Environmental Chemicals, Enzyme Function and Human Disease

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Environmental Chemicals, Enzyme Function and Human Disease
DAVID EVERED and GERALYN LAWRENSON

Chairman's introduction

T.A. CONNORS

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This is an opportune time for the discussion of environmental chemicals and their interaction with drug-metabolizing enzymes. Research in this area has, over the past few years, led us to revise our opinion of the safety of many chemicals to which human beings are exposed through the general environment (at work, for example, and in food additives, medicines and pollutants). We once thought these chemicals were innocuous because people could be exposed to them for long periods without developing any obvious symptoms of toxicity. However, extensive research, some of which we shall discuss at this symposium, has shown that many of these environmental agents may be associated, in the long term, with the development of cancer or other serious diseases. One of our symposium members, Dr Higginson, believes that we have reasonable evidence that most human cancers are related to the environment; we now know some of the hazards of the chemicals in our environment, and their interaction with drug-metabolizing enzymes.

A key discovery in this field was that chemically inert substances causing cancer in animals could be converted, by drug-metabolizing enzymes, into reactive metabolites (Miller & Miller 1974). This explained many observations that were not previously understood. About 40 years ago Sir Alexander Haddow commented that it was strange for the chemically reactive nitrogen-mustard, mechlorethamine, to share so many biological properties, including cancer induction, with what he called the 'inert' benzo[*a*]pyrene; all was explained once it was demonstrated that such 'inert' chemicals could be metabolized, *in vivo,* to electrophilic reactants. This discovery demonstrated a simple molecular mechanism whereby structurally dissimilar chemicals could initiate cancer via a common pathway. The reactive species produced could bind covalently with DNA to form promutagenic bases, and this constituted the initiating step in the process of cancer induction. A further conse-

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quence of the discovery was that the somatic mutation theory of cancer was strengthened; this led to the development of simple tests on the interaction of a chemical (or its metabolite) with DNA, by which the carcinogenic potential of the chemical could be predicted. At present many agents are *suspected* of being carcinogenic to humans but we do not yet have enough evidence to determine whether they are hazardous to humans under *normal* conditions of exposure.

However, I believe that we can already classify some chemicals as human carcinogens. The alkylating agents and related cytotoxic agents used in the treatment of cancer are reactive species and they bind extensively to DNA. These must be considered hazardous, and there is evidence that some members of the class have caused cancer in humans. The 5-nitrofurans are used to treat human infections, but they probably act only after reduction of the nitro group and generation of electrophilic reactants. Since mammalian enzymes can also reduce the nitro group, alkylation of DNA and initiation of cancer remains a possibility when the 5-nitrofurans are used therapeutically.

However, with most chemicals that are suspected of being carcinogenic, reactive species may be formed only by a minor pathway and under abnormal conditions. In these cases, reactive metabolites may have been detected, in the first place, only by highly sensitive analytical techniques or *in vitro* tests. Evidence for carcinogenicity in animals may have come only from bioassays in which large and sometimes toxic doses of the chemicals were administered for long periods. As more and more chemicals are suspected of being carcinogens, there will be increasing pressure on us to define more precisely the relationship between these tests and the risks to humans. Recently I searched for published reports of medicines that have been both used in the last 15 years and tested for carcinogenicity. Of 84 substances tested, 66 had produced evidence of carcinogenicity in animals. I hope that as a result of the discussions at this symposium we may gain a better understanding of the dangers to humans when these chemicals are used under normal conditions.

We shall be discussing how drug-metabolizing enzymes are influenced by a variety of endogenous and exogenous factors in both *in vitro* systems and controlled animal experiments. We shall then consider how these results apply to humans and we should identify the areas where more research is needed. I hope also that we may be able to formulate guidelines about the use of substances that are now suspected to be human carcinogens. What, for instance, is the relevance of animal experiments in which only high doses are carcinogenic? Also, how do we begin to extrapolate from animals to man in our attempts to quantify and predict risks of carcinogenicity? I hope these topics will lead to some fruitful discussion in the next three days.

Reference

Miller EC, Miller JA 1974 Biochemical mechanisms of chemical carcinogenesis. In: Busch H (ed) The molecular biology of cancer. Academic Press, New York, p 377-402

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DAVID EVERED and GERALYN LAWRENSON

The role of the drug-metabolizing enzymes

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Abstract Drug-metabolizing enzymes function in the biotransformation of both endogenous and exogenous lipophilic compounds. Phylogenetic studies indicate that the drug-metabolizing enzymes were a late evolutionary development. Stimuli for the evolution of these enzymes were probably movement to a terrestrial environment, a diet of higher plants and an increasing tissue specialization, with a consequential need for the formation and inactivation of hormones, bile salts etc. Most drug-metabolizing enzymes exist in multiple forms. Some are concerned solely with the metabolism of a very limited range of endogenous lipids; others such as 'phenobarbitone-type cytochrome *P*-450' seem to be concerned mainly with the metabolism of exogenous compounds. In mammals the liver and intestine have a major role in the biotransformation of exogenous compounds, whereas in other tissues the primary function of the drug-metabolizing enzymes appears to be the metabolism of endogenous lipids such as steroids, lipid-soluble vitamins and fatty acids.

The metabolism of exogenous lipophilic compounds (xenobiotics or foreign compounds) by higher animals occurs predominantly in the liver and intestine but is also brought about by other tissues. This metabolism is normally a two-stage process. In the first phase (phase 1) an electrophilic substituent group such as $-OH$, $-NH_2$, $-COOH$ or $-SH$ is inserted or revealed through the action of oxidation, reduction or hydrolytic enzymes. In the second phase (phase 2) this substituent group is conjugated, typically with a carbohydrate, amino acid or inorganic acid. The overall effect of this metabolism is to convert lipophilic compounds, which tend to pass readily into cells and bind to cellular components, into more lipophobic products which can be excreted actively or passively by cells. The enzymes carrying out these two phases of metabolism are commonly referred to as the *drug-metabolizing enzymes*. The name 'drug-metabolizing enzymes' is a misnomer for the enzymes which, in addition to transforming drugs, metabolize a very wide range of synthetic and

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naturally occurring xenobiotics. Many endogenous lipophilic compounds are also metabolized to more or less bioactive products by the single or combined action of oxidative, reductive, hydrolytic or conjugative enzymes. The enzymes that metabolize endogenous lipophilic compounds appear in many cases to be similar or identical to those concerned with exogenous compounds. Therefore the true function of the drug-metabolizing enzymes may be either to facilitate the clearance from the body of exogenous lipophilic compounds which it is unable to prevent from being absorbed, or to control the synthesis and degradation of bioactive endogenous lipophilic compounds such as sterols, steroids, prostaglandins, thyroxine, bilirubin and similar substances. The true function(s) of the drug-metabolizing enzymes may be derived from consideration of their distribution and activities under different circumstances.

EVOLUTIONARY DEVELOPMENT OF DRUG-METABOLIZING ENZYMES IN ANIMALS

Let us consider first the evolution of the enzymes that metabolize *exogenous* lipophilic compounds. In the simpler organisms, many xenobiotics are degraded by hydrolytic, reductive or oxidative enzymes to less complex structures which can be used wholly or partially in intermediary pathways of metabolism. In higher organisms this degradative ability is largely lost and as a consequence some means of clearing non-degradable exogenous lipophilic compounds from the body is more necessary. This is reflected, by and large, in a parallelism between the complexity of an animal and the range and total activity of its drug-metabolizing enzymes. The conjugating reactions appear to have developed considerably earlier than the forms of cytochrome *P*-450 with a broad substrate specificity (see Fig. 1) in spite of the fact that non-*P*-450 cytochromes are found in all animals. The *Platyhelminthes* (flatworms) are the simplest animals in which drug-metabolizing enzyme activity has been detected. As we ascend the phylogenetic tree, an increasing range and activity of conjugating enzymes is observed (Smith 1977). Interestingly, on the arthropoda branch, glucose is the main form of carbohydrate conjugate whereas on the vertebrate branch glucuronic acid is preferred. Oxidative activity of the cytochrome *P*-450-type is very low in crustaceans but is much higher in other arthropods, such as insects and arachnids. With the exception of glucuronic acid conjugation, insects appear to show all the major drug-metabolizing reactions observed in mammals. Reptiles, amphibia, fish, birds and mammals have qualitatively similar pathways of drug metabolism. However, considerable quantitative differences are apparent, the usual order

of enzyme activities being amphibia = fish < birds < mammals. In addition, mammals are considerably more versatile than fish and birds in the range of substrates metabolized effectively by individual phase I reactions. This difference is most marked in the case of cytochrome *P*-450-dependent reactions. Such differences might be explained by the development of additional isoenzymes in mammals; e.g. in mammalian liver, phenobarbitone is able to induce a form of cytochrome *P*-450 that has a particularly broad substrate specificity. This form of cytochrome *P*-450 is not induced in fish, in early mammalian fetuses or in de-differentiated hepatocytes in culture (Elcombe & Lech 1979, Guenther & Mannering 1977, Bridges & Fry 1978), whereas

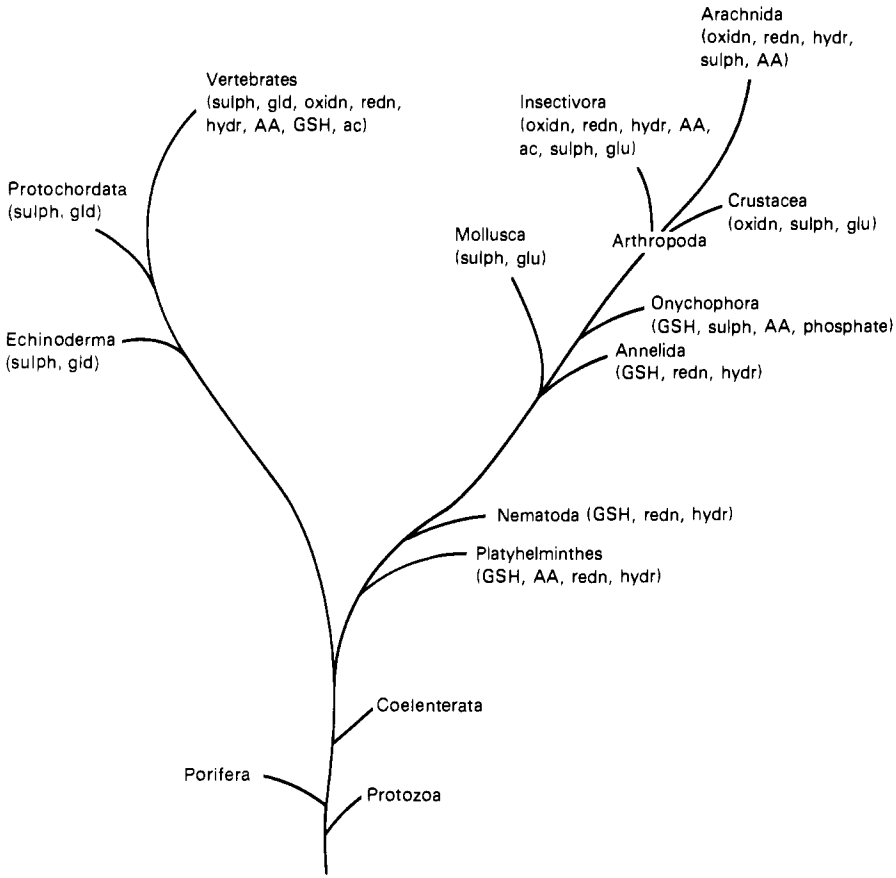

FIG. 1. Phylogenetic tree showing the occurrence of drug-metabolizing enzymes. (oxidn = oxidation, redn = reduction, hydr = hydrolysis, AA = amino acid conjugation, GSH = glutathione conjugation, ac = acetylation, sulph = sulphate conjugation, glu = glucose conjugation and gld = glucuronic acid conjugation).

3-methylcholanthrene, which promotes the synthesis of a form of cytochrome *P*-450 (*P*-448) with a much more limited substrate range, is an effective inducer in all these circumstances. The range of drug-metabolizing enzyme activities (expressed in nmoles·mg protein^{-1}·min^{-1}) among vertebrates for cytochrome *P*-450-dependent reactions, glucuronyl transferase (EC) 2.4.1.17) and epoxide hydrolase (EC 3.3.2.3), is greater than 1000-fold. Among both insects and the various classes of vertebrates there is a general tendency for flesh eaters to have lower drug-metabolizing enzyme activities than herbivores (Walker 1980, Brooks 1979). Carnivorous mammals are deficient in a number of important conjugation enzymes. The cat shows a much more limited range of glucuronic acid conjugation reactions than other mammals; dogs are deficient in arylamine acetylase (EC 2.3.1.5) and both cats and dogs have a poor ability to conjugate glycine in hepatic mitochondria.

A comparable study of the activity of the drug-metabolizing enzymes towards *endogenous* substrates over a wide species range has not been made. However, it is interesting that many of the endogenous substrates for the mammalian drug-metabolizing enzymes, e.g. sterols, steroids, thyroxine, bilirubin, bile salts, are either absent or occur at very low concentrations in lower animals. Thus, [C_{24}] bile acids are characteristic of higher vertebrates with calcified structures; steroids are important in insects and chordates but probably not in lower animals; while below protochordates the thyroid gland, and presumably thyroxine, are absent.

It may be concluded from the above data that the following major factors probably contributed to the development of the drug-metabolizing enzymes.

1. Higher animal forms. With increasing sophistication of organisms, specialization of tissue functions would become necessary. This would have three important effects, an increased need for inter-tissue communication through hormones and other mediators, an enhanced complexity of tissue components requiring removal from the body, and a reduced versatility in the degradation of ingested materials to nutrient sources.

2. Terrestrial environment. Transition of animal habitat from fresh water to land would necessitate water conservation and therefore the production of hypertonic excretory fluids. Excretion of significant amounts of lipophilic compounds would not be possible since they could not be concentrated in excretory organs because of their ability to pass readily across cell membranes back into the body. Therefore, such compounds would need to be metabolized to non-lipophilic forms in order to be excreted. Brodie & Maickel (1961) have suggested that fish and amphibia do not need detoxification enzymes

because they can excrete lipophilic substances through their gills and skin respectively. However, Adamson & Sieber (1974) have shown that fish are unable to clear such compounds readily through their gills. Metabolism followed by excretion in the urine and the bile seems to be an important clearance route (Guarino & Anderson 1976), even in aquatic vertebrates.

3. Diet. Carnivorous animals are exposed through their diet to a relatively narrow range of non-nutrient chemicals. In contrast, herbivores, particularly those with catholic tastes in higher plants, ingest an extremely complex range of chemical structures, e.g. flavonoids, alkaloids, quinones, phenols and terpenes. Many of these substances are potentially toxic (Harborne & Simmonds 1964) and require a means of effective inactivation.

4. Environmental chemicals. Within the last two hundred years, all organisms have become exposed to an increasing number of synthetic organic chemicals. Those animal species exposed to high doses of chemicals, and having a short period for the onset of sexual maturity, may have mutated to forms that had elevated activities of certain drug-metabolizing enzymes. In this context, it is interesting to note the wide range of insects that have developed resistance to pesticides, through an improved capacity to metabolize them (Brooks 1979) and also that pesticide-resistant strains of fish are emerging in heavily polluted waters (Wells et al 1973). These rapid evolutionary developments are likely to have increased the protection against acute toxins but probably not that against slowly acting toxins (e.g. carcinogens), many of which are effective towards the end of an animal's reproductive life.

From the data on species distribution currently available it is not possible to resolve which, if any, of the above factors was the primary influence in the development of the drug-metabolizing enzymes in animals.

TISSUE DISTRIBUTION AND SUBSTRATES OF THE DRUG-METABOLIZING ENZYMES IN MAMMALS

All the data from vertebrates that I have discussed so far were obtained from studies of the liver. In all the mammals that have been examined, the liver has proved to be the most active organ in the metabolism of exogenous compounds. The intestinal wall and, to a lesser extent, the lung are relatively versatile drug-metabolizing organs. Many other organs are significantly able to conjugate but appear to have limited oxidative enzymes active against exogenous model compounds. Cytochrome *P*-450-dependent aryl hydrocarbon hydroxylase (*P*-448, EC 1.14.14.1) is present at low concentrations in most

TABLE 1

Extrahepatic tissue distribution of microsomal cytochrome *P*-450 in the rat

	Cytochrome P-*450* *nmoles/mg protein*	*AHH Activity (pmoles· mg protein^{-1}· min^{-1})*	*Effective inducing agents*
Testis	0.06 – 0.1	2.5	–
Ovary	–	0.77	3-MC
Placenta	–	8.5[a]	3-MC
Adrenal	0.5 – 1.12	2167[a]	–
Mammary gland	–	4.6[a]	–
Kidney	0.11 – 0.20	2.5	3-MC
Spleen	0.023 – 0.025	16.6[a]	BP
Brain	0.025 – 0.051	0.08	3-MC
Pancreas	–	1.5	3-MC
Salivary gland	–	5.3[a]	BP
Prostate	–	9.6[a]	BP
Liver	0.86	1442	3-MC

[a]pmoles·g tissue^{-1}·min^{-1}. BP, benzo[*a*]pyrene; 3-MC, 3-methylcholanthrene; AHH, aryl (aromatic) hydrocarbon hydroxylase, i.e. benzo[*a*]pyrene hydroxylase (EC 1.14.14.1); –, not measured.

organs. This may indicate a particularly great need for the metabolism of exogenous aromatic hydrocarbons, or it could reflect the paucity of research on the metabolism of other foreign compounds, or it could simply indicate a minor activity of a form of cytochrome *P*-450 whose primary role is to metabolize endogenous lipids. However, most organs have significant levels of cytochrome *P*-450 (see Table 1) which are induced by 3-methylcholanthrene. In the steroidogenic organs, the predominant role of both the mitochondrial and microsomal cytochrome *P*-450 appears to be the formation and degradation of bioactive steroids. In other organs it is less easy to discern the major endogenous substrates of cytochrome *P*-450 (Table 2) (Connelly & Bridges 1980). ω- and ω-1 oxidation of fatty acids, metabolism of lipophilic vitamins, and formation and degradation of prostaglandins are possible candidates. The kidney for example, is active in ω- and ω-1 fatty acid oxidation and vitamin D hydroxylation, while platelet cytochrome *P*-450 acts in prostaglandin formation. The brain cytochrome *P*-450 may be responsible for the synthesis of catechol oestrogens, but the importance of these products is not understood. Flavin-dependent microsomal mixed function oxidase (EC 1.14.13.8, Ziegler's enzyme) which metabolizes many sulphur- and nitrogen-containing exogenous compounds is found in most mammalian tissues. According to Ziegler & Poulson (1977), the enzyme's true function may be to

TABLE 2

Cytochrome *P*-450-mediated metabolism of endogenous lipids

Steroids:	*Fatty acids:*
2α and 2β-hydroxylation	ω-oxidation
6α and 6β-hydroxylation	ω-1 oxidation
17α-hydroxylation	
21-hydroxylation	*Prostaglandins:*
C-17, 20-lyation	prostacyclin and/or
7α-hydroxylation	thromboxane formation and/or
aromatization	degradation?
11β-hydroxylation	ω-oxidation
14α-hydroxylation	ω-1 oxidation
15α-hydroxylation	
16α-hydroxylation	*Vitamins:*
18-hydroxylation	25-hydroxylation
oestrogen 2-hydroxylation	
cleavage of cholesterol side chain	
formation of bile salt (26-hydroxylation)	
14α-demethylation of lanosterol	

regulate cellular levels of cysteamine, and thereby to control formation of protein disulphide.

The natural substrates for epoxide hydrolase are even less certain. In insects, juvenile hormone is a substrate. However, the enzyme responsible is located in the cytoplasm, whereas epoxide hydrolase (which is responsible for metabolism of exogenous compounds) is almost solely microsomal. Steroids may be important substrates in mammals. The high concentrations of epoxide hydrolase in steroidogenic organs such as the testis would support this view.

The true function of the conjugation enzymes is less clear-cut than it at first appears. Among the conjugation reactions the importance of glucuronic acid in the conjugation of bilirubin, steroids and thyroxine is well established. Sulphation of steroids and bilirubin is also a major reaction, as is the amino acid conjugation of bile salts. In the case of glucuronidation, the available evidence suggests that its predominant role is to aid the biliary and urinary excretion of unwanted endogenous and exogenous lipophilic compounds. Evidence for assisted transport of glucuronides into bile supports this interpretation, although the widespread tissue distribution of β-D-glucuronidase (EC 3.2.1.31) is difficult to rationalize if this simplistic interpretation is accepted. Sulphation, in addition to aiding excretion, may have a significant role in the transport of certain bioactive forms of steroids between tissues.

Whether sulphation is necessary for the transport of other endogenous lipids (and possibly of exogenous compounds) between tissues remains to be established. Glutathione conjugation appears to be important in the inactivation of electrophiles which might otherwise react with cellular proteins, cofactors and nucleic acids. In view of the close association between glutathione transferases and glutathione peroxidase activity one might speculate that an important additional physiological role of these enzymes may be the binding and detoxification of organic peroxides such as peroxidized membrane lipids (Lawrence & Burk 1976). The role of other conjugation reactions is more difficult to identify. Amino acid conjugation has features that make it an atypical phase II reaction of drug metabolism: the enzyme that metabolizes simple aromatic acids is located in the mitochondria and it requires the activation of the substrate (through the aegis of ATP and coenzyme A) rather than the use of a readily available activated form of an endogenous acid (c.f. conjugation with glucuronic acid, sulphate and acetate, which employ UDPGA, phosphoadenosyl phosphosulphate (PAPS) and acetyl CoA respectively). Possibly the true role of amino acid conjugation is to protect mitochondria from aromatic carboxylic acids that interfere with the tricarboxylic acid cycle (which is the major pathway for producing the reduced cofactors essential for deriving cellular ATP). However, if this protective function operates it is hard to understand why the reaction should be largely confined to the liver and the kidney. The purpose of arylamine acetylation is also poorly understood. In many cases the product of the reaction, although less lipophilic, is also less water soluble, as demonstrated by the many cases of oliguria and anuria in humans treated with the early antibacterial sulphonamides.

FUNCTIONS OF THE DRUG-METABOLIZING ENZYMES OTHER THAN IN LIPID METABOLISM

A number of authors, attracted by the ability of cytochrome *P*-450 to combine with and metabolize oxygen, have suggested that this may be one of its major functions. Roles proposed for the enzyme include the retention and transport of oxygen, the detoxification of active oxygen species and the controlled peroxidation of lipid membranes to facilitate their regular turnover. Cytochrome *P*-450 could also act in a futile pathway for disposition of excessive reducing equivalents through the oxidation of NADPH. Although these suggestions are compatible with the established properties of cytochrome *P*-450, they are unlikely to be key functions because cytochrome *P*-450 has a rather low oxygen turnover and in most cells there are more effec-

tive alternatives, e.g. myoglobin, peroxidases and dismutases, to carry out these tasks. Takagi (1977) and Ohlsson & Jergil (1977) have suggested that rat hepatic cytochrome *P*-450 may facilitate the binding of polysomes to the endoplasmic reticulum. The physiological relevance of these findings remains uncertain. However, the results of these two research groups imply that cytochrome *P*-450 could control the synthesis of at least some proteins.

A logical extension of the argument in the preceding section on conjugation reactions is that the true role of the enzymes might be to insert a substituent onto an endogenous lipid or xenobiotic which can be recognized by cellular uptake and excretory mechanisms. Differential distribution of recognition sites would then lead to selective uptake and/or excretion of metabolites by cells of different tissues. If this is the case, one might speculate that the major role of the deconjugation enzymes associated with plasma membranes in normal healthy tissues is recognition, rather than hydrolytic cleavage. At present, however, there is little evidence to support such a concept. In various disease states, many of these deconjugating enzymes lose their tight compartmentalization and therefore have a greater opportunity to hydrolyse circulating conjugates of endogenous lipids and xenobiotics; the physiological or pathological significance of this remains uncertain.

MODIFICATION OF ENDOGENOUS LIPID METABOLISM BY EXOGENOUS COMPOUNDS

The majority of the drug-metabolizing enzymes that have been thoroughly investigated have been found to exist in multiple forms. The questions arise about whether endogenous and exogenous lipophilic compounds are metabolized by the same or by different forms of a particular enzyme, and whether exogenous enzyme inducers can modify the metabolism of endogenous lipids as well as that of xenobiotics. There is considerable evidence that phenobarbitone-type inducers increase the metabolism of vitamin D, bilirubin and cortisol in both animals and humans by increasing the concentrations of particular hepatic drug-metabolizing enzymes. Vitamins A and K, thyroxine and oestrogens may be other endogenous substrates whose metabolism is induced by drugs (Hunter & Chasseaud 1976). Inhibition studies also indicate the presence of interactions between endogenous and exogenous compounds at the level of the drug-metabolizing enzymes. Thus, steroids are potent competitive inhibitors of drug hydroxylases (Conney 1967), while metyrapone inhibits prostaglandin synthesis in platelets and steroid 17α-monooxygenase (EC 1.14.99.9) and C-17,20 lyase in testes and adrenals.

3-Methylcholanthrene and, presumably, other '*P*-448-inducers' increase the concentrations of cytochrome *P*-450 and glucuronyl transferase in many extrahepatic tissues. As the metabolism of endogenous substrates appears to be the predominant activity of the drug-metabolizing enzymes in most extrahepatic organs it might be expected that induction by 3-methylcholanthrene would lead to important modifications in metabolism of endogenous lipids (Connelly & Bridges 1980). Some changes in endogenous lipid metabolism undoubtedly occur, but whether chronic exposure to 3-methylcholanthrene and similar compounds produces toxicity upsetting lipid metabolism through an effect on drug-metabolizing enzymes remains to be established.

Therefore, certain forms of at least some drug-metabolizing enzymes operate on both endogenous and exogenous substrates. Certain forms of cytochrome *P*-450 seem to be exclusively concerned with the metabolism of endogenous lipids, e.g. the side-chain cleavage of cholesterol, 11β- and 18-hydroxylation of steroids, and 1-hydroxylation of 25-hydroxyvitamin D. Although it is unlikely that any form of drug-metabolizing enzyme is concerned exclusively with the metabolism of exogenous substances there is evidence that this may be the predominant function of some enzymes. Thus, addition of a range of aliphatic, aromatic and heterocyclic exogenous compounds to suspensions of hepatocytes from phenobarbitone-pretreated adult rats results in the rapid appearance of type I cytochrome *P*-450 binding spectra. Moreover, in most cases there is a close correlation between the binding constants in the intact hepatocyte and those in washed microsomes (see Fig. 2). These data indicate that a major form of the cytochrome *P*-450 present in hepatocytes from phenobarbitone-pretreated rats is largely, if not entirely, devoid of bound endogenous lipids. Another indication that phenobarbitone-type cytochrome *P*-450 may be primarily involved in exogenous lipid metabolism is its restricted distribution in tissues (liver and intestine) and in species (mammals and birds, but not fish) and during development (it arises late in fetal development). All of these distributions appear to correlate with exposure to exogenous compounds. Furthermore, phenobarbitone-type cytochrome *P*-450 rapidly disappears from adult rat hepatocytes when they are cultured unless exogenous substrates are provided continuously.

It may be concluded that multiple forms of cytochrome *P*-450 (and perhaps other drug-metabolizing enzymes) fall into three categories: (i) form(s) that exclusively metabolize endogenous compounds, (ii) form(s) that predominantly metabolize exogenous compounds and (iii) form(s) that readily metabolize both endogenous and exogenous lipophilic compounds. One might speculate that in the evolution of the drug-metabolizing enzymes the third form(s) (3-methylcholanthrene inducible forms?) may have developed first. Types of

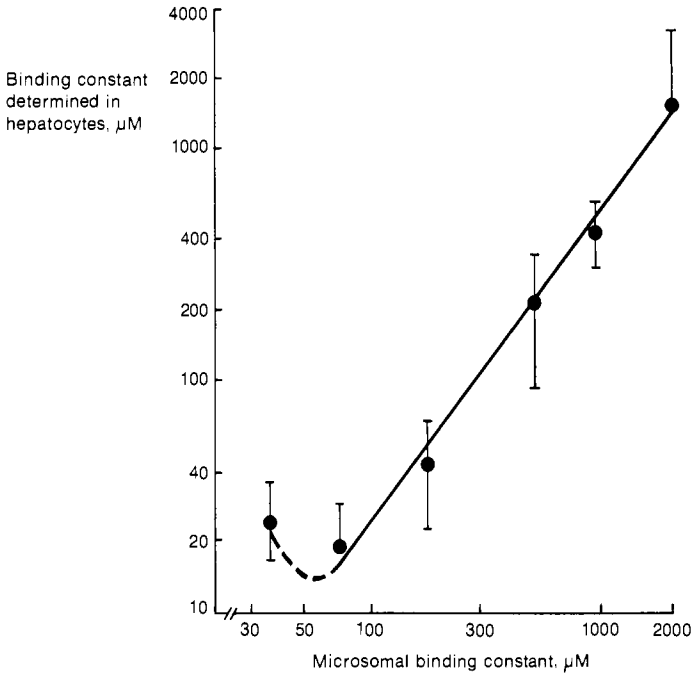

FIG. 2. The relation between the binding constants of *N*-alkyl carbamates interacting with cytochrome *P*-450 in hepatic microsomes and isolated viable hepatocytes from phenobarbitone-pretreated rats (N.S.E. Sargent, D. Upshall and J.W. Bridges, unpublished data).

drug-metabolizing enzymes that have evolved a specialized function in the metabolism of a restricted range of endogenous lipids are likely, because of evolutionary pressure, to show less species variation in their structure than those predominantly concerned with biotransformation of a wide range of exogenous compounds. In many cases, the enzymes that metabolize a selective range of substrates have quite different subcellular locations from the other enzymes and may be assumed to be under quite separate control mechanisms (see Table 3).

OTHER INDICATORS OF THE IMPORTANCE OF DRUG-METABOLIZING ENZYMES

A number of disparate pieces of information support the view that metabolism of xenobiotics may be a major function of hepatocytes. For example, when the liver is exposed to lipophilic exogenous compounds, cellular cofactors, e.g. NADPH and UDPGA, are diverted from intermediary metabolism to facilitate their biotransformation (Siess et al 1978). A number

TABLE 3

Sub-cellular distribution of 'drug-metabolizing enzymes'

Enzyme reaction	*Predominant location of enzyme responsible for metabolism of exogenous substrate*	*Endogenous substrate*	*Location of metabolism of endogenous substrates*
Mixed function oxidation	Endoplasmic reticulum	Steroids	Some in mitochondria of steroidogenic organs
Epoxide hydration	Endoplasmic reticulum	Not known in mammals, may be steroids	Cytosol
Glucuronidation	Endoplasmic reticulum	Bilirubin	Second glucuronidation in bile canaliculi membrane
Sulphation	Cytosol	Chondroitin and cerebrosides	Endoplasmic reticulum
Amino acid conjugation	Mitochondria	Bile salts	Endoplasmic reticulum

of drugs such as phenobarbitone inhibit glycogen synthesis and increase carbohydrate flux through the glucuronic acid pathway; after exposure of the body to allylisopropylacetamide, cytochrome *P*-450 appears to be resynthesized in preference to other haemoproteins, whereas administration of benzoic acid inhibits haem synthesis by competing successfully for the mitochondrial glycine pool (Tephly et al 1973).

CONCLUSIONS

Drug-metabolizing enzymes have an important role in the metabolism of both endogenous and exogenous compounds. It is not possible to identify the primary stimulus to their evolutionary development since the need to metabolize endogenous and exogenous lipophilic compounds probably arose in parallel. In mammals most of these enzymes exist in multiple forms. In mammalian liver and intestine, enzymes with the particular function of metabolizing *exogenous* compounds have evolved, whereas in other tissues the predominant role of the metabolizing enzymes appears to be the formation or degradation, or both, of *endogenous* bioactive lipid-soluble compounds. The relative importance of individual forms of drug-metabolizing enzymes in different tissues may change during the development of an organism or after its exposure to inducers.

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Discussion

Connors: You mentioned that if an enzyme is permanently induced, vitamin D metabolism may be altered. Is there any evidence from chronic experiments on animals that other biochemical pathways are affected – e.g. prostaglandin synthesis or the handling of steroids?

J. W. Bridges: As far as I know, long-term feeding of animals with inducers has not produced any evidence for chronic interference with prostaglandin metabolism. However, characterization of the interactions between prostaglandins and cytochrome *P*-450 is far from complete, so the type of interference we should be looking for is still unclear.

Davies: The problem is that the kinetic studies that have been done with prostaglandins are rather crude, especially those on humans. The technical problems involved in the measurement of concentrations of compounds like prostacyclin are enormous. There are very few studies of the kinetics of these compounds and I think it would be difficult to assess the effects of enzyme inducers on their metabolism. I would like to know what evidence exists for a link between cytochrome *P*-450 and the prostaglandins.

J. W. Bridges: There are several reports indicating that arachidonic acid and prostaglandins bind to cytochrome *P*-450 (e.g. Tan et al 1973, Greiner et al 1979). There is also evidence that some of the classical inhibitors of cytochrome *P*-450, such as metyrapone and carbon monoxide, interfere with the metabolism of arachidonic acid (Sylvester & McGowan 1978). Furthermore, there are a number of known cytochrome *P*-450 inhibitors, like the *N'*-alkylimidazoles, that appear to be particularly effective at blocking thromboxane formation (Moncada et al 1977, Tai & Yuan 1978). These data suggest that the cytochrome *P*-450 system is linked to prostaglandin metabolism.

Conney: Kupfer et al (1979, 1980) demonstrated that the metabolism of prostaglandins is inducible by phenobarbitone and by polycyclic aromatic hydrocarbons.

Orrenius: I agree. There is no question that cytochrome *P*-450 systems are involved with prostaglandin metabolism. However, I would be considerably more cautious in interpreting the evidence that these enzymes are linked to prostaglandin synthesis.

Selikoff: An essential question is how the biochemical studies relate to the clinical manifestations of exposure to foreign chemicals (xenobiotics). One might expect that people who have been heavily exposed to xenobiotics that are enzyme inducers would have an altered incidence of side-effects during drug treatment. But clinically, this doesn't seem to occur. We examined some 2500 people in Michigan who were exposed to polybrominated biphenyls

(powerful enzyme inducers) (Anderson et al 1979). Their incidence of side-effects to drugs did not seem to be at all unusual. The same was true for 350 capacitor workers who were very heavily exposed to polychlorinated biphenyls (Fischbein et al 1979). They, too, did not seem to have an altered incidence of side-effects to drugs although antipyrine studies showed that they did exhibit enzyme induction (Alvares et al 1977).

Conney: I'm not sure that there was clear evidence of enzyme induction in the two populations exposed to polybrominated biphenyls.

Selikoff: In the studies that Alvares did with us on the workers exposed to polychlorinated biphenyls, there was clear evidence of enzyme induction (Alvares et al 1977).

J. W. Bridges: What drugs were you looking at?

Selikoff: Some 30 different drugs, including steroids, antibiotics, analgesics, digitalis, antihistamines, antidepressants, diuretics and isoniazid.

Gillette: Since enzyme induction can lead either to an increase or a decrease in side-effects, it seems possible to me that induction might alter the *pattern* of side-effects without markedly changing the *incidence* of side-effects evoked by the substances in the environment.

Higginson: We must remember that drugs may be given at doses too low to allow identification of effects that might be demonstrable at higher doses.

J. W. Bridges: What was the incidence of side-effects in your control population, Professor Selikoff?

Selikoff: I don't remember the actual figures, but both control and test groups showed a similar incidence of side-effects. The Michigan population was very concerned about its health, and we expected frequent reports of side-effects but this did not occur.

Oesch: Were drugs with a small therapeutic range, such as anticoagulants, included in that study?

Selikoff: Yes. The relatively few people on anticoagulants were otherwise in good health and were able to attend for examination.

J. W. Bridges: With drugs like warfarin and tolbutamide there may have been compensation for the induction effect before the population was studied.

Selikoff: While our studies were relatively insensitive – albeit population-based – I suggest that examination of such populations might be useful for studying your hypothesis. There may be specific reactivity differences hidden within the overall picture.

Hunter: There are some examples of enzyme induction increasing the side-effects of paracetamol or of anaesthetics in the halothane group, e.g. methoxyfluorane (Hunter & Chasseaud 1976).

Breckenridge: I think that such changes are important. One can imagine

the situation in which the rate of prostaglandin synthesis is significantly altered and yet a clinical effect is not demonstrable because one wouldn't know which effect to look for.

Connors: I agree. However, some idiopathic diseases may be due to subtle changes in endogenous control mechanisms.

Davies: A clinically significant change in the therapeutic effect of a drug as a result of enzyme induction is usually due to a rapid alteration (over a period of days) in the rate of metabolism of the drug, as Professor Breckenridge and I have shown for warfarin (Breckenridge et al 1971).

