patient. Nerve conductions improved after 4 months. The amplitude of sensory and motor action potentials was decreased. The nerve excitability threshold showed a distal motor hypoexcitability. On repetitive nerve stimulation, decrement was present in all the patients [91].

Acute neurotoxic effects during AChE inhibition and delayed neurotoxicity appearing 2–3 weeks later are well recognized. An intermediate syndrome of neurotoxicity appearing 24–96 h after poisoning has been reported recently [77] (*see* Ch.12). EMG studies revealed fade on tetanic stimulation, absence of fade on low frequency stimulation and absence of post-tetanic facilitation, suggesting a postsynaptic defect.

Occupational exposure

The role of neurophysiological investigations to monitor occupational exposure to OPs has been investigated in several studies. In workers exposed to dimethyl phosphate esters, 50% were found to have EMG changes suggestive of NM dysfunction. EMG changes included low voltage and repetitive activity. Blood ChE was not inhibited, even in the workers with EMG signs; therefore the EMG was concluded to be superior to blood ChE monitoring [37]. In a similar study, in which 102 workers were exposed to a wide variety of pesticides, repetitive nerve stimulation revealed abnormality in 40%. Follow-up studies in 53 workers revealed that the EMG in 12 changed from normal to abnormal, while in 13 it changed from abnormal to normal. Blood ChE did not show significant difference between the subjects with normal and abnormal EMG records [24].

In 56 factory workers, 14 had EMG voltage <10 mV, but after 3 weeks vacation the voltage increased significantly in all the subjects. The mean EMG voltage in high and low exposure groups was significantly different. One of the mechanisms suggested was an increase in temporal dispersion of the various components of CMAP from reduction in motor conduction velocity. The workers were grouped into two groups: EMG voltage <10 mV, and those with >10 mV. The latter had normal nerve conduction velocity whereas the former had nerve conduction velocity 10% below normal. This difference was significant in both the fastest and slowest fibres (P <0.01) [73]. Slowing of conduction velocity in motor nerves from OP exposure was suggested to be related to a cholinergic link between axon and Schwann cells [93].

In a study on NM synaptic function in workers exposed to different OPs, ChE estimation, neurological examination and repetitive nerve stimulation were performed. No significant effects were seen.

Decrease in the amplitude of the evoked potentials, from NM synapse dysfunction, must be accompanied by a reduction in the muscle strength. EMG NM synapse testing and neurological examination were not sensitive enough to detect latent OP intoxication [39]. With 21 pesticide workers, motor and

sensory nerve conduction velocity and CMAP were normal. In ten subjects, summated action potential of thenar muscles after median nerve stimulation consisted of prominent initial negative phase and a smaller second negative phase, but this was also present in four controls and EMG techniques were not found to be useful in monitoring minor pesticides toxicity [42].

In 11 Swedish workers exposed to OPs, plasma ChE was significantly inhibited after work, while RBC AChE was unchanged. No disturbance of NM transmission was found with repetitive nerve stimulation or with jitter measurement on single fibre EMG. However, signs of subclinical neuropathy were revealed by a slight reduction in sensory nerve conduction velocity and increased fibre density [81]. In 24 workers engaged in spraying fenthion in India, motor and sensory nerve conduction velocity, F response, H reflex and repetitive nerve stimulation tests were studied. After this they were withdrawn from fenthion exposure for 3 weeks, following which the tests were repeated. There was no clinical evidence of neuropathy or muscle weakness. However, neurophysiological changes suggestive of subclinical NM dysfunction were present, which included change in motor conduction velocity ($P < 0.05$), terminal motor latency of median ($P < 0.1$) and peroneal nerve ($P < 0.05$), F minimal latency and H reflex latency ($P < 0.01$). In 29% of workers there was repetitive muscle activity and serum ChE showed significant change ($P < 0.01$). However, the neurophysiological changes were not significantly related to the change in AChE [61]. Variations in the results of neurophysiological monitoring in the occupationally exposed workers may be the result of variation in the extent of exposure, use of protective measures, difference in the neurophysiological techniques employed and the parameters evaluated in different studies.

Normal volunteer studies

Such studies have the advantage of standardized pre-exposure parameters. In 14 subjects, intra-arterial injection of 0.5–1.5 mg neostigmine resulted in progressive reduction in the amplitude and duration of action potential in response to successive stimuli. The average reduction in the amplitude of the first response following 1 mg neostigmine was 20% and that of fourth response was 70%. The injected arm became weaker. In all subjects repetitive firing to the first stimulus could be demonstrated. There were also local fasciculations. The smallest dose which produced these effects was 0.02–0.05 mg. Edrophonium produced similar changes, but its effect on initial potentials was greater. Following a large dose of edrophonium the potentials were sometimes completely abolished [32]. In another study with iv edrophonium (1–3 mg), the earliest change was a negative deflection occurring at the end of the muscle response, evoked by nerve stimulation. It did not occur after a second stimulus 30 ms later or immediately after 10 s of maximal voluntary contraction. Irregularities at the end of muscle response are common in healthy subjects. The behaviour of the potential should be studied following the above mentioned criteria to confirm that it is from repetitive activity [67]. With the higher dose of edrophonium the response to second shock (M2) at 30 ms was reduced in amplitude but M2 at 80 ms was unaffected. An even larger dose caused depression of M2 at 80 ms also and a decremental response to 50 Hz rate of stimulation. The amplitude of response to a single shock was unchanged throughout [67]. Mevinphos, 25 mg/kg, was administered daily to eight subjects for 28 days. At the end of exposure, 7% decrease in slow fibre conduction velocity and 38% increase in Achilles tendon reflex force were found. There was no effect on NM transmission. RBC AChE inhibition was 19% [92].

Central effects

Following acute OP poisoning, hypothalamic spikes have been reported to persist for 3 years [36]. In two patients with mild OP poisoning, EEG, disturbance of the temporal lobe, slowing and associated symptoms of depersonalization and déjà vu phenomena have been reported [13]. After occupational repeated exposure to OPs, EEG patterns showed medium voltage slow activity of 4–6 Hz during drowziness. Autocorrelograms showed less rhythmic activity which was from an increase in slowing [60]. A daily dose of carbaryl, 0.06 or 0.12 mg/kg, to male volunteers for 6 weeks produced no significant change in their EEG

[101]. Computed EEG was done in occupationally exposed OP workers using 4 channel EEG recording from frontal and occipital regions. EEG data were analysed by fast Fourier transformation and power spectra were calculated. The Bendor Visuomotor Gestalt test and part B of the trail marking test were significantly affected (P <0.05). The frontal regions showed slower alpha activity in the high chronic OP group. The neurophysiological tests and computed EEG data suggested an association between higher level of chronic OP exposure and frontal lobe impairment [46].

Event-related potentials have been evaluated in pest control operators whose mean blood ChE in low, medium and high exposure group was 92%, 86% and 88% respectively. A P3 component evoked by target stimuli was observed in all the subjects for each of the three exposures; however, a P3 component evoked by non-target stimuli was observed in 12, 7 and 6 subjects at low, medium and high exposure levels respectively. The amplitude of P3 evoked by target stimuli was higher than that evoked by non-target at all the three exposures. The prolongation of P3 latency evoked by target stimuli at high pesticide level was suggested to be from an increased OP effect mediated by significant increase in ChE level [88].

In a study on 32 workers spraying mainly fenthion for a mean duration of 10.5 years (range 1–14 years), cognitive functions and event-related potentials were studied. Significant changes were present in Benton Visual Retention Test, Memory quotient and Alexander's passalong test. Serum AChE level was inhibited by 27% compared with controls. P_3 component of event-related potentials was elicited in 27 of 28 subjects in whom the cognitive evoked potentials were evaluated. P_3 latency was prolonged in six subjects and the group difference was significant. These results suggest subtle effects of chronic OP exposure on the cognitive functions and event-related potentials in occupationally exposed OP workers (Misra and Prasad, 1991, unpublished data). Animal studies have also revealed that the medial septal area plays an important role in the generation of P_3 through the cholinergic component of the septohippocampal projection system [33a], suggesting the important role of cholinergic pathways in the generation of event-related potentials.

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41

Organophosphate poisoning in the UK: the National Poisons Information Service experience during 1984–1987

Sarah Weir, Neil Minton and Virginia Murray

Introduction

OP insecticides are effective as pesticides, but can cause severe toxicity and even death in humans and animals following accidental or deliberate exposure and subsequent absorption [32]. However, little is known about the frequency and severity of OP exposure in the UK or about the range of products involved in these incidents [21]. In addition, there are few data on the use of indirect laboratory diagnosis and the effect of this on clinical management of exposure to OPs. To answer some of these questions, this chapter reviews enquiries about cases of suspected OP poisoning reported to the National Poisons Unit's (NPU) Information Service (NPIS) at Guy's Hospital between 1984 and 1987. The incidence and severity of cases of OP exposure and poisoning is assessed together with methods of diagnosis and treatment of OP poisoning or suspected poisoning by hospitals and general practitioners who consulted the NPIS.

To determine the value of the data collected by the NPIS, the information from this study is compared with data on the incidence and severity of poisoning available from two surveillance and reporting schemes in the UK. These schemes are the Pesticides Incidents Appraisal Panel (PIAP) and the Office of Population Censuses and Surveys (OPCS). PIAP reviews incidents investigated by Her Majesty's Agricultural Inspectorate where the use of a pesticide during work may have affected the health of a person and the Office of Population Censuses and Surveys (OPCS) collects mortality data for England and Wales.

Methods

National Poisons Information Service (NPIS)

Source and method of managing enquiries

The NPIS started in 1963 to provide information and advice to doctors managing cases of acute or suspected acute poisoning [28]. Advice on management of OP exposure is given for each case and, where indicated, includes information regarding the use of gastric emptying procedures, supportive care including oxygen, antidote treatment including atropine, P2S (the oxime favoured in the UK) and diazepam, and estimation of blood ChE in symptomatic cases [21].

Information recorded at time of first enquiry

Each enquiry to the NPIS is recorded on a call sheet with details of the type of enquiry (emergency, recall, information or other), enquirer (hospital, general practitioner, emergency services, etc.) and patient. Information collected includes age and sex, agent, route of exposure, time since exposure, symptoms experienced, investigations performed and treatments administered. Since 1983, all data from the call sheets excluding the patients' and doctors' names, have been stored on computer, and are coded to facilitate data retrieval [6]. All compounds are coded according to type and usage. To obtain data relating to OP insecticides, the annual databases for 1984–1987 were searched.

Follow-up data collection

In addition to information taken at the time of the enquiry, written follow-up data is requested in selected cases. This is important for obtaining details of treatment and of value in determining final outcome, which was not ascertained at the time of the original enquiry. Follow-up questionnaires are sent about 1 month after the exposure has occurred. The criteria for follow- up are: (1) exposure to a large amount of OP, (2) moderate or serious symptoms present, (3) unusual or previously unreported symptoms, (4) new OP products implicated, and (5) unexpected increase of incidents involving reputedly safe products.

A database has been created to store the information returned from questionnaires. It includes further details about circumstances of exposure, onset and severity of symptoms, treatment, laboratory analysis performed and outcome.

The NPIS data discussed in this chapter are thus from two sources: the enquiry data and the follow-up data.

Pesticide Incident Appraisal Panel

The Pesticide Incident Appraisal Panel (PIAP) has been reviewing the incidents investigated by the Agricultural Inspectorate since 1980. The Panel assesses the likelihood of the reported clinical effects being associated with pesticide exposure.

Information from the Inspector's investigation reports of cases of suspected poisoning or injury caused by substances are presented. The data include the date of the incident and investigation, the notifier, the occupation of the injured person, accident details such as type of application and equipment involved; substances involved and information on exposure to the substance. For each incident the following parameters are recorded: age, sex, occupation, occupational and non-occupational exposure, route of absorption, clinical effects and severity of poisoning. From these data, the panel assesses the likelihood of poisoning having occurred, which is classified as confirmed, likely, unlikely or not confirmed.

Table 41.1 Total number of enquiries received by the National Poisons Information Service (NPIS) compared with total number of enquiries about cases of suspected OP poisoning, 1984–1987

	Enquiries to NPIS		
Year	*No. of cases of suspected poisoning*	*No. of cases of suspected OP poisoning*	*Percentage of total enquiries*
1984	39 024	400	1.0
1985	43 789	472	1.1
1986	47 463	395	0.8
1987	48 849	364	0.7
Total	179 125	1 631	0.9

Office of Population Censuses and Surveys (OPCS)

Mortality data for the period of this survey, gathered from death certificates returned to the Office of Population Censuses and Surveys (OPCS), are included for comparison with the results from the NPIS data. It is important to note that OPCS data cover England and Wales only: they do not include Scotland and Northern Ireland.

Results

National Poisons Information Service

Information recorded at time of first enquiry

During the 4 years from 1984 to 1987, the NPIS received 1631 enquiries relating to OPs, which comprised 0.9% of all emergency enquiries (179 125). Figures for 1984–1987 indicated a reduction in cases against a background of increasing enquiries (Table 41.1). Malathion, pirimiphos-methyl and dimethoate were most commonly implicated (Figure 41.1). The importance of malathion reflects its

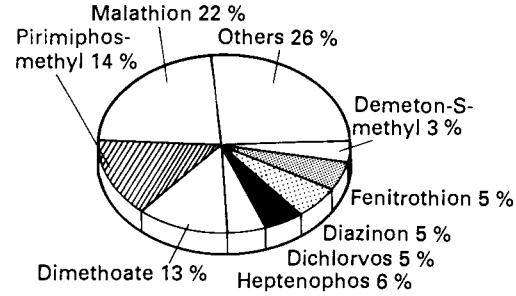

Figure 41.1 Range of OPs recorded from enquiries (n=1631) to NPIS between 1984 and 1987

widespread use in garden and agricultural products and domestic head louse preparations.

The 1206 enquiries concerning the eight most common OPs show that 55% of incidents involved household use, 36% agricultural, 5% pharmaceutical, 3% industrial and 1% miscellaneous. These data demonstrate that household preparations such as ant killers, fly sprays and garden chemicals, are readily available and are frequently implicated in human exposures. In all, 5% of enquiries were classified as medications containing OPs and included head louse shampoos and lotions, principally containing malathion.

The NPIS divides the age range of patients into three classes: <5 years, between 5 and 12 years, and >12 years. Analysis of 1206 enquiries showed that 42% of patients were <5 years old, 5% were aged between 5 and 12 years, and 47% were >12 years old, the remainder being unspecified.

Symptom severity is also recorded at the time of enquiry for the 1206 cases; 41% of patients had no symptoms, 33% had mild symptoms, 10% had moderate symptoms and only 1% had severe, life-threatening symptoms. No deaths were reported at the time of enquiry.

Follow-up data

Follow-up data were requested on approximately 500 cases. Questionnaires were returned on 244 cases, 15% of all OP enquiries occurring between 1984 and 1987. Possible reasons for return rate of only 50% include occasional delay in despatch of questionnaires from the NPIS and failure of hospitals to trace patient records.

From returned questionnaires, 74 different products were implicated and reflect the wide range of OP-containing products on the market. The five most frequent branded products identified were all for garden use except for a head louse lotion containing malathion. From the follow-up questionnaires, 68% of patients were male and 32% female. The age range shows that 27% were <5 years of age, 3% between 5 and 12 years and 64% >12 years, the remainder being unspecified. Compared with the age range of patients recorded at the time of the first enquiry for the eight most common compounds, there were fewer children under 5 years of age and more adults over 12 years. This can be explained by the fact that incidents involving children do not usually fall within the scope of our follow-up criteria.

Considering data by agent and symptom severity, malathion, again, was most commonly encountered (42 cases). Most patients had either no symptoms (79 cases) or mild symptoms (113 cases); there were moderate symptoms in 30 cases and severe symptoms in seven cases, and two deaths which were not revealed by the original enquiries to the information service. The most frequently reported symptoms and signs were nausea (24%), vomiting (23%) and sweating (15%). Other symptoms included abdominal pain (13%), blurred vision (6%), salivation (6%), ataxia (3%) and convulsions (one case). Some patients experienced more than one symptom.

Antidotes were used in only 27 (11%) of the 244 cases. Atropine was given in 25 cases (10%) and P2S in seven cases (3%), the latter including two (1%) where P2S was given without atropine. NPU has never recommended the use of P2S as the sole antidote and has always advocated previous treatment with atropine where necessary [21].

Measurements of ChE activity were reported in only 21 (9%) of the 244 cases. In three cases both plasma and RBC ChE activity were measured. In the remaining cases the method was not specified and, in addition, we noted a lack of standardization of units, making interpretation of results difficult. Where measurements were carried out only two cases demonstrated depression of enzyme activity, while it was within the normal range in ten cases. In the remaining nine cases there were insufficient data to interpret the levels given. The decision to measure ChE activity did not appear to be determined either by the route of exposure or by the severity of the symptoms. In cases where enzyme activity was estimated, two patients had no symptoms, 12 had mild symptoms, five had moderate symptoms and one had severe symptoms. Levels were not measured in either of the two fatal cases.

Between 1983 and 1986, there were ten reports of exposure of pregnant women to OPs. Analysis of these enquiries revealed four normal deliveries and six miscarriages. However, the contribution of the suspected OP

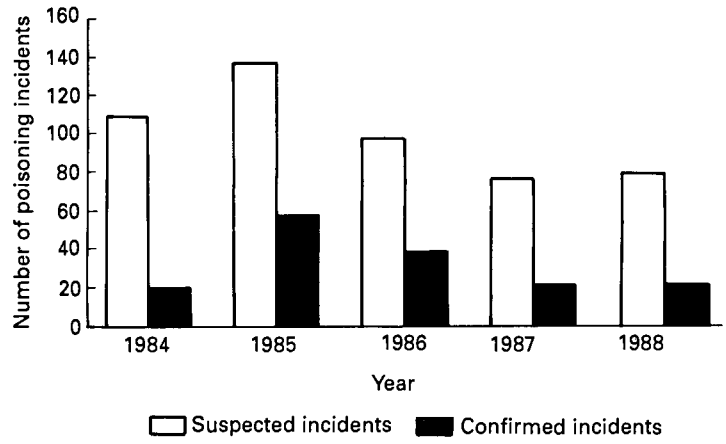

Figure 41.2 Number of incidents considered by Pesticide Incidents Appraisal Panel for the years 1984–1988. □, suspected; ■, confirmed

poisoning to the aetiology of the miscarriage is uncertain (personal communication).

Pesticides Incident Appraisal Panel

The total number of incidents investigated in the 5 years from 1984 to 1988 inclusive was 957, of which 500 were suspected poisoning incidents [12]. Of these incidents, 161 (32%) were classified as confirmed cases of poisoning by the Panel (Figure 41.2). The data from the Panel have been collated since 1981 but only published since 1986 [10–12], so poisoning incidents are only presented for the 3 years between 1986 and 1988. In 1986, there were ten suspected OP poisonings, in 1987 20 incidents, and in 1988 14 incidents. The substances involved in these incidents are listed in Table 41.2. The Panel confirmed OP poisoning in five incidents for 1987 and three incidents in 1988.

Table 41.2 Pesticide incidents investigated by Pesticide Incident Appraisal Panel involving OPs

Pesticide	*1986*	*1987*	*1988*
Chlorfenvinphos	2		
Chlorpyrifos	1	1	1
Demeton-S-methyl		5	6
Diazinon		1	1
Dichlorvos		1	
Dimethoate	2	4	3
Fenthion		1	
Heptenophos		1	
Omethoate		1	
Oxydemeton-methyl			1
Phorate			1
Phosalone	2		
Pirimiphos-methyl	1	4	
Triazophos	2	2	1
Total	10	21	14

Office of Population Censuses and Surveys (OPCS)

Using the International Classification of Diseases 9th Revision, information was obtained for deaths caused by or related to OP insecticides (codes E863.1, E950.6 and E980.7). The data for England and Wales from 1984 to 1987 in fact reveal only one definite death from accidental poisoning by insecticides of OPs (E863.1). However, the categories E950.6 and E980.7, include, for the years 1984–1987, a total of 135 deaths from suicide or injury related to agricultural and horticultural chemical and pharmaceutical preparations, of which an unspecified number may have been OPs.

Discussion

National Poisons Unit data

The number of enquiries to the unit regarding OPs was small (0.9%) compared with the total number of enquiries (Table 41.1) and the majority of cases reported were not severe poisonings. However, information collected by the unit is not a representative or complete

sample of poisoning in the UK. Cases of exposure or suspected poisoning will not be reported to the unit if the patient does not go to a general practitioner or a hospital or if the doctor does not suspect poisoning or recognize OP poisoning, a relatively uncommon clinical problem. Other reasons for failure of reporting include insufficient knowledge of the NPU and its functions, or the doctor not wanting to seek advice or information. There is also a geographical bias: NPIS receives approximately 50% of its enquiries from south east England [23].

Of the cases followed up few were children. Furthermore, most adult cases had either no symptoms or mild to moderate non-specific symptoms, not the characteristic muscarinic and nicotinic signs and symptoms noted in more severe cases. Reports of deliberate suicidal poisonings [4,5,18] have shown that such cases tend to produce more serious symptoms. These severe symptoms were infrequent (12%) in our follow-up data. Only two deaths were reported and these resulted from poisoning by pirimiphos-methyl and heptenophos.

Laboratory diagnosis by measurement of ChE activity was reported in only 9% of the cases followed up. This highlights the underuse of specialized laboratory facilities and reflects either an omission in requesting this important diagnostic test or its lack of availability. Although it is often difficult to determine the necessity for measuring plasma or RBC ChE activity, the NPIS recommends that it be measured in cases with significant signs or symptoms suggestive of cholinergic type poisoning. Furthermore, it is a useful marker of clinical recovery [3]. In occupational settings where chronic exposure may occur, ChE levels may be permanently depressed. Blood samples should be taken at intervals over several weeks or months to confirm exposure, ideally as part of an annual screening medical examination [9]. These measurements are also useful as a baseline level in the event of a subsequent exposure. However, data from previous tests were not available for those followed up by NPU, where 54 (22.1%) cases related to occupational exposure.

Results of ChE estimations in our survey indicate a general lack of standardization of methods for RBC and plasma ChE activity and moreover the variation in units and normal ranges makes interpretation of results difficult.

Methods of general supportive management were occasionally inappropriate. For example, gastric lavage or emetics were used where products contained organic solvents. Here there is the risk of aspiration and the development of pulmonary oedema. Antidotal treatment was used in only 27 (11%) of the 244 cases followed up. In two of these cases, P2S was incorrectly administered without previous atropine, contrary to accepted treatment [8,21,22,25].

The 50% return rate for questionnaires reflects the rate of return found in other studies of this nature [20,26]. Reasons for this include difficulty in tracing hospital records. In addition, relatively few of the OP enquiries satisfied the requirement for follow-up, explaining the small number of questionnaires sent out in relation to the total number of enquiries.

PIAP data

The Agricultural Inspectorate's remit covers the UK. The 1988 report includes data on the location of suspected pesticide incidents and these were reported from all 21 area offices of the Health and Safety Executive, with most occurring in the North Midlands (20%) and Northern Home Counties (17%). However, the number of incidents investigated was not found to follow the pattern seen in the Unit's enquiries. The substances involved in the suspected pesticide incidents show a different spectrum to those found in the NPU's enquiry, probably because PIAP records do not include domestic products.

PIAP data may also be limited by failure to obtain medical advice by the person concerned at the time of the incident. Some may have considered their symptoms too trivial and others may not have had ready access to their general practitioner. In addition, the notification of pesticide incidents to the Agricultural Inspectorate varies and frequently occurs via non-medical organizations such as the police and environmental health officers, or by a member of public. As a result, many of the clinical symptoms have disappeared by the time the Employment Medical Adviser has been informed of the incident. These findings

suggest that at least half the incidents followed up by the Agricultural Inspectorate are not comparable to the NPIS data as all but 5.8% of the NPIS enquiries are received from medical personnel [23].

Office of Population Censuses and Surveys data

Data from the OPCS for a comparable period suggests that our study may have underestimated the incidence of fatality from OP poisoning. However, data from the OPCS, contained within categories E950.6 and E980.7, do not specifically identify deaths from exposure to OPs. Reasons for under-reporting to the OPCs are complex but include the possibility that death may have occurred away from medical services. With representative and continuous samples of the population, mortality statistics are valuable in providing the incidence of OP poisoning [30]. Nevertheless, OPCS may have their own limitations vis-à-vis the other surveillance mechanisms. Thus mortality statistics are subject to errors which may arise from inaccuracies in diagnosis and patient information [2]. Vale *et al.* [27] showed inaccuracies in many instances of poisoning. They examined past medical history, circumstantial evidence, post-mortem reports and results of toxicological analyses, and found that patients had clearly died as a result of ingestion of a drug or drugs not recorded on their death certificates. By contrast, Onyon and Volans [24] looked at the number of deaths from paraquat poisoning between 1982 and 1984 which had been reported to the NPIS (n=106) compared with those reported to OPCS and the Registrar General of Scotland (n=111). They found few differences but reported that the degree of overlap remained unknown. Thus, taking into account the results of these and other studies, death certificates form a concise and convenient record for the purposes of epidemiological research [1], but the data relating to death from poisoning should be interpreted with care [30].

Other countries

When comparing data from the UK with that from other developed countries it was found that the USA had reported a similar incidence of OP exposure and poisoning. Litovitz *et al.* [19] showed that in 1986, 3% (36 541) of all poisonings reported to US Poison Centres were by pesticides. Of the total, 0.9% (12 142) involved OPs, which equates with the 0.9% OP enquiries to the NPU. By contrast, reports from developing countries such as Sri Lanka show a higher incidence with more fatal cases. In 1986, discharges from hospital following OP and CB poisoning were 40% (10 263) of all poison-related discharges, with 1022 (10%) fatalities [7]. An epidemiological study including a random sample survey showed a high fatality rate of 21.8% for OPs in 1981 [16].

Conclusion

Although PIAP and OPCS data have produced information not previously collected, neither of these schemes collects data from as many medical cases of acute or suspected acute poisoning [30]. Thus, reporting by Poison Centres of the incidence of OP poisonings or suspected poisonings could be used as a means of monitoring the size of the problem [17].

Guidelines on the role of Poisons Centres suggest that these centres should be able to carry out toxicovigilance, i.e. the identification, investigation and evaluation of the various toxic risks in the community with a view to taking measures to reduce or eliminate these risks [14]. However, in the UK, comprehensive data collection is difficult because several bodies collect data in different ways. They are the NPU, PIAP, the Regional Poisons Centres, OPCS, the Registrar General for Scotland and the Northern Ireland Department of Health. Standardized data collection and computerization should resolve this problem. On an international basis the joint IPCS/CEC working party on evaluation of antidotes used in the treatment of poisoning [13] is undertaking a prospective study with Poisons Centres to examine the clinical characteristics of OP poisoning as well as its treatment [15]. Additionally the WHO has published a protocol, currently being used for an epidemiological survey of OP poisoning [33].

Collaboration between Poisons groups and others concerned will reduce the problems of data collection and expand the information available on the incidence and severity of cases of OP exposure and poisoning, and the

methods of diagnosis and treatment of OP poisoning or suspected poisoning by medical personnel [29].

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42

Incidence, presentation and therapeutic attitudes to anticholinesterase poisoning in the USA

Alan H. Hall and Barry H. Rumack

The most common antiChEs causing poisoning the USA are OPs and CB pesticides. OPs are responsible for about 80% of pesticide-related hospitalizations [15,39,49]. OPs and CBs represent a large portion of the more than 900 US registered pesticides, sold under over 25 000 brand names and produced at more than one billion pounds annually [49].

OP inhibition of AChE is less easily reversible than that of CBs. Thus, CB poisoning tends to be less severe and shorter than OP poisoning [39], although poisoning from either class depends on the inherent toxicity of the specific compound, dose, duration of exposure, etc.

Incidence of antiChE poisoning in the USA

During the 1970s, the US EPA conducted a national survey of pesticide-related hospitalizations and fatalities, and estimated that approximately 3000 patients were hospitalized yearly in the USA because of pesticide poisoning, with 17 519 pesticide-poisoned patients admitted to hospitals between 1971 and 1976 [46,49]. OPs accounted for 31% of estimated hospital admissions from 1971–1973 and 25% from 1974–1976 [46]. Approximately 4875 of the pesticide-related hospital admissions during this period involved OPs [46]. Fatality rates in hospitalized poisoning cases were 50% in children and 10% in adults [49]. However, deaths from pesticide exposure in adults decreased from 152 to 52 cases annually between 1956 and 1974, while fatalities in pediatric pesticide poisonings decreased from 66% to 33% during this period [39].

The Annual Reports of the AAPCC National Data Collection System (NDCS) are major sources of recent data on the incidence of poison exposures reported to Poison Centres (PCs) in the USA, and are available for 1983–1987 [25–28,51] and later. However, as the number of PCs reporting to the NDCS has increased each year since 1983, annual changes in the incidence of exposure to particular categories of toxic agents cannot be taken as trends.

Between 1983 and 1987, a total of 4 147 583 cases of human poison exposure were reported to the AAPCC National Data Collection System (Table 42.1); categories are shown in Table 42.2. AntiChE pesticide exposures were recorded in 63 092 humans in the AAPCC database over the 5-year period (Table 42.2). Symptoms in patients are classified as *none* (asymptomatic), *minor* (non-life-threatening, resolved in 24 h, no residual disability), or *major* (life-threatening, lasted more than 24 h, or had residual sequelae) [28]. There were 18 489 antiChE-exposed symptomatic patients reported to the database during 1983–1987 (Table 42.3); 675 were classified as having major symptoms (Table 42.4), and 27 deaths were reported (Table 42.5).

Table 42.1 AAPCC National Data Collection System. Total cases of poison exposure reported, 1983–1987

Year	*No. of poison centres reporting*	*No. of human poison exposure cases*
1983	16	251 012
1984	47	730 224
1985	56	900 513
1986	57	1 098 894
1987	63	1 116 940
Total		4 097 583

Table 42.2 AAPCC National Data Collection System. Distribution by year and category of antiChE pesticide exposures

Category	*1983*	*1984*	*1985*	*1986*	*1987*	*Total*
CB alone	1085	3033	4504	4423	4458	17 503
OP alone	2853	5710	7266	8583	9023	33 435
OP with CB	552	1226	1773	2420	2478	8449
OP with chlorinated hydrocarbon	73	200	208	527	438	1446
OP with other pesticide(s)	128	352	376	612	791	2259
Total	4691	10 521	14 127	16 565	17 188	63 092

Table 42.3 AAPCC National Data Collection System. Symptomatic patients exposed to antiChE pesticides, 1983–1987

Category	*1983*	*1984*	*1985*	*1986*	*1987*	*Total*
CB alone	390	592	985	878	840	3685
OP alone	1190	1740	2292	2919	3032	11 173
OP with CB	208	315	509	719	695	2446
OP with chlorinated hydrocarbon	23	57	60	152	148	440
OP with other pesticide(s)	63	100	141	201	240	745
Total	1874	2804	3987	4869	4955	18 489

Table 42.4 AAPCC National Data Collection System. Patients with major symptoms exposed to antiChE pesticides, 1983–1987

Category	*1983*	*1984*	*1985*	*1986*	*1987*	*Total*
CB alone	78	15	14	12	7	126
OP alone	231	90	48	62	41	472
OP with CB	24	6	2	13	4	49
OP with chlorinated hydrocarbon	4	0	3	1	0	8
OP with other pesticide(s)	9	3	2	3	3	20
Total	346	114	69	91	55	675

Table 42.5 AAPCC National Data Collection System. Fatal cases in patients exposed to antiChE pesticides, 1983–1987

Category	*1983*	*1984*	*1985*	*1986*	*1987*	*Total*
CB alone	1	0	0	2	0	3
OP alone	1	9	6	4	3	23
OP with CB	0	0	0	0	0	0
OP with chlorinated hydrocarbon	0	1	0	0	0	1
OP with other pesticide(s)	0	0	0	0	0	0
Total	2	10	6	6	3	27

Table 42.6 AAPCC National Data Collection System. Patients with exposure to antiChE pesticides evaluated in a health care facility, 1983–1987

Category	*1983*	*1984*	*1985*	*1986*	*1987*	*Total*
CB alone	227	628	863	896	905	3519
OP alone	776	1712	2038	2650	2646	9822
OP with CB	107	242	370	518	479	1716
OP with chlorinated hydrocarbon	14	49	65	125	84	337
OP with other pesticide(s)	28	82	126	165	205	606
Total	1152	2713	3462	4354	4319	16 000

Table 42.7 AAPCC National Data Collection System. Administration of antiChE antidotes, 1983–1987

	No. of cases	
Year	*Atropine*	*Pralidoxime*
1983	131	25
1984	412	123
1985	388	91
1986	529	151
1987	541	123
Total	2001	513

Table 42.8 AAPCC National Data Collection System. Agents involved in 27 fatal cases, 1983–1987

Agent	*No. of fatal cases*
Unspecified CB	1
Unspecified OP	6
Unspecified OP with chlorinated hydrocarbon	1
Aldicarb	1
Carbophenothion	1
Chlorpyrifos	1
Diazinon	7
Fonofos	1
Malathion	5
Parathion	1
Parathion-methyl	1
'Phosphothioate'	1
Propoxur	1
Total	28[a]

[a]One patient ingested both diazinon and malathion

A total of 16 000 antiChE-exposed patients reported to the AAPCC database were evaluated in a health care facility during 1983–1987 (Table 42.6). Patients treated with antiChE antidotes may be predicted to have had a more serious poisoning; 2001 patients received atropine and 513 had 2-PAM (Table 42.7). Of the 27 fatalities reported, eight were <6 years old, one was in the 6–17-year-old group, and 19 were >17 years of age. Poisoning was accidental in 13 cases, suicidal in 13 (intentional misuse in one), and occupational in one case. The route was ingestion in 20 cases, inhalation in three, skin in three, parenteral in one, and unknown in five; five had multiple exposure routes. Coingestants were involved in five of the ingestion cases. The antiChEs involved in fatal cases are shown in Table 42.8.

AntiChE poisoning incidence and selected exposures, California

The California Department of Food and Agriculture (CDFA) collects data yearly on approximately 2500 cases of pesticide exposure through various mechanisms, including reviewing cases from selected PCs, direct reporting by physicians, collecting investigation reports from local and county health officials and reviewing pesticide-related reimbursement claims [31]. Selected CDFA occupational and accidental pesticide exposure cases from 1982–1986 have been reviewed [7,31].

A total of 283 cases of OP or CB related illness in agricultural pesticide applicators (Table 42.9) were reviewed by investigating county health officials during 1982–1985 [7]. The antiChEs associated more frequently with illness were not necessarily those most commonly used. Of these occupational exposures, 45 (16%) were accidents. Violation of either state health and safety codes or labelled requirements for safe handling accounted for 124 cases (44%). In 78 cases (28%), the illness was associated with pesticide exposure on three or more consecutive working days.

Maddy and Edmiston [31] reviewed 67 selected CDFA incidents involving 583 individuals from 1986; 37 incidents involved

Table 42.9 Specific agents associated with more than ten cases of illness. California Department of Food and Agriculture Data 1982–1985

Agent	*No. of cases*	*(%)*
Azinphos-methyl	12	(6)
Carbaryl	11	(5)
Diazinon	16	(8)
Dimethoate	16	(8)
Methomyl	47	(23)
Mevinphos	49	(24)
Oxydemeton-methyl	11	(5)
Parathion	44	(21)
Total	206	(100)

[a]Data from Brown *et al.* [7]

Table 42.10 AntiChE pesticides involved in 37 selected incidents reported to the California Department of Food and Agriculture in 1986

Agent	*No. of incidents*	*No. of individuals with illness*
Carbofuran	2	3
Chlorpyrifos	7	80
Chlorpyrifos with propoxur	1	12
Diazinon	7	32
Malathion	8	116
Methamidophos	1	26
Methidathion	1	13
Methomyl	2	11
Methomyl with methamidophos	1	1
Mevinphos	1	1
Oxydemeton-methyl	1	11
Parathion	2	50
Parathion with azinphos-methyl and carbaryl	1	1
Profenofos	1	13
Tetrachlorvinfos	1	1
Total	37	371

[a]Data from Maddy and Edmiston [31]

antiChE pesticides (Table 42.10). Suicide attempt was involved in only five of these.

Overspray exposure from agricultural pesticide application was responsible for illness in 81 individuals in four separate incidents. Use of antiChE pesticides in buildings caused illness in 165 occupants in ten separate incidents. Offices were involved in three incidents with eight persons, and schools in three incidents involving 119 individuals. Other indoor exposures were involved in four incidents affecting 38 persons. Drift of agricultural pesticides and indoor application were thus significant problems in the use of antiChEs [31].

Premature re-entry of workers into fields or orchards previously sprayed with antiChEs was responsible for four incidents involving 39 persons. Retail store employees, not wearing protective equipment while attempting to clean up spilled pesticides, were involved in three incidents causing illness in 24 individuals [31].

Overspray exposure with symptomatic poisoning in eight patients has been reported from aerial application of OP and CB pesticides to cotton fields in Israel; all became asymptomatic after the spraying season ended. All were women who were home alone during aerial spraying operations and lived within 150 metres of the cotton fields [41]. Together with the California experience, these incidents suggest that weather conditions and the presence of dwellings near fields should be taken into account before aerial pesticide application is undertaken.

Acute poisoning in office workers following fumigation of a building with chlorpyrifos and methylcarbamic acid has been described; five individuals developed a mild peripheral neuropathy [18]. In a separate incident, 18 mushroom workers were exposed to diazinon in a darkened room after only the entrance was sprayed [9]. Application of antiChEs in buildings should be done only by trained and experienced operators without occupants in the area. Established precautions should be carefully followed, potential occupants adequately warned, and adequate time for ventilation allowed before occupants return.

In four incidents in California between 1980 and 1983, 135 farm workers developed poisoning from exposure to OP pesticide residues on the foliage of lettuce and cauliflower plants [34]. In one instance, 23 agricultural labourers began working in a cauliflower field to which mevinphos and phosphamidon had been applied between 4 and 6 h earlier [34,35,53]. Loss of consciousness, bradycardia, excessive salivation, miosis and muscle fasciculations developed in some workers [34]. About 1 month after the incident, 18 labourers still had eye discomfort and 11 still complained of headaches and weakness [53]. Most symptoms cleared by 2–3 months, although blurred vision, discomfort while reading and photophobia were still present in 12 workers at 4 months [34,53].

In another incident, 31 agricultural labourers were exposed to mevinphos after beginning work in a lettuce field sprayed 2 h earlier [8,9]; 29 complained of persistent headaches, visual disturbances, nausea and vomiting. In some, headaches persisted for more than 10 weeks. Some family members exposed to contaminated clothing at home developed symptoms consistent with antiChE poisoning, and had persistent headaches and eye irritation for up to 4 weeks [8].

In the above incidents, mandated 48–72 h waiting periods before field re-entry were not followed, resulting in both acute and chronic illness from probably skin OP exposure [8,9,34,35,53]. Such incidents are completely preventable by observing established waiting periods.

Study of OP and CB poisoning in infants and children

Zweiner and Ginsburg [56] described 37 infants and children with antiChE pesticide poisoning. This was from ingestion of improperly stored pesticide in 28 cases (76%), while five were poisoned through skin contact after playing on home floors or carpets sprayed or fogged by unlicensed exterminators. The most common clinical effects were miosis (73%), excessive salivation (70%), muscle weakness (68%), lethargy (54%), tachycardia (49%), respiratory insufficiency (38%) and seizures (22%). Twenty-six children had both atropine and 2-PAM, four received only atropine and three received only 2-PAM, the majority responding promptly to treatment. Two children with OP poisoning required multiple atropine doses over 24 h. Tachycardia was common on presentation, but the heart rate decreased following atropine. All 37 children were hospitalized, 29 (78%) being admitted to intensive care. Endotracheal intubation and mechanical ventilation were necessary in 14 (38%). Ten had chest radiographic findings indicating atelectasis or pneumonitis, of whom six had ingested a petroleum distillate-based product. One patient with seizures on admission had persistent seizure activity and ataxia on hospital discharge. Early symptoms were often non-specific, resulting in initial misdiagnosis in 16 cases (43%). This may have been for several reasons: many children were of an age when incontinence of urine and stool is normal; excessive lacrimation may have been attributed to crying; and bradycardia and muscle fasciculations were present in only 19% and 22%, respectively. Nearly 25% had generalized seizures, a finding less common in adults.

Aldicarb

Aldicarb is well absorbed by ingestion, inhalation and skin contact in experimental animals, and is metabolized both by hydrolysis to inactive compounds and oxidation to the active antiChEs aldicarb sulphone and aldicarb sulphoxide [42].

Aldicarb has been responsible for antiChE poisoning in children [12], has caused death in agricultural workers by inhalation and skin exposure, and occupational exposure during manufacturing has resulted in toxicity [42]. Symptoms usually clear in about 6 h. An oral dose of 0.1 mg/kg produced signs and symptoms of mild antiChE poisoning and depressed RBC AChE activity in normal volunteers [42].

Because aldicarb is taken up by plants, food-chain concentration and food-borne poisoning may occur [13,14,21,36]. The first cases, reported in 1977 and 1978, involved 14 patients in two separate episodes involving hydroponic cucumbers grown in a local greenhouse [13].

In the first episode, nine patients developed diarrhoea, nausea, vomiting, diaphoresis, blurred vision, headache, abdominal pain, muscle fasciculations, seizures (one case) and dyspnoea within 15 min to 2.25 h following ingestion of cucumbers. The illness lasted 4–12 h; ChE levels were not measured. A CB was found on analysis of the cucumbers, but specific identification was not possible [13]. In the second outbreak, five individuals developed a similar illness between 30 min and 1 h after eating the cucumbers, which lasted for 3.5–5.5 h, resolving without specific treatment. Cucumbers grown in the greenhouse and removed from the local supermarket contained 6.6 and 10.7 ppm, a cucumber retrieved from a warehouse 9.9 ppm, the water- nutrient solution 1.8 ppm, and gravel from the cucumber beds 0.6 ppm of aldicarb. No aldicarb was

found in the greenhouse water well. The source of the pesticide was not determined [13].

Between June and September 1985, aldicarb-contaminated watermelons were responsible for the largest foodborne pesticide poisoning outbreak in the history of the USA [14,21,36]. Poisonings were initially noted in Oregon and California, and later reported from Alaska, Arizona, Colorado, Hawaii, Idaho, Nevada and Washington, and in Canada from Alberta and British Columbia [14,36]. In Oregon, five persons developed nausea, vomiting, diarrhoea, abdominal pain, blurred vision, excessive salivation, muscle fasciculation, dysarthria and paraesthesias of the lips and tongue about 30 min after eating watermelon [14]. In California, three persons had rapid onset of nausea, vomiting, muscle fasciculations and bradycardia [36]. A recall of watermelons was initiated in California, Oregon and Washington early during the outbreak. The implicated melons were most likely of Californian origin [14]. In California, 1350 cases were reported to state health officials [36]. Of these, 690 (51%) were believed probable aldicarb poisoning, 370 (27%) were classified as possible aldicarb poisoning, 235 (17%) were unlikely to have been aldicarb poisoning, and data were inadequate for classification in 55 (5%) [36]. In addition to the symptoms noted in the index patients, seizures, loss of consciousness, arrhythmias, hypotension, dehydration and anaphylaxis were seen in some cases [21,36]. Seventeen patients were hospitalized for treatment [36]. In California, 250 watermelons were assayed and 10 (4%) found to contain aldicarb [36]. Estimates of the amount of aldicarb producing illness were 0.0021–0.060 mg/kg [21], similar to that of 0.025–0.041 mg/kg which may have caused antiChE poisoning from ingestion of aldicarb-contaminated cucumbers [13].

In Oregon, 264 cases were reported [14]; 61 (23%) met a rigorous case definition and 43 (16%) met the established definition for a suspect case. Thirty-one watermelons were assayed and ten had aldicarb residues from 0.01 to 6.3 ppm.

Three possible mechanisms for contamination were considered: (1) sabotage, was rejected; (2) deliberate aldicarb misapplication on watermelons, a crop not registered for use; and (3) persistence of aldicarb in the soil after previous application on a properly registered crop [14]. Possible soil persistence has some credibility because an active metabolite, aldicarb sulphoxide can have a half-life of 360 days under certain laboratory conditions; the half-life of aldicarb in soil may be only 1–2 weeks [42]. Residues have been found in various crops grown on soil treated more than 400 days previously, although no aldicarb was detected in most soil samples [14].

Aldicarb is a groundwater contaminant in Suffolk County, New York [55]. Although experiments preceding registration of aldicarb for soil application indicated it would not reach groundwater, the contamination of sandy soil, above normal application dose, a shallow water table, and heavy rainfall over two years probably contributed to the contamination. No cases of human poisoning were attributed to aldicarb groundwater residues, but 1121 (13.5%) of 8404 wells tested had aldicarb levels exceeding the New York State recommended concentration of 7 ppb; one well contained 515 ppb. Aldicarb concentrations were highest in wells located within 1000 feet of previously treated fields, and decreased with increasing distance from application sites. Large activated charcoal filters removed most of the aldicarb residues. It was suggested that groundwater sampling for pesticides be done both before and during application in sensitive areas with special soil conditions and sole-source aquifers [55].

Chronic neurological sequelae following acute OP poisoning

A case-control study was carried out in 100 adults in Colorado and Texas, from a roster of patients with a documented history of acute OP poisoning [46]. The matched control cohort was from similar geographic areas. Most poisonings (96%) were occupational, primarily agricultural. Twenty-two patients had more than one acute poisoning episode, but the mean duration between the last toxic exposure and testing was approximately 9 years. The involved agents are shown in Table 42.11. Tests conducted included physical and neurological examinations, audiometric and ophthalmological studies, serum and blood chemistries, EEG and neuropsychological testing. No

Table 42.11 Agents responsible for OP poisoning in 100 patients examined for possible long-term neurological sequelae

Agent	*No. of cases*
Dicrotofos	2
Dioxathion	1
Disulfoton	8
Malathion	6
Mevinphos	5
Parathion	42
Parathion-methyl	54
Phorate	1
TEPP	2
S,S,S-tributylphosphorotrithioate (DEF)	1

[a]Some cases involved more than one pesticide
[b]Data from Savage *et al.* [46]

significant differences were found between the previously poisoned subjects and the controls other than for neuropsychological examinations [46]. The previously poisoned group had a significantly greater number of individuals with neuropsychological deficits characteristic of patients with documented cerebral lesions (24% compared with 12% in controls). Significant differences were noted in intellectual functioning and simple motor skills. The study subjects also had lower scores than the controls in such areas as language and communication skills, memory, intellectual functioning and perception [46].

Combined neurological and neuropsychological testing might be beneficial in evaluating patients presenting with possible organic neurological deficits following acute OP poisoning. Functional depression, however, could have been responsible for some or all of the findings in this study [46].

Miscellaneous observations on the presentation of antiChE poisoning

A pet groomer developed chronic periodic headaches, nausea, tiredness, blurred vision with pinpoint pupils and excessive sweating, associated with using a phosmet-based flea dip [37]. RBC AChE activity was within normal limits, but oral atropine caused symptomatic improvement. Symptoms recurred within 1 h of treating a dog with a chlorpyrifos-based flea dip. A second pet groomer experienced periodic dizziness, fatigue, blackouts, blurred vision, chest pain, excessive sweating, coldness and chills. After refraining from contact with OP-based flea dip products for 3 months, most symptoms cleared and RBC AChE activity increased by over 30%.

Similar complaints occurred during treatment of animals with antiChE flea dip products in 12 other surveyed pet groomers [37]. None routinely wore protective aprons or gloves.

Hyperglycaemia, metabolic acidosis and hypokalaemia were reported in cases of OP poisoning [19,32,38]. OP-induced acute pancreatitis has been postulated as the aetiology of the hyperglycaemia [38], and is supported by the development of hyperamylasaemia in some cases [32]. This, however, was not supported by a normal post-mortem appearance of the pancreas in one fatal case [19].

Intussusception was noted in a 14-month-old OP-poisoned child treated with atropine, 2-PAM and multiple doses of oral activated charcoal and magnesium citrate cathartic [10]. Possible immune-complex nephropathy with proteinuria and decreased urine output with amorphous crystalluria but no serum creatinine or BUN abnormalities have been described [1,52].

Uncommon neurological findings reported in acute OP poisoning were atypical ocular bobbing [17] and choreo-athetosis [22]. Prolonged diaphragmatic paralysis has been described [43] as well as bradycardia, cyanosis and diaphragmatic and skeletal muscle weakness [40].

A 75-year-old man developed non-specific symptoms consistent with antiChE poisoning and peripheral neuropathy after chronic exposure to carbaryl used as a home insecticide [4,5]. The patient was receiving cimetidine therapy for a gastric ulcer, and this was postulated as having a possible role by inhibiting oxidative conversion of carbaryl to its inactive metabolites [5].

Cardiac arrhythmias noted in OP poisoning include sinus bradycardia, A-V dissociation, idioventricular rhythms, multiform premature ventricular extrasystoles and polymorphic ventricular tachycardia [6]. Prolongation of the PR, QRS and QT intervals may also be seen [6]. QT interval prolongation and 'Torsade de Pointes' polymorphous ventricular arrhythmias have been reported in patients poisoned

with OPs in Israel [30]. Ventricular tachycardia with complete heart block has been documented in a patient from the UK [54].

Prolonged cholinergic effects following acute poisoning have been reported in adults and children [3,33]. Relapses may occur, and antiChE toxicity may rarely require 24–30 days to resolve, even with specific therapy [3,33].

Prolongation of suxamethonium (succinylcholine)-induced paralysis in a chlorpyrifos-poisoned child has been described [48]. Apnoea lasted for 3.25 h, most likely because of inhibition of serum BChE which normally rapidly metabolizes the drug. In a parathion-poisoned adult, suxamethonium produced 45 min of complete depolarizing NM blockade, while atracurium produced a non-depolarizing block of much shorter duration [2]. When suxamethonium is administered to antiChE-poisoned patients, a prolonged period of apnoea should be anticipated [48], and atracurium may be a more acceptable substitute [2].

A 58-year-old man applied a teaspoonful of diazinon to his genitalia to treat pubic lice [16]; coma, respiratory depression and copious pulmonary secretions made endotracheal intubation necessary, but he fully recovered following atropine and 2-PAM.

Therapeutic attitudes to antiChE poisoning in the USA

Decontamination: supportive therapy

Initial treatment of antiChE-poisoned patients involves preventing further absorption and providing supportive care. During the extrication, first aid and definitive treatment phases, rescuers and medical personnel must take care not to become contaminated. Protective clothing, gloves, and in some cases, respirators should be worn. Contaminated clothing, medical supplies and equipment may need to be isolated for either decontamination or proper disposal. Leather items (e.g. shoes) are difficult to decontaminate and may require incineration.

If inhalation is the exposure route, move the victim from the toxic environment and administer supplemental oxygen and assisted ventilation, if required. Exposed eyes should be thoroughly flushed with water or saline.

When skin exposure has occurred, remove contaminated clothing immediately. A thorough total-body wash with soap and water, repeated three times, and including such areas as the armpits, hair, genital area, umbilicus and between toes and fingers should follow. Older literature suggests that a soap-and-water wash, followed by a 95% ethanol wash, followed by a second soap-and-water wash may remove the maximum amount of OP from skin [11]. Tincture of green soap (containing 30% ethanol) may be used in skin decontamination [44].

For ingestions, emesis should not be induced because of the potential for rapid onset of coma or seizures, and the attendant risk of aspiration. The petroleum distillates, used as solvents, carry the risk of producing lipoid pneumonitis if aspirated [56]. Cautious gastric lavage with airway protection by endotracheal intubation or carrying out the procedure with the patient in the head-down, left lateral decubitus position with suction readily available, is the preferred method of stomach emptying. Following gastric lavage, activated charcoal and one dose of cathartic should be instilled. The efficacy of multiple doses of activated charcoal has not been shown and cathartics are not advised.

By any route of exposure, supportive therapy includes suctioning of oral secretions, maintaining airway patency and adequate oxygenation, monitoring of vital signs and ECG, and control of seizures with anticonvulsants. Diazepam, when given with atropine and oximes, increased survival in experimental animals with OP poisoning [20,23], and is the initial anticonvulsant of choice [44,45]. If cardiac arrhythmias occur which require intervention when the QT interval is prolonged, Type I antiarrhythmic agents should be avoided [6]. Lignocaine (lidocaine) bretylium, isoprenaline (isoproterenol), or overdrive cardiac pacing are appropriate [6].

Endotracheal intubation, supplemental oxygenation and assisted ventilation may be necessary. Non-cardiogenic pulmonary oedema may develop, and can necessitate positive end-expiratory pressure (PEEP) assisted ventilation. Drugs to be avoided include suxamethonium (succinylcholine), morphine, methylxanthines (caffeine, theophylline), cholinergic agents such as physostigmine and loop diuretics such as frusemide (furosemide) and ethacrynic acid [39,44,45,48].

Atropine

The specific treatment for muscarinic effects is atropine. The usual dose is 1.0–2.0 mg iv for adults and children older than 12 years. This dose may be repeated at 10–30 min intervals until reversal of cholinergic signs occurs [39, 44,45,49]. For children younger than 12 years, the initial atropine dose is usually 0.25 mg (0.01 mg/kg) followed by doses of 0.02–0.05 mg/kg, which may be repeated every 10–30 min [39,44,45,49].

These atropine doses are five to ten times those used therapeutically for other purposes. The end-point for atropine titration is drying of pulmonary secretions, which is more specific than either pupillary dilatation or development of tachycardia. In children, tachycardia is frequently observed, but the heart rate may decrease following atropine [56]. Although most patients require substantially less, atropine doses as high as 1000 mg per day have been required in unusually severe poisoning [49].

Repeated doses have been necessary in many cases, sometimes over several days [3,33], sometimes by continuous infusions [24, 43]. Initial infusion rates of 0.02–0.08 mg/kg per h have been suggested [49]. Care must be taken to avoid producing an iatrogenic anticholinergic poisoning.

Pralidoxime (2-PAM)

When central effects of coma or seizures and nicotinic effects such as muscle fasciculations or diaphragmatic weakness with resultant respiratory insufficiency are present, an oxime should be administered. The only oxime available for clinical use in the USA is 2-PAM, which should be administered as soon as possible to avoid the 'ageing' of AChE. Best results are obtained when 2-PAM is administered within the first 24–48 h following exposure.