Breckenridge: Another point that is always forgotten is the variability of these effects. We have recently examined the effect of various drugs on the metabolism of contraceptive steroids. Women exposed to the same dose of inducing agents can exhibit quite different changes in concentrations of plasma steroids. This variability is likely to occur in population studies like those reported by Professor Selikoff.

Selikoff: We particularly questioned people who had lost weight after illness or pregnancy because the hypothesized mobilization of polyhalogenated biphenyls from fat depots might have been greater in these people. We expected, but did not find, a change in their clinical status. Some symptoms of illness may be due to increased enzyme induction as a result of increased mobilization of inducing agents, and this requires further study.

J. W. Bridges: Did you examine blood chemistry in that study?

Selikoff: Yes we did, very extensively. Routine studies showed that there was little that was unusual.

Higginson: Can I ask a rather naive question? Could the excess sensitivity of certain people to alcohol and barbiturates be linked to *induction* of metabolizing enzymes?

Conney: Alcohol has two effects: given chronically, it stimulates drug metabolism, and acutely it inhibits drug metabolism (Conney, this volume p 147). Epileptics receiving large amounts of phenobarbitone and phenytoin have a stimulated drug metabolism, and I believe this population could be studied in more detail for possible adverse or beneficial effects of enzyme induction.

Connors: These patients take large quantities of drugs for many years. It is amazing that they don't have all sorts of diseases after what Jim Bridges has told us!

Rawlins: Perhaps Jim could suggest the specific diseases we should be looking for, and the biochemical tests that we should be using?

J. W. Bridges: There are no simple, universally applicable tests. I think we should aim to develop more sensitive analyses such as protein and peptide pro-

filing, to supplement or replace our present crude techniques which only detect liver damage that has become fairly severe.

McLean: I disagree. A mass of data about blood chemistry has no significance unless it can be correlated with a measurable change in the animal or person. Animals can be kept on phenobarbitone all their lives without any alteration in their life-expectancy or in their normal deposition of fat with increasing age. Feedback mechanisms must exist so that an organism can adjust to a massive input of xenobiotics, drugs or inducing substances by a change in its response. What we should study is the way environmental circumstances cause this adjustment to be inadequate, with the result that people become ill. Professor Higginson has just asked whether induction could alter our responses to ethanol. I think that almost all the human variation observed in response to ethanol has central rather than hepatic origins.

Smith: I disagree. We are overlooking the combination of variant forms of drug-metabolizing enzymes that human beings possess. Much of the variability in responses to ethanol can be explained by differences in metabolism due to the variant forms of alcohol dehydrogenase.

McLean: That could account for observed variations in plasma levels; but not for the very variable responses to equal plasma levels.

Breckenridge: In long-term studies of induction in humans, the rate of metabolism of the inducing agent can itself alter, and the resultant feedback may cause the effect of the inducing agent to be minimized.

De Matteis: The difficulty in interpreting changes caused by environmental chemicals is that some of these systems are homeostatic and if they are disturbed there is an adaptive compensation in the pathway where the inducer acts. Presumably the clinical symptoms may depend on the steady-state concentration of important body constituents, and if the rate of degradation of an important constituent is increased and, by homeostasis, the formation of it is also stimulated, there will be no overall effect. A change may be observed only in the turnover rate of a component or in the amount of a metabolite excreted in the urine, both of which could increase without any change in the steady-state plasma concentration of the parent compound and without any clinical symptoms.

Gillette: That point should be emphasized. Several years ago Bogdanski et al (1971) studied the turnover rate of corticosterone in rats, with and without induction. Although the turnover rate was markedly increased by phenobarbitone, the steady-state concentration of corticosterone in blood was unchanged.

Amos: Earlier, Dr Connors said that peculiar disease processes may be related to chronic enzyme induction. We should not forget that the im-

munological system, which is essentially a basic defence mechanism, is involved in the modulation of disease processes. I know of no evidence to relate chronic enzyme induction with products of immunological activation e.g. antibodies, lymphokines etc., but it is possible that chronic enzyme induction could have quite a serious effect on the immune response which, in turn, could influence the development of disease processes.

Selikoff: There is evidence that polybrominated biphenyls, at least among those more heavily exposed, have a marked effect on the number of T lymphocytes and on their activity (Bekesi et al 1978).

Amos: That's slightly different. There's plenty of evidence about the effect of chemicals on the production and function of T lymphocytes, but little has been done on lymphocyte metabolism for antibody product, or on the production of factors that help to generate the immune response, such as lymphokines and T cell cooperative factors.

Selikoff: That may be so, but the individuals with depressed T lymphocyte function had normal skin reactivity, although the T cell change was significant.

Amos: I think we need more specific evidence than that.

Selikoff: Yes. We have studied only polybrominated biphenyls in this respect.

Smith: Recent work shows an association between the immune system and the drug-metabolizing enzymes. Factors that 'perturbate' the immune system and drugs that affect interferon synthesis depress the drug-metabolizing enzymes. In addition, the plasma half-life of theophylline, measured as an index of its rate of metabolism, increases several-fold in individuals on the day after influenza vaccination. A relationship between the immune system and the drug-metabolizing enzymes has been suggested to explain this.

Selikoff: Some people show a lymphocyte change and others do not. It will be very useful for us to study their drug-metabolizing response simultaneously to see if interrelationships exist.

Connors: Patients given BCG vaccination also showed quite profound changes in their rates of metabolism of certain drugs (Lipton et al 1978).

Higginson: If we may return to the epileptics who were under treatment – their incidence of cancer was no different from that in control patients. The greater number of reports of liver cancer in Clemmesen's paper (1974) were due to Thorotrast and not to drug therapy. The populations examined in Denmark were not representative, but were selected groups living in hospital on standard diets and under medical supervision. I have reservations about accepting mild clinical phenomena as being significant in such groups, without further analysis.

Secondly, we must distinguish between changes in drug metabolism after administration of chemicals at low or almost physiological levels, and changes observed after pharmacological exposure. The two situations may be qualitatively and quantitatively different and should be clearly distinguished in reports of clinical side-effects. Further, patients receiving large doses of drugs may also be exposed to dietary modifications, and other factors associated with hospitalization, which may modify drug metabolism.

Connors: The situation is complex. It is worthwhile that we have started off by discussing detailed biochemical mechanisms but have immediately tried to apply the results to humans. I hope that eventually we shall have a much clearer indication of the dangers associated with these environmental chemicals.

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Environmental Chemicals, Enzyme Function and Human Disease
DAVID EVERED and GERALYN LAWRENSON

The influence of inducers on drug-metabolizing enzyme activity and on formation of reactive drug metabolites in the liver

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Abstract A variety of environmental agents can affect the rate of drug biotransformation in the liver by induction of drug-metabolizing enzymes. Both phase I and phase II reactions (the first and second stages of drug metabolism) may be influenced, and epoxide hydrolase, glucuronosyl transferases and glutathione-*S*-transferases are examples of enzymes which, in addition to the cytochrome *P*-450-linked monooxygenase system, are readily inducible by environmental agents. Phenobarbitone and 3-methylcholanthrene are the most widely studied representatives of two major classes of inducers.

Induction of hepatic drug-metabolizing enzymes is often associated with enhanced detoxification of drugs and other foreign chemicals that are metabolized by these enzymes. However, during recent years, the effect of induction on many compounds has been found to be the opposite, i.e. toxicity is increased. This is true for most hepatotoxic drugs and major groups of chemical carcinogens; experiments with carbon tetrachloride, bromobenzene and benzo[*a*]pyrene serve to illustrate this point in the present paper.

It is concluded that the toxicological significance of induction of drug-metabolizing enzymes may differ from one substrate to another, and that general conclusions about the beneficial or harmful effects of induction should not be drawn.

It is now well established that a great variety of environmental agents can induce drug-metabolizing enzymes in the liver and in several extrahepatic tissues. The enhanced rate of drug biotransformation resulting from enzyme induction was originally regarded primarily as a beneficial effect, since it could be shown to be associated with decreased toxicity of many drugs. However, more recently, the concept of toxicity through metabolic activation has become widely accepted, and it is now recognized that electrophilic metabolites of drugs and chemical carcinogens, rather than the parent compounds themselves, are often responsible for both acute and chronic drug toxicity. This has, in turn, led to a re-examination of the toxicological significance of induction of drug-metabolizing enzymes. During this process

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it has become clear that the effect of induction is often complex, and that its relevance to toxicity differs from one compound to another. I shall illustrate this with results from experiments on hepatotoxic drugs and a chemical carcinogen.

INDUCTION OF DRUG-METABOLIZING ENZYMES IN THE LIVER

The discovery and early characterization of the induction of drug-metabolizing enzymes in the liver occurred some 25 years ago, as part of the pioneering work on chemical carcinogenesis by James and Elizabeth Miller and their associates. In 1954, Brown et al reported the enhanced rate of oxidative demethylation of methylated aminoazo dyes in liver homogenates from rats and mice that had been pretreated with polycyclic aromatic hydrocarbons. There was a corresponding increase in the rate of reduction of the azo bond, and intact protein synthesis was required for the stimulatory effect to occur (Conney et al 1956). A correlation was found between the inducing effect of certain polycyclic hydrocarbons on oxidative demethylation and their ability to prevent the hepatocarcinogenicity of 3′-methyl-4-dimethylaminoazobenzene in young rats, which in turn was suggested to be due to induction of inactivating hepatic enzymes.

This work, contemporary with early studies on induction of tryptophan pyrrolase (EC 1.13.11.11) provided an example of substrate-induced enzyme synthesis in mammalian tissue and also produced the first evidence for the inducibility of the NADPH- and O_2-dependent enzyme system in liver microsomes. Subsequent work on the induction of benzo[*a*]pyrene monooxygenase (EC 1.14.14.1) by benzo[*a*]pyrene (Conney et al 1957) provided more direct evidence that the phenomenon was a substrate-induced enzyme synthesis. Studies with 2-acetylaminofluorene revealed that induction could increase the formation of reactive products (Lotlikar et al 1967), in addition to its accelerating effect on inactivation steps in the metabolic pathway.

The early work on induction of monooxygenase activities in liver microsomes by pretreatment with carcinogens was followed by the observation that phenobarbitone, and several other commonly used drugs, could also induce drug-metabolizing enzymes in the liver (Remmer 1959, Conney & Burns 1959). Further studies on induction by phenobarbitone revealed that the enhanced rate of hepatic metabolism of drugs was associated with proliferation of smooth endoplasmic reticulum in the hepatocytes (Remmer & Merker 1963) and increased concentrations of the microsomal constituents NADPH-cytochrome *c* reductase (EC 1.6.2.4) and cytochrome *P*-450 (EC 1.14.14.1) (Orrenius & Ernster 1964). An increase in hepatic microsomal

cytochrome *P*-450 was also produced by pretreatment of rats with 3-methylcholanthrene (Orrenius 1965). Detailed experiments subsequently revealed that the increase in haemoprotein concentration could be attributed to a form of cytochrome *P*-450 that had characteristic properties of light absorption and catalysis (Kuntzman et al 1968), and multiple forms of hepatic cytochrome *P*-450 have now been isolated (cf Lu et al 1971, Vatsis & Coon 1978).

It was soon realized that induction by phenobarbitone, 3-methylcholanthrene and an increasing number of other drugs affected not only the cytochrome *P*-450-linked monooxygenase system, but also increased the concentrations of several other drug-metabolizing enzymes (see Table 1, and Estabrook & Lindenlaub 1979). Like the cytochrome *P*-450 system, several of these enzymes have been found to respond differently to the two model inducers; epoxide hydrolase (EC 3.3.2.3), glutathione transferase (EC 2.5.1.18) and γ-glutamyl transferase (EC 2.3.2.2) are primarily inducible by phenobarbitone, whereas DT-diaphorase (NAD(P)H dehydrogenase (quinone) EC 1.6.99.2) is inducible by 3-methylcholanthrene. Other inducers, with much stronger effects on phase II reactions, have recently been discovered, e.g. *trans*stilbeneoxide (Oesch & Schassmann 1979, Seidegard et al 1980). Whether these agents also affect other phase II reactions, such as sulphate and glycine conjugation, which do not appear to respond to treatment with phenobarbitone or 3-methylcholanthrene, remains to be elucidated.

TABLE 1

Drug-metabolizing enzymes induced by phenobarbitone and by 3-methylcholanthrene

Compound or activity	*Inducer*	
	Phenobarbitone	*3-Methylcholanthrene*
NADPH-cytochrome *P*-450 reductase	+	−
Cytochrome *P*-450[a]	+	+
Epoxide hydrolase	+	+[b]
DT-diaphorase	−	+
Glucuronosyl transferases[a]	+	+
Glutathione transferases[a]	+	+[b]
γ-Glutamyl transferase	+	−

[a]Different forms or activities are affected differently by the two inducers
[b]Induction is either weak or questionable
+ inducible; − non-inducible

TABLE 2

Mechanisms involved in the induction by phenobarbitone and by 3-methylcholanthrene of the cytochrome *P*-450-linked monooxygenase system in hepatic microsomes.

Characteristics	*Inducer*	
	Phenobarbitone	*3-Methylcholanthrene*
De novo synthesis of enzyme protein	Yes	Yes
Stabilization of enzyme protein	Yes	No
Stimulation of mRNA synthesis	Yes	Yes
Cytosolic receptor protein involved	No(?)	Yes
Proliferation of SER	Yes	No
cAMP-system involved	Yes(?)	Yes(?)

SER, smooth endoplasmic reticulum

The mechanisms of induction of drug-metabolizing enzymes by phenobarbitone and 3-methylcholanthrene have been investigated extensively and are discussed in detail by other contributors to this symposium. Table 2 summarizes the pertinent results of these investigations (cf Estabrook & Lindenlaub 1979). Synthesis of new enzyme protein, preceded by mRNA synthesis, is obviously required in both cases. Induction by phenobarbitone also produces stabilization of enzyme protein, as revealed by decreased turnover of both NADPH cytochrome *c* reductase and cytochrome b_5. Proliferation of smooth endoplasmic reticulum in the hepatocyte is associated with induction by phenobarbitone, ethanol and other drugs (Rubin et al 1968), but not with induction by 3-methylcholanthrene. In contrast, a cytosolic receptor protein is involved in induction by 3-methylcholanthrene, whereas no evidence for this in induction by phenobarbitone has yet been presented. Finally, mechanisms relating to the cyclic adenosine-5′-monophosphate system have been postulated to be involved in induction of drug-metabolizing enzymes by both phenobarbitone and 3-methylcholanthrene. Whether cyclic nucleotides are directly involved however, remains to be established.

EFFECTS OF INDUCERS ON RATE AND PATTERN OF FORMATION OF DRUG METABOLITES IN THE LIVER

Fig. 1 represents a scheme of drug biotransformation in the liver. The monooxygenation of lipid-soluble substrates, catalysed by the cytochrome *P*-450 system, results in the formation of oxygenated products that are ex-

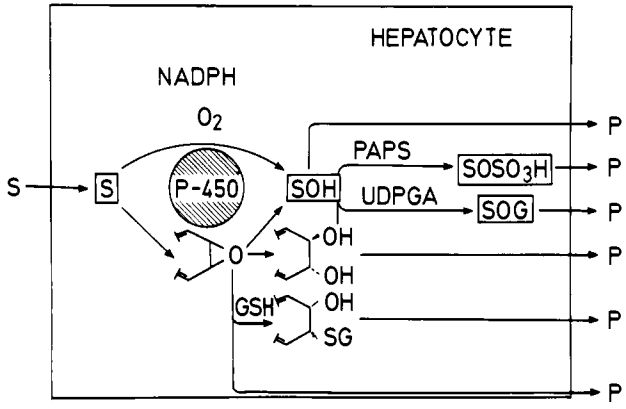

FIG. 1. Biotransformation of drugs by oxidation and conjugation in the liver. S, substrate; SOH, hydroxylated product; SOG, glucuronide; GSH, glutathione, reduced form; SG, glutathione conjugate; PAPS, 3′-phosphoadenosine-5′-phosphosulphate; UDPGA, uridine-5′-diphosphoglucuronic acid.

cretable. More often, however, the oxygenated products are further metabolized, before excretion, to hydrophilic conjugates, e.g. glucuronides and sulphates. Aromatic hydroxylations, on the other hand, can sometimes yield reactive epoxide intermediates which are converted to phenols by isomerization, to glutathione conjugates by non-enzymic or enzymic interaction with glutathione, or to *trans*dihydrodiols by epoxide hydrolase. Generally, these phase II reactions increase the hydrophilicity of the products, thereby making them more readily excretable. Often this is associated with decreased toxicity and therefore phase II reactions are frequently termed detoxification reactions.

As indicated above, the cytochrome *P*-450-linked monooxygenase system is readily inducible. Moreover, inducers affect the various cytochrome *P*-450 species differently, and the result of induction is often a combination of an increase in the total concentration of hepatic cytochrome *P*-450 and a change in the distribution of the individual species. Once this phenomenon had been recognized, it was utilized for purifying the different forms of cytochrome *P*-450 (Vatsis & Coon 1978).

The effects of most inducers, including phenobarbitone and 3-methylcholanthrene, on the cytochrome *P*-450 system are usually more pronounced on phase I than on phase II reactions. This is often manifested as a change in the ratio between oxygenated, unconjugated and conjugated drug metabolites after induction; the ratio of unconjugated to conjugated metabolites of 7-ethoxycoumarin, in isolated hepatocytes, changed from

~0.4 to ~9 after pretreatment of the animals with phenobarbitone and with 3-methylcholanthrene (Orrenius et al 1979). Moreover, pretreatment with inducers frequently increases the proportion of glucuronide conjugates formed. This increase is usually the result of a combined effect on the cytochrome *P*-450 and glucuronosyl transferase systems, i.e. stimulation of phase I reactions makes more oxygenated metabolites available for conjugation; and most of these will then undergo glucuronidation because of preferential induction of glucuronosyl transferases (Orrenius et al 1979). Access to sulphate limits the rate of sulphation and this also contributes to the predominance of glucuronidation in the induced state.

THE INFLUENCE OF INDUCERS ON FORMATION OF REACTIVE DRUG METABOLITES IN THE LIVER

When the toxicity produced by a drug is a property of the parent compound itself, induction of drug-metabolizing enzymes results in an enhanced rate of detoxification and may thus be regarded as a protective mechanism. For example, the toxicity of zoxazolamine, meprobamate, barbiturates and strychnine is markedly decreased by pretreatment with inducers (Conney 1971).

However, many toxic effects are now known to be due to formation of reactive metabolites, while the parent compound is usually biologically inert. This is analogous to results on major chemical carcinogens whose behaviour originally led to the concept of metabolic activation (Miller & Miller 1973).

Fig. 2 shows a scheme of metabolic activation and inactivation of en-

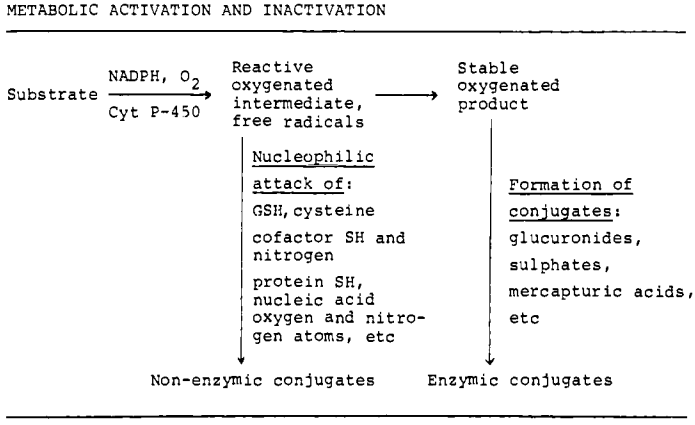

FIG. 2. Schematic representation of mechanisms for metabolic activation and inactivation of environmental chemicals.

vironmental chemicals in the liver. In the example given, reactive oxygenated intermediates or free radicals are produced from non-reactive substrates by cytochrome *P*-450-catalysed reactions. Due to their electrophilicity, the metabolites that are formed may attack nucleophilic sites in the tissue and thereby cause cell damage. Trapping of reactive metabolites by glutathione conjugation appears to be the primary defence mechanism against the harmful effect of reactive intermediates, although the metabolism of epoxides to *trans*dihydrodiols by epoxide hydrolase may contribute significantly to the inactivation of these species. As discussed below, removal of oxygenated metabolites by formation of conjugates with sulphate and glucuronic acid may also serve to decrease toxicity, particularly of metabolites which otherwise may accumulate, or recycle, or both, through the cytochrome *P*-450 system.

Thus, hepatic biotransformation of drugs involves a complex interplay of several reactions, all of which may affect the toxicity of a given compound. In addition, environmental inducers can influence this pattern by selectively affecting the rates of one or more of the reactions involved. There are several examples of hepatotoxic and carcinogenic agents which behave this way.

METABOLIC ACTIVATION OF THE HEPATOTOXIC AGENTS CARBON TETRACHLORIDE AND BROMOBENZENE

Extensive study of the hepatotoxic agents carbon tetrachloride and bromobenzene has shown that they each require metabolic activation by the cytochrome *P*-450 system before they can exert their cytotoxic effects (Jollow et al 1977). Metabolic activation of carbon tetrachloride proceeds by formation of the free radical CCl_3 (Recknagel & Glende 1973), while the highly electrophilic species bromobenzene-3,4-epoxide is probably the primary cytotoxic metabolite of bromobenzene (Jollow et al 1974, Jollow & Smith 1977). The available evidence indicates that carbon tetrachloride exerts its hepatotoxic effect by initiating lipid peroxidation, whereas the cytotoxic mechanism of bromobenzene depends on alkylation of nucleophilic groups in the tissue. In both cases the glutathione system serves as a primary defence mechanism by preventing the cytotoxic products from accumulating in the tissue (Jollow et al 1974, Högberg et al 1975, Thor et al 1978).

Fig. 3 relates the extent of lipid peroxidation and cell damage produced by carbon tetrachloride to the activity of the cytochrome *P*-450 monooxygenase system. This experiment, performed with isolated hepatocytes, showed that the cytotoxic effect of carbon tetrachloride is enhanced when the cytochrome *P*-450 system has been induced by pretreatment of the animals with phenobar-

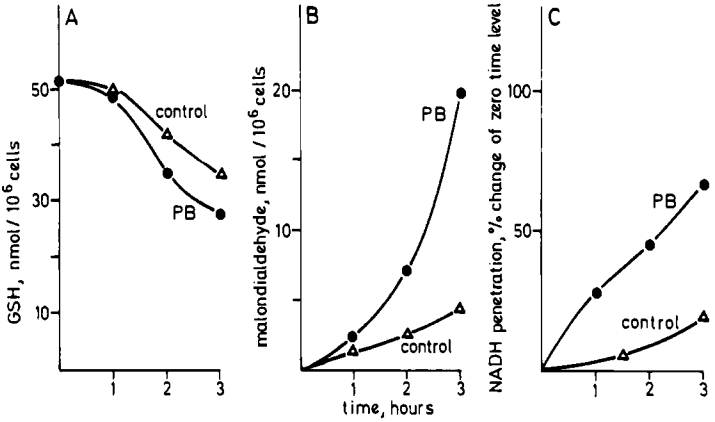

FIG. 3. Effect of incubation with carbon tetrachloride (1 mM) at time zero on intracellular glutathione (GSH) level (A), malondialdehyde formation (B), and NADH penetration (i.e. the percentage of cells permeable to NADH as a function of incubation time) (C), in hepatocytes isolated from control and phenobarbitone-pretreated rats. Hepatocytes were incubated at 37°C in rotating, round-bottomed flasks under an atmosphere of 93.5% O_2 + 6.5% CO_2 and at a concentration of 10^6 cells/ml in Krebs-Henseleit buffer (pH 7.4). GSH concentration and NADH penetration were measured as described by Thor et al (1978). Malondialdehyde was assayed according to Högberg et al (1975).

bitone. In this system carbon tetrachloride metabolism is associated with a slight decrease in cellular glutathione. The addition to the medium of antioxidant, e.g. α-tocopherol, protects the cells completely from the cytotoxic effect of carbon tetrachloride, as do inhibitors of the form of cytochrome *P*-450 that is inducible by phenobarbitone. In contrast, pretreatment of the animals with 3-methylcholanthrene does not increase the hepatotoxicity produced by carbon tetrachloride (Reid et al 1971).

Fig. 4 shows results from similar experiments with bromobenzene. Again, formation of the cytotoxic species is related to the phenobarbitone-inducible form of cytochrome *P*-450. The effect of bromobenzene on cellular glutathione level is much more dramatic than that of carbon tetrachloride, and the available evidence indicates that glutathione depletion precedes cell damage (Jollow et al 1974, Thor et al 1978). Induction of the cytochrome *P*-450 system by 3-methylcholanthrene also stimulates the rate of metabolism of bromobenzene by isolated hepatocytes. However, in contrast to the effect of phenobarbitone, there is no corresponding increase in cytotoxicity (measured as permeability of the cells to exogenous NADH). A possible explanation for this difference in the effects of induction by phenobarbitone and by 3-methylcholanthrene is the observation by Jollow & Smith (1977) that

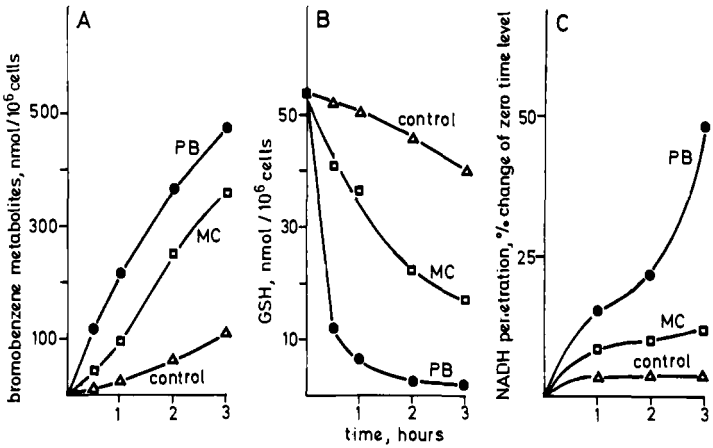

FIG. 4. Effect of incubation with bromobenzene on the production of bromobenzene metabolites (A), on the intracellular concentration of glutathione (GSH) (B) and on NADH penetration (i.e. the percentage of cells permeable to NADH as a function of incubation time) (C), in hepatocytes isolated from control, phenobarbitone and 3-methylcholanthrene-pretreated rats. Hepatocytes were incubated under the conditions described in Fig. 3 legend. Bromobenzene concentration was 0.6 mM. Bromobenzene metabolites were measured according to Zampaglione et al (1973). Concentration of GSH and NADH penetration were assayed as described by Thor et al (1978).

pretreatment with 3-methylcholanthrene produces a less reactive epoxide (bromobenzene-2,3-epoxide) as the major primary metabolite.

Thus, both carbon tetrachloride and bromobenzene are hepatotoxic agents whose effects are influenced not only by the concentration of the microsomal cytochrome *P*-450 system but also by a particular form of cytochrome *P*-450 which is inducible by phenobarbitone. Several cytotoxic chemicals show similar specificity, but the toxic effects of some compounds, e.g. paracetamol (acetaminophen) are increased not only by phenobarbitone but also by 3-methylcholanthrene induction. Finally, the metabolic activation of other xenobiotics, e.g. many chemical carcinogens, depends primarily on the 3-methylcholanthrene-inducible species of cytochrome *P*-450.

METABOLIC ACTIVATION OF THE CHEMICAL CARCINOGEN BENZO[*a*]PYRENE TO DNA-BINDING PRODUCTS, IN ISOLATED HEPATOCYTES

Fig. 5 illustrates possible mechanisms for the activation of the polycyclic aromatic hydrocarbon benzo[*a*]pyrene to DNA-binding derivatives (Yang et al 1978, Jernström et al 1980). The evidence indicates that the two major DNA-binding species of benzo[*a*]pyrene are formed by recycling of the primary metabolites, 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene and 9-hydr-

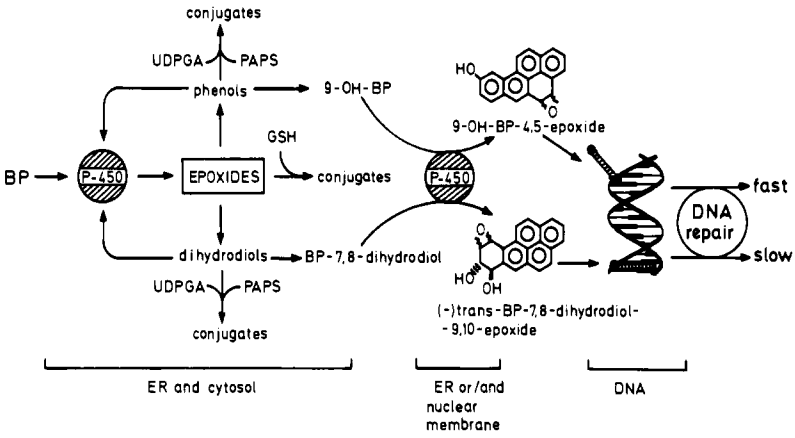

FIG. 5. Possible mechanisms involved in the formation and regulation of DNA-binding products of benzo[*a*]pyrene in hepatocytes isolated from 3-methylcholanthrene-pretreated rats. BP, benzo[*a*]pyrene; UDPGA, uridine-5′-diphosphoglucuronic acid; PAPS, 3′-phosphoadenosine-5′-phosphosulphate; GSH, glutathione, reduced form; ER, endoplasmic reticulum.

oxybenzo[*a*]pyrene, through the cytochrome *P*-450 system to yield the highly carcinogenic product benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (Sims et al 1974, Ivanovic et al 1976) and a less carcinogenic metabolite, probably 9-hydroxybenzo[*a*]pyrene-4,5-epoxide (Jernström et al 1980). As indicated in Fig. 5, cytochrome *P*-450 systems present in the endoplasmic reticulum and in the nuclear envelope are probably involved in the metabolic activation of benzo[*a*]pyrene. There is also evidence for differences, in both the type of interaction with DNA and the rate of removal of bound product from DNA, between the activated derivatives of benzo[*a*]pyrene-7,8-dihydrodiol and 9-hydroxybenzo[*a*]pyrene (Jernström et al 1980).

It is evident from Fig. 5 that the overall metabolism of benzo[*a*]pyrene involves a series of consecutive reactions, of which several may be influenced by induction. These include monooxygenation, hydration and conjugation as well as reactions associated with the repair of modified DNA. Benzo[*a*]pyrene is therefore a suitable model for study of the role of enzyme induction in the activation and inactivation of chemical carcinogens.

In contrast to the metabolism of carbon tetrachloride and bromobenzene, that of benzo[*a*]pyrene is affected primarily by 3-methylcholanthrene-induction. As shown in Table 3, pretreatment of rats with this inducer dramatically increases metabolism of benzo[*a*]pyrene by isolated hepatocytes, whereas pretreatment with phenobarbitone has only a minor effect. The rate of formation of both organic-soluble and water-soluble metabolites is increas-

TABLE 3

The effect of pretreatment with phenobarbitone and with 3-methylcholanthrene on the metabolism of benzo[*a*]pyrene by rat hepatocytes.[a]

Benzo[a]pyrene metabolites	*Inducer*		
	Control	*Phenobarbitone*	*3-Methyl-cholanthrene*
	Rate of production of metabolites nmol (1.5 × 10⁶ cells)⁻¹ (5 min)⁻¹		
Water soluble	0.53	0.91	10.00
Organic-soluble	1.37	0.16	20.60
	Distribution of organic-soluble metabolites, % of total concentration		
Polar metabolites	47	39	19
9,10-transdihydrodiol	5	7	16
4,5-transdihydrodiol	1	2	5
7,8-transdihydrodiol	3	20	15
Diones	18	22	9
9-OH-benzo[*a*]pyrene	8	4	9
3-OH-benzo[*a*]pyrene	18	7	17

[a]Freshly isolated hepatocytes were incubated at 37°C with 20 μM [^{14}C]benzo[*a*]pyrene in Krebs-Henseleit buffer (pH 7.4) containing 2% bovine serum albumin. The benzo[*a*]pyrene metabolites formed were analysed by high performance liquid chromatography (Burke et al 1977).

ed by 3-methylcholanthrene induction, and the pattern of distribution of the organic-soluble metabolites is also altered (Table 3). It is interesting that induction of both phenobarbitone and 3-methylcholanthrene is associated with an increased proportion of benzo[*a*]pyrene-7,8-*trans*dihydrodiol, which is probably the immediate precursor of the ultimate carcinogenic product of benzo[*a*]pyrene.

When benzo[*a*]pyrene is incubated with isolated hepatocytes from rats pretreated with phenobarbitone or with 3-methylcholanthrene, it yields DNA-bound products; results from chromatographic analysis of these are illustrated in Fig. 6. As expected from the metabolite assay (Table 3), induction by 3-methylcholanthrene yields large amounts of DNA-bound products of benzo[*a*]pyrene, while only a minor increase in DNA-binding can be observed after phenobarbitone induction. However there is no qualitative difference between the two systems; i.e. after each type of induction products derived from activation of 9-hydroxybenzo[*a*]pyrene (retention time ~41 minutes)

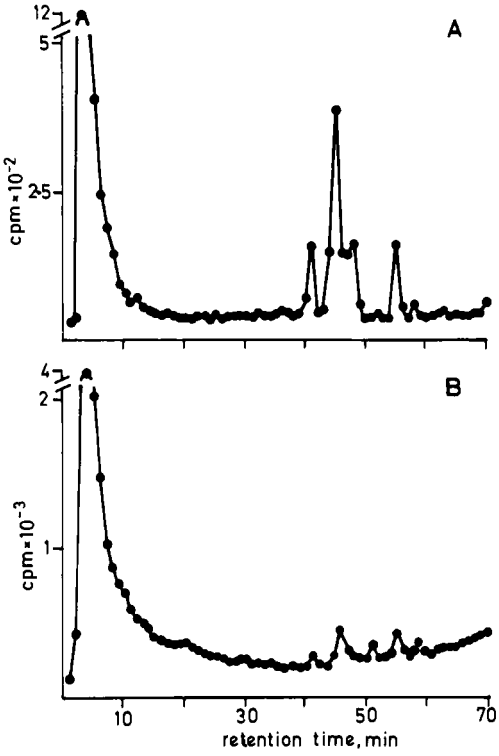

FIG. 6. Analysis by high-performance liquid chromatography of hydrolysed, benzo[*a*]pyrene-modified DNA from rat hepatocytes. (a) 2×10^8 cells, isolated from 3-methylcholanthrene-treated rats, were incubated with 90 μM [^{3}H]benzo[*a*]pyrene in 40 ml Krebs-Henseleit buffer for 10 min. DNA was isolated and hydrolysed to nucleosides (Jernström et al 1980). Hydrolysed DNA was analysed on a reversed phase column (C18) using a methanol/water gradient. Total benzo[*a*]pyrene modification of cellular DNA was estimated to be 20 pmoles/mg DNA. (b) Same conditions as above, except that the cells were isolated from phenobarbitone-treated rats and incubated with 20 μM [^{3}H]benzo[*a*]pyrene for 30 min. Total benzo[*a*]pyrene modification of cellular DNA was estimated to be 5 pmoles/mg DNA.

and benzo[*a*]pyrene-7,8-dihydrodiol (retention time ~46 minutes) can be identified (cf Jernström et al 1980).

Thus, by increasing the metabolic conversion of benzo[*a*]pyrene to DNA-bound derivatives, enzyme induction also modifies the carcinogenic property of the benzo[*a*]pyrene. As found for carbon tetrachloride and bromobenzene, this effect is attributable to induction of the cytochrome *P*-450 system. In contrast to activation of the other two agents, however, benzo[*a*]pyrene activation is mediated primarily by the form of the haemoprotein that is inducible by 3-methylcholanthrene. Simultaneous induction of inactivating en-

zymes, e.g. glucuronosyl and glutathione transferases, may decrease the harmful effect of cytochrome *P*-450 induction but the extent of this effect depends on the inducers and hepatotoxic agents used. In benzo[*a*]pyrene metabolism, the effects of inducers on epoxide hydrolase are particularly interesting in view of the dual function of this enzyme in both activating and inactivating metabolic pathways.

CONCLUSIONS

Induction of drug-metabolizing enzymes by environmental agents may be of considerable toxicological significance. However, the effects of induction are often complex and the implications for drug toxicity vary with different inducers and different drugs. If a toxic drug is metabolized to less toxic products, an increase in the metabolism as a result of induction will decrease its toxicity. However, if the reverse is true, induction may not always be associated with enhanced toxicity; induction by phenobarbitone increases, but that by 3-methylcholanthrene decreases, the hepatotoxicity of carbon tetrachloride and bromobenzene. Moreover, induction does not affect the cytochrome *P*-450 system alone; phase II reactions are also affected. With few exceptions, these are detoxifying reactions, and for any given drug their induction may thus counteract the effects of metabolic activation produced as a result of induction of the cytochrome *P*-450 monooxygenase system. It is therefore impossible to draw any general conclusions about the beneficial or harmful effects of the induction of drug-metabolizing enzyme activity.

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Discussion

Oesch: In your sequence of events of the toxicity produced by things that deplete glutathione, glutathione depletion is followed by covalent binding of the reactive metabolites to other cellular targets, since there is not sufficient glutathione available; and then cell damage, necrosis and toxicity occur. I'm sure this covalent binding to cellular biomacromolecules is important, because it will alter the functions of these molecules, which govern cellular biochemistry. But binding may not be the major cause of the toxicity that is observed. There is some recent evidence that the degree of toxicity produced by phenacetin and by an analogous compound depended on the extent to which each compound was covalently bound. The one that was bound more had much less toxicity. The absence of glutathione in this case could remove protection from other cell functions and other toxicities, like those mediated by lipid peroxidation, which might therefore be more important than covalent binding. What is the evidence that the sequence of events is governed predominantly by the covalent binding of the reactive metabolites; could it be that liver cell necrosis might be more dependent on other sequences of events, like lipid peroxidation, against which glutathione also provides protection?

Orrenius: It's difficult to generalize because bromobenzene is just one example, but our evidence with bromobenzene suggests that glutathione conjugation is extremely important, and a major defence mechanism. We have

tried various other nucleophiles including glutathione precursors like acetylcysteine and cysteine, and we have found that they exert their effects entirely by stimulating glutathione formation. This means that, compared to other nucleophiles, the glutathione system is superior, and when glutathione is present, most of the interaction with other nucleophiles is prevented. If paracetamol is used instead of bromobenzene, the situation is less clear-cut. A decrease in glutathione concentration is observed but toxicity is usually evident long before glutathione is depleted from the cells. Also, in the case of carbon tetrachloride, toxicity will arise long before glutathione is depleted. So there is a certain selectivity in the interactions of reactive metabolites with glutathione. I also agree that covalent binding is primarily a measure of metabolism and that there is no strict correlation between covalent binding and toxicity, since toxicity probably depends on interaction with specific macromolecules.

McLean: What doses of bromobenzene, paracetamol and carbon tetrachloride did you use, Professor Orrenius? Toxicity occurred within one hour, which is never found *in vivo,* and I wonder if your doses may be such that mechanisms quite different from those found *in vivo* are coming into operation. I also have a point about carbon tetrachloride. We have shown that if cysteine is given *in vivo,* protection against carbon tetrachloride does not occur (unpublished work). We found in liver cells that chelating agents will completely block lipid peroxidation, but the damage by paracetamol is not altered.

Orrenius: The concentrations we have used are 0.6 mM bromobenzene, 1.0 mM carbon tetrachloride and 20–90 μM benzo[*a*]pyrene. Further, I cannot claim that the isolated hepatocyte system represents the normal physiological situation. Our system for studying toxicity has, however, several advantages. It is a pure system; we can make it extremely sensitive, for example by induction treatments of the animals before cell isolation; we can manipulate the media; we can eliminate glutathione biosynthesis by omitting essential precursors, or by using inhibitors of glutathione synthetase. Thus, we can use the hepatocyte system as an extremely sensitive indicator of potential drug toxicity.

Gillette: I think the concept of covalent binding has been misinterpreted in many circles. When we originally started studying covalent binding, we did not use it as an indication of toxicity *per se,* but as an indirect measure of formation of chemically reactive metabolites (Gillette 1974). These metabolites react at different rates with different nucleophiles in the tissue. For example, we should expect that the relative importance of the reactions of glutathione and other nucleophiles with the reactive metabolites of bromobenzene,

paracetamol or *N*-hydroxy-2-aminofluorene would differ. Thus, there is not a straightforward correlation between the amount of binding of a reactive species and the extent of toxicity. For example, polycyclic hydrocarbons are usually more carcinogenic to lung than to liver but the amount of covalent binding of the metabolite in the liver is much greater than that in the lung.

Davies: I would like to return to the events leading to cell death. Originally, paraquat toxicity was thought to be due to lipid peroxidation, which was initiated by the superoxide ions formed from interaction between reduced paraquat and molecular oxygen. However, evidence for paraquat-induced lipid peroxidation has been difficult to find (e.g. Shu et al 1979) and it is now thought that depletion of NADPH may be a more important factor (Smith et al 1979). I would like to know Professor Orrenius's opinion on the role of NADPH depletion in paraquat toxicity and to ask whether direct alkylation was the mechanism of depletion of pyridine nucleotide in his studies.

Orrenius: Yes. When bromobenzene is used there is extensive alkylation of cofactors, which decreases the concentrations of pyridine nucleotides and of coenzyme A. This effect may therefore contribute to the toxicity.

Connors: Have you calculated both the number of reacting molecules and the number of molecules of cofactor? Are sufficient electrophilic reactants formed to deplete the cofactors significantly?

Orrenius: We have attempted to do this for bromobenzene. In the induced system, the metabolism of bromobenzene is rapid and presumably proceeds by way of formation of reactive intermediate(s). In the case of paraquat, which we have also studied, we have not seen a comparable effect on pyridine nucleotide concentrations, or on redox states of the pyridine nucleotide.

Davies: The depletion of nucleotides demonstrated by Smith et al (1979) was in the lung rather than in the hepatocyte.

Farber: I think we ought to distinguish clearly between cell damage and cell death. We understand very little about the critical factors that initiate cell death. Cellular toxicity can be defined usually only in qualitative terms whereas biochemical changes can be expressed much more quantitatively.

Conney: One important point that arises from your paper, Professor Orrenius, is the relative balance between the amounts of detoxification and activation, or between the amounts of conjugation and cytochrome *P*-450-mediated oxidation. Increasing the rate of conjugation relative to oxidation should be beneficial. Butylated hydroxyanisole and butylated hydroxytoluene were shown by Wattenberg et al (1977) to inhibit the carcinogenicity of a number of chemical carcinogens. These inhibitors are poor inducers of the *P*-450 system but they were recently shown to be potent inducers of conjugating systems. Epoxide hydrolase, glutathione transferase and glucuronyl

transferase are markedly induced in mice treated with butylated hydroxyanisole and butylated hydroxytoluene (Benson et al 1979, Cha & Bueding 1979). These compounds represent an important class of inducing agents. Have you tried them in your system?

Orrenius: We haven't yet, but I agree completely with you. In most cases we are faced with the situation where we produce reactive or toxic metabolites by means of the cytochrome *P*-450 system, and phase II reactions then decrease the toxicity of these products. The problem with most inducers studied is that they will affect the cytochrome *P*-450 system more readily than the conjugation reactions. On the other hand, as I have indicated, the compound *trans*stilbeneoxide is an example of the type of inducer that has a reverse effect, i.e. it stimulates the phase II reactions in preference to the *P*-450 reactions. This type of inducer could therefore be quite significant in reducing toxicity.

Gillette: We need to be cautious though, because there can be a species difference in inducibility. For example, the paracetamol toxicity is decreased in hamsters but increased in mice by pretreatment with phenobarbitone (Potter et al 1974, Mitchell et al 1973).

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Environmental Chemicals, Enzyme Function and Human Disease
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Effects of inducers and inhibitors on drug-metabolizing enzymes and on drug toxicity in extrahepatic tissues

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Abstract When a compound that is removed from the body by metabolism produces toxicity in extrahepatic organs directly, rather than via active metabolites, induction or inhibition of the drug-metabolizing enzymes simply will decrease or enhance, respectively, the toxic effects of the compound. On the other hand, the effects of chemicals whose toxicity depends on their activation by metabolism may be modified in a complex way by pretreatment with inducers or inhibitors of the enzymes; it may, therefore, be impossible to predict the effect of pretreatment on the metabolism and toxicity of a given compound. The major sources of such complexity are that (a) inducers and inhibitors can have multiple effects on pathways of drug toxification or detoxification, both in the liver and in extrahepatic tissues, (b) active metabolites can be formed both in the liver and at extrahepatic sites, and they may not be sufficiently stable for transport from one site to another, and (c) regardless of the effect of pretreatment on pathways of extrahepatic metabolism, the accompanying effects on hepatic metabolism may determine the extrahepatic distribution and site of metabolism *(in vivo)* of a protoxin or its active metabolite(s). A review of studies on pulmonary toxicity produced by three agents – monocrotaline, bromobenzene, and 4-ipomeanol – illustrates several of these problems, and also shows the value of using inducers and inhibitors in the experimental analysis of extrahepatic toxicity produced by reactive metabolites.

EXTRAHEPATIC DRUG-METABOLISM: GENERAL CONSIDERATIONS

A wide spectrum of drug-metabolizing enzyme activity is present in many extrahepatic tissues, especially in those with epithelial surfaces that can be exposed to drugs and other environmental chemicals, and also in organs and cells that metabolize hormones. As in the liver, there are many sources of the variations observed in endogenous drug-metabolizing enzymes in the extrahepatic tissues; these include both genetic and environmental factors such as species, strain, sex, age, diet, and exposure to chemicals.

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The contribution of extrahepatic drug metabolism to the overall disposition of compounds that are inactivated or eliminated by metabolism *in vivo* is usually small. The *liver,* because of its large mass and high concentrations of drug-metabolizing enzymes is largely responsible for the amounts of the parent compound, or its circulating metabolites, or both, that reach the extrahepatic tissues. Nonetheless, extrahepatic drug metabolism may be toxicologically significant. The metabolizing enzymes may act locally to remove an offending toxic agent or to yield toxic metabolites that produce cumulative or irreversible local effects on cells.

I have three specific aims: (a) to discuss the relative importance of hepatic and extrahepatic drug metabolism in determining susceptibility to chemically induced toxicity in extrahepatic target organs, (b) to consider modifications to drug metabolism by inducers or inhibitors, and (c) to illustrate some examples of (a) and (b), with the lung as a target organ for the toxicity produced by active metabolites.

Although this paper is focused primarily on acute 'toxicity' produced by active metabolites, it is also relevant to long-term toxicities, including carcinogenesis, in which active metabolites, rather than the parent compounds are responsible for initiating the pathological changes.

ROLES OF DRUG-METABOLIZING ENZYMES IN EXTRAHEPATIC TOXICITY

Parent compound versus active metabolites

It is imperative to distinguish between extrahepatic toxicity due to active parent compounds and that due to active metabolites before considering the

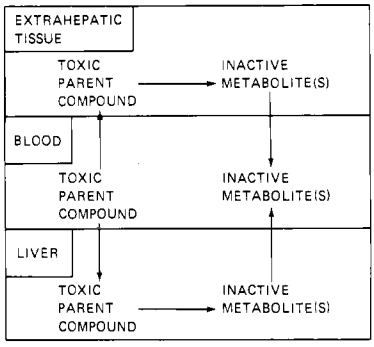

FIG. 1. Schematic representation of extrahepatic toxicity mechanism 'A'. Parent compound is ultimate toxin; metabolism serves detoxification function only.

factors that modulate the activity of drug-metabolizing enzymes. This may elucidate the kinds of extrahepatic toxicity that would occur with a particular agent. Figs. 1 & 2 illustrate schematically two classes of extrahepatic toxicity in which drug-metabolizing enzymes may play crucial roles.

Mechanism A. As shown in Fig. 1, the parent compound alone is the ultimate toxic agent responsible for damage to extrahepatic tissue in mechanism 'A'. The extrahepatic organ in which toxicity develops may depend upon selective exposure, preferential uptake or preferential concentration of the toxic agent in the target tissue, or upon the presence of specific receptors or other highly susceptible sites of action. Alternatively, there may be little selectivity of target organ; the toxic agent may affect the liver as well as many extrahepatic sites.

Mechanisms B. Fig. 2 depicts two types of mechanism in which active metabolites, and not the parent compounds, are responsible for toxicity in extrahepatic tissues. In 'B' mechanism I the toxic metabolites are formed *in situ* in extrahepatic target tissues or cells, whereas in 'B' mechanism II they are formed in the liver and transported to extrahepatic tissues by the circulation. As in A, toxicity produced by mechanisms B may show varying degrees of selectivity towards extrahepatic tissues. The particular organs in which B toxicity develops may depend on selective exposure to the protoxin (parent compound or proximate toxic metabolite), concentration of the protoxin at poten-

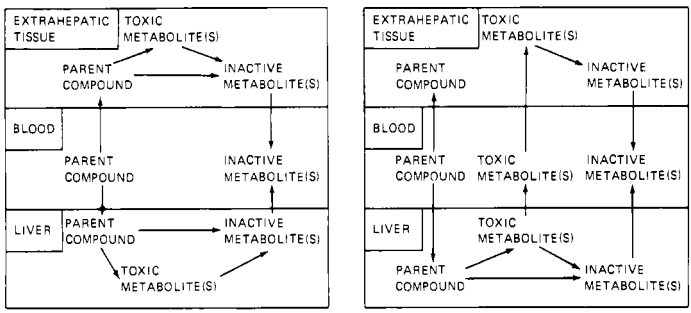

FIG. 2. Schematic representations of mechanisms 'B' showing potential relationships between toxification and detoxification pathways in extrahepatic toxicity produced by active metabolites generated *in situ* (mechanism I) or formed in the liver and delivered by the circulation (mechanism II).

tial sites for metabolic activation, or concentration of the ultimate toxic metabolites at their site(s) of action. It may also depend upon the presence of highly susceptible sites of action or specific receptors for the ultimate toxic metabolites. With mechanism I, the relative capacities of various extrahepatic tissues to produce the ultimate toxic metabolite(s) may be of crucial importance in determining the sites of selective toxicity.

It might be anticipated that toxicity produced by mechanism II rather than by mechanism I would be inherently less selective to extrahepatic tissues and cells. By definition, in mechanism II the toxic metabolites are sufficiently chemically stable to enter the circulation from the liver, and thereby to gain access to many extrahepatic tissues and cells where they may produce toxic responses. On the other hand, in mechanism I, ultimate toxic products might be generated more discretely, in specific extrahepatic tissues or cell types. It is unlikely that drug-metabolizing enzymes are randomly distributed throughout all cells in a particular extrahepatic organ; instead, they are more likely to be confined to relatively few types of cell which might therefore be especially susceptible to damage by compounds that require metabolic activation.

Hybrid mechanisms. In reality, mechanisms A, B-I, and B-II represent extremes, and an actual case of chemical toxicity may not be described completely by one particular model. Thus, many types of extrahepatic toxicity may fall into 'hybrid' categories, such as A/B (in which both the parent compound and its metabolite(s) are toxic) or B-I/B-II (in which toxic metabolites in extrahepatic tissue are carried from the liver by the circulation, and are also formed *in situ*). Nevertheless, for purposes of experimental analysis and discussion, the categorizations into mechanisms A and B are both useful and necessary.

Relative importance of detoxification and toxification pathways in various toxicity mechanisms

Mechanism A. When the parent compound alone is the ultimate toxin, metabolic transformations lead invariably to less toxic products (detoxification). Such detoxification reactions may occur in the liver or in the extrahepatic tissues or at both sites.

Mechanisms B and hybrid mechanisms. When toxicity is caused by active metabolites, metabolic transformations may produce the ultimate toxic products (by toxification pathways) or less active products (by detoxification pathways). Two kinds of detoxification pathways should be distinguished: (a)

those that metabolize and eliminate the parent compound solely through 'non-toxic' pathways and (b) those that perform transformations via a 'toxic' pathway (e.g. an active metabolite is formed initially, and undergoes a second transformation to a less toxic product which can be eliminated from the body). All these types of toxification and detoxification may occur in the liver or extrahepatic tissues or at both sites.

PREDICTIONS OF EFFECTS OF INHIBITORS AND INDUCERS OF DRUG-METABOLIZING ENZYMES ON EXTRAHEPATIC DRUG METABOLISM AND TOXICITY

In assessing the effects of pretreatment with a particular drug upon the metabolism and toxicity of another foreign compound, we must take into account the time course of administration of the pretreatment. When administered chronically, many 'inhibitors' of drug-metabolizing enzymes may act as inducers. Similarly, drugs which typically are viewed as 'inducers' may act as inhibitors if they are present in sufficiently high concentrations in the tissues at the time of administration of the toxic compound. These considerations apply to both A and B mechanisms.

If the mechanism of extrahepatic toxicity for a particular agent is not known, then a careful analysis of the effects of pretreatment with inhibitors and inducers upon the metabolism and toxicity of the agent *in vivo* may implicate a specific mechanism.

Conversely, in attempts to predict the effects of inducers or inhibitors upon the extrahepatic toxicity of a given agent, a knowledge of the toxicity mechanism is of considerable advantage. For example, it would be immensely important to know first if the toxicity were due primarily to the parent compound or to an active metabolite. If it were due to a metabolite, information about the chemical stability and potential site(s) of formation of the active metabolite(s) would be useful. However, predictions about the effects of pretreatment with inducers or inhibitors on mechanism A toxicity are relatively straightforward, whereas predictions about mechanism B toxicity may be more difficult.

Toxicity produced by mechanism A

If the parent compound is the ultimate toxin causing an extrahepatic lesion, and the rate of removal of the compound from the body or the target tissue does not depend upon metabolism, then the inhibition or the induction of drug-metabolizing enzymes is likely to have little or no effect on the toxicity of the compound. A possible exception to this could arise, however, if pretreat-

ment with an inducer brought into action a significant rate of metabolism-related drug removal that was not present in the uninduced animal.

On the other hand, when the parent compound alone is the ultimate toxin and the rate of its removal from the tissues *is* dependent upon metabolic detoxification, then the induction or the inhibition of drug-metabolizing enzymes will decrease or enhance, respectively, the toxicity of the compound.

Toxicity produced by B and hybrid mechanisms

When extrahepatic toxicity is caused by active metabolites, pretreatments with inducers or inhibitors of drug metabolism may produce complex effects. The *a priori* prediction of the effects of pretreatment upon the metabolism and toxicity of a particular agent *in vivo* may be difficult or impossible.

There are many potential sources of this complexity: (a) the effects of a given pretreatment on drug-metabolizing enzymes may vary among different organs and tissues; (b) most inhibitors and inducers are inherently non-selective; they frequently have simultaneous effects on toxification and detoxification pathways in liver and/or extrahepatic tissues; the net effect may depend upon the resulting 'balance' between these pathways at all potential sites of metabolism; (c) whether biotransformation produces toxification or detoxification depends upon the particular drug substrate and types of intermediates involved; many common xenobiotic biotransformation reactions (e.g. hydroxylation, epoxidation, sulphation, glucuronidation, nitroreduction) can serve either as detoxifications or toxifications, depending upon the chemical reactivity and/or the biological activity of the products; (d) a particular pretreatment simply may enhance or decrease the activity of metabolic pathways which are normally used in the non-pretreated animal or, alternatively, the treatment may result in altogether new pathways of metabolism; (e) there are many genetic and environmental sources of variability in response to inducers or inhibitors; moreover, the influence of such factors may vary between different tissues; (f) when toxicity is produced by hybrid mechanisms, the effects of pretreatments are potentially even more complex; nonetheless, the results of such pretreatment may implicate hybrid mechanisms.

In summary, the effects of pretreatment depend both on the type of pretreatment itself and on the toxic drug substrate. Thus, the emphasis is best placed upon analysis of the effects of *individual* inducers or inhibitors on the metabolism and toxicity of *specific* agents *in vivo*. In the following section the role of drug-metabolizing enzymes is considered in relation to the toxicity produced by active metabolites in the *lung*.

EFFECTS OF INDUCERS AND INHIBITORS ON TOXICITY OF ACTIVE METABOLITES (MECHANISMS B) IN THE LUNG

Toxicity of 4-ipomeanol: B mechanism I

4-Ipomeanol is a naturally occurring, toxic furan derivative. The compound was isolated originally from mouldy sweet potatoes. It appears to be responsible for the potentially lethal pulmonary injury that has occurred in cattle consuming these potatoes. It also has been detected in human food products, although the significance of this for public health is not known. A recent review (Boyd et al 1979a) summarizes other background information and the studies that led to the isolation and identification of this compound.

A characteristic lung lesion produced after ingestion or injection of 4-ipomeanol in several species (e.g. rat, mouse, and hamster) is necrosis of the non-ciliated bronchiolar (Clara) cells (Boyd 1977). The initial damage to this cell population appears to be highly selective and, in the early stages, no other toxic manifestations are apparent. Even the adjacent ciliated bronchiolar cells remain intact. Later manifestations of lung toxicity, observed after comparatively large doses of 4-ipomeanol, may include pulmonary oedema, pleural effusions, vascular congestion, haemorrhage and scattered necrosis of other types of lung cells. It is possible that some or all of these later changes occur as a result of the initial damage to Clara cells, but this needs further investigation.

4-Ipomeanol has proved to be an immensely valuable tool in experimental toxicology of the lung. In particular, studies with this pulmonary toxin have provided the first definitive evidence for a mechanism of pulmonary toxicity involving the generation of an active metabolite *in situ* (B mechanism I, Fig. 2). These studies have also shown that a specific population of lung cells, the Clara cells, activate 4-ipomeanol, and that these cells are an important site of cytochrome *P*-450 monooxygenase activity in the lung. Detailed reviews of this work are available (Boyd 1976, Boyd 1980a, b, Boyd et al 1979a, 1980) and therefore only a brief summary of the points relevant to this paper is provided here.

Role of metabolism in toxicity. The intraperitoneal administration to rats of toxic doses of radiolabelled 4-ipomeanol led to covalent binding of large amounts of radioactive product selectively to the lungs (Boyd et al 1975, Boyd & Burka 1978). Moreover, this product was shown by an autoradiographic technique to be located predominantly in the bronchiolar Clara cells.

In vitro studies (Boyd et al 1978) indicated that unless it was metabolized, 4-ipomeanol did not react with biological macromolecules. However, both

the liver microsomes and the lung microsomes of the rat contained cytochrome *P*-450 enzymes that mediated the covalent binding of 4-ipomeanol to microsomal protein. The chemical identity of the reactive intermediate in this reaction is not yet known. However, an epoxidation of the furan ring seems likely to occur, at least initially, and would produce a highly reactive oxygenated metabolite that could either react with nucleophiles or rearrange spontaneously to other reactive, or less reactive, products, or both processes could occur.

There is evidence that reactive metabolites of 4-ipomeanol may be detoxified by conjugation with reduced glutathione (Boyd et al 1978, Boyd & Burka 1978, Boyd et al 1979b, Buckpitt et al 1979, Buckpitt & Boyd 1980). Glucuronidation of the secondary alcohol group present in the molecule does not produce a reactive intermediate metabolite and is a potential detoxification pathway for 4-ipomeanol (Statham et al 1980).

Because the studies *in vitro* showed that 4-ipomeanol had to be metabolically activated in order to bind covalently to tissue constituents, it seemed probable that the product that bound covalently to the lung *in vivo* was also a reactive metabolite of 4-ipomeanol. Moreover, because the covalent binding and toxicity were highly selective, not only to the lung but also to a specific population of target cells, it seemed that the ultimate toxic metabolite of 4-ipomeanol was formed *in situ* within the lung. Studies with inhibitors and inducers of the drug-metabolizing enzymes provided strong confirmatory evidence for this view.

Inhibitor studies. Table 1 shows the effects of some inhibitors on the covalent binding of 4-ipomeanol in rat microsomes *in vitro* and on the covalent binding and toxicity of 4-ipomeanol in rats *in vivo* (data from Boyd

TABLE 1

Effects of inhibitors of drug metabolism on the covalent binding of 4-ipomeanol in rat microsomes *in vitro* and on the covalent binding and toxicity of 4-ipomeanol in rats *in vivo*

Inhibitor	*Effect on covalent binding in vitro*		*Effect on covalent binding in vivo*		*Effect on 4-ipomeanol toxicity in vivo*	
	Liver	*Lung*	*Liver*	*Lung*	*Liver*	*Lung*
Pyrazole	↓	↓	↓	↓	N.E.	↓
Piperonyl butoxide	↓	↓	↓	↓	N.E.	↓
SKF-525A	↓	↓	↓	N.E.	N.E.	N.E.
Cobaltous chloride	↓	↓	↓	↓	N.E.	↓

N.E., no effect

et al 1978, Boyd & Burka 1978). Three of the inhibitors tested (pyrazole, piperonyl butoxide, and cobaltous chloride) reduced the hepatic and pulmonary covalent binding of 4-ipomeanol *in vitro* and *in vivo,* and they were effective inhibitors of 4-ipomeanol lung toxicity *in vivo* (no liver damage occurred either in control rats or in the pretreated rats). Another agent, SKF-525A (β-diethylaminoethyl diphenylpropylacetate) inhibited both the hepatic and the pulmonary activation of 4-ipomeanol *in vitro,* and the covalent binding to the liver *in vivo.* However, neither the covalent binding *in vivo* nor the toxicity of 4-ipomeanol was substantially altered in the lung by SKF-525A. It is possible that this lack of effect was due to simultaneous inhibition of toxification and detoxification pathways by SKF-525A or, alternatively, that insufficient concentrations of the drug were achieved *in vivo* to inhibit the activation pathway in the lung.

Other inhibitor studies are also noteworthy. The concentrations of unmetabolized 4-ipomeanol in the blood and in the lung were much higher in rats pretreated with piperonyl butoxide than in the control rats (Boyd 1977). On the other hand, autoradiograms of the lungs showed that the amounts of 4-ipomeanol metabolite bound covalently in the bronchiolar Clara cells was markedly reduced in the pretreated rats. In other studies (Longo & Boyd 1979, Boyd et al 1980), it was found that 4-ipomeanol was actively metabolized *in vitro* in isolated whole lungs and in lung slices, and that a reactive metabolite of 4-ipomeanol was bound covalently in Clara cells in these preparations. The covalent binding of 4-ipomeanol to Clara cells *in vitro* was strikingly reduced in lung slices and in isolated whole lungs from animals pretreated *in vivo* with piperonyl butoxide.

The inhibitor experiments implicated an active metabolite in the pulmonary toxicity produced by treatment with 4-ipomeanol. However, it is studies with *inducers* of drug-metabolizing enzymes *in vivo* that have yielded the strongest evidence about the major site of formation of reactive metabolites of 4-ipomeanol which bind covalently and cause toxicity in the lung.

Inducer studies. Table 2 shows the effects of two inducers, phenobarbitone and 3-methylcholanthrene, on the covalent binding of 4-ipomeanol in rat tissue microsomes *in vitro* and on the tissue covalent binding and toxicity of 4-ipomeanol in rats *in vivo* (data from Boyd et al 1978, Boyd & Burka 1978). Pretreatment with either inducer markedly increased the covalent binding *in vitro* of 4-ipomeanol in the liver microsomes, but neither had an effect on the lung microsomes.

Pretreatment with either phenobarbitone or 3-methylcholanthrene markedly increased both the total metabolism (disappearance) and the production of

TABLE 2

Effects of inducers of drug metabolism on the covalent binding of 4-ipomeanol in rat microsomes *in vitro,* and on the covalent binding and toxicity of 4-ipomeanol in rats *in vivo.*

Inducer	*Effect on covalent binding in vitro*		*Effect on covalent binding in vivo*		*Effect on 4-ipomeanol toxicity in vivo*	
	Liver	*Lung*	*Liver*	*Lung*	*Liver*	*Lung*
Phenobarbitone	↑	N.E.	↓	↓	N.E.	↓
3-Methylcholanthrene	↑	N.E.	↑	↓	↑	↓

N.E., no effect.

non-bound polar metabolites of 4-ipomeanol in hepatic microsome preparations, but had little or no similar effect on the lung microsomes (M.R. Boyd, unpublished results). Moreover, the ratios of the amounts of covalently bound 4-ipomeanol to the total amounts of it metabolized were similar in microsomes from rat liver and lung ($\approx$ 0.3–0.5) and were not markedly affected by pretreatment with either inducer. Thus, there did not seem to be any major differences between a microsomal detoxification pathway (e.g. epoxide hydrolase, EC 3.3.2.3) for detoxification of a reactive 4-ipomeanol metabolite in lung and in liver preparations; similarly, there was no evidence that such a pathway might be subject to alteration by phenobarbitone or by 3-methylcholanthrene.

Other *in vitro* studies (Buckpitt et al 1979, Buckpitt & Boyd 1980) showed that the addition of reduced glutathione (GSH) to aerobic incubation mixtures containing lung or liver microsomes, NADPH, and 4-ipomeanol almost completely prevented the covalent binding of reactive 4-ipomeanol metabolites to microsomal protein, and yielded two distinct GSH–4-ipomeanol conjugates. These conjugates presumably were formed from the reaction of activated electrophilic metabolite(s) of 4-ipomeanol with the nucleophilic GSH. The two conjugates were produced in approximately the same ratios in pulmonary and in hepatic microsomes from control rats, and conjugate formation was not facilitated by enzymes in the supernatant fractions. Production of both conjugates was markedly increased in microsomal preparations from liver, but not lung, in rats pretreated with phenobarbitone or with 3-methylcholanthrene. However, neither pretreatment markedly altered the relative amounts of the two conjugates produced in either the hepatic or the pulmonary preparations. Thus, there seem to be no major differences in the

nature of the reactive metabolites of 4-ipomeanol that are produced by hepatic and by pulmonary microsomal activation; neither are there differences in the metabolites formed in each system after pretreatment with phenobarbitone or with 3-methylcholanthrene.

In contrast to its effect on the covalent binding of 4-ipomeanol *in vitro,* the effect of phenobarbitone *in vivo* was to decrease sharply both the pulmonary and the hepatic covalent binding of the compound. Correspondingly, the pulmonary toxicity of 4-ipomeanol was reduced in these pretreated animals (no liver damage occurred either in the control rats or in the phenobarbitone-treated rats). The pretreatment with 3-methylcholanthrene also reduced the pulmonary covalent binding and the toxicity of 4-ipomeanol *in vivo* but in contrast to the results obtained with the phenobarbitone-pretreated rats, 3-methylcholanthrene-pretreatment increased covalent binding of 4-ipomeanol in the liver. Consequently, centrilobular hepatic necrosis was frequently produced by 4-ipomeanol in the 3-methylcholanthrene-treated rats. (A similar shift in the specificity of target organ for covalent binding and toxicity of 4-ipomeanol has also been observed *in vivo* after 3-methylcholanthrene treatment of 'inducible' C57BL/6J mice, but has not been observed in the 'non-inducible' DBA/2J strain (Boyd & Dutcher 1978).)

The decrease in the pulmonary covalent binding and toxicity of 4-ipomeanol caused by pretreatment with both inducers *in vivo* appears to be due to enhanced rates of hepatic metabolism and removal of 4-ipomeanol from the circulation. Recent studies (C.N. Statham and M.R. Boyd, unpublished results) indicate that phenobarbitone enhances the hepatic metabolism of 4-ipomeanol primarily through a 'non-toxic' pathway (glucuronidation); conversely, 3-methylcholanthrene increases the rate of hepatic metabolism of 4-ipomeanol primarily through a 'toxic' pathway, leading to an increase in covalent binding of the reactive metabolite to the liver, and leading also to hepatic toxicity.

Since pretreatment with 3-methylcholanthrene *in vivo* increases both covalent binding and toxicity in the liver, but reduces each of them in the lung it is unlikely that a reactive metabolite of 4-ipomeanol formed in the liver could escape into the circulation and eventually become bound to the lung (cf pretreatments with monocrotaline and bromobenzene described in the following sections). Indeed these observations provide strong evidence that covalent binding of 4-ipomeanol in lung and liver is due to the formation of reactive metabolites of 4-ipomeanol *in situ* in the respective target tissues.

Toxicity of bromobenzene: hybrid B mechanism I/II

Bromobenzene has received considerable attention as a model hepatotoxin (for review, see Gillette 1975). However, in addition to causing hepatic necrosis, it may also damage the lungs. Reid et al (1973) reported that bromobenzene, and several other related aromatic hydrocarbon compounds, caused necrosis of the bronchiolar and bronchial epithelium in mice. Rats were much less sensitive to bromobenzene, and only infrequently showed definite morphological evidence of bronchiolar damage after administration of the compound. Reid et al (1973) did not attempt to characterize further the specific type of bronchiolar or bronchial cells damaged by bromobenzene.

Role of metabolism in toxicity. Numerous experiments have demonstrated that bromobenzene-induced liver necrosis in animals is caused by a highly reactive metabolite of bromobenzene produced by hepatic microsomal cytochrome *P*-450 enzymes (Gillette 1975). There is evidence that a 3,4-epoxide is the ultimate toxic metabolite of bromobenzene (Jollow et al 1974). This reactive metabolite can presumably form covalent bonds with tissue macromolecules and so lead to toxicity. Alternatively, the toxic metabolite may be detoxified by hydration or by conjugation with soluble nucleophiles like GSH, or it may rearrange spontaneously to less reactive products.

The observation that bromobenzene sometimes caused lesions not only in the liver but also in the kidney and the lung, led Reid et al (1973) to consider the role of metabolism in extrahepatic toxicity. *In vitro* studies revealed that the lungs, like the liver, contained microsomal enzymes that were capable of mediating the covalent binding of bromobenzene to tissue macromolecules. Microsome preparations from tissues that had not been shown to be targets for bromobenzene toxicity *in vivo* (e.g. spleen, heart) did not contain this enzyme activity. When toxic doses of radiolabelled bromobenzene were given intraperitoneally to mice or rats (Reid et al 1973), substantial amounts became covalently bound to liver and lung. Moreover, autoradiographic studies showed that the material covalently bound in the lungs was concentrated especially in the pulmonary bronchioles (although it was not possible to identify binding to specific bronchiolar cell types).

Therefore, because metabolism of bromobenzene was required in order to form products capable of binding covalently to tissues, and because it apparently became bound in the lung at a specific site of damage, it seemed likely that bromobenzene-induced bronchiolar necrosis was caused by a reactive

metabolite. Since the lung contained enzyme activity capable of mediating the tissue binding of bromobenzene, it seemed that the metabolite causing bronchiolar damage could have been formed in the lung. However, such an interpretation was potentially ambiguous because of other key observations, especially those concerning the effects of inhibitors and inducers on bromobenzene metabolism and toxicity. The latter studies suggest that the toxicity of bromobenzene to the lung was by a hybrid mechanism containing features of both B mechanisms I and II.

Inhibitor and inducer studies. Table 3 shows the effects of an inhibitor (piperonyl butoxide) and an inducer (phenobarbitone) on the covalent binding of bromobenzene *in vitro* in mouse microsomes, and on the covalent binding and toxicity of bromobenzene in mice *in vivo*. As expected, the inhibitor reduced and the inducer increased both the covalent binding of bromobenzene in hepatic microsomes *in vitro,* and its covalent binding and toxicity in the liver *in vivo*. However, neither the inhibitor nor the inducer affected covalent binding in lung microsomes *in vitro*. Nevertheless, the pulmonary effects of inhibition and induction *in vivo* were the same as those produced in the liver; piperonyl butoxide reduced and phenobarbitone increased the covalent binding of bromobenzene to lung macromolecules.

These results suggest that a reactive bromobenzene metabolite formed in the liver is sufficiently stable for a substantial amount of it to enter the circulation and subsequently bind covalently to the lung; pretreatment with an in-

TABLE 3

Effects of an inducer and an inhibitor on the covalent binding of bromobenzene *in vitro* in mouse microsomes and on the covalent binding and toxicity of bromobenzene in mice *in vivo*.

Pretreatment	*Effect on covalent binding in vitro*		*Effect on covalent binding in vivo*		*Effect on bromobenzene toxicity in vivo*	
	Liver	*Lung*	*Liver*	*Lung*[a]	*Liver*	*Lung*
Piperonyl butoxide (inhibitor)	↓	N.E.	↓	↓	↓	N.E.[b]
Phenobarbitone (inducer)	↑	N.E.	↑	↑	↑	N.E.[b]

Data from Reid et al (1973).

[a]Pretreatment produced similar effects in heart, spleen, and muscle, where little or no monooxygenase activity was present.

[b]Only bronchiolar necrosis was examined.

N.E., no effect.

hibitor and an inducer primarily affects the hepatic metabolism of bromobenzene. In addition, Reid et al (1973) showed that substantial amounts of bromobenzene metabolite were bound covalently to several other extrahepatic tissues (e.g. blood, heart, spleen, and muscle) that did not contain enzymes necessary for the metabolic activation of bromobenzene. The pretreatment with inhibitor reduced, and with inducer increased, the covalent binding of bromobenzene also in these other extrahepatic tissues.

Thus, these studies suggest that the toxicity of bromobenzene to the lung may be a hybrid B mechanism. However, such a conclusion must remain tentative until the relative toxicological significance of generation of a reactive bromobenzene metabolite *in situ,* and exposure of the lung to this metabolite through the circulation, have been elucidated fully.