2-PAM is usually administered iv in a dose of 0.5–2.0 g infused over 15–60 min [39, 44, 45, 49]. Some recommend repeating the initial dose 1–2 h later [39]; others suggest that the initial dose be repeated 3–4 times at 8–12 h intervals [44, 45 ,49]. The paediatric dose is 25–50 mg/kg, administered as above [39, 44, 45, 49]. Based on a computer pharmacokinetic simulation, continuous 2-PAM infusion at 500 mg per hour has been suggested as an alternate adult treatment regimen [50], but is not considered standard therapy. While side-effects of 2-PAM are usually mild, one case of recurrent asystole in conjunction with PAM infusion has been reported [47]. ECG monitoring should be done during 2-PAM infusions.

A controversy exists over the use of 2-PAM in CB poisoning. Older experimental animal studies contraindicate 2-PAM in CB poisoning. However, review of these studies revealed that only a few animals were used and that of the CBs tested, 2-PAM increased the toxicity of carbaryl only [45]. In paediatric CB poisoning where the diagnosis was initially uncertain, 2-PAM was administered without causing any harm, although clinical improvements could not be directly attributed to the oxime [12].

A consensus opinion of ten editors of a frequently-used poisoning treatment database concluded that six would administer 2-PAM in addition to atropine for severe, life-threatening CB poisoning, but four would not, and one mentioned two anecdotal cases in which it appeared that administration of 2-PAM was associated with worsening of the patients condition [45]. Some authors recommend 2-PAM in antiChE poisoning if the aetiology is unknown or when there is a combined OP-CB exposure [39,49].

Quaternary nitrogen oximes such as 2-PAM have been thought to be unable to penetrate the CNS. However, in a paediatric parathion poisoning case with coma and flaccid paralysis, 2-PAM infusion was begun during continuous EEG monitoring; within 2 min of beginning a 15 min 2-PAM infusion, a dramatic improvement in the EEG tracing was noted and was concomitant with return to a normal level of consciousness. No other therapeutic interventions could have accounted for the EEG or clinical improvement [29].

Conclusions

AntiChE poisoning is relatively common in both adults and children in the USA, and occurs in a wide variety of accidental, suicidal, environmental and occupational settings. The diagnosis may not always be straightforward, especially in mild to moderate cases with non-specific symptoms such as gastrointestinal complaints, headache and weakness. Initial treatment involves prevention of further absorption and provision of supportive care.

Rescuers and medical personnel should take appropriate preventive measures to avoid contamination during rescue and treatment. Specific therapy involves iv atropine and, at least in severe OP poisoning, 2-PAM.

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43

Incidence, presentation and therapeutic attitudes to antiChE poisoning in Asia

W-O. Phoon

Most countries in Asia are in the process of development. There is a rapid population increase, and food production fails to keep apace. Consequently, there is increasing use of chemicals to preserve crops. AntiChEs account for an appreciable volume of poisoning cases in Asian countries.

Incidence of antiChE poisoning in Asia

Information is extremely patchy. Persons poisoned by antiChEs may have difficulty in obtaining help from Western-trained health personnel; the latter abound in urban areas but are scarce in rural and remote areas where antiChE poisoning tends to occur. Sometimes proper treatment is given but cases are not notified to a central authority, and do not appear in official statistics. Also, health centres in rural areas are often ill-equipped in laboratory facilities and lack expertise for monitoring ChE levels either in prevention or treatment, so accurate diagnosis can be difficult.

Another problem in Asian countries is identification of a particular pesticide responsible for poisoning. This may be from ignorance of the victim or medical attendant. On other occasions it may result from the common practice of using pesticide mixtures; the composition may vary considerably from batch to batch.

In Korea, OPs have been increasingly deployed for agricultural and horticultural purposes. During 1975–80, for example, the quantity of agrochemicals used increased approximately 1.6 times. In 1980, the active ingredient quantity of agricultural chemicals used was about 16 000 tons. OP insecticides constituted about 47% and were responsible for about 50% of all poisonings from agrochemicals in 1980 (Table 43.1).

Table 43.1 Number of patients suffering from poisoning from agrochemicals in Chungcheong Bug Do in 1980

Chemical	*Male*	*Female*	*Total*
OPs	166	42	208
Halogenated hydrocarbons	31	8	39
Herbicides	29	15	44
Miscellaneous	13	2	15
Unspecified	83	22	105
Total	322	89	411

In 161 cases of antiChE poisoning in Korea, 45 were classified as mild, 81 as moderate and 35 as severe. Depression of ChE related to the severity of the signs and symptoms [12]. No information was given on the number of exposed.

In Indonesia, 52 workers in the packaging section of a pesticides formulating plant producing chlorpyrifos were studied. ChE levels were performed before exposure and then weekly up to the sixth week, when a production batch was completed. A post-exposure test was done 3 weeks later. Before exposure, the ChE activity was 5.94±1.29 units/ml, after 1 week 1.80±1.10 units/ml, and at the end of 6 weeks 1.20 units±0.87 units/ml; the difference between the first and the sixth week was statistically significant ($P < 0.005$). At the ninth week after exposure, the ChE activity was 3.7±1.49 units/ml; a statistically significant increase ($P < 0.005$). Two workers had mild intoxication, manifested as anorexia, nausea and muscle weakness.

In Singapore, all Parks and Recreation Department gardeners exposed to OP insecticides were studied. Five workers had low ChE

Table 43.2 Size of population, agricultural workers and pesticide users in the study areas

Country	*Total population*	*Agricultural workers*		*Pesticide handlers*	
		No. of cases	*Percentage of total population*	*No. of cases*	*Percentage of total agricultural workers*
Indonesia	5671	1349	23.8	469	34.8
Malaysia	4533	4351	96.0	4000	91.9
Sri Lanka	8885	3439	38.7	1317	38.3
Thailand	10557	4971	47.1	2060	41.1

Table 43.3 History of pesticide poisonings among users

Country	*Ever poisoned*		*Poisoned in 1983*		*Total pesticide users*
	No.	*(%)*	*No.*	*(%)*	
Indonesia	1	(0.2)	1	(0.2)	469
Malaysia	578	(14.5)	290	(7.3)	4000
Sri Lanka	157	(11.9)	94	(7.1)	1317
Thailand	404	(19.4)	—	—	2080

Table 43.4 Analysis of hospital admissions for pesticide poisoning according to area of domicile

Country	*Within study area*		*Outside study area*		*Total*
	No.	*(%)*	*No.*	*(%)*	
Indonesia	—	—	107	(100)	107
Malaysia	24	(87.5)	4	(14.3)	28
Sri Lanka	47	(50.0)	47	(50.0)	94
Thailand	6	(13.6)	38	(86.4)	44

Table 43.5 Major chemicals causing poisoning among hospital admissions

Country	*OPs*	*CBs*	*Bipyridyls*	*Copper*	*Others*
Indonesia	72.3	7.2	1.2	19.3	—
Malaysia	53.6	10.7	25.0	—	3.6
Sri Lanka	69.1	—	—	—	27.7
Thailand	22.2	14.8	25.9	—	37.0

(a)Values are percentages

Table 43.6 Rates of pesticide poisoning

Country	*Any previous history of pesticide poisoning*	*History of pesticide poisoning in 1983*	*Admissions to local hospital for pesticide poisoning per 1000 general population in 1983*
Indonesia	13.3	0.7	—
Malaysia	132.8	66.7	5.7
Sri Lanka	45.7	27.3	5.2
Thailand	81.3	—	0.6

(20–60% of normal) but were asymptomatic [18].

In Sri Lanka, 15 000 patients were admitted yearly into government hospitals for the years 1975, 1976 and 1977, an annual morbidity rate of approximately 100 cases per 100 000 population. Almost 1000 patients died during each of the 3 years. The proportions of cases from antiChE pesticides were not described, but must have been significant. The problem is highlighted by the fact that during 1987 there were only two deaths in Sri Lanka from malaria. In the same year, the total number of deaths from poliomyelitis, tetanus, diphtheria, whooping cough and malaria together was 646, much smaller than the number due to pesticide poisoning in the same year. The author noted that many cases of pesticide poisoning were attempted suicide rather than occupational exposure. Others were from accidents because of unsatisfactory storage conditions, inadequate or absent labels, misuse of pesticide containers, or failure to recognize pesticides as poisons [5].

In Thailand, a sample of 2298 households in an agricultural area was studied. Of 10 557 individuals in the sample, 48.5% worked full-time in agriculture. Among pesticide users, 19.52% had poisoning, with 33.3% occurring among those who mixed the pesticide. From hospital records, 44 cases of poisoning were admitted in 1984, of which 61.3% were suicide attempts. Non-pesticides were responsible for half of the non-occupational poisonings, 20% of occupational poisonings, and 40% of suicidal attempts. Bipyridyls were next in frequency and OPs were third. It was concluded that the incidence rate of pesticide poisoning was 8.26% among the total sample of the agricultural population studied. Hospital records were very inadequate [19].

Table 43.7 Causes of pesticide poisoning among hospital admissions in 1983

Country	*Occupational*	*Non-occupational (accidental)*	*Suicidal*	*Homicidal*	*Other*
Indonesia	1.9 (2)	—	62.6 (67)	—	35.5 (38)
Malaysia	14.3 (4)	17.9 (5)	67.8 (19)	—	—
Sri Lanka	31.9 (30)	28.7 (27)	36.2 (34)	3.2 (3)	—
Thailand	13.6 (6)	9.1 (4)	61.4 (27)	—	15.9 (7)

[a]Values are percentage of cases in each country; the values in parentheses are actual number of cases

Table 43.8 Classification of poisoning cases by severity

Group	*Clinical manifestations*
Mild	Fatigue, headache, dizziness, nausea and vomiting, excessive sweating and salivation, tightness in chest, abdominal cramps or diarrhoea
Moderate	Inability to walk, difficulty in talking, miosis, muscle fasciculations and symptoms as under 'mild'
Severe	Coma, marked miosis and loss of pupillary reflex to light, flaccid paralysis, excessive secretions from mouth and nose, rales in lungs, respiratory difficulty and cyanosis

To correct the lack of data on pesticide poisoning in Asian countries a combined study was launched in four areas in Indonesia, Malaysia, Sri Lanka and Thailand (Table 43.2) [6]. Records from local hospitals near each project area were analysed to estimate admissions for acute pesticide poisoning during 1983. The pesticide responsible and the reason for poisoning were obtained from clinical records.

In Indonesia 3.8% of pesticide users said they suffered from one or more episodes of occupational pesticide poisoning (Table 43.3). An analysis of data from patients admitted to hospital is shown in Table 43.4.

In Indonesia, Malaysia and Sri Lanka OPs were the most frequently identified pesticides responsible for poisoning (Table 43.5).

The extent of pesticide poisoning as an occupational hazard is best estimated as the incidence of poisoning per 1000 pesticide users. Hospital admissions for pesticide poisoning in the areas studied are given in Table 43.6. The cause of acute pesticide poisoning among patients in 1983 was mainly attempted suicide (Table 43.7).

From this study it appears that in Malaysia 7.3% and in Sri Lanka 7.1% of the pesticide users believed they had suffered an episode of poisoning. This is a disturbingly high rate. Conversely, it is difficult to explain the low rate in Indonesia. Information for episodes in 1983 is not available for Thailand.

In Japan, 70 male operators exposed to OPs in companies engaged in pest and termite control were studied. Eighteen had severe depression of blood ChE but no clinical signs or symptoms of OP poisoning [3].

Presentation of antiChE poisoning in Asia

In Israel, massive application of methomyl in the 1970s resulted in hundreds of intoxications requiring emergency treatment. Protective masks and clothing were not used in many cases. Exposure ranged from 0.03 mg/m^3 to 2.60 mg/m^3 during spraying and 0.03– 0.15 mg/m^3 immediately after (ACGIH TLV 2.5 mg/m^3). Despite brief (<2 h) and mostly subthreshold exposures, six sprayers complained of symptoms, including fatigue, weakness, nausea, vomiting, stomach cramps, diarrhoea and excessive sweating. These usually appeared 1–2 h after spraying.

In Korea, 190 cases of OP poisoning admitted to hospital were studied; all lived and worked in farming areas. Cases were classified into mild, moderate and severe by clinical criteria (Table 43.8).

In 56 mild cases of OP poisoning, all except six cases showed depression of ChE activity; about 70% had values below 62.5% of normal and 41% were below 50% of normal.

Among 92 moderate cases, all except one showed depression of ChE activity. About 62% were below 50% of normal and 22% had complete ChE inhibition.

All of 42 severe cases had depression of ChE activity; about 74% showed complete ChE inhibition.

In all three groups, blood ChE activity recovered completely in the first week after intoxication [12].

In Thailand, 44 cases of hospitalized pesticide poisoning in 1984, 61.3% were suicide attempts [19].

In India, Chhabra *et al.* [2] studied 35 cases of malathion poisoning. ECG changes occurred in 37%. In nine, right axis deviation was found; in five of these it reverted to normal, indicating pulmonary oedema as its cause. ST segment and T wave changes were present in 13 (37%); in all except five, the changes reverted to normal within 1 week. Ventricular arrhythmias were observed in nine cases; three with persistent abnormalities died. In the fatal cases, myocardial damage was found at autopsy. It was suggested that transient ECG changes could result from ion disturbances, but persistent changes were a direct myocardial toxicity. ECG monitoring was advocated for early detection and management of malathion poisoning.

In another study in India [9], the effects of occupational exposure to phorate of 40 male formulation workers was described. Exposures ranged from 2 to 19 years. Signs and symptoms of toxicity were present in 60% of workers; including neurological, gastrointestinal, skin and eye symptoms, and bradycardia. There was significant depression of whole blood and plasma ChE activity, but no evidence that clinical manifestations of toxicity were correlated with the degree of ChE inhibition. No cardiac damage was seen.

In Sri Lanka, 23 patients hospitalized with acute pesticide poisoning were studied. The locally-marketed (Endrex 20) insecticide was a combination of endrin (an organochlorine) and parathion. (The data presented suggest that most cases were probably OP poisoning.)

More than half developed symptoms either during spraying or within 1 h of stopping work (Table 43.9). All patients were aware of symptoms within 4 h after spraying. There was no apparent relationship between the severity of poisoning and time of onset of symptoms. One died during the acute phase.

Table 43.9 Onset of symptoms

Time	*No. of patients*
While spraying to < 30 min	7
30 min to < 1 h	6
1–4 h	10
Total	23

In 19 of 22 survivors examined 1 year after discharge from hospital, ChE activity was normal. Only four had continued to use pesticides.

However, EMG on 18 subjects indicated that seven had abnormalities. These included significant reduction in motor recruitment pattern produced during maximal attempted voluntary muscular contraction, and a reduction in the mean voltage and duration of motor unit potential identified with evidence suggesting fragmentation of motor units. Although some gave a history of fasciculations, this was not observed clinically; EMG showed no characteristic giant motor units. There was no evidence of median or ulnar nerve conduction defects. All EMG abnormalities occurred among workers exposed to Endrex 20 [8].

The authors noted that abnormal EMG patterns were found among workers exposed to parathion in Denmark (unpublished data).

From Japan, a case of delayed neurotoxicity was described in a 70-year-old woman who ingested 40 ml of fenitrothion emulsion [15]. No toxic symptoms were apparent initially, but appeared after 48 h. Impaired consciousness, fasciculations and muscle weakness were noted. Plasma and urinary 4-nitro-3-methylphenol (NMC) reached a maximum. Neither atropine sulphate nor PAM was effective. For 3 weeks the patient required ventilatory support. Muscle strength and neurological functions recovered with falling NMC levels.

There is scarcity of information on the presentation of antiChE poisoning in other Asian countries. In the Pacific region, however, which is increasingly identified with Asia itself, a few cases have been recently published.

In one report, two patients were described in whom asthma was precipitated by exposure to synthetic OPs. One had occupational exposure to fention; a 21-year-old man with a history of intermittent mild asthma controlled by bronchodilators. He worked in an abattoir with sheep skins which were sprayed monthly with powdered insecticide. Asthmatic symptoms followed a work-related pattern. Bronchoprovocation testing implicated the insecticide as causal [1].

A case of paralysis following malathion intoxication in an 18-month old boy was described; hypertension and glycosuria also occurred. Paralysis occurred weeks after intoxication, was not related to the anti-ChE activity of malathion, and followed the pattern of an ascending demyelination [4].

Another case of OP poisoning was of a 19-year-old worker in Australia, who spilled a concentrated solution of monocrotophos on his chest and arms, which he merely washed with water. Symptoms first appeared after 24 h, consisting of muscle weakness, blurred vision, blackouts, headache, constricted pupils and sweating. Whole blood ChE activity varied from 10% after one and a half days to 74% after 46 days. He was treated with atropine and PAM methiodide, and recovered four days after exposure [16].

Therapeutic attitudes to antiChE in Asia

In Malaysia, cases of pesticide poisoning generally initially seek treatment from Western-type general practitioners. In a defined community 42% of males and 25.8% of females preferred hospitals, while 18.3% of males and 11.2% of females preferred self-medication. Only 0.5% of males and 0.3% of females said they would seek help from traditional (indigenous) healers [11].

In Indonesia, 69.9% of agricultural workers in a defined community said that they would go to the hospital for treatment of pesticide poisoning and 24.1% said they would seek help at a health centre, manned by Western-type health personnel [17].

Table 43.10 Choice of medical attention for pesticide poisoning

Type of medical attention	*Male No. (%)*	*Female No. (%)*	*Total No. (%)*
Hospital	1681 (42.0)	1031 (25.7)	2712 (67.8)
Western-type general practitioner	732 (18.3)	448 (11.2)	1180 (29.5)
Indigenous practitioner	20 (0.5)	12 (0.3)	32 (32.5)
Self-medication	47 (1.17)	29 (0.72)	76 (1.89)
No medication	—	—	—

Discussion

The actual incidence of antiChE poisoning in Asia is still largely unknown. Often aggregate figures for pesticide poisoning are not available. The situation is worse for information specific to antiChE poisoning. Sometimes, the victim, his family, his workmates, and his employer are illiterate and cannot describe details of the pesticide involved. Sometimes, the trade name but not the chemical composition is known. In most Asian countries, legislation for compulsory disclosure of the chemical composition of pesticide formulations is still limited. On many occasions, different pesticides are mixed. The mixture may vary considerably, depending on factors such as the availability of pesticides.

Little or no protective clothing is used, resulting in both dermal absorption and inhalation. The tropical or subtropical climate may make the use of personal protective equipment unbearable [13].

From the available evidence there is little doubt that antiChE poisoning abounds in Asia, even if the precise extent is unknown.

Importation of pesticides into developed countries in Asia occurs without sufficient safeguards. Development authorities, farm managers and individual farmers are often not sufficiently aware of the hazards of antiChEs. Sometimes usage is excessive, thereby enhancing risks. Often, disused containers containing residues fall into wrong hands and poisoning results. Pesticide poisoning is one of many penalties exacted by neglect to take the necessary occupational health and community health precautions [14].

In Asian developing countries, one common problem is that those affected may not be aware of the need to seek prompt treatment. Information from Indonesia and Malaysia, cited earlier, is somewhat reassuring. It indicates that farmers believe they should seek help from Western-type medical practitioners, rather than traditional medicine men who do not have sufficient knowledge about antidotes. A common problem, however, is that antiChE poisoning is mistaken as something else by the victim or his family and treatment is either not sought or delayed.

Therapeutic regimens used in Asia follow conventional lines; atropine, PAM salts and

other oximes, are complemented by supportive treatment.

Control of antiChE pesticides and prevention of antiChE poisoning are urgent priorities for most countries in Asia. At the governmental level, importation, manufacture, and usage of pesticides should be comprehensively licensed and controlled by one national central authority. Currently, either no licensing system exists or is not adequately enforced; when several government agencies collaborate to control the problem there may be poor coordination or communications are inadequate.

In agriculture, the storage of pesticides should be strictly supervised to minimize improper uses by unauthorized personnel. The formulation, distribution, and usage of pesticides should be supervised by trained and competent persons. Equipment for spraying should be regularly checked for leakage. Light but effective personal protective equipment appropriate for the local climate is required.

Agricultural communities should be made aware of poisoning; including routes of absorption, early signs and symptoms, the need to seek prompt treatment from qualified practitioners, and preventive measures. Pesticide handlers should be educated to follow safety and hygiene precautions, including the proper use of personal protective equipment, washing, and changing into clean clothes after spraying. Eating, drinking and smoking in a work area should be strictly prohibited; and washing hands before these activities enforced.

Medical practitioners and other health personnel need training in the diagnosis, treatment and prevention of antiChE poisoning. At clinics and hospitals, especially in agricultural areas, laboratory facilities to measure ChE activity should be available on a 24-h basis.

In some Asian countries, pesticides in liquid form are sold in containers similar to those for soft drinks. Accidental and suicidal poisoning may be prevented by adding an emetic and/or stenching agent to liquid pesticides.

Monitoring persons exposed to antiChEs requires pre-exposure baseline blood ChE. In 1383 randomly selected subjects in Indonesia, Malaysia, Sri Lanka and Thailand, Jeyaratnam, Lun and Phoon [7] found no relationship between blood ChE and age or sex of workers. However, in Sri Lanka female sprayers had significantly lower ChE levels (57.2% had values <50.0%) which was attributed to concomitant anaemia.

Residues of pesticides may accumulate in food chain or be washed from treated agricultural land and contaminate surface water. The general community can therefore be exposed to antiChEs by these sources [10].

Accidental poisoning by antiChEs can be reduced by restricting access to authorized personnel, keeping pesticide stores under lock, maintaining inventories, and proper disposal of disused containers. Promotion of safety education is of paramount importance. For example, an Asian farmer poured an antiChE into an empty cup in preparation for usage. A few moments later, he poured some coffee into a similar empty cup. Because of inadequate lighting, the two cups were mistaken for each other, and the farmer drank the insecticide instead of the coffee with fatal results. This kind of mishap is not rare in Asian countries.

The common use of antiChEs for suicide is difficult to prevent. Limiting access to unauthorized personnel may alleviate the problem.

Summary

Most Asian countries are developing and the struggle to provide enough food for an expanding population often results in increasing use of antiChE and other pesticides, sometimes without adequate safeguards.

Information on the incidence of antiChE poisoning in Asia is extremely patchy, and many cases are missed or not reported. Studies in different Asian countries in recent years suggest that pesticide poisoning is a major problem in agricultural communities. A four-country study showed rates per 1000 agricultural workers with a history of pesticide poisoning of 13.3 for Indonesia, 132.8 for Malaysia, 45.7 for Sri Lanka and 81.3 for Thailand. Within a single calendar year, 0.2% of pesticide users considered themselves to have suffered an episode of poisoning, in contrast to 7.3% for Malaysia and 7.1% for Sri Lanka. These subjective assessments are difficult to evaluate in view of the discrepancies between the prevalences reported from Indonesia in contrast to those of the other countries studied. Admission records in hospitals in Sri

Lanka and several Asian countries demonstrate that poisoning from antiChEs is a major health problem in workers and the general community.

The presentation of antiChE poisoning in Asia follows what has been described in other parts of the world. Therapeutic attitudes toward antiChE poisoning among Asians favour seeking help from Western-type health personnel. However, there is still widespread ignorance about the early diagnosis of pesticide poisoning and the necessity of seeking prompt treatment.

Enforcement of regulations concerning the manufacture, distribution, storage and disposal of antiChEs, the education of agricultural communities and health personnel regarding pesticide poisoning, the inculcation of safe and hygienic habits among pesticide users, and the monitoring of the work environment and exposed workers will reduce, if not eradicate, antiChE poisoning in Asia.

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Incidence, presentation and therapeutic attitudes to anticholinesterase poisoning in Egypt

Essam Enan

Many compounds in use today, including dichlorvos, dimethoate and malathion in household products in Egypt have an acute antiChE effect but do not produce delayed lesions [6,7,10].

The insecticides cyanofenphos and leptophos are known to be poor AChE inhibitors, because they are phosphonothioate derivatives, and produce delayed neurotoxic effects [4,5,9].

A major hazard of insecticides in Egypt exists in the application of agrichemicals to cotton plant, the main cash crop, where concentrations of aerosols of the applied compounds are often sufficient to create an inhalation hazard to those in the immediate vicinity. Skin absorption often represents a hazard to workers. Approximately 150 000 seasonal workers and 20 000 permanent staff of the Egyptian Ministry of Agriculture are employed in the application of insecticides for 3 months a year. In addition, approximately 35000 seasonal workers and 10 000 employees of the Ministry of Health of Egypt are employed for vector control throughout the year.

Incidence of antiChE poisoning in Egypt

In 1979 two cases of acute poisoning from exposure to diazinon were recorded in Alexandria, Egypt. Their illustrative clinical reports were as follows:

Case 1. A 33-year-old man had sprayed diazinon for >18 months. He used a back sprayer but did not use a mask or gloves. He sprayed approximately 1200–1500 l of diluted diazinon once a week from March to October. On 14 April 1979 he sprayed an area for housefly infestation in Seedy Beshr, Alexandria, Egypt, with the new 'tin' containers of 601 EC diazinon after the normal dilution (approximately 0.1%). Before spraying was completed at noon he complained of nausea and vomiting. He became weaker and noted muscle twitching of the arms and legs. He became excited and breathing was difficult; he was persuaded by his colleagues to go to the University Hospital, arriving at approximately 13.30 hours. He was given 1 mg of atropine sulphate (im) at approximately 13.30 hours which was repeated after 2 h. He remained at the hospital until the next day, and was discharged without another examination.

Case 2. A 50-year-old man sprayed diazinon for more than 3 years as described for case 1. On 12 April 1979, he was spraying an area for housefly infestation in Moharam Bek, Alexandria, Egypt, using the same containers as in case 1. In the afternoon, after completing spraying, he complained of nausea and vomiting but went home. He tried to eat dinner but vomited again. Later he complained of burning eyes and blurred vision, and difficult breathing. In the evening, he became weaker and breathing became difficult. From Friday to Sunday he had a severe headache and remained at home. On Monday April 16, he went to work and reported his illness.

On 16 April 1979, the director of the Hygiene Department sent a report describing the incident, along with a sample of the diazinon used, to the Pesticide Toxicology Department Laboratory for examination and analysis.

Table 44.1 Activity of AChE in two poisoned patients

Time after poisoning incident (days)	*Residual ChE activity (%)*			
	Case 1		*Case 2*	
	Plasma	*RBC*	*Plasma*	*RBC*
2	68.7	42.1		
4			68.1	62.1
8	75.4	45.3		
10			76.8	70.3
15	80.3	51.2		
17			85.8	74.6
18	86.1	57.8		
20			98.3	92.8
28	94.2	95.6		

Table 44.2 Cases of pesticide poisoning admitted to Alexandria University Hospital during 1980–1987

Year	*No. of cases*	*AntiChE poisoning cases*
1980	150	90 (60)
1981	128	85 (66)
1982	140	100 (71)
1983	200	120 (60)
1984	145	70 (48)
1985	141	78 (55)
1986	130	62 (48)
1987	116	60 (52)
Total	1150	665 (58)

Figures in parentheses are percentages

Table 44.3 Cases of pesticide poisoning admitted to Gamal Abdel Nasser Health Insurance Hospital during 1980–1987

Year	*No. of cases*	*AntiChE cases*
1980	51	18 (35)
1981	54	20 (37)
1982	44	15 (34)
1983	48	21 (44)
1984	53	18 (34)
1985	50	17 (34)
1986	41	19 (46)
1987	42	12 (29)
Total	383	140 (37)

Figures in parentheses are percentages

Table 44.4 AChE activity of antiChE poisoned cases in the two hospitals in Alexandria during 1987

Month	*AChE inhibition (%)*	
	University Hospital	*Insurance Hospital*
April	10.5	8.0
June	22	14
August	28	20
October	25	19

Percentage inhibition calculated as the average of the monthly cases

The ChE activities of the two workers are reported in Table 44.1.

From chromatographic analysis, it was found that the sample had virtually completely converted. Sulfotep and monothiono-TEPP were two identified products; both are much more toxic than diazinon [14].

During 1980 to 1987 an inventory of cases admitted to the University Hospital of Alexandria was prepared (Table 44.2). Over the last 8-year period, 1150 cases of pesticide poisoning were recorded, of which 665 cases were caused by antiChE insecticides (58%). An inventory of cases admitted to Abdel Nasser Health Insurance Hospital in Alexandria during the same period (1980–1987) are summarized in Table 44.3. It was found that over this time, 140 of 383 cases of pesticide poisoning admitted suffered from antiChE insecticide poisoning, representing 37% of the series. The cause for the lower number of cases admitted to the Abdel Nasser Health Insurance Hospital is that they are workers taking a certain amount of precautions. On the other hand, cases admitted to the University Hospital are farmers and other poisoning incidents in individuals taking minimal degrees of health precautions and having no health education.

AChE activity of antiChE cases was measured during 1986 [3] in both Hospitals (Table 44.4). Maximum inhibition of AChE use was during August, the intensive spraying season for cotton in Egypt.

In Egypt, many studies have been carried out to investigate the side-effects of pesticide application in either agricultural or public health practices. In 1982 Enan *et al.* [9] measured the activities of ChE, alkaline phosphatase (AP), aspartate aminotransferase (AspAT) and alanine aminotransferase (AlAT) in a team of 175 persons actively working in pesticide spraying programmes in El-Behera governorate. Results showed that highly exposed workers (127 persons) had significantly greater enzyme changes denoting liver dysfunction and ChE inhibition than less exposed persons.

Ahmed *et al.* [1] studied the effect of occupational exposure to OP insecticides on serum ChE and lymphocyte NTE activities. There were significant effects on the activity of these enzymes among male workers at a pesticide formulation plant in Egypt. Also, smokers

averaged higher levels of NTE activity than non-smokers. ChE activity tended to be higher among older workers.

In 1984, Shamy [13] studied ChE activity among agricultural workers occupationally exposed to pesticides in three Governorates of Egypt. He reported equivocal effects of pesticides on ChE activity in Behera, induction occurred in Monefia while no effect was found in Assiut. The effect of some antiChE insecticides on certain enzymes in different blood groups of agricultural workers occupationally exposed to pesticide was studied by Enan *et al.* [10]. Profenofos, fenitrothion, malathion and methomyl inhibited AP, AspAT, AlAT and lactate dehydrogenase (LDH) by different magnitudes depending on blood group. Also induction of creatine phosphokinase (CPK) and glucose-6-phosphate dehydrogenase (G6PD) was revealed after exposure to the four OPs. Significant differences were found for each enzyme when comparing group A and B or group B and O.

In 1986 Enan and Berberian [8] investigated 119 male workers involved in agricultural pest control, identified according to their exposure to antiChEs during handling and spraying. The results showed a decrease in the activities of serum ChE and monoamine oxidase (MAO), and increased acid phosphatase activity. All effects were more pronounced among the high exposure group (75 subjects) than the low exposure group (44 subjects). Berberian and Enan [2] also studied other effects in persons occupationally exposed to antiChEs. The results showed a significant increase in blood adrenaline but no significant change in noradrenaline. The activities of serum ChE and MAO were decreased. Systolic blood pressure was increased. The fasting blood sugar was significantly increased compared with an unexposed group. The results indicated a high chronic exposure level.

In 1987, Noweir *et al.* [12] studied pesticide residues in some Egyptian foods. They reported that the initial residues of chloropyrifos and phospholan were higher, indeed approximately double methomyl residues, owing to the better management in the application of the last compound. The degradation of chloropyrifos deposits in tomatoes was lower during autumn than in summer, which was attributed to differences in ambient temperature and humidity. In vegetables collected from markets chlorpyrifos was still detectable at 12 and 14 days in summer and autumn, respectively while methomyl still could be detected after 3–5 days. It has been suggested that phospholan should not be used on foods. However, no inhibition of ChE could be detected in a sample of 27 consumers.

Table 44.5 Amounts of pesticides used during the period from 1982 to 1985 for vector control programme operations in Cairo and Alexandria

Pesticide	*1982*	*1983*	*1984*	*1985*
Malathion (1%)	950 l	1000 l	800 l	800 l
Malathion (57%)	—	—	3000 l	4086 l
Dimethoate	—	—	6925 l	8500 l
Fenitrothion	8905 kg	9740 kg	—	—

In 1987 Enan investigated 30–40-year-old workers occupationally exposed to pesticides. Eight were employed by the insect control unit of West Cairo Medical region, and ten belonged to the insect control unit of Alexandria. Exposure characteristics are given in Table 44.5. ChE activity was inhibited 26% and 23% in the workers of Cairo and Alexandria, respectively, during the spraying season. Liver function tests showed elevation of AspAT and reduction of AlAT activities. AP activity was increased among the workers in both cities. Triglycerides, total lipids, and β-lipoproteins were altered, and K^+ and Cl^- were significantly reduced, but no significant change in copper was noted.

Clinical presentation

AntiChEs, used in Egypt, produce a typical intoxication sharply characterized by muscarinic, nicotinic and CNS features. The clinical picture depends on the severity of the poisoning. In mild intoxication mainly parasympathetic symptoms are evident, while in moderate and severe intoxication nicotinic and CNS symptoms appear.

In 90% of antiChE intoxications in Egypt, it was found that the interval between exposure to these insecticides and the onset of signs and symptoms was from 10–15 min. Typically there is blurred vision, headache, salivation, lacrimation, sweating, abdominal colic, nausea, vomiting, diarrhoea, and tachycardia. After an hour,

the condition progresses rapidly and patients show intense miosis and difficult breathing.

Treatment of acute intoxication

Generally, treatment consists of: (1) decontamination, (2) the maintenance of respiration, (3) administration of atropine and PAM, and (4) other supportive measures [11].

In Egypt, at the phase of poisoning and even before suspicion of insecticide intoxication, gastric lavage is performed followed by administration of 2 mg of atropine. The patient is then transported to hospital or to the Medical Poisoning Centre where the following explorations and investigations are carried out: (1) arterial blood pressure, (2) phonocardiography, and (3) laboratory investigations. These include chromatographic analysis of the patient's gastric contents and urine, haematology and plasma ChE. After diagnosing anti-ChE intoxication, the patient is given activated carbon and further atropine. Physical examination is continued daily with other investigations until the patient becomes free from signs of poisoning.

Conclusion and recommendations

Strict legislation is required to limit exposure to hazardous materials. Also, work should continue to develop less hazardous antiChEs and formulations. Research should be directed towards early prediction of chronic side-effects of new compounds, particularly delayed neuropathy and carcinogenesis. Hazards of insecticides to pesticide workers may be controlled by: (1) preplacement medical examination for proper selection of workers which should include investigations of the central and peripheral nervous systems, lungs, skin, eyes and liver; (2) periodic medical examination for early detection of health impairment; (3) proper training of workers for safe use and handling of pesticides; (4) proper use of personal protective equipment; (5) provision of emergency first aid services; and (6) proper labelling of pesticides in the Arabic language. The label should include: description of contents, toxicity and hazards, storage and handling precautions, and first aid for intoxication and contact with skin and eyes.

The variability in susceptibility to intoxication of blood group (ABO) status requires further study. Such variations in human susceptbility to toxicants should be taken into consideration in establishing safety measures and maximum tolerated levels. These variations should also be considered in the government regulations.

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Anticholinesterases used in the USSR: poisoning, treatment and preventative measures

Y. I. Kundiev and Y. S. Kagan

In the USSR, antiChEs are widely used as pesticides and medications. For industrial hygiene purposes, toxicity is classified by LD_{50} into classes I, II, III and IV, having LD_{50}s <50, 50–200, 200–1000 and >1000 mg/kg, respectively. World data suggest that 86% of poisonings are caused by class I pesticides [55]. Because of this, pesticides with LD_{50}s <50 mg/kg have been banned in the USSR. Such compounds include important OPs, for example demeton, demeton-methyl, TEPP, phorate, disulfoton, mevinphos, schradan, parathion, azinphos-methyl, azinphos- ethyl, phosacetim, dicrotophos, monocrotophos, mipafox and O,O-diethyl O-(4-methyl-2-oxo-2H-chromen-7-yl) phosphorothioate (Potasan). Some of them, including demeton, demeton-methyl, schradan and parathion were withdrawn in the 1950s and 1960s but an exception was made for selected pesticides such as terbufos, carbofuran and oxamyl that were recommended for agricultural use as granular formulations. While the application of parathion-methyl is temporarily allowed, the ban has significantly decreased the number of poisonings.

Causes of poisoning

OPs are responsible for 73.4% of pesticide poisonings [54]. The remainder are from organochlorines (12.6%) as well as arsenicals, mercurials and others. Noor and Loogna [53] analysed 1600 cases of acute poisoning caused by caustics, gases and pesticides and found that 5% of all poisonings were caused by OPs. In other reports, the majority of OP poisonings were caused by parathion, parathion-methyl, demeton, demeton-methyl or malathion [22,59]. Because class I pesticides have been banned in the USSR, most cases have been from trichlorfon and malathion [46]. Poisoning by CBs has been infrequent.

OP poisoning may be classified as occupational or non-occupational. Most OP occupational poisonings are agricultural, occurring during spraying or dusting of crops for vector control. The reason was insufficient training of operatives or inadequate adherence to preventative measures. Hot weather tends to increase the number of poisonings as it makes individual protective equipment uncomfortable. Failure to adhere to re-entry conditions is another cause of intoxication [22,38,59]. Inadequate pesticide storage or application contribute to domestic poisonings. The former is particularly important with intoxication in children.

Route of entry

The routes of entry into the body for OPs and CBs are the gastrointestinal tract, skin and respiratory tract.

Suicidal poisonings are usually oral, in which case the severity of the poisoning depends on the swallowed substance, its toxicity and the delay before the institution of treatment.

The percutaneous entry of OPs has been studied by Kundiev [39]. Sweat gland ducts may contribute to absorption [62]. Dusting or spraying of crops can give rise to a transdermal hazard, as can harvesting fruit immediately after trees have been treated [39]. The majority of acute occupational intoxications

have been observed after application of highly toxic liquid formulations, that are absorbed through the skin. Thus, Gulyamova and Maksudova [19] reported that 50.9% of all poisonings were recorded after demeton spraying. In the majority of cases this was from working in demeton-treated areas, while a small number of poisonings were caused by demeton-contaminated water. Half the reported poisonings from cotton spraying with OPs were reported to be in cases where work was being carried out in treated areas. The most hazardous operation was sucker removal, when sap containing the pesticide makes contact with the unprotected skin of the hand. In 222 cases of parathion-methyl intoxication, 70% were in farm workers cultivating cotton, and in these cases the skin was the most probable means of entry into the body [59].

The third mode of entry of OPs is by inhalation as aerosols or vapours and absorption may take place in all regions of the respiratory tract. The possibility of secondary evaporation of OPs, after contact with crops and the soil, should always to taken into consideration especially where the environmental temperature is high.

It is probable that, in many cases, both percutaneous and inhalation poisoning contribute to the total intoxication of workers in treated fields [38,67]. The effect of simultaneous entry of OPs by different routes should always be considered as the toxicity may be increased [65].

Clinical forms and pathogenesis of poisoning

Poisoning by OPs and CBs may be divided into acute, subacute and chronic. Acute and subacute poisonings are characterized by ChE inhibition and cholinergic overactivity. Although the effects of all antiChEs are broadly similar, there are differences caused by variation in the extent of central and peripheral muscarinic and nicotinic effects, while the dynamics of the intoxication are dependent on the individual antiChE. The clinical signs and symptoms of antiChEs may be classified into three groups, namely muscarinic, nicotinic and central [17,22,58]. Luzhnikov [46] drew attention to a fourth group of symptoms and signs associated with a curare- like effect of antiChEs. Muscarinic effects include salivation, sweating, miosis, bronchospasm and bronchorrhoea and are alleviated by atropine. The nicotinic effects such as skeletal muscle effects and hypertension are not affected by atropine but are alleviated by antinicotinics such as aprophen, as well as ChE reactivators [18,22]. The central action of OPs and CBs are mediated through receptors in the CNS and may produce respiratory insufficiency, tremor, convulsions, impairment of consciousness, coma and neuropsychiatric manifestations. Salikov [60] examined 241 patients with acute intoxication with malathion, trichlorfon, parathion-methyl or dichlorvos. Trichlorfon and dichlorvos were reported to bring about effects mostly associated with muscarinic receptors, whereas malathion and parathion-methyl produced signs of nicotinic receptor excitation. According to Luzhnikov [46], the clinical signs and symptoms of acute antiChE poisoning represent two main phases of intoxication: the toxicogenic from ChE inhibition and the somatogenic, which is associated with bodily adaptation to low levels of enzyme activity. Particularly in oral poisoning, disturbances in the CNS, manifested by emotional disorders and EEG changes, may occur. This results in the asthenic syndrome of headache, dizziness, anxiety and fear, while the development of intoxication psychosis and coma is possible. The EEG shows irregular α-activity replaced by irregular β-activity and diffuse slow waves. Intoxication psychosis is characterized by psychomotor excitation, a feeling of panic, fear and disorientation in time and space. Coma is characterized by a decreased pupillary light reflex as well as corneal reflex and muscular tone with diminished tendon and abdominal reflexes. Epileptiform convulsions may occur with the EEG showing high frequency β-activity [46]. Akimov [2], who examined 438 patients with acute malathion intoxication, found that 24 of them died within 4–30 days. The authors noted the development of myasthenia, coma, psychosis, spasticity and cerebral, extrapyramidal and meningeal signs. Muscle paresis, respiratory disorders of neurological origin, autonomic effects and polyneuropathy were also reported.

Myosis and visual disturbances are characteristic of antiChE poisoning: in severe

intoxication, the pupil is miotic and its reaction to light is absent.

Effects of antiChEs on the PNS are myasthenia, decrease in muscle tone and the presence of fasciculation. Later there may be paresis and respiratory paralysis. In severe poisoning, paresis of respiratory muscles may be observed for 5–7 days.

The origin of respiratory insufficiency is in fact multifactorial, effects on the PNS, including the NM junction, being an important factor in the development of acute respiratory insufficiency and complications such as pneumonia. However, central effects also play a part [68]. In the initial phase, there is hypertonicity of respiratory muscles, which gradually changes to respiratory muscle paralysis. In malathion intoxication, changes in lung surfactant have been identified [5]. Bronchorrhoea may also contribute to respiratory insufficiency. The secretions contain 8–10% protein [46] and the resultant film occludes the airways, producing cyanosis.

Cardiovascular disturbances are manifested as hypertension, with systolic blood pressures of up to 200–250 mmHg and diastolic pressures of 150–160 mmHg. This is associated with high levels of circulating adrenaline (epinephrine). Bradycardia develops gradually, and the pulse may fall to between 20 and 40 beats/min. Intraventricular conduction slows and atrial and/or ventricular fibrillation may develop. The occurrence of shock is characterized by pallor, diminished arterial pressure, respiratory disorders and disturbance of consciousness. The decrease in stroke and minute volumes and blood circulation is marked. There is an increased tolerance to heparin. However in the case of deep shock, impaired coagulation and fibrinolysis may develop [46]. The concentrations of catecholamines in the myocardium change [20]. Impairment of cardiac activity was observed in 18–42.8% of cases [61] (*see* Ch.14).

Gastrointestinal effects are caused by spasm of the smooth muscle and the absorptive capacity is impaired [57]. Symptoms include nausea, vomiting, sharp abdominal pains, intestinal colic and diarrhoea, which may need to be distinguished from conditions such as appendicitis [46]; see later.

Non-specific signs and symptoms of poisoning may involve changes in the immune state [6,9,69] (*see* Ch.9). Disturbance of non-specific humoral immunity, including a decrease of lysozymal activity in serum and saliva as well as in serum complement activity, has been observed after OP exposure [3].

Laboratory investigations

Significant decrease in RBC AChE activity or plasma BChE activity is most useful [15,17,22]. Other useful studies may include chemical identification of the agent in washings of the affected person's skin or clothes or in their saliva, vomit, blood or urine using TLC, GLC, HPLC and enzymatic methods such as ELISA [31,56].

Grading of poisoning

Intoxication may be graded as follows: mild, an affected person does not lose motor function; moderate, disorders of the central and autonomic nervous system occur, together with respiratory, cardiovascular and gastrointestinal signs; and severe, there is paralysis, disturbance of consciousness, clonic-tonic convulsions and respiratory failure [15,16,22].

Luzhnikov [46] distinguished three stages of poisoning. The first is the stage of excitement, the second consists of hyperkinesia and convulsions and the third is characterized by paralysis. In the first stage only trace concentrations of OP are present, but in the second and third stages, patients had blood levels of 5–296 mg/l of trichlorfon, 1–30 mg/l of malathion or 3 mg/l of parathion-methyl. In the latter two stages, RBC AChE is significantly depressed and may fall to 5–10% of normal.

Chronic poisoning

Chronic poisoning by antiChEs during production or application has been noted [10, 21, 30]. Such poisoning features general debility, cardiovascular disorders of vagotonic character, polyneuropathy, radiculoneuritis and diencephalic syndromes, sometimes encephalopathy and psychological disorders. Allergic reactions may develop [7].

Delayed effects

Adverse delayed effects of antiChEs may be neurotoxic, gonadotoxic, teratogenic, mutagenic

or oncogenic. Polyneuropathy, polyneuritis, paresis and persistent paralysis were observed in persons exposed to trichlorfon and malathion [2,44]. Delayed neurotoxic effects resulting from demyelination in the spinal cord and peripheral motor nerves has been described following exposure to tri-o-cresyl phosphate, leptophos and mecarbam [27,28,35,36,48] (*see* also Ch.10). The early manifestation is a decrease in the speed of propogation of impulses in motor nerves, impairment of high frequency impulse conduction: this is accompanied by a decrease in activity of neuropathy target esterase in brain and lymphocytes [26,28]. Such effects cannot be demonstrated in the case of OPs, such as heptenophos and O-(2,4-dichlorophenyl) O-ethyl S-propyl phosphorothioate (Etaphos), which do not induce peripheral neuropathy or with CBs such as carbofuran. A number of biochemical changes have been observed in the nervous system after exposure to OPs causing peripheral neuropathy [42].

Embryotoxic [12,13,43] and reproductive effects [66] have been noted. In animal studies, phosmet has been shown to have teratogenic effects [1,23,64]. Carbaryl has been shown to have gonadotoxic effects, and studies in rats have shown an effect on reproduction [63]. Slight cytogenic activity was established for trichlorfon, malathion, parathion- methyl and dimethoate [41]. The potential and real hazard posed by the delayed effects of antiChEs, especially OPs, has not been studied adequately.

Treatment

There are three main components to treatment of antiChE poisoning: (1) measures to remove the poison from the body, (2) antidote therapy, and (3) symptomatic therapy.

Emergency care aims to prevent further absorption of the antiChE, to eliminate the non-absorbed fraction, to bind and inactivate the poison by administration of antidotes and to support vital body functions. As with all poisons, the earlier first-aid is undertaken, the more effective it is. Gastric lavage with large quantities of water is continued until the lavage fluid is clean and any odour of poison is no longer present. For removal of substances from the skin alkaline solutions are generally used. To hasten elimination, forced diuresis, haemoperfusion, haemodialysis or peritoneal dialysis may be used [37,45,47]. In view of the lipophilic nature of many antiChEs and their penetration into tissues, extracorporeal methods of elimination should be used as early as possible after intoxication. The use of extracorporeal methods of toxicant elimination is indicated by severe poisoning and marked decreases in AChE and BChE. The efficacy of extracorporeal methods of treatment in general is as follows: haemoperfusion using activated charcoal > haemodialysis > peritoneal dialysis [5,14,16,46]. Thus the clearance rate in parathion-methyl poisoning by charcoal haemoperfusion is 90.5 ml/min, by haemodialysis, 40.2 ml/min and by peritoneal dialysis, 26.8 ml/min [46]. Haemodialysis should be performed for at least 8 h for maximum effect, while peritoneal dialysis should be continued until there is a complete absence of the poison in the peritoneal fluid. A further acceptable method for elimination of OPs from blood is intestinal lavage with saline [49].

In OP and CB poisoning, large doses of anticholinergics such as atropine are the mainstay of treatment. Between 15 and 180 mg atropine sulphate may be needed. In mild poisoning treatment begins with 1–2 mg atropine im, while moderate cases will require 2–4 mg. In severe cases, 4–6 mg is administered iv. Atropine injections are repeated every 3–8 min until full atropinization occurs [11,22]. Intensive atropinization during the first hour is intended to counteract the muscarinic actions of OPs. The doses for intensive atropinization are for mild, moderate and severe cases, 2–3 mg, 20–25 mg and 30–50 mg respectively [46]. Subsequently, atropine is given at lower doses during the second to fourth day at doses of 4–6 mg, 30–40 and 100–150 mg, for mild, moderate and severe cases [46]. Concurrently with intensive atropine therapy, ChE reactivators are used during the first day. In mild cases TMB-4 is given as im injections of 150 mg, up to a total of 450 mg. Moderate cases will require injections every 2–3 h, with a total dose of up to 2 g. Severe cases will require TMB-4 at 30–40 min intervals until a total of 3–4 g has been administered [46]. In the USSR, a ChE reactivator having a central action is used. This is dietixim (diethyxime). Dietixim is extremely

effective in poisonings with trichlorfon, parathion-methyl, phosalone and dimethoate. Moreover it is five to seven times less toxic than TMB-4. Dietixim reactivates ChE in tissues rapidly and reverses the neurological changes induced by dichlorvos. Favourable results have been obtained using a combination of dietixim and TMB-4 [22,25,33]. Dietixim, which is supplied in ampoules of 10% solution, is used at a dose of 7–10 mg. In experimental animals, inducers of the liver monoxygenase system have been used effectively [25,29,33]. Inducers, such as phenobarbitone (phenobarbital) or flumecinol (zixorine, zixoryn), enhance the detoxication of most lipophilic substrates [34]. If these substances are used, care should be taken at the stage at which toxic OP metabolites are being formed [24,28]. Generally, however, enhancement of detoxication of the active metabolite exceeds its rate of formation. Supportive therapy, such as artificial ventilation is particularly important [68]. Additionally, it is necessary to ameliorate spasms and psychomotor excitement. In patients with prolonged bronchorrhoea and respiratory disorder of central origin, low tracheostomy with artificial ventilation is recommended, while in the case of muscle spasms, muscle relaxant drugs are indicated. Therapy with antibiotics may be necessary to prevent pneumonia, while other measures such as the use of hormones, cardiovascular supportive therapy, magnesium sulphate and chlorpromazine may be required [46]. Vitamin combinations that may give benefit include those containing vitamins C, B1, B2 and methionine. To correct hypokalaemia, potassium compounds will be needed [8]. Physiotherapy is also to be recommended [2].

Prevention

Preventative measures involve the review of pesticides before their introduction into use, followed by supervision of their application. All new preparations are studied from the point of view of their acute, subacute and chronic toxicities, their delayed effects, etc. In the USSR, the data recommendations are laid down in *Methodological Instructions on the Hygienic Appraisal of New Pesticides* [50] and *Instructions of the Pharmacological Committee of the Ministry of Public Health of the USSR* [52]. Handling, application and transport of pesticides is carried out in accordance with regulations adopted by the Chief State Medical Officer of the USSR. The most important of these regulations relate to maximum allowable concentrations (MACs) in the workers environment [4], while others relate to concentrations of pesticide in the atmosphere, water and soil. Regulations also prescribe maximum allowable food residues and concentrations in animal feedstuffs. Values are given by Kagan [25,26]. Methods of pesticide application are discussed by Klisenko [32]. The problems of occupational health and hygiene and environmental protection in relation to the application of OPs and CBs are discussed by Kagan [21,22], Kundiev [38] and the Reference Book of Pesticides [50].

Biological monitoring is carried out using the measurement of blood ChE of potential exposees. A decrease in whole blood ChE greater than 25% indicates the need for withdrawal of the person from exposure.

The experience of the USSR with antiChEs is similar to that of other countries but there are minor differences in emphasis, particularly in antidote usage, which reflect local conditions and traditions of treatment.

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46

Acute anticholinesterase poisoning in China

Xue Shou-Zheng

Introduction

Registration reports in China indicate that 80–90% of current intoxications are caused by OPs, with parathion being dominant. The occupational cases are usually mild; the non-occupational cases, mainly intentional ingestions, have serious clinical features.

AntiChEs produced and used in China

Parathion, demeton and sulfotep prevailed in the 1950s and early 1960s; malathion, trichlorfon, dimethoate, dichlorvos and parathion-methyl were used until the late 1960s; methamidophos and omethoate have become the major OPs used since the early 1980s. Carbaryl production and usage has not reached the expected levels. Carbofuran, imported since the mid-1970s, has become the prevailing CB insecticide.

In China the incidence of pesticide poisoning has increased with their use (Table 46.1). The situation has been exacerbated by the absence of strict regulation of toxic chemicals, and by lack of instruction on safe use and public health services.