Although Reid et al (1973) did not attempt to measure the pulmonary damage produced by bromobenzene in mice, they noted that the large alterations in pulmonary covalent binding of bromobenzene caused by inducer or inhibitor pretreatment were not accompanied by similar changes in the extent of pulmonary bronchiolar damage (Table 2).

Other investigations (Boyd 1977) have indicated that the non-ciliated bronchiolar (Clara) cells are a site of cytochrome *P*-450 enzyme activity in lung. Therefore, these cells may be major sites of formation and toxicity of a reactive bromobenzene metabolite in the lung. It is conceivable that cells adjacent to Clara cells (e.g. ciliated bronchiolar cells) also might be damaged by a reactive bromobenzene metabolite that is generated predominantly in Clara cells but which is sufficiently stable to pass from one cell to another. In addition, other specific populations of lung cells may also be capable of mediating the formation of a reactive metabolite of bromobenzene.

The toxicological significance of the blood-borne portion of the bromobenzene metabolite that binds to the lung *in vivo* remains to be determined but may be elucidated by study of other potential sites of toxicity and binding (e.g. endothelial cells) for bromobenzene metabolites in the lung, and by study of the subsequent effects of induction and inhibition at these sites. However, reactive metabolites of bromobenzene formed in the liver and eventually bound to the lung may also be bound non-specifically to many pulmonary cells and structures, with few toxicological consequences.

Toxicity of monocrotaline: B mechanism II

Monocrotaline is a member of the pyrrolizidine family of compounds which occur naturally in many plants worldwide. Several of these compounds are highly toxic, and their ingestion, especially by grazing animals, has caused

numerous outbreaks of poisonings. They are also potentially toxic to man. A detailed account of the occurrence and toxicological features of these compounds has been provided by McLean (1970).

A prominent target organ for damage by pyrrolizidines is the liver. However, some pyrrolizidines are also toxic to extrahepatic tissues. In particular, oral or intravenous administration of monocrotaline to animals causes severe lung injury, in addition to hepatic damage. The initial site of damage is the pulmonary vascular endothelium, but other pathological changes, including pulmonary hypertension and right heart failure may follow.

Role of metabolism in toxicity. There is considerable evidence that pyrrolizidines must undergo metabolic activation *in vivo* to produce toxicity (for reviews, see Mattocks 1972, Boyd 1980a). Mattocks (1972) showed that toxic pyrrolizidines were dehydrogenated *in vitro* and *in vivo* to highly reactive pyrrole derivatives. This reaction appeared to be mediated by a cytochrome *P*-450 enzyme system present in hepatic microsomes. Dehydrogenation is an unusual reaction to be catalysed by microsomal enzymes, but Mattocks (1972) envisaged that an initial ring-hydroxylation yielded an unstable oxygenated product, which could degrade spontaneously to form the dehydropyrrolizidine (or pyrrole) derivative.

The dehydro-alkaloids may react with soluble nucleophiles (e.g. GSH) to form less reactive conjugates, or may undergo hydrolysis to form less reactive pyrrole alcohols,

e.g. monocrotaline pyrrole $\xrightarrow{H_2O}$ dehydroretronecine.

It is possible that this type of reaction may detoxify pyrrolizidine metabolites *in vivo*. Relatively less reactive products, such as the pyrrole alcohols, might undergo a second activation (e.g. epoxidation) and thus produce other manifestations of pyrrolizidine toxicity.

Studies *in vitro* (Mattocks & White 1971, Guengerich 1977) indicated that pulmonary microsomal enzymes were either incapable of dehydrogenating toxic pyrrolizidines, or were only present in low concentrations in the preparations. Although they act as potent alkylating agents toward nucleophilic cellular constituents, some of the dehydropyrrolizidines are sufficiently stable that they can be synthesized chemically and kept in non-aqueous media. Thus, Butler and co-workers (1970) were able to study the pulmonary toxicity induced by the pyrrole intermediate of monocrotaline. Injection of the pyrrole directly into the pulmonary arteries of rats damaged the pulmonary endothelial cells in a similar way to the damage produced by feeding or injecting rats with the parent compound, monocrotaline.

In summary, monocrotaline toxicity can be attributed to B mechanism II

(Fig. 2) because (a) the reactive derivatives of pyrrolizidine are produced by hepatic metabolism, (b) the enzymes necessary for this reaction are absent from the lung, and (c) there are similarities between the endothelial toxicity produced by dehydromonocrotaline and by monocrotaline in the lung. The results from experiments with inhibitors and inducers of drug-metabolizing enzymes also support this mechanism.

Inhibitor and inducer studies. Table 4 shows the effects of an inhibitor (chloramphenicol) and an inducer (phenobarbitone) on the formation of reactive pyrrole derivatives from monocrotaline *in vitro* in rat microsomes, and on pyrrole concentrations and toxicity produced by monocrotaline *in vivo* in rats.

Chloramphenicol reduced the pyrrole concentration and toxicity of monocrotaline *in vivo* in both liver and lung while phenobarbitone increased these factors. Chloramphenicol inhibited and phenobarbitone increased pyrrole formation catalysed by hepatic microsomes *in vitro*. Therefore, since pulmonary microsomes could not activate the pyrrolizidine, this suggests that an active metabolite was responsible for the vascular endothelial damage produced in the lung by monocrotaline and also that the active metabolite responsible for this toxicity was formed primarily in the liver.

TABLE 4

Effects of an inducer and an inhibitor on the formation of reactive pyrrole derivatives from monocrotaline *in vitro* in rat microsomes and on pyrrole concentrations and toxicity produced by monocrotaline *in vivo* in rats.

Pretreatment	*Effect on pyrrole concentrations produced in vitro*		*Effect on pyrrole concentrations in tissue in vivo*		*Effect on monocrotaline toxicity in vivo*	
	Liver	*Lung*	*Liver*	*Lung*	*Liver*	*Lung*
Chloramphenicol (inhibitor)	↓	[a]	↓	↓	↓	↓
Phenobarbitone (inducer)	↑	[a]	↑	↑	↑	↑

Data from Mattocks & White 1971, Allen et al 1972, and Chesney et al 1974

[a]Pyrroles not produced in detectable amounts in lung microsome preparations *in vitro*.

Summary

In this paper I have emphasized the usefulness of categorizing mechanisms responsible for extrahepatic toxicity according to whether the parent com-

pound or its metabolites are active. Although the categories do not represent absolute 'mechanisms' they provide a foundation on which to evaluate the potential effects of inducers or inhibitors on drug-metabolizing enzymes and on drug toxicity in extrahepatic tissues.

It is relatively easy to predict the effects of inducers and inhibitors on toxicity produced by compounds subject only to metabolic detoxification. It is more difficult, however, when compounds can be metabolized through 'toxic' or 'non-toxic pathways. Nevertheless, experiments with inducers and inhibitors can be useful. They may implicate an active metabolite in the toxicity produced by a particular agent, or may identify the site of formation of a toxic metabolite *in vivo*.

The examples presented here illustrate these points and emphasize that endogenous drug-metabolism, and its modification by exogenous inducers or inhibitors, can control the specificity of attack of certain compounds to particular target organs. Hepatic drug metabolism and its modification may dictate the susceptibility of certain extrahepatic tissues to attack by active metabolites, even when the active metabolites are generated primarily *in situ* in the extrahepatic target tissue.

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Discussion

Breckenridge: Dr Boyd, what is the evidence that the same toxic metabolite is formed from the compounds that you have used in both the liver and the lung, and what is the influence of the route of administration of these compounds on the type of toxicity that is found?

Boyd: We see selective pulmonary alkylation and toxicity with 4-ipomeanol in rats whether we give the compound orally, intraperitoneally, or intravenously. As far as the nature of the toxic reactive metabolites is concern-

ed, we cannot isolate these directly, so we have developed ways of measuring them indirectly, when they have formed conjugates with nucleophiles, such as glutathione. We have devised a high-pressure anion-exchange technique (Buckpitt et al 1979) which allows us to separate and quantitate the glutathione conjugates formed with reactive metabolites of furan compounds (Buckpitt & Boyd 1980). Two conjugates were formed in incubation mixtures containing microsomes from rat lung or liver, NADPH, 4-ipomeanol, and glutathione. Soluble-fraction preparations from homogenates of rat lungs or livers did not enhance the corresponding microsome-mediated production of either conjugate, suggesting that glutathione transferase activities did not play a major role in the formation of conjugates between glutathione and reactive 4-ipomeanol metabolites. The amounts of both conjugates were increased greatly in hepatic, but not pulmonary, microsome preparations from animals pretreated with phenobarbitone or 3-methylcholanthrene. However, the ratios of the amounts of the two conjugates produced were similar in hepatic and in pulmonary preparations from control rats, or from rats treated with either inducer. So these results indicate that the reactive 4-ipomeanol metabolites formed during pulmonary and during hepatic microsomal metabolism are similar. Moreover, the increase in hepatic covalent binding and toxicity of 4-ipomeanol caused by pretreatment with 3-methylcholanthrene, but not phenobarbitone, is not likely to be due to a major change in the kinds or ratios of reactive 4-ipomeanol metabolites produced by hepatic microsomal metabolism.

Meyer: Could you speculate on the functional significance of the well developed cytochrome *P*-450 system in the Clara cells of the lung?

Boyd: We do not know the physiological significance, but the systems we have developed should enable us to study that.

Connors: Does any other chemical concentrate selectively in the Clara cells?

Boyd: Let me emphasize that it is not 4-ipomeanol itself which is concentrated in the Clara cells. It is the covalently bound 4-ipomeanol metabolites that accumulate. These metabolites, which are generated by the cytochrome *P*-450 system in Clara cells, react avidly, as they are formed, with nearby cell constituents. Extrapolating from our studies with the furans, of course, we would expect that covalently bound metabolites of other compounds that could be metabolized to reactive products in Clara cells may also accumulate in the cells.

Selikoff: In which cells are polychlorinated biphenyls localized in the bronchial mucosa?

Boyd: These compounds apparently may accumulate to some extent in the

bronchial epithelium, but I do not believe it has been established definitively in which specific bronchial or bronchiolar cell type(s) this occurs, nor whether these compounds are metabolized in the cells.

Selikoff: It would be interesting to know. Workers exposed to these compounds show defects in pulmonary function tests (Warshaw et al 1979).

Connors: The Clara cell should be a major target site for many compounds because of its high enzyme content.

Boyd: Yes, that is worth emphasizing. Our work, e.g. with 4-ipomeanol, showed that the Clara cells contain an extraordinarily active cytochrome *P*-450 monooxygenase system (Boyd 1977, Boyd et al 1978, Boyd & Burka 1978, Boyd et al 1980). The studies with 4-ipomeanol also confirmed the toxicological significance of the system. Because many other kinds of toxic or carcinogenic compounds also require metabolic activation by a cytochrome *P*-450 enzyme system, we proposed that the pulmonary Clara cells might be an important site for the bioactivation of many such compounds (Boyd 1977). The Clara cells would therefore be especially susceptible to the toxic or carcinogenic effects of such chemicals (Boyd 1977). Studies with a variety of compounds, including other furans and related agents, aromatic hydrocarbons and their halogenated derivatives, carbon tetrachloride, and certain nitrosamine derivatives, have provided confirmatory evidence for this hypothesis (for review, see Boyd 1980). These chemicals cause toxic changes, probably as the result of their bioactivation, in the Clara cells.

Nitrosoheptamethyleneimine, a nitrosamine derivative, binds irreversibly and selectively to the pulmonary Clara cells *in vivo* in hamsters. The resultant pulmonary tumours not only contain cells morphologically similar to Clara cells, but also retain the capacity to bind the nitrosamine (Reznik-Schüller & Lijinsky 1979). Thus, the alkylation and tumorigenesis produced by nitrosoheptamethyleneimine may depend upon its metabolic activation, although further experiments are needed to explore this possibility. It will be of interest to test these and other pulmonary tumours for their ability to metabolize and covalently bind 4-ipomeanol, since this activity would implicate a Clara cell origin for the tumours. The possibility also should be explored that potent cytotoxins such as 4-ipomeanol may exhibit tumour-directed alkylation and cytotoxicity *in vivo* in tumours derived from Clara cells, and therefore might be useful therapeutically (Boyd 1977).

Selikoff: The Clara cells are also sites of predilection for the effect of vinyl chloride.

Boyd: Vinyl chloride is, of course, activated metabolically by the cytochrome *P*-450 system.

Bend: Devereux et al (1979) have demonstrated that Clara cells isolated

from rabbit lung have relatively high monooxygenase activity. With benzo[*a*]pyrene as substrate, the cells have 2.5 to 3 times the activity of alveolar type II cells (L.M. Ball, T.R. Devereux, J.R. Fouts, J.R. Bend, unpublished work). Moreover, Dr. Philpot and his colleagues have demonstrated a higher concentration of pulmonary cytochrome *P*-450 in Clara cells than in alveolar type II cells by using a specific antibody–immunofluorescence procedure (Serabjit-Singh et al 1980).

Boyd: Our work showed that there was relatively little metabolism and covalent binding of 4-ipomeanol in the type II cells. This may indicate either that the amounts of cytochrome *P*-450 are lower in the type II cells than in Clara cells or that 4-ipomeanol is a poor substrate for a different cytochrome *P*-450 system in the type II cells. However, the type II cells are generally less susceptible than the Clara cells to toxic chemicals that require metabolic activation. So it seems that the Clara cells have an extraordinarily active monooxygenase system in comparison to other pulmonary cells.

Orrenius: I do not doubt that the Clara cell is a site of generation of active metabolites by the cytochrome *P*-450 system in the lung, but I want to add a word of caution. When alkylation of macromolecules in the lung is compared with that in the kidney and in the liver, a much greater effect is often observed in the lung than in the other two organs. This may represent differences in activation, but the glutathione system, the glutathione content and the glutathione transferase activity also differ considerably between the various tissues: in liver and kidney the concentrations of glutathione are between 5 and 10 mM, whereas in lung much less is present, and glutathione transferase activity is also much lower.

Boyd: With 4-ipomeanol we have much evidence that the selective alkylation and toxicity in the Clara cells is due to the selective activation of the compound (e.g. see review by Boyd 1980). Glutathione (GSH) apparently provides a detoxification pathway for 4-ipomeanol *in vivo,* by forming less reactive and less toxic conjugates with the metabolites of 4-ipomeanol (Boyd et al 1979). However, this reaction is not facilitated by the transferase enzymes (Buckpitt & Boyd 1980). Moreover, the depletion of pulmonary GSH (to less than 20% of control levels) by pretreatment of animals with diethylmaleate markedly increases the pulmonary toxicity and the amount of 4-ipomeanol metabolites bound covalently in the lungs *in vivo,* but it does not alter the Clara cell selectivity (Boyd et al 1979, M.R. Boyd, unpublished results). Similarly, in lung slices taken either from control or diethylmaleate-treated animals, 4-ipomeanol was metabolized and covalently bound selectively *in vitro* in the Clara cells (the relative amounts bound were of course much

higher in the tissues from the pretreated group) (Longo & Boyd 1979, M.R. Boyd, unpublished results).

J.W. Bridges: Dr Boyd, you referred to relatively reactive metabolites passing from the liver to the lung where they cause toxicity. Since the heart is exposed to significant levels of these metabolites one might expect the heart itself also to become damaged; is this the case?

Boyd: There is evidence that the pyrrolic derivatives of the toxic pyrrolizidines can induce vascular necrosis in any vascular bed into which they are injected, but I do not know of any reports of similar toxic effects on the heart itself. We may speculate that this is because of the difference in surface area between the heart and the larger vascular bed of tissues such as the lungs. Right heart hypertrophy and eventual cardiac failure frequently accompany chronic pyrrolizidine poisoning, but these effects result primarily from the severe pulmonary hypertension which occurs secondarily to the pulmonary vascular damage (e.g. see McLean 1970).

Garner: We have studied aflatoxin B_1, another furan-containing compound, and have some interesting results on the relevance of microsomal techniques to predictions about *in vivo* responses. We used liver preparations from various animals to study their activity in the *Salmonella*/microsome assay (Garner 1980) with aflatoxin B_1 as the mutagen. Aflatoxin alone induced no mutations in *Salmonella typhimurium* TA100, but in conjunction with liver monooxygenases it was a potent mutagen. With monooxygenase from control rat liver 340 and 503 mutants/plate were induced by 1 and 2 μg aflatoxin/plate respectively; with liver from phenobarbitone-pretreated animals there were 397 and 673 mutants/plate and with hamster liver, 673 and 974 mutants/plate. The mutagenicity of aflatoxin B_1 depends on its oxidation by liver monooxygenase to yield an epoxide, the 'ultimate' carcinogen. Hamster liver *in vitro* therefore has the greatest ability to produce this metabolite. These results contrast strikingly with the reduction of carcinogenicity of aflatoxin by phenobarbitone *in vivo* (McLean & Marshall 1971) while the hamster is resistant to aflatoxin's carcinogenic action (Herrold 1969).

If on the other hand one compares binding of radiolabelled aflatoxin B_1 *in vivo,* or in liver slices *in vitro,* different answers are obtained. When liver slices from male and female rats are incubated with 0.5 μg/ml aflatoxin B_1 in phosphate–Ringer medium, 35 ng/mg of carcinogen is bound to DNA but only 1 or 2 ng/mg is bound to protein. When slices from phenobarbitone-pretreated rats are used, only 14 ng/mg of carcinogen binds to DNA, while for hamster liver slices, the value is 11 ng/mg. In mouse liver slices only 1 ng/mg of radiolabelled aflatoxin B_1 is bound. Results from DNA binding in

the whole cell (liver slice) system therefore correlate better than results from microsomal mutagenicity studies with carcinogenic activity. In other words, the balance between activation and detoxification of aflatoxin B_1 is reflected by slices but not by microsomes. We must be cautious, therefore, about extrapolating from microsomal data to what happens *in vivo*. I would like to make a further point about induction, which arose from Professor Orrenius's paper. Induction by phenobarbitone increases the toxicity of carbon tetrachloride (Garner & McLean 1969), but this is the only example I know of an increase in toxicity with an inducing agent. Generally, inducing agents tend to decrease the toxicity by increasing detoxification at the expense of activation.

McLean: That is true in carcinogenesis, but in cases of acute toxicity there are many examples of induction increasing the toxic effect, e.g. of paracetamol (acetaminophen).

Higginson: Yes. In these discussions we should not use the terms 'toxicity' and 'carcinogenicity' interchangeably but distinguish between acute, subacute and chronic toxicity, and carcinogenesis.

Hunter: In Thorpe & Walker's work (1973) in the mouse, phenobarbitone increased the incidence of liver tumours. The mouse may be unusually susceptible, but this is a well described example of increased carcinogenicity as a result of phenobarbitone *per se*.

De Matteis: I would like to ask Dr Garner to provide an explanation for the disparity between results from cell-free systems and those from liver-slice (or whole cell) systems. Is it the ratio between activation and detoxification pathways that is different in these two situations, or is it the ratio between activations at two different sites (e.g. microsomal and nuclear) that has changed?

Garner: I cannot give a complete explanation for this. More research is needed before we can explain this discrepancy. In experiments with microsomes one tends to examine activation pathways far more than detoxification pathways because one supplements the assays only with oxidation cofactors (NADP, glucose-6-phosphate) and not with detoxification cofactors (UDP-D-galactose, 3′-phosphoadenosine 5′-phosphosulphate (PAPS) etc.). In addition, some parts of the cell that might be responsible for detoxification reactions are discarded in microsomal studies.

Connors: Do you think that data from microsomal systems are less relevant than whole cell data to the situation *in vivo*?

Garner: No, but attempts could be made to reproduce more physiological conditions in terms of choice of substrate, and concentrations of cofactors.

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Environmental Chemicals, Enzyme Function and Human Disease
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Induction of enzymes involved in DNA repair and mutagenesis

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Abstract Three phenomena examined here have been claimed to reflect the operation of inducible repair systems. It has been postulated that 'SOS repair' involves the induction by DNA-damaging agents of an error-prone repair system that is capable of affecting the replication of damaged DNA. This system works with bacteriophages and animal viruses, in which it is possible to separate the effects of DNA damage on the viral DNA from that on the host cell. Whether this system also operates in repair of the cell's own DNA is, however, controversial. The system appears to have little effect on survival of bacterial cells and its operation in cellular mutagenesis is still not proven, at least in bacteria with otherwise normal repair capacity.

'Adaptation' is the response of bacteria to low doses of methylating agents. Adapted bacteria are more resistant to the lethal and mutagenic action of alkylating agents. The process includes the induction of an enzyme that 'removes' O^6-alkylated bases from DNA and which, unusually, is consumed during the course of the reaction. The reaction itself is also unknown; it does not depend on a nuclease, glycosylase or demethylase, but could use a transmethylase. There is some evidence that an analogous process occurs in animals.

The third process affects the synthesis of high molecular weight DNA in cultured mammalian cells that have been exposed to split doses of DNA-damaging agents. It has been postulated that this system is inducible and error-free, but detailed analysis suggests that the observed effect is an artifact arising from an abnormal distribution of sizes of nascent DNA after the second dose, and as a result of exposure to the first dose.

Inducible DNA repair systems may be expected to influence kinetics of the dose–response relationships obtained after exposure of cells to mutagens and carcinogens. The interactions between these effects and those produced by inducible pathways of metabolic activation and detoxification are discussed.

Many of the contributors to this symposium will be familiar with the metabolic activation and detoxification of mutagenic and carcinogenic (DNA-damaging) chemicals and also with the influence that such processes may have

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on dose–response relationships. This paper may seem out of place because it deals with the response of cells whose DNA has already been damaged. However, the activity in pathways of DNA repair and mutagenesis may be equally important in determining the dose–response relationships. In this paper I shall describe three inducible DNA repair pathways, postulated or established, and I shall consider the influence on dose–response relationships of interactions between inducible or saturating repair systems and drug-metabolizing systems. General consideration of DNA repair processes is given by Lehmann & Bridges (1977).

SOS REPAIR

Many years ago Weigle & Dulbecco (1953) found that when u.v.-irradiated bacteriophages (phage) were allowed to infect host bacteria, more of the phage survived if the bacteria had themselves been exposed to u.v. irradiation. This increased survival was, moreover, accompanied by a high frequency of phage mutants induced by u.v. These phenomena have become generally known as Weigle- or W-reactivation and W-mutagenesis respectively, and have been demonstrated with both single and double strand phages. A comparison between phage mutagenesis and bacterial mutagenesis led Defais et al (1971) to suggest that W-reactivation and W-mutagenesis reflect the operation of an error-prone repair system (SOS repair) induced by the DNA damage in the bacterial chromosome. The SOS repair theory, as applied to repair of the bacterial DNA itself, is attributed to Radman (1975). Rather against my will, I have often been placed in the role of Devil's Advocate for the SOS theory, so the reader should bear this in mind in what follows!

Damage to the DNA of *E. coli* (for example, by u.v. irradiation) can trigger three effects on phage, as shown by the experimental results in Fig. 1. First, mutations are induced in the unirradiated phage in proportion to the u.v. dose to the host bacteria. Secondly, this effect is observed to a greater degree in phage which have themselves been irradiated (W-mutagenesis), but it disappears, or at least saturates (being masked by the spontaneous mutator activity), at doses to the host of 100 J m^{-2} or higher. Thirdly, the fraction of surviving, irradiated phage increases at doses of 50 or 100 J m^{-2} irradiation to the host (W-reactivation), but this effect disappears at 150 J m^{-2}.

Originally, Radman postulated that SOS repair involved induction of a new enzyme (e.g. a DNA polymerase or a terminal nucleotidyl transferase) which, unlike known bacterial polymerases, could randomly insert bases opposite a non-coding lesion, such as a pyrimidine dimer, during DNA replication. This would simultaneously enhance survival and cause errors in the newly syn-

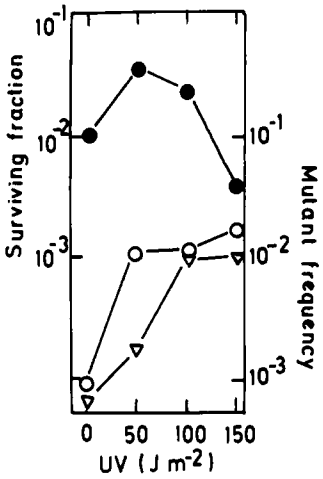

FIG. 1. Effects on phage λ of different doses of u.v. irradiation to the host bacterium *Escherichia coli* K-12 W3110. Frequency (per viable phage particle) of clear plaque mutants of unirradiated phage λ (▽) and of phage λ after 150 Jm^{-2} u.v. irradiation (○). Surviving fraction of u.v. irradiated phage (●). (Clear plaque mutants were assayed on a lawn of NO483 which detects both pure and mixed clones.)

thesized DNA. However, it has subsequently become clear that the explanation must be more complex. There is, for example, one bacterial strain that shows W-mutagenesis but not W-reactivation (Kato et al 1977).

The induction by u.v. of both phage and bacterial mutagenesis, together with the phenomena of W-reactivation, post-replication repair and others, depend on the bacterial genes *recA* and *lexA*. After u.v. and many other treatments that damage DNA there is a massive synthesis of the *recA* gene product. This protein binds to single-stranded DNA and catalyses both the ATP-dependent re-annealing of complementary DNA strands and the invasion of superhelical, double-stranded DNA by the homologous single-stranded DNA (for references see Bridges 1979). One can readily envisage that the *recA* protein plays a role in the stabilization of configurations involving DNA gaps, so facilitating DNA repair. But what is the evidence that inducible repair is significant for the bacteria themselves?

We have done three studies on the operation of a hypothetical, inducible and error-prone pathway in bacteria. First, we introduced into cells a u.v.-irradiated plasmid as the inducing agent. We then assayed the induced error-prone activity by using u.v.-irradiated phage λ and we observed, in the cell itself, the number of mutants induced by a low dose of u.v. We found (Bridges et al 1973) that although both W-reactivation and W-mutagenesis of

phage λ were induced in this indirect way (confirming the work of George et al 1974) there was no effect on the frequency of u.v.-induced mutation in the bacteria themselves. This experiment therefore failed to supply any positive evidence for an inducible mutagenic repair pathway operating on bacterial DNA.

Our second study was begun as a result of a report that cell-free extracts of temperature-sensitive *tif-1* bacteria, believed to express SOS repair at 42°C, could incorporate 'wrong' bases at a higher rate with synthetic polynucleotide templates than *tif*$^+$ or *tif-1* bacteria at 34°C (Radman et al 1976). After many trials and tribulations with this potentially useful research system we found that the most important factor was the presence of a DNA-dependent NTPase, which grossly changed the concentrations of nucleotide precursors and thus changed the relative amount of incorporation of bases. When breakdown of the nucleotide precursors was prevented, there was no evidence for the induction of a misincorporation activity in *tif-1* strains at 42°C (McGarva et al 1979). In retrospect, this result was not surprising since polymerizing activity in the cell-free extract was almost entirely due to DNA polymerase I, whereas we have shown that u.v.-induced mutations *in vivo* are due to DNA polymerase III.

Our third study was more complex. Mutations induced by u.v. in excision-deficient bacteria arise *after* DNA replication, as in W-mutagenesis. In excision-proficient bacteria, on the other hand, post-replication repair at most loci is much less error-prone, and mutations arise largely during excision repair events, *before* DNA replication. Sedgwick (1975) showed that mutagenesis during post-replication repair is chloramphenicol-dependent in excision-deficient bacteria, and this is consistent with the operation of an inducible process (but see below).

Mutations in excision-proficient bacteria usually arise during only about 5% of excision repair events, but these events probably cover a long tract, of perhaps 1000 nucleotides, in DNA. Since this long-patch repair is chloramphenicol-sensitive, is the associated mutagenesis therefore inducible? Addition of chloramphenicol after u.v.-irradiation will not provide a simple answer to this question because the drug causes a massive loss of viability, which cannot be attributed solely to inhibition of long-patch repair. When DNA replication and protein synthesis are halted by addition of chloramphenicol before irradiation, the chloramphenicol present after u.v. treatment does not promote cell death, nor does it inhibit the fixation of mutations (Bridges & Mottershead 1978a). We therefore conclude that the error-prone process itself is constitutive and that protein synthesis is not required for its operation. Protein synthesis may, notwithstanding, be required to maintain

the 'viability' of long-patch repair under conditions of normal growth. Perhaps this provides a role for the *recA* protein.

Although the mutagenic process occurring before replication is basically constitutive it can appear to be inducible, since protein synthesis may be required to maintain cellular viability. By analogy, therefore, although induction of mutation during post-replication repair may be chloramphenicol-sensitive, the present evidence does not exclude the possibility that post-replicative mutagenesis is also basically constitutive. Indeed, reasonable models can be made to explain SOS repair in terms of dose-dependent loss of 'immunity' to a constitutive, error-prone repair process at sites that are potentially mutagenic (Green 1977).

If there is no good evidence that an inducible pathway leads to cellular mutation, is there any stronger evidence that an inducible pathway leads to enhanced survival? Does W-reactivation work on the bacterial chromosome as well as on an infecting phage particle? Evidence to suggest that it does not comes from the properties of a temperature-resistant revertant of a *dnaE* strain (Bridges & Mottershead 1978b): this strain lost the ability to carry out W-reactivation of phage T3, yet its own u.v. sensitivity was almost unaffected.

Mr A. von Wright and I (unpublished results) therefore tried to imitate W-reactivation with the bacterial chromosome. A male Hfr strain was irradiated with u.v. light and mated either into a female histidine-requiring recipient that had been exposed to u.v. or into one that had not. The 'survival' of the incoming Hfr chromosome was followed by assaying for the recovery of recombinants that carried a donor marker (*his*$^+$). This experiment showed (Fig. 2) that when W-reactivation of phage λ occurred in the recipient exposed to u.v., the 'survival' of the *his*$^+$ marker from the donor chromosome was the same as that in the recipient which had not been exposed, and was also the same as the survival in a *lexA* recipient, which cannot perform W-reactivation. We therefore found no evidence that the W-reactivation pathway has a significant effect on the bacterial chromosome itself.

More definitive evidence to support this conclusion has come from studies with a most elegant system devised by Salaj-Šmic et al (1980). They used λ*c*1857 *ind red* lysogens, which are non-inducible by u.v. but which can be induced by a rise in temperature from 30°C to 42°C. With this system the survival of an infecting phage can be compared with that of a phage that has been integrated into the bacterial chromosome and allowed to lyse the cell at a subsequent stage.

Salaj-Šmic et al (1980) first studied W-reactivation in non-lysogens and showed that the inducible activity reached its peak 60 min after

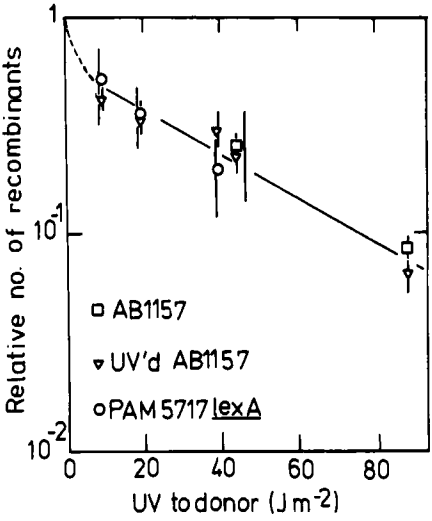

FIG. 2. Yield of *his*$^+$ recombinants in crosses between three recipient strains of *E. coli* and a u.v.-irradiated donor strain, *E. coli* JC5088. The results are expressed as a fraction of the number of recombinants obtained with an unirradiated donor. Recipients: K-12 AB1157, unirradiated (□); K12 AB1157, irradiated with 50 Jm^{-2} u.v. (▽); PAM 5717 *lexA* (incapable of W-reactivation), unirradiated (○). The absence of a difference in relative number of recombinants between the three recipients indicates that the W-reactivation pathway in AB1157 has no significant effect on the incoming bacterial chromosome.

u.v.-irradiation and then declined. They reasoned that, in a lysogen, a prophage that was allowed to remain integrated in the bacterial chromosome for 60 min before being induced to replicate by a temperature shift should survive much better than if it were so induced immediately after irradiation, provided that the inducible W-reactivation activity also worked on the bacterial chromosome. Contrary to this hypothesis, however, there was no effect in an excision-deficient strain, and the small effect which was observed in a wild-type strain could reflect the greater role of excision in determining bacterial survival compared with its role in phage survival. These workers concluded that the phenomenon is significant only when the doses given to the phage are considerably different from those given to the bacteria. This can also be seen from our data in Fig. 1, where the phage was given 150 J m^{-2} u.v. irradiation. When the cells were given the same dose of radiation the survival of the phage was reduced rather than enhanced. Therefore, I must conclude that W-reactivation, as a manifestation of SOS repair, is of little or no significance for bacterial survival. It will be interesting to apply the techniques of Salaj-Šmic et al (1980) to the study of mutagenesis, to see whether a similar conclusion can be drawn.

W-reactivation and W-mutagenesis of viruses have also been reported in cultured mammalian cells (for references see Sarasin 1978) but there is at present little evidence on the action of SOS repair on the mammalian chromosomes themselves.

ADAPTATION

Our knowledge of the phenomenon termed adaptation has expanded enormously in the two years since Samson & Cairns (1977) reported it. They initially observed that when *Escherichia coli* was grown in continuous culture, with a low concentration (1 μg/ml) of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), mutations were induced only during the first hour, although the culture was examined for mutations for seven days. The significance of a similar observation, previously made by Jiménez-Sánchez & Cerdá-Olmedo (1975) had not been appreciated. Samson & Cairns (1977) demonstrated that bacteria exposed to low concentrations of MNNG became resistant to both its lethal and its mutagenic actions. The development of this resistance was inhibited by chloramphenicol and presumably required *de novo* protein synthesis. Samson & Cairns (1977) concluded that 'adaptation' (as it was later termed) reflected the operation of an inducible and relatively error-free repair system.

Jeggo et al (1977) examined the effect of various DNA repair genes and concluded that adaptation was distinct from SOS repair. It had a rather general effect on simple alkylating agents but it was not induced by, nor did it affect damage produced by, ultraviolet light or 4-nitroquinoline 1-oxide. Jeggo et al (1977) also showed that the effect on mutagenesis could be distinguished from that on killing because mutagenesis required the genes *polA*$^+$ and *dam*$^+$. However, both types of adaptation could occur in the absence of DNA polymerase II, and therefore it was no longer relevant that MNNG, in the presence of chloramphenicol, had increased by five-fold the activity of DNA polymerase II in a *polA* strain of *E. coli* (Miyaki et al 1977).

The biochemical basis of adaptation was first uncovered by Schendel & Robins (1978), who showed that adapted bacteria rapidly removed O^6-methyl guanine (O^6-MG) from their DNA. However, the removal process was saturated as the challenge dose of MNNG was increased, and once saturation had occurred O^6-MG accumulated in the DNA and mutations were induced. It was envisaged that constitutive and inducible removal of O^6-MG were engaged in a race against two mutagenic processes (Schendel et al 1978). At low doses, miscoding by O^6-MG during replication was predominant while at high doses, error-prone repair, dependent upon the *lexA* gene, was more important.

The fate of the O^6-MG during adapted repair is obscure. Karran et al (1979) have recently shown that although a crude enzyme preparation from adapted cells removes O^6-MG from methylated DNA, there is no detectable release of methyl groups, alkylated bases or nucleotides. The preparation does not, therefore, contain a simple nuclease glycosylase or demethylase. Karran et al (1979) suggested that the methyl group might be transferred to another, biologically 'safer' site in DNA, but not to the N^7-position of guanine, which had been monitored. An alternative possibility is that O^6-MG might be converted to a non-coding residue, e.g. by cleavage of the pyrimidine ring, to generate a substituted imidazole residue that remains bound to DNA. Transfer of a methyl group or ring-opening might also render the lesion amenable to other repair systems. However, any biochemical model must account for the distinction, demonstrated genetically, between the effects of MNNG on adaptation against killing and those on adaptation against mutation. Induction of O^6-MG-removal can be demonstrated in *polA*$^-$ bacteria yet they do not show adaptation against killing. Conversely, a mutant, uncharacterized at present, and capable of adaptation against killing, cannot remove O^6-MG (P. Jeggo, personal communication).

The property of rapid saturation has recently been pursued by Robins & Cairns (1979) who concluded that the mediator of O^6-MG-removal is consumed during the reaction and that each molecule of the mediator may be able to eliminate only one O^6-MG residue. They particularly noted the parallel with some restriction enzymes, whose targets are also methylated bases and which act only once.

P. Jeggo (personal communication) has now identified a gene, *ada,* that blocks the adaptation phenomenon. Another gene, *alk,* has been shown by Yamamoto & Sekiguchi (1979) to block repair of methylated DNA. This second gene controls a pathway that is dependent on the *polA* gene and not the *recA* gene. Therefore the pathway may be linked with the one involved in adaptation. Clearly, further genetic and biochemical experiments will be needed to resolve this.