Incidence of pesticide poisoning

In parts of China prevention and personal prophylaxis has resulted in dramatic reductions in pesticide intoxications and the incidence of fatalities. An example is provided by Xue *et al.* [46] for one Chinese prefecture (Table 46.2). Although large scale surveys may be complicated by population movements and other confounding variables, longitudinal studies are useful in understanding the trend of pesticide poisoning with time. The frequency of poisoning in a Chinese province, with a population of 80 million with 70% rural-dwellers and 70% engaged in agricultural pursuits, was studied by Xue *et al.* [46]. Despite fluctuations both in total cases and in deaths, it is clear that since 1981 both have decreased (Table 46.3). In other provinces where comparable data are available the trend appears similar. Yin [47] analysed pesticide poisoning in He-Bei province which has a population of 53.6 million with 88% engaged in agriculture. In the 1980s, 15 000 tons of pesticides were used, of which 8000 tons were

Table 46.1 Incidence of poisoning related to amount of pesticide sold in a Chinese Prefecture (Gan-Zhou)

Period	*Pesticides sold (g/Mu[a])*	*Incidence of poisoning (per 100 000 of population)*
1954–1957	1.50	0.61
1958–1967	18.62	5.23
1968–1975	112.39	20.11
1976–1984	110.99	19.33

[a]Mu is a unit of area = 666.6 m^2

Table 46.2 Reduction of pesticide poisoning in a Chinese Prefecture

Calendar year	*No. of exposures (in millions)*	*Cases of pesticide poisoning*	*Rate of poisoning (per 1000)*	*No. of deaths*
1981	2.05	14 632	7.14	90
1982	2.38	1749	0.74	5
1983	3.26	671	0.21	3
1984	3.30	326	0.10	0
1985	2.56	187	0.07	0
1986	2.20	73	0.03	0
1987	2.56	70	0.03	0
Total		17 708		98

Table 46.3 Occurrence of poisoning and deaths from pesticide poisoning in a Chinese province

Calendar year	*No. of the occupational cases*	*No. of deaths*	*Death rate (per 1000)*
1973	13 597	42	3.09
1974	9 087	69	7.59
1975	10 158	54	5.32
1976	7 397	45	6.08
1977	6 746	33	4.89
1978	16 154	85	5.26
1979	13 486	74	5.49
1980	8 788	23	2.59
1981	40 466	110	2.72
1982	19 093	80	4.19
1983	24 363	86	3.53
1984	14 165	62	4.38
1985	17 939	70	3.90
1986	6 868	14	2.03
1987	6 398	45	7.03
1988	4 370	29	6.64
Total	219 165	921	4.48
Annual average	13 698	58	

Table 46.4 Occurrence of occupational pesticide poisoning in He-Bei province

Calendar year	*No. of poisonings*	*No. of deaths*	*Death rate (per 1000)*
1981	9 344	47	5.03
1982	4 628	33	7.13
1983	946	11	11.63
1984	1 142	5	4.38
1985	1 126	8	7.10
Total	17 186	104	6.05
Average	3 437	21	

Table 46.5 Reduction of pesticide poisoning and deaths in Ji-Ning Prefecture in Shang-Dong province

Calendar year	*Occupational poisoning*		*Non-occupational poisoning*	
	No. of cases	*Deaths*	*No. of cases*	*Deaths (%)*
1981	2832	6	3249	515 (15.9)
1982	1746	4	2089	350 (16.8)
1983	474	1	2749	269 (9.8)
1984	223	0	1810	102 (5.6)
1985	95	0	1807	101 (5.6)

OPs, 4000 tons CBs and 1200 tons pyrethroids. In He-Bei, there has been a dramatic fall in the number of poisonings and deaths over the period 1981–1985 (Table 46.4). The overall annual incidence is only 0.0725% of the agricultural population. This does not represent a remarkable social hazard, although the absolute figure is not negligible and there is still room for improvement. In a prefecture in Shang-Dong province, there was a very impressive reduction in pesticide poisoning over 1981–1985. Not only did occupational cases decrease by 98% but the frequency of non-occupational ones fell to 55% (Table 46.5). Another encouraging report emanated from Liao-Ning province where 5086 cases of acute pesticide poisoning were registered in 1983 [7]; this was one-tenth of the highest number recorded in the past. OPs, particularly parathion, figured frequently (Table 46.6).

Table 46.6 Types of acute pesticide poisoning

Category of pesticide	*Poisoning No. of cases (%)*	*Deaths No. of cases (%)*
OPs	4631 (91.0)	53 (100.0)
Parathion	3844 (75.6)	46 (86.8)
CBs	243 (4.8)	0
Pyrethroids	95 (1.5)	0
Chlorinated hydrocarbons	17 (0.3)	0
Miscellaneous	100 (2.0)	0

The foregoing examples show that attention to education, good working practices, individual physical protection, and public health measures can bring about rapid reductions in pesticide poisoning. The statistics describe the situation for one-third of the Chinese people, namely those living in the most developed part of the country. Another third of the population inhabits areas where agriculture is not well-developed and the use of pesticides is limited; pesticide poisoning is not a serious problem. The remaining third of the population lives in areas where agricultural practice is ranked as intermediate. Pesticide use is less than in the developed third, but instruction in safe use and the public health service are both unsatisfactory. The extent of the problem is uncertain as records are incomplete. However, sample surveys are not reassuring. Thus, in a large province the rate of acute pesticide poisoning among applicators was 1.11%; the death rate may be as high as 4.5%. The number of acute pesticide intoxications was 7.5-fold the number of legally-defined occupational diseases over the same period. Deaths from occupational pesticide poisoning was more than 100-fold that of acute chemical intoxication in industry.

Thus, while in many areas of China the problem of pesticide poisoning is diminishing, in other areas the situation needs improvement.

Acute antiChE poisoning

The incidence of poisoning among OPs applicators in the field has been reduced from several percent (in the early 1960s) to less than one per 1000 [34]. Substitution of parathion and demeton with malathion, trichlorfon and dimethoate was the major reason. Safety education and training courses also played important roles [34]. Cases of carbofuran intoxication were recorded from spraying the suspension formulation, but disappeared with a change to scattering the dry fine granules.

Common clinical features of OP poisoning

The clinical features of OP poisoning have been typical [31]. Atropine and oxime reactivators are given in combination with supportive treatments [7,8]. Oxime reactivators were used in domestic trials in the 1960s [57]. Because they are safer than atropine, 2-PAM and obidoxime are widely used by rural doctors.

Most occupational cases have been treated by rural doctors in villages. The remainder were referred to country hospitals after preliminary treatment. For the convenience of rural doctors, a simplified schedule for the recognition and treatment of cases was provided (Table 46.7).

Peculiar features of dimethoate poisoning

Dimethoate has not been under strict regulation owing to its low mammalian toxicity, and could be easily purchased. The same is true for dichlorvos for controlling domestic insects. These are the commonest insecticides used for suicide in China. The course of dimethoate poisoning in China is atypical. About 10 min after swallowing a mouthful of dimethoate, the victim becomes unconsciousness, but awakens spontaneously during transport to hospital. Narcosis reappears, but recovery occurs without treatment. Blood ChE is still normal at this time, and no cholinergic symptoms are present. Typical cholinergic features appear on the second and third day. Treatment with oximes is ineffective. The victim recovers after gastric lavage, atropine, and supportive therapy on the third or fourth day. Some patients died on the third to fifth day without any warning, and some died after discharge. This tragedy, seen hundreds of times, has been referred to as the 'rebound death' of dimethoate.

The first coma is caused by the solvent benzene or toluene (50% emulsion), and the second narcosis is the effect of dimethoate. Dimethoate is metabolized by amidase, an enzyme with marked species differences;

Table 46.7 Schedule for recognizing and treating OP poisoning

Factor	*Degree of severity*		
	Mild	*Moderate*	*Severe*
Latency from exposure to onset	Long > 4 h	Intermediate 2–4 h	Short < 2 h
Visited doctor	Walking by themselves	Walking with help of others	Carried by others
Talking to doctor	Clear	Speech blurred (muscular twitching)	Unconscious
First aid	Decontamination, atropine in regular dose	Relieve symptoms and signs, then decontamination	Maintain respiration and circulation; atropinize; send to hospital
Atropine im (1 mg/ampoule)	1 per 4 h	2 per 2-hourly	4 iv, 4 per h as necessary
Oximes iv (0.4 g/ampoule) No. of ampoules	1	2 repeat 2-hourly later as needed	4, repeat in 1 h when necessary

humans possess lowest activity. The presence of trimethylphosphorothioate impurities in technical products of OP pesticides highlighted the possibility of pulmonary insufficiency being responsible for fatalities [38]. However, neither clinical examination nor at autopsy were significant pulmonary lesions found. Clinicians suggested cardiac failure as the probable cause of sudden death. Low dose atropinization, keeping the cardiac rate fast in the evening and at night for 1 week, greatly reduced deaths.

In the early 1970s, trichlorfon (5 mg/kg po for 10 days) was substituted for potassium antimony tartrate in the treatment of schistosomiasis. Cardiac complications (1–2%) were noted among elderly patients, including arrhythmia, frequent premature beat, bigeminy, trigeminy, Adam-Stoke syndrome, etc. [31]. Small doses of atropine reversed these effects. Liu Jin-Xin reported two cases of auricular fibrillation from phosamidophos which were reversed by oxime and atropine in 1.5 h [21].

Five cases of transient mania and delirium after inhaling dimethoate vapour from sewage were seen in our affiliated teaching hospital. Blood ChE was normal, and no cholinergic signs developed. They recovered without sequelae after 1 week of treatment with sedatives.

Uncommon features or complications of acute OP poisoning

'Sympathetic' predominance

Among young strong adolescents with mild to moderate intoxication, some cases occurred with symptoms of tachycardia and hypertension, and dry and flushing skin, instead of the usual bradycardia. At first, overdose of atropine was suspected; however, they denied having received treatment. Blood ChE was lowered. Small doses of atropine were beneficial. 'Sympathetic type' of poisoning was suggested, possibly from the predominance of nicotinic effects on the sympathetic ganglia.

Neuropsychiatric sequelae

Zhao [54] reported 200 cases of neuropsychiatric sequelae after parathion and demeton poisonings; most were hysteria and severe neurosis, which recovered after several years. Yu [49] reported nine cases of psychiatric sequelae after OP intoxication, of which eight cases recovered with sedation during the 3-year follow-up. Collective hysteria was reported in a team of female farmers, about ten of whom stepped into a paddy field where parathion had been applied 2 days previously. The concentration of parathion residue in the water was below the safety limit. They recovered quickly after being told there was no poisoning of the field water.

Pancreatitis

Ma [22] reported six cases of acute pancreatitis within 24–48 h of dichlorvos poisoning, a rate of 6.3% among his series of moderate to severe intoxication.

Allergic effects

One case of allergic purpura caused by dimethoate was reported by Jiang [15]. One case of haemolytic anaemia after dichlorvos poisoning was reported by Li [16]. Allergic and contact dermatitis (36 cases) and bronchial asthma (five cases) were reported by Li [17]. Xue *et al.* [44] reported 30 cases of dermatitis in two plants processing dichlorvos. Allergic dermatitis (type IV delayed reaction) has been demonstrated in animal experiments by Xue *et al.* [44].

Ophthalmological complications

Changes (macular bleeding) in the ocular fundus after OP poisoning, resolved by retrobulbar injection of atropine for 3 days, were reported by Chen [6].

Miscellaneous complications

Misdiagnosis of OP poisoning as diabetes mellitus, because of temporary glycosuria, has been reported by Pan *et al.* [28]. Rare cases, including haematuria, haemoglobinuria, and weakness of neck muscles (trapezius) resulting in drooping of the head, have been reported.

Acute CB insecticide poisoning

A review of recent Chinese medical journals produced several reports of acute poisoning from CB insecticides (Table 46.8). It is

Table 46.8 Acute poisoning from CBs

Causative chemical	*No. of cases*	*Exposure*	*Symptom, treatment, recovery*	*Reference*
Carbaryl	18 mild	Unloading	Atropine < 10 mg	Zhu [56]
Isoprocarb	5 moderate	Application	Atropine 5–20 mg im	Su [36]
Isoprocarb	1 M	Ingestion (20 ml) (vomited)	Atropine 10 mg iv	Jiang [14]
Isoprocarb	1 F	Ingestion (20 ml)	Gastric lavage (4 h later) Atropine 370 mg	Jiang [14]
Isoprocarb	1 M	Ingestion (200 ml)	Lavage, 930 mg atropine Died	Jiang [14]
Tsumacide[a]	24: M 14 F 10	Accident in production	Atropine dose unclear Recovered in 4 h	Mao [25]
Carbofuran	112: M 42 F 70	Spraying 3% granule in suspension	Coma (6), muscular twitching (5), others mild; all recovered Atropine 0.5–1 mg im	Li and Liu [18]
Carbofuran	15: M 8 F 7	Occupational: 5 Ingestion: 10	Mild, atropine, recovered One death after giving 3 g 2-PAM, respiratory arrest Others recovered, atropine 18–29 mg	Lu and Jia [20]
Carbofuran	33: M 12 F 21	Occupational: 11 Ingestion: 22 (10–300 ml, 3% formulation)	Mild, atropine about 4 mg, all recovered 8 deaths from respiratory failure, lung oedema, 12 h gastric lavage, atropine	Niu [27]
Carbofuran	1 M	Grinding powder	Atropine 17 mg Recovered on third day	Ma [23]
Carbofuran	325 (10 deaths)	All causes (a review)	like OP poisoning but mild Giving smaller doses of atropine oximes contraindicated	Ding [8]
Carbofuran	10: mild (8), moderate (2)	Processing granules	Latency 0.5–2 h RBC ChE lowered Atropine 0.6–1 mg Recovered within 24 h	Zhang [51]
Carbofuran	156: mild (99), moderate (57)	Processing granules	RBC ChE 70–80% ECG findings (31 cases) Atropine: mild 0.9 mg × 2–3 daily; moderate 1.5 mg iv × 3–4 daily	Mao [26]
Carbofuran	7, 1 unconscious	Unloading 30 ton; hot and humid climate	Mild cases (3), skin wash, atropine 0.6–2 mg; severe cases (4), atropine 2–10 mg iv, recovered 11–36 h	Wang [42]

F, female; M, male
[a]m-tolyl methylcarbamate

believed that the list is incomplete, because mild individual cases will not be reported.

'Subacute poisoning' in OP packers

In factories producing OP pesticides, the clinical features are more prominent among packers. Typically, they fill small bottles manually or semi-manually. The bottles are easily broken, contaminating the working environment.

Although protected by plastic gloves and aprons, skin contamination is a serious problem. The overall prevalence reached 3.17% in a nationwide survey covering 168 plants and 17 000 employees (1979–1981).

Exposure is correlated with the effect/response; symptoms increased and blood ChE activity decreased with length of years of exposure. Clinical features were cholinergic in nature; they rarely progressed to a serious condition, principally because of frequent sick leave breaking the continuity of exposure.

The illness develops within 2 weeks of the initial exposure. After removal from work, it subsides within weeks. Oxime treatment is ineffective. The dose of atropine used for relieving symptoms is smaller than for acute OP poisoning. This type of poisoning was referred to as 'chronic poisoning' in China. It is distinct from what western literature cites as chronic poisoning and delayed neuropathy.

Table 46.9 Delayed neuropathies after OP poisoning

Causative chemical	*No. of patients*	*Exposure*	*Severity of poisoning*	*Delay to onset (days)*	*Recovery*	*Reference*
Methamidophos Parathion Dichlorvos Malathion Dimethoate	4 2 2 1 1	Occupational (5) Non-occupational (5)	Severe All comatose	Average of 14.6	Complete (8) Disabled (2)	Fang *et al.* [11]
Methamidophos	1	Spraying in the field	Coma	10	Myoatrophy	Sha [33]
Methamidophos	1	Ingestion (30 ml)	Severe	10	Incomplete	Cai [3]
Methamidophos Dimethoate Dichlorvos	5	—	Severe	7–15	Like lateral sclerosis (3); like spinal myoatrophy (2)	Zhang [53]
Methamidophos	3	Ingestion (30–80 ml)	Severe	14–19	Partially recovered (2) Disabled (1)	Zhou [55]
Methamidophos	1	Ingestion (60 ml)	Severe	13	Myoatrophy	Yu [50]
'Bao-mian-fon'	1	Ingestion (20 ml)	Severe	15	Paralysis of lower extremities	Lo [19]
Methamidophos	1	30 ml	Severe	15	Paralysis	
Not specified	8	—	—	7–21	Good	Wang [42]
Methamidophos	4	Ingestion (3) Spraying (1)	Coma	10	Incomplete	Shu [35]
Methamidophos	30	Men spraying 50-fold concentrated solution	Mild (6) Moderate (9)	—	Mild neuropathy All recovered	Qian [30]

Chinese occupational physicians called it 'subacute poisoning'. Indications of low level continuous exposure to OPs resulting in this type of poisoning have been reported in studies on pesticides packers [13,28,29]. These observations are consistent with the results of animal experiments reported by Brodeur and DuBois [2] and Schwab and Murphy [32].

Delayed neuropathy

Reports in the Chinese literature in recent years are listed in Table 46.9.

Experimental toxicology

Many laboratory studies conducted in China have not been published abroad or even in the domestic literature. The following summarizes some of these studies.

Toxicometry

A candidate new pesticide or formulation has an acute peroral LD_{50} assay routinely conducted. Sometimes the results are used to give guidance for selecting better ways of synthesis and processing, and they also plays an important role in prevention. For example, ethion was synthesized in the late 1950s, and duplicated reports on its acute toxicity gave the LD_{50} as 4–10 mg/kg, compared with that cited in reference books of 1200 mg/kg. The technical preparation was abandoned owing to its high toxicity. A similar situation occurred with methamidophos, although it is still produced and used in China. The LD_{50} of many samples ranged 5–12 mg/kg, against its published value of approximately 30 mg/kg. It was believed that pesticide poisoning among applicators could be reduced by substituting parathion with methamidophos. Unfortunately, this turned out not to be the case. The same problem occurred with malathion. The LD_{50} value range was 500–800 mg/kg instead of 1400 mg/kg as reported abroad. The critical factor in all these cases may be the presence of impurities produced during synthesis.

Experimental therapy

Oxime reactivators were investigated by a comprehensive collaboration among Chinese scientists in the early 1960s. Obidoxime was

discovered, synthesized, and tested under our name of DMO–4 in 1964 [57]. Many clinical trials were carried out and published in the domestic literature but not reported in the world literature until 1985 [45].

Synergism of combined formulations of OPs and CBs (parathion and carbaryl as an example), was studied by Tang [37], and provided fundamental knowledge to explain the complex clinical feature of acute poisoning caused by mixed formulations. In the treatment of such cases, oximes are contraindicated (one case of death was cited earlier in this chapter). The dose of atropine needs initially to be small, and then increased later (about 2–4 h, according to the dose of OP absorbed).

Standard setting

The hygienic standards for pesticides includes regulations for controlling the residues in food, reducing pollution, and protecting the health of employees. Most are based on 'threshold' or 'no effect levels' determined in laboratory animals with 90 day to 6 months assays.

Toxicokinetics

The toxicokinetics of omethoate in the rat was studied by Cao *et al.* [4]. The absorption by human and animal skin was studied extensively both *in vitro* and *in vivo*. This work provided an important basis for prevention of acute poisoning through skin contact [43].

Comparative studies on the toxicokinetics of the inhibition of ChE between monocrotophos and parathion were conducted by Zhang [52]. Bimolecular rate constants of the interactions between 15 phosphoroamidothioate compounds and RBC ChE was reported by Ma *et al.* [24]. The isoenzymes of ChE and their role in OP poisoning was studied by Wang [39,41]. Aspects of interaction of OP with cellular membranes have been studied by Ding and Liu [10].

Studies on long-term effects

The potential of OPs to induce delayed neuropathy is routinely studied. However, methamidophos caused many cases of neuropathy, but the results of tests were negative [12].

The carcinogenicity of OPs or CBs has been considered, and work done in long-term animal assays. The preliminary analyses of morbidity and mortality of employees exposed to them, and the thousands of victims of severe poisoning by attempted suicide and those recovered from occupational intoxications, showed no carcinogenic effects. The outcome from genetotoxic monitoring and epidemiological surveys may offer more practical and decisive information in this area.

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47

Clinical presentation and diagnosis of acute organophosphorus insecticide and carbamate poisoning

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Introduction

Toxic exposure to both OP insecticides and CB pesticides produces a clinical picture of cholinergic excess. However, unlike OPs, CBs such as carbaryl do not cause relatively irreversible inhibition of AChE because spontaneous hydrolysis of the carbamylated AChE enzyme occurs. As a result, CB poisoning is usually less severe and of shorter duration than OP poisoning.

OP insecticide poisoning

The mortality rate in patients severely poisoned with OPs admitted to an intensive care unit may be as high as 18% [2, 14, 21, 30, 52]. These figures are in sharp contrast to an overall mortality rate of less than 2% in other types of poisoning admitted to an intensive care unit [23]. Patients with OP poisoning have average stays in intensive care units of 7 days [2,52].

The clinical presentation and severity of OP poisoning is dependent not only on the pesticide and the magnitude of exposure but on several other factors including: (1) the route of exposure, (2) the age of the patient, (3) whether exposure was a suicidal attempt, and (4) the solvent in the formulation.

Route of exposure

OP poisoning in humans can result from ingestion or absorption via the skin or mucous membranes. Exceptionally, it may follow parenteral administration [40]. Dermal absorption can occur as a result of local injury and, if appropriate protective clothing is not worn, both dermal and inhalational exposure may result. Poor work practice has been shown to increase the occupational risk of decreased plasma ChE activity among pesticide production workers in Taiwan [62]. A recent study revealed that chronic occupational exposure to pesticides had a direct bearing on the respiratory impairment identified in exposed workers [47]. In one case reported by Peiris *et al.* [44], acute respiratory failure occurred in a 32-year-old man after the contents of a 100 ml bottle of monocrotophos spilled on to a 5 cm laceration above the left eyebrow.

Age of the patient

It is likely that the elderly (who may also be chronically ill) are at greater risk from exposure to pesticides. It is also probable that the amount of OP passing to the fetus in a pregnant woman exposed to an insecticide is quite small [25]. Neonates have small stores of ChE and may be more susceptible to a given level of enzyme inhibition [36]. Furthermore, because of the presence of immature detoxification enzyme activity, neonates may activate OPs, resulting in enhanced toxicity [55].

It has only been recognized recently that severe toxicity may result in young children from dermal and inhalational exposure, which may occur after a child has been playing on a recently sprayed carpet. Such exposure can result in an atypical presentation so that an incorrect diagnosis is made initially [51].

Attempted suicide

Suicide is often the prime cause of severe OP poisoning. In South Africa, 40–70% of severe cases of OP poisoning resulted from attempted suicide [2,10,21]. In practical terms, the physician should assume that severe poisoning will follow any suicidal attempt as large amounts of OP are likely to have been ingested. Szajewski and colleagues (personal communication), in a study of 848 patients treated in Warsaw found that the best predictor of outcome was the volume of pesticide ingested. Ingestion of less than 25 ml of insecticide concentrate is unlikely to result in death irrespective of the class or type of OP. In contrast, the mortality rate may be as high as 45% in patients ingesting more than 50 ml concentrate. Patients ingesting between 25 and 50 ml have a mortality rate of approximately 6%.

Solvent in the formulation

OP toxicity may also be enhanced by the presence of the solvent in the pesticide formulation. The ingestion of a large quantity of organic solvent may not only induce vomiting, with risk of aspiration pneumonia, but also clouding of consciousness as well. Enhanced skin absorption of an OP may occur if the formulation contains xylene [46].

Clinical features of acute poisoning

The diagnosis of OP poisoning is based on the patient's history, clinical presentation and laboratory tests. In a patient with a positive history, a typical smell on the breath, characteristic symptoms and depressed plasma or RBC ChE activity, the diagnosis is not difficult to make. Unfortunately, a history is often unobtainable and in one study [21] was missing in 36% of cases. Moreover, the clinical features of OP poisoning may not be recognized as such if the patient presents with heart block, gastroenteritis, convulsions or diabetic ketoacidosis. An awareness of this diversity of presentation is the first step to diagnosis.

The typical features of OP poisoning are those of cholinergic poisoning and may be conveniently divided into muscarinic, nicotinic and central effects. The symptoms vary in severity and rapidity of onset depending on the route of entry into the body and the amount absorbed. Symptoms can present within 5 min of massive ingestion and almost always occur within 12 h. Muscarinic symptoms never present later than 24 h after ingestion [43] although it must be remembered that these symptoms can reappear if therapy with PAM and atropine is discontinued too early (*see* Chs 51 and 52).

The acute clinical features of OP poisoning are shown in Table 47.1 which is divided into muscarinic and nicotinic features and CNS features. Muscarinic features appear first and characterize mild to moderate poisoning but are not always present. In one study [24] no single symptom was noted in more than 60% of cases; miosis, although the most prevalent specific sign was found in only 44% of cases. Bardin *et al.* [2] observed miosis in 82% of 61 cases.

In moderate to severe cases of poisoning the nicotinic features appear first: muscle twitching, similar to that seen after intravenous suxamethonium [15], affects the eyelids, tongue, face and calf muscles; the respiratory muscles (diaphragm) then become involved and generalized muscle weakness ensues. Tachycardia is likely to be present and mydriasis [63] may be observed, particularly if atropine is given.

Table 47.1 Acute manifestations of organophosphate poisoning

Effect	*Signs and symptoms*
Muscarinic	Cough, wheezing, dyspnoea, bronchoconstriction, bronchial hypersecretion, pulmonary oedema, cyanosis
	Rhinitis, salivation, lacrimation, diaphoresis
	Urinary and faecal incontinence
	Nausea, vomiting, abdominal cramps, diarrhoea, tenesmus
	Bradycardia, hypotension
	hyperaemia, blurred vision, miosis
Nicotinic	Muscle fasciculation, including diaphragm muscle weakness
	Tachycardia, pallor
	Mydriasis
	Hyperglycaemia
CNS	Anxiety, insomnia, nightmares, headaches, drowsiness, confusion, tremor, ataxia, dysarthria, dystonic reactions
	Hypotension, respiratory depression
	Convulsion, coma

[a]Adapted from Grob and Harvey [17] and Tafuri and Roberts [53]

In very severe cases of poisoning a triad of muscarinic, nicotinic and CNS symptoms will be apparent. This combination was found in 16.9% of patients in one series [24], although in a further study of more severely poisoned patients requiring ventilatory support, these features occurred in 60% [14].

Two factors predictive of ventilatory support are a PaO_2 <8 kPa and an abnormal chest radiograph [2]. Pulmonary function changes associated with OP poisoning include a decrease in dynamic lung compliance and an increase in total pulmonary resistance and an alveolar arterial oxygen gradient [34]. Respiratory symptoms can be expected to be more severe in older people with a history of respiratory disease [16,18]. In some patients, mechanical ventilation may be required for several weeks, particularly if a highly lipophilic OP, such as fenthion, has been ingested.

The immediate cause of death in severely poisoned untreated cases of OP poisoning is respiratory failure with pulmonary oedema from hypersecretion. However, after patients have been admitted to an intensive care unit and mechanical ventilation has been instituted, the clinical problems are mainly cardiac (Table 47.2). The effects of OP on the heart are not exclusively cholinergic. Heart block [61], S-T changes, peaked T-waves and QT prolongation [9,21] are commonly seen. In one unusual presentation, a 66-year-old man presented with a cardiac arrest and heart block [6].

Ventricular arrhythmias are a common cause of death and tachyarrhythmias of the torsade de pointes type [31,35,52] may progress to ventricular fibrillation and/or asystole. Thus, ECG monitoring is essential and a temporary pacemaker should be inserted in all patients with ventricular extrasystoles [14]. Histopathology at post mortem may show myocarditis [1] with local areas of partial or extensive lysis of myofibrils and various degrees of Z-band abnormalities [45] *see* Ch. 14). Such changes may explain why some ECG abnormalities persist after recovery.

Although the presentation of OP poisoning is often classic, children may present with less characteristic features, including severe CNS depression, dyspnoea and flaccidity [51]. Small children often present with tachycardia [11] and seizures. Almost 25% of children had seizures [68] compared with only 2.4% of adults in another study poisoned with OP.

A number of attempts have been made to grade patients based on the severity of poisoning. One widely cited classification is that of Namba *et al.* [43]. Patients were categorized into four groups (latent, mild, moderate and serious). For practical purposes such a classification is inadequate [2] because it depends on a mixture of signs and symptoms as well as an estimation of plasma ChE activity, which is not immediately available in most centres. Furthermore, the severity of OP poisoning correlates poorly with initial plasma ChE activity. It is, therefore, more important to identify certain clinical criteria known to be associated with severe toxicity and a poor prognosis, such as a low arterial oxygen tension (PaO_2), opacification or pulmonary oedema on chest radiographs or ventricular arrhythmias (*see* Ch.49).

Laboratory findings

Glycosuria and hyperglycaemia are commonly seen in OP poisoning [37,43] which can lead to a mistaken diagnosis of diabetic ketoacidosis [63]. Ketones are, however, not usually found in the urine. Leucocytosis and low-grade

Table 47.2 Cardiac manifestations of OP poisoning

Bradycardia, tachycardia	
ECG	S-T changes, peaked T waves
	A-V block
	Q-T prolongation
	Ventricular arrhythmias: torsades de pointes
	Ventricular fibrillation
	Asystole
Myocarditis	Lysis of myofibrils
	Z-band abnormalities

pyrexia are frequently noted; a fever may persist for a week, even in the absence of infection [21]. As therapy with atropine and oxime may be expected to increase survival, total creatine kinase activity can be expected to be high in severely poisoned patients [1] (*see* Ch.51). A low PaO_2 and metabolic acidosis will be seen in severely poisoned patients; monitoring blood gases and pulse oximetry are most helpful in respiratory care.

The direct measurement of OPs or their metabolites in body fluids has little place in the immediate diagnosis or early management of OP poisoning. In many cases, rapid hydrolysis prevents the detection of the parent compound, although urinary metabolites may persist for several days [32]. Many commonly used OPs, such as parathion, are metabolized to p-nitrophenol, which can be detected in the urine, but many patients will present before this metabolite appears in the urine [21]. The measurement of metabolites may be most helpful as a qualitative measure of low level chronic exposure [54].

Measurement of ChE activity

ChE activity provides a useful screening test. Difficulties in interpretation do however occur because of fluctuations in ChE activity caused by disease, medication, pregnancy and genetic disposition [20]. A low plasma ChE should be confirmed, if possible, by the measurement of RBC AChE activity. RBC ChE activity is depressed more slowly and recovers more slowly following acute poisoning than plasma ChE activity and may not be initially depressed even in severe poisoning. AChE regenerates at a rate of about 1% per day taking an average of 66 days to return to 'normal' [7], but this presumably depends on the rate of spontaneous reactivation (*see* Ch.49). Typically, plasma ChE activity recovers within 1–3 weeks. In acute poisoning, symptoms usually occur when ChE activity is reduced to 50% of 'normal' and a diagnosis of OP poisoning should be suspected [66]. Following chronic exposure to OP, ChE activity can be depressed gradually to very low levels without clinical effects.

The lack of correlation between the severity of initial presentation and ChE activity [67], means that the measurement of ChE activity is of little value in immediate management. However, repeated measurement of plasma ChE does sometimes correlate with the clinical course [26], although ChE activity alone should not be used in determining when to terminate an atropine drip [33]. The use of ChE activity as a tool for the biological monitoring of agricultural workers exposed to pesticides is discussed in Chapter 49.

Subacute toxicity after acute poisoning

In 1987, Senanayake and Karalliedde [49] published a paper describing ten patients with an 'intermediate syndrome' developing 24–96 h after poisoning. The clinical picture was characterized by a paralysis of limb muscles, neck flexors and cranial nerves, and developed after resolution of cholinergic symptoms. A similar picture of late onset limb weakness and areflexia has been described by Wadia *et al.* [56]. Such a clinical presentation has been reported predominantly in patients who have ingested highly lipophilic OPs such as fenthion, and it is these patients in whom relapse is most commonly observed [3,8,10,21, 33,39,58,60,64]. Intensive management is essential in the prevention of relapse; of the ten patients reported by Senanayake and Karalliedde, only four received intermittent positive pressure ventilation; of the six who did not, two died. Only small doses of both atropine and PAM were used; no measurement of ChE activity was undertaken.

The weakness of proximal musculature observed probably represents nicotinic effects resulting from an accumulation of ACh at the motor endplate with persistent depolarization; similar muscle groups remain weak after general anaesthesia with muscle relaxation. These effects cannot be reversed by atropine. A more optimal use of PAM may prevent this clinical picture developing which is more likely to be an expression of the natural course of undertreated OP than an 'intermediate' syndrome (*see* Ch.12). Some degree of rhabdomyolysis may also contribute to the weakness observed on the second to fourth days after intoxication with long-acting OP [5].

Long-term sequelae

Neurological

Delayed mixed sensorimotor peripheral neuropathies can result from acute exposure to certain OPs including mipafox, leptophos, fenthion, trichloronat and methamidophos (*see* Ch.10). Symptoms often begin 1–2 weeks after acute exposure and include paraesthesia in the distal lower extremities, fatigue and cramps. Gait disturbances can develop and remain for several months, occasionally with permanent injury. Other transient neurological sequelae have also been reported after acute ingestion, including chorea [27,28], ataxia [38] and in one case opisthotonos [50]. Convulsions are common events in children (see earlier) and often respond to atropine therapy.

Psychiatric

Both transient and persistent behavioural abnormalities have been associated with OP toxicity. These include depression, irritability, confusion and emotional lability. Most symptoms resolve within a year. Formal neuropsychological testing is useful in the evaluation of suspected OP-induced psychopathology [48].

CB insecticides

CB insecticides are reversible antiChEs. Although the immediate clinical picture of CB poisoning is similar to that of OPs, reversible inhibition with spontaneous hydrolysis of the carbamylated AChE moiety results in less severe and less prolonged toxicity. Between 1966 and 1977, 193 cases of carbaryl intoxication were reported in the USA [12]. Carbamate toxicity can occur through inhalation, ingestion or following percutaneous exposure. The half-life of carbaryl after toxic exposure is short, about 1.3 h [22], and symptoms usually disappear within 6–8 h of ingestion. Fatal cases have been reported and death occurred within 6 h after ingestion of 500 ml 80% carbaryl [13]. CBs penetrate the blood-brain barrier poorly, and therefore the CNS effects seen in OP poisoning are absent or minimal [42]. Seizures are uncommon, and if they occur, should alert the clinician to seek another cause [19,41]. Symptoms do not usually persist beyond 24 h.

Measurement of ChE activity is unlikely to be helpful because of the rapid course of the intoxication. On the contrary, results may be misleading since ChE actually may be normal initially despite the presence of cholinergic signs [20]. Furthermore, most laboratories are not acquainted with the special procedure required to assay RBC ChE activity in the presence of CBs [59]. Although CBs are associated with fewer prolonged sequelae than OPs, a chronic neurological syndrome has been reported after carbaryl exposure [4]. This syndrome has been ascribed to a cimetidine-induced inhibition of carbaryl metabolism, producing an accumulation of carbaryl with enhanced toxicity [57].

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Pseudocholinesterase deficiency and anticholinesterase toxicity

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From an anaesthetist's point of view plasma ChE (PsChE, pseudocholinesterase) is important because it hydrolyzes suxamethonium (succinylcholine). In spite of several rather serious side-effects this depolarizing NM blocking agent is still extensively used owing to its rapid onset and short duration of action. However, in patients with decreased PsChE activity, respiratory insufficiency for hours may be seen following suxamethonium administration. The causes of PsChE deficiency and the effect of these on the duration of action of suxamethonium-induced blockade are discussed later. Furthermore, the clinical importance of PsChE in the hydrolysis of two new drugs mivacurium and bambuterol is evaluated.

Ps ChE: general properties

Plasma ChE (acylcholine-acylhydrolase E.C. 3.1.1.8.) is a glycoprotein synthesized in the liver [41]. It consists of four identical peptide chains each with a molecular weight of approximately 85 000 kDa [24]. The four subunits form two dimers each with one intersubunit disulphide bond. Each subunit consists of about 574 amino acids, hence many variants are possible even if only one amino acid is altered [25]. ChE is found in both the central and peripheral nervous system of all animals, in cerebrospinal fluid [20] and especially in liver and in blood plasma [41]. The physiological function of PsChE is not known.

The biosynthesis of PsChE is controlled by at least four allelic genes at locus E_1: (1) the E^u_1 (usual) responsible for the biosynthesis of the usual or normal form, (2) the E^a_1 (atypical), (3) the E^f_1 (fluoride resistant), (4) the E^s_1 (silent) gene, not producing any PsChE. These four allelic genes can be combined to form one normal $E^u_1E^u_1$ and nine abnormal genotypes: $E^u_1E^a_1$, $E^u_1E^f_1$, $E^u_1E^s_1$, $E^a_1E^f_1$, $E^f_1E^s_1$, $E^a_1E^s_1$, $E^a_1E^a_1$, $E^f_1E^f_1$ and $E^s_1E^s_1$.

Recently three more allelic genes at locus E_1 have been described: E^j_1, E^k_1 and E^h_1 [15,34, 42].

Decreased ChE activity

There are four main causes of PsChE deficiency: (1) physiological variation, (2) disease, (3) iatrogenic changes, and (4) genetically determined changes.

Physiological variation

The PsChE activity in a normal healthy adult is stable when measured at regular intervals, but the activity is known to vary with age, sex and with other parameters such as plasma lipids or lipoprotein fractions. Males have higher PsChE activities than females. During the first trimester of pregnancy the PsChE activity decreases, often to 70–80% of the prepregnancy level. This decrease in activity is maintained until 2–4 days after delivery [41]. The PsChE activity in the newborn is about 50–60% of that in healthy adults. At the age of 3–4 years the activity has increased to approximately 30% higher than the activity in young adults. The activity falls to the adult level at puberty. During the rest of life there seems to be a progressively slow decline in activity [38].

Reduced activity from disease

PsChE is released from the liver to plasma immediately following its synthesis, the concentration in plasma reflecting the rate of synthesis. In 50–75% of all patients with prolonged liver disease such as hepatitis, liver abscess and cirrhosis of the liver, a substantial reduction of PsChE activity is seen. Often the reduction is to 50% or even 25% of the initial level. During the acute phase of hepatic disease the PsChE activity is normal, presumably because the biological half-life is approximately 12 days [33]. In patients with acute or chronic renal disease PsChE may be reduced. Often a level of 30–35% of normal is seen, which may be from impaired liver synthesis, but chronic dialysis appears to increase the activity [38]. Patients with malignant tumours tend to have low PsChE activity, probably owing to a more selective hormonal effect of cancer cells either suppressing the biosynthesis in the liver or producing ChE-inhibiting substances. Often a PsChE activity of 60–65% of healthy normal adult values is seen, while in some patients it may fall to below 25% of normal [38]. In patients with burns PsChE activity decreases during the first few days after the trauma. The lowest level is normally found 5–6 days after the trauma and may reach 20% of normal values depending on the degree of injury and extent of the burns. This low level of PsChE activity may persist for months after the injury [38].

Iatrogenic causes

Many drugs can reduce PsChE activity. Two major factors may be responsible for this reduction: (1) inhibition of the enzyme in plasma and (2) reduced synthesis of the enzyme in the liver. Inhibitors are usually classified as reversible or irreversible.

Inhibitors of PsChE include many drugs used in daily clinical practice, i.e. metoclopramide, antibiotics, cytotoxic drugs, hormones and drugs used in psychiatry (Table 48.1). The clinically most important drugs are ecothiopate (echothiophate) eyedrops, pesticides and the new bronchodilator drug bambuterol. Bambuterol is a bismethyl carbamate derivative of terbutaline. It is an inactive prodrug which is hydrolyzed by PsChE to the active drug terbutaline. Thus PsChE is needed for the prodrug to become active. However, hydrolysis of bambuterol is normally slow because CB is built into the molecule. Bambuterol thus inhibits its own hydrolysis by inhibiting PsChE. Following bambuterol 30 mg orally the maximum depressant effect (41–97%) is seen after 2–3 h (Table 48.1) [2].

Inherited abnormal ChE activity

Because of a number of different genetic mutations, PsChE activity varies from essentially no activity in some subjects to very high levels in others, and more importantly, the enzyme differs qualitatively because of these inherited abnormal genes.

Determination of ChE activity and genotypes

Determination of the different PsChE genotypes has for many years been based on biochemical investigations, the clinical information available and comprehensive family studies.

Numerous methods have been described for determination of PsChE activity. Most commonly used today is ultraviolet spectrophotometry measuring the rate of hydrolysis of a substrate (an ester) catalyzed by PsChE. Often benzoylcholine or propionylthiocholine is used.

It is not possible to differentiate the various genotypes of PsChE by measuring the PsChE activity alone because the activity of different genotypes overlap. The qualitative difference in PsChE was first demonstrated by Kalow and Genest [18] in 1957. Based on the differences in sensitivity to inhibition by the local anaesthetic dibucaine hydrochloride, they developed a simple test to classify the type of esterase into usual, intermediate or atypical. From the percentage inhibition of PsChE (dibucaine number or DN) by dibucaine 10^{-5} M under specific experimental conditions, the usual esterase was inhibited about 80%, and the atypical esterase was inhibited about 20%. Patients heterozygous for the normal and the atypical gene showed inhibition values of about 60%. It is not possible to differentiate all genotypes solely by the DN. Numerous other inhibiting chemical compounds have therefore been used, i.e. sodium fluoride and

Table 48.1 Drugs that can inhibit PsChE

Drug	*Drug name*	*Inhibition of ChE*	*Clinical significance*	*Reference*
Anaesthetic agents				
Inhalational	Enflurane Halothane Isoflurane	Minor	Small decrease in PsChE activity is probably of little clinical significance. However, owing to other mechanisms of action the inhalational agents may prolong the duration of action of suxamethonium. At least halothane facilitates the development of a phase II block	Kaniaris *et al.* [21] Miller [27] Viby-Mogensen [39]
Non-depolarizing relaxants	Pancuronium Vecuronium	5–15% 5–10%	When pancuronium is used for precurarization the onset time and the duration of action of suxamethonium are slightly prolonged. However, if suxamethonium is given to a patient who has been given pancuronium the effect is unpredictable, i.e. the degree and duration of block may be enhanced or counteracted. The clinical importance of vecuronium's PsChE inhibiting effect is small	Mirakhur *et al.* [29] Viby-Mogensen [39]
AntiChEs	Edrophonium Neostigmine	Minor Inhibition varies between 10 and 100% depending on the dose given. It may last for hours	Duration of action of suxamethonium may range from 30 to 80 min. In patients with renal failure prolonged NM block following suxamethonium has been seen as long as 6 h after the administration of pyridostigmine	Sunew and Hicks [36] Mirakhur *et al.* [30] Bishop and Hornbein [6] Viby-Mogensen [39] Mirakhur [28]
Antibiotics	Penicillin Streptomycin	15–20%	Effect of suxamethonium may be enhanced	Viby-Mogensen [39]
Cardiovascular	Quinidine	60–80%	Duration of action of suxamethonium is prolonged and a phase II block may be seen	Kambam *et al.* [19]
	Esmolol	Significant	Duration of action of suxamethonium may be prolonged	Barabas *et al.* [4]
Cytotoxic	Cyclophosphamide	50–70%	Slightly prolonged duration of action of suxamethonium is to be expected	Dillman [10]
	Thiotepa	35–70%	Minor prolongation	Viby-Mogensen [39]
Eyedrops	Ecothiopate longlasting	70–100%	Duration of action of suxamethonium is prolonged 10–20 min	Cavallaro *et al.* [8] Viby-Mogensen [39]
Hormones	Corticosteroids	50% following 50–100 mg/day	Minor	Foldes *et al.* [14]
	Contraceptive pills	20–30%	Minor	Viby-Mogensen [39]
Organophosphate antiChE	Pesticides	100% irreversible	Duration of action of suxamethonium may be increased by 20–30 min. No clear correlation between activity and duration of action of suxamethonium has been documented	Viby-Mogensen [39]
Psychotropics	Phenelzine (monoamine-oxidase inhibitor)	Significant	Duration of action of suxamethonium may be prolonged	Bodley *et al.* [7]
	Lithium	Slight	Prolonged block following suxamethonium may be seen	Choi and Derman [9]
Respiratory	Bambuterol	40–100%, depending on dose. Maximum effect 2–3 h following oral administration	Recovery of suxamethonium-induced blockade will be 2–3 times prolonged and a phase II block may be seen. In some patients with abnormal genotype the duration of action of suxamethonium may be prolonged 2–3 h	Fischer *et al.* [13] Bang *et al.* [2,3]
Miscellaneous	Metoclopramide	Minor	Duration of action of a small dose of suxamethonium is prolonged a few min; a more prolonged effect may be seen following larger doses	Kao and Turner [22]

Table 48.2 Biochemical characteristics of the various ChE variants (locus E_1)

Genotype	*No. of patients*	*ChE activity U/l*	*Dibucaine number*	*Fluoride number*	*Urea number*
$E^u_1E^u_1$ [a]	970	690–1560	79–87	55–56	41–53
$E^u_1E^a_1$ [a]	745	433–1197	55–72	40–53	54–69
$E^a_1E^a_1$ [a]	207	190–732	14–27	16–30	86–100
$E^u_1E^s_1$ [a]	78	329–870	78–86	56–66	42–52
$E^a_1E^s_1$ [a]	33	146–450	16–27	19–30	86–100
$E^s_1E^s_1$	9	0–48	–	–	–
$E^u_1E^f_1$ [b]	21	514–1150	74–80	44–53	61–71
$E^a_1E^f_1$ [b]	12	318–777	45–52	25–33	75–100
$E^f_1E^s_1$ [b]	2	351–509	63–64	26–38	90–91

[a]Indicates that 2.5 and 97.5 percentiles are given
[b]Indicates that ranges are given
[c]From the Danish Cholinesterase Research Unit [38]

urea [16,17]. It is usually possible to determine a patient's genotype by combining the dibucaine, fluoride and urea number. Patients with heterozygous occurrence of the silent gene can, however, only be diagnosed by family studies and patients homozygous for this gene will have no or very little PsChE activity in plasma [23]. The biochemical characteristics of the various well-established PsChE variants of locus E_1 are given in Table 48.2.

To identify the new alleles j, k and h the Ro2-number [Ro2-0683, the dimethylcarbamate of (2-hydroxy 5-phenyl benzyl) trimethylammonium bromide] is now widely used [12,42]. The k- and the j-genes are associated with about 33% and 66% reduction in the PsChE activity, respectively. Both variants can be identified with certainty only when they occur together with the atypical variant, E^a_1 E^k_1 and E^a_1 E^j_1. The new h allele segregating with the atypical gene seems to be associated with an even more drastic reduction in enzyme activity.

New methods of identifying PsChE genotypes

Molecular biological studies are the basis for the new, specific, diagnostic methods to identify PsChE variants. In 1987 Lockridge *et al.* [25] succeeded in determining the complete amino acid sequence of usual human PsChE. They were then able to compare DNA sequences coding for the usual form of PsChE with the analogous DNA coding for a number of variant forms of the enzyme, and hence deduce the amino acid alterations in the protein. The following symbols are used for the DNA bases: adenine (A), cytosine (C), guanine (G) and thymine (T). A point mutation at nucleotide 209 (GAT to GGT), which changes codon 70 from Asp (aspartate) to Gly (glycine) was found by McGuire *et al.* [26] (Figure 48.1). This mutation seems to be a reasonable explanation for the altered properties of the atypical ChE variant. The DNA codings responsible for the amino acid alterations in the silent and the k-variant have also been identified. The silent mutation is explained by a frame shift at nucleotide 351 (GGT to GGAT) and it has a stop codon at position 129. Therefore, no functional enzyme is produced [26]. The k-variant has a threonine alanine polymorphism at position 539 in the enzyme (GCA = Ala, ACA = Thr) [5].

Studies are in progress of other PsChE variants to identify the DNA mutations responsible for their structural and functional alterations.

These new, specific, diagnostic methods are now being developed using blood DNA, the polymerase chain reaction (PCR) amplification, allele-specific probes and calorimetric (non-radioactive) detection methods. Future studies using these methods will increase our understanding of the clinical and physiological importance of ChE and its variants with respect to suxamethonium and other ester-containing drugs (or prodrugs) hydrolyzed by this esterase.

Plasma ChE activity and the reaction to suxamethonium

Normally, suxamethonium is hydrolyzed in plasma in a few minutes by PsChE and only

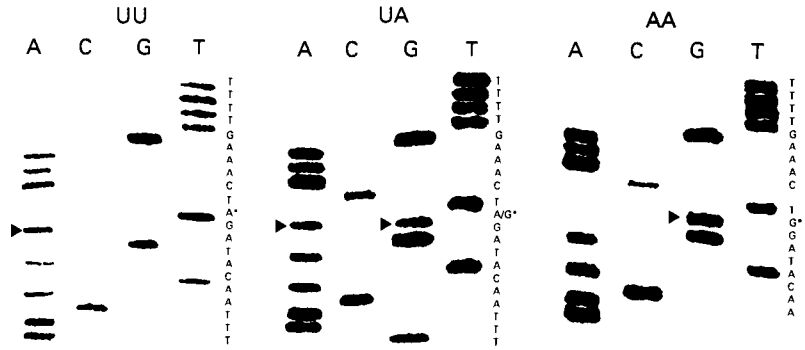

Figure 48.1 DNA sequence of usual ($E^u_1E^u_1$) heterozygous ($E^u_1E^a_1$), and atypical ($E^a_1E^a_1$) ChEs showing the nucleotide substitution in atypical ChE. Total genomic DNA from three individuals was amplified by the polymerase chain reaction with oligonucleotides corresponding to the coding region of the ChE gene. Both strands of the double-stranded, amplified DNA were sequenced simultaneously. The sequences are identical except for the nucleotide marked with a star. Codon 70 is GAT in E^uE^u, GAT and GGT in E^uE^a, and GGT in E^aE^a. Reproduced from McGuire *et al.* [26] with permission

5–10% of the injected drug reaches the NM endplates. A reduction in the rate of hydrolysis of suxamethonium causes an increased amount of the drug to reach the receptor site and this may cause prolonged NM block. Usually the degree of NM block during anaesthesia is evaluated by clinical criteria such as spontaneous muscular movements, coughing and spontaneous respiration. These clinical criteria are, however, uncertain and often the anaesthetist must in addition evaluate the muscle contraction in response to indirect nerve stimulation. The most common method used is stimulation of the ulnar nerve supplying the adductor pollicis muscle at the wrist, and evaluation of the contractions of the thumb. Most often train-of-four (TOF) stimulation is used, which consists of four supramaximal stimuli at 0.5 s intervals repeated every 12th s [1]. The amplitude of the fourth response of the muscle in relation to the first is defined as the TOF ratio and its used as a guide in detecting the transition from a phase I to a phase II block or dual block (see later). In genotypically normal patients, a typically depolarizing block with equal inhibition of all four responses in the TOF is seen following suxamethonium injection (Figure 48.2). Following repeated or larger doses of suxamethonium a phase II block, characterized by fade in the TOF response and prolonged duration of action of suxamethonium may be seen. The mechanism of this shift from a depolarizing to a non-depolarizing block is not known. The development of a phase II block depends on the amount of suxamethonium that reaches

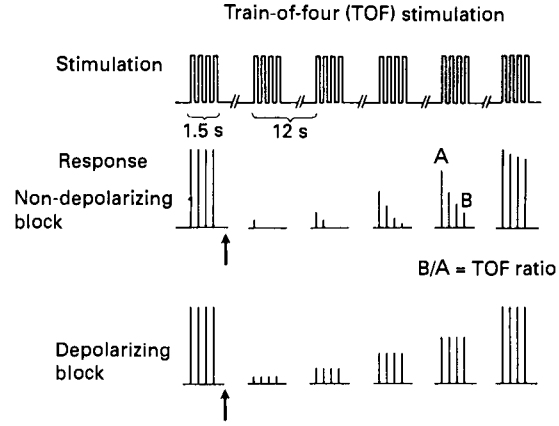

Figure 48.2 Train-of-four nerve stimulation designates four supramaximal stimuli at 0.5 s intervals over a 2-s period (2 Hz) [1]. The train-of-four is usually repeated every 12th s. The amplitude of the fourth muscular contraction in relation to that of the first contraction is defined as the train-of-four ratio. The train-of-four ratio in non-depolarizing block is reduced by comparison with the train-of-four ratio observed in depolarizing block. The reduction in the train-of-four ratio in non-depolarizing block is described as 'fade in train-of-four response'. In depolarizing block the four amplitudes ideally remain the same. When a phase II block develops following suxemethonium a reduction in train-of-four ratio is seen. Reproduced from Viby-Mogensen [40] with permission

the NM endplate and this of course depends on the PsChE activity and the dose of suxamethonium.

Genotypically normal patients

Following an intubation dose of suxamethonium (1 mg/kg) in a patient with normal PsChE activity and genotype the duration of apnoea is 5–10 min and the time to 100% twitch height recovery is 10–15 min [38]. If the PsChE activity is decreased the duration of action of suxamethonium increases. However, even ChE activities as low as 400 U/l cause only a moderate prolongation of the block [38].

Most drugs that inhibit PsChE activity prolong the duration of action of suxamethonium only moderately (20–25 min) (Table 48.1). However, because of its very pronounced PsChE inhibiting effect, bambuterol (see earlier) causes a clinically very significant prolongation of the duration of action of suxamethonium [13]. The effect of bambuterol on the PsChE activity has been found to be maximal 2–3 h after oral administration and to remain low for about 8 h. If suxamethonium is injected during this period the NM block is prolonged 3–4 times, and often a phase II block develops causing a very prolonged NM blockade [2].

Genotypically abnormal patients

In patients heterozygous for one normal and one abnormal gene, i.e. $E^{u}_{1}E^{a}_{1}$, $E^{u}_{1}E^{s}_{1}$ and $E^{u}_{1}E^{f}_{1}$ a depolarizing block is seen following normal doses of suxamethonium and, depending on the PsChE activity, the duration of action may be normal or slightly prolonged up to 10–25 min [38]. Precurarization with a small dose of non-depolarizing relaxant, i.e. pancuronium and a corresponding increase in the dose of suxamethonium to 1.5 mg/kg to facilitate tracheal intubation may, however, cause a phase II block and prolonged respiratory insufficiency. This may also occur if the PsChE activity is low because of pregnancy, disease or the administration of a drug that depresses the enzyme activity. Thus, in patients heterozygous for the usual and the atypical gene, 20 mg of bambuterol given orally causes a two to threefold prolongation of the duration of action of suxamethonium. In some patients the respiratory insufficiency has lasted as long as 2–3 h [3].

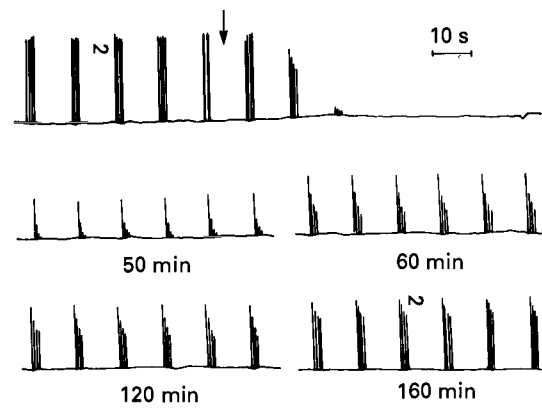

Figure 48.3 Reaction to train-of-four (TOF) nerve stimulation after iv administration of suxamethonium 1 mg/kg (arrow) in a patient homozygous for the atypical gene ($E^{a}_{1}E^{a}_{1}$). Anaesthetic thiopentone (thiopental), N_2O/O_2, halothane. The NM blockade is markedly prolonged compared with that seen in normal patients and there is pronounced fade in the TOF response (phase II block). Reproduced from Viby-Mogensen [38] with permission

In patients heterozygous for two abnormal genes, i.e. $E^{a}_{1}E^{f}_{1}$ or $E^{f}_{1}E^{s}_{1}$, a phase II block is seen already following the first dose of suxamethonium 1 mg/kg, and the time to 100% twitch height recovery is moderately prolonged (30–35 min) [37]. Patients heterozygous for the atypical and the silent gene ($E^{a}_{1}E^{s}_{1}$) and patients homozygous for the atypical gene ($E^{a}_{1}E^{a}_{1}$) show a mean duration of apnoea of 50–60 min following suxamethonium 1 mg/kg, and time to full spontaneous recovery of muscle power is 140–150 min (Figure 48.3). These patients always develop a phase II block following a normal dose of suxamethonium [38].

Diagnosis and treatment of prolonged NM blockade following suxamethonium

Prolonged apnoea following suxamethonium is not always caused by an abnormal PsChE. In the clinical situation it is therefore important to exclude other possible causes of prolonged apnoea, i.e. central respiratory depression or hyperventilation. This is done by the use of a nerve stimulator. In suxamethonium apnoea

the most important thing to do is to keep the patient ventilated and anaesthetized.

If the patient has a normal genotype the prolonged apnoea may be the result of an overdose of suxamethonium or to decreased PsChE activity. Because these patients normally have some PsChE activity there will be no suxamethonium left in plasma 10–15 min after an iv injection. Only suxamethonium bound to the receptors persists, resulting in the characteristic fade seen in the TOF response. The phase II block, seen in these patients as a 'pure' phase II block, is reversible by antiChEs [11,38]. Contrary to this, in patients with abnormal genotypes the effect of a ChE inhibitor is unpredictable. In patients homozygous for the atypical gene the quantity as well as the quality of ChE are changed and as a result the suxamethonium will persist for longer time in plasma and at the NM junction. A mixed block exists with both a depolarizing and a non- depolarizing element. A rational procedure is then to treat the depolarizing part with human ChE and the non-depolarizing part with antiChEs.

Patients who have shown a prolonged response to suxamethonium should have blood samples taken for measurement of PsChE activity and genotype. Warning cards should be issued to patients with abnormal genotypes [38].

Plasma ChE and mivacurium

Until recently PsChE activity has only been considered important in relation to anaesthesia if suxamethonium was administered.

Recently, however, a new non-depolarizing NM blocking agent, mivacurium, has been introduced. To develop a drug with the advantages of suxamethonium, i.e. with a rapid onset and short duration of action, mivacurium has been designed to undergo hydrolysis by PsChE. Other routes of metabolism do exist for mivacurium, but the clinical significance of these is not known [35]. Clinical studies in patients with normal genotype and low or normal ChE activity have shown an inverse correlation between plasma PsChE activity and the duration of action of mivacurium [31]. In patients heterozygous for the atypical gene for plasma PsChE the duration of action of mivacurium is prolonged approximately 20–30% [32]. Patients with homozygosity for the atypical PsChE gene are extremely sensitive to mivacurium [31a].

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Clinical management of acute organophosphate poisoning: an overview

Martin K. Johnson and J. A. Vale

Introduction

Management strategy

The successful management of OP insecticide poisoning depends on:

(1) The clinician possessing an understanding of the mechanisms of OP insecticide toxicity.
(2) Good first aid care being instituted.
(3) Accurate diagnosis and assessment of the severity of intoxication being made.
(4) Further absorption of the OP insecticide being minimized.
(5) Maintenance of vital body functions and adequate clinical monitoring.
(6) Appropriate use of atropine, PAM and diazepam.

Mechanisms of OP toxicity and implication for therapy

An understanding of the mode of action of OP insecticides is essential if treatment is to be maximized to prevent a fatal outcome in severe cases. As the mechanisms of toxicity have been reviewed in Chapters 4 and 6 only a brief summary will be given here and emphasis will be placed on those aspects that have implications for management. Extensive lists of OP insecticides according to structural class can be found in WHO [28] and Morretto and Johnson [18]. Nomenclature of OPs is also discussed in Ch.1. OP insecticides are toxic because they or some of their metabolites inhibit neural AChE leading to excessive accumulation of ACh and over-stimulation of the cholinergic nerve system.

The toxicokinetics of most OP insecticides have not yet been established although limited animal and human data indicate that these compounds are widely distributed in the body and may be eliminated slowly. Moreover, the effects of intoxication, particularly on the cardiovascular system, will alter the distribution and elimination of the parent OP compound and of active metabolite(s). The slow continuing production of active metabolite(s) capable of inhibiting AChE may be responsible for clinical relapses seen after apparent recovery from acute intoxication.

It should be noted that:

(1) The interaction of OP insecticides with neural AChE and with some other esterases such as serum ChE all proceed in similar fashion. However, the rates of each step differ according to the structure of the OP and from enzyme to enzyme. In every case the initial step of inhibition requires that the compound be in the oxon (P=O) form.
(2) Although phosphates are themselves active as ChE inhibitors, the P=S phosphorothioates need biotransformation into their phosphate analogues to become biologically active. As a result, signs of intoxication may be delayed. Moreover, as phosphorothioates are more lipophilic and chemically stable than phosphates, prolonged intoxication may result or there may be a recurrence of clinical features after apparent recovery from poisoning. However, as many P=S phosphorothioate formulations contain traces of oxon generated by oxidation, it is difficult to predict with certainty the time of onset of symptoms.

(3) The extent of inhibition of AChE by an OP oxon following intoxication will depend not only on the rate constant for the reaction but also on the time that AChE is exposed to the oxon. As a result, inhibition is likely to increase progressively (with a consequent increase in the severity of intoxication) until most of the pesticide is degraded *in vivo* and/or cleared from the body. The persistence time varies greatly from compound to compound. Thus, dichlorvos may be cleared within a few hours while inhibitory oxon from chlorpyriphos or demeton-S-methyl may persist for many days, particularly after massive ingestion of the insecticide.

(4) The rate of spontaneous reactivation of alkyl-phosphorylated AChE depends on the chemical structure of the side chains bound to the phosphorus atom. Most of the commonly used OP insecticides carry either two methyl or two ethyl ester groups attached to the phosphorus atom so that dimethyl phosphorylated AChE or diethyl phosphorylated AChE respectively will be generated. The probability is that an intoxicated patient will present with one of these two defined chemical species of inhibited AChE.

(5) Spontaneous reactivation of human dimethyl phosphorylated AChE proceeds quite rapidly so that, in a case of poisoning, spontaneous reactivation of the patient's AChE should occur with consequent improvement in the condition of the patient. There is no such expectation of rapid recovery for patients intoxicated with diethyl phosphoryl insecticides. A few insecticides (e.g. prothiofos) have one alkyl group linked to phosphorus through a sulphur atom instead of oxygen and the rate of spontaneous reactivation of the inhibited enzyme is faster still [4]. It is believed that no spontaneous reactivation occurs after inhibition of AChE by the few N-alkyl phosphoramidate insecticides.

(6) The spontaneous rate of reactivation can be accelerated by certain oximes which have a molecular structure which 'fits' the surface of the inhibited AChE. The extent of reactivation depends on: (a) the chemical form of inhibited AChE, (b) the nature and concentration of reactivator (oxime) which is present at the site, and (c) the length of time that the reactivator is present.

(7) The phosphorylated oxime products of the induced reactivation reaction may be potent AChE inhibitors themselves and thus, potentially, a temporary exacerbation of symptoms might occur in intoxicated patients. In reality, because patients receiving oximes will also be given atropine, deterioration is unlikely.

(8) Oximes can only be of benefit as long as some of the inhibited AChE remains in the 'unaged' form. It is commonly, but erroneously, believed that within 1 day of intoxication with a dimethyl OP insecticide, virtually all the inhibited AChE will be in the 'aged' form so that oxime therapy commenced after that time would be useless. This belief is based on *in vitro* studies which suggest that 97–99% 'ageing' will have occurred in <1 day. Such experiments are unlikely to represent the case *in vivo* for the following reasons:

   (a) If the body AChE were totally inhibited it is likely that the patient would be dead. Clinical signs of intoxication become severe before inhibition of neuronal AChE is complete, at 75–90%. At such a point, levels of ACh are increased and tend to compete with the OP oxon for the active sites of remaining uninhibited molecules of AChE. This process will reduce the rate of further progressive inhibition to a marked but undefined degree. Thus, the state of complete inhibition is more difficult to reach *in vivo* than in a study of AChE *in vitro*.

   (b) Timing should not be made from the point at which first signs of intoxication are seen; these may appear while about 50% of AChE remains uninhibited.

   (c) Even when signs of intoxication are marked, some spontaneous reactivation (and even some little synthesis of fresh enzyme) will be going on: reinhibition by persistent inhibitor may be less rapid than *in vitro*.

   (d) As soon as an effective concentration of oxime is achieved *in vivo* the balance of ageing and reactivation reaction rates for the inhibited AChE is altered in favour of the latter. Thus,

progress towards complete inhibition may be slowed markedly even if the desired objective of reversal of such progress is not achieved at the first treatment.

It is probable that benefit will ensue even if oxime therapy is commenced or continued several days after intoxication has occurred. Oximes are of particular value in cases of intoxication from diethyl phosphates because the rate of spontaneous reactivation is very slow.

(9) Being ionized compounds, oximes do not penetrate the CNS easily although there are some indications that useful amounts may reach inhibited enzymes in the area of the respiratory centre in the medulla oblongata [28]. In addition, oximes can be highly effective in restoring some active AChE and normal respiratory function at the diaphragm where the nicotinic effects of excessive AChE are not antagonized by atropine.

(10) Traditionally, perhaps because of data generated in studies investigating short-lived nerve agents, oximes have been administered for only a few hours after OP exposure. Such a dosage regimen may well be enough to reactivate sufficient AChE to allow 'normal' functioning in mild cases of intoxication but, in severe cases of OP insecticide poisoning, it must be emphasized that a continuous therapeutic battle must be fought for as long as the OP insecticide and its active inhibitory oxon remains in the body. Thus, therapeutic concentrations of oxime should be maintained to regenerate as much active enzyme activity as possible until the OP compound has been eliminated. As a consequence, oxime therapy may need to be continued for days to prevent relapse or death and to bring about a lasting improvement in the clinical condition of the patient.

Management

First aid

The casualty should be removed from the contaminated environment without the rescuers themselves being placed at risk. If clothing is contaminated this should be removed, if necessary, by first-aid personnel or by nursing or medical staff, who are protected against contamination. The skin should then be washed with soap and water.

Making an accurate diagnosis and assessment of severity of intoxication

In some European countries, clinicians see so few cases of OP insecticide intoxication that they tend to over-react therapeutically when confronted by a suspected case. It is by no means uncommon in these circumstances for inexperienced physicians to give many milligrams of atropine (in 2 mg boluses) to patients who are not significantly intoxicated with an OP insecticide; the management of severe atropine poisoning can be quite a challenge!

Thus, it is wise, unless the patient is *in extremis* or the characteristic clinical features of severe poisoning are present, to confirm the diagnosis analytically by measuring the inhibition of ChE. It is preferable that both RBC AChE and plasma ChE activities be measured; the RBC test is usually more specific but some OP compounds (e.g. chlorpyrifos, demeton, malathion) depress plasma ChE activity to a greater degree, thus producing a useful sensitive monitor [28]. Few clinicians have the support of a laboratory which can quantitatively or even qualitatively

Table 49.1 Assessment of severity of OP insecticide poisoning

Grade 0 Suggestive history but no features of intoxication are present
Grade 1 Patient is alert and awake and has:
Increased secretions
Fasciculation +
Grade 2 Patient is drowsy and has:
Severe bronchorrhoea
Fasciculations +++
Crackles/wheezes on auscultation
Hypotension (systolic BP <90 mmHg)
Grade 3 Patient is comatosed and has all the features of severe intoxication. Increased F_IO_2 needed but not mechanical ventilation
Grade 4 Patient is comatosed and has all the features of severe intoxication. PaO_2 <8 kPa despite F_IO_2 >40%; $PaCO_2$ >6 kPa. Mechanical ventilation required. Abnormal chest radiograph (circumscribed or diffuse opacities, pulmonary oedema)

determine the insecticide responsible for the intoxication.

The clinical features of OP insecticide poisoning are summarized in Chapter 47 and attention will be directed here to the determination of the severity of intoxication (Table 49.1). It is generally true that the presence of certain clinical features is more helpful in determining the severity of intoxication and prognosis than measurement of ChE activities alone.

Patients who are severely poisoned (>Grade 2; Table 49.1), as shown by drowsiness, hypotension, severe bronchorrhoea and marked muscle fasciculation, should be moved to an intensive care unit as further deterioration may occur and mechanical ventilation may be required. Respiratory complications are the major cause of death.

Minimizing further absorption of the OP insecticide

If the insecticide has been ingested it is customary to administer an emetic, such as syrup of ipecacuanha, or to undertake gastric lavage or to give activated charcoal in the hope of reducing the absorption of the OP compound. In fact, there is no good clinical evidence to support the use of any of these three methods in OP poisoning.

Syrup of ipecacuanha should be avoided as emesis is dangerous in a patient whose level of consciousness might deteriorate; aspiration pneumonia is a well recognized complication in these circumstances. Moreover, aspiration can also occur in conscious patients given syrup of ipecacuanha [1] and is more likely to happen if hydrocarbons are present in the pesticide mixture. The capacity of activated charcoal to adsorb most OP compounds has not yet been demonstrated.

As there is some evidence that absorption of OP insecticides may be slow [29], it may be beneficial to undertake gastric lavage in all potentially serious cases on admission to hospital. Lavage should be undertaken with care and with an endotracheal tube *in situ* if the level of consciousness is depressed. The value of 'continuous' lavage for several days, although advocated [29], remains unproven.

Maintenance of vital body functions and adequate clinical monitoring

All patients suspected of being poisoned by an OP should be treated as an emergency by experienced medical and nursing staff available to monitor changes in the patient's vital functions and to institute appropriate therapy. All severely poisoned patients (>Grade 2; Table 49.1) should be nursed in an intensive care unit, if available.

Most severely poisoned patients will have increased salivation, tachypnoea (although a few will be hypoventilating), lung crackles/wheezes on auscultation, tachycardia (although some may have bradycardia), hypotension, vomiting, diarrhoea, disturbance of consciousness, miosis and muscle fasciculations.

Bronchorrhoea requires prompt relief with intravenous atropine (see later) and supplemental oxygen should be given to maintain the PaO_2 at >10 kPa. If these measures fail, the patient should be intubated and mechanical ventilation (with positive end expiratory pressure (PEEP)) should be instituted. Careful attention must be given to fluid and electrolyte balance and adjustments to infusion fluids made as necessary.

In severely poisoned patients (Grade 4 severity; see Table 49.1), it may be necessary not only to infuse plasma expanders but to use an inotrope, e.g. dobutamine 5–40 μg/kg per min, to maintain cardiac output, and also to give low-dose dopamine (2.5 μg/kg per min) to increase urinary output. In such cases, resuscitation may be required because of the onset of an arrhythmia; this should be treated conventionally. The management of convulsions and muscle fasciculation is discussed later.

Heart rate, blood pressure and ECG should be monitored routinely. If facilities are available, arterial blood gas measurement is preferable to the use of a spirometer or measure tidal/minute volumes as additional information on acid-base status is also provided. Most conveniently, blood pressure may be measured directly using an indwelling arterial catheter which also allows repeated access for blood gas measurement. A chest radiograph should be taken daily, or more frequently, if clinically indicated.

Daily haematological and biochemical profiles should be performed to exclude hepatorenal dysfunction and cultures should be undertaken if septicaemia is suspected.

Appropriate use of atropine, oxime and diazepam

These aspects of therapy are reviewed extensively in Chapters 50–52.

Atropine

Atropine is beneficial in: (1) reducing bronchorrhoea and reversing changes in ventilation-perfusion inequalities resulting from uneven distribution of ventilation caused by ACh-mediated airway constriction [22]; and (2) counteracting the convulsive effects of OP compounds [16,17], an effect not related to muscurinic blockade. This benefit may be increased by the concomitant use of diazepam and PAM [2].

Atropine may also be of value in treating OP-induced acute dystonic reactions and primary position upbeat nystagmus [12–14,23]. Many different atropine regimens have been advocated and there is no general agreement as to dosage even among experienced clinicians. Although animal studies suggest strongly that synergism between oximes and atropine occurs, atropine has often been administered without concomitant oxime therapy or inadequate doses of oxime have been given (see later):

The two main determinants of therapy are: (1) sufficient atropine should be administered to ensure that bronchorrhoea and bronchospasm are reduced or eliminated, and (2) oximes should be given for as long as atropine therapy is required.

In patients where the diagnosis is strongly suggested by characteristic clinical features, atropine should be given initially in 2 mg bolus doses to reduce bronchorrhoea. Repeated injections of atropine may have to be given over the first few hours of therapy; the dose should be titrated against peripheral muscarinic signs, principally bronchospasm and bronchorrhoea. In severe cases, particularly when oximes are not administered, several thousand milligrams of atropine may be required.

Oximes

The first use of 2-PAM in man was in 1956 to treat OP insecticide poisoning [19]. It was not until 1964, however, that 2-PAM was licensed for use in the USA [11], while other PAM salts are used outside the USA. Obidoxime chloride is a bis-pyridinium oxime which, in high dose for several days, produces hepatoxicity [6; unpublished data submitted to WHO/IPCS]. Consequently, obidoxime cannot be advocated in the management of severe OP insecticide poisoning.

Although many animal studies (*see* Ch.52) have confirmed the value of oximes, particularly if employed conjointly with atropine, some physicians who have wide experience of OP insecticide poisoning believe that oximes are of little, or no, benefit and instead employ high doses of atropine together with supportive measures with apparently excellent results [7,8,14,30].

Moreover, there is considerable disagreement regarding the dose and frequency of oxime administration and this could account for many supposed treatment failures.

Therapeutically effective oxime concentrations

Early experiments in anaesthetized cats given lethal doses of iv sarin and im PAM established that plasma PAM concentrations of 4 mg/l were required to counteract NM block, bradycardia, hypotension and respiratory failure [25]. Crook *et al.* [5] gave dogs oral P2S and PAM-lactate, 30–115 mg/kg body-weight, 1–5 h before exposure to sarin vapour. Atropine 5 mg/kg body-weight was administered 1 min after the dogs were exposed to sarin. The authors extrapolated from this study in dogs to man and concluded that a plasma PAM concentration of at least 3 mg/l would be required 'for reasonably protective attenuation of the toxic effects of organophosphorus anticholinesterases'.

The relationship between plasma PAM concentrations and protection against sarin poisoning in rats has been investigated by Shiloff and Clement [20] and Bokonjić *et al.* [2]; their data support the recommendation that plasma PAM concentrations of the order of 4 mg/l are required for effective protection against sarin.

However, a plasma PAM concentration of 4 mg/l may not be optimum (or even very effective) for dimethyl or diethyl phosphates. Studies to establish what are the clinically effective doses of oxime in such cases are urgently needed.

If the general conclusions of these studies are correct, how may plasma PAM concentrations of at least 4 mg/l be achieved?

There is a wealth of human kinetic data available largely derived from studies in healthy non-poisoned subjects. However, as there is recent evidence from studies in animals [9,10] and man [15] that OP compounds can alter PAM kinetics in a complex manner, it may be inappropriate to extrapolate the results of volunteer studies to the treatment of severely intoxicated patients. Jovanović [15] has demonstrated that the mean plasma PAM concentrations after 1 g 2-PAM were almost one and a half times higher in patients than in volunteers; thus, oxime concentrations above 4 mg/l were maintained for a mean of 239 min in patients compared with a mean of 137 min in volunteers. The explanation for this observation is unclear, but a decrease in cardiac output and in the effective renal blood flow is known to occur in patients severely poisoned with OP compounds. Because PAM is primarily eliminated via the kidneys either altered regional blood flow or alterations in active tubular transport mechanisms which are known to occur [26] could increase oxime concentrations.

Notwithstanding these preliminary and, as yet, unconfirmed observations of Jovanovi´c [15], it would seem reasonable to take the human volunteer kinetic studies as a basis for determining oxime dose. It is possible, however, that the dosage regimens so calculated may be higher in some cases than necessary. Further clinical studies will be required to confirm this.

PAM dosage

It has not yet been established whether it is more advantageous clinically to given iv PAM therapy by bolus injection (i.e. to produce high peak concentrations) or by continuous infusion. It is apparent, however, that lower total doses of PAM are needed with the continuous infusion regimens.

Intravenous administration: bolus doses

A summary of data generated in volunteer studies following the administration of iv PAM is shown in Table 49.2. This shows that therapeutically effective oxime concentrations are reached in less than 5 min and, if 15–30 mg/kg body-weight of 2-PAM is administered, a concentration of oxime >4 mg/l is maintained for at least 6 h. Studies by Calesnick *et al.* [3] suggest that P2S may be less effective than 2-PAM in producing sustained oxime concentrations so that 4-h dosing may be required. These dosage regimens need to be confirmed in clinical practice using refined analytical techniques.

Intravenous administration: continuous infusion

The use of a continuous infusion of PAM has also been advocated in cases of severe intoxication. A dose of 500 mg/h has been proposed and it is claimed (on the basis of computer-simulated model) that such an infusion produces oxime concentrations of >4 mg/l in

Table 49.2 Intermittent iv therapy with PAM

Administered dose (mg/kg)	*PAM salt*	*Mean time (min) plasma concentration > 4 mg/l*	*Mean C_{max} (mg/l)*	*Study*
5	2-PAM	25	10	Sidell and Groff [21]
10	2-PAM	55	30	Sidell and Groff [21]
15	2-PAM	360	21	Calesnick *et al.* [3]
30	2-PAM	360	18	Calesnick *et al.* [3]
45	2-PAM	360	29	Calesnick *et al.* [3]
20	P2S	>90	50	Sundwall [24]
45	P2S	240	19	Calesnick *et al.* [3]

<15 min and a steady state concentration of approximately 14 mg/l [27]. Assuming that the volume of distribution of PAM at steady state is 0.76 l/kg (this is based on work in volunteers), the elimination rate constant (K_{el}) is 2.58/h and the patient weighs 70 kg, an infusion rate of 500 mg/h would only produce an oxime concentration of 3.6 mg/l. An infusion rate of >550 mg/h would be required to produce a steady state concentration of 4 mg/l though this concentration would not be reached for some 6.5 h if one assumes a half-life of approximately 1.3 h. As a consequence, two 30 mg/kg body-weight bolus doses of PAM (chloride or mesylate salts) would have to be administered 4 h apart to cover the period before satisfactory PAM concentrations were achieved by the use of the infusion.

Therapeutic PAM dosage regimens

Based on the above data, patients severely poisoned with OP compounds can be treated first by the iv administration of 2-PAM or P2S in doses of 30 mg/kg body-weight. There is some evidence that 2-PAM can be administered at 6-h intervals compared with the 4-h regimen required for P2S. Second, it is possible that the continuous iv infusion of PAM at a rate of 8 mg/kg per h (550 mg per h) will produce satisfactory oxime concentrations but with such a regimen there is also the need to administer two initial iv bolus doses of a PAM salt (30 mg/kg body-weight) 4 h apart.

Diazepam

Diazepam may be of benefit in OP-poisoned patients by: (1) reducing anxiety and restlessness, (2) reducing muscle fasciculation, (3) arresting seizures, and (4) reducing morbidity and mortality when used in conjunction with PAM and atropine. Diazepam 5–10 mg iv (preferably the emulsion Diazemuls, which reduces the risk of thrombophlebitis) will reduce anxiety, restlessness and visible motor activity. Short term, diazepam 10–20 mg is also an effective anticonvulsant but if repeated doses are required to suppress seizure activity, phenytoin should be considered as an alternative. Potentially, respiratory depression may result from the repeated use of diazepam although this is unlikely to be a significant clinical problem unless OP-induced respiratory impairment is also present and the patient is breathing spontaneously. Midazolam has been employed as an alternative benzodiazepine but offers no advantages over diazepam.

Conclusion

Severe cases of acute OP intoxication present a considerable clinical challenge and the mortality rate may be as high as 25% even in centres that treat a large number of patients each year. This may be in part, because many patients are currently receiving suboptimal oxime therapy. It is our hope that a better understanding by clinicians of the mechanisms of toxicity outlined in this chapter will lead to changes in management. Specifically, we recommend the use of PAM in sufficient dose to produce and maintain adequate plasma oxime concentrations for as long as the patient exhibits the signs of OP poisoning. It is our contention that such a therapeutic approach is likely to result in a more favourable outcome even in those severely poisoned with an OP insecticide.

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Anticholinesterase poisoning: an overview of pharmacotherapy

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Introduction

Clinical management of antiChE poisoning, including drug therapy, has been described in detail by Durham and Hayes [12], Grob [18] and Namba *et al.* [31]. With regard to its general principles, the treatment outlined by these authors is still applicable today. However, modifications to the dose regimens of the antidotes, on the basis of a better knowledge of their pharmacokinetics and of the toxicokinetics of the antiChE agents, are appropriate.

Antidote therapy

Oximes

Clinical efficacy

Oximes reactivate the OP-AChE complex. Their efficacy in the reactivation of CB blocked enzyme is at the very least controversial. *In vitro,* oximes have a limited or moderate effect on CB-inhibited AChE [19]. *In vivo,* oximes seem to be inactive in humans, but they increase the LD_{50} of CBs in mice [32,37].

One would expect this causal treatment to reverse completely the intoxication syndrome, but clinical experience shows that this is not always the case [13,33,39,41,42]. Different reasons may be responsible for this: (1) limited lipid solubility and variable but poor penetration into the brain [18,24,25,27,29], and (2) non-uniform activity against all OP-enzyme complexes [24], (3) ageing of the OP-enzyme complex, (4) formation of a complex which in itself is a potent antiChE, and (5) interruption of oxime administration before the concentration of the toxic agent has declined below toxicologically significant levels.

Table 50.1 Oxime dosage schemes by iv administration

1	2-PAM (pralidoxime chloride, Protopam chloride®, Ayerst) [31]	
	Level 0:	None
	Level 1 and 2:	1 g
	Level 3:	1 g 1 g after 15–30 min if no improvement, 0.5 g per h if still no improvement
2	Pralidoxime methylsulphate (Contrathion®, Specia, *see* Data Sheet, Specia)	
	Level 1 and 2:	400 mg 200 mg after 30 min, 4, 6, and 12 h
	Level 3:	500 mg 500 mg after 30 min 200 mg in repeated doses up to 2 g in 24 h
3	Obidoxime chloride (Toxogonin®, E. Merck) [13]	250 mg, and 250 mg within 2 h if necessary or 3–6 mg/kg 1 to 2 times shortly after poisoning

Table 50.1 gives a conventional dosing schedule for the three commercially-available oximes. These dose schemes should be critically evaluated with regard to the theoretical notion of therapeutic plasma concentrations, the pharmacokinetics of the oximes, and the toxicokinetics of the antiChE (Figure 50.1).

'Therapeutic' plasma concentrations

As for other drugs, a therapeutic plasma concentration has also been proposed for oximes. On the basis of *in vitro* (rat diaphragm) and *in vivo* (cat) experiments with methanesulphonate (P2S), it was suggested that the effective plasma concentrations should desirably be >4 mg/l. At and above this concentration P2S counteracts *in vivo* bradycardia, hypotension and respiratory failure, and reverses *in vitro* a NM block of 50% in approximately 4 min. However, the *in vivo* results were based on experiments with the

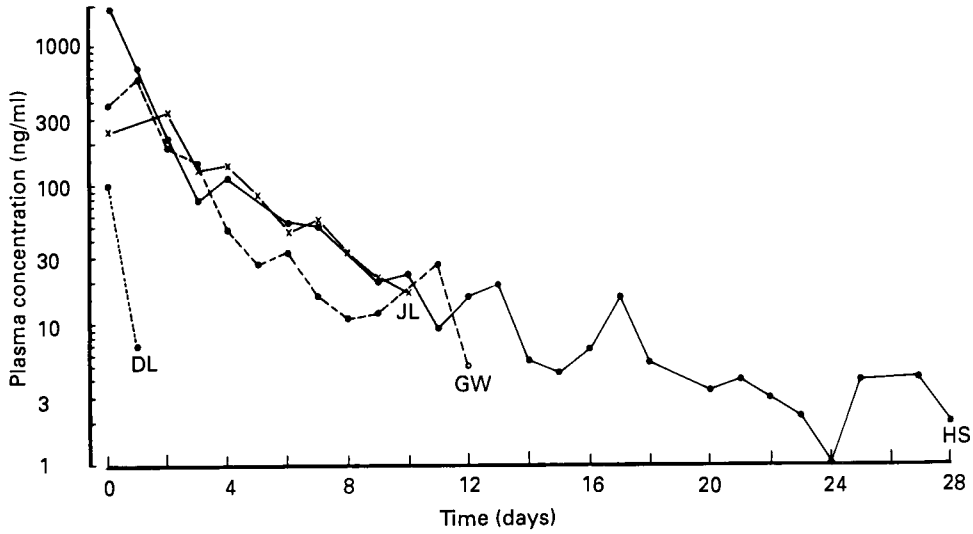

Figure 50.1 Parathion (●) and trichloronate (×) plasma concentrations in four patients who took a commercial preparation of these compounds orally in an unknown amount. Data from Braeckman [Doctoral Thesis, University of Ghent School of Pharmacy, 1982]

inhibitor methylisopropoxyphosphoryl thiocholine only, whereas the *in vitro* results were obtained with the same inhibitor and with sarin. Under the same *in vitro* conditions, P2S was much less active against NM inhibition induced by tabun [38]. The level of 4 mg/l is therefore not applicable to all oximes against one inhibitor or to one oxime against different inhibitors. Nevertheless, the figure of 4 mg/l has been used as a reference for calculating oxime doses to be given to humans. It seems acceptable to use, as a first approximation, the same therapeutic plasma levels for PAM as for P2S. To obtain the same therapeutic molar concentration, however, plasma concentrations of 2-PAM should be about 3 mg/l. For obidoxime chloride a therapeutic level of 4 mg/l was in fact initially used. The molar concentration is lower than for P2S but obidoxime is more potent *in vitro*. Boelcke *et al.* [3] proposed a therapeutic plasma concentration for this oxime of 1 mg/l, on that basis, for use in parathion and paraoxon intoxications.

Plasma concentration simulations with different dose regimens

Simulations [16] for 2-PAM, P2S or PAM methylsulphate, as well as obidoxime chloride on the basis of the dose regimens proposed for level 3 poisonings and of the mean human kinetic parameters computed by Sidell *et al.* [35] are shown in Figure 50.2. In this and the following simulation the pharmacokinetic data of P2S are applied to the dose schedule of PAM methylsulphate.

An alternative approach, within the concept of therapeutic effective plasma concentrations, is the administration of the drug as a continuous infusion preceded by a loading dose given as a bolus. On the basis of the therapeutic levels given earlier, loading and maintenance doses have been calculated for the three oximes. The calculated doses are given in Table 50.2 and, as an example, the theoretical plasma curves for P2S are given in Figure 50.2. The simulated curves for the other oximes are nearly identical. A similar approach has been published for 2-PAM [40].

Whether the use of loading and maintenance doses, as calculated in Table 50.2, will give better clinical results than the conventional dose regimens mentioned in Table 50.1, needs to be tested very carefully. Indeed, the simulations are based on mean pharmacokinetic values, and differences between individuals exist. Moreover, the reference therapeutic plasma concentration of 4 mg/l remains subject to discussion. Finally, in severe poisonings not only will the toxicokinetic behaviour of the inhibitor change but

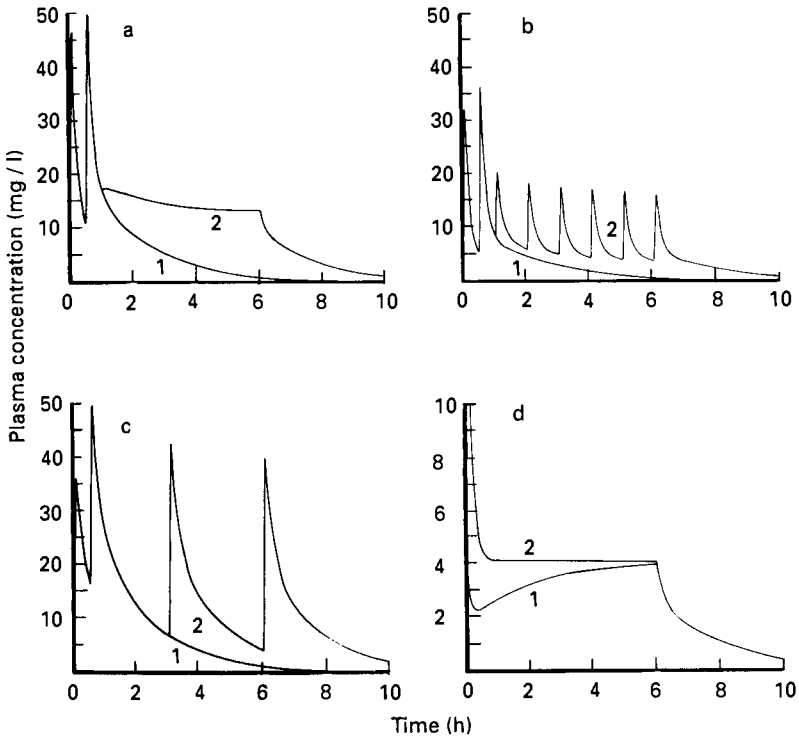

Figure 50.2 Simulations of concentration-time profiles in the central compartment (plasma concentration) for three oximes given in different iv dose regimens to an adult of 70 kg. (a) Pralidoxime chloride is given at time 0 and 0.5 h at dose of 1 g, at a rate of 400 mg/min; at 1 h, an infusion of 0.5 g/h is started. (b) Pralidoxime methanesulphonate is given at time 0 and 0.5 h at dose of 0.5 g, at a rate of 400 mg/min, then hourly doses of 0.2 g are given at the same rate. (c) Obidoxime chloride is given at time 0, 0.5, 3 and 6 h, doses of 0.25 g are given at a rate of 200 mg/min. For a, b and c, trace 1 simulates the first two doses, and trace 2 the full dose sequence. (d) Pralidoxime methanesulphonate at time 0, for trace 1, D* = 0.78 mg/kg, trace 2, D* = 4.42 mg/kg. For both a continuous infusion of 2.14 mg/kg per h was started at time 0

so will the pharmacokinetic behaviour of the antidotes, as a consequence of mutual interferences [7,17,24,28,43].

Duration of oxime therapy

It has been proposed that the dose regimen of obidoxime chloride should be limited to one or two doses of 3–6 mg/kg [13]. The first reason for this limitation was the inefficiency of oximes in the case of ageing of the enzyme-inhibitor complex, which occurs within 24–48 h in insecticide poisoning [2,42]. This reason is, however, not entirely valid. In actual fact, earlier persistent inhibition of serum ChEs is not only by ageing but also may indicate persistence of toxicologically relevant concentrations of the inhibitor in the organism (Figure 50.1). In that case the continuing administration of oxime might, when counterbalancing the inhibitor concentration, reactivate newly inhibited enzymes not yet showing the phenomenon of ageing and speed up the clinical recovery of the patient.

A second argument was the possibility of formation of an oxime-OP complex with strong ChE inhibitory properties [10]. This possibility has been suggested on the basis of *in vitro* experiments with obidoxime and dimethoate [14], and obidoxime and PAM with soman [34]. It remains doubtful, however, whether this occurs in humans with the usual dose regimens. Among a series of OP and CB antiChEs, only the toxicity of the CB carbaryl

Table 50.2 Calculated infusion rates and loading doses for the three oximes

	Css (*mg/l*)	*Ko* (*mg/kg per h*)	*D** (*mg/kg*) *CssVc*	*CssVd*$_\beta$
Pralidoxime chloride (Protopam chloride®)	3[e]	1.68	0.81	3.18
Pralidoxime methanesulphonate (P2S)	4	2.14	0.78	4.42
Obidoxime chloride (Toxogonin®)	4	0.48	0.40	0.80
	1[f]	0.12	0.10	0.20

[a]Css: steady state plasma concentration
[b]Ko: infusion state
[c]Vc: volume of the central compartment
[d]Vd_β: volume of distribution during the β phase
[e]3 mg/l pralidoxime chloride is equimolar to 4 mg/l P2S
[f]Proposed as therapeutic level by Boelcke *et al.* [4]

Table 50.3 Atropine dose regimen

Level 0:	None
Level 1:	1 mg sc
Level 2:	1–2 mg iv every 30 min until atropinization
Level 3 and 4:	5 mg every 30 min until atropinization
For levels 2, 3 and 4: maintenance dose of 0.5–1 mg every 3–4 h, depending on the clinical picture	

[a]Data from Durham and Hayes [12], Grob [18] and Namba *et al.* [31]

was increased by obidoxime in *in vivo* experiments in the mouse [37].

Finally, the most important argument to limit the administration of obidoxime chloride was the suggestion that it produces hepatic toxicity. Hepatic toxicity is readily seen in the first days after acute OP poisoning, but a secondary increase in liver enzymes or the appearance of cholestatic icterus has been observed in several severe cases treated with high doses of obidoxime, e.g. with 6.75 g given in 3 days or 3.75 g in 5 days [2], 3 g in 5 days [4], 10 g in 5 days [22] and 2.75 g in 5 days [11]. If a total dose of 3 g is taken as the upper limit, the dose regimen shown in Figure 50.2 should be stopped after the 12th injection, i.e. after 30 h. With the highest loading and maintenance doses given in Table 50.2 this occurs after 87 h. It remains possible that the hepatic toxicity is not unique for obidoxime; in a severe parathion case, treated for 1 day with 2.25 mg obidoxime followed by 17 g of 2-PAM in 7 days, cholestatic jaundice occurred on the 8th day.

Anticholinergic drugs

Besides causal therapy, symptomatic treatment with anticholinergic drugs is also important. Both therapeutic principles, oxime administration and anticholinergics, should always be combined because their actions are synergistic [18].

The increased secretions and bronchoconstriction, hampering respiration and pulmonary gas exchange, are blocked by muscarinic antagonists, of which atropine is the most widely used. Atropine also antagonizes other muscarinic symptoms, e.g. the cardiovascular effects of the cholinergic crisis, decreasing the likelihood of cardiac arrhythmias.

There is some discussion whether atropine also antagonizes the CNS symptoms of the cholinergic crisis, the most important of which is the depression of the respiratory centre and the occurrence of convulsions. A central component in the protective effect of atropine has, however, been clearly demonstrated experimentally in the rat [8,9.24].

Table 50.3 gives the atropine dose regimen proposed by Namba *et al.* [31], with additionally a maintenance dose regimen for use over a few days once atropinization has been obtained [12,18]. Only scanty and contradictory results regarding the pharmacokinetics of atropine are available in man. They indicate, however, that there is no direct relationship between plasma concentrations (central compartment) and effect on, e.g. heart rate, but that there is some relationship between this effect and atropine tissue concentrations (peripheral compartment) [1,21].

With intramuscular atropine, maximum heart rate increase occurs at about maximal plasma and, presumably, tissue concentrations (20–50 min) [18,20,30,36]. The increase in heart rate disappears with a half life of 70–90 min.

After the initial doses, titration of further atropine doses in the individual patient has to be carried out on the basis of the most relevant effects for a favourable clinical outcome, i.e. decrease in bronchial constriction and secretions as judged by auscultation and blood gas analysis. Heart rate changes are less important but easier to follow, and a mild tachycardia of 80 beats or more per min should be maintained.

In recent years attention has been drawn to the possibility that some of the signs and symptoms seen in the course of antiChE poisoning, such as disorientation, hallucinations, motor agitation and resistance to ventilation, are actually from atropine overdose and intoxication [11,23]. It seems, therefore, advisable to allow a sufficient time interval between successive atropine doses, taking into account its relative long persistence in the tissue compartment (Table 50.3). To diminish the possibility of overdosing with ensuing CNS toxicity, methyl atropine, which does not cross the blood-brain barrier, has been used regularly. It seems, however, more logical to use atropine, very carefully titrated in limited doses, because of its protective action on the respiratory centre as well as against convulsions.

Anticonvulsants

Atropine protects only partially against convulsions and the resulting brain damage in severe poisonings [8,9]. Complementary treatment, including diazepam, phenytoin and/or pentobarbitone should be applied when necessary [11,18,31]. Diazepam is also of interest in agitated patients [2,41].

It has been shown in experimental soman poisoning in rhesus monkeys that diazepam antagonizes the convulsive action of soman [26] and that addition of diazepam to the basic treatment scheme greatly improves morbidity and mortality, independent on its anticonvulsive effect [5] (*see* Ch.53). Whether the same applies to OP or CB poisoning, in the absence of agitation or convulsions, is not known. Diazepam, or newer water-soluble anticonvulsants, are now incorporated in drug treatment schemes made available by different armed forces to their soldiers, to be applied immediately after exposure to OP warfare agents, among which soman is a very likely candidate [15].

Clonidine, which also has some protective action against soman [6], has been used together with flunitrazepam against atropine overdose in severe antiChE poisoning [23].

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Atropine in the management of anticholinesterase poisoning

Andrew J.W. Heath and Tim Meredith

Introduction

Atropine was first isolated from belladonna by Mein in 1831 [120]. It is a muscarinic cholinergic blocking agent which competitively inhibits ACh and other muscarinic agonists at parasympathetic, postganglionic nerve endings. Atropine has little effect at nicotinic receptor sites; large doses of atropine produce only partial block of autonomic ganglia and have almost no effect at the NM junction.

Atropine is a specific antidote for antiChE poisoning caused by either OP or CB compounds. Although other anticholinergic agents (such as dexemetide) with different distribution kinetics may have advantages [69], the place of atropine in the treatment of antiChE poisoning is essentially unchallenged. However, while the need for muscarinic blockade in this situation is clear-cut, there is controversy as to the most appropriate dosage regimen for atropine.

If titrated correctly, atropine has few serious side-effects when used in OP poisoning. However, care needs to be taken with the use of the drug in warm climates because of inhibition of sweating. In addition, hypoxic patients may be at risk of developing ventricular tachycardia or fibrillation if given atropine; use of the drug should therefore be avoided if possible before resuscitation with oxygen or, if necessary, artificial ventilation [44,76].

Physicochemical properties

Atropine ($C_{17}H_{23}NO_3$) is a naturally occurring antimuscarinic tertiary amine (Figure 51.1) [72, 121]. The chemical name of atropine is benzeneacetic acid, α(hydroxymethyl)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester endo -±-, and for its sulphate salt, benzeneacetic acid, α(hydroxymethyl)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester endo -±-, compounds, sulphate (2:1) (salt). The Chemical Abstracts Number is 51-55-8 for atropine and 55-48-1 for atropine sulphate [19]. As a parenteral antidote, atropine is available as the sulphate salt from several manufacturers (including Abbott, LyphoMed, Elkins-Sinn, and Astra) [72]. The molecular weight of atropine is 289.38 and that of the hydrated sulphate salt is 694.82 [121].

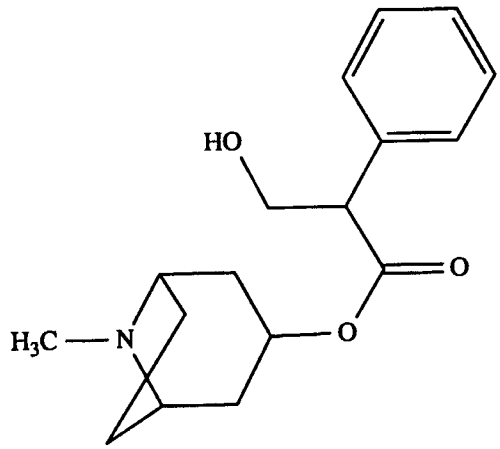

Figure 51.1 Structural formula of atropine

Atropine occurs as optically inactive white crystals or as a crystalline powder. Atropine has a solubility of 2.17 mg/ml in water and 0.5 g/ml in alcohol at 25°C. Atropine has a pKa of 9.8, and the pH may be adjusted to 3 to 6.5 with sulphuric acid [72]. Atropine effloresces when exposed to dry air and is slowly affected by light [72]. When heated to decomposition, very toxic fumes of NO_x and SO_x are emitted [100]. Atropine sublimes under high vacuum at

93–110°C and has a melting point of 114–116°C. Hydrated atropine sulphate has a melting point of 190–194°C [121]. There are no data available concerning the specific gravity or refractive index of atropine or its salts.

Synthesis and formulation

Atropine is a racemic mixture of *d*- and *l*-hyoscyamine [72]. The drug is an organic ester which may be prepared synthetically by combining tropine and tropic acid, but is usually extracted from the plants *Atropa belladonna* (deadly nightshade), *Datura stramonium* (jimsonweed), or *Duboisia myoporoides* [72]. Extracted atropine is a combination of *d*- and *l*-hyoscyamine, and both isomers bind to muscarinic receptors [10]. However, the pharmacological activity of atropine is almost entirely due to *l*-hyoscyamine [72]. Atropine sulphate is available as a sterile solution in normal saline or water for injection. The preservative parabens may be found in the injectable product.

Atropine sulphate should be stored in single- or multiple-dose containers between 15 and 30°C and protected from light. Freezing should be avoided [72]. The shelf-life is 24 months from the date of manufacture (personal communication, Abbott Laboratories) if kept under the above conditions.

Pharmacology

Atropine is a competitive cholinergic blocking agent. Small doses depress sweating and salivary and bronchial secretion. Atropine is particularly useful in relieving bronchoconstriction and salivation induced by antiChEs. Doses required to inhibit gastric secretion are invariably accompanied by a dry mouth and ocular disturbances. The heart rate increases as the effects of vagal stimulation are blocked. When given alone atropine has little effect on blood pressure, although it can completely block the hypotensive and vasodilatory effects of choline esters. Larger doses decrease the normal tone and amplitude of contractions of the bladder and ureter, inhibiting micturition. Atropine inhibits both the tone and motility of the gut, reducing peristalsis.

Unlike scopolamine, small doses of atropine have little depressant action on the CNS. However, in toxic doses, atropine initially causes central excitation (exhibited as restlessness, confusion, hallucinations and delirium) followed, after even larger doses, by central depression with coma and death. Both atropine and scopolamine shift the EEG to slow activity, reducing the voltage and frequency of the alpha rhythm. Atropine normalizes increased EEG activity caused by isoflurophate (DFP) [68].

Large doses of atropine dilate the pupil and blur vision because of impaired accommodation. The normal pupillary response to light or on convergence may be completely abolished, ocular effects which may be seen after oral, systemic, or local administration [120].

Pharmacokinetics

Absorption and distribution

Atropine is rapidly absorbed from the gastrointestinal tract and mucous membranes; in adults, atropine is absorbed mainly in the duodenum and jejunum rather than the stomach. Using [^{3}H]atropine, Beerman *et al.* [8] found maximal radioactivity at 1 h after an oral dose. Atropine is also absorbed rectally. In children, the relation between im and rectal peak plasma concentrations is 3.2 : 1, i.e. comparable to that between oral and im doses [87]. Atropine is less readily absorbed through the skin; absorption may take place through the lacrimal ducts [91], although only limited absorption occurs through skin. After an im injection, peak concentrations are reached at 30 min. Concentrations after 1 h equate with those seen 1 h after iv administration. Studies in anaesthetized monkeys show that mean plasma concentrations after administration intraosseously correspond to those found 2 min after iv administration. Both the intraosseous and endotracheal routes are acceptable means of administration if iv access is delayed [89]. The intraosseous route is an emergency route of last resort in treating children, whereas the endotracheal route may be of value in a military setting.

After iv dosing, only 5% remains in the circulation after 5 min [10], with an initial distribution half-life of around 1 min [58].

Elimination kinetics can be fitted to a two-compartment model after therapeutic doses. The apparent volume of distribution (V_D) is 1–1.7 l/kg with a clearance of 5.9–6.8 ml/kg per min and a half-life of 2.6–4.3 h in the elimination phase [1,59,116].

Atropine crosses the placenta rapidly; in one study, concentrations in the umbilical vein were 93% of the maternal level 5 min after an iv injection [62]. Penetration into human lumbar CSF is less complete, particularly after a single iv injection [116]. It has been suggested that the CSF represents a 'deep' compartment with slow drug penetration [58]. Nevertheless, atropine penetration into the CNS is greater than that into lumbar CSF, compatible with the known central anticholinergic effects of the drug.

The elimination half-life of atropine is longer in children under 2 years of age and in the elderly [58]. In children, this is due to an increased V_D, increasing the half-life up to 5–10 h in the neonate. In the elderly (70 years and older), the half-life may be prolonged up to 10–30 h because of reduced clearance. These changes do not appear to be sex-related. Furthermore, not only the pharmacokinetic but also the pharmacodynamics may change with age, making both the younger and older patient more sensitive to a given dose [9,109].

Metabolism and excretion

Atropine is metabolized in the liver by microsomal monooxygenases. HPLC separation of urine has identified five compounds: atropine, noratropine, tropine, equatorial N-oxide, and tropic acid [115]. Thus, atropine is partly metabolized and partly excreted in the urine. Since faecal excretion is negligible, the hepatic plasma clearance of 519±147 ml/min represents metabolism. Hepatic blood clearance and extraction ratio were 476±136 ml/min and 0.32, respectively. The elimination of atropine is, therefore, partly flow-dependent [47]. Following iv injection, 57% of the dose is found in the urine as unchanged atropine and 29% as tropine. As the renal plasma clearance (656± 118 ml/min) was found to approach the renal plasma flow (712±38 ml/min), significant tubular excretion may occur. Thus, both liver and renal disease can be expected to influence the kinetics of atropine [47].

Pharmacodynamics

There is a clear temporal relationship between the plasma concentrations of atropine after iv injection [10], and the time course of the cardiac-accelerating effect of the drug [104]. This does not hold true for all pharmacological effects; for example, the antisialogue effect is more delayed, peaking at 100 min after an im injection [85]. The effect of atropine on pupillary dilatation and near point vision reaches a maximum only 6 h after administration [82]. Furthermore, studies in both adults [105] and children [106] show no correlation between a single serum atropine concentration and both subjective and objective responses. The relationships between pharmacokinetics and dynamics is clearly complex, where the effect compartment is different from the blood sampling site [58].

Toxicology

Poisoning can occur following oral, ocular, respiratory or parenteral exposure. There are numerous case reports of atropine poisoning from plants from antiquity to the present. In a case of jimsonweed (*Datura stramonium*) poisoning, a 4-year-old boy presented confused, with hallucinations, ataxia and tachycardia. Symptoms developed 3 h after ingestion. Recovery took 2 days [101]. In another case, a 25-year-old woman and her husband developed mild toxicity from drinking a strong burdock root tea [15]. In a 65-year-old man, 3 mg of atropine produced not only peripheral atropinization but a central anticholinergic syndrome with restlessness, hyperactivity and dysphasia. Symptoms resolved within 24 h with symptomatic therapy [122]. Mild atropine toxicity, with a central anticholinergic syndrome, may also occur in a hospital setting after 'normal' dosing in the elderly. This prolonged half-life of atropine with age puts the older patient at risk, particularly if more than one anaesthetic is given in a day, e.g. when reoperation is necessary [7].

Arthurs and Davies [4] reported three children overdosed with atropine following a 1000-fold error in dosage. During the first 12 h, the patients were sedated and disorientated. They became increasingly restless and a

central anticholinergic syndrome persisted for 2 days, requiring large quantities of diazepam for sedation; physostigmine was unavailable. The pupils remained dilated for a week.

In a review of nine cases of accidental poisoning with eye drops (Eumydrin®, atropine methonitrate), Meerstadt [77] found toxicity with atropine dosages ranging from 0.39 to 3.55 mg/kg. One patient, a 6-week-old boy, presented with fever, irritability, warm dry skin, inspiratory stridor, and dilated and unresponsive pupils. Recovery was uneventful.

In one case study following an accidental oral overdose of 0.3 mg/kg atropine in two small children, Saarnivaara *et al.* [94] reported maximum serum levels of 29 and 15.6 mg/l at 2–2.5 h, concentrations normally found in the distribution phase following an iv bolus. Toxic symptoms resolved uneventfully within 8 h. In children, death has been reported following doses of between 1.6 and 100 mg [33], although one patient recovered following an estimated ingestion of 1 g [3].

Antidotal efficacy in OP poisoning

Animal studies

The study of potential antidotal therapies in a controlled clinical situation is precluded by the very nature of OP pesticide poisoning. Thus evaluation of the efficacy of atropine and other forms of therapy must be based in part on animal experiments. This section reviews animal work relevant to the management of OP poisoning with atropine alone, atropine in combination with oximes, and atropine in combination with other therapeutic agents.

Atropine alone

Atropine is a proven antidote in OP poisoning, both in experimental [96,97] and clinical studies. Nevertheless, Stein and Neill [111] have, from the results of experiments using atropine to treat dimethoate toxicity, questioned the value of atropine in OP poisoning. Their conclusions, however, may be invalid, as the dose of atropine used was inadequate for the species studied [98]. Another hazard in extrapolating animal data to humans is that many animal models evaluate 24-h mortality after a single injection of an antidote given just after the intoxication, a model not mimicked in clinical practice. Nonetheless, in animal models, injections given later may have little effect on outcome [93].

In calves poisoned with iv dichlorvos, atropine was shown to reverse the respiratory effects of the OP [65]. The toxin-induced reduction in dynamic lung compliance and arterial oxygen tension and increase in total pulmonary resistance, viscous work of breathing and alveolar arterial oxygen gradient were reversed by atropine. Atropine may therefore reverse changes in ventilation perfusion inequalities resulting from uneven distribution of ventilation caused by ACh-mediated airway constriction [107]. Atropine had no effect on muscle fasciculation or plasma ChE inhibition. In monkeys, Lipp [67] investigated the effect of atropine on soman-induced respiratory depression. An immediate increase in heart rate was accompanied by a gradual increase in respiratory rate. One potential non-invasive method of measuring vagal tone in OP poisoning is with a vagal tone monitor [27]. Only for protection against low doses of OP is there a relationship between the antimuscarinic and therapeutic activity of atropine pretreatment [41]. Large doses of atropine counteract the convulsive effects of massive poisoning, an effect not related to muscarinic blockade.