There is, as yet, no published evidence of an adaptation phenomenon in cultured mammalian cells but recent work on the rat indicates that a process analogous to adaptation can occur in the whole animal. Montesano et al (1979) have shown that the livers of rats treated with daily doses of 2 mg dimethylnitrosamine/kg could not only remove O^6-MG from DNA faster but could also synthesize DNA faster than untreated animals. A similar effect of 3,3-dimethyl-1-phenyltriazene in brain tissue has recently been suggested by Cooper et al (1978).

The phenomenon in rat liver may be different from that in bacteria since

pretreatment of rats with dimethylnitrosamine does not affect the removal of O^6-MG produced by subsequent exposure to methylnitrosourea; bacterial adaptation does not appear to show such specificity. By analogy with other DNA repair processes the underlying mechanism is likely to be considerably more complex in animals than in bacteria. One obvious complicating factor is that in large doses, dimethylnitrosamine itself inhibits O^6-MG-removal in the liver (Pegg 1978).

The most intriguing aspect of the work of Montesano et al (1979) is its relation to carcinogenicity data. There is a wealth of evidence (reviewed by Montesano et al (1979)) that the organs in which tumours most frequently arise *in vivo* after treatment with dimethylnitrosamine, ethyl- or methylnitrosourea, or 1,2-dimethylhydrazine, are generally those in which most O^6-alkylguanine persists. Why, then, should chronic low doses of dimethylnitrosamine, which induce O^6-MG-repair in the liver, give rise largely to liver tumours, while single high doses produce kidney tumours instead? Montesano et al (1979) suggest that the higher DNA synthesis in chronically treated liver is the source of additional miscoding. It is worth emphasizing that in u.v.-irradiated *E. coli* most mutations arise as errors during the process of excision repair itself and are not induced by photoproducts that remain unexcised (see Bridges & Mottershead 1978a). However, if excision is inhibited or blocked genetically then persisting photoproducts will induce mutations at a much higher rate. The correction of strand-directed mismatch, which operates after semi-conservative replication in bacteria, may not function as well at sites of excision repair; this could account for the higher error rate in repair-replication than in normal semi-conservative replication.

POST-REPLICATION REPAIR IN MAMMALIAN CELLS

When damaged DNA in mammalian cells is allowed to replicate, the newly synthesized DNA has a molecular weight that is lower than normal. It is still unclear whether a gap is left opposite the damage, as in bacteria, or whether termination of replication occurs at the replicon concerned. Subsequently, the molecular weight of the DNA is restored to normal, which may reflect either gap-filling or synthesis beyond the lesion. This process is called post-replication repair (PRR). Whether PRR enzymes may be induced in response to DNA damage is controversial.

Several papers have shown that the chasing of low molecular weight DNA into larger DNA can be enhanced in cells from Chinese hamster and from humans with xeroderma pigmentosum by prior treatment of the cells with a small dose of u.v. light or *N*-acetoxyacetylaminofluorene. This effect was not

observed when cells were incubated with cycloheximide between the split doses of u.v. light (for references see d'Ambrosio et al 1978). This has been interpreted as evidence for an inducible component in PRR and Chang et al (1978), on the basis of mutagenesis in Chinese hamster cells after fractionated doses of u.v., have concluded that this inducible repair is error-free.

Painter (1978), while confirming the phenomenon, interprets the concept of inducibility differently. He suggests that previous interpretations failed to take into account the damaging effect of the first exposure, which causes an abnormal distribution of different sizes of nascent DNA molecules at the time of the second irradiation. The existence of an inducible PRR component need not be invoked to explain the observed results.

Therefore, the case for inducible PRR in mammalian cells is not convincing at present, and further careful studies will be needed if Painter's argument (1978) is to be refuted.

DOSE–RESPONSE CURVES

On the basis of the stochastic effects such as tumorigenesis that are observed experimentally at high doses, we frequently need to predict the risks associated with low doses. These risks cannot usually be determined by direct experimentation. The question usually considered is whether the dose–response curve displays a threshold or quasithreshold (Fig. 3a) or is linear (Fig. 3b). However, there is a third possibility, depicted in Fig. 3c, in which a steep curve at low doses flattens to a more shallow curve at high doses (i.e. a saturating effect). Such a curve provides a tolerable fit to the data on carcinogenesis obtained with vinyl chloride in rodents.

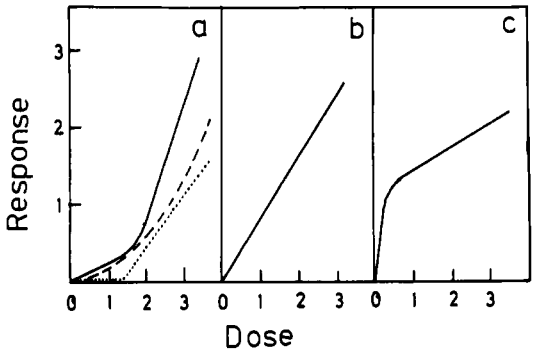

FIG. 3. Hypothetical dose response curves (a) threshold (. . . .), quasithreshold (———) or cumulative (– – –), (b) linear, (c) saturating.

A progressively saturating system of detoxification could generate a threshold or quasithreshold curve, but so too could an inducible activation pathway. Conversely, the saturating curve could reflect an inducible pathway of detoxification or a progressively saturating activation pathway. Similar curves could, however, be equally well generated by differential repair. Threshold or quasithreshold curves could be generated by either an inducible error-prone pathway or a progressively saturated error-free pathway; the third type of curve could be the consequence of an inducible error-free system or a progressively saturated error-prone system.

Effects due to metabolic or repair functions may be expected to be superimposed upon one another; they may differ between species, between individuals, between organs and between cell types. Effects may be different even with structurally related substances. Therefore, useful predictions of the kinetics of the dose–response relationship are practically impossible with our present knowledge.

Consequently, any extrapolation from the effects at high doses to those at low doses must be made on the basis of an empirical model. I would favour a linear model, not on theoretical grounds which are usually indefensible, but because it is a good compromise between the curves of Fig. 3a and 3c. Others may prefer different empirical models, e.g. that of Mantel et al (1975). Whatever model is used, the predicted effect may be a considerable underestimate or overestimate of the real effect. Control over the effects of potentially carcinogenic drugs and other chemicals therefore requires continuous and vigilant monitoring of exposed populations.

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Discussion

Garner: With regard to inducible repair in rat liver, Buckley et al (1979) have recently reported that pretreatment of rats with acetylaminofluorene induces repair of the O^6-methylguanine formed when a small dose of dimethylnitrosamine is given; this means that the inducing agent need not be the one that induced the damage in the first place. This may have wide implications for *in vivo* adaptation when organisms are exposed to many different chemicals. Another point is that O^6-methylguanine removal may not be representative of the DNA damage induced by other agents. You mentioned, for instance, that adaptation in bacteria was not seen with 4-nitroquinoline-1-oxide.

B.A. Bridges: The O^6-methyl and O^6-ethyl forms of guanine seem to be interchangeably removed in bacteria.

Garner: But they are not interchangeable in terms of biological effect.

Farber: And of course the kinetics of the reactions are different in whole animals.

Garner: Damage to DNA by ethylnitrosourea may be repaired by different mechanisms than those used after damage by methylnitrosourea and therefore the behaviour of structurally similar agents is not necessarily similar (Garner & Pickering 1979, Warren & Lawley 1980).

B.A. Bridges: Yes, there are clear differences between those compounds in rat liver and also in bacteria.

Gillette: When you use low concentrations of the alkylating agent do the kinetics of repair fit a first-order reaction, or is the repair a saturable process?

B.A. Bridges: This is complex and there is not enough information to allow generalizations to be made. In the skin, for example, the removal of pyrimidine dimers produced by u.v. light appears to be first order, but is saturable. In cultures of skin fibroblasts a small dose of u.v. leads to virtually no death, and does not induce mutations. As the dose is increased the excision process still occurs, but begins to saturate. Both cell death and induction of mutation begin to be observed, and dimers persist in the DNA for longer and longer periods of time. The skin therefore has an efficient process for removing that type of DNA damage. Whether the same saturation is also true for

chemical damage is controversial. One school of thought believes that the cell deals with 4-nitroquinoline-1-oxide damage in the same way as it deals with u.v. damage, and the other believes that the two are seen by the cell in totally different ways.

Conney: After pretreatment of bacteria with nitrosoguanidine, in low concentrations, how long does the enhanced removal of O^6-methylguanine last? Is there any information about the duration of enhanced repair in mammalian systems?

B.A. Bridges: I don't know the answer for the rat liver system – but for the bacterial system it is around 1–2 h. The bacteria have a short cell cycle and dilution of the O^6-methylguanine would therefore be expected to occur quite rapidly.

Conney: You indicated that if rats are pretreated with a low dose of dimethylnitrosamine followed by a high dose of methylnitrosourea, there is not an enhanced removal of O^6-methylguanine. Does enhanced repair occur if pretreatment with methylnitrosourea itself precedes a high dose of methylnitrosourea?

B.A. Bridges: I have not yet seen any published results from experiments of that nature. If we had more data from studies on mammalian cells, rather than from whole animals, we could construct critical hypotheses that could be thoroughly tested in the whole animal.

Garner: Do you use human or rodent cells in your cultures?

B.A. Bridges: Peter Karran in my laboratory is using human cells at the moment but rodent cells, or even hepatocytes, may also be worth investigating.

Farber: Isn't there a problem with the use of hepatocytes?

B.A. Bridges: Not particularly. We have removed the hepatocytes from rats, and used them immediately, as a primary culture, for activation purposes. They last for about 24 hours.

J.W. Bridges: In the repair system, do mutations occur only because saturation occurs or could chemicals act selectively to damage the repair system?

B.A. Bridges: Workers in this field have concluded that the phenomenon is generally one of saturation rather than one of inactivation of the repair enzymes. There is, however, some evidence that chemicals are capable of modifying repair enzymes: nitrosoguanidine, for example, is believed to modify a bacterial polymerase, thus making it more error-prone (Jiménez-Sánchez 1976).

J.W. Bridges: Do we know enough about the repair enzymes to suggest what sort of agents might damage them?

B.A. Bridges: No. The enzymology of DNA repair is still developing and much remains to be explained, but these enzymes presumably can be damaged in the same way as any other proteins.

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Environmental Chemicals, Enzyme Function and Human Disease
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Induction of drug-metabolizing enzymes by polycyclic aromatic hydrocarbons: mechanisms, and some implications in environmental health research

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Abstract The mechanisms by which administration of polycyclic aromatic hydrocarbons (PAH) stimulates drug-metabolizing enzymes are summarized. The hepatic monooxygenase system of marine and freshwater fish is also induced by PAH-type compounds, including chlorinated dioxins and certain polychlorinated and polybrominated biphenyl isomers, and these chemicals are, or are suspected to be, toxic to humans. Consequently, there is considerable interest in whether or not PAH-type induction in fish can be used as a sentinel or early warning indicator for certain classes of toxic pollutants in the aquatic environment. We have investigated various aspects of PAH-type induction in marine fish and have found that approximately 50% of the winter flounder *(Pseudopleuronectes americanus)*, captured near Mount Desert Island, Maine, appear to have induced hepatic monooxygenase systems, which suggests that they are exposed to PAH-type chemicals in the environment. The potential advantages and limitations of using PAH-type enzyme induction as a sentinel system for pollutants in aquatic ecosystems are discussed.

The cytochrome *P*-450-dependent monooxygenase system in microsomes oxidatively metabolizes a large variety of exogenous chemicals (drugs, pesticides, pollutants, food additives) and endogenous chemicals (steroids, hormones, vitamins, fatty acids). This enzyme system, which is present in hepatic and in many extrahepatic tissues, takes part in both detoxification and metabolic activation (conversion of a substrate to a more toxic product). It has been known for some time that the administration of various xenobiotics, including many polycyclic aromatic hydrocarbons (PAH), to animals can increase monooxygenase activity in both hepatic and extrahepatic tissues (see review by Conney 1967). There are multiple forms of cytochrome *P*-450 which can vary in their substrate specificities and in the relative proportion of oxidation at various positions of the same substrate molecule (e.g. benzo[*a*]pyrene as substrate). Consequently, induction of the monooxygenase

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system can play an important role in xenobiotic-mediated toxicity by dramatically, and sometimes preferentially, increasing the amount of a compound that is biotransformed to chemically reactive (toxic) metabolites or to non-toxic products. Owing to the potential contribution of monooxygenase induction to chemical carcinogenesis, mutagenesis, teratogenesis, and toxic drug–drug, drug–pollutant and pollutant–pollutant interactions it is important to understand the mechanism of this process. In this respect, more is known about PAH-type enzyme induction than about induction caused by other classes of xenobiotics, typified by phenobarbitone. Since induction of these enzyme systems was the topic of a recent symposium (Estabrook & Lindenlaub 1979), the mechanisms of PAH-mediated enzyme induction are summarized only briefly here. That PAH binds stereospecifically and with high affinity to a cytosolic receptor has been demonstrated in rat and mouse liver (Poland et al 1976) and in four different cultured cell lines (Guenther & Nebert 1977). The specific nuclear uptake of the PAH appears to be mediated by this cytosolic receptor protein since the inducer–receptor complex is translocated from the cytoplasm into the nucleus (Greenlee & Poland 1979). Once in the nucleus, this complex initiates the transcription and translation of a gene (or genes) which code for aryl hydrocarbon hydroxylase (AHH) activity (EC 1.14.14.1), cytochrome *P*-448, cytochrome P_1-450, UDP glucuronosyltransferase (EC 2.4.1.17), glutathione transferase B (EC 2.5.1.18 ligandin), aldehyde dehydrogenase (EC 1.2.1.3), DT-diaphorase (EC 1.6.99.2), δ-aminolaevulinate synthase (EC 2.3.1.37), epoxide hydrolase (EC 3.3.2.3), and possibly other enzymes that metabolize xenobiotic chemicals, by a mechanism that is not understood. However, earlier studies with cultured cells demonstrated that gene transcription was required for the PAH-mediated induction of AHH activity. Thus, inhibition of DNA-dependent RNA synthesis prevented increases in enzyme activity (Nebert & Gelboin 1970), and the early stages of this PAH-dependent enzyme induction were also dependent upon the synthesis of heterogeneous nuclear RNA (Wiebel et al 1972). Recent studies with other structural genes, such as mouse β-globin, suggest that considerable post-transcriptional modification of the heterogeneous nuclear RNA is required before the formation of functional mRNA, because genes are encoded in a discontinuous manner that requires removal of the DNA-inserts that do not code for the protein (Tilghman et al 1978). After the transport of the specific functional mRNA to the cytoplasm, translation is initiated. The dependence of PAH-mediated enzyme induction on protein synthesis was also demonstrated several years ago in studies which showed that increases in enzyme activity were sensitive to inhibitors of protein synthesis during the entire induction process (Nebert & Gelboin 1970). Even

though much work has gone into investigation of the mechanism of PAH-initiated enzyme induction, further study is necessary for an understanding of the manner in which the cytosolic receptor protein–inducer complex initiates transcription within the nucleus.

A current question, relevant to environmental health research, that falls within the scope of this symposium (i.e. the interactions between environmental chemicals and xenobiotic-metabolizing enzymes), is whether or not PAH-type enzyme induction can be used as a sentinel or early warning indicator for certain classes of chemicals that pollute the aquatic environment. The purpose of the study outlined here was to investigate various aspects of PAH-type enzyme induction in marine fish, including the possibility of using this enzyme response as a sentinel system for certain pollutants.

METHODS

Adult fish (winter flounder, *Pseudopleuronectes americanus;* little skate, *Raja erinacea;* sheepshead, *Archosargus probatocephalus*) were captured near Mount Desert Island, Maine or near Marineland, Florida and were acclimated at the laboratory (in seawater) for at least 24 h before use. Whole liver homogenate and washed hepatic microsomes were prepared as previously described (Bend et al 1979). The procedures used for determination of cytochrome *P*-450 content, and activities of AHH (EC 1.14.14.1) (in the presence and absence of 7,8-benzoflavone), 7-ethoxyresorufin deethylase, 7-ethoxycoumarin deethylase and benzphetamine *N*-demethylase (all EC 1.14.14.1), have also been described elsewhere in detail (Pohl et al 1974, Bend et al 1979). The protein content of microsomal and whole homogenate preparations was determined according to the method of Lowry et al (1951). Statistical analyses, including analysis of variance and multiple regression, were performed using standard procedures (Snedecor & Cochran 1967).

RESULTS AND DISCUSSION

There are several properties of PAH-type enzyme induction in fish liver which suggest that this response may be suitable for use as a sentinel system for pollutants of certain chemical classes, although there are also several limitations and potential problems in this approach. These properties are listed here.

(a) The hepatic monooxygenase system of both marine and freshwater fish is induced by PAH-type chemicals, including halogenated dioxins, and isomers of polychlorinated biphenyls (PCB) and polybrominated biphenyls

(PBB), but not by phenobarbitone-like inducing agents (Bend & James 1978, Elcombe & Lech 1979).

(b) Fish extensively accumulate lipophilic compounds from the water and the liver is normally a major storage site for this accumulation. Even though liver and several extrahepatic tissues of fish do contain cytochrome *P*-450-dependent monooxygenase activity (Bend & James 1978) fish normally metabolize xenobiotics much more slowly than do mammalian species. For example, 2,4,5,2′,5′-pentachlorobiphenyl has a half-life of approximately 2.5 days in the rat (Matthews & Anderson 1975) but is much more persistent in the dogfish shark, where 90% of an intravenous injection can still be present in the liver one week after administration (Bend et al 1976). Consistent with this observation is the finding that PAH-type enzyme induction is more persistent in fish than in mammals, and that it occurs at lower doses than in the mouse or rat. Thus, near maximal induction of hepatic AHH activity occurs after a 2 mg/kg dose of 3-methylcholanthrene in the sheepshead (a Florida teleost) and the enzyme activity remains maximally induced for approximately thirty days during winter (James & Bend 1980).

(c) Many of the chemicals that are widely distributed in the environment and cause PAH-type enzyme induction are or are suspected to be toxic to mammals, including humans.

(d) Within certain chemical classes the potency for enzyme induction has been correlated with acute toxicity in mammalian systems (Poland et al 1979).

An observation at our marine laboratory in Maine initially interested us in PAH-type enzyme induction in wild fish. In early studies, we demonstrated that the hepatic monooxygenase system of the little skate, a marine elasmobranch, was induced after treatment with PAH-type agents, such as 1,2,3,4-dibenzanthracene (DBA). Some of the properties of the monooxygenase system from untreated and DBA-induced little skates are summarized in Table 1. Under conditions where there was about a 40-fold induction of AHH activity in DBA-treated skate, there was no increase in the content of hepatic microsomal cytochrome *P*-450 and no hypsochromic shift in the difference spectrum of the haemoprotein (i.e. no obvious formation of cytochrome *P*-448). However, upon digestion and partial purification of the cytochromes *P*-450 from DBA-induced fish, a cytochrome *P*-448 species was identified (Bend et al 1979). *In vitro*, 7,8-benzoflavone *inhibited* the AHH activity of a reconstituted monooxygenase system that contained skate cytochrome *P*-448 from *DBA-treated* skate liver, whereas the addition of 7,8-benzoflavone, *in vitro, increased* the AHH activity of a reconstituted monooxygenase system that contained cytochrome *P*-450 from *untreated* skate liver (Bend et al 1979). These data agreed with those obtained with

TABLE 1

Effect of pretreatment with 1,2,3,4-dibenzanthracene on the hepatic microsomal monooxygenase system of the little skate, *Raja erinacea*[a]

Variable	*Untreated fish*	*DBA-induced fish*
Cytochrome *P*-450 content (nmol/mg protein)	0.24 ± 0.08 (14)[b]	0.15 ± 0.05 (3)
AHH activity (FU/min/mg protein)	0.18 ± 0.18 (14)	7.9 ± 3.9 (3)
AHH activity (without 7,8-benzoflavone)	0.23[c]	5.2[c]
AHH activity (+ 10^{-4} M 7,8-benzoflavone)	0.73[c]	1.2[c]
7-ethoxycoumarin deethylase activity (nmol/min/mg protein)	0.64 ± 0.49 (14)	2.3 ± 0.3 (3)

[a]Data from Bend et al 1979, James & Bend 1980.
[b]All values given in this way are mean ± 1 SD(n).
[c]Data from a single experiment. Experiment was repeated twice and similar data were obtained each time.
FU, fluorescent unit; AHH, aromatic hydrocarbon hydroxylase (EC 1.14.14.1). 1 FU is the fluorescent intensity of a solution of 3 μg quinine sulphate per ml 0.1 N H_2SO_4 at excitation wavelength 400 nm and emission wavelength 525 nm.

liver microsomes from DBA-induced and control skates; 7,8-benzoflavone inhibited the AHH activity of microsomes from PAH-induced fish and increased the AHH activity of hepatic microsomes from untreated skate (Table 1).

However, when similar experiments were performed with winter flounder, a marine teleost, somewhat unexpected results were obtained (for example, see Table 2). Those fish that were pretreated with PAH-type agents all had elevated hepatic microsomal AHH activity which was inhibited by benzoflavone *in vitro,* as would be consistent with PAH-type induction. However, some of the untreated fish invariably also had high AHH activity, suggesting they were pre-induced, possibly due to exposure to PAH-type compounds in the environment. To test this possibility we studied a larger population of flounder and determined AHH activity in the presence and absence of 7,8-benzoflavone and 7-ethoxyresorufin deethylase (7-ERF deethylase) activity in the livers of individual fish. We modified our procedures so that these enzyme activities could be measured in whole homogenate, enabling us to study a larger number of fish, and we verified this approach by comparing these enzyme activities in whole homogenate with those in microsomes (Bend et al 1978).

Over the last two summers more than 200 fish have been sampled. Large variations (greater than 40-fold) were observed in hepatic AHH and 7-ERF

TABLE 2

Effect of 7,8-benzoflavone *in vitro* on aromatic hydrocarbon hydroxylase activity in hepatic microsomes from untreated and dibenzanthracene-pretreated winter flounder *(Pseudopleuronectes americanus)*

	AHH activity (FU/min/mg protein)	
	Without 7,8-benzoflavone	*With 10^{-4} M 7,8-benzoflavone*
Untreated fish	0.05	0.19
	0.40	0.19
	1.76	0.83
DBA-treated fish[a]	1.11	0.63
	1.67	1.01
	1.84	0.78

[a]Treated with 10 mg/kg 1,2,3,4-dibenzanthracene (DBA) on days 1, 2 and 3, assayed seven days after last injection.
FU, fluorescent unit.

deethylase activities in both years, indicating that this heterogeneity is a recurring phenomenon. When AHH activity was plotted against the change in activity due to the addition of 0.5 mM 7,8-benzoflavone *in vitro* (i.e. AHH activity in the presence of 7,8-benzoflavone minus AHH activity in the absence of 7,8-benzoflavone) the data obtained in 1979 closely approximated a straight line, as shown in Fig. 1. It is obvious that the higher the AHH activity, the greater the inhibitory effect of 7,8-benzoflavone *in vitro* while the lower the AHH activity, the greater the likelihood of observing stimulated AHH activity with 7,8-benzoflavone.

Fig. 2 shows that there was also a positive correlation between hepatic AHH activity and 7-ERF deethylase activity in the flounder studied in 1978. This data (Fig. 1 & Fig. 2), together with what is known about PAH-mediated enzyme induction in fish and rodents, suggests that the flounder with high hepatic AHH and 7-ERF deethylase activities were induced by exposure to a PAH-type enzyme inducer (or inducers) in the environment, and that this induction is related to the *de novo* synthesis of a new form or forms of cytochrome *P*-450, commonly designated cytochrome *P*-448 or cytochrome P_1-450.

Studies with benzphetamine, which is normally a poor substrate (i.e. has a low turnover number) for PAH-induced forms of cytochrome *P*-450, also supported this conclusion. Benzphetamine *N*-demethylase activities varied only about two-fold in liver homogenate from eight flounder, whereas AHH activity in the same fish varied 35-fold. Moreover, benzphetamine

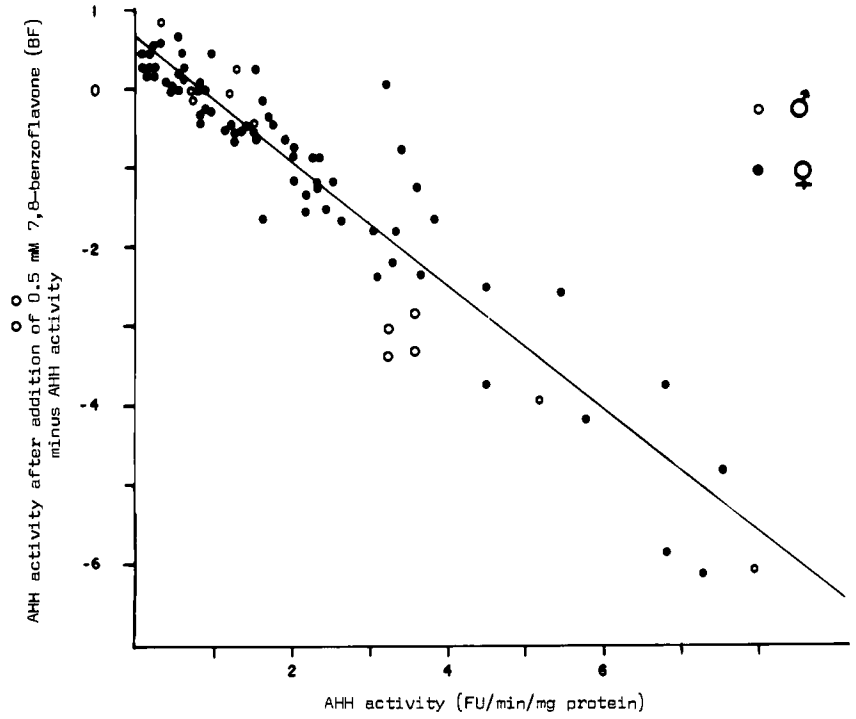

FIG. 1. Specific benzo[*a*]pyrene hydroxylase (AHH) activity (EC 1.14.14.1) in liver homogenate of male and female winter flounder *(Pseudopleuronectes americanus)* plotted against AHH activity after *in vitro* addition of 0.5 mM 7,8-benzoflavone minus AHH activity in the same homogenate. The line was fitted to the data by multiple regression analysis. (r = 0.95; n = 81)

TABLE 3

Cytochrome *P*-450 content and aromatic hydrocarbon hydroxylase activity of hepatic microsomes from several winter flounder

Cytochrome P-*450* *(nmol/mg protein)*	λ Max *(nm)*	AHH *(units/nmol cytochrome* P-*450/min)*
0.60	448.4	6.4[a]
0.25	451.0	0.7[b]
0.23	450.4	1.1[a]
0.18	450.2	3.1[a]
0.16	450.8	1.1[a]
0.12	450.2	1.8[a]
0.12	450.0	0.9[a]

[a]AHH activity inhibited by addition of 7,8-benzoflavone *in vitro* (0.5 mM).
[b]AHH activity increased by addition of 7,8-benzoflavone *in vitro* (0.5 mM).

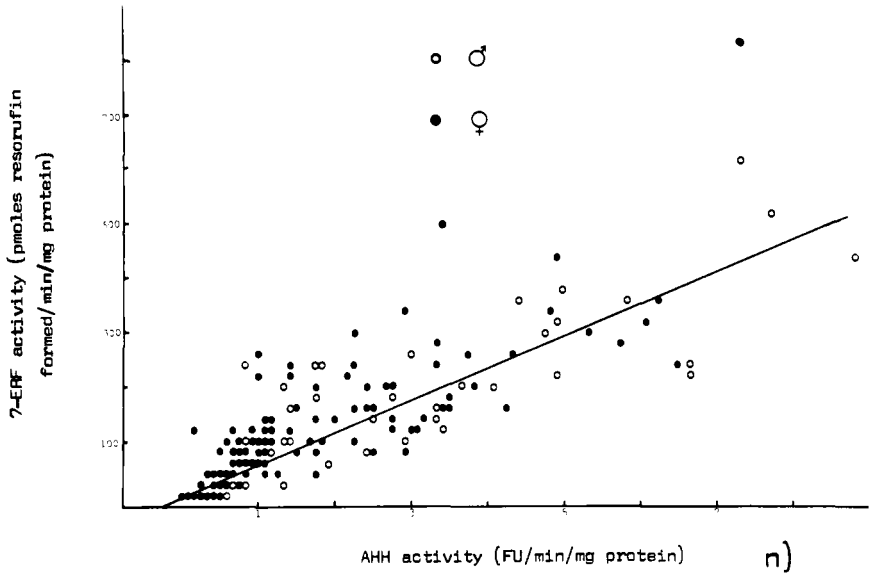

FIG. 2. Specific benzo[*a*]pyrene hydroxylase (AHH) activity (EC 1.14.14.1) in liver homogenate of male and female winter flounder *(Pseudopleuronectes americanus)* plotted against 7-ethoxyresorufin deethylase (7-ERF) activities in the same homogenate. The line was fitted to the data by multiple regression analysis. (r = 0.83; n = 172)

N-demethylase activity was independent of AHH activity (Bend et al 1980).

This argument for PAH-mediated enzyme induction is strengthened by the data presented in Table 3, where the cytochrome *P*-450 content, the maximum absorption wavelength (λ) of cytochrome *P*-450 (in the carbon monoxide-ligated and reduced form), and the AHH activity in hepatic microsomes from several winter flounder are compared. In the single fish with highest AHH activity and highest microsomal cytochrome *P*-450 content, the maximum absorption λ occurred near 448 nm, which indicates the presence of large amounts of cytochrome *P*-448 in the microsomes. In several of the other fish, however, AHH activity was elevated without an obvious hypsochromic shift in the absorption peak of cytochrome *P*-450 (which occurs near 450 nm). This is similar to results obtained after administration of PAH-type chemicals to fish, when there can often be large increases in hepatic AHH activity that are inhibited by 7,8-benzoflavone added *in vitro,* but where there is neither a discernible shift in the maximum absorption λ of the microsomal cytochrome *P*-450 spectrum nor an increase in the specific cytochrome *P*-450 content. For

this reason, we have routinely used a combination of monooxygenase activities (7-ERF deethylase activity and AHH activity in the presence and absence of 7,8-benzoflavone) to test for PAH-type enzyme induction in fish populations, instead of using the amount of cytochrome *P*-450 or its maximum absorption λ.

Enzyme activities were also studied in kidney homogenate from 31 flounder, on the assumption that if the hepatic enzyme response was due to exposure to PAH-like inducers present in the marine environment, renal activities in fish with induced hepatic AHH activities might also be increased (data not shown). However, the renal monooxygenase system of only one fish tested appeared to be induced even though the hepatic monooxygenase system of 85% of these particular flounder was induced. Thus, if PAH-type chemicals are responsible for the hepatic enzyme response, then either these chemicals must be largely sequestered in the liver and unavailable at sufficient concentrations in the kidney to exert PAH-type enzyme induction of the renal monooxygenase system, or the renal system may not be inducible.

It was previously reported by Stegeman (1977) that sexually mature male rainbow trout *(Salmo gairdneri)* contain more hepatic microsomal cytochrome *P*-450 and about three-fold higher aminopyrine demethylase activities (EC 1.14.14.1) than do females during spawning. This suggested that spawning and age (sexual maturity) of the winter flounder might be related to the wide variations in 7-ERF deethylase and AHH activities that we observed in this fish population. Consequently, AHH activity was compared to the gonad wt./body wt. ratio in female fish. Relatively large variations were noted in gonad/body wt. ratios (0.8–2.3%), which were consistent with different levels of ovarian function in these fish. The correlation coefficient for the relationship between AHH activity and gonad/body wt. ratio was only 0.34 (n = 72), which indicated no relationship between the two variables (Bend et al 1980). Moreover, as shown in Fig. 1, there was no difference in AHH activities, either in the presence or in the absence of 7,8-benzoflavone, between the male and female flounder assayed. Thus, it appeared that spawning and sexual maturity made only a minor contribution to the widely divergent AHH and 7-ERF deethylase activities. This is consistent with the induction of the hepatic monooxygenase system in some of these fish by a PAH-type environmental pollutant.

If elevated AHH and 7-ERF deethylase activities and inhibition of AHH activity by 7,8-benzoflavone *in vitro* are used as indices for induction, then more than 50% of the winter flounder sampled in Maine over a two-year period had induced hepatic monooxygenase systems (Fig. 1). Since these fish were all captured in the same general area, four miles seaward of Sand Beach, Mount

Desert Island, Maine, and since they were inhabiting a similar environment, it was interesting that all of the fish did not show the response. Because the flounder with low AHH and 7-ERF deethylase activities could be induced by treatment with PAH (Table 1), they do not represent a subpopulation that fails to respond to exposure to PAH-type chemicals. Some of the flounder studied at each period between June and August had induced monooxygenase systems and hence this enzyme response was not time-dependent, at least within the summer season (Bend et al 1978). The possibility exists, of course, that the induced fish were exposed to environmental pollutants elsewhere and that they then migrated into this relatively pristine area.

The data reported here demonstrate that about half of the winter flounder captured near Mount Desert Island, Maine, have hepatic monooxygenase systems that are characteristic of PAH-type enzyme induction. The reason for this enzyme induction has still not been elucidated although the observations are consistent with the enzyme response being caused by an exogenous factor, such as the presence of a polycyclic hydrocarbon-like inducer in the environment. However, the work that we have completed to date has shown that additional detailed studies are required before pollutants can be associated definitively with the increased hepatic monooxygenase activities in these Maine flounder, and especially, before enzyme induction can be used as a biochemical monitor for toxic environmental chemicals, at least with this species.

CONCLUSIONS

Much is known about the mechanism by which polycyclic aromatic hydrocarbons induce enzymes that metabolize xenobiotics, including the cytochrome *P*-450-dependent monooxygenase system. However, further study is required to elucidate certain aspects of this process, including the manner in which the cytosolic receptor–inducer complex initiates transcription within the nucleus.

The question of whether or not PAH-dependent enzyme induction in fish can be used successfully as a routine biochemical monitor for toxic pollutants in the aquatic environment is still not settled. In spite of some theoretical considerations that support this suggestion, there remain several limitations and practical problems to be resolved. Major advantages for such a biochemical monitoring system would be the sensitivity to potent PAH-type enzyme inducers, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and the fact that such repetitive assays are inexpensive. A difficulty is that certain constituents of the aquatic environment, other than toxic pollutants, may cause

PAH-type enzyme induction. Obviously, a thorough characterization should be made of the hepatic monooxygenase system in untreated and PAH-treated fish of any selected species, under carefully controlled laboratory conditions, before field observations can even be contemplated. Consideration should be given to studies of routes of administration and dose–responses with selected chemicals that are important contaminants of the marine environment. The time required for maximal induction at various water temperatures and the duration of induction are also important considerations. The hepatic enzyme response should be correlated with tissue concentrations (particularly in the liver) of the various compounds used as inducing agents. A necessary part of any field study should be the identification and quantification, if possible, of classes of pollutant chemicals that both cause PAH-type induction and occur as residues in the liver. These contaminants, and their concentrations, should be compared in fish with highly induced enzymes and in fish with uninduced hepatic monooxygenase systems. Such experiments are in progress with Maine flounder. Thorough investigations of this type are required to determine whether, and under what specific conditions, enzyme induction in fish can be used as a biochemical monitoring system for toxic pollutants. Our data show that many of the flounder that inhabit a relatively unpolluted area of coastal Maine have PAH-type induced monooxygenase systems. This induction also occurs in another Maine teleost, the killifish *(Heteroclitus americanus)* (G.L. Foureman & J.R. Bend, unpublished data).

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Discussion

Connors: The amount of enzyme induction observed in these fish may be correlated either with the concentration of pollutant or with the age of the fish. If induction is continuous, how can you quantitate the effects of these variables?

Bend: Since about 95% of lipophilic chemicals, e.g. 2,4,5,2′,5′-pentachlorobiphenyl, are localized in the liver of marine fish in Maine (where the

summer water temperature is 10–15°C), we are presently testing for a correlation between the variable hepatic enzyme activities (induced versus normal) and the amount of polycyclic aromatic hydrocarbon (PAH) and polychlorinated biphenyl residues in livers of similarly sized fish.

Farber: Dr Stich in Vancouver (personal communication) has studied development of epidermal papillomata in fish populations in bays that have varying degrees of pollution from industrial wastes. Have you attempted to correlate presence or absence of induction with formation of such focal hyperplasia?