The effects of atropine in OP poisoning are clearly far more complex than muscarinic blockade. In a study in rats, Pazdernik *et al.* [88] investigated the effect of atropine pretreatment on local cerebral glucose use during seizures induced with soman. High dose atropine (10 mg/kg) was found, like diazepam, to reduce local cerebral glucose use and thus reduce brain damage.

Support for an anticonvulsant property of atropine has been presented by McDonough *et al.* [71] who found that atropine pretreatment prevents the development of convulsions and brain damage induced by soman or VX injected directly into the amygdala. Brain damage caused by OPs is probably a result of seizures rather than a direct neurotoxic effect.

Soman increases plasma creatine phosphokinase (CPK) activity (Sket, D., Vreca, I., Brzin, M., personal communication) when 60–70% activity of skeletal muscle AChE is inhibited. Atropine pretreatment resulted in the survival of animals with very high inhibition of AChE

(over 90%). Therefore, in atropine-pretreated animals the CPK-MM and CPK-MB fractions were even higher than in controls.

Chronic exposure to certain OPs may induce changes in pharmacodynamics, influencing the response of the animal to atropine therapy. Atropine has been shown by Fernando *et al.* [34] to produce myoclonus and stereotyped responses in rats (related to enhanced serotonergic and dopaminergic activity) following DFP. In rats challenged 6–72 h after single doses of sarin or soman, myoclonus was markedly enhanced [35], suggesting a rapid hypersensitivity to antiChEs.

Furthermore, repeated DFP administration causes a specific decrease in muscarinic receptors and [^{14}C]choline uptake in the striatum and ileal longitudinal muscle of guinea pigs [125], a change associated with a more than 50% depression of tissue AChE. The concept of a down regulation of muscarinic receptors after chronic soman administration has also been corroborated by Modrow and McDonough [83], who, using a behavioural model in rats, found a supersensitivity to atropine. There is a clear need for more information on the effects of acute and chronic poisoning with OPs with different pharmacological properties on therapy with atropine and oximes.

Atropine in combination with oximes

Several oximes are effective in treating OP poisoning. However, their value in poisoning with slow-acting compounds which produce largely irreversible inhibition (such as dimethoate) can be questioned. The established concept is that the synergism between oximes and atropine depends largely on whether the enzyme inhibition is reversible [99].

Although the role of atropine in OP poisoning is clear, our knowledge of the kinetics and dynamics of atropine, and of the oximes when combined in treating a case of poisoning, is very limited. In one recent study in mice, Clement *et al.* [23] noted that soman increased the T1/2 and V_D of the oxime HI-6. Atropine increased the clearance of HI-6 with no effect on V_D. These changes in oxime kinetics were probably the result of haemodynamic changes.

An experimental study of sarin poisoning in rats [103] compared the efficacy of HI-6, obidoxime and 2-PAM as antidotes when combined with atropine. HI-6 was found to be most efficacious, followed by 2-PAM and obidoxime. These conclusions were based however on a single dose regimen of sarin and of atropine. This may be important, as the optimal combination of atropine with an oxime may depend upon the severity of the poisoning. Response surface methods, which provide an assessment of the entire dose response surface for all inherent variables, were used to optimize treatment therapies in soman intoxication [17]. These workers showed in guinea pigs that the level of soman exposure altered the nature of the atropine/PAM interaction. At low exposure levels, the optimal treatment is atropine alone; as soman toxicity increases, PAM becomes important and the optimal dose of atropine required initially decreases slightly but then again increases when soman is given in high doses.

Atropine in combination with other therapeutic agents

Carbamates

In animal experiments, both physostigmine [2,28,42,43]; and pyridostigmine [12,27,55] pretreatment has been shown to reduce atropine and PAM requirements. CBs that cross the blood-brain barrier may protect against OP toxicity by reducing the OP-induced rise in total brain ACh, thereby restoring neural function [43]. The optimal atropine/PAM dose combination has been shown to be a function of the challenge level of soman and pyridostigmine [55]. The protective effect of CBs may involve several mechanisms other than ChE inhibition. It is of interest that physostigmine, in addition to its antiChE activity, interacts with multiple sites at the ACh receptor [2]. The synergistic effect of atropine following pretreatment with physostigmine has been studied by Deshpande *et al.* [28] in an experimental study in the rat. These workers found that physostigmine pretreatment 30 min before injection of sarin reduced mortality to 28%; when coadministered with atropine, mortality fell to 4%.

Diazepam

The anticonvulsant effect of diazepam has been shown in rats treated with soman [88]

and in the monkey [66]. In quinalphos-poisoned rats, pretreatment with atropine and diazepam decreased the acute toxicity of the insecticide 3.3 times [13]. This effect was further enhanced by a continuous infusion of PAM, which maintained PAM concentrations between 1 and 5.4 mg/l. Later treatment with diazepam may not be as effective; in a study of rats poisoned with fluostigmine (DFP) and pretreated with atropine and diazepam, the subsequent administration of diazepam had no effect on the course of the poisoning [93].

Clonidine

In a recent study in mice, Buccafusco and Aronstam [16] showed that pretreatment with the centrally acting α-2 adrenergic agonist clonidine protects against several of the centrally-mediated toxic effects of soman, increasing survival rates. Furthermore, there was a synergistic effect on survival when clonidine was combined with atropine. At protective doses, clonidine not only blocks ACh release but non-competitively inhibits AChE activity. It also inhibits ligand binding to cortical muscarinic receptors. Clearly, clonidine has multiple effects and should be considered for further experimental study.

Ca^{2+} channel blockers

This group of drugs, together with phenytoin (but not other anticonvulsants), may also offer protection against OP toxicity [30]. In mice, pretreatment with phenytoin, verapamil, nifedipine, nitrendipine or nimodipine elevated the LD_{50} of DFP. The mechanism of action enhancing the effect of atropine and PAM may be the protective action on central respiratory centres and peripheral nicotinic sites.

Other agents

The effects of antidotal therapy, on neuronal RNA content have been studied by Doebler *et al.* [29]. Soman produced a virtually complete inhibition of AChE activity and a moderate decline in neuronal RNA content. Atropine pretreatment together with PAM reduced RNA levels but increased AChE levels. Thus no precise relationship exists between the restoration of neuronal RNA and AChE levels. The effects on neuronal RNA metabolism may reflect alterations in ACh sensitivity; if this is so, then manipulation of ACh responsiveness may be a further mechanism for therapeutic intervention. Sterling *et al.* [112] studied two drugs found to inhibit presynaptic ACh synthesis *in vitro*. In a rat model, pretreatment with N-hydroxyethylnapthylvinylypyridine and N-allyl-3 quinocludinol were found to protect rats from soman toxicity, enhancing the effects of atropine and PAM.

Thus the effects of atropine, PAM and other drugs which synergistically decrease OP toxicity are complex and as yet not elucidated. Although there are promising new therapeutic approaches, the clinical impact of clonidine, Ca^{2+} channel blockers and presynaptic blockade of ACh synthesis is not yet known.

Clinical studies

The first reference to atropine as an antidote against AChE inhibitors was by Fraser in 1870 [49,50] who used atropine to counteract the effect of physostigmine on the pupil. Although the clinical efficacy of atropine in OP poisoning is well known [6,20,22,31,33,36,46,48,61,73, 79, 81, 86, 92, 102, 114, 128, 129] because of the nature of the poisoning there are no controlled prospective studies in the literature. This evaluation is therefore based on case reports and retrospective case studies.

The peripheral antimuscarinic effects of atropine may not be the only antidotal property of the drug in OP poisoning. Atropine may also be of value in treating acute dystonic reactions and primary position upbeat nystagmus occasionally observed in acute OP poisoning [54,56,57,108,119]. Patients with extrapyramidal symptoms have abnormally low plasma and RBC ChE levels, producing an excess of ACh relative to dopamine [119]. There is no clinical evidence available to confirm any possible anticonvulsive effects of atropine in humans and at what dose this may occur.

Dose and duration of atropine therapy

Atropine should preferably be given promptly [36] and iv as a bolus injection, but on occasion (see later) it can be given by continuous iv infusion. Recommendations for the initial iv

dose range from 1 mg in adults and 0.01 mg/kg in children as a 'test dose' [63] to up to 5 mg in adults [37,61,64], and 0.05 mg/kg in children [14,32,70,84]. In one adult; up to 15 mg has been given as a single bolus [123]. Large doses may, however, be necessary and the initial dose of 1–2 mg in adults, and 0.05 mg/kg in children, should be repeated every 10 min or less until the desired clinical response is achieved. From a retrospective study of 37 paediatric patients, Zweiner and Ginsburg [129] found that one-third of patients required at least 0.05 mg/kg before any decrease in cholinergic activity was observed. Indeed large quantities of atropine may need to be given over a prolonged period of time, in some cases up to 5 weeks [64]. Most cases of mild to moderate OP poisoning require a total of 5–50 mg atropine [48]. However, it is also clear that a small number of patients require massive quantities, in particular, those poisoned with highly lipid-soluble compounds, such as fenthion [14]. In two cases of severe OP poisoning, 600 mg of atropine sulphate were given in a day to one patient and 1600 mg to another without adverse effects occurring attributable to the drug. Total doses as high as 240 mg [127], 453 mg [80], 850 mg [92], 2620 mg [53], 3911 mg [118], 11 442 mg [51] and 19 590 mg [40] have been given in severe cases in which the patients recovered, although one died later from an unrelated cause. Wycoff *et al.* [124] have also reported the case of a 14-year-old girl who had largely recovered from parathion poisoning, following treatment including 1122 mg of atropine, but who died after the tracheostomy tube eroded the innominate artery.

It is of interest that echocardiographic findings in OP poisoning treated with atropine have recently been reported [75]. Left ventricular function was examined in 12 patients before and after atropine treatment (average total dose 900 mg). There were no apparent changes in myocardial contractibility, cardiac conduction or rhythm except for sinus tachycardia. Relapse during therapy appears to be more common with highly lipophilic OPs [14,31,46,64,79,118,123,126]. Because of risk of relapse, patients should be weaned slowly [37]. If large quantities of atropine sulphate are given, care should be taken to avoid using formulations containing preservatives such as chlorbutanol or benzyl alcohol.

Route of administration

To give large doses of atropine can present practical difficulties. The need for larger ampoules, containing 10–100 mg of atropine, has been addressed [11]. Another approach is to give an iv infusion rather than intermittent bolus doses. There are several reports advocating infusion therapy [6, 14, 20, 31, 60, 64] although it has not always been successful [95]. This approach seems logical, both saving time and producing less fluctuation in plasma atropine concentrations. However, it should be remembered that the half-life of atropine (up to 4 h) necessitates using a bolus dose as well as adjusting the drip rate if a rapid increase in the degree of atropinization is required.

In an emergency situation, it may be necessary to give atropine before iv access can be established. As noted earlier, access to the systemic circulation can be rapidly achieved through the intraosseous route in children, and through the endotracheal route [89]. Atropine was given endotracheally to a 16-month-old child with a CB overdose [38]. The child responded rapidly to 1.0 mg and a total of 2.5 mg atropine was given by this route.

Atropine can also be administered orally. This route may be useful when the patient has been on iv therapy for several days or weeks, is stable and needs slow weaning [123].

Assessment of optimal atropinization

An evaluation of adequate atropine dosing can be assessed in two ways: with biochemical parameters such as plasma atropine concentrations or RBC or plasma ChE levels, and clinically, noting a decrease in cholinergic (muscarinic) symptoms.

Although knowledge of plasma atropine concentrations and kinetics could be useful in the individual patient, assay of plasma atropine concentrations is not often possible. One other approach is to assess toxicodynamics (rather than kinetics), by analysis of RBC or plasma ChE levels. For centres with access to rapid analysis, both RBC and plasma ChE levels can be useful in the diagnosis of OP and CB poisoning [33]. Furthermore, sequential post-exposure plasma ChE concentrations may confirm a diagnosis in the absence of baseline values [24]. More controversial is whether ChE levels relate

to the severity of the poisoning. In 1971, Namba *et al.* [86] proposed a four-stage grading of severity, partly based on plasma ChE levels. Recently, Jaeger *et al.* [52], reported a study of 18 patients. In five of these patients, daily plasma ChE levels were measured. A correlation was found between the total dose of atropine and PAM required, the clinical course, and a plasma ChE level (using Ellman's technique) of <400 units/l. Other workers have found plasma ChE concentrations less useful. Bardin *et al.* [6], from a retrospective study of 61 patients, found no correlation between the severity of the poisoning and plasma ChE. Zilker *et al.* [128], reporting 45 patients treated for parathion poisoning, found no significant difference at admission in the plasma ChE concentrations when related to the outcome. From the day of admission onwards, plasma cholinesterase levels in mildly poisoned patients recovered more rapidly; although the levels may reflect the course of the poisoning, they appear to be of little value in determining the immediate atropine requirement, or when to wean [64]. Furthermore, few centres have access to immediate plasma ChE analysis.

Thus the decision on whether more atropine is required, or whether the patient can be weaned, is essentially clinical. The degree of atropinization can be titrated against pupil size, heart rate and dry skin [79,102]. However, both tachycardia and mydriasis may be unreliable methods, because they may result from nicotinic stimulation in more severely poisoned patients [37,48,123]. Mann [74] noted that 10% of OP-poisoned patients do not exhibit miosis. Nonetheless, pupil constriction which dilates following atropine does appear to be a reliable indicator [38]. However, the pupillary response to atropine may be only partial or temporary in cases of severe OP poisoning [18,90,110]. Atropine is not contraindicated in the symptomatic patient who has tachycardia. Indeed, the heart rate may fall paradoxically [129]. ECG changes may be seen to regress as the dose of atropine is titrated [5]. The single most sensitive measure of adequate atropinization is a repeated evaluation of the quantity of secretions [6,25,31,45]. Even so, atropine dosage regimens vary from country to country. In some, high doses of atropine are used for long periods, guided by signs of maximal atropinization. In others, much smaller doses of the drug are used, titrated against peripheral muscarinic signs, principally bronchospasm and bronchorrhoea. In either case, when weaning patients who have been treated for several days, atropine should be continued for at least 24 h after symptoms have subsided [6].

Adverse effects

Over-atropinization can occasionally be observed; the frequency clearly depends on how aggressive an approach to therapy has been taken. One study of 61 patients [6] noted at least one occasion of atropine toxicity in 16 patients (26%); Hirschberg and Lerman [48], in a retrospective, multicentre study, noted an overdose in three of 232 cases. Churkin and Sadovnikova [21] reported the development of psychosis attributable to the use of atropine in OP pesticide poisoning. Clearly, the dose of atropine should be reduced if the patient shows signs of atropine toxicity such as fever, delirium or fasciculations [84].

One concern when administering atropine in an emergency situation is the risk of developing ventricular tachycardia or fibrillation [44, 76] in the cyanotic patient. In this situation, atropine should be given at the same time as the patient is oxygenated [127]. Another problem reported following prolonged atropinization is prolonged paralytic ileus [31]. Whether prolonged atropine therapy can desensitize a patient to produce a spastic rigidity on stopping an infusion is still open to question. In a case reported by LeBlanc *et al.* [64], rigidity was observed for up to 10 days following weaning after a 5-week period of therapy. Atropine toxicity should not be confused with the development of an 'intermediate syndrome' [102,117]. Finally, care should be taken with the use of atropine in warm climates because of inhibition of sweating. The body temperature should be monitored carefully and cooling measures may be necessary.

Other anticholinergic agents

Both N-methylatropine and glycopyrrolate bromide have been used in OP poisoning in man. DeKort *et al.* [26] reported a case where 69 mg of N-methylatropine-nitrate was used. As this drug is a quaternary derivative it does not penetrate the blood-brain barrier to the

same extent as atropine sulphate [113]; because a central antimuscarinic effect is of value, atropine sulphate should be preferred. Gerkin and Curry [39] report using 5850 mg of atropine sulphate and 124 g PAM in a case of parathion-methyl poisoning. In an attempt to use an anticholinergic with a longer duration of action, 510 mg glycopyrrolate bromide was given. The large amount of bromide involved resulted in an elevated plasma bromide concentration. Thus the risk of bromide intoxication restricts the use of large quantities of glycopyrrolate in OP poisoning. Atropine sulphate is the antimuscarinic agent of choice.

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52

Efficacy, toxicity and clinical use of oximes in anticholinesterase poisoning

C. Bismuth, Robert H. Inns and Timothy C. Marrs

Introduction

Together with atropine the oximes represent the most important component in the armoury of drugs used against OP poisoning. Essentially, they remove the phosphoryl group from the OP-inhibited enzyme, thus restoring the ChE to normal function.

Wilson [230] found that nucleophilic agents such as hydroxylamine reactivated ChEs, inhibited by TEPP or diisopropyl phosphorofluoridate (DFP), more rapidly than spontaneous regeneration. Wilson reasoned that selective and greatly improved reactivation of the enzyme was likely to be achievable using a site-directed nucleophile, wherein interaction of a quarternary nitrogen with the negative subsite of the active centre would take the nucleophile in close apposition to the phosphorus. In the years following, a number of related compounds were investigated and then discarded [10,11].

Pyridoxime-2-aldoxime (pralidoxime, PAM) salts, introduced by Wilson and Ginsburg [231] were the first reactivators of ChE to achieve clinical use on any scale and, to a larger extent realized the goal of a site-directed nucleophile. Reactivation with PAM salts occurs about a million times faster than with hydroxylamine. Bis-pyridinium oximes were studied in the form of TMB-4, but the next major advance was the introduction of obidoxime by Lüttringhaus and Hagedorn [152] and Erdmann and Engelhart [76]. In the case of many OPs these bis-pyridinium oximes are more effective than PAM salts.

The most recent oximes studied have been introduced primarily for use in chemical defence especially against soman poisoning. They include HI-6, HS-6, HGG-12, HGG-42 and pyrimidoxime.

Chemical structure

The oximes fall conveniently into two groups, the mono-pyridinium oximes (Figure 52.1) and the bis-pyridinium oximes (Figure 52.2). Of the former, two salts of PAM are of clinical importance, 2-PAM and P2S. Pyrimidoxime, an imidazo-alkylpyridine aldoxime, has been studied on behalf of the armed forces of

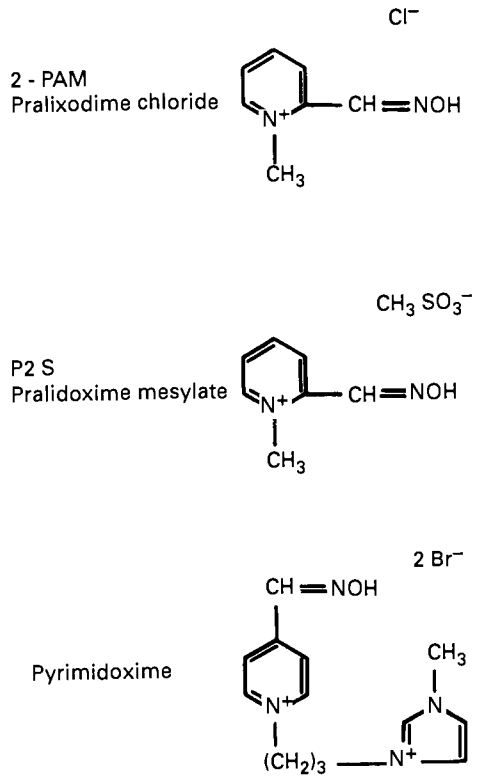

Figure 52.1 Monopyridinium oximes: 2-PAM and P2S are both salts of pralidoxime chloride and methanesulphonate respectively. Contrathion (not shown) is the methylsulphate of pralidoxime

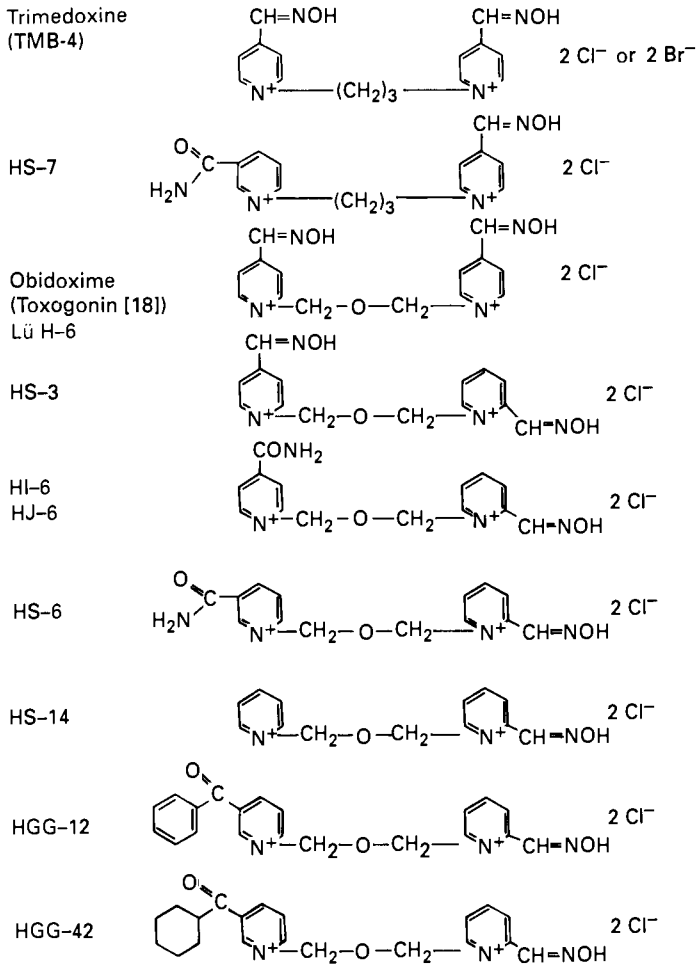

Figure 52.2 Bispyridinium oximes

France and is in some ways more akin to a bis-pyridinium oxime. Although the bis-pyridinium oximes are the most potent reactivators of AChE so far introduced [109], only obidoxime has been used clinically. Many bis-pyridinium oximes have been studied in recent years, especially HI-6, but investigation of mono-pyridinium oximes has, with the exception given above, largely been confined to PAM salts [10]. The use of oximes in OP poisoning is one of the most heavily investigated areas in pharmacology and many attempts have been made to correlate both physical and chemical properties and the structure of oximes with enzyme reactivating potency [22,31,54,96,111,154]. In recent years some novel oximes have been studied, probably because of the special problem of soman poisoning: these have included methylthio derivatives of TMB-4 and obidoxime [30] and oximes containing 4,4'-bipyridyl or 2,2'-bipyridyl groups [43].

Mode of action

The essential feature of the oximes is their ability to reactivate ChEs from various sources, which have been inhibited by OPs: this process is accompanied by restoration to normal function. As generally visualized, the oxime is orientated proximally to exert a nucleophilic attack on the phosphorus of the enzyme inhibitor complex. Intermediate in the

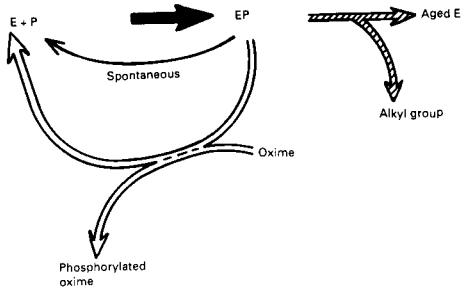

Figure 52.3 Reaction of ChE (E) with OP (P) to form enzyme-OP complex. Spontaneous or oxime-mediated reactivation may occur or, with certain antiChEs, formation of aged enzyme.

reactivation is a complex formed between the phosphorylated enzyme and the reactivator [198]. The oxime-phosphate complex, which may have its own toxic effects, is then split off, leaving the regenerated enzyme (Figure 52.3). Mechanistic studies of the action of the oximes have generally shown that two binding sites were involved. Thus, the different effects of obidoxime, TMB-4 and HS-6 on the hydrolysis of acetyl-β-methylcholine or acetylcholine by AChE were interpreted by Kuhnen [142] as showing that the reactivators react at the active site of the enzyme and at a secondary binding site. Reactivation by P2S has been studied by Harvey *et al.* [104,105], who came broadly to a similar conclusion.

The enzyme reactivation effect proceeds at different rates on inactivated antiChEs present at different sites in the body [8,101,128,157, 178,239]. Thus RBC AChE is most easily reactivated and plasma ChE less so [100]: there are considerable differences in tissues such as the brain, and it is frequently the case that blood ChE activity can be restored while that in the tissues cannot. Furthermore, drug distribution effects may cause the rate of reactivation to differ with the different oximes. Thus 2-PAM is inferior to pro-PAM in CNS penetration and central nervous AChE reactivating power [133; see later]. Possibly as a result of this difference in action at different sites, while oximes reverse cholinergic effects satisfactorily at peripheral nicotinic sites, they are less effective at muscarinic ones and their effects on CNS-mediated symptoms are probably clinically insignificant [216; see later].

Despite their breadth of efficacy in terms of species, including OP target species, such as insects [58], the oximes have a narrow spectrum of activity, even within antiChEs. They are, for example, probably not useful against CB ChE inhibitors (see later) and against the lethal effects of the pesticide impurity O,S,S-triethyl phosphorodithioate [222].

Oximes and ageing

Ageing is the result of loss of one alkyl or alkoxy group. This leaves a monoalkylphosphoryl or monoalkylphophonyl ChE, that is much more stable because the negative charge of the monoalkylphosphoryl group repels the oxime, thereby increasing stability to nucleophilic attack [27,79]. Although there are some reports that oximes may retard the ageing of inhibited antiChE [63], it seems likely that none of the oximes at present in use can do so to any meaningful degree. Thus it is still true that the major area in antiChE poisoning where oximes are ineffective is in poisoning with those OPs associated with rapid ageing [109]. Although the most serious problem is presented by the nerve agent soman, poor reactivation by oximes of ChE inhibited by certain OP pesticides, e.g. profenofos [94] and dichlorvos [99] has been attributed to ageing. In fact, ageing occurs to some extent with most OPs. The data of Hobbiger [108] were interpreted as showing a dependence of the process on the whole structure of the OP rather than its dialkylphosphoryl group. Nevertheless, it has been stated that rapid ageing will occur and oxime reactivation be less effective with dimethyl and diisopropyl but not diethyl substituted OPs [99], while Aldridge [6] stated that phosphonates containing tertiary alkoxy groups are more prone to give rise to ageing than those that contain secondary or primary congeners. Further, linkage of residual alkyl groups to phosphorus through sulphur, as opposed to oxygen increases the rate of ageing (and spontaneous reactivation) of inactivated ChEs [55]. Because the mechanism of ageing is C–O fission, the rate of ageing depends on the ease with which the alkyl group can form a carbonium ion. Metabolic activation steps can make attempts to determine structure-activity relationships remarkably difficult; French *et al.* [82] showed that R(+)-ethyl S-propyl methylphosphonothioate is bioactivated by rats and by the isolated liver to a compound whose ChE

adduct is not amenable to oxime reactivation, unlike that of the parent compound and its S(–) enantiomer. Studies *in vitro* reported in the same paper showed that reactivation profiles of the R and S isomers of VX were different under some conditions. Some of the apparently conflicting data on ageing rates may be explicable by species differences such as have been observed by French *et al.* [83]; they found, after inactivation by soman, that rat ChE dealkylated more slowly than that of the guinea pig. Oxime reactivation of aged enzyme is not enhanced by atropine [143], but atropine may retard ageing of human RBC AChE.

In the absence of ability to reactivate aged enzyme the outcome of oxime treatment is largely determined by the relative rates of ageing and reactivation and this has been demonstrated both for pesticides [125] and chemical warfare agents. Influence of OP structure on rate of ageing has been discussed earlier, while the velocity of reactivation by oximes, at least in the case of PAM, parallels the rate of spontaneous reactivation. It is greatest for dimethylphosphoryl ChE, less for diethylphosphoryl ChE, less for diisopropylphosphoryl ChE and so forth.

The problem of ageing could be attacked by using oximes, such as HI-6 or pyrimidoxime, that reactivate AChE very rapidly, but with certain OPs (e.g. soman) this becomes logistically difficult. The only effective method available to counter ageing, in such cases, is pretreatment with a CB (*see* Ch.56).

Oximes and CBs

Some experimental results have supported the view that oximes might decarbamylate ChE [176,181]. Thus both mono- and bis-pyridinium oximes are reported to accelerate the decarbamylation of carbamylated AChE formed when CBs are used to protect animals against OP poisoning [62]. If, as Harris *et al.* [103] suggest, oximes decarbamylate the enzyme, in the case of ChE protected from ageing with CBs, the implication is that oximes could decarbamylate the enzyme in CB poisoning. The bulk of experimental evidence is that the oximes are not active against CB antiChE intoxications [42], and spontaneous decarbamylation, accelerated or not by the presence of free CB [28] seems the most likely explanation of the work referred to above. Indeed oximes may aggravate CB insecticide poisoning [47] and Natoff and Reiff [176], who found some activity of oximes against certain CBs, showed that oxime treatment increased the toxicity of carbaryl.

Non-antiChE inhibitor activity

In addition to their ability to hydrolyze the enzyme inhibitor complex, it is probable that oximes have some hydrolytic power against the active inhibitor. Furthermore, some of the earliest used oximes possess anticonvulsant activity (e.g. diacetylmonoximes) [87] and anticonvulsant action may have produced benefit that was ascribed to ChE reactivation; there is evidence that some of the newest oximes, e.g. HI-6, may have some direct anticonvulsant properties [146]. Other non-ChE reactivating properties may be therapeutically important. Thus it seems likely that some of the activity observed with certain bis-pyridinium oximes against soman is because of such actions. For example, in the guinea pig, Inns and Leadbeater [115] observed that the three non-oxime bis-pyridinium compounds, bis(pyridinium-1-methyl) ether, SAD 128 and P-65 (Figure 52.4), given as therapy, all conferred some protection on soman-poisoned guinea

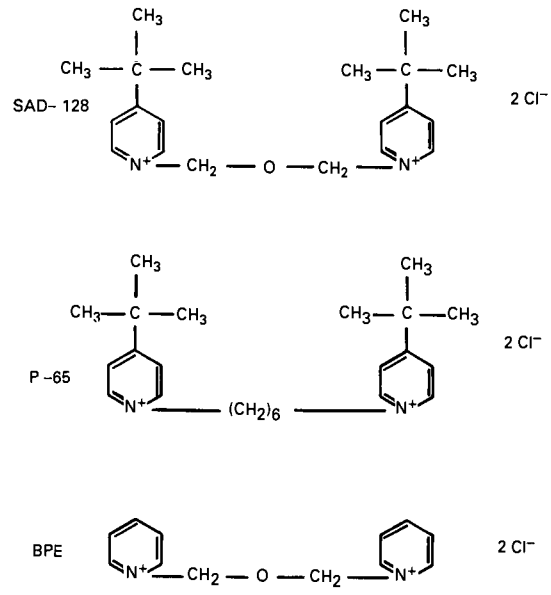

Figure 52.4 Bispyridinium non-oximes

pigs. Schoene *et al.* [197] found that, *in vitro*, these compounds did not affect the rate of detoxification of soman, but could competitively inhibit AChE and thus protect the enzyme from soman inhibition. This finding does not completely explain the therapeutic action of the non-oximes because it is doubtful if sufficient enzyme could be protected in time, in view of the rapid ageing of soman-inhibited enzyme. Schoene and Wulf [199] reported that various bis-pyridinium compounds reduced the rate of ageing of soman-inhibited enzyme and Kuhnen *et al.* [143] thought that atropine, which is, of course, used in most of these studies, could retard ageing. However, while this would provide an explanation for bis-pyridinium oxime reactivation of unaged enzyme, it would not explain the beneficial action of the structurally-related non-oximes, which do not reactivate the enzyme, whether aged or not [83]. French *et al.* [83] found that an appreciable proportion of the activity of TMB-4, but not P2S or obidoxime, in soman-induced neuromuscular blockade of the isolated rat diaphragm, was the result of direct action. By contrast, in the same preparation, but from the guinea pig, all the tested oximes had some direct action. That of TMB-4 was a greater proportion of the total activity than that of obidoxime, which in turn was greater than that of P2S.

Early reports suggested that oximes could produce ChE inhibition and atropine-like effects [29], presumably because they bind weakly at the active site of the enzyme. However, in human volunteers, PAM derivatives exerted no antiChE activity [44]. Moreover, unlike atropine, obidoxime appears to be a non- competitive inhibitor of the muscarinic receptor [144]. The direct action of oximes on the enzyme was studied by Kuhnen [140,141] who found that obidoxime and other bis-pyridinium oximes had activating or inhibitory effects on bovine RBC ChE *in vitro*, depending on their concentrations and also that of ACh. They suggested that the compounds each reacted with separate but interdependent receptors.

Ganglion-blocking, hemicholinium-like, antinicotinic and antimuscarinic effects have all been ascribed to oximes. Lundy and Tremblay [151] found that some of the bis-pyridinium oximes had ganglion blocking effects. Robineau and Coq [185] found that P2S produced a dose-dependent depression in NM transmission in the isolated rat phrenic nerve diaphragm preparation, while NM blockade by HI-6 was demonstrated by Clement [48] and attributed to a hemicholinium-like effect. Fossier *et al.* [80] reported that both obidoxime and TMB-4 had a depressive action on cholinergic transmission, with a curare-like effect at postsynaptic receptors, while Clement [50] showed that HI-6 had both antimuscarinic and antinicotinic activity. The importance of nicotinic receptor binding for a larger number of bis-pyridinium oximes was emphasized by Su *et al.* [214] as possibly supplying an explanation for some part of their activity in some poisonings. Caratsch and Waser [46] carried out a number of interesting studies on the effect of obidoxime at the frog NM junction. They concluded that this oxime had a weak direct depolarizing effect and potentiated ACh-induced depolarization. After sarin-induced depolarization, obidoxime had the reverse effect, i.e. it decreased ACh-induced depolarization. Other studies have been carried out on the NM junction of the frog; for example, Alkonden *et al.* [7] showed that 2- PAM and HI-6 both weakly inhibited AChE and demonstrated a direct molecular interaction with the natural agonist and with the ACh receptor-ion channel complex.

2-PAM is said to exert marked effects on the brain neuronal network, which is unrelated to ChE reactivation [69]. Further, Borbely *et al.* [39], working with mice, reported that obidoxime reduced brain ACh levels, even in non-poisoned mice. Valdes *et al.* [218] observed that PAM-methiodide and HI-6 could interact directly with the rat brain muscarinic cholinergic receptor. Similarly, Amitai *et al.* [9] reported that many of the bis-pyridinium oximes bind to mouse brain muscarinic receptors and also exert a mild antiACh activity in the guinea pig ileum.

The sympathetic effects of the oximes have been less studied, but Stavinoha *et al.* [211], investigating the effects of 2-PAM on the arterial blood pressure of dogs concluded that the observed pressor effect was mediated through an effect of noradrenaline on peripheral α-adrenergic receptors. The same workers observed potentiation of the effect of adrenaline and noradrenaline on the isolated

rabbit aortic strip. The mechanism of the effects of oximes on steroid and catecholamine levels in the rat, studied by Benesova *et al.* [25], have yet to be fully explained.

The non-ChE-reactivating effects of oximes are a somewhat confused area and in particular the beneficial activity of the non-oxime bis-pyridinium compounds in nerve agent poisoning is currently unresolved. It is clear that the oximes have widespread and diverse pharmacological actions, but it is less clear if they are of practical importance in the therapy of human poisoning. Extrapolation from animal studies to humans is made more difficult because major species differences have been described [83,220]. It is noteworthy that the direct action of HI-6 and HS-6 seen on rat and guinea pig intercostal muscle and diaphragm was not seen in human intercostal muscle [209,237].

Delayed neuropathy and oximes

There is no evidence that oximes prevent the development of the delayed neuropathy associated with certain OPs; indeed there is evidence that treatment with oximes and other drugs may allow experimental animals to survive acute poisoning only to develop delayed neuropathy [95,227,228,233].

Efficacy

Many studies have been carried out on the efficacy of oximes in the treatment of OP poisoning, both *in vivo* and *in vitro*. Some of the former are difficult to interpret because of species differences (for review of choice of animal model in the study of OPs *see* Calabrese [45]). These problems are particularly complex when studying bis-pyridinium oximes in soman poisoning. Furthermore, it is particularly important to choose methods for ChE determination which do not suffer from interference by oximes where ChE reactivation is employed as a measure of reactivating potency [93].

P2S and 2-PAM and other PAM salts

PAM-methiodide in combination with atropine was studied by Askew [12] and Fournel [81]. Among the earliest experiments which established the usefulness of PAM-methiodide were those of Hobbiger [108]. In the mouse, it was found that while 25 mg/kg was fairly ineffective against some OPs (DFP, paraoxon and 3-(diisopropoxyphosphinyloxy)-pyridine), it was active against TEPP, 3- (diethoxyphosphinyloxy)-N-trimethylanilinium methylsulphate and 3-(diethoxyphosphinyloxy)-N-methylquinolinium methylsulphate. A generally-held belief is that oximes are not particularly effective used without atropine (but *see* Ligtenstein [145]). In a cat intercostal muscle preparation, Koelle [138] demonstrated, by an histochemical technique, that PAM-methiodide reactivated DFP-inhibited AChE in ganglia and motor endplates. Using a behavioural paradigm in rats Rosić [188] found that 2-PAM by itself was not very effective against DFP or Armin (ethyl-4-nitrophenyl ethylphosphonate) and Serrone *et al.* [203] found that while 2-PAM would reverse the whole blood ChE inhibition produced in Rhesus monkeys by administration of a single dose of parathion it did not prevent death. On the other hand, PAM-methiodide, unaccompanied by atropine, was a satisfactory antidote in goslings to experimental poisoning with diazinon: the treatment was given after the poisoning when the birds were unable to walk [205]. Nevertheless, the study of Hobbiger [108] and subsequent workers showed clearly the potentiating effects of the administration of atropine with the oximes. Bethe *et al.* [29] investigated the activity of PAM-methiodide in parathion, paraoxon and DFP poisoning of guinea pigs and found it highly effective with atropine, and Wilson and Sondheimer [232] found PAM-methiodide administered with atropine effective in mice against TEPP, sarin and probably parathion. In the same species, the drug was active against dichlorvos [117], but a very important point was soon apparent, namely that the efficacy of PAM/atropine combination was much less against tabun than other OPs [232]. The probable reason for this is that the nucleophilic attack of PAM on the inhibited enzyme is less effective with tabun, the P–N bond causing the phosphorus atom to be less prone to attack as a consequence of back donation of electrons. Matsubara and Horkoshi [161] showed that, in the rat, PAM-methiodide was effective in poisoning with

fenitrothion, although Yamanaka and Nishimura [239] found that this oxime was incapable of reactivating ChE activity in rats after a sublethal dose of the same OP. In the same species, Robinson and Beiergrohslein [186] showed that 60 mg/kg PAM-methiodide increased the ip LD_{50} of methamidophos from 15 to 52 mg/kg when the oxime was given 5 s after the OP.

P2S is a much more soluble compound than the methiodide [61] and was shown to be effective when given with atropine in sarin poisoning of mice, rats and rabbits by Davies *et al.* [60]. Crook *et al.* [57] showed that PAM and P2S were both effective against lethal concentrations of sarin vapour in dogs. Johnson and Stewart [119] investigated the effect of 2-PAM on rabbits treated with sarin; with previous administration of atropine, 2-PAM will reverse respiratory blockade and 2-PAM is also effective against VX in rats [102]. Soman poisoning presents a number of special challenges, partly because of the rapidity of ageing of the inhibited enzyme but also because there is evidence of significant species differences in response to oxime treatment. Inns and Leadbeater [115] state that the P2S/atropine combination is largely ineffective against soman, unless combined with pyridostigmine pretreatment, or diazepam, or both (*see* Ch.56). In mouse soman poisoning 2-PAM is not very effective [40], and in the rat this oxime together with atropine can reverse soman-induced central neuronal RNA depletion, but AChE levels are not restored [70].

Acuña *et al.* [2] showed that 50 mg/kg 2-PAM almost completely reversed parathion-methyl inhibition of dog plasma and RBC ChE. Ganendrin and Balabaskarin [89] found that PAM-methiodide was of low efficacy in reactivating human whole blood ChE inhibited by dicrotophos. The same workers [90] found PAM-methiodide of low effectiveness against malathion and malaoxon-inhibited ChE. However, in buffaloes the clinical manifestations of experimental malathion toxicity were reversed within 15 min, and atropine alone was less effective [98]. Glickman *et al.* [94] reported that P2S reactivated brain AChE from chicks poisoned with methamidophos but not those poisoned by profenofos; similar findings were reported for eel AChE inactivated by methamidophos and bioactivated profenofos respectively. In experiments *in vivo* in chicks, P2S with atropine increased the LD_{50} of profenofos from 1.9 mg/kg to 5.9 mg/kg; atropine alone increased it to 2.0 mg/kg. By contrast no benefit accrued from P2S and atropine to mice over that from atropine alone. Both these studies used a prophylactic protocol.

Because it penetrates the eye poorly, 2-PAM is effective in elevating RBC AChE levels after ecothiopate, without interfering with the ocular hypotensive action of the latter drug [148].

2-PAM is effective against the glycogenolytic effect of malathion [160], and protects against the production of skeletal anomalies by parathion in birds [163–166]. Gauna and Orias [92] reported that PAM was inactive against the antidipsogenic activity of parathion, whilst Agarwal and Matin [3] state that 2-PAM can prevent the rise in blood glucose that accompanies malathion poisoning in rats. *In vitro*, it has been reported that 2-PAM can reactivate dichlorvos-inhibited ChE to a greater extent in plasma from non-pregnant women, than either from pregnant women or human fetuses [23].

Other monopyridinium oximes

The efficacy of monopyridinium oximes other than the PAM group has also been studied by several workers, e.g. Hobbiger *et al.* [111], Benschop *et al.* [26], Engelhart and Erdmann [74], Gajewsi and Owczarczyk [88] and Serafin *et al.* [200]. Pyrimidoxime (Figure 52.1) and certain analogues, have been studied in France [130,131]. Pyrimidoxime shows promise *in vitro* against OPs that bring about rapid ageing.

The use of pro-PAM, the tertiary amine N-methyl-1,6-dihydropyridine-2-carbaldoxime hydrochloride which corresponds to the quaternary structure of PAM [34,35], is intended to overcome the pharmacokinetic barrier between the CNS and the rest of the body [212]. Pro-PAM penetrates into the CNS [34,41,48,204], whereas PAM derivatives do so only to a very limited extent. Rump *et al.* [190] showed that pro-PAM significantly increased the activity of DFP-inhibited brain AChE, partially normalized the ECG and increased the dose of DFP needed to bring about convulsions, while Clement [48], in mice and guinea

pigs, found that when used prophylactically pro-PAM was definitely superior to 2-PAM either alone or with atropine, in DFP and sarin poisoning, and slightly so in soman poisoning. However, combined pro-PAM and CB prophylaxis and atropine treatment in soman intoxication was inferior to a similar regimen using 2-PAM. Boscković [41] showed that the protective effects of pro-PAM were unexpectedly less against paraoxon in mice than 2-PAM, in spite of the greater CNS penetration and reactivation of brain AChE. Thus the benefit to be derived from pro-PAM is unexpectedly questionable, suggesting that inhibition of central ChEs is less important than sometimes thought.

Obidoxime

The efficacy of obidoxime as an antidote to paraoxon in mice was shown by Erdmann and Engelhart [76]. The therapeutic index was more favourable than for PAM salts. Bisa *et al.* [32] showed that obidoxime was superior to 2-PAM in parathion, paraoxon and DFP-poisoned rats. Except in the post-poisoning treatment of paraoxon intoxication, this was true whether the oxime was given before, after or simultaneously with the OP. In dogs, Hahn and Henschler [100] showed that obidoxime reactivated parathion-inhibited RBC AChE even when administered 12 h after poisoning, less reactivation was achieved with malathion and dimethoate and only a minimal degree with fenthion. Obidoxime (as well as PAM-methiodide) was effective against dichlorvos poisoning of mice when given with atropine [117]. The efficacy of atropine and obidoxime in sarin poisoning of mice was studied by Borbely *et al.* [39]. These authors found that high doses of both antidotes were required for appreciable therapeutic effect, and that the CNS action of atropine was required. Obidoxime reversed malaoxon-induced inhibition of isolated rat diaphragm cholinesterase; when 20 mg/kg ip of obidoxime was given ip with atropine, the LD_{50} of mice was raised 5.1 times [1]. Studying the effects of obidoxime on pig ChE inhibited by the pesticides, trichlorfon, dichlorvos and coumaphos, Gyrd-Hansen and Kraul [99] showed that there were marked differences in the reactivability of the inhibited ChEs; dichlorvos-inhibited enzyme could not be reversed. Schoene *et al.* [195] showed that pretreatment with obidoxime and atropine was effective in rats against inhaled sarin, but not soman. On the other hand, in combination with other drugs, obidoxime (and HS-6) was more effective in soman poisoning of mice than 2-PAM or TMB-4. However, Junod *et al.* [123], who found obidoxime and atropine effective when administered after sarin to mice, reported that the same therapeutic combination was ineffective against soman. Inns and Leadbeater [115], using guinea pigs, found obidoxime only effective against soman after pyridostigmine pretreatment. Obidoxime and the other 4-substituted bis-pyridinium oxime, TMB-4, were found by Inns and Leadbeater [115] to be more effective than P2S in tabun poisoning of guinea pigs, and according to Heilbronn and Tolagen [107], in mice obidoxime is only slightly less effective than TMB-4. Hopff *et al.* [114] concluded that from data on obidoxime in sarin poisoning, guinea pigs behaved similarly to mice and rats.

Numerous studies of the activity of obidoxime against particular aspects of antiChE intoxication have been carried out. Fusek *et al.* [85] observed normalization by oxime ChE reactivators of the inotropic response of the isolated guinea pig heart treated with O-ethyl-S-(2-dimethylaminoethyl) methylphosphonothioate. Reversal by obidoxime of alterations of hepatic metabolism produced in DFP poisoning have been studied *in vitro* by Kleinrok and Rajtar [136], and in addition, this oxime together with atropine has been observed to antagonize the effects of paraoxon on perfusion rates of various organs of rats [223].

Besides being more effective than PAM salts, it has been said that the CNS penetrating power of obidoxime is greater than that of PAM salts [75]. Thus it might be expected that obidoxime would better reactivate ChE in the vital centres; however it prevents the rise in brain ACh seen in DFP poisoning in rats through its peripheral action on ChE [162,170] and in poisoning of mice with parathion, parathion-methyl, paraoxon and O-ethyl O-(p-nitrophenyl) phenylphosphorothioate, it was observed that, although obidoxime provided a great degree of prophylactic protection, a protective effect on brain AChE was not seen [184]. Similarly obidoxime was observed not to

prevent the rise in ACh in rat brain [171]. By contrast, using a histochemical technique, de la Manche *et al.* [67] were able to show reactivation by obidoxime of cerebral enzyme inhibited by paraoxon. Furthermore, Vasić *et al.* [221] observed that obidoxime inhibited the rise in ACh content in the rat pons and medulla induced by Armin, and produced satisfactory reactivation of the target enzyme in the pons and medulla. In parathion intoxicated dog brain, reactivation of ChE showed considerable regional variation [128,129], and this may be the explanation of the conflicting results obtained by others.

Rump *et al.* [189,191] studied the effect of the timing of therapy in relation to poisoning with DFP, finding that 40 mg/kg obidoxime, with atropine, was much more effective administered at 2 h than at 24 h. They also investigated the effect of multiple injections of obidoxime in rats poisoned with DFP. Second doses of 40 mg/kg obidoxime with atropine given at 90 min, the first dose being given 30 s after intoxication, failed to improve survival. Clearly therefore the temporal relationship between OP and oxime dosing and the precise treatment regimen is critical. Many studies where a prophylactic protocol has been employed, e.g. Ramakrishna and Ramachandran [184], may give an exaggerated impression of the efficacy which the oxime would possess when used as an after-the-event treatment.

Other bis-pyridinium oximes

Hobbiger and Sadler [110] reported that TMB-4 was considerably more potent a reactivator of ChE than PAM-methiodide and the efficacy of TMB-4 is well-established in animal studies [107]. Hobbiger and Vojvodić [112] found that TMB-4 was 1.15 and 1.4 times more active as a reactivator of diethyl phosphoryl and diisopropyl phosphoryl-AChE prepared from washed human RBCs, than obidoxime. TMB-4 was reported by Vojvodić and Milosević [225] to be more effective than 2-PAM at reversing the cardiovascular effects of soman. Beneš *et al.* [24] studied the effect of the same oxime together with atropine on the plasma and RBC ChE of sheep poisoned with trichlorfon. Bajgar *et al.* [17], using rats, found that this oxime, with atropine was better at reactivating blood ChE inhibited by O-ethyl-S-(2-dimethylaminoethyl) methylphosphonothioate than obidoxime at the same molar dose. In the mouse, TMB-4 was reported by Schoene and Oldiges [196], to be especially effective against tabun. Bis-pyridinium oximes have been reported to be effective in a number of *in vitro* systems, e.g. the study of Fusek *et al.* [85], discussed earlier in connection with obidoxime, also investigated the effectiveness of TMB-4 and methoxime. The former was also studied in the isolated sympathetic ganglion of the cat by Lukomskaya *et al.* [150] while De Jong *et al.* [65] showed that P2S was less effective than TMB-4 and obidoxime in reactivating methamidophos-inhibited electric eel AChE. Other studies vouch for the efficacy of TMB-4. Thus Maksimović [155] reported that this oxime was a better reactivator of rat diaphragm ChE than any of a large array of oximes including 2-PAM, obidoxime and HS-6, while, in the immobilized enzyme preparation of Trammel *et al.* [217], TMB-4 was a better reactivator of DFP-inhibited enzyme than PAM-methiodide. Interestingly, Klemm [137] reported that TMB-4 accompanied by atropine, but not 2-PAM, was effective against neostigmine in mice. Another oxime, methoxime, was reported to be more effective than either obidoxime or TMB-4, when given with atropine to sarin poisoned mice [18], but neither TMB-4 nor methoxime has acquired clinical significance.

An attraction of the 'Hagedorn' oximes [74,76,152] is their relatively good efficacy against soman in animal studies [75,179,193, 194,236]. Thus high efficacy of HI-6 and HS-6 was reported in soman poisoning of mice and rats [126] and in these experiments, HI-6 seemed preferable. Schoene *et al.* [195] found that, accompanied by atropine, HI-6 but not obidoxime, was effective as a treatment against inhaled soman in rats. De Jong and Wolring [64] studied the reactivating effects of HI-6, HS-6, HGG-12 and HGG-42 on bovine RBC soman-inhibited AChE. They reported that these oximes, particularly HI-6 and HS-6, had greater reactivating potency than the conventional oxime antidotes, P2S and obidoxime, but they also showed that HI- 6 had no effect on soman-aged enzyme. The beneficial effect of this oxime is thus probably its extremely high reactivating potency. As it

seems clear that brain ChE activity is not indicative of the protective effect of HI-6, it is reactivating effects at sites outside the brain, which are important [49– 52,54]. In the mouse, Clement [50] found that HI-6 was the most efficacious oxime in soman poisoning, as well as the least toxic of a group including 2-PAM, obidoxime and TMB-4. Comparing HI-6 and HS-6 with some novel bis- pyridinium oximes given with atropine to mice poisoned with soman, Clement and Lockwood [54] concluded that HI-6 was one of the least toxic and the most efficacious. Similarly, Schoene *et al.* [195], using rats found HI-6 more effective than obidoxime: pretreatment of rats with atropine and HI-6 increased the LCt_{50} of soman by seven, while obidoxime was ineffective. Although from an efficacy point of view many animal studies have shown the superiority of HI-6 and HS-6 over obidoxime, the choice between HI-6 and HS-6 is difficult; in the monkey studies of Lipp and Dola [147], it was not possible to distinguish between the therapeutic effects of HI-6 and HS-6 on soman intoxication, when they were given with pyridostigmine, clonazepam and atropine. However these workers stated that hypotension was worse with HS-6. Before these results are extrapolated to support the efficacy of HI- 6 in soman poisoning, the results of van Helden *et al.* [233] must be considered. It was demonstrated that HI-6 was much less effective in muscle preparations derived from marmosets and humans than from rodents and dogs. Furthermore, Inns and Leadbeater [115] found that when given with atropine in soman poisoning of guinea pigs, neither HI-6 nor other bis-pyridinium oximes were much more active than atropine alone. Furthermore, in the same study, only obidoxime and TMB-4 were effective in tabun poisoning (see earlier). The last-named drug was deleterious at high dose, in combination with pyridostigmine, atropine and diazepam, in soman poisoning. Supplementing the atropine/oxime therapy with pyridostigmine pretreatment or diazepam gave a protective effect with HI-6, HS-6 and other bis-pyridinium oximes. Further evidence for the inefficacy of HI-6 against tabun, is provided by the work of Clement [52] and Cetković *et al.* [47a]. Clement found that this oxime reactivated sarin-inhibited brain and diaphragm AChE, but not tabun-inhibited enzyme, while in the rat, HI-6, as well as HGG-12, together with atropine, were relatively ineffective against the lethal toxicity of tabun [47a]. The treatment of tabun poisoning by oximes is indeed a problem, and it has been addressed from a structure-activity point of view by Mager and Das Gupta [154]. It has to be concluded that the inactivity of HI-6 against tabun- inactivated ChE is a major disadvantage of this oxime.

HS-6 is effective in VX poisoning of rats [102] as is HI-6 in guinea pigs [115], but in the chemical warfare field its ineffectiveness against tabun to some extent counterbalances its effectiveness against soman, and in any case the latter may not extend to man (*see* Ch.56).

Used experimentally, HI-6 is effective against many pesticides but not necessarily more so than other oximes. Thus in a study in chicks [94] the LD_{50} of profenofos was increased from 1.9 to 3.8 mg/kg by atropine; addition of HI-6 or P2S increased the LD_{50} by 4.9 and 5.9 respectively, demonstrating that in this situation HI-6 was inferior to P2S. However, HS-6 was more effective than either of these oximes giving an LD_{50} with atropine of 9.3 mg/kg. In mice neither HS-6 nor P2S was more effective than atropine alone. *In vitro*, both HI-6 and HS-6 were incapable of some reactivation of bioactivated profenofos-inhibited eel AChE, but they were able to reactivate that inhibited by methamidophos. In rats, HI-6 was effective against quinalphos poisoning; using constant blood levels of oxime, maintained by osmotic minipumps, very considerable degrees of protection were attained [38], but with dimethoate it did not seem effective [122].

Other oximes that have been investigated include HGG-12, HGG-42 and HS-14. Weger and Szinicz [226] found that when together with atropine and benactyzine a number of oximes were given 6 min after $5\times LD_{50}$ of soman, HGG-12, HGG-42 and HI-6 were the most effective in soman poisoning of dogs. Clement [53] studied the efficacy of various mono- and bis-pyridinium oximes together with atropine against soman, sarin and tabun poisoning in mice. HI-6 gave the best protection against sarin lethality, while HGG-42 was most effective against soman and tabun. 2-PAM was generally less effective than the bis-pyridinium oximes. Kirsch and Weger [133]

thought that HGG-12 differed from HGG-42 in having an affinity for ganglionic muscarinic receptors, but both have ganglion blocking properties, which are important to their therapeutic efficacy [132]. Because these oximes did not reactivate aged phosphonylated enzyme, Hauser *et al.* [106] ascribed their efficacy in soman poisoning to ganglion blockade. Jovanović [121] found that in the *in vivo* isolated phrenic nerve-diaphragm preparation of the rat, HGG-12 was inferior to HI-6 in sarin, tabun and VX poisoning while the two reactivators were equieffective against soman-induced NM blockade. In the isolated diaphragm preparation, HS-6, HI-6 and HS-14 were superior to P2S in reversing soman-induced NM blockade of the rat diaphragm, but in the guinea pig diaphragm preparation all four oximes produced good reversal [83].

Other bis-pyridinium oximes have been studied by Maksimović *et al.* [156] and Simeon *et al.* [206]. Convincing data on the therapeutic advantage of these compounds were not obtained.

In spite of occasional reports suggesting better efficacy of a monopyridinium oxime to a bispyridinium one at reactivating certain inhibited ChEs [173], the preponderance of evidence is that in most circumstances obidoxime, TMB-4 and HI-6 are more effective than 2-PAM.

Toxicology of oximes

Space does not permit a detailed discussion of the toxicology of the oximes. However the acute toxicity is relevant to their safety of use. The LD_{50}s of the various oximes have been the subject of numerous estimations often in the course of efficacy studies. Important points to note are that TMB-4 is more toxic than most oximes and that the toxicity of oximes has sometimes been ascribed to cyanogenesis. This was particularly the case with the earliest oximes [13], and storage of P2S can result in cyanide production [56]. There may also be a potential problem with HI-6, an oxime whose poor stability may militate against its introduction into clinical use [86]. Eyer *et al.* [77] noted this oxime's cyanogenic potential at pH 7.4. However, cyanogenesis is unlikely to contribute to the toxicity of presently used oximes; Ballantyne *et al.* [19] gave rabbits an im LD_{90} of P2S and was unable to detect cyanide immediately after death.

The repeated dose toxicity of the clinically used-oximes has been the subject of several studies. Subchronic administered PAM [4,5,61] is notable for producing damage at injection sites and to mucous membranes when given orally. Changes in serum protein levels and the white cell count have also been observed. Repeated dose toxicity studies on obidoxime; have been carried out [76,207,208]. In these studies death appeared to result from NM paralysis. There has been some question that obidoxime may be hepatotoxic [20,32,235]. Whether this is a genuine effect of obidoxime is controversial [36,37].

Reproductive studies (Bradshaw *et al.*, unpublished data) and the Ames test (Morris, unpublished data) have indicated neither a reproductive hazard for PAM nor the likelihood of mutagenicity. HI-6 and HS-6 were negative in the standard overpour Ames test, while HS-6, but not HI-6, was positive in *Salmonella typhimurium* strain TA 100 in the concentration range 50– 100 μg/plate in a liquid preincubation test. For a more detailed review of the toxicology of oximes *see* Marrs [158].

Clinical use of oximes

Insecticide poisoning

Oximes have been recommended in the treatment of OP pesticide poisoning since about 1960, but have not been the subject of a randomized study in humans. Their use in the early stages of intoxication, together with atropine, alleviates the cholinergic symptoms and clinical signs, and reduces the amount of atropine needed to ensure survival. The pyridinium oximes are reputed to enhance the anticholinergic power of atropine about five-fold, so that *in vivo* they behave as though they potentiate the effect of atropine in intoxicated subjects. Nevertheless, the efficacy of oximes in OP poisoning is difficult to assess on a biological basis because of the poor correspondence between ChE depression and clinical signs of OP poisoning. Thus, with miotic eye drops, for example, ChE activities near to zero may coexist with clinical normality

(Bismuth, unpublished data). Thus, the appraisal of oximes in OP insecticide poisoning must remain a clinical appreciation.

Central effects

In humans, as in animals the effects of oximes on the central nervous effects of OP antiChE pesticides remains debatable. The clinically-used oximes, being quarternary nitrogen-containing compounds, penetrate the CNS of experimental animals poorly, if at all, and limited evidence suggests that the same is true of humans; thus PAM was not found in the spinal fluid of a healthy man after infusion at a dose of 44 mg/kg [118]. Nevertheless, there are occasional clinical reports in which central effects of intoxication are described as having been ameliorated [84,116,174,182]. Thus successful use of oximes has been reported with a number of pesticides, where central nervous signs were present. For example Gaultier *et al.* [91] noted the reversal of a convulsant coma after suicidal ingestion of parathion, when, after the failure of 4.75 mg atropine, PAM-methylsulphate was added. Willis [229] reported three cases of OP poisoning responsive to PAM therapy after prolonged coma. He suggested that the reactivator might be useful up to as much as 2 days after the intoxication and that high doses could be tolerated (182.5 g PAM over 47 days). Lotti and Becker [149] reported a case of a 3½-year-old comatose child severely poisoned by parathion and treated with a high dose of 2-PAM under EEG monitor. The dramatic effect on cortical electrical activity represented, for the authors, the effects of a direct action of 2-PAM on central nervous activity. On the other hand Kissel *et al.* [134] noted the failure of 2-PAM and atropine to reverse a severe intoxication with parathion. Death occurred after a coma of 12 h. Cardiac arrhythmia had nevertheless been corrected with oxime.

None of these reports, however, provides objective evidence for a central nervous ChE reactivating effect. On the other hand, it is not clear what levels of ChE inhibition in the CNS are required to produce central effects, but best estimates are that they are very high and close to lethal levels [139]. Therefore, even slight reactivation might have very dramatic clinical effects [73]. Alternatively, the reactivating effect might be more marked in selected vital areas of the brain, because it is known that oximes have selective access to different regions of the CNS [78,109,113,187]. A complicating factor is that OPs increase the access to the brain of other chemicals [175]. Thus Firemark *et al.* [78] found that 2-PAM penetrated the CNS to a greater extent in rats poisoned with trichlorfon than in unpoisoned animals. On the other hand Streichenberg and Waser [213] found that obidoxime distribution in mice was not altered by sarin. It is therefore unresolved whether the distribution of oximes could be different during treatment.

Peripheral effects

The effects of oximes on peripheral ChEs is less controversial in both humans and animals. It is not easy to exclude an effect of oximes not only in reactivating inhibited ChE but also in favourably altering the equilibrium between inhibited enzyme and the increase in acetylcholine levels [21]. However, the expectation of successful reactivation of ChE depends on the enzyme not having aged. Although this phenomenon is most well known with the nerve agent soman it is of importance in the pesticide field. Moreover, being time-dependent, ageing is a reason why oxime treatment should be given fast (ideally within 1 h). A possible way around the need to give oximes early to combat ageing is the use of prophylaxis in exposed populations (*see* later and Ch.56). This was proposed by Quinby [183] in the form of oral PAM 1 g three times per week. This produced a more stable ChE level in pesticide workers than in a corresponding group of workers that was untreated. The efficacy of oximes against the late peripheral effects of OPs (peripheral neuropathy) is generally discounted, but the success of PAM-methylsulphate in human tricresyl phosphate poisoning was attested by Dezoteux [68].

Side-effects and drug interactions

High doses of 2-PAM and related compounds can themselves cause NM blockade and other effects, including inhibition of ChE. Such actions are minimal at clinically-used doses (1–2 g iv). If this oxime be given at a rate more rapid than 500 mg/min, it can cause tachycar-

dia, muscle rigidity, NM blockade, hypertension and laryngospasm, these effects being dose and rate of administration-related [72]. Oximes are contraindicated in patients with myasthenia gravis being treated with anti-ChEs; in this condition they may precipitate a myasthenic crisis. Toxic levels of PAM may accumulate in the presence of renal dysfunction, while obidoxime may cause liver problems.

Preparations

In accidental or suicidal poisoning with OP pesticides, several oxime preparations are available.

Pralidoxime chloride, 2-PAM, molecular weight 173, (Protopam) [14,15] is the only reactivator generally available in the USA. It is dispensed in sterile 1 g quantities for solution in 20 ml sterile water before use. In the absence of contraindications such as pulmonary oedema, 1–2 g should be given by infusion in 100 ml saline over 15–30 min. If infusion is not possible, 1–2 g in adults or 20–40 mg/kg in children should be administered iv over not less than 5 min. The manufacturer states that stability tests justify a 6-month expiration period at 25°C, for Protopam chloride injectible [16]. Tablets of 2-PAM 500 mg were also available.

P2S, pralidoxime methanesulphonate or mesylate, molecular weight 232, is available in the UK as a 20% solution in 5 ml ampoules from the Department of Health (England), the Scottish Home and Health Department and the Department of Health (Northern Ireland). Dosage is similar to 2-PAM (allowing for the difference in molecular weight). Use of P2S is largely confined to the UK.

Pralidoxime methylsulphate, molecular weight 248 (Contrathion, SERB) is used in France [33]. It is presented as bottles containing 200 mg lyophilizate, accompanied by ampoules containing 10 ml isotonic aqueous sodium chloride. The solution is prepared at the time of use by adding the contents of one ampoule to one bottle. In severe intoxications, 400 mg (two bottles) is given by slow iv injection. Subsequently 200 mg may be given at 3, 6 and 12 h and then 4-hourly, depending on the patient's progress [201]. These doses are, allowing for molecular weight, much below those recommended with 2-PAM. In Anglophone countries and possibly elsewhere there has been some confusion about this PAM salt. According to the Merck Index [168], Contrathion is a synonym for P2S, but Martindale [159] states that is has one more oxygen atom and is the methylsulphate [*see* also 202].

Other pralidoxime salts are found in certain national pharmacopoeias including the iodide [159].

Obidoxime chloride (Toxogonin, E Merck) is used in some countries, including Germany and Sweden [180]. It is available in 1 ml ampoules containing 0.25 g obidoxime chloride [167]. It is more potent than 2-PAM, the recommended dose being 1 ampoule or 3–6 mg/kg injected iv over 5–10 min (recommended dosing schedules vary slightly [120]).

Other oximes that have been studied experimentally are generally unavailable. Diacetyl monoxime, which was recommended at an iv dose of 1 g, at a rate of 200 mg/min, penetrates the blood-brain barrier and thus is capable of reactivating ChE in the brain. TMB-4 and the newer oximes (HI-6, HS-6 and pyrimidoxime) designed for use against OPs that cause rapid ageing of ChE are not available for civilian use.

Use in pregnancy

Limited experimental evidence from animal studies suggests that there is no special hazard from the administration of 2-PAM in pregnancy. Case reports from human poisonings with OPs tend to confirm these findings [124].

Position of oximes in the treatment of antiChE poisoning

Current opinion holds that the cornerstone of successful treatment of OP poisoning is the concomitant use of oximes and an anticholinergic such as atropine, although some groups reporting large series of poisonings have stated that oximes did not appear beneficial [71].

Immediate life-threatening clinical signs and symptoms result from weakness of the respiratory muscles, central depression of respiration, bronchospasm, bronchial secretions and pulmonary oedema, all of which result in hypoxaemia. Endotracheal intubation and assisted ventilation are then necessary to

Table 52.1 Relative reactivating potencies of oximes against certain OP compounds

	PAM	*Obidoxine*	*HI-6*	*HGG-12*	*Pyrimidoxine*
Tabun	0	++	0	+	++
Sarin	+	++	++	++	++
Soman	0	0	+	+	+
VX	+	+	+	0	?
Paraoxon	+	++	+	?	++
DFP	+	++	?	?	?

Table 52.2 OP pesticides against which oximes have limited efficacy

Crotoxyphos (Ciodrin)	Morphothion
Demeton	Schradan
Dimethoate	Prothoate
Dimefox	Triamiphos
Methyl-phenkapton	

Data from Tufuri and Roberts [215], Bismuth *et al.* [33] and Ayerst Laboratories [15]

Table 52.3 Factors affecting reactivation of particular OP inhibited-ChEs

Pharmacokinetics of OP
Pharmacokinetics of oxime
Rate of ageing of OP-enzyme complex
Rate of oxime-induced reactivation of enzyme
Accessibility of enzyme to oxime
Adequacy of recommended dose of oxime

maintain adequate oxygenation. Convulsions may require benzodiazepines.

Atropine antagonizes both the muscarinic and central nervous effects of OP poisoning [72] and thus alleviates excessive bronchial secretions, salivation, anorexia, nausea, epigastric 'tightness', abdominal cramps, vomiting and bradycardia. Atropine has little or no effect on muscle weakness or respiratory failure in severe poisoning as this drug does not reactivate ChEs. High doses are often necessary (2 mg in adults or 0.01 mg/kg in children every 15 min). The drying of secretions or full atropinization, rather than dilated pupils, is the effective end-point of atropine titration.

Oxime reactivators must be given concurrently with adequate atropine doses and may be combined with other measures such as haemoperfusion and haemodialysis [240]. Because of the phenomenon of ageing, oxime treatment must be started as early as possible after exposure, ideally within 5 min to 2 h. Although administration of oximes is usually advised in poisoning by any OP AChE, there is considerable variation in effectiveness with the OPs (Table 52.1). Oximes are reportedly effective in exposures to parathion, parathion-methyl, EPN, TEPP, dicrotophos, dichlorvos, dimethoate and mevinphos. Individual case reports have suggested effectiveness with coumaphos [224] and demeton-S-methyl [219] and methidathion [127]. In diazinon poisoning oximes were reportedly ineffective [192]. However, the patient discussed in the latter report has rather unusual symptomatology and Lyman [153], citing case reports and experimental work, stated that 2-PAM was indicated in diazinon poisoning. In the case of parathion both PAM-methylsulphate [135] and obidoxime [59,234] seem effective although some reports, e.g. Zilker *et al.* [241], suggest inefficacy. Differences between 2-PAM and obidoxime, in pesticide poisoning are less clear than with nerve agents, although there are some reports of superior efficacy of obidoxime [238]. Soman-like therapeutic failure seems unlikely, because pesticide-ChE complexes do not usually age rapidly. However, tabun-like failure with PAM-salts remains a possibility and this might provide an explanation for superiority of obidoxime in some instances. Before it be inferred that, on the basis of efficacy studies with tabun, any advantage is sure to lie with the bis-pyridinium oximes, it is worth noting the findings of Bokonjić *et al.* [38] in rats. These workers observed that 2-PAM was consistently superior to HI-6 in experimental quinalphos poisoning. It has been said that the reactivating effects of oximes in poisoning by OPs that form the same type of phosphorylated or phosphonylated ChE can vary greatly. To the medically-qualified toxicologist, it appears that this is an area that is more confused than necessary because authors have failed to state which poison was present or else a mixture was used [71,177,210,238]. Often, of course, the precise OP responsible for a

poisoning cannot be determined. There is some evidence that quite important pesticides are less amenable to treatment with oximes (Table 52.2). Many are phosphorothioates. In nerve agent poisoning oximes are effective except with soman and, in the case of 2-PAM and P2S, tabun. Factors that affect the performance of oximes often cannot be elucidated (Table 52.3).

There is evidence to suggest that, with some OPs, patients may benefit from the more prolonged oxime therapy than has been given in the past [66,169,172] (*see* Ch.12). If this is so, the necessity for such treatment probably depends on the dose, toxicokinetics and lipophilicity of the particular OP.

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Anticonvulsants in anticholinesterase poisoning

Åke Sellström

AntiChE intoxication may give rise to a variety of symptoms depending on the severity of the intoxication. In this chapter symptoms such as muscle fasciculations and convulsions are of particular interest. Namba *et al.* [51] described the symptoms encountered in 47 parathion-intoxicated patients. Approximately 40% of the patients had muscle fasciculations and 20% had cramps. When compiling data on symptoms encountered in 105 patients intoxicated with OPs in Rhodesia (now Zimbabwe), Hayes *et al.* [22], also showed 40% to have muscle fasciculations and 6% to have convulsions. By contrast, the more common symptoms, such as abnormal pupils, vomiting, sweating and salivation were encountered in 60–90% of patients, in both these investigations.

Because anticonvulsants are used as adjuncts to anticholinergics and enzyme reactivators in antiChE poisoning, only those muscle fasciculations and convulsions persisting after atropine and oxime therapy have been treated with anticonvulsants. There are no statistical data to indicate what this figure may actually be. The number of case reports indicating the use of an anticonvulsant in the treatment of antiChE poisoning is, however, low (Table 53.1). Nevertheless, in the course of intoxication before the more severe symptoms of an 'over-stimulated' brain or 'over-stimulated' muscles become visible, the intoxicated patient will normally encounter a phase of anxiety and restlessness [5,6,21,24,49,64] (Table 53.2). The report of Hayes *et al.* [22] cited earlier showed, for example, 9% of their intoxicated patients to become restless.

Effect of antiChEs on muscle and nerve cell activity

AntiChEs increase the activity of the cholinergic synapse and of the motor endplate. In

Table 53.1 Anticonvulsants used in antiChE therapy

Intoxicating agent	*Anticonvulsant*	*Primary therapy*	*Reference*
Parathion	Phenobarbitone 3 × 100 mg po (against anxiety)	Atropine	Menzel and Wessel [49]
Paraoxon	Phenytoin 200 mg (once 0–3 h; once 3–12 h)	Atropine Pralidoxime Obidoxime	Wender and Owsianowski [69]
Parathion	Diazepam 160 mg	Atropine Obidoxime	Barckow *et al.* [4]
Demeton-S-methyl	Diazepam 10 mg im	Atropine Pralidoxime	Vale and Scott [68]
Fenthion	Diazepam and phenytoin	Atropine Pralidoxime	Merril and Mihm [50]
Dimethoate	Diazepam, phenytoin and phenobarbitone (iv)	Atropine Pralidoxime	LeBlanc *et al.* [33]
Thionazin	Phenytoin iv (prophylactic treatment)	Atropine Obidoxime	Sepulcri *et al.* [63]
Carbofuran	Diazepam 20 mg iv, 5 mg/h iv	Atropine Pancuronium	Poirier *et al.* [57]

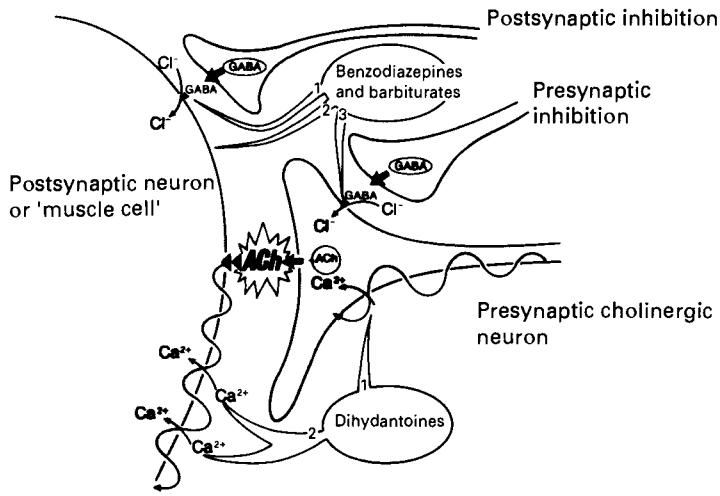

Figure 53.1 Schematic drawing of the configuration: cholinergic neuron, GABAergic neuron and muscle. The over-stimulation caused by excess ACh could be compensated for by the action of benzodiazepines and barbiturates on the presynaptic (3) and postsynaptic (1) inhibition. Benzodiazepines may also interfere more directly with the excitable membrane, probably via a Ca^{2+}-mediated mechanism (2). Dihydantoins may via 'inhibition of' voltage-gated Ca^{2+}-channels, presynaptically (1) or postsynaptically (2), compensate for the ACh induced over-stimulation

the periphery this gives raise to various symptoms among which respiratory arrest and circulatory collapse are considered the most serious. The effect of antiChEs, in low doses or early in the course of intoxication with higher doses, is probably relatively specific to the cholinergic neuron and may without obvious symptoms influence the ACh turnover and reduce the 'threshold' for excitation. Relatively soon, however, the intoxication will result in altered behaviour and symptoms such as tension, anxiety and restlessness will be encountered [5,6,20,24,49,64]. With increasing degrees of intoxication, the cholinergic neuron will start spontaneous repetitive firing, which further develops into generalized seizures that can be observed by EEG. At this stage of intoxication involuntary muscle activity (fasciculations or convulsions) can also often be observed [19]. If the antiChE-induced overactivity of the CNS is allowed to proceed for some time, it has been shown to cause pathological changes in the morphology of the brain [32,34,45]. A development parallel to that in the nervous system may be outlined at the level of the motor endplate. Mild intoxication will affect ACh turnover and the excitability of the muscle. The developing intoxication will give rise to spontaneous activity. More importantly it may, however, also activate the neuronal output to the muscle. The combined effect of a reduced threshold for the muscle and an increased activity of the motor nerve will result in visible muscular activity. Of particular importance may be spasms of the respiratory muscle and of the glottis [19]. In addition, a sustained antiChE-induced activity

Table 53.2 AntiChE intoxication

Mechanism (in order of increasing severity)	*Symptoms*	
	CNS	*Muscle*
AChE inhibition ↓ AChE buildup ↓ Over-activity of the cholinergic neuron ↓	Abnormal EEG	
Over-activity of other neurons ↓	Abnormal EEG Anxiety Restlessness	Muscle twitches Muscle fasciculations
Over-activity of major brain areas	Abnormal EEG Unconsciousness	Convulsions

of the muscle has been shown to cause pathological changes, both in the heart (for review *see* McLeod [44]) and skeletal muscle (for review *see* Hudson *et al.* [25]). The different symptoms and their probable mechanism are summarized in Table 53.2.

Brief survey of anticonvulsants

Severe antiChE intoxication may give a clinical picture comparable to status epilepticus and it has accordingly been treated with anticonvulsants such as benzodiazepines, barbiturates and/or hydantoins. Attempts in the literature to explain the mechanism of action of anticonvulsants often pertain to the facilitation of the inhibitory GABAergic nervous system. The effect of anticonvulsants may, however, be mediated through several mechanisms. Facilitation of the GABAergic nervous system and blockade of Ca^{2+} channels will briefly be discussed later as likely mechanisms of action for these anticonvulsants (Figure 53.1).

Benzodiazepines

The benzodiazepines potentiate the action of the inhibitory transmitter γ-aminobutyric acid (GABA) at its receptor [13]. Accordingly, benzodiazepines will potentiate the endogenous control of the nervous systems against over-excitation. The benzodiazepine diazepam has been shown to reduce the excitability of the neuron [55]. The benzodiazepines, however, can also inhibit voltage-dependent Ca^{2+} channels [59]. In the cholinergic nervous system diazepam probably decreases the synaptic release of ACh [66]. This causes a dose-related increase in ACh and a decrease in the Ch concentration [65,66] and accordingly a decrease in the ACh turnover [39]. Obviously the benzodiazepines may serve as reducer of the overall activity of the cholinergic neuron. Using local cerebral glucose utilization as an index of neuronal activity in general, Ableitner *et al.* [1] showed that diazepam significantly reduced this parameter in some brain areas. Benzodiazepines are not widely used for long-term management of epilepsy because tolerance develops to their anticonvulsant efficacy (for review *see* Haigh and Feely [21]). Diazepam is, however, the preferred drug against status epilepticus and is thus recommended against similar conditions in antiChE intoxication. Furthermore, the antiChE-induced convulsions could be expected to have a relatively limited duration (see paragraph on clinical therapy). Serum concentrations of at least 0.5 μg/ml are recommended in the therapy of status epilepticus [7], which may be achieved by a slow iv injection of 10 mg of diazepam. Clonazepam [10] and midazolam [15] have been suggested as alternatives to diazepam. Midazolam has the advantage of rapid and efficient uptake after im administration [48].

Hydantoins

Phenytoin (diphenylhydantoin) is believed to exert its action on the neuronal control of ion fluxes. There is evidence to indicate that this action is to reduce the activity of the voltage-dependent Ca^{2+} channel [18,43,59]. In view of the apparent similarity between hydantoins and calcium-channel antagonists, the ability of the latter to reduce ACh- release from the nerve terminal becomes an interesting observation [53] (Figure 53.1). The effect of the hydantoins on the voltage-dependent Ca^{2+} channels may explain why phenytoin, in contrast to the benzodiazepines and the barbiturates, does not elevate the threshold for seizure. It does, however, restore abnormally increased excitability towards normal [58]. Accordingly, phenytoin does not depress respiration or the degree of alertness as much as the benzodiazepines and the barbiturates [11]. Phenytoin may, however, have effects on ventricular automaticity and should thus be given with great caution to patients with sinus bradycardia, sinuatrial block, serious A-V block or Adams-Stokes syndrome [11]. Phenytoin is recommended for the long-term control of convulsive activity.

Phenobarbitone

Phenobarbitone and other barbiturates with anticonvulsant activity are believed to exert their effects via the GABA-operated chloride channel [3,54]. Like the benzodiazepines they increase the threshold for excitation via a potentiation of the inhibitory activity of GABA and will therefore, also, have the same

Table 53.3 Anticonvulsants used in antiChE research

Anticonvulsants	*Intoxicating agent*	*Prophylactic or therapeutic*	*Adjunct to*	*Observed parameter*[a]	*Species*	*Reference*
Benzodiazepines						
Diazepam	Soman	T	—	EEG (+)	Monkey	Lipp [35]
	DFP	P/T	—	EEG (+)	Rabbit	Rump *et al.* [60]
	Soman	P/T	—	EEG (+)	Monkey	Lipp [36]
	Soman	T	—	Muscle activity (+)	Rat	Johnson and Lowndes [26]
	Soman	P	Atropine	Survival (+)	Rabbit	Johnson and Lowndes [26]
	DFP	T	Atropine, obidoxime	Survival (+)	Rat	Rump and Grudzinska [61]
	DFP	T	—	Muscle activity (+)	Rat	Rump and Grudzinska [61]
	Soman	T	Atropine	Heart and respiratory activity (+)	Rabbits	Johnson and Wilcox [27]
	DFP	P	Atropine, obidoxime	Survival	Mouse	Kleinrok and Jagiello-Wojtowicz [29]
	Soman	P	Pyridostigmine	Survival (+)	Mouse	Heimbürger and Heilbronn [23]
	Tabun		Atropine			
	VX		Obidoxime			
	Soman	P	Pyridostigmine, atropine, obidoxime	Survival (+)	Guinea pig	Heimbürger and Heilbronn [23]
	Soman	P	—	Convulsions (+) Gaba metabolism (+)	Rat	Lundy *et al.* [41]
	Soman	P	—	cGMP levels (+)	Rat	Lundy and Magor [40]
	DFP	T	Atropine, obidoxime	Survival (+)	Rat	Grudzinska *et al.* [20]
	Soman	P	Atropine, TMB-4, HI-6, BDB-27	Survival time (+)	Rat	Bosković [8]
	Physostigmine	P	—	Survival (+) Pupil diameter (+)	Rat	Niemegeers *et al.* [52]
	Soman	P	—	Brain lesions (+)	Rat	Martin *et al.* [46]
	Paraoxon	P	Atropine	Survival (+)	Rat	Krutak-Krol and Domino [31]
	Soman	P	—	Local glucose (+)	Rat	Pazdernik *et al.* [56]
	Physostigmine	P	Atropine	'Physical output' (+)	Rat	Matthews *et al.* [47]
Clonazepam	Soman	P	—	Seizures and convulsions (+)	Monkey	Lipp [37]
	Soman	T	Pyridostigmine	EEG (+)	Monkey	Lipp and Dola [38]
			Atropine	ECG (+)		
			HS-6	Respiratory activity Blood pressure (+)		
	Soman	P	—	Convulsions, cGMP (+)	Rat	Lundy and Shaw [42]
Midazolam	Paraoxon	P	Atropine	Survival (+)	Rat	Krutak-Krol and Domino [31]
Barbiturates						
Phenobarbitone	Paraoxon	P	—	GABA levels (+)	Rat	Kar and Martin [28]
	DFP	P	Atropine, obidoxime	Survival	Mouse	Kleinrok and Jagiello-Wojtowicz [29]
	Physostigmine	P	—	Survival (+) Pupil diameter	Rat	Niemegeers *et al.* [52]
	DFP	T	Atropine	Survival (+) Survival time (+)	Mouse	Klemm [30]
	TOCP	T	—	EEG (+)	Chicken	Ershova and Kokshareva [17]
	DFP	P	Atropine, obidoxime	Survival	Rat	Grudzinska *et al.* [20]

Table 53.3 (continued)

Anticonvulsants	*Intoxicating agent*	*Prophylactic or therapeutic*	*Adjunct to*	*Observed parameter*[a]	*Species*	*Reference*
Pentobarbitone	DFP	P	Atropine, 2-PAM	Survival	Mouse	Dretchen *et al.* [14]
	Physostigmine	P	—	Survival (+) pupil diameter	Rat	Niemegeers *et al.* [52]
Dihydantoines						
Phenytoin	DFP	P	Atropine, obidoxime	Survival	Mouse	Kleinrok and Jagiello-Wojtowicz [29]
	DFP	P	Atropine, obidoxime	Survival	Rat	Grudzinska *et al.* [20]
	DFP	P	Atropine, 2-PAM	Survival (+)	Mouse	Dretchen *et al.* [14]
Valproic acid	Soman	P	Atropine, TMB-4, HI-6, BDB-27	Survival time (+)	Rat	Bosković [8]
Local anaesthetics	DFP	T	Atropine	Convulsions (+/–)	Rat	Rump & Kaliszan [62]
'Na-channel blockers'	DFP	T	Atropine	Survival (+) Survival time (+)	Mouse	Klemm [30]
Ca-channel blockers	DFP	P	Atropine, 2-PAM	Survival (+)	Mouse	Dretchen *et al.* [14]

[a] (+) Denotes a positive effect of the anticonvulsant on the parameter was observed, i.e. normalization of EEG, increased survival, increased survival time, etc.

effect as the benzodiazepines on respiration and alertness. Phenobarbitone is recommended for the long-term control of convulsions in patients where phenytoin is ineffective or where phenytoin is not used because of its effects on cardiac function [11].

Anticonvulsants used in the experimental therapy of antiChE poisoning

Although anticonvulsants alone provide little protection against the lethal effects of OP intoxication, it has repeatedly been shown that different anticonvulsants are beneficial as adjuncts to the atropine/oxime treatment, both in suppressing or retarding the onset of symptoms and in protecting against lethal effects (Table 53.3).

Benzodiazepines

Diazepam was first shown to suppress the seizure and convulsive activity induced by the antiChE soman in the rhesus monkey by Lipp [35]. He also showed the combination of atropine and diazepam to be more effective for control of convulsions and seizures than atropine alone. A year later Lipp [37] and Rump *et al.* [60] showed diazepam to be effective against seizures in monkeys and rabbits induced by soman and DFP, including when diazepam was used as a pretreatment. This observation was extended by Lipp [37] to include another benzodiazepine, clonazepam, in this case given 2 h before soman. Clonazepam in higher doses (0.15 mg/kg) may have potentiated the respiratory depression induced by soman.

In an attempt to understand the mechanism whereby diazepam was active Johnson *et al.* [26,27] showed diazepam to counteract the over-activity normally associated with skeletal and heart muscle following soman intoxication. It was also shown, however, that diazepam enhances the respiratory depression produced by soman in the pentobarbitone-anaesthetized rabbit. Recently more attention has been given to the protection afforded by diazepam against OP-induced effects on the CNS. Accordingly, Martin *et al.* [46] showed that prophylactic treatment with diazepam prevented or

retarded the development of central neuropathy in soman intoxicated rats. Furthermore, Pazdernik *et al.* [56] showed that diazepam normalized local cerebral glucose utilization in the soman intoxicated rat. Diazepam, together with atropine, has also been shown to restore the performance and the thermoregulatory decrements that are induced by the CB physostigmine in the rat [47].

The main emphasis in experimental work has so far been on diazepam. Additionally, however, more recently developed drugs such as the water-soluble benzodiazepine, midazolam, have been studied [31]. Both diazepam and midazolam protected against paraoxon-induced lethal effects when given together with atropine. Midazolam was more potent than diazepam, which may be explained by its better absorption after im injection [48].

Hydantoins

Wills [70] suggested that phenytoin was of little value in treating symptoms of intoxication with OPs. Grudzinska *et al.* [20] confirmed that phenytoin added no protection to an atropine/obidoxime treatment of DFP intoxication. Niemegeers *et al.* [52] showed phenytoin to be ineffective in the symptomatic treatment of physostigmine poisoning.

Despite these negative results, compounds thought to interact with the ion channels of the neuron such as local anaesthetics and Na^+-channel and Ca^{2+}-channel antagonists have been successfully tried as anticonvulsants in antiChE intoxication. Accordingly, Rump and Kaliszan [62] showed that lidocaine and prilocaine could temporarily abolish the convulsive activity induced by DFP. Klemm [30] reported that drugs like ketamine, phenobarbitone, lignocaine, morphine, prednisolone and lithium, which were claimed to have the common ability to block Na^+ channels, protected against DFP-induced lethal effects in mice. Also, some protective effects were reported when a number of Ca^{2+} channels blocking agents with anticonvulsive effects were tried for their effects against DFP-induced death [14].

Barbiturates

Barbiturates have also been used with some success to block the convulsions and epileptiform activity of antiChEs [19]. Barbiturates most certainly exert a protective effect via a normalization of antiChE-induced central overactivity [17]. There is, however, an additional component of the protective effect of the barbiturates, which may be their effect on hepatic metabolism [2,9,12].

Anticonvulsants, diazepam in particular, may accordingly, in experimental studies, decrease the acute toxicity of antiChEs. This may be achieved by suppression of convulsions, i.e. by suppression of neuronal and muscular activity. The effect on neuronal activity will also suppress or retard the onset of behavioural signs and symptoms which are difficult to observe in the animal model.

Anticonvulsants used in the clinical therapy of antiChE poisoning

In Table 53.1 some case reports, involving the use of an anticonvulsant in antiChE intoxication are tabulated. The reports are relatively few, which may mean that relatively few patients intoxicated with OPs have convulsions following atropine/oxime treatment. The anticonvulsants used are phenytoin, phenobarbitone and diazepam (Table 53.1). With one exception [49] the anticonvulsants were used as a complement to the atropine/oxime therapy, i.e. to control those muscle symptoms that still remain after treatment with atropine and oxime. The anticonvulsants may, however, play a more important role in the therapy of intoxications with CBs than with OPs because the use of oximes is not recommended in CB intoxication.

Although benzodiazepines, unlike other anticonvulsants, are effective against all seizure types, they are, except for their preferential use in status epilepticus, still considered as only a second line or adjunct therapy [21]. Nevertheless Vale and Scott [68], who state that antiChE-induced convulsions are reduced by large doses of atropine, suggest that if convulsions continue to interfere with respiration, 5–10 mg diazepam should be administered iv. In view of the limited usefulness of benzodiazepines the clinician may use alternatives such as the hydantoins or the barbiturates (Table 53.1). Whether they represent an improvement is open to question: the major

argument against the use of benzodiazepines as anticonvulsants is the tendency for patients to develop tolerance [21]. The period of treatment with anticonvulsants could, however, be expected to be relatively short, 1–6 days in most cases. Sepulcri *et al.* [63], who used phenytoin as prophylactic treatment for 35 days, are the only workers to report on the use of anticonvulsants in antiChE intoxication for an extended period of time. Such extended usage is rarely necessary, which means that the development of tolerance to the anticonvulsant effect of benzodiazepines normally may be ignored, and that benzodiazepines, particularly diazepam may thus be advocated as the first line choice against antiChE-induced convulsions.

In some cases intoxication with antiChE gives rise to residual or slowly disappearing mental and motor disturbances. Although, the motor disturbances have essentially been attributed to the neurotoxic esterases, a more direct effect of the antiChE on the neuron or on the muscle itself cannot be ruled out [16]. Unfortunately, it is not possible from the existing case reports to arrive at any conclusion concerning the benefit of using anticonvulsants in this respect, i.e. if there are 'unwanted' neuronal and muscle activities not manifesting themselves as seizures or convulsions, but still harmful, that may be suppressed by an anticonvulsant. Phrased differently: is there a case where long-lasting motor or mental disturbances in patients may have been prevented by a more frequent use of an anticonvulsant? Recent advances in experimental toxicology suggests that the prophylactic or therapeutic use of diazepam may, indeed, reduce some such pathological changes [46,47,56].

Intoxicated patients on medication normally have one or several recurrent cholinergic crises [33,49,50]. These crises are described as following the pattern outlined in Table 53.2 More severe symptoms such as fasciculations and convulsions are announced or preceded by increased anxiety or an increased restlessness [5, 6, 24, 49, 65]. It is suggested that such symptoms should be used as indications for the use of an anticonvulsant and that such prophylactic treatment of the cholinergic crisis would cause less damage to the brain and the muscles.

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Calcium channel blocking agents in the management of acute anticholinesterase poisoning

Kenneth L. Dretchen, Theresa R. Henderson and Arthur Raines

Introduction

ChE inhibitors are used in medicine, as agricultural and household insecticides, and represent an inexpensive 'low tech' means of waging war on military and civilian populations. The need for antidotes to antiChEs is obvious, as accidental or military exposure may prove fatal. Death results from respiratory failure from a combination of central and peripheral actions.

The development of antidotes for CB or OP poisoning has moved slowly. The last significant advance was about 30 years ago with the demonstration that 2-PAM effectively reduced mortality produced by OPs [58]. The mechanism involves dephosphorylation of the inactivated phosphorylated enzyme. Although effective, the drug does not readily penetrate the blood-brain barrier because of its quarternary ammonium structure so CNS toxicity is not ameliorated.

Before the development of 2-PAM, the only effective intervention was large doses of atropine. Overdose with atropine has not been a major concern. However, nicotinic blockade has not proven a feasible intervention in overdose with antiChEs, as antinicotinic agents produce NM paralysis and or ganglionic blockade. Even optimal doses of atropine combined with 2-PAM provide limited effectiveness, e.g. only a 45% increase in the LD_{50} of DFP occurs in the mouse [9]. Other species, such as the guinea pig (see later), are more extensively protected by 2-PAM and atropine. Therefore, the need for pharmacological interventions with novel mechanisms of action is great.

Protection of animals against poisoning by OP antiChEs

The mechanisms for neural activation by ChE inhibitors appears to involve at least two separable actions. (1) The alteration of nerve terminal structures so that they fire repetitively in response to a single activation [40] thus leading to enhanced ACh release and consequent post synaptic hyperactivation. (2) An effect to inhibit ChE with resultant accumulation of ACh at cholinergic neuroeffector junctions. Clearly, these two effects act synergistically, as prejunctional neurotransmitter is spared its usual rapid destruction. Thus it appears that an intervention directed at either site would prove useful in reducing the cholinergic hyperactivity observed after exposure to antiChEs.

The capacity of phenytoin and Ca^{2+} channel antagonists to block repetitive discharges and fasciculations originating in nerve terminals [12,36,37] has led to evaluation of these compounds as protective agents. Several drugs with known Ca^{2+} channel blocking properties were examined on DFP-produced lethal effects in the presence and absence of optimal doses of atropine and 2-PAM. In a recent report [9] an attempt was made to protect mice against lethal doses of DFP using pretreatment with several Ca^{2+} channel antagonists alone and in combination with atropine (1 mg/kg) and 2-PAM (10 mg/kg). The protective effects of maximally effective doses of the Ca^{2+} antagonists studied appear in Figure 54.1. Verapamil (3.0 mg/kg), nifedipine (0.1 mg/kg), nitrendipine (0.1 mg/kg), nimodipine (1.0 mg/kg) and

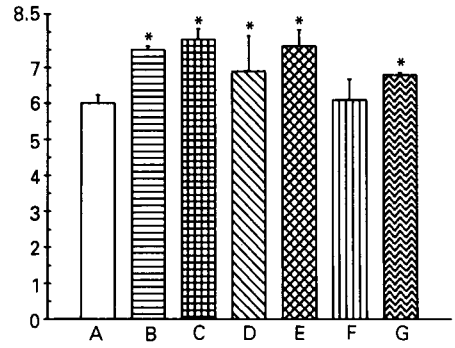

Figure 54.1 Protective effects of various Ca^{2+} channel blocking agents against DFP toxicity in the mouse. A, control. The Ca^{2+} channel blockers: B verapamil (3.0 mg/kg); C nifedipine (0.1 mg/kg); D nitrendipine (0.1 mg/kg); E nimodipine (1 mg/kg); F diltiazem (10 mg/kg) and G phenytoin (15 mg/kg) were administered ip 30 min before exposure to several doses of DFP and the LD_{50} of the OP was determined. Mean (s.e.). * Significantly greater than the LD_{50} of DFP alone; $P<0.05$

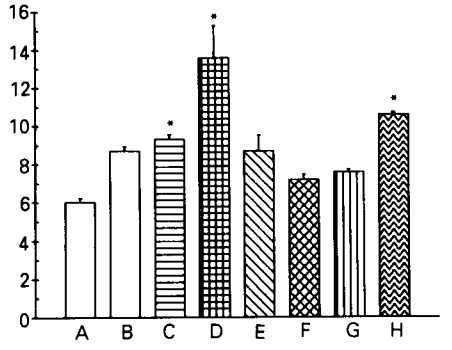

Figure 54.2 Protective effects of various Ca^{2+} channel blocking agents given in combination with atropine (1 mg/kg) and PAM (10 mg/kg) against DFP toxicity in the mouse. A, controls; B atropine and 2-PAM. The Ca^{2+} channel blockers: C verapamil (2.5 mg/kg); D nifedipine (0.1 mg/kg); E nitrendipine (0.1 mg/kg); F nimodipine (2.5 mg/kg); G diltiazem (2 mg/kg) and H phenytoin (25 mg/kg) were administered ip 30 min before exposure to several doses of DFP and the LD_{50} of the OP determined. Mean (s.e.). * significantly greater protection than atropine and 2-PAM alone; $P<0.05$

phenytoin (15 mg/kg), an anticonvulsant which also blocks Ca^{2+} movements [47,52], all provided significant protection against the lethal effects of DFP. Diltiazem failed to protect in doses of 2–10 mg/kg. The protection afforded by the effective Ca^{2+} channel antagonists was comparable to the optimal protection produced by atropine (LD_{50} raised from 6.0±0.23 to 7.3±0.09 mg/kg). Interestingly, 2-PAM was more effective than atropine (LD_{50} increased to 8.1±0.24 mg/kg) and the combination of atropine and 2-PAM was more effective than either agent alone (LD_{50} elevated to 8.7±0.25 mg/kg DFP).

The addition of atropine and 2-PAM to an effective dose of a Ca^{2+} channel blocking agent produced a still higher level of protection (Figure 54.2). Of the agents studied, the most effective drug combinations were either phenytoin or nifedipine combined with atropine sulphate and 2-PAM. The effectiveness of these agents cannot be attributed to an anticonvulsant effect of these drugs, as carbamazepine, phenobarbitone and diphenylbarbiturate have no protective effect by themselves and do not increase the protective effects of atropine and 2-PAM.

Similarly, Karlsson and Sellstom [24], studying soman toxicity in C57 mice, reported that nimodipine was effective when used in a pretreatment regimen in enhancing the protective effects of pyridostigmine and atropine. A novel investigational calcium blocker, 1-methyl-4-(1-napthylvinyl) piperdine hydrochloride (B120) [20], also enhances the protective actions of atropine and 2-PAM against OP antiChEs. In mice, the LD_{50} of sarin was observed to be 164 μg/kg. This value was increased to 201 μg/kg by a combination of atropine sulphate 16 mg/kg and 2-PAM, 25 mg/kg. The addition of B-120, 35 mg/kg, further increased the LD_{50} dose of sarin to 285 μg/kg [17]. In guinea pigs we observed an LD_{50} of soman of 27 μg/kg. Pretreatment with pyridostigmine (130 μg/kg), atropine (16 mg/kg) and 2-PAM (25 mg/kg) raised the LD_{50} to 206 μg/kg soman. The addition of B120, 35 mg/kg to the above pretreatment regimen further increased the soman LD_{50} to 854 μg/kg. On the other hand, Milovanović *et al.* [30] did not observe protection when they added nimodipine to a standard protection treatment consisting of HI-6, atropine and diazepam against soman in mice. It is to be emphasized that these workers used a treatment intervention rather than a pretreatment-prevention experimental design. Thus it appears, that pretreatment with several different agents with the capacity to block Ca^{2+} movements through excitable membranes exert protective actions against OP poisoning in mice and guinea pigs.

Actions of antiChEs and Ca^{2+} channel blockers on motor nerve endings

It has been known since the studies of Masland and Wigton [29] and Feng and Li [16] that neostigmine and physostigmine modify motor nerve terminals so they respond to a single stimulus with a high frequency repetitive burst. This event can be monitored as antidromically conducted action potentials recorded from decentralized ventral rootlets; these presynaptically generated bursts correlate with fasciculations. Thus, in the absence of treatment with these agents, the application of a supramaximal shock to a peripheral motor nerve leads to the appearance (after an appropriate latency) of an antidromically conducted single spike from motor fibres. After treatment by drugs capable of modifying nerve terminals so as to produce these stimulus bound repetitive potentials (SBR), the application of a single supramaximal shock leads to the arrival in ventral root filaments of stimulus-evoked response followed by a variable number of additional action potentials originating in the nerve endings and signaling the production of SBR. The production of SBR by edrophonium is shown in Figure 54.3.

The orthodromic transmission of the SBR leads to augmented postjunctional responses (Figure 54.3) each of which is a brief tetanic contraction (i.e. driven by a train of neurally produced stimuli) rather than simple twitches (driven by a single prejunctional action potential). Clearly, this augmentation of response cannot be produced by recruitment of motor units in supramaximally stimulated preparations, and are fully explained by the conversion of a simple twitch to a tetanic response. SBR produced by physostigmine, neostigmine, edrophonium and other agents account (at least in part) for the reversal of NM blockade by curare and similar agents.

Interestingly, the modification of nerve terminals so as to produce SBR is a property of diverse drug classes, including antiChEs [42, 51], tetraethylammonium ion (TEA) [40], hydroxyanilinium ions [40,41,43], penicillin [35] and agents capable of increasing intracellular cAMP [12]. The production of SBR does not appear to be linked to ChE inhibition [40]; TEA lacks activity on ChE [25,26]. Theophylline, DBcAMP, NaF, PGE1, agents which raise intracellular cAMP do not inhibit ChE [8]; to our knowledge penicillin has not been reported to inhibit ChE. Furthermore, junctional ACh accumulation acting on ACh receptors does not appear to play a role in SBR production, as ACh fails to reproduce SBR both qualitatively and quantitatively [10,38,39].

Moreover, it is possible to simulate SBR by using a high frequency conditioning stimulation of the motor nerves of slow muscles.

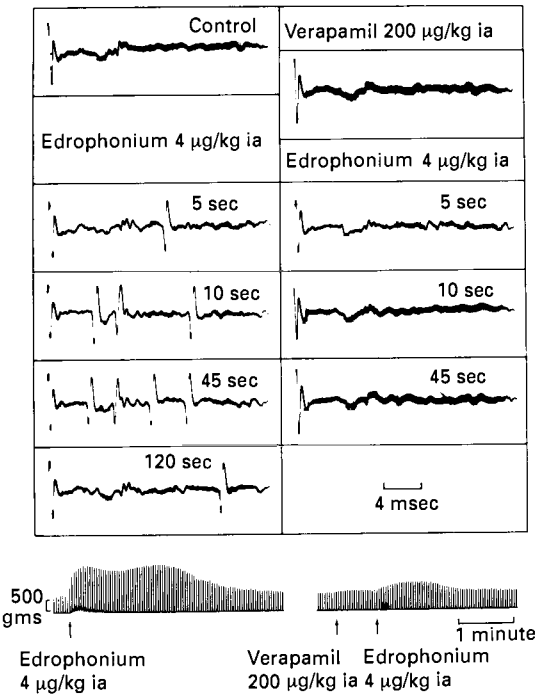

Figure 54.3 Top: effect of verapamil (200 μg/kg intra-arterially) on repetitive activity in cat soleus motor axons produced by the administration of edrophonium. In the left column, the first trace shows the response to a single stimulus applied to the sciatic nerve before edrophonium, 4 μg/kg intra-arterially; subsequent traces show the responses to similar stimuli at the noted time intervals after edrophonium. The right column shows the responses to edrophonium in the same fibre after the administration of verapamil. Bottom: effect of verapamil on the potentiation of the force of contraction produced by edrophonium. The record on the left represents the response to edrophonium before the administration of verapamil; the right is the response after the administration of the Ca^{2+} channel blocker. The baseline of both records reveals fasciculations which can be observed immediately after edrophonium. Note that verapamil depresses SBR, its expression in the form of muscle facilitation as well as fasciculations

That is, subsequent to such stimulation, in the post-tetanic period, a single stimulus applied to the motor nerves produces repetitive bursts of action potentials [50] indistinguishable from SBR which appear in Figure 54.3. The post-tetanic repetitive activity in the nerve (PTR) is likewise transmitted to the muscle producing an augmented post-tetanic response.

The mechanisms whereby a single impulse is converted into a high frequency repetitive train of action potentials appears to involve an intraneuronal potential difference in which the nerve terminals experience action potentials of longer duration than the contiguous axon [51]. Thus, at a time when the axon has recovered from a wave of excitation, the nerve terminal is still partially depolarized and this cathodal focus creates a flow of current leading to depolarization somewhere in the area of the last node of Ranvier. Depolarization in the nodal area creates additional action potentials which invade both axonal and terminal structures and lead to anti- and orthodromically conducted discharges.

Hubbard and Schmidt [21] showed that rat motor nerve terminals experience prolonged negative after-potentials in the presence of neostigmine. Werner [56,57] has proposed that a prolonged cathodal focus in feline nerve terminal structures occurring after physostigmine serves as the sink for a current source leading to SBR. The ionic species carrying this current appears to be Ca^{2+}, as SBR can be evoked in the presence of tetrodotoxin but is blocked by Ca^{2+} channel blockers. Thus, we have previously reported [46] that verapamil and methoxy-verapamil (D-600) block SBR produced by a variety of agents that increase intracellular cAMP. Similarly, verapamil blocks SBR produced by edrophonium [10]. This is shown in Figure 54.3.

An additional, but related phenomenon to SBR is the production of fasciculations; in the presence of neostigmine, *asynchronous* presynaptic repetitive discharges are responsible for the fasciculations observed after treatment with this agent [48,49]. These events are blocked by the Ca^{2+} channel blockers verapamil, nitrendipine and nifedipine [36]. The efficacy of verapamil in suppressing fasciculations in the rat is apparent from the records presented in Figure 54.4.

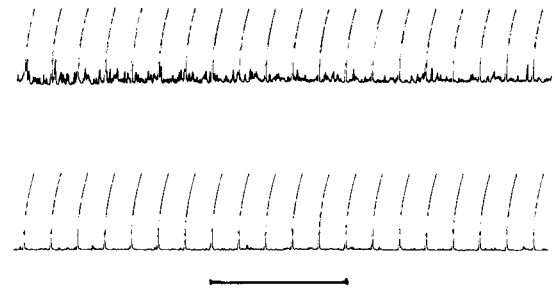

Figure 54.4 Effect of verapamil on muscle fasciculations produced by neostigmine. The upper polygraph record shows the response of the rat gastrocnemius-soleus muscle to supramaximal indirect stimulation, 2 min after the administration of neostigmine 20 μg/kg, iv. Note the fasciculations which appear in the baseline. The lower record was obtained after treatment with verapamil, 8 mg/kg, iv, followed by neostigmine as above. Note the marked reduction of fasciculations. This rat was pretreated with atropine sulphate 1.0 mg/kg, iv

Phenytoin, an anticonvulsant agent which likewise interferes with Ca^{2+} movements through excitable membranes [19,32,52,59,60] also has actions on the motor nerve endings similar to Ca^{2+} channel blockers. For example, phenytoin blocks SBR produced by agents which increase intracellular cAMP [12]. Similarly, the drug blocks fasciculations produced by suxamethonium [18]. Phenytoin blocks fasciculations produced by neostigmine [36]. Phenytoin and the Ca^{2+} blockers likewise block PTR and the resultant twitch augmentation (post-tetanic potentiation) [12,37].

Thus, the antiChE and other agents can alter nerve terminals so as to augment neurotransmitter release. This action appears to be mediated by a presynaptically generated flow of Ca^{2+} ions and is prevented by pretreatment with phenytoin or more conventional Ca^{2+} channel blocking agents. Finally, this action of the antiChEs is not a manifestation of ChE inhibition, nor does it result from interaction with cholinergic receptors.

Effect of Ca^{2+} channel blockers on NM transmission

Ca^{2+} is essential for neurotransmitter release and several workers have examined the influences of Ca^{2+} entry blocking drugs on neurosecretion of ACh at the NM junction.