Bend: As the epidermal papilloma does not occur in marine flatfish from Maine, we have not correlated induction with hyperplasia. Some recent work by Dr Clyde Dawe and his colleagues at the National Cancer Institute has shown that 'X cells' are unicellular parasites, probably amoebae (Dawe et al 1979). Consequently, it is believed that those tumours, such as epidermal papillomata of Pacific flatfish, that are composed largely of X cells, are caused by parasitic diseases which only simulate neoplasms. So the tentative correlation that has been made between incidence of epidermal papillomata in flatfish and concentration of PAH (or other chemical carcinogens) in the environment may only be coincidental.

Hunter: In general terms, does the presence of induction modify the health of the fish in any other way?

Bend: We have been unable to detect any differences in health between the induced and normal fish. However, we have never observed them in the laboratory for more than a few weeks.

Oesch: It is known that some compounds are potent enzyme inducers without being carcinogenic, and that there is no correlation between enzyme induction and carcinogenicity when polycyclic hydrocarbons are administered to rodents (Arcos et al 1961). However, you stated that within any class of compounds, carcinogenicity was indeed related to the degree of induction in the fish. Would you include the polycyclic hydrocarbons as one of these groups of compounds?

Bend: No. My original comment was that within certain classes of compounds that cause PAH-type induction, there is a correlation between induction and acute toxicity. Polycyclic hydrocarbon toxicity is relatively complicated because secondary, tertiary, and even quaternary metabolism can be responsible for production of potent carcinogens or mutagens. In studies of benzo[*a*]pyrene metabolism in perfused lung preparations, we found (Ball et al 1979) that primary and secondary oxidative metabolites (phenols and dihydrodiols) formed in the lung accumulated, thus facilitating further metabolism to more toxic products (e.g. benzo[*a*]pyrene

7,8-dihydrodiol-9,10-epoxides), which may be at least partially responsible for the lung's susceptibility to PAH-type carcinogens. The complexity of the metabolism of PAH to carcinogens may be responsible for the lack of correlation between induction by PAH and carcinogenesis. For example, products from minor metabolic pathways may cause cancer but I doubt that such minor metabolites contribute much to the induction process.

Breckenridge: F.S. Pagan and R.D. Pagan (personal communication, 1977) demonstrated a seasonal effect on induction in human lymphocytes, with differences between people who remain in Buffalo and those who go to visit Florida. Does the same thing happen in fish?

Bend: The effect is reproducible from one year to another and is not like inducibility of benzo[*a*]pyrene hydroxylase activity in lymphocytes. These fish have their highest hepatic monooxygenase activities during the time of year (June–August) that we examine them. Fish often feed only once a week or less, and we recently tested for any correlation between induced hepatic enzyme activity and intestinal contents (as an indication of recent feeding) in winter flounder, but we found no relationship between them. Fish with induced benzo[*a*]pyrene hydroxylase and 7-ethoxyresorufin deethylase activities were found to be almost equally distributed between the two groups (J.R. Bend, E.R. Stockstill, G.L. Foureman, unpublished work). However, in fish under laboratory conditions in Florida, PAH-mediated hepatic enzyme induction did not depend on the time of the year.

Breckenridge: Is the induction temperature-dependent?

Bend: Yes. Hepatic monooxygenase induction occurred in sheepshead *(Archosargus probatocephalus)* acclimated to either warm (26°C) or cold (14°C) water, after treatment with 3-methylcholanthrene (10 mg/kg). Maximum induction of benzo[*a*]pyrene hydroxylase activity occurred four days after treatment in warm water and eight days after treatment in cold water; the induction was considerably more persistent in the cold water fish (M.O. James, J.R. Bend, unpublished work). We attribute this to the more rapid metabolism of the inducing agent in warmer water.

Smith: Did you observe a wide range of values, which might suggest that metabolism is polymorphically controlled?

Bend: Yes, we observed a wide range of enzyme activities but we do not know yet how dependent this is on genetic heterogeneity and how much on enzyme induction. We are fairly certain that the results obtained are not due only to genetic heterogeneity since the flounder with the low benzo[*a*]pyrene hydroxylase activities are all induced after treatment with PAH-type agents. Also, the properties of the hepatic monooxygenase system in the wild fish with elevated enzyme activities are analogous to those in fish given PAH.

Smith: What is the genetic structure of a shoal of fish; is it an inbred or a wild population?

Bend: I am not certain, but I believe that in the repeated sampling that we have conducted over three summers we would have tested *some* genetically different flounder. However, with killifish *(Fundulus heteroclitus),* which tend to return to the same estuary on the incoming tide and which also show the same pattern of enzyme induction in the wild as flounder, there is a greater likelihood of inbreeding.

Smith: Each shoal may therefore constitute a separate genetic population.

Bend: I suppose that is possible but the flounder are more migratory than the killifish and so I do not think this is likely.

McLean: Dr Bend, you have been testing the efficacy of this system as a means for measuring pollution by polycyclic substances in the environment, but Wattenberg (1975) has demonstrated induction of benzo[*a*]pyrene hydroxylase activity in the gut by many natural compounds.

Paine & McLean (1974) showed that riboflavin and light in tissue culture could produce as powerful an induction as that produced by a maximum inducing dose of 3-methylcholanthrene. Therefore, there are very many inducers, of natural origin, which may play a part in the natural food chain.

Bend: The points you make are excellent, and we have taken them into consideration. Actually, we have been trying to determine the reason for the observed hepatic enzyme induction in marine teleost fish; we have not been testing its efficacy for measuring polycyclic hydrocarbon substances in the environment. It is well known that certain natural products, such as flavonoids, are potent inducers of hepatic microsomal monooxygenase activities but these chemicals are not known to be widely spread in the aquatic environment. On the other hand, polychlorinated biphenyls and PAH are quite widely distributed and occur as tissue residues in fish species.

Rawlins: We have examined the effect of corticosteroids on the induction of benzo[*a*]pyrene hydroxylase in epidermal cells of human skin, and we observed that hydrocortisone, prednisolone and betamethasone are equipotent (in molar terms), and have dose–response curves that are almost superimposable. Thus, there is dissociation of biological potency between corticosteroid activity and inducing activity.

Higginson: Many environmentalists are searching for simpler indices of pollution, possibly among fish in polluted water. Your work suggests that such a simple system does not exist.

Bend: Some people in North America (Payne 1976) and Europe claim that elevated hepatic benzo[*a*]pyrene hydroxylase activities in fish can be used as an index of petroleum pollution in water but, given the nature of our data, we

disagree with that assumption and feel that more information is necessary before this inductive response should be used in field studies.

Connors: What advantages do biological systems for pollution detection have over simple physico-chemical measurements?

Bend: There are two potential advantages. The first is that very potent chemicals, such as the halogenated dioxins, would give biological effects at concentrations that are very near or below the detectable levels. A second advantage could be the relative expense involved; biological tests could be cheaper. However, before biological end-points are used for detection of water-borne pollutants, the cause(s) of the effect must be clearly elucidated. This is currently what we are investigating.

J.W. Bridges: Your argument regarding differences between elasmobranchs and bony fish intrigues me. Is methylcholanthrene a more potent and persistent inducer in bony fish because of inherent features of the induction mechanism(s) in these species or is it just that the elasmobranchs metabolize polycyclic hydrocarbons more rapidly, or concentrate them less, because they possess different binding proteins?

Bend: 3-Methylcholanthrene (3-MC) injection produces hepatic enzyme induction in the elasmobranchs, and we have isolated cytochrome *P*-448 from little skates treated in this manner (Bend et al 1979). It is possible that the little skate metabolizes and excretes 3-MC more quickly but we have not studied either that or the persistence of induction in the little skate after administration of 3-MC so I cannot answer that part of your question.

Hunter: Which effect is more important in producing natural induction in your fish – is it the water it swims in, or the food it eats?

Bend: I think both are important; the relative importance would depend upon the amount of inducing agents absorbed from food and the amount absorbed from the water.

Hunter: Is there a difference in the diet of your elasmobranchs and teleosts?

Bend: The teleosts (winter flounder) and elasmobranchs (little skate) live in the same areas and have very similar diets, but this is an important question.

Connors: It seems, therefore, that the fish system is not a perfect monitor of environmental pollution!

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Environmental Chemicals, Enzyme Function and Human Disease
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Induction of drug-metabolizing enzymes by phenobarbitone: structural and biochemical aspects

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Abstract Two aspects of the induction of microsomal monooxygenases by phenobarbitone have been investigated. First, structural associations between mitochondria and single cisternae of rough endoplasmic reticulum (mitochondria–RER complexes) may operate as functional units in the biosynthesis of cytochrome *P*-450. This was deduced from (i) studies on the subcellular distribution of the phenobarbitone-induced incorporation of leucine into microsomal proteins including apocytochromes *P*-450 and (ii) the incorporation of labelled δ-aminolaevulinic acid into the haem prosthetic group of cytochrome *P*-450. Secondly, in hepatocytes from chick embryo in primary monolayer culture, induction of cytochrome *P*-450-haemoproteins was markedly influenced by changes in the proliferative activity of hepatocytes. Inducibility of cytochrome *P*-450 by phenobarbitone and by β-naphthoflavone was decreased in cultures with 'spontaneous' or experimentally increased proliferative activity of hepatocytes. Treatment with inhibitors of DNA synthesis increased the induction response.

The phenomenon of induction of microsomal mixed function oxidases (or monooxygenases) by drugs and other chemicals or by endogenous compounds has been known for over 25 years, yet we still do not understand the molecular events within the cell that trigger the induction process. Of the three essential components of the monooxygenase reaction, NADPH-cytochrome reductase (EC 1.6.2.4), cytochrome *P*-450 and phospholipid, the haemoproteins collectively called cytochrome *P*-450 play a key role in oxygen activation and substrate binding and they determine the overall substrate specificity of the enzyme system (for review see Ullrich et al 1977).

Compounds that induce hepatic microsomal monooxygenase activities usually have been divided into two major classes: one typified by phenobarbitone, which induces several molecular forms of cytochrome *P*-450 and monooxygenase activities directed toward a wide variety of substrates, and the other typified by the polycyclic aromatic hydrocarbon 3-methylcholanthrene,

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which induces one (or possibly two) different subspecies of cytochrome *P*-450 and a more limited group of associated enzyme activities. A number of additional differences between these two classical inducers has been demonstrated, including absence of any marked increase in membrane proliferation or cell hypertrophy, and absence of any increase in phospholipid synthesis after exposure of the organism to 3-methylcholanthrene. However, these differences are not consistently present in all species (Buynitzky et al 1978).

The above 'dichotomy' of the inducer–response relationship appears somewhat artificial and too simplistic in the light of recent developments. There is now ample evidence for at least six forms of cytochrome *P*-450 which have different catalytic, spectral, immunological, structural and probably, also, catabolic properties (for review see Ullrich et al 1977). In fact, one of the puzzling questions about microsomal cytochrome *P*-450 in the liver is whether this small number of distinct forms of the enzyme can account for the multitude of reactions catalysed, or whether a much larger number of isoenzymes is involved. Moreover, the different molecular species of cytochrome *P*-450 have overlapping substrate or inhibitor specificity, i.e. all cytochrome *P*-450 species are capable of metabolizing all substrates but they possess different affinities for each substrate. Each of the several hundred drugs, carcinogens, steroids and other chemicals that induce their own, and the metabolism of other, substrates apparently stimulates a particular 'profile' of molecular forms of cytochrome *P*-450. This change in profile may result in different ratios of inactive, active or toxic metabolites being produced from the same substrates and it can thereby modify drug action and toxicity. The similarity between these profiles or patterns of induced or decreased activities of cytochrome *P*-450-haemoproteins (and associated monooxygenase activities) may still allow distinction of several classes of inducers, but there is clearly more diversity in the induction response than was previously suspected.

While induction of monooxygenases by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene is preceded or regulated by binding of the inducer to a specific cytosolic receptor and by the consequent hepatic nuclear uptake of the inducer (Poland et al 1976, Greenlee & Poland 1979, Guenther & Nebert 1977), no cytosolic receptors have been identified for phenobarbitone or other drugs or steroids. The initial intracellular event that produces the signal for differential induction of cytochrome *P*-450-haemoproteins by these compounds is unknown and one can only speculate on the potential mechanisms that would allow the *de novo* synthesis of the multiple enzyme proteins (Nebert 1979).

In this paper, we review two of the many aspects of the phenobarbitone-

mediated increase in hepatic microsomal cytochrome *P*-450-haemoproteins. First, we describe the structural and functional interaction between the endoplasmic reticulum and mitochondria during synthesis of cytochromes *P*-450, and the possible role of these organelle interactions in membrane biogenesis. In the second part, we discuss how changes in the proliferative state of hepatocytes affect the induction response. These latter studies also demonstrate the usefulness of primary culture of hepatocytes in the study of genetic, nutritional, hormonal and other factors that modify the induction of drug metabolism.

EXPERIMENTAL PROCEDURES

Subcellular fractions were prepared from male Sprague-Dawley rats (150–200 g) after perfusion of the liver *in situ* with ice-cold 0.9% NaCl and homogenization in 0.35 M sucrose, 2.5 mM Mg acetate, 10 mM Tris acetate, pH 7.4. Subfractions of endoplasmic reticulum were prepared as described by

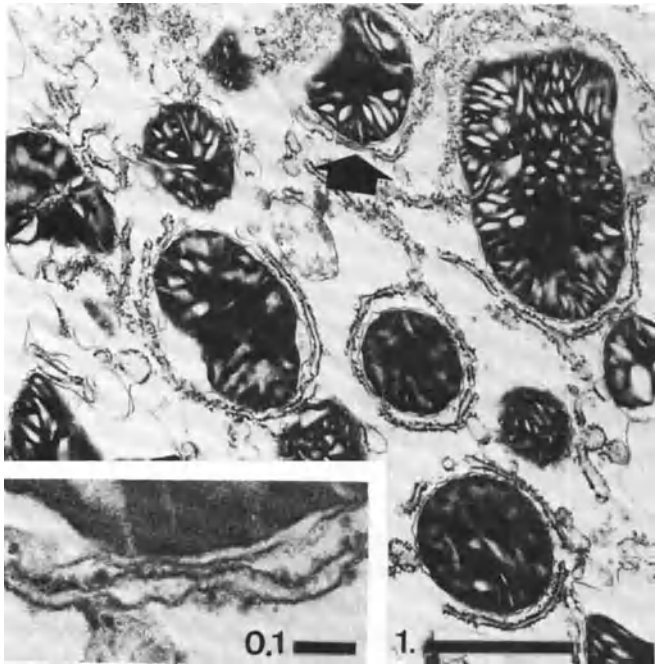

FIG. 1. Electron micrograph of 'Mitochondria–rough endoplasmic reticulum (RER)' fraction isolated from nuclear pellets. Inset and arrow point to close associations between RER and mitochondrial membrane.
—— 0.1 μm (inset), and 1.0 μm

Dallner (1974). Mitochondria with associated rough endoplasmic reticulum (RER) membranes were isolated by modifying the techniques described by Lewis & Tata (1973). These RER membranes were separated from mitochondria by hypotonic–hypertonic treatment (P.J. Meier, M.A. Spycher and U.A. Meyer, unpublished work).

For the primary monolayer culture, hepatocytes were isolated by collagenase (EC 3.4.24.3) digestion of livers from 15 day old chick embryos (Shaver hybrids). The cells were maintained in Williams E medium in the absence of serum except for an initial two to four hour attachment period (Althaus et al 1979). Drug metabolism in monolayers *in situ* was demonstrated by histofluorimetry, with ethoxyresorufin as substrate (Burke & Hallman 1978, U. Giger & U.A. Meyer (unpublished work)). Induction of cytochrome *P*-450 and drug metabolizing activity was studied after 16 to 18 hours of exposure of the preparations to phenobarbitone or β-naphthoflavone, exposure being started at different times after culture. DNA synthesis was demonstrated by autoradiography *in situ* of cultures that were seeded in 35 mm plastic dishes and exposed to 6-[^{3}H]thymidine for periods of 12 or 24 hours (0.2 μCi/ml; 20 Ci/mmol).

RESULTS AND DISCUSSION

Structural and functional interaction between mitochondria and rough endoplasmic reticulum during synthesis of cytochrome P-*450-haemoproteins*

Induction of cytochrome *P*-450-haemoproteins by phenobarbitone in the liver requires *de novo* synthesis of cytochrome *P*-450 apoproteins and of the prosthetic group haem (iron–protoporphyrin IX) and is associated with the proliferation of membranes of the endoplasmic reticulum. Apocytochrome *P*-450 is synthesized on membrane-bound polysomes of rough endoplasmic reticulum (RER; Fujii-Kuriyama et al 1979, Craft et al 1979) whereas the final steps of haem synthesis are catalysed by mitochondrial enzymes (Meyer & Schmid 1978). The obligatory functional interaction of mitochondria and RER suggested to us that the intimate structural associations between mitochondria and single cisternae of RER observed in rat liver *in vivo* (Jones & Emans 1969) could be important in the regulation of the synthesis of apocytochrome *P*-450 or haem or in the final assembly of holocytochromes *P*-450. Intact structural complexes of mitochondria and RER (MITO–RER complexes) were therefore isolated from crude nuclear pellets of rat liver homogenates (Fig. 1) and the cytochrome *P*-450 concentration, as determined by its carbon monoxide-binding spectrum, was found to be higher in RER

that had been isolated from these MITO–RER complexes than in RER that had been prepared from postmitochondrial supernatants (Table 1). Moreover, dissociation of synthesis of apocytochrome *P*-450 and availability of haem, by concomitant treatment of rats with $CoCl_2$ and phenobarbitone, resulted in an apparent accumulation of 'free' apocytochrome *P*-450 predominantly in MITO–RER complexes (Correia & Meyer 1975, Meier et al 1978, P.J. Meier & U.A. Meyer, unpublished work).

TABLE 1

Cytochrome *P*-450 concentration in subfractions of endoplasmic reticulum isolated from mitochondria–rough endoplasmic reticulum (RER) complexes and from postmitochondrial supernatants (PMS)

	Cytochrome P-450 nmol/mg protein[a]
Rough endoplasmic reticulum (RER) separated from mitochondria–RER complexes	0.531 ± 0.032[b]
Rough microsomes (RER isolated from PMS)	0.453 ± 0.029
Smooth microsomes (SER isolated from PMS)	0.921 ± 0.108

[a]Data are given as mean ± S.D.; $n = 6$
[b]Different from rough microsomes ($P < 0.05$) and smooth microsomes ($P < 0.001$)

We used the double isotope technique described by Dehlinger & Schimke (1972) in order to examine the effect of phenobarbitone on the relative incorporation of leucine into microsomal proteins including apocytochromes *P*-450. To detect the primary site and maximal rate of protein synthesis, we chose a short labelling period of 30 minutes (Table 2). Three hours after phenobarbitone administration, the isotope ratio was highest in rough microsomes, or in RER membranes not associated with mitochondria, which suggests a high phenobarbitone-mediated incorporation rate of leucine into microsomal protein of this subfraction. However, when the labelled haem precursor, δ-aminolaevulinic acid, was administered to rats, the specific activity of haem extracted and crystallized from subfractions of protease-digested membrane was higher in RER associated with mitochondria as compared to that associated with rough and smooth microsomes (Table 3).

These preliminary data suggest the following sequence of subcellular events during the formation of holocytochrome *P*-450 (Fig. 2): After an inductive stimulus, primary synthesis of apocytochrome *P*-450 occurs at RER membranes that are not associated with mitochondria in the intact hepatocytes. These apoprotein-loaded RER membranes may then associate with mitochondria to accept the prosthetic group haem for assembly of holocytochrome

TABLE 2

Incorporation of [^{3}H]leucine and [^{14}C]leucine into proteins of molecular weight 48 000–57 000 of different subfractions of endoplasmic reticulum

Subcellular fraction	*Phenobarbitone (d.p.m. of [^{3}H])*	*NaCl (d.p.m. of [^{14}C])*	*[^{3}H]/[^{14}C] ratio*
Homogenate	4852	1583	3.07
RER separated from mitochondria–RER complexes	910	228	3.99
Rough microsomes	2528	490	5.16
Smooth microsomes	3275	700	4.68

Three pairs of control (NaCl) and phenobarbitone-treated (80 mg/kg i.p.) rats were injected with 50 μCi [^{14}C]leucine and 200 μCi [^{3}H]leucine respectively, 30 minutes before decapitation, at 3 hours after phenobarbitone or NaCl administration. Homogenates were pooled, combined and subcellular fractions were prepared and subjected to sodium dodecyl sulphate–polyacrylamide electrophoresis. Radioactivity was determined in protein bands of apparent molecular weight 48 000 to 57 000. In addition, the radioactivity in a trichloroacetic acid precipitate of the homogenate was measured (Dehlinger & Schimke 1972).
d.p.m., disintegrations/min

P-450. The free apocytochromes may either trigger the extramitochondrial transport of haem by their high affinity for haem or, by decreasing the postulated regulatory haem pool, may be responsible for secondary induction of δ-aminolaevulinate synthase (EC 2.3.1.37) and the consequent coordinative increase in mitochondrial haem synthesis (Meyer & Schmid 1978).

TABLE 3

Incorporation of [^{14}C]δ-aminolaevulinic acid (ALA) into haem of cytochrome *P*-450 in various subfractions of endoplasmic reticulum

	Radioactivity of haem isolated from protease-digested endoplasmic reticulum subfractions	
	d.p.m./nmole cytochrome P-450-haem	
	Experiment 1	*Experiment 2*
RER separated from mitochondria–RER complexes	2711	5081
Microsomes (total)	1251	3694
Rough microsomes	1667	4170
Smooth microsomes	1478	3641

Rats (150–180 g) were injected with 4-[^{14}C]ALA (7.5 or 10 μCi) and the animals were killed 4 hours after injection. Isolation of haem from protease-digested particles and determination of its radioactivity were done as described by Meyer & Marver (1971).

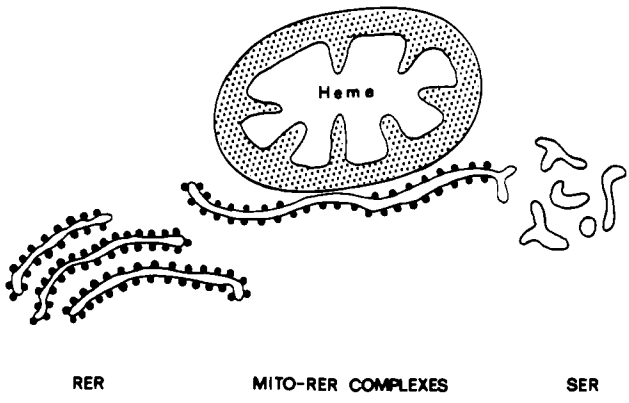

FIG. 2. Hypothetical scheme for the subcellular events during synthesis of cytochrome(s) *P*-450. Synthesis of apocytochrome *P*-450 is initiated in rough endoplasmic reticulum (RER) not associated with mitochondria (as isolated fraction, this RER corresponds to rough microsomes). These membranes structurally associate with mitochondria (MITO–RER) and incorporate mitochondrial haem. The final result is smooth endoplasmic reticulum (SER) with a high specific activity of holocytochrome(s).

Holocytochrome(s) *P*-450 may then be translocated into the intracisternal space of RER and pass to the smooth endoplasmic reticulum (SER; Craft et al 1979, Fujii-Kuriyama et al 1979), which is the final and definite site of their metabolic function. These data generally support the concept of a functional heterogeneity of RER along its lateral plane (for review see Depierre & Dallner 1976). Whether the proposed sequence of events during the stepwise formation of holocytochrome *P*-450 is a prerequisite for the parallel proliferation of SER is an open question.

Induction of cytochrome P-*450 haemoproteins in primary cultures of chick embryo hepatocytes*

Sex, age, species, diet, hormones, biorhythmicity and the ability of the *P*-450 haemoproteins to be induced differentially by a large variety of different drugs and chemicals all play an important role in modulating the overall activity and the metabolite pattern of hepatic drug metabolism. In addition, all these modifying factors are expressed against a background of significant genetic differences in the activity and inducibility of microsomal monooxygenases in both animals and humans.

One major determinant of the induction response may be the 'proliferative state' of hepatocytes. This is suggested by the model of the regenerating liver,

in which the basal concentrations of cytochrome *P*-450 decrease to 30–50% of normal values and 'inducibility' of the drug-metabolizing system by phenobarbitone is delayed. These effects depend on the respective temporal sequence of drug addition and partial hepatectomy (for review, see Schulte-Hermann 1974).

Hepatocytes cultured under chemically defined conditions offer a potent tool in the study of the complex interaction of the factors that modify drug metabolism. Our experience includes the culture of hepatocytes from adult rats (Bissell et al 1973, Guzelian et al 1977), adult mice (F.R. Althaus, H. Arnheiter, U.A. Meyer, unpublished work) and chick embryos (Althaus et al 1979). Of these three systems, only chick embryo hepatocytes in primary monolayer culture maintain the capacity for differential induction of several molecular species of cytochrome *P*-450-haemoproteins by drug substrates in the initial phase of culture. In the present study we tested the effect of changes in proliferative activity of chick embryo hepatocytes on induction of cytochrome *P*-450-haemoproteins.

Proliferative activity of cultured hepatocytes from chick embryo and induction of cytochrome P-*450 haemoproteins*

From 9 to 11% of the hepatocyte nuclei were labelled after a 12 h exposure to tritiated thymidine from the fourth to the sixteenth hour of culture. More than half of the labelled nuclei were considered to be postmitotic because of the close proximity and similar (smaller) nuclear size as well as similar grain density of a pair of nuclei. Labelled metaphase figures were also regularly observed. In the subsequent 12 h labelling periods the proportion of labelled hepatocytes was always below 1%. Thus, there is considerable proliferative activity of the hepatocyte population during the first few hours in culture, despite the absence of serum, but proliferation rapidly declines thereafter. Indeed, phenobarbitone-mediated inducibility of cytochrome *P*-450 was lower in the first day of culture than during the second and subsequent days. Further evidence that proliferative events affect induction of cytochrome *P*-450 was derived from experiments in which inhibitors of DNA synthesis were given at inoculation of cells and throughout the period of phenobarbitone induction. Hydroxyurea, arabinocytidine and formamidoxime (a close analogue of hydroxyurea) all increased the induction response by up to a factor of three. The effect was concentration-dependent, with maximally effective concentrations of 10^{-4} M, 4×10^{-6} M and 2×10^{-2} M respectively. DNA synthesis, as measured by the percentage of [^{3}H]thymidine-labelled hepatocyte nuclei determined in the early culture period (4th to 16th hour),

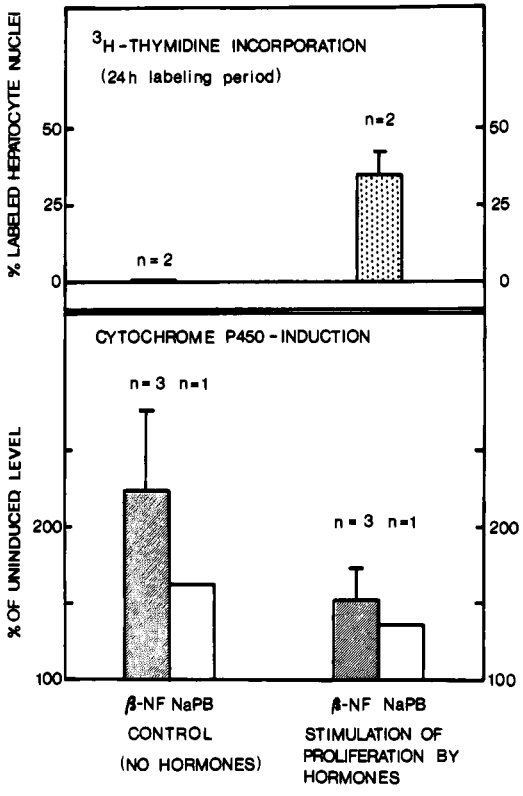

FIG. 3. Effect of stimulation of cell proliferation by hormones and inosine on DNA-synthesis and drug-mediated induction of cytochrome *P*-450 in primary cultures of chick embryo hepatocytes. Insulin, hydrocortisone, triiodothyronine, glucagon, inosine and dialysed heat-inactivated fetal calf serum (12% v/v) were added at 16–19 h after inoculation (Leffert et al 1977). Labelling with thymidine for 24 h began 24–27 h after start of hormone treatment. Phenobarbitone (1.57 mM) or β-naphthoflavone (22 μM) were present for 18 h, beginning 26–36 h after start of hormone treatment. Cytochrome *P*-450 concentration in control cultures was 118 ± 55 nmol/mg protein. Above columns: number of experiments.

was decreased (by 20–30%) at these concentrations of inhibitors. Interestingly, the glucocorticoid, dexamethasone, seemed to mimick the effect of the inhibitors of DNA synthesis. Over an extremely wide range of concentrations (10^{-20}–10^{-4} M) dexamethasone, when present from the beginning of culture, increased the phenobarbitone-mediated induction of cytochrome *P*-450 (phenobarbitone present from 20–38 h). The effect of dexamethasone was concentration-dependent in the range 10^{-12}–10^{-6} M, with 2.5×10^{-8} M being the optimum concentration. DNA synthesis, determined in parallel cultures concomitantly with the induction period, was inhibited also in a concentration-

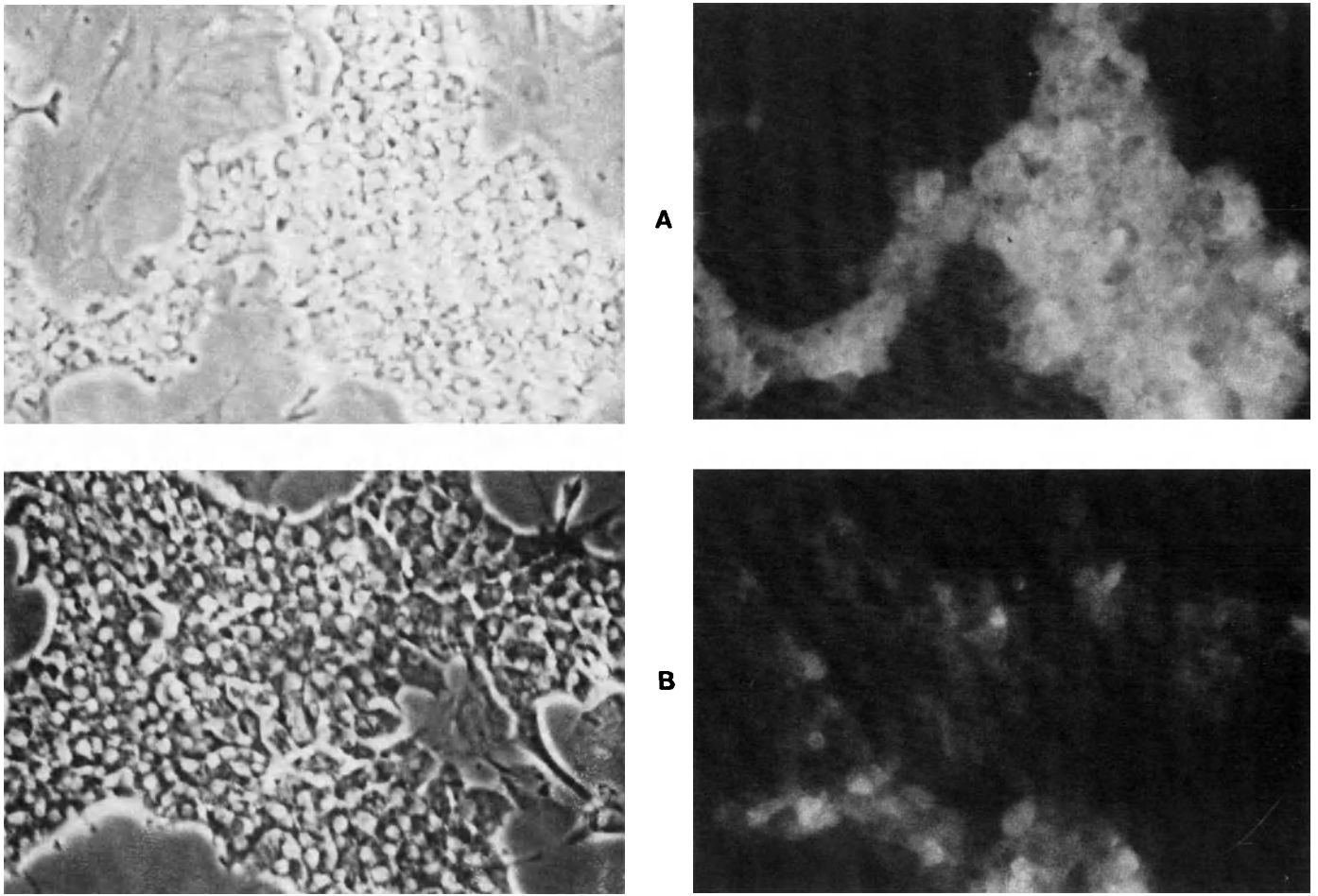

FIG. 4. Effect of stimulation of cell proliferation by hormones and inosine on the fluorescence of cultured chick embryo hepatocytes which were exposed to ethoxyresorufin (Burke & Hallman 1978, U. Giger & U.A. Meyer, unpublished work). Phase contrast micrographs (left side) and fluorescence micrographs (right side) in hepatocyte monolayers exposed to β-naphthoflavone (18 hours, 22 μM):
A: Control culture (no hormones).
B: Stimulation of proliferation by exposure to hormones and inosine (see Fig. 3 for details).

dependent manner (H. Hirsiger, F.R. Althaus, U. Giger, U.A. Meyer, unpublished work).

Cultured hepatocytes from fetal and adult rats can be stimulated to progress from a 'quiescent' stage (1–2% labelled cells after a 12–24 h exposure to [^{3}H]thymidine) to enter the stage of DNA synthesis. Division of the hepatocytes can be stimulated by addition to the medium of combinations of inosine and several hormones, including insulin, hydrocortisone, triiodothyronine and glucagon (for review, see Leffert & Koch 1978). When we added a mixture of these hormones, inosine and fetal calf serum to our hepatocyte cultures from chick embryos, at 16–19 h (when DNA synthesis was at 'resting' level), a dramatic increase in the number of [^{3}H]thymidine-labelled nuclei was observed, to a maximum of 35% during the second day of treatment (24 h labelling periods were used). Drug-mediated induction of cytochrome *P*-450 was determined during the second day of hormone and inosine treatment, and was found to be lower than in untreated cultures (Fig. 3).

Concomitant histofluorimetric estimation of *O*-deethylation of ethoxyresorufin showed that in control cultures all cells exhibited strong and relatively uniform fluorescence after stimulation with β-naphthoflavone, whereas hormone and inosine treatment decreased the fluorescence intensity in most cells (Fig. 4). Unfortunately, no histofluorimetric test was available for evaluation of phenobarbitone-type induction.

In summary, changes in the proliferative activity of chick embryo hepatocytes in primary monolayer culture influenced the induction of cytochrome *P*-450-haemoproteins by phenobarbitone and β-naphthoflavone. Inducibility of cytochrome *P*-450 was decreased in cultures with increased proliferative activity of hepatocytes. Treatment with inhibitors of DNA synthesis, including dexamethasone, increased the induction response.

CONCLUSIONS

The mechanisms regulating the differential induction of the various molecular species of cytochrome *P*-450 haemoproteins in mammalian liver by phenobarbitone and by other drugs are poorly understood, but seem to involve genetic, hormonal, nutritional and numerous other factors. In particular, the subcellular events that lead to the initial induction signal are unclear. Two approaches to a study of the induction process have been pursued:

Our investigations on the subcellular organization of synthesis of haem and apocytochrome suggest that formation of holocytochrome *P*-450 is compartmentalized within the endoplasmic reticulum of hepatic cells. Structural

complexes between mitochondria and rough endoplasmic reticulum may mediate the coordination of haem and apoprotein synthesis by influencing extramitochondrial transport of haem or insertion of haem into the free apocytochrome, or both, and by insertion of holocytochrome into the membrane. The use of these specialized structural units in the study of the biosynthesis of cytochrome *P*-450 *in vitro* (Craft et al 1979, Bhat & Padmanaban 1978) will be of interest.

Tissue culture systems offer the promise that *in vitro* approaches will continue to provide information on the complex interaction of various factors during induction of drug metabolism. Our data from primary cultures of chick embryo hepatocytes suggest that a major determinant of the induction response may be the proliferative state of hepatocytes.

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Discussion

Rawlins: We have demonstrated that enhanced cell division influences benzo[*a*]pyrene activity in human epidermis. If the turnover of epidermal cells is increased by 'stripping' off the keratin with tape, the benzo[*a*]pyrene hydroxylase activity is reduced by 50% after 48 h, when compared to control activity. After incubation with 100 μM benzanthracene, the induced activity is also 50% of control, although the induction ratio (induced activity: basal activity) is unaltered.

Farber: You mentioned the rapid drop that has been observed in cytochrome *P*-450 activity in regenerating liver. In some current work with

Fischer 344 rats, we have observed only a small decrease (10–15%) in the activity of cytochrome *P*-450 in regenerating liver.