The first experimental approaches examined the influence of Ca^{2+} channel blockers on muscle twitch response to indirect stimulation. Sato and Ono [45] reported that diltiazem, nifedipine, and verapamil produce an increase rather than the expected decrease in muscle response by *in situ* intra-arterial injection into the blood supply to the canine anterior tibial muscle. Because the augmentation of response was greater than that to direct muscle stimulation, they concluded that these Ca^{2+} channel blockers actually exerted a facilitatory action on NM transmission. A similar facilitatory effect by verapamil (2-10μM) was observed by Asai *et al.* [3] in the isolated mouse phrenic nerve-diaphragm preparation. Kraynack *et al.* [27,28], using concentrations of verapamil 1000-fold greater than Asai *et al.*, reported that verapamil produced a dose related depression in twitch amplitude in the isolated frog sciatic nerve/sartorius muscle preparation.

Other workers have observed an augmentation of twitch strength elicited by either nerve or muscle stimulation, suggesting that the effect is being exerted on the muscle directly. For example, Chang *et al.* [7] demonstrated that verapamil, diltiazem and nifedipine produced augmentation in the isolated phrenic nerve-diaphragm preparation, whether the contractions were elicited directly (in the presence of curare) or indirectly. A similar augmentation was observed with either direct (curarized) or indirect stimulation of the cat triceps surae in the presence of nitrendipine [11]. Finally, Skirboll *et al.* [46] showed that verapamil and D-600 augmented twitch strength of both directly and indirectly stimulated feline soleus or gastrocnemius muscles; furthermore, this augmentation was associated with a 30–40% prolongation of the muscle action potential. Prolongation of the muscle action potential is known to be an adequate cause for muscle twitch augmentation [44].

Phenytoin, despite the fact that doses of 5–20 mg/kg iv in the cat profoundly depresses SBR and PTR, fails to diminish the response of muscle to indirect stimulation in doses as great as 80 mg/kg [37]. Phenytoin, like the other Ca^{2+} channel blockers, was observed to augment the twitch strength of indirectly or directly stimulated (curarized) muscle [37] and appears to prolong action potential duration in muscle.

Microelectrode studies have failed to demonstrate a significant prejunctional depressant effect of Ca^{2+} channel blockers on normal NM transmission. Nachshen and Blaustein [31] reported no effect of verapamil or D-600 on miniature endplate potential (MEPP) frequency in the frog sartorius muscle. Similarly, Publicover and Duncan [34] found only a slight decrease in MEPP frequency with 10 μM verapamil in the frog cutaneous pectoris muscle. However, at concentrations of ≥100 μM, there was an increase in MEPP frequency; furthermore, they concluded by monitoring EPP amplitudes that there was no effect on quantal content. Bregestovski *et al.* [5] evaluated the effects of D-600 on the frog sartorius muscle preparation; they found an increase rather than a decrease in MEPP frequency and no effect on EPP amplitude, indicating no effect on quantal content. Chang *et al.* [7], studying the isolated mouse phrenic nerve-diaphragm reported that verapamil, diltiazem and nifedipine failed to influence MEPP frequency; however, they observed that verapamil and diltiazem had a slightly greater effect in depressing EPP amplitude than MEPP amplitude, suggesting a modest inhibition of quantal content; nifedipine was without effect on these parameters. Lastly, Edeson *et al.* [15] studying the isolated frog sciatic nerve-sartorius muscle preparation monitored miniature endplate currents (MEPcs) and EPcs and concluded that verapamil had no effect on spontaneous transmitter release.

Data on pharmacologically relevant drug concentrations on transmitter dynamics are lacking. Yaari *et al.* [59,60] and Pincus *et al.* [33] reported that phenytoin (100 μM) reduces EPP amplitude in the frog isolated sartorius nerve-muscle preparation. They reported a reduction of presynaptic evoked transmitter release and an increase in spontaneous MEPP frequency. These data are difficult to evaluate as they are at least tenfold above therapeutic concentrations in mammals. Thus the Ca^{2+} channel blockers appear to have little, if any, significant prejunctional actions in the course of normal NM release.

Therefore the above results demonstrating little or no prejunctional effects of Ca^{2+} channel blockers under conditions of normal indirect stimulation, and the profound effects of these drugs under conditions of neostigmine

exposure (and other facilitatory drugs or conditions) indicate that under the latter circumstances, a critical Ca^{2+} current susceptible to the dihydropyridines, verapamil and phenytoin is activated. This current appears to be the immediate cause of prejunctional repetitive neural discharges responsible for fasciculations and facilitatory drug (or post-tetanic) effects. It is possible that cAMP plays a role in the induction or modulation of this current. This Ca^{2+} current is to be distinguished from the Ca^{2+} current associated with excitation-secretion coupling, as Ca^{2+} channel blockers and phenytoin fail to interfere with neuromuscular transmission at even the highest achievable *in vivo* concentrations.

Interaction of Ca^{2+} channel blockers with NM blocking agents

Substantial experimental evidence and clinical experience indicates that Ca^{2+} entry blockers intensify the NM blockade produced by a variety of non-depolarizing (curare-like) and depolarizing NM blockers. Studying the rat phrenic nerve-diaphragm preparation, Wali *et al.* [55] reported that verapamil potentiates the NM block produced by atracurium. Similarly, Bikhazi *et al.* [4] reported that nifedipine and verapamil significantly decreased the IC_{50} and IC_{90} (concentration to inhibit NM transmission by 50 and 90% respectively) of curare, pancuronium, vecuronium and atracurium. Ilias and Steinbereithner [22], also studying the rat phrenic nerve-diaphragm, reported that verapamil, D-600, nisoldipine and diltiazem potentiate pancuronium-induced NM blockade. In the cat, Anderson and Marshall [2] reported that verapamil, bepredil and nifedipine potentiate NM paralaysis produced by vecuronium in the anterior tibial muscle.

Similar results were reported on the interaction between verapamil and pancuronium by Carpenter and Mulroy [6]. Hartman *et al.* [18] reported that phenytoin intensifies the NM blockade produced by suxamethonium (succinylcholine) in the cat soleus NM preparation. Interestingly, the fasciculations produced by suxamethonium were blocked by phenytoin. Anderson and Marshall [2] also noted that bepredil blocked suxamethonium induced fasciculations. Using another *in vivo* preparation, Durant *et al.* [13] described a potentiation of the NM blockade produced by pancuronium and suxamethonium in the rabbit gastronemius and anterior tibial muscles. Studying the isolated frog sciatic nerve-sartorius muscle, Kraynack *et al.* [26,28] reported that verapamil potentiated pancuronium-induced NM paralysis. Using microelectrodes in the frog cutaneous pectoris muscle, Adam and Henderson [1] reported that nicardipine, bepredil and verapamil potentiated the suxamethonium induced depression of endplate currents. Similarly the potentiation of curare-like agents by verapamil has been reported in a few clinical cases [23,53,61].

It appears that the capacity of the anticurare drugs neostigimine and edrophonium to antagonize NM blockade is impaired by the presence of Ca^{2+} channel blockers. In several of the above studies [4,23,28,52]neostigmine either failed to reverse a curare-like agent or only partially exerted an effect. Alternatively, Carpenter and Mulroy [6] and Wali *et al.* [55] reported that the ability of either neostigmine or edrophonium to reverse curare is not impaired.

The previously described intensification of the effects of curare-like agents on muscle responses to nerve stimulation appears to be accounted for by actions of the Ca^{2+} blockers on postjunctional elements. Thus, nifedipine, verapamil and diltiazem depress ACh-induced muscle contractures in an *in vivo* canine anterior tibial muscle [45]. Similarly, bepredil potentiates vecuronium antagonism of ACh-induced contractions in the cat anterior tibial muscle [2]. Bregestovski *et al.* [5], using the frog sartorius muscle, described the reduction of ACh-induced endplate currents by D-600. The Ca^{2+} blocker also reduced the amplitude and decay time constant of miniature endplate currents. Wachtel [54], studying endplate currents in the mouse diaphragm muscle, described a dose-dependent reduction in average channel lifetime after treatment with either verapamil or diltiazem. These drugs also reduced the frequency of open channel spontaneous events. Lastly, Edeson *et al.* [14,15] reported that verapamil blocks nicotinic receptors in isolated frog sartorius muscles. These workers studied endplate currents and reported that the drug produces a closed channel block and shortens the open channel lifetime.

Thus, the lack of depression of nerve terminal release of ACh under conditions of isolated stimuli applied in the absence of neostigmine (or similar agents), together with the data indicating a potentiation of either stabilizing or depolarizing NM blocking agents and that demonstrating direct actions on endplate structures, indicate that the Ca^{2+} blockers exert significant postjunctional actions which are at least additive with NM blockers.

Summary

The research reviewed indicates that ChE inhibitors exert effects on nerve terminal structures which appear unrelated to enzyme inhibiting properties. These presynaptic events appear to be triggered by Ca^{2+} currents and are expressed as augmentations in transmitter release secondary to high frequency discharges. The latter can be either synchronous, and lead to an organized bombardment of postsynaptic elements producing enhanced tissue responses, or it may be asynchronous leading to fasciculations. Although at this time only NM and ganglionic tissues have been studied, it is likely that other sites (perhaps in the CNS) behave similarly. Drugs with the capacity to block critical Ca^{2+} movements appear capable of suppressing SBR, PTR and fasciculations. Furthermore, and most significantly, this action is able to antagonize some of the toxic manifestations produced by OP and CB antiChEs. The action of these Ca^{2+} channel blockers appear to be relatively selective, in that Ca^{2+} movements associated with normal neurotransmitter release do not appear to be modified significantly.

The Ca^{2+} channel blockers often intensify the NM actions of curare-like drugs. This is most likely a result of a postjunctional action of these agents to block motor endplates, resulting in an additive NM paralysis. The action of the Ca^{2+} channel blockers seems, under some circumstances, to interfere with the anticurare action of neostigmine and edrophonium. This is not surprising as the anticurare action of these latter agents appears to be the result of prejunctionally produced high frequency neural discharges and these discharges are suppressed by Ca^{2+} channel blocking agents.

The protective action of the Ca^{2+} channel blockers appears to be independent of and additive to, the protective actions of atropine and 2-PAM against poisoning by OPs and, perhaps CBs. The contribution of the Ca^{2+} channel blockers does not involve anticholinergic, anticonvulsant or enzyme reactivation actions and represents an advance in the management of poisoning by antiChEs. Thus, it appears that Ca^{2+} channel blockers offer substantial promise as adjuncts to atropine and 2-PAM in exposure to OP poisoning.

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Acetylcholinesterase sequestration of organophosphate intoxicants

Alan David Wolfe

Introduction

Specific groups of enzymes, for example, carboxylesterases (EC 3.1.1.1; CEs), occur in animal sera and sequester small quantities of many OP chemical warfare agents [4,9]. Their action suggests that the administration of large quantities of enzymes which covalently bind OPs might constitute an effective pretreatment regimen by means of which OPs could be removed from the circulation before they reach their biochemical and physiological target, AChE. The rapidity and affinity with which AChEs from higher organisms react with OPs is similar [12] and therefore this enzyme, in particular, appears uniquely suited to act as an OP scavenger [21,25]. CEs have also been proposed for use as OP scavengers [9]. In addition, specific phosphoryl phosphatases hydrolyze OPs, including DFP and soman, and have been suggested for use as OP detoxification agents [13,17].

This chapter is devoted to recent research on the use of a mammalian AChE to sequester circulating OPs. Such sequestration has been demonstrated experimentally in mice [21,24, 25], although serum BChE has been used clinically [10] to treat parathion intoxication. Thus experimental and clinical data indicate that ChE detoxification of OPs is possible and experimental data [21,24,25] show that ChE sequestration of OPs occurs and is governed strictly by the stoichiometry between the quantity of circulating AChE and the challenging OP. This stringent condition results in a surprising but logical anomaly: for any specific LD_{50}, the more toxic the OP the less AChE required for detoxification. In addition, the more toxic the OP, the more rapidly it reacts with AChE. Complete detoxification of one LD_{50} of DFP in monkeys will hypothetically require more than 20 times as much AChE as complete detoxification of one LD_{50} of soman, since the molecular ratio of their LD_{50}s is 20:1 (280 μg/kg [18] compared with 14 μg/kg [14]. In view of their bimolecular rate constants, the reaction of AChE with soman will also be more than 1000 times as rapid as its reaction with DFP (1.0×10^8 M^{-1} min^{-1} [12] compared with 7.7×10^4 M^{-1} min^{-1} [22]). Thus a potential new agent for OP detoxification is being evaluated; exogenous and endogenous AChE react equally rapidly with OPs, but sequestration of OPs by the exogenous enzyme occurs in the circulatory system and thereby protects the endogenous AChE.

Experimental results

Pharmacokinetics

The most serious impediment to the development of an agent to sequester OPs has been the inability to obtain sufficiently large quantities of AChE to test the scavenger hypothesis. This impediment was eliminated through the development of a batch procedure for the procainamide affinity gel purification of a globular AChE [6,20] from fetal bovine serum (FBS). The ability to obtain AChE in mg quantities led first to an assessment of enzyme tolerance and clearance in mice [21,25]. Figure 55.1 [21] shows the pharmacokinetics of FBS-AChE in mice after iv (tail vein) and ip administration of enzyme. Peak levels accounting for 90% of the injected AChE occur immediately after iv injection and this activity decreases in 1 h to 75% of the total enzyme injected. The iv administration of enzyme presents the

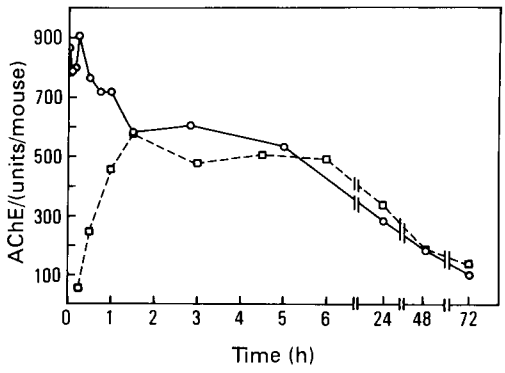

Figure 55.1 Average whole blood AChE levels following iv or ip administration of FBS-AChE. ○, mice administered 920 units/mouse by iv route (n=2). □, mice administered 760 units/mouse by ip route (n=3). Variations among individual animals administered with the same amount of AChE was <15%. Blood volume was assumed to be 7.5% of body-weight. All AChE activity measurements presented in this review utilized a variation of a colorimetric method [8], in which acetylthiocholine (ATC) is used as the substrate. Reactions were conducted at 25°C at pH 8.0 in 1 mM phosphate buffer

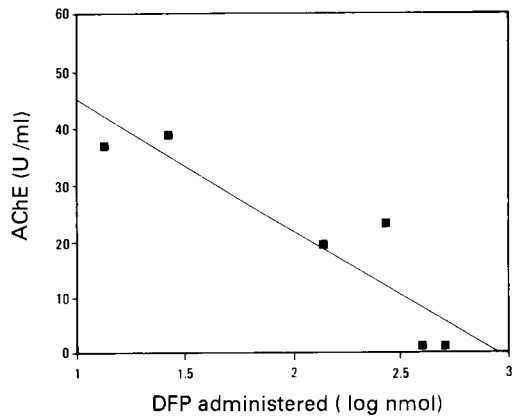

Figure 55.2 *In vivo* titration of injected FBS-AChE with graded quantities of DFP. FBS-AChE (875 units) was injected iv into mice and 1 h later DFP was injected im. Blood samples were taken 1 h later and serum analysed for ATC hydrolyzing activity. The ordinate indicates the average enzyme activity per ml of serum after challenge by DFP, plotted on the abscissa as the log of the nmol of DFP injected. The respective doses of DFP were (in nmols) 13.5, 27, 35, 270, 405 and 540. Each point represents the average derived from paired mice

opportunity for optimal ,OP sequestration. Peak levels of AChE which result from ip injection occur in 90 min, represent nearly 80% of the injected dose, and appear to be

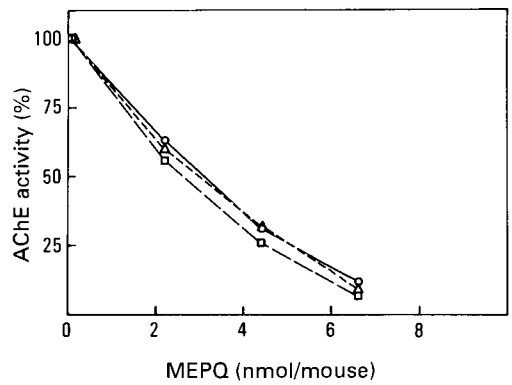

Figure 55.3 *In vivo* titration of blood AChE in mice pretreated with FBS-AChE. Consecutive OP injections were administered at intervals of 15 min, followed by assay for blood AChE residual activity. Representative results shown are for three individual mice with initial blood AChE levels of: □, 4.17 nmol; △, 4.11 nmol; ○, 4.78 nmol. Titrations were initiated 2–3 h after administration of FBS-AChE, with the time profiles of blood AChE levels shown in Figure 55.1

maintained for >6 h. Regardless of route used to administer the enzyme, a slow, identical decrease in active circulating AChE follows over 48 h. Approximately 30–40% remains in the circulation 24 h after administration. Thus both routes of administration will protect mice from appropriately scaled OP challenges 1 day after enzyme injection and this suggests an ability to protect biological systems for a longer period than that obtained by drug therapy. Mice injected with AChE by either route showed normal behaviour despite the extremely large quantities of AChE administered.

OP challenge

Fundamental to the scavenger concept is the ability of injected AChE rapidly and quantitatively to bind OPs. Figure 55.2 and 55.3 [21, 25] show titrations of injected AChE by DFP and by 7-methylethoxyphosphinyloxy-1-methylquinolinium iodide (MEPQ). Each dose of MEPQ caused a stoichiometric decrease in the quantity of circulating AChE until exogenous activity disappeared. MEPQ titrations were conducted 2–3 h after enzyme administration to obtain a relatively stable baseline. These experiments demonstrate the ability of exogenous AChE to bind OPs and suggest the

potential efficacy of enzyme pretreatment against this class of chemical warfare agents.

A direct test of the scavenger hypothesis against the nerve agent VX is shown in Table 55.1 [25]. Mice pretreated by ip administration of FBS-AChE were challenged 20 h later with graded LD_{50}s of VX. Mice were protected from challenge doses up to 3 LD_{50}s of the OP; in a second experiment, the challenge dosage was increased to obtain the new, AChE modulated LD_{50}. The experiments were consistent and showed that <1 mg of AChE, administered ip 20 h previously, could protect a mouse from approximately three LD_{50}s of VX.

Similar tests of the scavenger hypothesis were conducted with MEPQ. This OP has not been proposed for use as a chemical warfare agent, but its bimolecular rate constant with AChE (2.44×10^8 M^{-1} min^{-1} [21] and its LD_{50} in mice (30 μg/kg; [21]) indicate a toxicity equivalent to such compounds. Titration of iv administered FBS-AChE with MEPQ produced a linear relationship between the total quantity of circulating enzyme and MEPQ, while nearly identical protective ratios were produced experimentally or calculated from the quantity of enzyme administered in comparison with the MEPQ challenge dose (Table 55.2; Figure 55.4). In addition, mice pretreated with enzyme and challenged with MEPQ were treated (Table 55.3; [21]) with a reactivating oxime, TMB-4, and the quantity

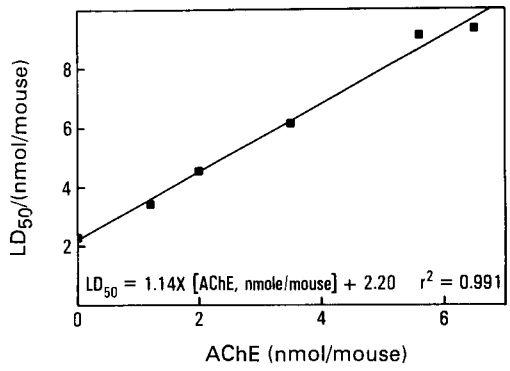

Figure 55.4 Correlation between iv LD_{50} of MEPQ and blood AChE levels expressed in nmoles/mouse. Mice were challenged with MEPQ 15 min following iv administration of FBS AChE. Each dosage group contained 12 mice. Linear regression analysis produced the depicted result. The LD_{50} of MEPQ in unprotected mice was found to be 2.27 nmoles/mouse weighing 30 g assuming a blood volume of 7.5 of the body weight

Table 55.1 Protection of mice from VX[a]

Experiment 1[b]		*Experiment 2*[b]	
Dose (LD_{50})	*Survivors* (+AChE)	*Dose* (LD_{50})	*Survivors* (+AChE)
1.0	5/5	2.12	5/5
1.5	5/5	3.00	4/5
2.0	5/5	4.24	1/5
3.0	4/5	5.99	0/5
		8.46	0/5

[a]Mice were injected with 10.89 nmols fetal bovine serum. AChE given approximately 20 h before challenge with VX
[b]No added AChE: 1 LD_{50} = 56.3 nmols/kg VX (95% confidence interval: 49.4–64.3 nmols/kg)
Added ChE: 1 LD_{50} = 201.3 nmols/kg VX (95% confidence interval: 168.8–240.1 nmols/kg)
Protective ratio: 3.6 (confidence interval: 2.9–4.5; $P<0.001$)

Table 55.2 Protective ratio conferred by AChE against 7-methylethoxyphosphinyloxy-1-methylquinolium iodide (MEPQ) poisoning[a]

AChE[b] *(units per mouse)*[g]	*LD_{50} (iv) (μg/kg)*[c]	*Protective ratio*	
		Found[d]	*Calculated*[d,e]
Unprotected mice	30 (28–33)[f]	1.0	—
474 ± 7 (1.2)	46 (38–56)	1.5	1.5
808 ± 23 (2.0)	60 (53–69)	2.0	1.9
1387 ± 19 (3.5)	80 (66–96)	2.7	2.5
2246 ± 46 (5/6)	121 (111–132)	4.0	3.5
2606 ± 48 (6.5)	124 (108–141)	4.1	3.9

[a]MEPQ was administered iv 15 min after iv AChE
[b]Average ± s.e.m. blood AChE activity before MEPQ injection; $n = 12$
The figures in parentheses are nmol/mouse (assuming 1 nmol = 400 units)
[c]95% confidence limits
[d][LD_{50}-treated mice]/[LD_{50}-untreated mice]
[e]Calculated on the assumption of 1:1 stoichiometry of MEPQ sequestering by AChE and an average LD_{50} of 2.27 nmol MEPQ per mouse
[f] MEPQ toxicity in CBDP-treated mice: LD_{50} = 9.3 (8.2–10.4) μg/kg (determined 90–120 min after iv CBDP 5 mg/kg)
[g]Values in parentheses are nmol/mouse

Table 55.3 *In vivo* reactivation of AChE by TMB_4 in mice challenged with MEPQ[a]

Mouse	*AChE activity (U/ml)*			*In vivo reactivation (U/ml) (%)*
	Initial[b]	*Before TMB_4*[c]	*After TMB_4*[d]	
1	964	90	328	57 (575)
2	881	96	296	51 (580)
3	930	78	324	59 (549)
4	980	48	235	46 (511)
5	921	114	330	70 (471)

[a]Mice were given an average iv dose of 2246 ± 46 U AChE per individual mouse. 1–5 min later an iv injection of 3.5–4.0 × LD_{50} of MEPQ was given
[b]AChE activity in U/ml of whole blood following AChE injection
[c]1.5–2 h from administration of MEPQ. TMB_4 given im at a dose of 12.5 mg/kg
[d]Assayed 90 min after the administration of TMB_4
[e]Percentage reactivation relates to maximum reactivatible AChE of each individual blood sample after 16 h of incubation at 25°C with 1 mM TMB. Figures in parentheses are maximum activity of FBS-AChE in U/ml

Table 55.4 Protection of mice from soman[a]

Group (n = 5)	*Serum AChE activity (U/ml)*	*Treatment given[b]*	*Soman dose*	*Survivors 2 h*	*72 h*
1	None	—	2 × LD_{50}	0/5[c]	—
2	None	A + P	2 × LD_{50}	3/5	0/5
3	807	AChE	2 × LD_{50}	2/5	1/5
4	763	AChE + A + P	2 × LD_{50}	4/5[d]	3/5

[a]Ten male Swiss white ICR mice were injected via the tail vein with 0.125 ml (3.3 mg; 41 nmol) of an AChE solution of approximately 26.3 mg/ml (specific activity 2800 U/mg protein) in 0.05 M phosphate buffer, pH 8.0 (groups 3 and 4), and 5 control mice were injected with 0.125 ml physiological saline (group 1). After 24 h 2 × LD_{50} of a soman solution (208 μg/kg, or approximately 5.2 μg total (31 nmol/mouse) was injected im. At 10 s after soman challenge, 5 mice which received enzyme (group 3) and an additional 5 mice (group 2) were given im a formulation containing atropine and 2-PAM at dosages of 25 mg/kg and 11.2 mg/kg respectively. Serum AChE activity was assayed before soman challenge

[b]A, atropine; P, 2-PAM

[c]Mice died within 120 s

[d]The probability is <0.05 that 4/5 mice in group 4 would survive 2 × LD_{50} of soman for 2 h in comparison with untreated mice (group 1)

Table 55.5 Protection of mice from soman by intraperitoneal pretreatment with FBS-AChE[a]

Pretreatment regimen	*Soman toxicity (LD_{50}) mg/kg (confidence limits)[b]*	*nmol/kg*
None	113.0 (94.6–135.2)	620
CBDP	9.6 (6.0–12.6)	53
CBDP + FBS-AChE	21.8 (17.9–25.0)	120

Protective ratio = 120/53 = 2.26[c]

[a]Mice were treated in groups of 5, and results analysed by means of a single tailed Student's *t* test. FBS-AChE (257 nmol/kg) was given ip 16 h before administration of cresyl benzodioxaphosphorin oxide (CBDP), and 1 h after CBDP administration the animals were challenged with im soman. Immediately before CBDP injection a blood level of 160 U/ml was found, and 140 U/ml was measured 1 h later. Mice were challenged with 6 graded soman doses appropriate to their specific pretreatment (i.e. with or without CBDP), and LD_{50} values were determined by probit analysis of mortality data after 24 h

[b]Confidence limits at $P<0.01$

[c]A second experiment, identical in design to the above, produced a protective ratio of 1.96 ($P<0.01$)

of active enzyme increased to 50–70% of the maximum reactivatible enzyme, thus showing that (1) the FBS-AChE sequestered the MEPQ, and (2) an oxime could restore the activity of the exogenous circulating AChE.

Use of VX and MEPQ to determine the potential efficacy of the sequestration process appeared encouraging, but initial experiments with the most lethal OP, soman, failed to produce substantial protection [25]. The most reasonable explanation for this appeared to be the disparity between the LD_{50}s of soman and VX in mice. Additional factors were that: (1) both VX and MEPQ are relatively hydrophilic compared with soman, and (2) of these three OPs, only soman ages. Thus the first evaluation [25] of the scavenger technique using soman gave equivocal results (Table 55.4). Large quantities of AChE, administered without supporting drugs, provided little protection against 2 LD_{50}s of soman. However, it is of interest to note that the enzyme, in conjunction with the drugs atropine and 2-PAM, were able to protect mice synergistically.

The LD_{50} of soman varies in different animals. For example, the LD_{50} of soman in rhesus monkeys is approximately 14 μg/kg [14] one-seventh the LD_{50} in mice. This disparity may arise from the presence of comparatively large quantities of CEs in the serum of mice [5]. These observations were recently confirmed by using the specific CE inhibitor, cresylbenzodioxaphosphorin oxide (CBDP; [3]), to demonstrate that in the absence of active CEs, mammals have nearly identical soman LD_{50} values [4,16].

When CBDP was used to decrease the soman LD_{50} value in mice from approximately 113 μg/kg to 9.6 μg/kg, pretreatment with AChE [24] administered ip doubled the LD_{50} of soman (Table 55.5). This increase was commensurate with the quantity of active enzyme remaining in the circulation 16 h after administration, and showed that protection of relatively long duration could be provided against a lethal dose of soman. Administration iv of a slightly increased quantity of enzyme, followed by immediate challenge with soman, resulted in AChE protecting mice as much as 6.5 LD_{50}s of this OP (Table 55.6). This experiment was similar to those with MEPQ in that a stoichiometry approximating one was obtained when consideration was given to the racemic composition of the soman and the recognition that toxicity resided in the P(–) enantiomeres [2]. Table 55.6 reveals that

Table 55.6 Soman titration of intravenously administered FBS-AChE[a]

Mouse	*AChE dose (nmol/kg)*	*Racemic soman dose* LD_{50}	*nmol/kg*	*(AChE) Soman*	*AChE[b] (nmol/kg)*	*Observation*
1	None	5.0	325	—	—	Died in 90 s
2	333	5.0	325	1.02	144	Survived
3	333	6.5	422	0.79	68	Survived
4	333	8.0	520	0.64	5	Died in 30 min

[a]CBDP was injected sc into mice. At 1 h later FBS-AChE was given iv, and 10 min after that mice were challenged with im soman. The LD_{50} of soman in the experiment was 65 nmol/kg. A second experiment yielded similar results when mice were challenged with 2 and 5 × LD_{50} of soman
[b]Residual blood levels of FBS-AChE 30 min after soman challenge

sequestration is an active process which occurs as soman enters the circulation after im administration. The quantity of enzyme remaining in the circulation 30 min after soman challenge was inversely related to the soman dose; when the circulating enzyme had virtually disappeared, the mouse died. Thus experimental data show that mice may be protected from the chemical warfare nerve agents soman and VX, and the equally toxic MEPQ.

Discussion

The present experiments have shown that AChE can be used to protect mice from multiple LD_{50}s of lethal chemical warfare nerve agents. Many advantages are intrinsic in such pretreatment. Mice protected by AChE exhibit few, if any, symptoms of OP intoxication, a result to be expected from a protective procedure which removes OP from the circulation before it reaches its physiological target. Use of AChE presents a scavenger which has an affinity spectrum identical to endogenous AChE, in contrast to the CEs, for example, which do not appear to react extensively with VX [3]. In addition, administered AChE appears to have a relatively long half-life, at least in mice, offering the hope that extended protection against OPs may be possible. Finally, enzyme protection against OPs has been achieved without administration of drugs which themselves influence the nervous system and cause performance deficits.

However, such preliminary investigations only suggest the ChE pretreatment potential. Many pharmacokinetic, pharmacodynamic, neurological and immunological variables await assessment and evaluation, both in mice and in higher mammalian species. The safety and efficacy of this pretreatment remains to be evaluated in primates, much less humans, although enzymes destined for prophylactic or therapeutic intervention may be treated with polyethylene glycol or polyvinylpyrrolidone to reduce antigenicity, or to produce an even more extended half-life [1]. In addition, many different ChEs bind OPs, and therefore await evaluation as potential scavengers, particularly BChE, because it is a normal component of human blood and has been used successfully in a case of parathion intoxication [10]. It may even be possible to protect humans against OP through stimulation of endogenous BChE synthesis. Antimuscarinic agents such as atropine, and reactivating oximes such as 2-PAM and TMB-4, have proven value, and combinations of enzyme and drug also await evaluation. However, the use of an exogenous AChE as an effective, experimental pretreatment method against OPs has been demonstrated, and these observations now await exploitation. The current progress being made toward elucidation of the structure and catalytic mechanism of the ChEs [7,11,15,19,23] offers hope that catalytically active synthetic peptides, capable of sequestering OPs, may ultimately become widely available for use as a single, highly efficient pretreatment agent capable of preventing OP intoxication without the occurrence of side-effects.

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Prophylaxis against anticholinesterase poisoning

Robert H. Inns and Timothy C. Marrs

Introduction

Certain groups within the population have the potential of being exposed to antiChEs. These include agricultural workers, employees in the chemical industry and soldiers; more prosaically gardeners and householders may be involved. Although in such groups prophylaxis may have its attractions, it implies that incidents are anticipated and morbidity among such groups is more appropriately prevented by physical protection and by minimizing the opportunity for exposure. Moreover, most OP pesticides are not refractory to therapy with oximes and can satisfactorily be treated with atropine and an oxime (*see* Ch.52). Therefore the special problems that exist for military medical services, with regard to the therapy of the nerve agent soman, do not generally apply. Although prophylaxis of OP pesticide poisoning has occasionally been advocated [39], it is the opinion of the authors that prophylaxis has no place in civilian countermeasures against OP poisoning.

Military use of prophylaxis

In countermeasures against chemical warfare agents, drug prophylaxis has a number of attractions. The main attractions are that the minimum lethal and incapacitating doses of OP nerve agents may be raised, thereby minimizing the effectiveness of any use of such agents. Furthermore, drug prophylaxis should not have the incapacitatory effect that is a consequence of full physical protection with impermeable suits and respirators. However this does not imply that prophylactic antidotes are likely to replace protective clothing and the main impetus behind prophylaxis is probably the lack of adequate therapy against soman after poisoning. This nerve agent gives rise to rapid ageing (dealkylation, *see* Ch.52) of the OP ChE complex, a phenomenon which renders oximes ineffective (Table 56.1).

Treatment of soman poisoning

In actual fact there are a number of possible approaches to the problem of soman poisoning, including both therapeutic and prophylactic approaches (Table 56.2). However to date, with the exception of prior protection of the enzyme, none has shown convincing evidence that it is likely to be successful in humans. The most promising prophylaxis for preventing formation of an aged enzyme-inhibitor complex is to protect the ChE from inhibition with a reversible ChE inhibitor such as a CB. These compounds can only be used as pretreatments: they are ineffective as therapies [6]. The other main approach to the prevention of ageing is pretreatment with oximes. The grounds for this avenue of treatment is that no oxime indisputably reactivates the aged complex. This means that success is only likely, with this group of drugs, if the oxime is present and able immediately to reactivate the enzyme before ageing has time to occur. Like oximes, antimuscarinic, antinicotinic and anticonvulsant drugs have been studied mostly as post-poisoning therapies. However, there is experimental work to suggest they could be used prophylactically. The intention would be that they should be present before poisoning, immediately to counter the build-up of ACh, rather than, more familiarly, to reverse a pre-existing intoxication. It must be emphasized

Table 56.1 Protection afforded by P2S and atropine against an oxime sensitive nerve agent, sarin, and a rapidly ageing nerve agent, soman, in guinea pigs compared with pyridostigmine pretreatment

	Protection ratio (95% confidence limits)	
	Sarin	*Soman*
Atropine	<3	1.5 (1.2–1.9)
Atropine and P2S	38 (27–52)	1.7 (1.5–1.9)
Atropine, P2S and diazepam	70 (45–109)	2.5 (1.9–3.1)
Pyridostigmine, atropine, P2S and diazepam	45 (25–81)	14 (10–20)

[a]Data from Inns and Leadbeater [26]
[b]Pyridostigmine iodide 0.32 μmol/kg im was given at 0 min and sarin or soman sc at 30 min
[c]Atropine sulphate 50 μmol/kg im at 31 min
[d]P2S 130 μmol/kg im at 31 min
[e]Diazepam 25 μmol/kg im at 31 min

Table 56.2 Possible antidotal approaches to soman poisoning

- Prevention of ageing
  - By protection of ChE
    - CBs
    - OPs
    - Choline esters
    - Local anaesthetics
  - Prior administration of oximes
  - Very rapidly-active oximes (HI-6)
- Improvements to measures against ACh accumulation
  - Anticholinergic
    - Muscarinic
    - Nicotinic
  - Anticonvulsants
  - Muscle relaxants
- Injection of ChE
- Soman antibodies

that many drugs have been studied for a role in prophylaxis in experimental animals. However, only the CBs and oximes have been studied sufficiently thoroughly to assure safety and efficacy in humans.

General problems with prophylaxis

It must be appreciated that drugs that are useful in therapy after poisoning will not necessarily be useful in prophylaxis even if efficacy can be shown in animal studies. This is because one may accept a certain degree of toxicological hazard when treating an acute poisoning, but not when giving a drug to normal men [24].

Animal studies on prophylaxis

ChE inhibitors

A number of substances that will occupy binding sites on ChEs have been studied for protection against OP poisoning. Those effective were antiChEs such as CBs [24] and certain OPs. Choline esters, such as carbachol and methacholine [6,37] and certain local anaesthetics [4] were effective. The extent of the usefulness of these compounds probably depends on the time during which they remain bound to the enzyme: if it is too short, as with the choline esters, protection against soman is not very effective.

Carbamates

Koster [31] showed that the reversible anti-ChE physostigmine (Figure 56.1) could protect ChEs against inactivation by OPs in cats, while Koelle [30] showed that the same drug or neostigmine could protect ChE against inactivation by diisopropyl phosphorofluoridate (DFP) *in vitro*. The reason for the efficacy of CBs is that they form fairly stable complexes with ChEs, which are refractory to phosphorylation or phosphonylation, but which decarbamylate spontaneously, leaving functional enzyme. The kinetic study of Green [20] concurs with this explanation.

Berry and Davies [6] pointed out that CB pretreatment should be effective against any OP ChE complex and that CBs should therefore work in soman poisoning. Using guinea pigs, they found that physostigmine, at the maximum dose that was not associated with clinical disturbance in that species, could, when administered with atropine before poisoning, raise the LD_{50} of soman by a factor of about 8. In the same species, these workers also studied the effect of different time intervals between physostigmine and the soman and found that the protective effect was only observed after prophylactic administration of the CB. Gordon *et al.* [19] studied a number of CBs in guinea pigs to determine their ability to protect against soman poisoning, The best of these, physostigmine, pyridostigmine (Figure 56.1), mobam and decarbofuran, all increased the LD_{50} of soman by a factor of

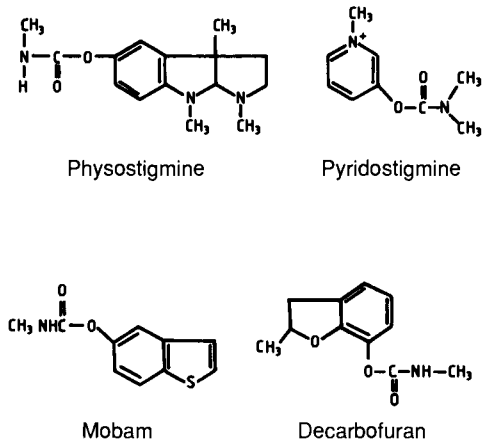

Figure 56.1 CBs of use or studied in the pretreatment of OP antiChE poisoning.

6.5–8. The CB was given at the maximum clinical sign-free dose at an interval before poisoning depending on the time taken for the minimally toxic dose of the CB to produce clinical signs. Pralidoxime methanesulphonate (P2S) and atropine were necessary to achieve protection. While CB pretreatment did not prevent the signs of soman poisoning, certain CBs hastened recovery. Physostigmine pretreatment produced the fastest recovery but animals pretreated with the quaternary CB, pyridostigmine, showed intermittent relapse in condition. However, a single injection of pyridostigmine gave the longest duration of protection (4 h) when compared with the other three CBs. The effects of the regimen was also studied in rabbits and rats and, as with the study of Berry and Davies cited earlier, rats were relatively unresponsive. The treatment regimen with CB, P2S and atropine was also effective against other nerve agents, but while the centrally-acting CB, mobam, had some beneficial activity by itself, the quaternary CB, pyridostigmine had none. In these studies the toxic effects of the CBs did not appear to summate with those of the OP. These authors emphasized the effectiveness of CB pretreatment and oxime- atropine therapy against any OP antiChE.

The two most investigated CBs in soman prophylaxis have been pyridostigmine and physostigmine. The former does not pass the blood-brain barrier [8] and presumably exerts its main beneficial effects at the NM junction [14,16]. Interestingly, French *et al.* [16] suggested that the failure of CB pretreatment noted in some studies using rats, resulted from the fact that the lethal action of soman in the rat, unlike some other species, was central rather than at the NM junction. In rabbits, Heyl *et al.* [23] showed that orally administered pyridostigmine at a dose of $\frac{1}{8}LD_{50}$, was active against soman poisoning of rabbits, with atropine and chlorpromazine pretreatment. Pyridostigmine and physostigmine were studied in mice by Deyi *et al.* [12]. Only physostigmine protected brain ChE but the duration of activity of physostigmine, as measured by inhibition of blood ChE, was shorter than that of pyridostigmine. This appears to be the main advantage of pyridostigmine [19], and it has militated against the introduction of physostigmine despite the advantages of therapy with a drug that is active in the CNS.

In view of the clear evidence of species differences among laboratory animals in the efficacy of CB pretreatment, the effectiveness of prophylaxis in primates is of profound importance in extrapolation of the efficacy studies to humans. Accordingly it is reassuring that pyridostigmine given prophylactically, with atropine administered after the poisoning, protects against soman in both marmosets and rhesus monkeys [13].

A number of drug inter-relationships were studied by Inns and Leadbeater [25], investigating the treatment of soman poisoning in guinea pigs. As with other studies, atropine and oxime alone were not active against soman. However, a regimen including prophylactic pyridostigmine, and atropine and diazepam given 1 min after poisoning, was more effective than one omitting the diazepam, while the further addition of P2S, HS-6, or the bispyridinium non-oxime SAD-128 further increased the effect.

In a study employing both lethal effects and decrease in performance of a swimming test, both in guinea pigs, Leadbeater *et al.* [22] found that pyridostigmine given prophylactically, together with atropine, P2S and diazepam given after poisoning, protected against the lethal effects of soman and sarin. The treatment was less effective in preventing decrease in swimming performance. Substitution of pyridostigmine by physostigmine provided

extra protection against both lethal effects and incapacitation, as measured by the swim test. A small but significant protective effect of physostigmine alone was noted, in most cases, against the lethal and incapacitory effects of sarin and soman, but this was absent when pyridostigmine was substituted for physostigmine.

A number of other CBs have been studied. Thus Heyl *et al.* [23] investigated, as well as pyridostigmine and physostigmine, isopropyl methylphenyl CB, neostigmine and benzpyrinium in rabbits. No convincing advantage of the latter three CBs was demonstrated. Karlsson *et al.* [28] studied pretreatment with a CB derivative of ferrocene in soman poisoning of mice.

Although there is some suggestion that CBs can protect against the delayed neurotoxic effects of certain OPs, perhaps by reversible carbamylation of neuropathy target esterase analogous to that which occurs at the NM junction [26], it also is possible that successful CB prophylaxis, together with anticholinergic and oxime treatment, may allow survival of nerve agent poisoning at doses that will cause delayed neuropathy [18]; this is notably the case with sarin.

Eseroline

Eseroline, a polycyclic aminophenol derived from physostigmine, was shown by Galli *et al.* [17] to have some protective effect in mice against DFP poisoning and rather less effect against physostigmine poisoning.

Mechanistic studies on CBs

While there is little doubt that reversible carbamylation adequately describes the protective action of CBs in OP poisoning, the reader should be aware of recent research into the direct actions of CBs at the nicotinic receptor-ion channel. Albuquerque *et al.* [2] found that pyridostigmine had weak agonist activity at the nicotinic-ionic channel complex and has desensitizing properties [1,41]. This should decrease the amplitude of the ACh-induced response and therefore be beneficial in antiChE poisoning. Albuqueque *et al.* [2] found physostigmine to act as an open channel blocker. Sherby *et al.* [42] found that physostigmine, pyridostigmine and neostigmine acted as partial agonists and potentiated nicotinic receptor desensitization at high doses. However at low doses, such as those used in myasthenia gravis, which are more comparable to doses considered efficacious in nerve agent prophylaxis, they suggested that the main action of CBs was inhibition of AChE. Subsequently, Kawabuchi *et al.* [29] has found that the (+) enantiomer of physostigmine, despite being markedly less potent an inhibitor of ChE, to be as effective as the (−) enantiomer in the pretreatment of antiChE poisoning. For a review of some of this work, *see* Aracava *et al.* [3].

OP compounds

A number of animal studies have shown that certain OPs protect against certain effects of other OPs. Some of these are primarily of mechanistic interest and are unlikely to be exploited therapeutically. For example, soman, because it produces an inhibited but unaged form of neuropathy target esterase, protects hens against DFP-induced delayed neuropathy [27].

Theoretically, an oxime-responsive OP antiChE, administered prophylactically at a sublethal dose, should protect against poisoning by soman. This is because the AChE-inhibitor complex so-formed would not be expected to age. After the use of an OP in this fashion, it is clearly essential to use an oxime to reactivate the enzyme. Such OP pretreatment might possess the advantage of a longer duration of protective action than that bestowed by the CBs, but it would have the disadvantage that oximes would subsequently have to be administered to achieve the protective effect. The use of OPs in the pretreatment of nerve agent poisoning was investigated by Berry *et al.* [7]. These workers found that tetraethyl pyrophosphate (TEPP), paraoxon and ethyl 4-nitrophenyl methylphosphonate, when given prophylactically, with atropine and oximes given after the poisoning, protected guinea pigs against soman. O-(3-(trimethylammonio)phenyl)-1,3,2-dioxaphosphorinane 2-oxide iodide (TDPI) appears to be analogous in mode of action to physostigmine [5], because an inhibited AChE broke down with a t½ of about 10 min; it is the rapid breakdown of the enzyme-inhibitor complex that is the key to the action.

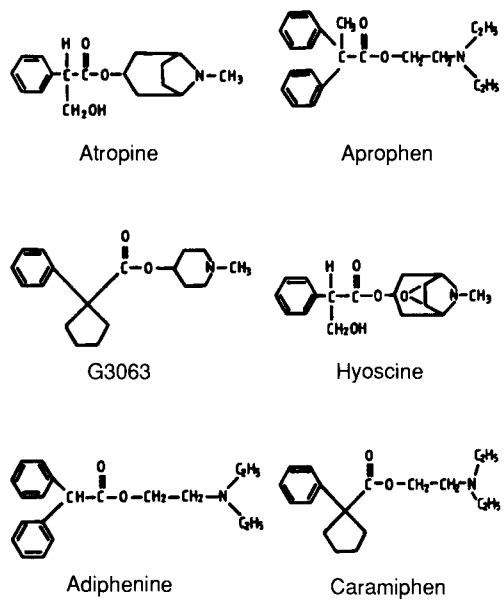

Figure 56.2 Anticholinergic compounds of use or studied in the pretreatment of OP antiChE poisoning.

Anticholinergics

Acting at muscarinic receptors

In most studies on prophylaxis, CBs have been used as the pretreatment while atropine (Figure 56.2), or another anticholinergic drug, has been administered after poisoning. In a few studies, however, anticholinergics have been used prophylactically. Thus, as long ago as 1957 [11], previous atropinization was shown to increase resistance to sarin 1000-fold in the monkey, 150-fold in dogs, by smaller factors in cats and rabbits and, to a minor degree only, in mice. The rationale for using anticholinergics as prophylaxis is to preserve some function after CB pretreatment and OP-poisoning where for a short time, a very large proportion of the ChE will be inhibited reversibly and irreversibly. In the study of Karlsson *et al.* [28] in mice, where 5.5 mg ferrocene-CB was given 30 min before soman and 20 mg/kg atropine was given 10 min before, the LD_{50} of soman was increased by a factor of 6. In an experiment otherwise similar, but where the atropine was given 1 min after soman, the LD_{50} of soman was only increased by a factor of 3. These findings suggest that prophylactic atropine is more effective than therapeutic atropine.

Although atropine is usually considered to be relatively ineffective at peripheral nicotinic sites, as was shown in a number of early experiments [9], Patterson *et al.* [38] have shown that atropine sulphate or methylnitrate has some effect in preventing DFP-induced muscle fibre necrosis. Atropine methylnitrite, a quaternary atropine salt, has been regarded as a useful way of increasing the atropinization of the poisoned subject, to counter the peripheral respiratory effects without excessive and incapacitating atropinization in the CNS. Coleman *et al.* [10] studied a number of tertiary and quaternary anticholinergic drugs, including atropine, administered with P2S 15 min before sarin injected sc into mice and rats. Except in the case of Trasentin-6H methylbromide, quaternization made little difference to the activity of the anticholinergics used instead of atropine. There was some evidence of benefit from the simultaneous use of tertiary and quarternary compounds. In mice exposed to sarin, quaternary atropine salts were less effective than the sulphate. This study has implications for ideas on the predominant site at which atropine exerts its antidotal activity.

Quaternary salts are unlikely to gain significant access to the CNS and the fact that they nevertheless had beneficial, but not marked, activity suggests that some peripheral component was, at least partially responsible. Brimblecombe *et al.* [9] measured the central and peripheral anticholinergic activity of the anticholinergic drugs, atropine, hyoscine, caramiphen, an analogue thereof and three esters of glycollic acid, by estimating their ability to produce mydriasis or to block oxotremorine-induced salivation. In tests on their lethal effects the drugs were given alone and in conjunction with P2S, 15 min before sarin and the effect measured on the LD_{50} of sarin. None of the drugs, by itself, was of great benefit but better results were obtained when atropine was also used. When atropine and P2S were given to rats the LD_{50} was raised 20 times and even better results were achieved with some other anticholinergics. However, no correlation was observed between central and peripheral anticholinergic activity and ability as an antidote to sarin.

Germane to the same problem, experiments were performed in which the effect of pretreatment with atropine was studied on rats subsequently poisoned with one of two V-type alkylaminothioate nerve agents. It was found that atropine was beneficial in the case of the centrally-acting agent, but not in the case of that with predominantly peripheral action [34]. It was therefore concluded that the peripheral actions of atropine were not those responsible for the therapeutic activity of this drug in the rat. Leadbeater *et al.* [32] investigated the effect of sign-free doses of anticholinergics on protection against the lethal and incapacitating effects of sarin or soman. When aprophen (2-diethyl-aminoethyl α-diphenylpropionate (Figure 56.2), a broad-spectrum anticholinergic drug was given in conjunction with either pyridostigmine or physostigmine pretreatment, significant protection was observed, especially with the latter. This study was extended to include a number of anticholinergics with a variety of pharmacological actions. When tested as a pretreatment with physostigmine, supported by P2S, atropine and diazepam therapy, atrophen was found to give the highest level of protection against both the lethal and incapacitating effects, followed by G3063 and hyoscine, caramiphen, dicyclomine, atropine and adiphenine (Trasentine).

Acting at nicotinic receptors

The preponderant effects of atropine are at muscarinic receptors. However, many deleterious effects of OPs are probably mediated at nicotinic receptors, notably in the CNS and at the NM junction. Therefore, cholinergic blockers acting at nicotinic receptors have attractions in the prophylaxis and treatment of OP poisoning. Pentamethonium was observed to have some beneficial action, when, with atropine, it was given prophylactically [11]. Harris *et al.* [22] studied the effects of a number of pretreatment regimens, some of which included the anticholinergic, mecamylamine, on soman poisoning. In the rat, a combination of mecamylamine and either neostigmine or physostigmine was not effective. However, triple combinations of atropine and mecamylamine with either neostigmine or physostigmine were strikingly effective and more so than the same regimens omitting the mecamylamine. Inns (unpublished data) found that mecamylamine pretreatment raised the protection afforded to mice by P2S/ atropine therapy by a factor of 7 in the case of VX and a factor of 2.1 in the case of soman.

NM blocking drugs

The use of d-tubocurarine, alone and in combination with atropine sulphate or methylnitrate, was studied for the prevention of DFP-induced muscle fibre necrosis. While all three agents were, to some extent, protective, a combination of d-tubocurarine and atropine methylnitrate was most effective [38].

Oximes

Being reactivators of OP-inhibited ChEs, the various PAM salts or obidoxime are, with atropine, the standard therapy for OP poisoning (*see* Ch.52). An attraction of oximes used prophylactically is that, with OPs that give rise to an inhibited enzyme that ages rapidly (i.e. soman), they might be able to reactivate the enzyme before a significant amount of ageing has time to occur. On this basis, P2S tablets were issued to the British Services for some years before replacement by pyridostigmine pretreatment. In their study on the effects of P2S and anticholinergics, Brimblecombe *et al.* [9] found that the oxime given alone was relatively ineffective. In an inhalation study by Schoene *et al.* [40], pretreatment with atropine (10 mg/kg) and HI-6 (13 mg/kg) 10 min before poisoning, raised the LCt_{50} (lethal concentration killing 50% of the animals × time of exposure) of soman by a factor of 7. A combination of atropine and obidoxime was not effective. Against sarin both oximes were effective when combined with atropine.

Anticonvulsants

Numerous studies have been carried on the anticonvulsant effects of various CNS depressants. The subject is discussed elsewhere in this book (*see* Ch.53) and only studies of prophylaxis will be cited here. DeCandole and McPhail [11] found that prophylactic pentobarbitone had no effect on the toxicity of sarin in mice. The much more promising

benzodiazepines have been much studied in therapeutic situations, but less often for prophylaxis. However, Lipp [35] found that clonazepam, given 2 h before exposure to soman, prevented the EEG abnormalities associated with soman intoxication in monkeys. Lundy *et al.* [36] studied the effects of pretreatment with another benzodiazepine, diazepam, as well as aminooxyacetic acid and n-propylacetic acid on the severity of convulsions in rats. All three were capable of reducing the severity of the convulsions. Of the drugs, diazepam was the most effective. On the other hand, Tonkopii *et al.* [43] were unable to demonstrate any benefit from pretreatment with diazepam in mice poisoned 30 min later with OPs. After carrying out studies which showed that pentobarbitone, pentobarbital (or atropine sulphate) prevented convulsions and consequent damage to the integrity of the blood-brain barrier in rats Ashani and Catravas [4] suggested that convulsions might increase the CNS penetration of antiChEs. This provides another possible explanation for the efficacy of anti- convulsant pretreatment, in those instances where it has been demonstrated experimentally.

Other prophylactic agents

AChE

Fetal bovine serum AChE was effective when given 20 h before a challenge of up to 3 LD_{50}s of VX in mice [45] .

Monoclonal antibodies

Monoclonal antisoman antibodies given ip to mice were found to prolong survival of mice poisoned 2 h later with soman [3]. A probable disadvantage of monoclonal antibodies is the narrow spectrum of their activity against perhaps a single OP or a few of close similarity in structure.

Steroids

Pretreatment with the steroids, triamcinolone and deoxycorticosterone have been shown to have some beneficial effect against OP-induced delayed neuropathy [15]. However, this effect was probably not a prophylactic one, since dosing was continued afterwards.

The place of prophylaxis in clinical practice

As explained earlier, there is no place for anti-ChE prophylaxis in civilian medical practice; the need for such would imply that workplace practices were unsatisfactory. In any case physical protection would be more appropriate. Thus chemical warfare is the only likely scenario in which prophylaxis would be desirable. The CB antiChEs are not candidate or credible chemical warfare agents, so that prophylaxis means, in effect, prophylaxis of OP poisoning.