Meyer: I was referring to the observations published by Hilton & Sartorelli (1970) and Chiesara et al (1970), as well as by Barker et al (1969) and many others. These authors used phenobarbitone as the inducer and found delayed inducibility of the drug-metabolizing enzyme system.

Farber: Our experiments did not include phenobarbitone; we measured cytochrome *P*-450 levels per unit protein as a function of time after 2/3 partial hepatectomy.

Amos: Presumably, Professor Meyer, the work which has failed to demonstrate a cytosol receptor for phenobarbitone induction was done in rats?

Meyer: The pioneering work was done by Alan Poland, who identified a binding protein for polycyclic aromatic compounds, in the cytosol of rats and mice, which has the binding properties predicted for induction of at least one molecular species of cytochrome *P*-450 (Poland et al 1976). Phenobarbitone showed no affinity for this receptor protein, even at very high concentration.

Amos: There is a difference between chickens and rodents in whether or not phenobarbitone can act as an inducer. Has any *in vitro* work been done on this comparison?

Meyer: Our present experience suggests that different inducers produce similar responses in chick embryos and in rodents. The only *in vitro* work that I know on the cytosolic receptors was published by Guenthner and Nebert (1977). These investigators tested four established tissue culture lines, derived from rat or mouse liver and monkey kidney. They found that the presence of the cytosolic receptor by itself does not necessarily ensure inducibility of benzo[*a*]pyrene hydroxylase. Phenobarbitone, even at 1000-fold excess concentration, did not displace active inducers from the cytosolic receptor.

Several research groups are searching for phenobarbitone-type inducers that are more potent (on a molar basis) than phenobarbitone. This would help to identify the binding species, if there is one, for phenobarbitone-type drugs.

Farber: Dr Butler of Imperial Chemical Industries recently informed me (personal communication) that dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin or TCDD), when used in very small doses, produced giant cells in the liver of rats. This suggests that the liver might respond to dioxin in a complex manner.

Meyer: Data on the binding of TCDD and other halogenated aromatic compounds to a cytosolic receptor protein are well correlated with induction,

but it has been difficult to purify the receptor protein, which is heat-labile and believed to have a molecular mass of approximately 200 000 daltons (see Poland et al 1976).

De Matteis: We know that DDT (dichlorodiphenyltrichloroethane) is fairly good as an inducer of δ-aminolaevulinate synthase (EC 2.3.1.37) in chicken (De Matteis 1971), but which kind of cytochrome *P*-450 do you think might be induced by DDT?

McLean: In rats, DDT gives the phenobarbitone-type of induction.

De Matteis: But DDT would not be as powerful as TCDD.

Meyer: It is obvious that more research is necessary for the recognition of molecules which potentially could act as cytosolic receptors.

Connors: Have structure–activity relationships been studied? How would the chemist start to look for active molecules?

Meyer: I would propose that the chemist look for molecules with phenobarbitone-type induction that are very potent inducers, and then make congeners which are poorly metabolized, and test their binding properties in hepatic cytosol.

J. W. Bridges: Even before TCDD came on the scene, a structure–activity relationship for the 3-methylcholanthrene-type induction was apparent. Dr Conney (1967) pointed out the rather specific structural requirements for aromatic hydrocarbons to act as inducers of cytochrome *P*-448. However, the evidence that there is a specific receptor or receptors for induction by phenobarbitone is far less convincing. There appears to be an association between the inducing ability of phenobarbitone-type compounds and the extent to which they bind and persist on one or more cytochrome *P*-450. This might indicate that a form of cytochrome *P*-450 is itself the receptor, but the hypothesis has yet to be tested critically.

Meyer: Dr Alan Paine (1978) has proposed that a common intermediate may be produced during metabolism of various substrates and that it may cause induction. However, this would make it difficult to explain the different patterns of induced proteins obtained after administration of different inducing compounds. Theoretically, at least, there could also be a common initial step, followed by translational modifications by the inducing compound.

There is another interesting aspect. One of the several reasons why we have begun to study the effect of cell proliferation on induction of drug-metabolizing enzymes is the indication that the induction process is dependent on the phase of the cell cycle (Guillouzo et al 1978).

Connors: Does Dr Poland know why dioxin is so good in terms of its structure?

Meyer: Yes. At least for the halogenated aromatic inducers one finds a well defined structure–activity relationship (Poland & Glover 1977).

Oesch: But when cells are proliferating, they go more often through the G_1 phase.

Meyer: Yes. In the normal rat liver, most cells are probably in the so-called G_0 phase of the cell cycle. One would speculate that perhaps phenobarbitone moves them into or prolongs the G_1 phase and then additional mechanisms are responsible for induction. That type of action may be one of the initial requirements of an inducer; but this is speculation. During stimulated proliferation, the duration of the G_1 phase may be shortened.

Farber: Did you observe mitosis in your *in vitro* system?

Meyer: Yes; in the initial phase of the culture, and after addition of hormones and inosine, mitotic cells could be observed.

Farber: Do you think that the thymidine incorporation could be related more to DNA repair than to DNA replication?

Meyer: No. The time course of the labelling and the occurrence of mitoses provide evidence for true replication in DNA synthesis.

Farber: How long does this continue, and is there a net increase in the number of cells?

Meyer: So far we have observations only from 2nd–4th day of culture. Microscopical examination of the cultures, and particularly the presence of mitotic and postmitotic cells, provide evidence for an increase in the number of cells; however we have not quantified this yet. Leffert has published similar data for rat liver (Leffert & Koch 1978). Our own experiments are different from those of Leffert in that we have used *embryonic* hepatocytes from a *different species*. By using the same mixture and the same concentrations of hormones and inosine as Leffert's group, we observed both a marked increase in the incorporation of thymidine into DNA and a considerable percentage of postmitotic cells.

Gillette: When cytochrome *P*-450 is reconstituted by the addition of haem to the apoprotein, is the re-incorporation of haem fast or slow?

Meyer: As I reported, the maximal increases in cytochrome *P*-450 concentration, determined by CO-(carbon monoxide-)binding spectra, are observed after 10–20 min incubation of the liver homogenate with 4–8 μM haemin at 37°C. If we use subcellular fractions such as the mitochondria–RER complexes, addition of 105 000 $\times$ *g* supernatant is required before haem will affect the CO-binding spectra. We have preliminary evidence that under the conditions used, i.e. pretreatment of rats with cobaltous chloride and phenobarbitone, reduced glutathione (GSH) and/or phospholipids are required for 'incorporation' of haem, because we can replace the supernatant

with GSH and also, partially, with phospholipids (P.J. Meier & U.A. Meyer, unpublished work).

Orrenius: Does this occur in the presence of excess haem?

Meyer: Yes. But we assume much non-specific binding of haem to the various proteins in the system.

McLean: When Colin Garner and I (1974) added haem to lipid membranes we found that there was a peak at 420 nm, just like cytochrome *P*-420 and this was reducible by NADPH. Did you observe that?

Meyer: Yes, we always see increased absorption at 420 nm in the CO-binding spectrum when haem is added to microsomes in suspension.

Gillette: When the serum albumin–haem complex, in reduced form, is combined with carbon monoxide, does absorption occur at around 420 nm?

McLean: It's at about 414 nm; but when the complex is attached to the lipid membrane it is indistinguishable on spectroscopic grounds from the denatured *P*-420 peak. But did you say originally, Professor Meyer, that the reduced CO-complex absorbs at 450 nm in your experiments?

Meyer: Yes, the change is at 450 nm and the difference at 420 nm can be explained because of excess haem (Meier & Meyer 1977).

Gillette: But to return to my original point, it is still a very slow reaction.

Meyer: Yes, it is not an immediate effect.

De Matteis: I would like you to clarify some details of the isotopic experiments. You described the uptake of 5-aminolaevulinate into different fractions, and the appearance of maximum specific radioactivity after 4 h. Was this in a cytochrome *P*-450 pool?

Meyer: The time course was indeed surprising to us. We assume that we determined the specific activity of the haem of cytochrome *P*-450, but obviously we cannot exclude a small, rapid turn-over haem pool of high specific activity trapped between RER and mitochondria, or in the outer mitochondrial membrane. Unfortunately, we cannot isolate haem from highly purified cytochrome *P*-450s yet.

De Matteis: That's surprising, because if you look in the finished product which, presumably, according to your scheme, is the smooth endoplasmic reticulum, previous studies (Levin et al 1970) have shown that the maximum specific activity of haem after a tracer dose of δ-aminolaevulinic acid is achieved within ½–1 hour.

Meyer: Yes, but these studies were done with different subcellular fractions and the haem was not crystallized to constant specific activity.

De Matteis: In these studies (Levin & Kuntzman 1969, Levin et al 1970) the so-called CO-binding particles were isolated, but these may still contain several pools of haem.

Meyer: Our results suggest that the incorporation of δ-aminolaevulinic acid into the haem of cytochrome *P*-450 does *not* correlate with the early peak for labelled haem observed 1 h after injection of glycine in hepatic microsomes, but it *does* correlate with the second peak observed at 4.5 h (Yannoni & Robinson 1976).

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Substrate-dependent irreversible inactivation of cytochrome *P*-450: conversion of its haem moiety into modified porphyrins

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Abstract 2-Allyl-2-isopropylacetamide and other drugs containing either an allyl, a vinyl or an ethynyl unsaturated side chain are metabolized by liver cytochrome *P*-450 to reactive derivatives that irreversibly inhibit the haemoprotein by a suicidal type of inactivation. The main target is the haem moiety of cytochrome *P*-450 which is converted into abnormal porphyrins. These have been isolated from the liver of treated rats, extensively purified and compared with model porphyrins. The abnormal porphyrins incorporate metal ions *in vitro* much more readily than does their parent porphyrin, protoporphyrin. They are also much more basic than protoporphyrin, and on titration with a strong acid they readily give rise to a porphyrin monocation which then requires relatively large amounts of acid for conversion to the porphyrin dication. In all these respects and also in the intensity of their bathochromic shifts these abnormal porphyrins closely resemble *N*-alkylated porphyrins and they markedly differ from porphyrins that are substituted at one of their *meso*-carbon positions or which bear electron-withdrawing substituents at the β-positions of the pyrrole rings. This suggests strongly that reactive derivatives of the unsaturated drugs act as electrophilic reagents and alkylate one of the pyrrole nitrogen atoms of cytochrome *P*-450.

A model centred on the apoprotein of cytochrome *P*-450 is considered for the degradation of liver haem caused by unsaturated drugs. The apocytochrome may accept exchangeable pools of liver haem for degradation, leading to a state of haem depletion and to activation of δ-aminolaevulinate synthase (EC 2.3.1.37).

The endoplasmic reticulum of the liver cell contains an enzymic system concerned with the oxidative metabolism of a number of lipid-soluble substrates both of endogenous and exogenous origin. The system is organized around a group of inducible haemoproteins, collectively known as cytochrome *P*-450. The various cytochromes of this group have been shown to differ in molecular weight and substrate specificity, as well as in their response to inducers of the

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drug-metabolizing system or to the effect of selective inhibitors. The majority of exogenous chemicals, when they are metabolized by the cytochrome *P*-450 system, are rendered more water-soluble and more easy to excrete so that their biological activity is greatly decreased. There are cases however when metabolism leads to increased reactivity, with the appearance of functional or morphological manifestations of toxicity.

In the present paper, which is concerned with one example of liver toxicity mediated by oxidative metabolism of foreign chemicals, compounds containing one or more unsaturated side-chains will be discussed. They are metabolized by cytochrome *P*-450 to reactive derivatives and therefore they irreversibly inhibit the haemoprotein by a suicidal type of inactivation. The species of cytochrome *P*-450 that is inducible by phenobarbitone is preferentially inactivated in all cases, and, within the haemoprotein, the haem moiety is preferentially attacked. The main purpose of this paper is to summarize and discuss the latest findings: the reader is referred to recent reviews for a detailed coverage of the background aspects to these problems (Ortiz de Montellano et al 1978a, De Matteis 1978).

ALTERATIONS OF THE PORPHYRIN NUCLEUS OF CYTOCHROME *P*-450 IN THE LIVER CAUSED BY TREATMENT WITH DRUGS CONTAINING UNSATURATED SUBSTITUENTS

Drugs containing allyl substituents have been known for some time to cause loss of liver cytochrome *P*-450 and conversion of its haem moiety into modified porphyrins, henceforth referred to as green pigments (De Matteis 1970, 1973, Levin et al 1973). Similar findings have been reported with Fluroxene (2,2,2-trifluoroethyl vinyl ether) and with vinyl testosterone, two drugs containing the unsaturated vinyl grouping (Ivanetich et al 1975, 1976, White 1978) and also with compounds bearing the unsaturated ethynyl ($-C{\equiv}C-$) substituent, including ethynyl-substituted steroids, 1-ethynylcyclohexanol and acetylene ($HC{\equiv}CH$) itself (White & Muller-Eberhard 1977, White 1978, Ortiz de Montellano et al 1979a). With all these drugs the presence of at least one unsaturated substituent was found to be essential for the destruction of the cytochrome and the production of green pigments in the liver. These pigments, originally discovered by Schwartz & Ikeda (1955) can be extracted from the liver of treated animals after methylation and can be separated from haem and from normal porphyrins by thin-layer chromatography (t.l.c.).

Treatment with either 2-allyl-2-isopropylacetamide, secobarbitone (5-allyl 5-(1-methylbutyl) barbiturate) or ethynyl-substituted compounds, each

resulted in the appearance of one main band and various minor bands, none of which was present in the controls and which migrated less than the major haem band on t.l.c. All these bands appeared green or green-brown to the naked eye and showed red fluorescence under ultraviolet (u.v.) light, but some variability was encountered in their number and R_F values. The most intense of these bands, when extracted in chloroform, exhibited a visible spectrum of the aetio-type* (Fig. 1a) with a bathochromic** shift of all absorption maxima, compared with the absorption maxima of the native porphyrin of cytochrome *P*-450, protoporphyrin (Table 1): this indicates that the green pigments are modified porphyrins (McDonagh et al 1976, Unseld & De Matteis 1976, White 1978, Ortiz de Montellano et al 1979a, De Matteis & Cantoni 1979). A three-banded visible spectrum was also seen (Fig. 1b) especially after

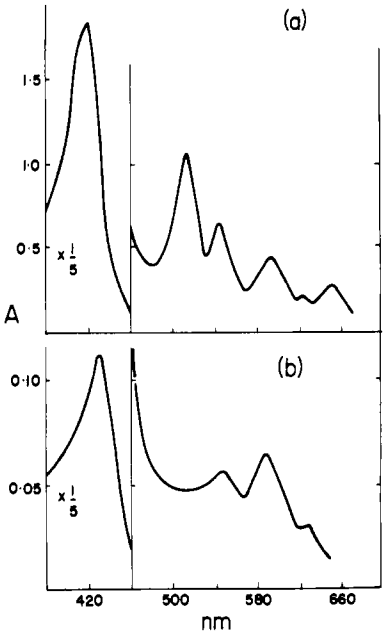


FIG. 1. Two types of spectrum exhibited by the green pigments isolated from liver of 2-allyl-2-isopropylacetamide-treated rats after methylation and thin-layer chromatography (t.l.c.) (Unseld & De Matteis 1976): (a) aetio-type spectrum of the porphyrin free base showing a Soret band at 417 nm and the following additional bands with maxima in parentheses: band IV (512 nm); band III (545 nm); band II (593 nm); band Ia (623 nm); and band I (651 nm). (b) Second type of spectrum with Soret at 430 nm and additional bands at 548, 590 and 631 nm.

*In the aetio-type spectrum the four bands of the visible spectrum of a porphyrin free base decrease in intensity from shorter to longer wavelength (i.e. bands: IV > III > II > I).
**A bathochromic shift is an increase in the wavelength of an absorption maximum.

TABLE 1

Absorption spectrum of protoporphyrin and of octaethylporphyrin and bathochromic shifts from their absorption maxima seen in various porphyrins obtained by modification of these two parent porphyrins.

Porphyrin	*Absorption maxima or bathochromic shifts (nm)*						*Reference*
	Soret	*IV*	*III*	*II*	*Ia*	*I*	
Absorption maxima of:							
A) Protoporphyrin IX methyl ester	407	505	541	575	603	630	Falk (1964)
B) Octaethylporphyrin	400	498.5	534	566.5	594	620	De Matteis & Cantoni (1979)
Bathochromic shifts seen in:							
2-Allyl-2-isopropylacetamide-green pigment (from A)	10	7	3	18	20	21	De Matteis & Cantoni (1979)[a]
Secobarbitone-green pigment (from A)	9	7	2	19.5		22	De Matteis & Cantoni (1979)
Norethisterone-green pigment (from A)	10	7	5	15		16	Ortiz de Montellano et al (1979a)
1-Ethynylcyclohexanol-green pigment (from A)	9.5	7	4	17	17	18	Present work[b]
N-Methylated octaethylporphyrin (from B)	12	9.5	5	19.5	22	22	De Matteis & Cantoni (1979)
α-Formylated octaethylporphyrin (*meso*-substituted) (from B)	8	8.5	7	10.5		5	Inhoffen et al (1966)
2,4-Diformyldeuteroporphyrin (from A)	28	21	21.5	20		21	Falk (1964)

[a]Similar bathochromic shifts can be calculated from the data published by McDonagh et al (1976).

[b]Similar bathochromic shifts can be calculated from the data published by Ortiz de Montellano et al (1979a).

All porphyrins listed here exhibited an aetio-type spectrum and were examined dissolved in chloroform. Protoporphyrin, 2,4-diformyldeuteroporphyrin and the various green pigments were all methyl esters. The absorption maxima of the Soret and of the visible bands are given above for protoporphyrin (A) and for octaethylporphyrin (B), together with the bathochromic shifts (from the absorption maxima of the two parent porphyrins A or B) calculated for the green pigments and for other modified porphyrins from data given in references above.

purification of one of these pigments by multiple t.l.c. runs (Unseld & De Matteis 1976). This second type of spectrum has also been described by Ortiz de Montellano et al (1978b), who have identified the derivative responsible for it as the Zn^{2+} chelate of the modified porphyrin. Comparison of the published data indicates that the absorption maxima of both types of spectrum are strikingly similar regardless of the drug used to produce the green pigments; however, differences in chromatographic mobility have been described between green pigments produced by treatment with different ethynyl-substituted compounds (White 1978). Differences are also consistently found between the main band produced by 2-allyl-2-isopropylacetamide and that produced by secobarbitone (Table 2), whether they were produced by administration of the drugs to the rat *in vivo* or by incubation of the rat liver microsomes with the drugs *in vitro*. This indicates that green pigments produced by different drugs must in some way differ in structure.

Evidence has been obtained in different laboratories that these unsaturated drugs have all to be metabolized in order to cause destruction of cytochrome *P*-450: the suggestion has therefore been put forward that reactive derivatives (e.g. epoxides) produced during the metabolism of these drugs by cytochrome *P*-450 may attack its haem moiety, so giving rise to the green pigments (De Matteis 1970, 1973, Levin et al 1973, White 1978). Two important lines of evidence have recently been obtained in support of this view: (1) after treatment with two of these drugs, 2-allyl-2-isopropylacetamide and norethindrone (17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one), radioactivity from the drug was recovered irreversibly bound to the green pigments in a 1:1 stoichiometry (Ortiz de Montellano et al 1978b, 1979a). (2) When the pigments produced by

TABLE 2

Chromatographic behaviour of the main green pigment produced by treatment with either 2-allyl-2-isopropylacetamide or with secobarbitone

Drug	*R_F values*	
	Pigments produced in vivo	*Pigments produced* in vitro
2-Allyl-2-isopropylacetamide	0.47 – 0.56[a]	0.50
Secobarbitone	0.09 – 0.21[a]	0.21

Green pigments were either produced *in vivo*, by treating rats with 2-allyl-2-isopropylacetamide or with secobarbitone (De Matteis & Cantoni 1979), or *in vitro*, by incubating the microsomal fraction of rat liver with either drug in the presence of NADPH. After methylation the pigments were chromatographed on silica gel t.l.c. plates using the system described by Unseld & De Matteis (1976) and the R_F values of the major band were determined.

[a]Range of values obtained in 4 experiments.

treatment with these two drugs were analysed by mass spectrometry, the mass of the molecular ion obtained for either of them could be accounted for almost exactly by the sum of the protoporphyrin plus the respective drug (Ortiz de Montellano et al 1979a, 1979b).

Still unknown are (1) the nature of the reactive derivative of the drug that is responsible for the alkylation of the haem of cytochrome *P*-450; (2) the site within the porphyrin nucleus where the attack takes place; and (3) the chemical reaction involved; but a better understanding of the structure of the green pigments should help to clarify these points.

SPECTRAL STUDIES OF THE GREEN PIGMENTS

We have now carried out spectral studies of the green pigments and have compared them with other modified porphyrins whose chemical structure is known and which also exhibit a bathochromic shift in their absorption maxima (as compared with the absorption maxima of their respective parent porphyrins). The green pigments were obtained after treatment of phenobarbitone-induced rats with an unsaturated drug and were isolated as described by Unseld & De Matteis (1976) and by De Matteis & Cantoni (1979). The methods for titration of porphyrins with trifluoroacetic acid and for incorporation of Co^{2+} into porphyrins *in vitro* have also been reported (De Matteis & Cantoni 1979).

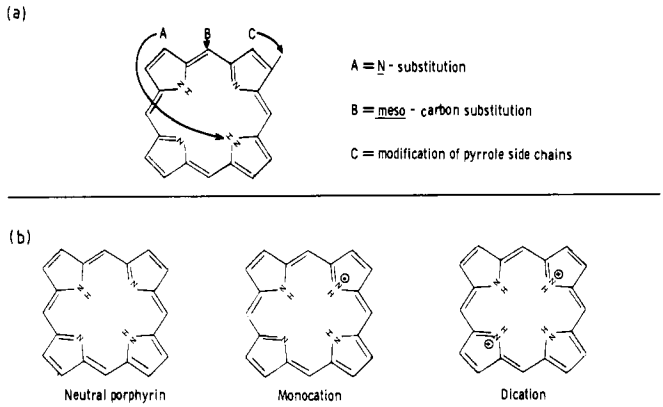


FIG. 2. (a) Sites within the porphyrin nucleus where electrophilic attack by a reactive metabolite of an unsaturated drug might conceivably take place: substitution or modification of structure at these sites is known to lead to a bathochromic shift in absorption maxima. (b) Protonation of the two imino-type nitrogen atoms of the porphyrin nucleus leading from the porphyrin free base (or neutral porphyrin) to the porphyrin dication, through the intermediary porphyrin monocation species.

There have been described three different types of modification of the porphyrin structure that lead to a bathochromic shift, while retaining an aetio-type spectrum. They are (Fig. 2a): (A) substitution at the pyrrole nitrogen atom; (B) substitution at the bridge *meso*-carbon atom; and (C) presence of electron-withdrawing side-chains at the β-positions of the pyrrole rings. The reactive derivative of an unsaturated drug could conceivably alkylate the porphyrin nucleus of cytochrome *P*-450 at any of these positions. Table 1 shows the absorption maxima of protoporphyrin and of octaethylporphyrin and also the bathochromic shifts from the absorption maxima of these two parent porphyrins seen in the green pigments and in modified porphyrins of known structure, belonging to the three classes mentioned above. The bathochromic shifts observed with green pigments obtained by treatment with four different unsaturated drugs all closely resembled those exhibited by *N*-alkylated porphyrins in order of intensity (I > Ia > II > Soret > IV > III) and also in absolute values; they differed in both respects from those of porphyrins that were substituted at the *meso* bridge carbon or that carried strong electron-withdrawing groupings at the periphery of their pyrrole rings.

The two imino-type nitrogen atoms (=N–) of the porphyrin nucleus are capable of accepting protons, and their progressive protonation to monocation and dication porphyrin species (Fig. 2b) can be followed spectrally when strong acid is added to them. When trifluoroacetic acid was added to a chloroform solution of green pigments (obtained with 2-allyl-2-isopropylacetamide) these pigments were found to give rise very readily to the porphyrin monocation, and this could then be converted into the porphyrin dication after addition of further acid (Fig. 3). Similar findings have been

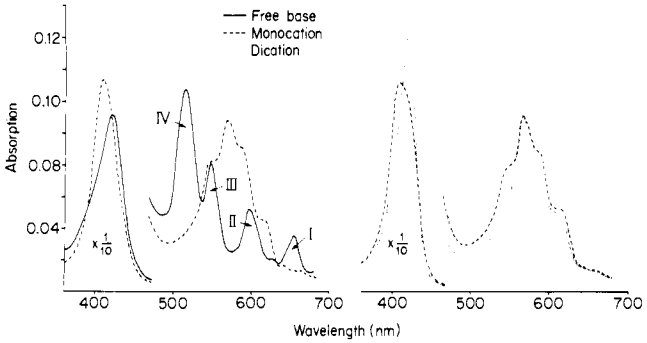


FIG. 3. Spectrum of the green pigment methyl ester. The main green pigment obtained after treatment with 2-allyl-2-isopropylacetamide was eluted from the t.l.c. plate and dissolved in chloroform. Its spectrum was determined before (in the free base form) and after addition of trifluoroacetic acid to a final concentration of 0.4 mM (monocation) or 6 mM (dication).

reported for *N*-alkylated porphyrins (Neuberger & Scott 1952, Jackson & Dearden 1973).

It will be noticed from Fig. 3 that band IV decreased when conversion of the free base into the monocation occurred; band III decreased when the monocation was converted into the dication. The absorption at the wavelength maxima of these two bands was therefore followed at different concentrations of trifluoroacetic acid and the titration curves obtained for green pigments were compared with those obtained with protoporphyrin (Fig. 4). With the latter porphyrin, bands IV and III decreased together, because the dication was directly formed and no intermediary monocation could be demonstrated: the most likely explanation for this is that in the case of the parent porphyrin the pK_a values of the conjugated acids are relatively close to one another (Neuberger & Scott 1952). In contrast, with the green pigment, band IV decreased rapidly at a very low concentration of acid but band III decreased slowly and only when relatively large amounts of acid were added. The titration curves obtained with octaethylporphyrin and with *N*-methylated octaethylporphyrin were found to resemble those obtained respectively with protoporphyrin and with green pigments. We can therefore conclude that, like *N*-methylated porphyrins (Neuberger & Scott 1952, Jackson & Dearden 1973), the green pigments are more basic than their parent porphyrins and that the pK_a values of their conjugated acids are sufficiently apart to allow the existence of the monocation even after addition of a strong acid. Green pigments produced by secobarbitone and by 1-ethynylcyclohexanol behaved on titration similarly to those obtained with 2-allyl-2-isopropylacetamide.

The same titration experiment was carried out with 2,4-diformyl-

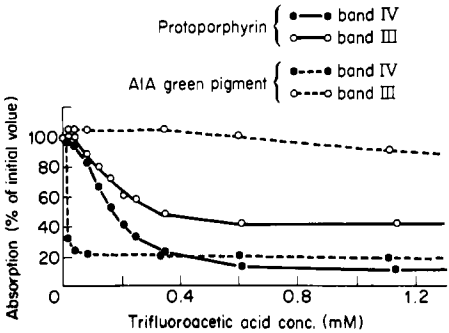


FIG. 4. Titration of green pigments (produced by 2-allyl-2-isopropylacetamide) and protoporphyrin with trifluoroacetic acid. The absorption at the wavelengths of band IV and band III maxima was followed for both porphyrins at increasing concentrations of acid and is expressed as a percentage of the initial value of the free base (from De Matteis & Cantoni 1979, reproduced with permission of Biochem J).

deuteroporphyrin and with formyl- and hydroxymethyloctaethylporphyrins (both *meso*-substituted) and although the former porphyrin was found to be less basic than protoporphyrin, and the latter two more basic than octaethylporphyrin (their respective parent porphyrins), with none of them could a clear monocation spectrum be demonstrated. Therefore in this respect, too, the green pigments resembled *N*-alkylated porphyrins, but differed from the two other classes of modified porphyrins.

During the purification of the 2-allyl-2-isopropylacetamide-green pigments by t.l.c., Unseld & De Matteis (1976) observed the appearance of a derivative with a three-banded visible spectrum, later identified as the Zn^{2+}-chelate of the green pigments (Ortiz de Montellano 1978b). This suggested increased chemical reactivity of the green pigments with divalent cations. The *in vitro* incorporation of Co^{2+} into green pigments and into other porphyrins was therefore studied by dual wavelength spectrophotometry. The green pigments produced by treatment with three different unsaturated drugs were all found to incorporate Co^{2+} much more readily than did their parent porphyrin, protoporphyrin, and in this respect also they resembled *N*-alkylated porphyrins; but they differed from the other two types of modified porphyrins (Table 3).

TABLE 3

In vitro incorporation of cobalt ions by green pigments produced by treatment with 2-allyl-2-isopropylacetamide or 1-ethynylcyclohexanol. Comparison with model porphyrins.

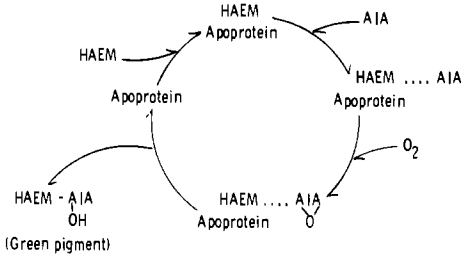
Porphyrin	*Concentration of* Co^{2+}	*Time required for complete disappearance of band IV (min)*
Octaethylporphyrin	3.6 mM	83.6 ± 15.3[a]
α-Formylated octaethylporphyrin (*meso*-substituted)	3.6 mM	41.6 (40.0, 43.2)
Protoporphyrin	3.6 mM	370.0 ± 64[a]
2,4-Diformyldeuteroporphyrin	3.6 mM	1020 ± 179[a]
N-Methylated octaethylporphyrin	80 μM	0.6 ± 0.02[a]
Green pigment (2-allyl-2-isopropylacetamide)	80 μM	10.2 ± 2.4[a]
Green pigment (1-ethynylcyclohexanol)	80 μM	1.2 (1.0, 1.39)

The various porphyrins were dissolved in chloroform to a concentration giving an absorption of 1–1.5 at their Soret maximum. A methanol solution of cobaltous acetate was added and the rate of disappearance of band IV was measured at room temperature by dual wavelength spectrophotometry; the times required for complete disappearance of band IV were calculated from the initial rate, and are given above as average ± SD of 3 determinations or as average, with individual observations in parentheses. With some porphyrins a higher concentration of metal ions was required to obtain a measurable rate of incorporation.

[a]Data from De Matteis & Cantoni (1979).

CONCLUSIONS

The present work has shown that green pigments produced by several unsaturated drugs are much more basic than their parent porphyrin, protoporphyrin, and that on titration with a strong acid they give rise readily to a porphyrin monocation, which then requires relatively large amounts of acid for conversion to the porphyrin dication. They can also incorporate metal ions chemically, *in vitro,* much more readily than does protoporphyrin. In all these respects, and also in the intensity of their bathochromic shifts, the green pigments closely resemble *N*-alkylated porphyrins and markedly differ both from porphyrins bearing electron-withdrawing substituents at the β-positions of the pyrrole rings and from porphyrins substituted at one of their *meso*-carbon positions. If we take into account the report that radioactivity from 2-allyl-2-isopropylacetamide or from norethindrone can be recovered bound to the respective green pigments in a 1:1 stoichiometry (Ortiz de Montellano 1978b, 1979a), the most likely interpretation for our findings is that a reactive derivative of these drugs acts as an electrophilic reagent and alkylates one of the pyrrole nitrogen atoms of cytochrome *P*-450. Several bands of green pigments can be detected during t.l.c. and some of these may represent



Overall stoichiometry

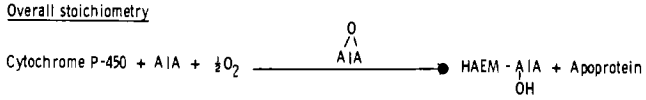


FIG. 5. The apoprotein of cytochrome *P*-450 as a catalytic centre for conversion of exchangeable pools of haem to green pigments in the liver. The following successive steps can be visualized (proceeding from the top of the figure in a clockwise direction): (1) 2-allyl-2-isopropylacetamide (AIA) or another unsaturated drug binds the oxidized cytochrome *P*-450 at or near its haem moiety (see Sweeney & Rothwell 1973); (2) the haem moiety is then reduced, binds molecular oxygen and the allyl group of AIA is metabolized to a reactive derivative, for example an epoxide; (3) the haem moiety is alkylated to produce the green pigment and this leaves the apoprotein site in the microsomal membranes (see De Matteis 1973); (4) fresh haem is then taken up by the free apoprotein to reconstitute the cytochrome and to undergo degradation (see Unseld & De Matteis 1978; Correia et al 1979). Note that although an epoxide has been illustrated here as the reactive derivative of AIA responsible for alkylation of haem, the evidence in favour of an epoxide is only indirect and some other reactive metabolite may instead be involved.

isomeric porphyrins, in which different nitrogen atoms have been alkylated. It is less likely that a reactive metabolite of one of these drugs alkylates protoporphyrin either at one *meso*-carbon position or at one of the vinyl groups (as suggested by Ortiz de Montellano et al 1978a, 1979b).

The unsaturated drugs that have been considered in this paper depress both the concentration of liver cytochrome *P*-450 and the activity of the drug-metabolizing enzyme system. They also stimulate the pathway of haem biosynthesis by enhancing the activity of the enzyme, δ-aminolaevulinate synthase, and by causing the accumulation of the intermediates of the pathway and the biochemical feature of porphyria. With 2-allyl-2-isopropylacetamide, which has been studied extensively, there is evidence that the action of the drug is not confined to the haem of the pre-existing cytochrome *P*-450 but it may extend to other pools of haem in the liver (De Matteis 1973) including exogenous haem added *in vitro* to liver microsomes (Unseld & De Matteis 1978) or given *in vivo* to animals treated with the drug (Correia et al 1979). It is possible that readily exchangeable free haem has to gain access onto the apocytochrome *P*-450 in order to be degraded to green pigments by 2-allyl-2-isopropylacetamide and by other unsaturated drugs.

The apoprotein of the cytochrome may therefore be visualized as a catalytic centre for degradation of exchangeable pools of haem, including the pool of 'regulatory' haem concerned with the regulation of δ-aminolaevulinate synthase (Unseld & De Matteis 1978, Correia et al 1979). This mechanism, schematically illustrated in Fig. 5, would be expected to lead to a decreased negative feedback control on the activity of the synthase and in this way would contribute to the stimulation of the enzyme (Marver et al 1966) and to the induction of hepatic porphyria (Goldberg & Rimington 1955, White & Muller-Eberhard 1977), both of which are seen after treatment with unsaturated chemicals. The present findings contrast with the results obtained after carbon disulphide (De Matteis 1978), where evidence has been obtained for the preferential damage of the apoprotein moiety.

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Discussion

Selikoff: Viscose rayon workers may be heavily exposed to carbon disulphide, and they exhibit significant acute and subacute toxic symptoms, including cerebral symptoms sometimes sufficiently severe to lead to mistaken diagnosis of mental illness and admission to a psychiatric hospital. Has enzyme induction been sufficiently studied in these people?

De Matteis: Yes, Mack et al (1974) reported inhibition of the oxidative demethylation of aminopyrine in humans by low doses of inhaled carbon disulphide. In their studies, the concentration of carbon disulphide in air was as low as 20 p.p.m., the current threshold limit in industry.

Gillette: You may be observing two separate effects of carbon disulphide: it may cause the destruction of cytochrome *P*-450 and it may also lead to the induction of cytochrome *P*-450 synthesis. Our studies with thio steroids reveal effects similar in many ways to the effects that you have observed with carbon disulphide. The injection of deacetylated spironolactone to guinea pigs and dogs causes a decrease in the 17α-hydroxylation and *C*-21-hydroxylation of progesterone by adrenal microsomes. Paralleling this decrease in enzyme activity is a loss of cytochrome *P*-450 and haem and a preferential decrease in one of the protein bands associated with the cytochrome *P*-450 in adrenal microsomes (Menard et al 1979a). Thus, unlike the effect of carbon disulphide on liver microsomal cytochrome *P*-450, deacetylated spironolactone does not appear to induce the synthesis of adrenal cytochrome *P*-450. Studies *in vitro* have revealed that in the presence of NADPH, deacetylated spironolactone causes the preferential decrease in 17α-hydroxylase activity with little decrease in *C*-21-hydroxylase activity. Paralleling this decrease in activity there was a loss of cytochrome *P*-450 haem, but no loss in the apoprotein. Nevertheless, when [^{35}S]-labelled deacetylated spironolactone was incubated with guinea pig adrenal microsomes, the [^{35}S]-label became associated with the apoprotein rather than with the haem fraction; the [^{35}S] however was preferentially bound to one of the apoproteins associated with adrenal

cytochrome *P*-450 (Menard et al 1979b). Thus, the *in vitro* effects appear to be highly selective. We have also studied the effects of preincubation of 7α-thiotestosterone with NADPH and liver microsomes on the steroid hydroxylases, and we found that preincubation of 7α-thiotestosterone inhibited the hydroxylation of testosterone but did not inhibit the hydroxylation of benzo[*a*]pyrene (Menard et al 1979b).