In a military context prophylaxis is dealt with in Chapter 34. It is clear that, of the possible prophylactic approaches mentioned earlier, only CB pretreatment forms a major part of anti-OP measures at present in use. This is largely a result of a lack of other therapeutic approaches to the problem of ageing and the consequent refractoriness of soman-inhibited ChEs to oxime reactivation.

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Role of poison control centres in the recognition and management of anticholinesterase poisoning

Edward P. Krenzelok

Insecticide poisoning is a common problem reported to poison control centres (PCCs). During 1985–1989 PCCs participating in the American Association of Poison Control Centers (AAPCC) Cooperative Data Collection System responded to 190 350 human cases [5–9] involving insecticide poisoning. Nearly 50% were exposures to antiChEs either alone or in combination with other insecticides. Relative to other toxins, this represents a 1.5% poison exposure incidence for antiChEs, a substantial portion of the PCCs activity. Therefore, it is apparent that PCCs are being utilized as an information resource in the recognition and management of antiChE poisoning.

What is the role of PCCs in the treatment of this intoxication? What services do PCCs offer to the professional seeking advice about exposure to an antiChE? What resources are available in PCCs? Are data available to direct treatment decisions and assist in the recognition of these emergencies? The responses to these questions are varied since the poison control system within the USA is not standardized.

The *Physician's Desk Reference* [10] lists 520 poison control centres! The AAPCC, the association of professionals involved in dissemination of poison information, lists a membership roster of 112 PCCs and differentiates 37 PCCs as Regional Poison Centres. The AAPCC defines the function of the poison centre 'to provide poison information, telephone management and consultation, collect pertinent data, and deliver professional and public education' [13] and to provide these services to individuals seeking assistance regarding poisonings. Not all of the 520 PCCs or 112 PCCs provide all of these services or the same quality of service. Although many non-regional PCCs provide excellent service, only the designated Regional Poison Centres are known to provide consistent and standardized poison information service.

A study by Thompson *et al.* [12] strongly suggested that AAPCC-designated regional poison centres provided more consistent and better information than their non-regional counterparts. A survey of regional and non-regional centres by Geller *et al.* [3] noted differences between the two types of centres especially in the manner they complied with the AAPCC standards for regionalization. Although outcome data were not measured, the study implies that regional centres are more qualified to respond to poisoning emergencies. Therefore, professionals seeking assistance regarding exposure to an antiChE insecticide should utilize the services of an AAPCC Regional Poison Centre. If a designated centre is not conveniently available, the local centre should be evaluated against the following criteria [13] in anticipation of an antiChE poisoning emergency.

Criteria for certification as a regional poison centre

Determination of a region

The PCC should serve a distinct region with a population base of at least one million individuals and not in excess of ten million. It is anticipated that this will provide a minimum of

10 000 human exposure calls annually, ensuring that PCC personnel maintain clinical competency. With this case volume a PCC would respond to approximately 150 exposures to antiChEs annually. The larger the service area, the more experience the PCC gains in antiChE poisoning.

Regional Poison Information Service

Service must be provided by telephone and available 24 h per day and every day of the year. The PCC must have comprehensive poison information resources and appropriate patient management protocols. The staff should consist of a physician Medical Director and a Managing Director with expertise in clinical toxicology. Poisoning inquiries should be responded to by AAPCC Certified Poison Information Specialists (CPIS) who are either registered nurses or pharmacists. Additional consultative and educational staff are also mandated. Active quality assurance must be evident. Meeting these criteria will assure expertise in addressing the information and treatment needs regarding victims of antiChE poisoning.

Regional treatment capabilities

The regional PCC must be aware of the capabilities of treatment facilities within their region; this includes analytical toxicology, availability of extracorporeal services, hospital triage and referral patterns, the availability of antidotes, etc. Although, as discussed later, most antiChE exposures do not require extraordinary medical intervention, some are medical emergencies and referral to appropriate treatment facilities is critical. Therefore, the PCC should be aware of the ability of hospitals within its region to respond to antiChE poisonings.

Regional data collection system

Documentation of all exposure calls by approved medical documentation procedures (problem oriented approach) is required. All cases must be documented on the AAPCC Cooperative Poison Centre Report Form and these data must be submitted to the AAPCCs National Data Collection System. The data can be used to identify trends and potential epidemics, to assess the outcomes of patients exposed to antiChEs, and to direct education efforts.

Professional and public education programmes

Professional and public education programmes must be available to everyone in the centre's region. The majority of poisonings, including antiChE exposures, can be prevented through poison prevention education programmes. Nearly 99% of all exposures to antiChEs are accidental [5–9]. Through active education they should be preventable.

The Regional Poison Centre provides all the above services in a high quality fashion. Some non-regional centres may provide the same level of service but it is not consistent from centre to centre.

Role of regional poison centres in antiChE poisonings

The primary functions of the regional PCC are twofold: (1) to provide information which will aid in confirming or excluding the diagnosis, and (2) to recommend treatment methods.

Substance toxicological information

PCCs are a reservoir of toxicological information whose proper use is critical to the diagnosis of antiChE poisoning. For a PCC to provide appropriate information it is incumbent upon the enquirer to exchange patient information with the poison information service CPIS. The PCC provides more than mere information. The trained CPISs combine their experience with poison information and patient variables to generate patient-specific poison information. Therefore, the CPIS will request information on the sex, weight and age of the patient; past medical history; a history of the exposure, including the amount and route of exposure as well as extensive information on the specific antiChE, and when the exposure occurred. Additional information is required on the patient's current medical condition including signs, symptoms, analytical results, etc. The patient's name is also important to enable the

PCC to follow patient progress and comply with the medicolegal requirements of documentation. Given this information, the CPIS can then gather specific toxicology information on the antiChE in question.

The sources of information utilized may include computer databases, reference texts and literature searches.

The most common tool used by regional PCCs is the computer database. A variety of commercial programs are available and may provide product component information as well as specific toxicology information. Poisindex® [11] is the most common computer database used by PCCs to respond to enquiries about insecticide poisoning. It provides ingredient information on commercial products such as antiChEs and provides rapid accessibility to information within seconds [4]. This information generally includes the name of the specific antiChE in the insecticide as well as excipients such as solvents and emulsifiers, and lists their respective percentages or amounts. In addition to ingredient listing, which is an important starting point since many antiChE product labels do not provide comprehensive ingredient information, the Poisindex® system provides details on the treatment of antiChE poisoning. Components of the treatment protocol include clinical effects of antiChE exposure, the range of toxicity, pharmacokinetics, toxicology, physiochemical properties, treatment, references, etc. The reference list is extensive and allows the CPIS further to resolve a specific question about antiChE poisoning. Protocols are written and reviewed by experts in the field of antiChE toxicology.

Chemtox® [1] another computer database used by PCCs does not have the clinical applicability of Poisindex® and focuses more on the antiChE compound's chemical properties, environmental impact of spills and transportation requirements. Although health hazard information is included it is not extensive. An advantage of this system is that it allows user access by a variety of chemical synonyms or alpha-numeric identifiers such as the Chemical Abstracts Service (CAS) number, RTECS number, STCC number, DOT number and the EPA number. Thus, the PCC can assist in identifying which specific antiChE was involved in a transport accident when only one of these product identifiers is available. As with Poisindex®, Chemtox® is menu-driven, user friendly and permits rapid access to information.

Before the development of advanced technology to store and retrieve archival information using computers, the PCC relied upon reference texts and the medical literature to respond to enquiries. Reference texts, which provide more extensive and detailed information on antiChEs than the computer databases, remain the cornerstone of PCC operations. Specific information on mechanisms of action, cellular toxicity, previous case reports, etc. can be found in the texts. If information is not readily available on the computer database, CPIS will resort to using the texts or current medical literature.

Reference texts rapidly become outdated. Similarly, computer databases rely on medical literature for updates and there is a hiatus between publication in the literature and its appearance in computer databases and reference texts. Since the toxicology of antiChEs is kinetic, the PCCs rely on constant surveillance of the current medical literature to provide updated information on antiChE poisoning and treatment. Some PCCs can conduct online literature searches using databases such as Medline® and Toxline® which can expedite resolving a challenging question on antiChE poisoning.

Consultants are a viable information resource for the PCC and ultimately the caller. Most PCCs have consultants available who have expertise in antiChE toxicology and/or experience in the treatment of poisonings. Consultants are usually used by PCC to help resolve an information or treatment problem, and are frequently available to speak directly with the caller when necessary.

Customarily, toxicological information is provided via telephone or occasionally in written form. Since patient care may involve several health professionals who are not all able to discuss the case with the PCC, it may be valuable to provide written information with specific toxicological information for the sake of consistency. Expeditious transmittal of this information may be difficult because of the distance between the PCC and the hospital. Some PCCs have facsimile machines [2] which enable them to transmit written information to

the treatment facility over conventional telephone lines. Therefore, the caller has the advantage of verbal communication with the CPIS and the opportunity to receive literature and written consults. PCCs utilize the facsimile equipment to share information with other centres and to rapidly retrieve literature from medical libraries.

Through these resources the PCC can provide information on various topics. Although the majority of calls to a PCC on antiChE poisoning concern human exposures, the PCCs are also consulted about veterinary exposures to antiChEs. PCCs provide information about the effects of antiChEs on small and large animals, and on the location of storage depots for antidotes such as atropine, PAM and activated charcoal which are used in voluminous amounts for treatment of a herd of cattle having antiChE poisoning. A very limited number of PCCs serve as an antidote depot for veterinarians within their region. The most commonly requested information relates to acute and chronic sequelae from exposure to antiChEs. Not uncommonly, the PCC is requested to interpret animal LD_{50}s in perspective with their clinical experience and a literature consensus. Also, the PCC maintains information on spill and fire cleanup and containment which may be useful to hazmat teams, fire departments, and governmental and industry officials responsible for monitoring cleanup activities after inadvertent environmental release of antiChEs. Teratogenicity and carcinogenicity information may also be available from the PCC. If the PCC does not have immediate access to the above information they generally know where it may be obtained and where to refer the caller.

In summary, it is essential that the professional making an enquiry of the PCC supply as much patient-specific information as possible so that the CPIS can provide not merely generic toxicological information on antiChEs, but also specific clinical toxicological information which is oriented to a specific patient.

Specific treatment information

CPISs who staff regional PCCs are health professionals (registered nurses and pharmacists) with clinical assessment and management skills as well as experience in information retrieval and evaluation. They are always supported by clinical and medical toxicologists if they have questions on the treatment of a patient exposed to an antiChE.

Some PCCs are located in hospitals and PCC personnel participate in the management of the patient, especially in the emergency department. However, most recommendations are made without being present clinically to evaluate the patient or to assess response to therapy. This disadvantage can be significantly reduced through the free exchange of information between the CPIS and the professional managing the patient. It is customary for PCCs to conduct routine follow-up calls to assess the patient's progress and compliance with treatment recommendations. PCCs share the liability associated with patient care and must assure that the patient is being appropriately treated. All information is extensively documented by accepted medical documentation standards. Most PCCs utilize the problem-oriented approach of medical documentation. Extensive quality assurance programmes exist in regional PCCs to ensure that standardized care is provided.

The PCC makes recommendations about patient care but the health professional must take the ultimate decision. Medical backup is available from all regional PCCs so information on basic life support can be provided directly by the CPIS or from the on-call physician for the PCC. The PCC specializes in providing patient-specific information, e.g. recommended doses or infusion rates for iv atropine. In conjunction with the patient information supplied, CPISs can assist the treating physician in deciding to use PAM in conjunction with atropine based on the presence or absence of nicotinic symptomatology. Advice is available on the necessity for analytical tests such as RBC AChE activity, where the sample can be analysed, how to interpret the results and whether they affect patient therapy.

The PCC has a wealth of clinical toxicology and treatment information on the recognition and management of antiChE poisoning, much of which is generated through cases which they respond to through the data collection programme. PCCs utilize their data to formulate treatment protocols, to develop research projects, to influence legislation and to develop education programmes for both the lay

and professional public. The use of the PCC in antiChE poisoning increases the database which can be converted into useful patient care or awareness information.

Data collection/analysis

Role of data collection

Each actual or alleged exposure case responded to is documented as described above. This information is uniformly collected by all regional PCCs and non-regional centres that participate in the AAPCC Cooperative Data Collection System. The result is to produce specific and generic data on the incidence, treatment, outcome and demographics of antiChE poisoning. Each PCC has their own data and the national data for comparative purposes. The data can meet several needs: (1) early recognition of trends of epidemics, (2) provide information on patient outcome, (3) provide a basis on which to conduct research, and (4) direct educational efforts.

Table 57.1 Incidence of insecticide poisoning by age from 1985 to 1989

		Percentage of cases		
	Total	*<6 years*	*6–17 years*	*>17 years*
All insecticides	190 350	53	8	39
AntiChEs	87 687	47	8	46

Data from Litovitz *et al.* [5–9]

Table 57.2 Outcome of insecticide poisoning from 1985 to 1989

	Outcome (toxicity)				
	None (%)	*Minor (%)*	*Moderate (%)*	*Major (%)*	*No. of fatal cases*
All insecticides	62.3	33.7	3.6	0.4	49
AntiChEs	58.9	35.9	4.5	0.6	32

Data from Litovitz *et al.* [5–9]

Table 57.3 Insecticide poisonings and fatalities compared with all exposures and fatalities from 1985 to 1989

	All poison exposures (fatal)	*All insecticide exposures (fatal)*	*All antiChE exposures (fatal)*
1985	900 513 (328)	26 171 (6)	14 127 (3)
1986	1 098 894 (406)	36 541 (10)	16 565 (6)
1987	1 166 940 (397)	37 856 (9)	17 197 (6)
1988	1 581 540 (590)	48 283 (12)	20 860 (8)
1989	1 368 748 (545)	41 499 (12)	18 948 (9)
Totals	6 116 935 (2266)	190 350 (49)	87 697 (32)
Percentage	0.04	0.03	0.04

Data from Litovitz *et al.* [5–9]

Recognition of trends

If a PCC is properly utilized within its region, the majority of antiChE exposures should be reported to that PCC. Through data surveillance and analysis the PCC may recognize early poisoning trends from ChEs, e.g. inappropriate use of insecticides with the subsequent development of toxicity by applicators, their children and even pets. Recognition of these problems can direct education efforts through the media, etc. to reduce the incidence of such poisonings. Chronic trends can also be detected and appropriate action taken.

A pooling of AAPCC data from 1985–1989 [5–9] on exposures to antiChEs alone or with other insecticides reveals that there were 87 687 exposures to antiChEs over the period compared with a total of 6 116 635 reported exposures to all substances (1.4%). This ratio has not changed over the period. Similarly, there has not been a notable change in the number of antiChE exposures in various age groups. Children <6 years of age (46.6%) and adults (45.8%)

account for approximately 92% of exposure to antiChEs, and adolescents (6–17 years of age) account for the balance (Table 57.1).

Patient outcome data

How effective is the care provided to victims of antiChE poisoning? The same data cited above reveal that nearly 94% (Table 57.2) of all antiChE exposures resulted in either no or only minor symptoms (not life-threatening and self-limited). This compares with 96% for all insecticide exposures. Fatalities from antiChEs (Table 57.3) were responsible for 1.3% of all deaths for the 5-year period compared with 2.2% for all insecticides, i.e. antiChEs were responsible for 60% of all fatalities caused by insecticides. However, put into perspective, there were but 32 fatalities out of nearly 88 000 reported exposure cases. This data is only available because PCCs were consulted about patients exposed to antiChEs.

Research

Data of the above type provokes questions about the epidemiology of antiChE poisoning and the value of treatment methods. It also provides large case series to analyse treatments and outcomes, and ultimately to publish the information to improve our understanding of antiChE poisoning and treatment protocols. It provides a basis to seek financial research support. Scientists interested in antiChE toxicology should not overlook the PCC as a source of research data.

Education

The data gives direction to educational efforts. Awareness of which specific antiChEs are producing toxicity and in what age groups allows the development of strategies to prevent poisonings through education. Similarly, knowing treatment trends and patient outcomes permits PCC staff to educate professionals in the recognition and management of antiChE poisoning.

Summary

The PCC, especially the regional PCC, can be an invaluable asset to the professional seeking information on the recognition and management of antiChE exposures. Working with consulting health care professionals, the CPIS can provide information on antiChE product composition and specific toxicology which can assist the professional in confirming or excluding a diagnosis of antiChE poisoning. Information is retrieved from extensive literary and computer databases combined with the experience of the PCC staff. The clinical staff of a PCC can provide information on the management of poisoning and make recommendations on the use of antidotes. The PCC extensively documents each exposure case, resulting in a medical record which allows the PCC to follow patient progress and protect the medicolegal interests of the patient, care provider and the PCC. The medical record also services as the basis for data collection which is used by the PCC to improve patient care through surveillance, research and education.

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Forensic aspects of acute anticholinesterase poisoning

Bryan Ballantyne

This chapter summarizes some aspects of acute antiChE poisoning that may have legal implications.

Causes of poisoning

Exposures to antiChEs may occur under the following circumstances.

Incidental

Controlled exposures occur in the workplace and as residues in foodstuffs. Most developed countries have regulations governing permissible exposures. Incidental exposure in the domestic environment occurs from permitted use patterns, e.g. insecticidal vapour and spray preparations.

Accidental overexposure and misuse

Where antiChEs are used or stored there is a potential for harmful overexposure by accident and intentional or unintentional misuse.

Occupational

Most developed countries have guidelines and strict regulations for the protection of the worker. The assessment and communication of chemical hazards in the workplace has been reviewed elsewhere [34]. In any complex industrial situation accidents may occur leading to overexposure to chemicals, but providing adequate emergency forward planning procedures have been developed the effects can be minimized. In contrast, criminal neglect, leading to harmful effects in the worker, may result from poor hazard evaluation, bad communications, poor training, inadequate protective and precautionary measures, lack of monitoring of the workplace, inadequate medical or biological monitoring of workers, poor or no auditing procedures, and even the deliberate concealment of information. Most industries in the West have developed auditing programmes to ensure that the likelihood of these events occurring is minimized. However, on occasions circumstances may arise resulting in adverse effects in workers requiring both legal and regulatory investigation.

Potentially lethal sources of overexposure include plant leaks, accidents during transfer operations, transportation accidents, drift during aerosol spray applications and inadequate protective and precautionary measures. For example, pilots contaminated with antiChEs before or during spraying operations may develop effects, particularly in the eye, conducive to accidents [19]. Accidents, unsafe industrial practices, or mistakes in industry or commerce may lead to poisoning in the general population. Waste materials discharged into rivers or unsupervised disposal, may lead to the presence of antiChE materials in drinking water. With uncontrolled crop spraying operations, run-off may also result in drinking water contamination. Several mass outbreaks of antiChE poisoning have resulted from heavy accidental contamination of foodstuffs [36].

Domestic

Most domestic accidents are the result of not reading label or insert instructions, inadequate disposal of containers or deliberate misuse. For example, high concentrations of antiChEs may develop in poorly ventilated spaces by

dispersal of powders or aerosols [1]. Small containers of antiChEs in solution may be mistakenly drunk, particularly by children. Non-disposal of poorly decontaminated containers is a significant source of poisoning. Additional factors leading to poisoning are poor container design, lack of safety containers for domestic use and inadequate label warnings. A particular problem is children finding improperly disposed containers [15]. Loss of the label from unwashed containers has led to accidents in their use for other storage purposes and even for cooking [20].

Deliberate poisoning

AntiChEs have been used for suicide, and to a lesser extent for homicidal purposes. Ready availability, and general knowledge about toxicology, is mainly responsible for this abuse. Additionally, highly toxic OPs are agents for chemical warfare operations.

Pesticide, including antiChE, poisoning is common in some areas because of uncontrolled availability, poor control of use and local economic conditions. For example, Sri Lanka has one of the highest suicide rates in the world, with 29 cases per 100 000 of population in 1980. The most common mode of suicide is by the ingestion of liquid pesticides. In an analysis of 157 autopsies of suicides in Colombo in 1985, 57% were from pesticide poisoning. Suicide is significantly greater than death from accidental overexposure to pesticides. In a review of 407 pesticide-related deaths in an agricultural area of Sri Lanka, 92% were from suicide and 7% from occupational exposure [4].

Changing patterns of antiChE exposure occur with regulation of antiChE pesticides. For example, in Ethiopia with increasing importation of pesticides, the incidence of poisoning increased, and displaced therapeutic drugs as the major cause of poisoning. Factors responsible included widespread and uncontrolled availability, lack of education and harsh environmental conditions [32]. As mandatory regulation increases, the incidence of accidental poisoning decreases [10].

Regional variations in the incidence of pesticide poisoning occurs. For example, in Japan during 1985 there were 3800 deaths from poisoning; 53% were from carbon monoxide and 38% from pesticides (paraquat 66% and OPs 23%) [23]. In contrast, analysis of 210 deaths from toxic substances from 1982 to 1985 in Prima County, Arizona, showed carbon monoxide to be the leading cause of death, but none was attributable to pesticides [14].

Most lethal accidental and deliberate antiChE poisoning is from OPs, although several CB fatal poisonings have been reported, e.g. methomyl [20,24], oxamyl [15], propoxur [7] and carbaryl [12].

The oral route is common in intentional poisoning, domestic accidents and foodstuff accidental contamination. Occupational exposure may involve inhalation and/or percutaneous absorption. Inhalation may be a route of exposure by misuse in domestic circumstances. Consideration of the route is important in forensic toxicology, because the blood levels, metabolites produced and different tissue concentrations may vary significantly by absorption through different routes.

Diagnosis of acute lethal antiChE poisoning

Clinical features

Signs and symptoms of a cholinergic crisis before death may be strongly suggestive of antiChE poisoning. The time of poisoning may provide valuable information. For example, if the antiChE is known, the time to death may indicate or confirm the route of exposure. Also, time to onset of signs and death is more rapid with CBs and directly-acting OPs, than with OPs requiring metabolic activation.

Autopsy

The cause of death is usually ascribed to asphyxia, resulting from laryngospasm, bronchospasm, bronchorrhoea, respiratory muscle paralysis and central depression of respiration. Cardiac failure may be a contributing factor. Autopsy features, not diagnostic, may suggest a cholinergic crisis, e.g. foam in the nose and mouth, increased tracheobronchial secretions, pulmonary oedema, tracheopulmonary congestion and haemorrhage, and cerebral oedema.

ChE activity

With CBs, owing to rapid reactivation of ChE, a depression of ChE activity may not be measurable.

For OPs, BChE may not be a valuable guide. Normal BChE activity is influenced by liver disease, various drugs, steroid hormone levels, malnutrition and infections. AChE activity, in blood and brain is the most appropriate biochemical index of acute antiChE poisoning.

To confirm antiChE poisoning, histochemical methods are available. In experimental studies, using endplate AChE, Bergner and Durlacher [5,6] found that various materials, including carbon monoxide, potassium cyanide and arsenic, did not cause a histochemically-demonstrable depression of enzyme activity. Following death from DFP, prostigmine, physostigmine, parathion, TEPP and schradan, there was partial or complete inhibition of AChE activity. In normal mice, ChE activity was still demonstrable 48 h after death. In human intercostal muscle, enzyme activity was present for at least 26 h at room temperature, and in refrigerated bodies for 180 h. Moore and Petty [25] demonstrated ChE activity in refrigerated muscle for up to 157 h, and for a similar period in tissue from bodies within 24 h of embalming. Petty and Moore [27] reported a 23-year-old male fatally poisoned with Systox. There was a significant reduction in the intercostal muscle ChE activity by a histochemical procedure. Biochemically, RBC AChE activity was stated as 0.38 μM ACh (RBC AChE for 97 males dying in non- poisoning circumstances averaged 1.52 μM ACh, range 0.74–2.38 μM ACh). Finkelstein *et al.* [13] studied postmortem AChE by a quantitative histochemical procedure in the brains of two acute parathion poisonings; intervals between death and autopsy were 16 and 32 h. The largest decreases in brain AChE activity were in cerebellum, some thalamic nuclei, and cortex (60–85% decrease). Moderate decreases (10–30%) were detected in substantia nigra and basal ganglia, with no effects in white matter. Thus, histochemical determination of CNS AChE activity is useful in confirming acute antiChE poisoning, but several regions should be examined.

Parent compound and metabolite identification

Biochemical measurement of AChE activity is of diagnostic value in acute antiChE poisoning, but a definitive diagnosis requires measurement of OP, CB, and/or metabolite(s) in blood and tissues.

In suspect cases, or when multiple antiChEs are present, screening methods are available to separate antiChEs and obtain preliminary information on OPs or CBs present. Urine screens for dialkyl phosphates may be useful for OPs. Reed and Watts [28] described a method involving the detection of urinary dialkyl phosphates residues. Reverse phase thin layer chromatography appears useful, and for which a series of R_f values have been published for various OPs and CBs [38]. The use of immobilized ChE electrodes as a screening method has been described [11].

Definitive identification of OP, CB and metabolite(s) requires a separation/clean-up procedure followed by analysis, usually by gas chromatography-mass spectrometry. Negative ion chemical ionization has several advantages in forensic applications [8]. It gives group specific peaks in the spectrum, allowing narrowing down of candidates of an unknown OP. Hattori *et al.* [17] recommend both negative ion chemical ionization and positive ion electron impact: the former provides proof of an OP and gives group structure, and the latter gives molecular peaks. Final identification requires comparison of a positive electron impact mass spectrum between the suspect chemical and an authentic specimen. Negative ion chemical ionization is highly sensitive [30], and applicable to small samples [26].

Because CBs are usually unstable at the high temperatures needed for gas chromatographic analysis, high-performance liquid chromatographic analyses were developed [33,35]. A thin-layer chromatographic method has been developed [18]. For certain CBs gas chromatographic and gas chromatographic-mass spectrometry techniques have been developed [9,37]. Suzuki *et al.* [31] have published a useful paper describing positive ion impact, positive ion ionization and negative ion chemical ionization mass spectra for several CBs.

Problem areas

In planning the analytical approach to diagnosing antiChE poisoning, possible confounding factors need to be taken into account. Examples are given below.

Old specimens

Some cases may involve the use of liquid pesticides kept under poor storage conditions, where high environmental temperature may result in the conversion of parent material to a complex, lethally toxic mixture. This may present analytical problems in analysing both the container contents and what is appropriate to measure in body fluids and tissues. For example, Sovocool *et al.* [29] described a case of acute antiChE poisoning where intact OP could not be detected on gas chromatography of container fluid. Using field ionization, field desorption, chemical ionization, exact mass measurements at high resolution, and gas chromatography-low resolution mass spectrometry, they found the composition consistent with a virtually complete conversion of diazinon to at least 26 products or impurities. This was verified by model decomposition studies.

Body decomposition

When there has been a significant interval between death and discovery of the body, analysis of fat and stomach may be helpful. Gunatilake and Goff [16] suggest that analysis of larvae feeding on decomposing remains is useful; they successfully applied this to a case of malathion poisoning.

Previous treatment

It is not expected that pre-mortem atropine and/or oxime will have a significant influence on OP or CB measured at post mortem. However, factual information in this area is limited. Life support measures, including mechanical ventilation, result in lowered antiChE concentrations in blood and tissues.

Impurities

Some low toxicity OPs contain potentially lethally toxic impurities. For example, the acute peroral LD_{50} (rat) of diazinon in 1964 was reported in the range 76–108 mg/kg, and in 1971 as 250–466 mg/kg. The higher lethal toxicity of earlier samples was believed to be from highly toxic pyrophosphates, most probably monothio-TEPP (acute peroral LD_{50} (mouse) 4 mg/kg [21,36]). Poor control of production may result in potentially hazardous toxic impurities being present. This has occurred in less well developed countries. If an inappropriately low dose of an antiChE has caused death, the analyst may require to consider the possibility of highly toxic impurities in the formulation.

Another example of inappropriately low doses causing death is the presence of impurities which inhibit tissue carboxylesterase and thus potentiate the toxicity of OPs detoxified by these enzymes. Malathion is a particular example. Prime potentiating agents include isomalathion and O,S,S-triethyl phosphorodithioate [2,22]. Although most commercial formulations have concentrations not producing significant potentiating effects, they may increase during storage; major determinants include time, elevated temperature and formulating agents [36]. Deaths from malathion potentiation are reported [3]. A similar hazard may exist with phenthoate, whose low mammalian toxicity results from a hydrolyzable ethoxycarbamylester bond. If carboxylesterase-inhibiting impurities, such as the S-methyl ester and trimethyl phosphorothioates, increase, then a possibility for potentiation of phenthoate toxicity may exist. The above considerations highlight the need for the forensic toxicologist to be prepared to examine for impurities, particularly if apparently inappropriately small amounts of OP have caused death.

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Aerial application and spray drift of anticholinesterases: protective measures

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Introduction

Aerial spraying of OPs is carried out wherever there is a need for rapid application of these pesticides to large or remote land areas. Aerial application has produced a relatively low reported number of work intoxications per tonne applied; in California, aerial spray workers have applied 60 per cent of all pesticides but have caused only 10 per cent of total reported pesticide illness episodes [11]. These advantages have been offset by the hazards: for pilots, crash injury and death from behavioural effects of low-level exposures; for ground crew workers, direct contact associated with transport, preparing, mixing, formulating, loading, disposal and equipment maintenance; and for field workers, contact with residues when working in sprayed fields. Additionally there is potential for community exposure to pesticide drift near residential areas.

Spray formulation and application: conventional and ultralow volume

Drift downwind from aerial application of sprays is four- to fivefold higher than that produced by high clearance ground sprayers. Dry dusts also produce much more downwind drift than wet sprays; this is why pesticides applied by aircraft are in the form of sprays and not dusts [23].

In aerial application, drift spraying releases small droplets from high altitude which remain suspended, while placement spraying releases larger drops from low altitudes to narrow swathes. Drift spraying is for control of airborne pests or for covering extensive areas of ranchland or forestry; placement spraying is for crop pest control.

Table 59.1 shows data which compare the potential for drift downwind for droplets, especially aerosols (e.g. < 50 μm) from conventional and ultralow volume (ULV) spraying. ULV sprays contain highly concentrated preparations of pesticide and require less volume per hectare. This means that very large areas can be treated with each spray load.

The use of ULV results in less mass and volume of respirable pesticide made available for downward drift but the tenfold higher concentration of ULV pesticide (Table 59.1) means that the drift sent downwind is more hazardous. On the other hand, with conventional spraying the mass of active pesticide in

Table 59.1 Conventional and ULV sprays: dose-volume-mass relationships[a]

Aerial application technique	*Dose-Volume rate*		*Pesticide concentration*	*VMD (μ)*	*Pesticide <50 μm*		
			(g/l)		*Volume*		*Mass*
	(g/ha)	*(l/ha)*			*(%)*	*(l)*	
	(a)	*(b)*	*(b/a)*		*(c)*	*(b×c)*	*(b/a)×(b×c)*
Conventional hydraulic nozzle	500	20	25	200	4.0	0.8	20
ULV (atomizer)	500	2	250	100	0.7	0.014	3.5

[a] From Coutts [4] by permission of Elsevier

droplets of < 50 μm available for drift out of the spray area is six times greater than for ULV. This is because the volume spectra for conventional sprays are much larger despite their larger median volume diameters.

Exposure and effects in aerial spray workers

In pilots, functional impairment from pesticide intoxication could easily lead to accidents; double vision, in particular, may result from direct contact of pesticide with the eye without evidence of systemic absoption [19]. The role of OP exposures in causing crashes in aerial spray pilots was suggested in the 1960s when depressed ChE levels were found, sometimes at post mortem, in pilots after crashes [2, 14] although in some cases exposure may have been after the crash and caused by spillage. In one case a pilot who had previously had extreme miosis and depressed ChE levels crashed [16]. California data indicated that visual impairment ranked fourth (after mechanical or engine failure, obstructions during flight, and loss of control) as an attributed cause of crashes [11]. These reports triggered concern that cockpit and ground exposure to OPs, even if not producing acute signs or depression in ChE levels, might insidiously impair pilot vision and overall alertness, skill and performance. In studies carried out in the 1960s, Durham and Wolfe [6] and Wolfe et al. [25] observed respiratory exposure to parathion in pilots averaging 0.02 mg/h and skin exposures averaging 13 mg/h; higher skin and respiratory exposures were found among flaggers and plane loaders. Depressed levels of ChE in air and ground crews, correlating with increased amounts of p-nitro phenol (PNP) in urine, have also been described [5].

In Israel, there is intensive aerial application of pesticides to fields adjacent to residential communities. Each summer, some 1000 tons of OPs are aerially applied to a crop area (some 400 km^2) of cotton fields and orchards nationwide [7, 18]. A network of regulation, data collection and medical surveillance has enabled the exposure problems of the various workers and community groups at risk to be documented and some of the possible solutions tested.

Increases in the crash and incident rate per 1000 hours of flying time in Israeli spray pilots led to an assessment of air and skin exposures to parathion aerosols and vapours in agricultural spray pilots and ground crews in the 1970s [3]. Exposure-absorption resulted from inadequate environmental conditions and work practices, probably similar to those seen in many other countries. Pilot workload during peak season was heavy with early work hours and prolonged flight times. Data from these studies (Table 59.2 and 59.3) showed the wide range of cockpit exposure levels measured from 0 to 437 μg/m^3; lower values with longer sampling periods appeared to result from time dilution of peak exposures from runs through aerosol streams. Inhalation exposure rates per hour, based on a breathing rate of 25 l/min, were usually < 19 μg/h, although one case of 620 μg/h was measured. Total skin exposure ranged from 5 μg/h to 200 μg/h, of which 10 per cent was estimated to have been absorbed [16]. Wearing clean whole-body overalls reduced the estimated surface area for skin absorption to 2000 cm^2, but high temperature, sweating, prolonged exposure and delays before showering may have led to enhanced penetration.

Cockpit concentrations of parathion were especially high when wind direction was either parallel to the spray line or wind was absent but not when wind direction was 45–135° to the spray line. Additional exposures resulted from sprays, mists, dusts and vapours depositing residues in the cockpit at the landing site during washdown, maintenance and reloading. Air sampling at this site detected levels as high as 350 μg/m^3. Exposed pilots had blurred

Table 59.2 Parathion exposures in Israel during 1977 for pilot cockpit exposures in air[a]

Parathion concentration (μg/m^3)		*Sampling time* <30 min (11–21)	>30 min (35–260)
<100	No. of cases	5	17
	Median (μg/m^3)	9	14.1
	Range (μg/m^3)	(0–92)	(0–85)
	Rate (μg/h)[b]	14	21
>100	No. of cases	7	2
	Median (μg/m^3)	221.5	265.5
	Range (μg/m^3)	(158–437)	(121–410)
	Rate (μg/h)[b]	331	397.5

[a] Based on Cohen *et al.* [3]
[b] Odds ratio for likelihood that concentration >100 μg/m^3 when sampling <30 min: 11.9

Table 59.3 Parathion exposure in Israel during 1977 per ground crew: exposures in air[a]

Location	*Site*	*No. of readings*	*Sampling period (min)*	*Concentration (μg/m³) median (range)*
Field site	Loading area	9	32–218	15.9 (4.5–63.5)
	Storage unit	4	170–287	2.4 (1.9–25)[b]
	Office	1	221	3.2
Central maintenance and washing	Workshop	1	100	19.4
	Washing area	2	85–108	324.5 (292–357)
	Garage	2	107–110	54.8 (12.6–97)
	Office	1	97	43

[a] Based on Cohen *et al.* [3]
[b] Puddles on the floor

vision, dizziness, nausea, headache, weakness and thirst, and reported a subjective deterioration in skill level as the spray season progressed. Odour was not a sensitive indicator of exposure. That parathion pentrated the cockpit other than by the ventilation shuttle was suggested by the fact that exposures were detected even when the shuttle was closed.

Ground crew workers

Ground crew workers are exposed to contaminated dust blown from unpaved ground strips, mist from washdowns, direct contact with the contaminated surface and parts of the aircraft, and the volatilization of puddles of contaminant [3]. At loading sites without washdown, maximum parathion concentrations for aircraft loaders were usually $< 70\ \mu g/m^3$. At a central site for washing down of aircraft, levels as high as $350\ \mu g/m^3$ were measured. Skin exposure, measured by pads, ranged from 40 to 5000 μg/h; absorption was estimated at 10 per cent. Hexane handwashes showed up to 4000 μg from contamination from preparing and loading, holding loading pipes, and cleaning cockpit windshields without gloves.

Total absorption estimates and exposure-effect relationships: aerial sprayers

Total absorption estimates based on the above air and skin exposure studies, a 5-h work day, and 100 per cent airway and 10 per cent skin absorption led to the following conclusions. Some pilots absorbed parathion by inhalation alone at levels exceeding the acceptable daily intake (ADI) of 350 μg [12], but in most cass the ADI from the sum of the inhalation and percutaneous routes was below this threshold. Even so, many pilots reported symptoms attributable to antiChEs which directly interfered with their flying ability. For ground crew workers, parathion absorption occurred predominantly via the skin. Total daily estimated intakes sometimes were as high as 2500–3000 μg. Ground crew workers were at greater risk for acute intoxication and depressed levels of ChE, especially in the vicinity of aircraft washdowns.

Environmental control measures

For reducing exposure, effective environmental measures are: paving the landing ara, drainage, NaOH neutralization and washdown of puddles, separate loading, unloading and washdown sites, bottom-loading systems for spray planes, and closed-system loading and mixing systems. Neutralization of waste runoff may require special treatment, as described elsewhere [9]. Personal control measures include protective clothing, boots and gloves, and the proper use, storage and maintenance of masks. To control pilot airborne cockpit exposures, absolute filters are required to prevent aerosol penetration, and air ventilation and air cooling are needed to decrease pilot fatigue from heat stress [8]. Flying in box pattern rather than hairpin pattern avoids flying back into the spray plume. For pilots, the danger of diplopia from eye surface contact with droplets is itself an indication for

preventive action, even without evidence of systemic absorption. Lockers and showers for use immediately after work hours are mandatory. Vigorous scrubbing with soap and water within 30 min of application removes 80 per cent of a test dose of parathion as against 60 per cent after 5 h [16]. A patient was seen who complained of losing his direction driving home whenever he threw his contaminated clothes into the back seat of his car; this was confirmed by a trails test. Equipping pilots with atropine-oxime syringes may prevent death in the case of sudden contamination following a crash. Another possibility is the use of OPs of low acute toxicity.

The increasing use of ultralight aircraft without cockpits has introduced a new set of safety problems requiring surveillance and exposure assessment.

Routine monitoring of ChE levels or, preferably, urine alkyl phosphates, is especially important for pilots and ground crews, although normal levels do not exclude local eye toxicity. Intoxication should be suspected in persons who complain of headache, dizziness, nausea, breathing difficulties, runny nose, mucus secretion, abdominal pain and diarrhoea, or numbness of the extremities. Air and skin sampling should be reserved for periodically assessing the relative role of skin and air adsorption in new or difficult situations; this complements but is no substitute for biological monitoring.

Flagmen are notoriously prone to overspray, contamination and poisoning. The practice of using adults or children instead of flags on poles for this dangerous and unnecessary measure should be banned.

The case for incorporating all these measures into an international code of practice of pre-crash, crash and post-crash phase preventive measures is self-evident.

Low-level exposures

Even with substantial cleanup and reduction in exposure, workers when asked may still report complaints such as headache, fatigue, dizziness and limb paraesthesiae. ChE levels may be depressed < 15–30 per cent from baseline and in-season fluctuations may not give a clearcut picture of what is happening. Furthermore, pre-season 'baseline' ChE levels may also be depressed from cumulative previous exposure [1]; this compromises the sensitivity of in-season tests. Studies of sural and peroneal nerve peak conduction amplitude may show depressions within normal limits [21].

When accurate baseline values for ChE representing true pre- (or post) exposure situations are available, it may be possible to use a Cusum statistical technique to plot systematic cumulative trends in ChE depression. This simple technique, which any health worker can be taught, makes it possible to extract and amplify systematic trends from within-normal limit seasonal fluctuations. It has been found that persons with within-normal depressions in ChE levels which are amplified by the Cusum technique are more apt to complain of headaches, dizziness, fatigue, weakness and tingling in legs and arms compared with persons in whom Cusum analysis shows that ChE activity does not drop from the baseline [21]. Such findings suggest that symptom questionnaires together with Cusum-analysed ChE levels may be a reasonable low-cost substitute for alkyl phosphate determinations in monitoring. Weakness and tingling in the legs may suggest a possible NTE effect (see Chapters 9, 10 and 11).

Hazard for residents living near sprayed areas

Data are not readily available on alkyl phosphate measurements in persons exposed to drift.

In 1969, Mizrahi [13] reported episodes of skin rashes, burning eyes, nausea, runny noses, vomiting and dizziness, together with depressed plasma or RBC ChE levels in children from migrant labour camps exposed to OP drift. Sprays were applied to fields as close as 3 m from residential areas, and complaints persisted in residents for up to 14 days; ChE activity in children of migrant workers living in ranch houses supplied by the grower was depressed, but none was critically abnormal. Symptomatic children had lower 'within-normal' limit values compared with asymptomatic children from the same group (Table 59.4). It was difficult to interpret the fact that asymptomatic children themselves

Table 59.4 ChE levels in children of farm labourers in California[b]

	Symptomatic[c] (n=29)		*Asymptomatic (n=29)*		*Normal values[d] in adults[e]*
	Mean	*(s.d.)*	*Mean*	*(s.d.)*	*Range*
BChE	3.46	(0.86)	4.18	(0.87)	3.6–6.8
RBC AChE	11.22	(2.32)	12.42	(1.17)	11.1–16.0

[a] Values are μM/min/ml
[b] Parental report
[c] Method and normal values from Nabb and Whitfield [15]
[d] Normal values not available for children
[e] Based on Mizrahi [13]

had a lower range of normal ChE levels compared with normal adults values from non-exposed persons because of the lack of population-based normal values for ChE levels in children. Reduced ChE levels were seen in children who did not work in fields as well as those who did (Table 59.5). Only a third of children with symptoms attributable to OP toxicity had seen a doctor for their symptoms. Frequently, there was exposure to drift from fields which were nearby but belonged to owners other than the employers or patients of the involved children.

Mizrahi's observations indicated that toxic effects were associated with residential exposures to residues in drift, although field work and ingested fruits and vegetables were suggested as additional sources of exposure. His findings also questioned the adequacy of thresholds for levels of depressed ChE for protecting children when based on data from adult field workers. In these same children, blood residues of DDT and DDE generally exceeded normal values. This indicates that multiple exposures to several types of pesticides may have been occurring.

In Israel, there have been widespread complaints of headache, dizziness, diarrhoea, nausea, skin eruptions, difficulty in breathing and worsening of pre-existing respiratory disease among adults and children of kibbutzim and moshavim (small collective and cooperative settlements) which were attributed to aerial spray drift (unpublished data). In one report, nine women and two men who lived near fields which were crop-dusted had complained of weakness, dizziness, irritability, cramps, nausea and diarrhoa; ChE levels were depressed 30–60 per cent compared with post-season levels, when their symptoms abated [17].

Table 59.5 ChE levels in children related to work status in California during 1969

ChE levels	*Worked in fields*				
	Yes		*No*		*Total*
	No. of cases	*(%)*	*No. of cases*	*(%)*	
Depression >25%	3	17.6	2	4.9	5
Below lower limit of normal	7	41.2	8	19.5	15
Normal range	7	41.2	31	75.6	38

During the 1980s, OPs were heavily used for pest control in orchards and cotton fields bordering small settlements of some 500 adults and children. Aerial spraying was mainly used for applying insecticides to fields within a radius of 50 – 1000 m from residential areas. In a 1-month survey (July 1983) in one settlement surrounded on all sides by cotton fields, dispensary records were tabulated with data on aerial spray date, location of field sprayed and agent sprayed (OP, organochlorine or liquid fertilizer). Total visits on 'spray days' for each agent were compared with those on two types of control days: the previous day and the same weekday of the previous week. This study design compensated for the confounding effect on hospital visits associated with day of the week. Table 59.6 shows that on OP spray days; there were pronounced increases in respiratory complaints, and in addition headache, eye problems and 'other problems' (a category including cardiovascular and blood pressure) [20]. Sequential ChE levels in this

Table 59.6 Visits to infirmary (July 1983), type of compounds sprayed and duration of spraying (preliminary data)

Category of complaints	*No. of visits*								
	Organophosphate (3 days)			*Organochlorine (3 days)*			*Liquid fertilzer (2 days)*		
	S–0[a]	S–1[a]	S–7[a]	S–0[a]	S–1[a]	S–7[a]	S–0[a]	S–1[a]	S–7[a]
Respiratory complaints (cough, shortness of breath, sore throat)	22	7	3	14	5	5	6	1	4
Gastrointestinal (diarrhoea)	5	3	0	4	5	2	1	2	1
Skin (rash, itching)	3	2	0	3	0	1	1	0	0
Headache	7	1	1	2	0	0	0	0	0
Neurological (other than headache)	3	2	4	3	1	1	0	0	0
Eye (irritation, conjunctivitis)	7	1	0	2	2	0	1	0	1
Other (including cardiovascular problems)	8	5	1	3	1	1	3	0	2

[a]S–0, spraying days; S–1, the day before spraying; S–7, the same weekday of the previous week without spraying

Table 59.7 Baseline[a] and in-season ChE activities in field workers and residents

Workers (n = 23–26)[b]				*Residents exposed to spray drift?*					
ChE activity (U/l)	*Baseline (mean ± s.d.)*	*In-season (mean ± s.d.)*	*Change (%)*	*Yes (n = 11)*			*No (n = 7)*		
				Baseline (mean ± s.d.)	*In-season (mean ± s.d.)*	*Change (%)*	*Baseline (mean ± s.d.)*	*In-season (mean ± s.d.)*	*Change (%)*
Plasma	4.99 (±1.01)	4.68 (±0.892)	–6.2	5.19 (±0.52)	4.81 (±0.55)	–7.3	4.44 (±0.61)	4.36 (±0.61)	–1.8
RBC	34.7 (±2.92)	34.06 (±2.82)	–1.8	34.9 (±2.7)	33.2 (±3.3)	–4.8	33.1 (±(5.3)	32.40 (±3.60)	–2.1
Whole-blood	4.98 (±0.81)	4.33 (±0.88	–13	5.00 (±0.31)	4.76 (±0.36)	–4.8	4.59 (±0.34)	4.50 (±0.19)	–2.0

[a] Baseline: at least three pre-season or post-season (i.e. late autumn) determinations
[b] For workers, $n = 26$ for plasma, 23 for RBC and 24 for whole blood, respectively

population were compared with levels from field workers and residents in a kibbutz not exposed to spray drift. Table 59.7 shows that in sentinel individuals (aged 14–17 years) from the kibbutz exposed to spray drift, there were in-season drops which were slightly greater than in residents from the kibbutz not exposed. These drops were in the range approaching the precision of the method for ChE [24].

In 1987, the Israel Ministry of Health Laboratory for Drug Standards examined mid-day urine specimens from field workers and residents from several kibbutzim whose residents were exposed to drift from sprayed fields (methidathion, parathion, pirimiphos-methyl) in the Huleh valley [10]. In urine specimens from field workers and residents, there were wide variations in levels of diethyl and dimethyl phosphate (DEP and DMP), but mean and median levels were more than one log order of magnitude higher than 0.01 mg/1, the threshold of detection. That metabolites

Table 59.8 Urine alkyl phosphates versus changes in ChE levels in Israel during 1987

DMP and DEP levels[a] versus change in ChE (whole blood) from baseline					
DMP (mg/l)	*n*	*ChE Mean drop*	*DEP (mg/l)*	*n*	*ChE Mean drop*
>0.5	8	–8.4%	>0.5	5	–8.4%
<0.5	20	–6.1%	<0.5	23	–6.4%
>0.1	20	–6.4%	>0.1	21	–7.4%
<0.1	8	–7.5%	<0.1	7	–4.8%

[a] Corrected for creatinine concentration
[b] Urine specimens taken on same day that bloods were drawn for cholinesterase (August 1987)
[c] Agents sprayed: parathion, pirimiphos-methyl, methidathion

were found in some residents post-season is puzzling but may reflect direct contact with residues and their oxidized intermediates which persist for substantially longer periods in the cooler autumn and winter weather. In residents with concurrent ChE and DEP and DMP determinations, the degree of depression

Table 59.9 DMP and DEP concentrations in the urine of five residents with symptoms[a] and five asymptomatic residents (matched for age, sex and day of visit)

Matched Pair	*Symptomatic residents*				*Asymptomatic residents*			
	Age (years)	*Sex*	*DMP (mg/l)*	*DEP (mg/l)*	*Age (years)*	*Sex*	*DMP (mg/l)*	*DEP (mg/l)*
1	27	F	1.00	ND	22	F	0.07	0.06
2	40	F	0.47	0.180	44	F	ND	1.00
3	65	M	0.71	0.930	50	M	ND	0.02
4	15	F	ND	0.170	15	F	ND	0.02
5	8	M	0.59	0.910	7	M	0.49	0.40
Mean			0.55	0.418			0.11	0.15
Median			0.59	0.180			–	0.10
Range			ND–1.000	ND–0.93			ND–0.49	0.02–0.4

[a] Patients presented with nausea and abdominal pain (n=5), diarrhoea (n=4), headache (n=2) and dizziness (n=2)
[b] ND, not detected

in ChE (whole blood) levels was greater in those with DMP or DEP levels of > 5 mg/l compared with those with lower levels (Table 59.8). When a cut-off point of 0.1 mg/l was set, a similar difference for ChE was seen with DEP, but not DMP. Metabolites were detected in the urine of residents living 500–1000 m from sprayed fields; this questions the adequacy of the 120 metre guideline recommended by the regulatory authorities.

In a small case-control study, median and mean DMP and DEP levels in the urine of five residents with non-specific complaints were higher compared with five age 6 years date-matched residents without complaints (Table 59.9). The odds ratio for an association between DMP or DEP levels of > 0.1 – 0.5 mg/l and having one or more of the above symptoms was of the order of 5 – 7.5 (13 symptomatic versus 13 asymptomatic) (Table 59.10).

Table 59.10 Threshold for DMP and DEP levels in relation to symptoms[a] in Israel during 1987

DMP or DEP (mg/l)	*At least one symptom*	*No symptom*	*Odds ratio*	*P*
>0.5	5	1	7.5	<0.07
<0.5	8	12		
>0.3–0.4	7	2	6.5	<0.05
<0.3–0.4	6	11		
>0.2	8	3	5.3	<0.05
<0.2	5	10		
>0.1	10	6	5.8	<0.05
<0.1	3	7		

[a] Urine collected on day on which patient complained of symptoms
[b] DMP and DEP levels corrected for creatinine concentration
[c] Symptoms (headache, dizziness, nausea, abdominal pain, diarrhoea) in 13 patients and 13 comparison individuals exposed to drift

Monitoring potential exposures

Variations in local topographic, weather and wind conditions, type and amount of spray, and height of application make it difficult to establish absolute guidelines for safe distances between aerial spray swathes and residential area. Therefore, the adequacy of a distance threshold has to be evaluated wherever such a threshold is promulgated and implemented. To monitor potential exposure to drift, Anderson samplers, glass plates and drift pads can be used for passive collection [23]. Bee colonies are vulnerable to OP-containing aerial spray drift and can be used for sentinel environmental monitoring, but the optimum remains biological monitoring based on ChE levels, urine alkyl phosphates, and medical history and examination.

Preventative measures and their evaluation

Positive actions by spray pilots, farmers and populations themselves are required to ensure freedom of exposure of residential populations from drift.

The case for action is clear when residents have complaints of headache, dizziness, chronic fatigue, abdominal pain, diarrhoea and breathing problems on days when there is drift from aerial spraying. Proof of exposure to pesticide drift is provided when urine alkyl

phosphates are found in symptomatic or asymptomatic residents or when plasma, RBC or whole blood ChE levels are depressed in comparison with baseline measurements or levels in unexposed persons. Preventative action to protect populations includes reducing the use of antiChEs, increasing the time interval and space between the spray and the exposed populations, and modifications of spray technology to reduce drift.

In individual children and adults in settlements exposed to pesticide drift, the differential diagnosis of patients presenting with headache, fatigue, dizziness, sudden changes in blood pressure, abdominal pain, diarrhoea and allergy-type breathing problems should include low-level exposure of OPs. This also applies for other more persistent complaints, insomnia, changes in mood, and arrhythmias. Complaints suggestive of sensory neuropathies should also be evaluated. DMP or DEP levels of > 0.1 mg/l should indicate a causal relationship between exposure to OPs and symptoms when specimens are collected within 24 h of illness (Table 59.10). High residues may be present 2–3 days later. ChE levels may remain within so-called normal limits even when substantial levels of alkyl phosphates are found in the urine and individuals are symptomatic. One recommended threshold for depressed ChE levels for triggering preventive action in workers is a depression of > 30 per cent below a well-established baseline, until the value rises to 80 per cent of the pre-exposed baseline [22]. This threshold was not established on the basis of medical or epidemiological data showing a no-effect level with smaller falls, but represents the drop from a baseline level based on three determinations reaching a level of statistical significance of $P < 0.05$ [24]. This threshold cannot be considered adequate for the protection of residents exposed to drift containing antiChEs against acute and subacute effects associated with the depression of ChE in the nervous system.

After exposure to drift containing CBs, carbamylated ChE can be reactivated during storage of blood, and further inhibition or partial recovery after sampling and before assay is an artefact which may compromise the sensitivity of ChE for CBs. Therefore, reliance on this test to rule out exposures to CBs may result in a false sense of security.

A definitive statement on a recommended no-effect level awaits the outcome of long-term follow-up studies of workers and residents who have had repeated low-levels of exposures to OPs. Until such data becomes available, prudence dictates measures to reduce all exposures to levels as low as possible. Urine alkyl phosphate levels should be < 0.1 mg/l and as close as possible to the threshold of detection (0.01 mg/l) in populations under surveillance. If the alkyl phosphate assay is not available, the use of ChE data analysed by the Cusum method appears to be a reasonable substitute, provided the laboratory meets quality control requirements for accuracy and reproducibility [24].

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[Square brackets contain text abbreviations]