De Matteis: This is very interesting because it would suggest that the carrier molecule confers specificity of target. Williams (1959) suggested that oxidative desulphuration might be implicated in the toxic effects of phenylthiourea in the rabbit. A number of sulphur-containing chemicals are now known to be potentially toxic. They may all require a similar type of metabolic activation but the preferential target of their toxicity appears to differ: α-naphthylthiourea attacks the lung (Boyd & Neal 1976), liver phosphorothionates and CS_2 attack the liver (Seawright et al 1976, Bond et al 1969) and spironolactone attacks the testis (Stripp et al 1973). Perhaps the carrier molecule determines the site of activation and hence the site of toxicity.

Connors: Would allylisopropylacetamide (AIA) act in a whole animal by selectively binding to the haem nitrogen?

De Matteis: Yes; the toxic effect appears to be selective because other microsomal components are not decreased.

Connors: In a previous discussion Dr Garner (p 64-65) mentioned that aflatoxin B_1 binds to DNA.

Garner: Aflatoxin B_1 binds to RNA as well, but only to a small extent to protein.

Connors: So with aflatoxin very little protein binding occurs, yet with AIA there is selective binding to haem.

Gillette: The question arises as to whether the specificity is due to a high rate constant of inactivation (relative to the rate constant of dissociation of the complex between the enzyme and the chemically reactive metabolite) or to a high affinity of the enzyme for the chemically reactive metabolite. If the specificity is due to a high rate constant of inactivation, then only those forms of cytochrome *P*-450 that catalyse the formation of the chemically reactive metabolite may be inactivated. But if the specificity is due to a high affinity of the enzyme for the chemically reactive metabolite, then the chemically reactive metabolite may be formed by one of the cytochrome *P*-450 enzymes and may inactivate other forms of cytochrome *P*-450.

De Matteis: In the haem molecule, all four pyrrole nitrogens are coordinated to iron but one of them must in some way become accessible to the reactive derivative of an unsaturated drug, for *N*-alkylation to take place. As the activated oxygen species involved in monooxygenase reactions is thought

to be an iron-bound oxene, then the oxygenated reactive metabolites of these drugs are likely to be generated in close proximity to the pyrrole nitrogens.

J.W. Bridges: Do other allyl compounds also bind only to the pyrrole nitrogen, or do the more bulky ones bind to proteins as well?

De Matteis: Our work suggests that the relatively bulky drugs also bind to the pyrrole nitrogen. It is possible that covalent binding onto microsomal protein also takes place; however, as far as I know, no other microsomal enzyme has been shown to be inhibited.

Breckenridge: Long-term treatment with these unsaturated compounds could have three possible effects: it could either increase or decrease drug metabolism, or it could have no effect at all. You have shown that secobarbitone can destroy cytochrome *P*-450, yet we and others have shown that the same compound is an effective inducing agent (Breckenridge et al 1973). How can you explain this, Dr De Matteis?

De Matteis: When compounds of this type are administered in a single dose to the rat, there is first a decrease in cytochrome *P*-450 concentration and then an increase to above control levels (De Matteis 1971). It is likely that the apoprotein of cytochrome *P*-450 is not greatly affected in the process of haem destruction and that it can use newly synthesized haem to reconstitute the complete cytochrome. So, at any given time, the amount of functionally active cytochrome *P*-450 will depend on the balance between two different reactions, (1) the supply of haem by the biosynthetic sequence and (2) the degradation of haem due to metabolism of the unsaturated drug to a reactive derivative.

McLean: Do you mean that even *after* the secobarbitone has gone, the damaged apoprotein may destroy cytochrome *P*-450, or are you speaking of the period when the drug is still *present?*

De Matteis: The drug must be present because it participates as a reactant in the process of haem destruction: i.e. it destroys haem by becoming bound to its porphyrin nucleus.

McLean: Is this a stoichiometric process (one haem, one secobarbitone), or is some of the secobarbitone going into non-damaging pathways? What controls the balance between the amount of secobarbitone metabolized and the amount of porphyrin produced?

De Matteis: I don't know. Complicated experiments would be necessary to resolve that, because a balance sheet of the secobarbitone metabolism would be required.

Farber: If an animal is given small doses of carbon tetrachloride, it is protected against the effects of larger doses of carbon tetrachloride. If the endoplasmic reticulum is destroyed by the toxic metabolites of CCl_4, activation

would be inhibited. Such a process could be occurring in your experiments with carbon disulphide.

De Matteis: Yes, small doses of carbon disulphide will protect, like small doses of carbon tetrachloride (see De Matteis & Seawright 1973).

Farber: Do you get any green compound with carbon disulphide?

De Matteis: No, not after carbon disulphide. The situation is apparently different after carbon tetrachloride, when degradation of haem to certain green compounds occurs, but because of lipid peroxidation there is also bleaching and the green compounds cannot be detected easily when this has taken place.

Gillette: It seems to me that there are two possible mechanisms which could account for your data, Dr De Matteis. In the mechanism you suggest, the covalent binding of the reactive metabolite to haem occurs before the reactive metabolite dissociates from the enzyme, and then the metabolite–haem conjugate comes off the enzyme and is replaced by exogenous haem. However, it seems equally plausible to me that the chemically reactive metabolite could dissociate from the enzyme and then react directly with exogenous haem. Do you have any evidence that would distinguish between those two mechanisms?

De Matteis: I have no direct evidence, but I would favour the idea that the reactive metabolite of an unsaturated drug reacts with haem that is bound to cytochrome *P*-450. It is possible that if the reactive metabolite does not react with haem when it is being produced (i.e. when it is so near the pyrrole nitrogen), then it will lose its chance.

Gillette: The reaction of the chemically reactive metabolite with haem could occur whether the haem was attached to the apoenzyme or not.

De Matteis: In cytochrome *P*-450 the spatial arrangement of the haem, the unsaturated side-chain to be metabolized and the iron-bound oxygen may be such that the reactive metabolite is generated very close to the site where alkylation takes place; perhaps there is a transient and very reactive iron-bound intermediate very close to the pyrrole nitrogen.

Gillette: How the chemically reactive metabolite reacts with haem is another story. I like your idea that the intermediate may initially combine with the iron in the haem. In our studies with spironolactone, we were surprised to find that the haem was destroyed without causing the formation of the green pigment that you observed with the allylic compounds. We are beginning to believe that the chemically reactive metabolite of spironolactone extracts the iron from the haem, although we have no convincing evidence to support this view. Perhaps the reactive metabolite of the allylic compound acts by a similar mechanism, i.e. one molecule of the reactive metabolite extracts the iron and another molecule reacts with the pyrrole ring of the protoporphyrin.

Smith: Many drugs in widespread use contain a thione (C = S) group. Is this structure considered to be a general toxicological hazard? Secondly, the toxicity of sulphur-containing drugs is characterized by variations in the organs affected, and variations also depend on species and strain of animal. Is there any unifying explanation for the wide range of toxic effects that result from metabolic activation of these drugs?

De Matteis: The first question is difficult to answer, but it appears that several sulphur-containing compounds of this type are toxic under the right circumstances. In answer to the second question, it is possible that the basic mechanism of toxicity is similar and that some form of reactive sulphur is produced in all cases; but the site of its production may depend on the carrier molecule.

Davies: Dr Gillette (personal communication) has demonstrated that after incubation of spironolactone with adrenal microsomes, specific binding of sulphur to one form of cytochrome *P*-450 occurs. What happens with carbon disulphide?

De Matteis: There is evidence that the cytochrome *P*-450 that is induced by phenobarbitone is preferentially lost as a result of carbon disulphide administration (Bond & De Matteis 1969).

Meyer: A point we may have missed in the discussion of these mechanisms is that AIA, and other allyl-containing chemicals, may affect cytochrome *P*-450-haemoproteins by two or three different mechanisms. For example, AIA, in addition to its property of forming an adduct with the haem of cytochrome *P*-450, is a phenobarbitone-type lipid-soluble inducer of cytochromes *P*-450. By inducing cytochrome *P*-450 apoproteins, it induces both δ-aminolaevulinate synthase (EC 2.3.1.37) and haem formation in a dose-dependent way. However, a different dose range is necessary for its effects on haem 'destruction' and on haem and apoprotein synthesis. Thus, we are dealing with dual effects on cytochrome *P*-450 concentration.

De Matteis: Yes, I agree with you entirely.

Meyer: At low doses, induction of cytochrome *P*-450 may be the predominant effect and 'destruction' of cytochrome *P*-450-haem may be negligible. Perhaps this is clinically important, because doses of secobarbitone that are used therapeutically may have little or no effect on destruction of cytochrome *P*-450 – but this should be investigated.

Gillette: There is recent confirmation of that in humans, for spironolactone (Stripp et al 1975, Pita et al 1975). At normal doses there is virtually no destruction. The side effects of spironolactone are not related to this destruction of haem but to the affinity of the spironolactone for the receptor sites for testosterone.

J. W. Bridges: Dr De Matteis, did you imply that if enough AIA is present, haem will be transferred from peroxidases (EC 1.11.1.7) and from tryptophan 2,3-dioxygenase (tryptophan pyrrolase EC 1.13.11.11) to cytochrome *P*-450, so that tryptophan 2,3-dioxygenase and peroxidase activities will become depressed?

De Matteis: A loss of catalase activity (EC 1.11.1.6) has been reported after AIA and other allyl-containing drugs (Schmid et al 1955, Abbritti & De Matteis 1971), and the haem saturation of tryptophan 2,3-dioxygenase is also significantly reduced by these drugs (Badawy & Evans 1973). These findings can be explained by a rapid destruction and depletion of the precursor pool of haem (which is taken up by the apoprotein of cytochrome *P*-450 and then converted into green pigments), so that the supply of haem for catalase and for tryptophan 2,3-dioxygenase may become limiting.

J. W. Bridges: Is the cell, as a consequence, more vulnerable to the effects of peroxides?

Orrenius: It should be, because the catalase system plays a very important role in the inactivation of peroxides. In particular, is it a starvation effect, i.e. not enough haem is available for synthesis of all haem proteins, or can haem be taken out of one cytochrome and used for cytochrome *P*-450?

De Matteis: It is unlikely to be a starvation effect. Schmid et al (1955) investigated both starvation and carbon tetrachloride to see whether the loss of catalase was an aspecific effect, perhaps an index of aspecific liver injury. Neither starvation nor carbon tetrachloride cause a significant loss of catalase.

Orrenius: Is destruction of haem in cytochrome *P*-450 stoichiometric with cytochrome *P*-450, i.e. is it possible to design an experiment in which haem is added to microsomes and then the added haem is destroyed by alkylation?

De Matteis: I do not know of any stoichiometric experiments on this.

Gillette: The mechanism is important here in interpretation of the results in the two different ways that I mentioned before. The metabolite may be sufficiently stable to dissociate from the enzyme and then to react with the exogenous haem.

Farber: Does carbon disulphide protect against carbon tetrachloride, Dr De Matteis?

De Matteis: Yes, it does. This has been investigated by Seawright et al (1973) in sheep that received carbon tetrachloride as a treatment for liver flukes. Seawright et al found that when carbon disulphide and carbon tetrachloride were given together, the liver damage caused by the latter agent was prevented but there was still an effect on the parasite.

Farber: Is induction required before necrosis occurs with carbon disulphide?

De Matteis: Carbon disulphide is far less effective than carbon tetrachloride as a necrogenic agent. In our Porton strain of rats there is mostly hydropic degeneration with very limited focal necrosis and, for these changes to appear, prior induction is required. However, Dr V.J. Cunningham and collaborators (personal communication) have just found that in certain strains of rat CS_2 can be considerably necrogenic.

Farber: Is this correlated with levels of enzymes?

De Matteis: I don't know.

Amos: Dr De Matteis, you mentioned the use of Sedormid (allylisopropylacetylurea) which can cause platelet aggregation; can you speculate on how platelets should be involved, e.g. is there covalent binding between the drug and the haem moiety of the cytochrome *P*-450 system?

De Matteis: Some recent evidence (Van der Ouderaa et al 1979) suggests that prostaglandin endoperoxide synthetase is a haem-dependent enzyme, and there is also a report that platelets may contain cytochrome *P*-450 (Cinti & Feinstein 1976). Perhaps platelets aggregate when their prostaglandin-type enzyme system is interfered with, or when they are damaged because of metabolism of the allyl group of Sedormid to a toxic derivative. This is entirely speculative, however.

J.W. Bridges: But wouldn't that cause the opposite effect, namely inhibition of both thromboxane formation and platelet aggregation?

De Matteis: Yes, I think you are probably right.

J.W. Bridges: Dr De Matteis, many drugs generate these reactive epoxides and yet there are relatively few that appear to produce significant damage to cytochrome *P*-450. Is that because access to the pyrrole nitrogen is difficult, or are there other explanations?

De Matteis: There are several possible explanations. First, the reactive metabolite of these unsaturated drugs may not be an epoxide. A suicidal-type inactivation of cytochrome *P*-450 has recently been described with an entirely different class of compounds, cyclopropylamines (Hanzlik et al 1979) but it is not yet known whether with these compounds the haem or the apoprotein moiety is attacked. Alternatively, it is possible that for an epoxide to bind the pyrrole nitrogen, it must be an epoxide of an olefine (as contrasted with an arene oxide) that possesses the right characteristics of size and shape.

J.W. Bridges: Is it known whether benzo[*a*]pyrene, which is metabolized via epoxides, interferes with cytochrome *P*-450 activity by binding to either the haem or the apoprotein?

De Matteis: I don't know.

Conney: Perhaps the absence of a constant rate of product formation during the course of incubation with some drugs may be caused by the formation

of reactive intermediates that destroy cytochrome *P*-450.

De Matteis: Yes, it could be so, but the loss of drug-metabolizing activity during incubation of microsomes is usually ascribed to lipid peroxidation.

Gillette: Our evidence does not suggest that epoxides cause the destruction of cytochrome *P*-450 (Thorgeirsson et al 1976). It is often possible to distinguish between a selective destruction of cytochrome *P*-450 and cell death in general by measurements of the ratio of the cytochrome *P*-450 to the cytochrome b_5 present in liver microsomes. Cell death results in the loss of the endoplasmic reticulum. Thus both cytochrome b_5 and cytochrome *P*-450 are decreased, but there is little change in the ratio.

Higginson: Obvious clinical or functional evidence of chronic liver toxicity in humans is comparatively rare. This is probably due to the relatively low doses of chemicals to which humans are generally exposed, and to the enormous reserves of the liver. Experimentally, the pre-existing state of the individual liver can influence the degree of toxicity observed. Thus, a fatty liver will take up three times more carbon tetrachloride than a non-fatty liver (P.S. Quinn & J. Higginson, unpublished work).

Gillette: That should be irrelevant really.

Higginson: Yes, but the concentration per cell would be different under those circumstances.

Gillette: But the total concentration is irrelevant. The rate of formation of a metabolite at any given time depends on the unbound concentration of carbon tetrachloride; thus, the clearance of a substance by an organ is entirely independent of reversible binding or localization of carbon tetrachloride in fat within the organ. It is also important to realize that the total amount of reactive metabolite formed in a given cell depends on the dose of carbon tetrachloride, the clearance for the formation of the chemically reactive metabolite and the sum of the other clearances by which carbon tetrachloride is eliminated from the body. Of course, high concentrations of carbon tetrachloride in cells of a fatty liver may reflect the presence of cells that are already damaged, and such cells may be more susceptible to the toxic effects of a given amount of chemically reactive metabolite. But that is another aspect of the problem, because the localization of carbon tetrachloride in fat would then be a *symptom* of the predisposition of such cells to manifest the toxicity, rather than being the *cause* of the toxicity.

Farber: We know that the liver activates benzo[*a*]pyrene and other polycyclics, thus generating potentially damaging epoxides, and yet there is no obvious cell damage in the liver (Tsuda et al 1980). There is no question about the compounds actually reaching the liver, since covalent binding of the compounds to form DNA adducts is readily demonstrable.

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Environmental Chemicals, Enzyme Function and Human Disease
DAVID EVERED and GERALYN LAWRENSON

General discussion I

J.W. Bridges: When we compare and contrast induction by phenobarbitone with that by 3-methylcholanthrene, the 3-methylcholanthrene-type induction is seen in most cells and in a wide range of species. Phenobarbitone-type induction, on the other hand, seems to be mainly confined to the liver and to the gastrointestinal tract, and is seen in relatively few animal phylla.

Ought we to regard induction by both types of inducers as normal physiological responses of cells to challenge by a chemical, or should we consider induction by phenobarbitone to be *physiological,* and that by methylcholanthrene to be *pathological?* Poland et al (1979) have argued that for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, or dioxin), there is a relationship between the extent of induction and toxicity. When a new drug is developed which exhibits a pattern of induction more similar to the 3-methylcholanthrene-type than to the phenobarbitone-type, should this be a cause for concern *per se?*

Oesch: This problem was behind my earlier question to Dr Bend (p 95), who equated higher inducing potency with higher toxicity; I think the answer depends entirely on the compound that is being examined. In some instances, 3-methylcholanthrene can induce pathways that produce less toxic metabolites (F. Oesch, unpublished results).

Conney: An important question is whether induction by a variety of different inducers is good or bad for the individual. The answer to this question depends on the environment. Enzyme induction by phenobarbitone protects rats from the acute toxicity of warfarin, pentobarbitone and strychnine and from the carcinogenic effects of aminoazo dyes and aflatoxin B_1, presumably by enhancing detoxification pathways relative to pathways that form toxic metabolites. However, enzyme induction by phenobarbitone increases the hepatotoxicity of paracetamol (acetaminophen) and increases the car-

cinogenicity of safrole (5-(2-propenyl)-1,3-benzodioxole), presumably by increasing metabolic activation pathways relative to detoxification pathways. Although induction produced by phenobarbitone increases the hepatotoxicity of bromobenzene, the induction of different cytochrome *P*-450s by 3-methylcholanthrene is associated with protection of rats from the hepatotoxicity of bromobenzene. Phenobarbitone enhances the metabolism of steroid hormones, bilirubin and other normal body constituents in human beings (Conney & Kuntzman 1971), but it is not known if the overall effects of induction are good or bad. Neonatal jaundice can be prevented by treatment of the expectant mother with phenobarbitone for two weeks before delivery (Maurer et al 1968), and in some populations, e.g. on the isle of Lesbos in Greece, phenobarbitone has been used prophylactically to lower bilirubin concentrations because this population normally exhibits a high incidence of neonatal jaundice. This is an example of induction being beneficial in a population under very special circumstances. There are, however, certain risks associated with the administration of phenobarbitone. These include alterations in the metabolism of endogenous substrates, such as steroid hormones, and also the possibility that phenobarbitone may be a tumour promotor.

Farber: Under certain conditions, phenobarbitone administration at an early stage in cell toxicity can serve a protective function in the development of carcinogenesis (Peraino et al 1971).

Conney: Yes, but it does depend very much on the timing of phenobarbitone administration.

J. W. Bridges: Aside from effects of inducers on the metabolism of exogenous chemicals what other consequences does induction have in hepatic and other cells?

Oesch: In the family of substances that induce a pattern similar to that produced by 3-methylcholanthrene, there are some relatively non-toxic substances, such as β-naphthoflavone.

J. W. Bridges: Induction by phenobarbitone and by 3-methylcholanthrene presumably involves switching on different blocks of genes, thus giving rise to a number of proteins, not all of which are drug-metabolizing enzymes. In addition, with 3-methylcholanthrene, induction occurs in a wide variety of extrahepatic tissues – should this itself be a matter for concern?

Higginson: It would be useful if there were a baseline to which reference could be made in experimental descriptions of enzyme induction, e.g. the amount of induction in germ-free inbred animals over several generations could be used as a baseline. Could enzyme imprinting cause a difference between generations, either in normal or in germ-free animals?

McLean: Essentially there is no difference in enzyme activity between germ-free and conventional animals (Short & Davis 1969). However there is a fundamental controlling effect of diet, and a baseline of enzyme activity can be produced by the progressive removal of everything except highly purified components from the diet. In general, the concept of a baseline is useful, not because induction is necessarily pathological, but because a baseline would enable us to detect environmental influences on induction. We have some initial data, but we need more before such a baseline can be established more certainly. We could then examine a number of conventional environments, e.g. the market food-basket, which gives us some measure of the nutritional 'pressures' in the human environment. We could create an environment similar to that in the year 1900 by selecting the kinds of food used then, and we could compare the 'inducing pressures' that occurred then with those we encounter in our present environment. I doubt that we would observe any difference. The inducing pressure that exists in natural food substances has probably been with us from evolutionary times, ever since the first dinosaur suffered from osteosarcoma and finished up in the British Museum (Natural History)!

Farber: It may be that that 'inducing pressure' has survival value, if we could only understand it.

McLean: Perhaps there is no single optimum level of drug-metabolizing enzyme activity and the health effects just depend on the other chemicals in the environment. More background data on induction pressures in the diet would enable us to monitor our environment more effectively. We have a better-stored, more sophisticated diet, and therefore we may be moving downwards in the induction scale because we have fewer bacterial and fungal products. Alternatively, we may be moving up the scale because we are using more exotic foods and additives. We ought at least to establish where we are on the induction scale.

J. W. Bridges: Could I return to the question: is there any evidence that induction is, in itself, harmful? Should the drug industry try to avoid introducing new drugs that induce liver enzymes?

Davies: The safest drugs are obviously those that produce only the desired effect, and without enzyme induction or any other undesirable effect.

Hunter: But the reason why we don't, in practice, use a drug that causes enzyme induction, if we can avoid it, is the risk of modification to drug efficacy, and the resulting complications and interactions, and not because of the drug's inherent effects on the liver.

Amos: Is drug induction an all-or-none phenomenon or does it differ in degree between individual people?

Breckenridge: There are many examples of a wide range of inducibility between different human individuals. In this respect, humans are different from the elasmobranch fish, as we have heard already, and from inbred strains of rats.

McLean: There's a dose–response relation that covers a wide range of doses (McLean & Driver 1977). For most drugs in use at the moment, the amounts required for any induction to occur are around 50–100 mg/day. Some potent drugs, like the benzodiazepines, are used clinically in doses of only 1–2 mg, so it is unlikely that they give rise to any induction effect.

Connors: In the development of a new drug today, then, any preparation that acted as an enzyme inducer in humans or in animals would be unlikely to reach the market?

Breckenridge: If it was a drug to cure cancer, enzyme induction would be a less major disadvantage!

Meyer: One of the drugs with well established enzyme-inducing properties in humans is rifampicin, a potent anti-tuberculosis drug.

Conney: A potential risk with inducers of monooxygenase activity is the possibility of their having a promoting effect on the tumorigenic action of chemical carcinogens. When a low dose of 2-acetylaminofluorene (2-AAF) is administered to rats, it does not cause many liver tumours. If an enzyme inducer, e.g. phenobarbitone or DDT, is administered at a later time, increased tumour formation is observed.

Garner: But that's an artificial situation because you are separating exposure to the carcinogen from exposure to the inducing agent; in real life, exposure to both probably occurs at the same time.

De Matteis: I would like to comment on the possible relationship that Jim Bridges has mentioned (p 141) between the powerful inducing ability of TCDD and its marked toxicity. Recent work by Sweeney et al (1979) suggests that iron deficiency protects against the hepatic and general toxicity produced by TCDD. An interpretation of this is that toxicity is somehow linked with iron metabolism.

J. W. Bridges: This could be related to the fact that normally TCDD induces other proteins besides drug-metabolizing enzymes. Iron deficiency could modify the properties of some, but by no means all, of these induced enzymes and other proteins.

Higginson: How, then, does individual susceptibility affect enzyme induction? Are differences in induction levels between individuals due predominantly to previous exposures to environmental factors or to genetic factors? The only group of animals studied for long periods under controlled conditions are germ-free animals. We need to know whether they show varia-

tions in induction similar to those seen in conventional animals.

Conney: In humans, identical twins respond more similarly than do fraternal twins, with regard to induction by phenobarbitone (Vesell & Page 1969). The most likely explanation for this is probably genetic, yet identical twins tend also to eat similar diets, and to live in similar environments, so it is possible that environment may play some role in the similar responses of identical twins to enzyme inducers.

Idle: Presumably, the B6 and D2 inbred strains of mice also eat similar diets, and the reason why they differ in response to inducers is a single genetic one (Nebert 1979). It is hard to envisage why humans should be different from mice in this respect.

Smith: Yes. For humans, we should consider different base-lines of induction that depend on individual genetic structure.

Selikoff: What order of magnitude of induction would exist in humans at serum levels of 10 p.p.b. of polychlorinated biphenyls (which is the average concentration suggested by large scale studies in the United States)?

Breckenridge: It is difficult to estimate whether induction has occurred in humans because we cannot define a suitable control rate of drug metabolism for comparative purposes.

Hunter: Professor Meyer has mentioned indirect ways of studying enzyme induction; e.g. the urinary excretion of 6β-hydroxycortisol or D-glucaric acid has been shown to reflect the activity of hepatic microsomal enzymes (Hunter et al 1972). Workmen making dieldrin and aldrin in Holland have greater serum concentrations of these chemicals than the normal population. It was not possible to show any increase in induction in these men by means of the indirect methods of measurement (Hunter et al 1972), so I suppose that humans are not normally in an induced state, and I doubt that 10 p.p.b. of polychlorinated biphenyls could initiate induction.

McLean: But you are suggesting that induction is an all-or-none phenomenon, and I would dispute that. All the members of the human population lie on a normal distribution curve of enzyme activity, and natural inducers will shift that curve up a little. So in those terms we might reiterate your previous comment by saying that we normally fit in a low position on the dose–response curve. However, if any of us were put on to a highly purified diet, without both vegetables and meat, our hydroxylation activity would probably be reduced.

Hunter: I accept that. The chemicals (e.g. terpenes and flavoids) that we take from our food probably have the greatest effect on our enzyme activity. Eating a lot of cabbage will probably have more effect on hepatic enzyme activity than will polychlorinated biphenyls or DDT!

McLean: And here we return to the importance of attempting to define baseline levels of induction. We could then provide a guideline, e.g. that the population levels of drug metabolism should not rise more than an arbitrary 10% over the present levels – rather in the same way that we deal with radiation. However, the difference is that we know that radiation is harmful; with enzyme induction we are unsure of the consequences.

Farber: We have exactly the same problem in defining the acceptable levels of carcinogens, which are with us all the time.

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Environmental Chemicals, Enzyme Function and Human Disease
DAVID EVERED and GERALYN LAWRENSON

Regulation of human drug metabolism by dietary factors

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Abstract Several dietary factors influence the oxidative metabolism of chemicals in humans. Increasing the ratio of protein to carbohydrate or fat in the diet, feeding cabbage and brussels sprouts or feeding charcoal-broiled beef for several days stimulates human drug metabolism. The chronic ingestion of ethanol stimulates drug metabolism whereas the chronic ingestion of methylxanthine-containing foods inhibits drug metabolism. In contrast, an increase in the ratio of fat to carbohydrate in the diet of normal subjects or the fasting of obese individuals for several days has little or no effect on drug metabolism. Flavonoids in edible plants influence the metabolism of foreign chemicals by human liver *in vitro*. The addition of flavone, tangeretin or nobiletin to human liver microsomes activates both the hydroxylation of benzo[*a*]pyrene and the metabolism of aflatoxin B_1 to mutagens. On the other hand, quercetin, kaempferol, morin and chrysin, which are also normally occurring flavonoids, inhibit the hydroxylation of benzo[*a*]pyrene by human liver microsomes.

Drugs are metabolized by oxidative pathways at markedly different rates in different people and this individuality in metabolism contributes to differences in drug response. There is a similar individuality in the response to environmental carcinogens which are metabolized by the same enzymes that metabolize drugs. Both genetic and environmental factors control the rates and pathways of chemical biotransformation in human beings, but it is difficult to quantify the relative contributions of genes and environment. Interindividual variations in the *in vivo* metabolism of antipyrine, phenylbutazone, and nortriptyline are greater in fraternal than in identical twins (Vesell & Page 1968, 1969, Alexanderson et al 1969), which suggests that genetic factors are important in the control of oxidative drug metabolism. The importance of genetic factors in the regulation of human drug metabolism has also been emphasized by recent studies on the oxidative metabolism of debrisoquine (Woolhouse et al 1979) and sparteine (Eichelbaum et al 1979).

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TABLE 1

Effect of dietary protein (PRO), carbohydrate (CHO) and fat on human drug metabolism

Study	Dietary regimen	*Antipyrine*		*Theophylline*	
		Half-life (h)	*Metabolic clearance rate (ml min^{-1} kg^{-1})*	*Half-life (h)*	*Metabolic clearance rate (ml min^{-1} kg^{-1})*
1	High protein (44% PRO, 35% CHO, 21% FAT)	9.6 ± 0.4	0.76 ± 0.04	5.2 ± 0.4	1.00 ± 0.06
	High carbohydrate (10% PRO, 70% CHO, 20% FAT)	15.6 ± 1.7	0.52 ± 0.06	7.6 ± 0.7	0.69 ± 0.04
2	High carbohydrate (10% PRO, 80% CHO, 10% FAT)	12.3 ± 0.5	0.57 ± 0.02	7.9 ± 0.4	0.76 ± 0.06
	High fat (10% PRO, 20% CHO, 70% FAT)	11.7 ± 0.6	0.59 ± 0.02	7.9 ± 0.4	0.74 ± 0.04
	High protein (50% PRO, 20% CHO, 30% FAT)	9.9 ± 0.5	0.71 ± 0.05	5.8 ± 0.3	0.98 ± 0.08
3	High unsaturated fat (15% PRO, 25% CHO, 60% FAT)	10.2 ± 0.6	0.69 ± 0.04	6.5 ± 0.6	0.95 ± 0.10
	High carbohydrate (15% PRO, 60% CHO, 25% FAT)	10.0 ± 0.5	0.74 ± 0.04	6.7 ± 0.3	0.91 ± 0.06
	High saturated fat (15% PRO, 25% CHO, 60% FAT)	10.0 ± 0.6	0.72 ± 0.03	6.0 ± 0.5	0.98 ± 0.09

Six subjects in study 1, six subjects in study 2, and nine subjects in study 3 were each fed an isocaloric diet for 10 or 14 days before administration of antipyrine or theophylline respectively. Each value represents the mean ± S.E. Taken from studies by Alvares et al (1976), Kappas et al (1976), and Anderson et al (1979).

However, environmental factors can also regulate the metabolism of chemicals. The use of drugs, exposure to pesticides, ingestion of alcohol, smoking of cigarettes, and changes in the diet are examples of environmental factors that influence human drug metabolism (Conney 1967, Rubin et al 1970, Misra et al 1971, Conney et al 1977a, 1977b, 1979, Iber 1977). The amount of intraindividual variability in the biotransformation of chemicals in unrestricted, normal volunteers who are pursuing their usual life styles has been used as an index of the effect of environment on the regulation of human drug metabolism (Alvares et al 1979). Although appreciable intraindividual differences in the metabolism of some drugs occur when they are administered to healthy human subjects on several occasions, only small intraindividual differences occur with other drugs (Alvares et al 1979).

Our diet is a major source of interaction with the environment. Changes in the ratio of protein to carbohydrate or fat in the diet, and ingestion of charcoal-broiled beef, cabbage and brussels sprouts, xanthine-containing foods, or ethanol are among the dietary factors that can influence human drug metabolism.

EFFECT OF PROTEIN, CARBOHYDRATE AND FAT CONTENT OF THE DIET ON HUMAN DRUG METABOLISM

Three studies were initiated on the effects of dietary protein, carbohydrate and fat on drug metabolism in normal volunteers aged 21–30 years (Table 1). Heavy drinkers of alcoholic beverages and smokers were excluded, and the subjects were not permitted to take any drugs for 3 weeks before or during the experiments, except for an occasional aspirin. All dietary regimens were administered for 14 days and contained 2500 kcal/day (1.046×10^4 kJ/day). The diets were prepared by the Metabolic Diet Kitchen of the Rockefeller University Hospital. Smoked food, brussels sprouts, cabbage and other known inducers or inhibitors of drug-metabolizing enzymes were excluded from the diets. The high-carbohydrate diet was enriched with sucrose, cereal products, fruits, vegetables, sherbet and candy. The high-protein diet was enriched with egg white, chicken, fish, cottage cheese, milk, soya nuts and a protein-rich liquid supplement (Sustacal). The high-fat diet contained relatively large amounts of lipid-rich food such as beef and pork, dairy products, corn oil and walnuts. The effects of the relative amounts of polyunsaturated and saturated fats in the diet were considered only in the third study described in Table 1. In the polyunsaturated fat diet, 80% of the fat content was in the form of additive-free corn oil. In the highly saturated fat diet, which was otherwise identical to the highly unsaturated one, 80% of the fat was in the form of butter instead of corn oil. The plasma half-lives of orally

administered antipyrine (18 mg/kg) or theophylline (5 mg/kg) were determined on days 10 and 14, respectively, of each study period. Further details of these studies are given elsewhere (Alvares et al 1976, Kappas et al 1976, Anderson et al 1979).

The average half-lives of antipyrine and theophylline increased 63% and 46% respectively when six subjects were shifted from the high-protein, low-carbohydrate diet to the isocaloric high-carbohydrate, low-protein diet (Table 1, study 1). Interestingly, there was considerable individuality in response to this alteration in diet. The increase in antipyrine half-lives among the six subjects ranged from no change in one subject to 111% in another subject. The increase in theophylline half-lives ranged from 14% to 71%. Significant decreases in the metabolic clearance rates of antipyrine and theophylline were also observed when the subjects were shifted from the high-protein, low-carbohydrate diet to the low-protein, high-carbohydrate diet. These results indicate that increasing the protein content of the diet at the expense of carbohydrate stimulates human drug metabolism. A similar stimulation of antipyrine and theophylline metabolism was observed when the amount of dietary protein was increased at the expense of fat (Table 1, study 2). However, an increase in the amount of either saturated or unsaturated fat in the diet at the expense of carbohydrate had no effect (Table 1, study 3).

EFFECT OF DIETARY CHARCOAL-BROILED BEEF ON HUMAN DRUG METABOLISM

Polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene, dibenz[*a,h*]anthracene and benz[*a*]anthracene are widespread in our environment as products of incomplete combustion. These compounds induce the synthesis of liver enzymes that metabolize drugs and carcinogens in rats (Conney 1967, Conney et al 1956, 1957). High concentrations of polycyclic hydrocarbons are present in tobacco smoke and even higher amounts are present in charcoal-broiled beef. Exposing rats to cigarette smoke or feeding them a diet containing charcoal-broiled beef enhances, by several-fold, the metabolism of benzo[*a*]pyrene and phenacetin *in vitro* (Welch et al 1971, Harrison & West 1971, Pantuck et al 1974, 1975). In humans, cigarette smoking not only stimulates the activity of placental enzymes that metabolize several foreign chemicals (Welch et al 1968, 1969, Conney et al 1977a) but it also lowers the plasma concentrations of orally administered phenacetin by enhancing phenacetin metabolism in the gastrointestinal tract and/or during its first pass through the liver (Pantuck et al 1972, 1974). When we studied the effects of dietary charcoal-broiled beef on phenacetin metabolism in humans, we administered 900 mg of phenacetin, two hours before breakfast, to nine healthy subjects

after they had been fed (1) a control diet for seven days, (2) the control diet for three additional days and then a charcoal-broiled beef diet for four days, and (3) the control diet again for seven days. All the diets contained hamburger at lunch and steak at dinner, and these meats were cooked over burning charcoal. For the control diet, aluminium foil was placed between the meat and the charcoal during cooking; for the test diet, the meat was exposed directly to the burning charcoal. Further details of this study are given elsewhere (Conney et al 1976, Pantuck et al 1976a).

Feeding charcoal-broiled beef to volunteers for four days markedly lowered their mean plasma concentration of phenacetin (Fig. 1) and decreased the area under the curve for plasma concentration of phenacetin against time. The concentration of phenacetin's major metabolite, *N*-acetyl-*p*-aminophenol, was measured in the plasma of the subjects after they had received phenacetin. Feeding the charcoal-broiled beef diet did not alter the total plasma concentration of conjugated and unconjugated *N*-acetyl-*p*-aminophenol. The ratio of the mean concentration of total *N*-acetyl-*p*-aminophenol to the mean concentration of phenacetin in plasma at each time after administration of phenacetin was markedly increased when the subjects had been fed charcoal-broiled beef, and the ratio at each time was decreased when the subjects returned to the control diet. The results suggest that feeding charcoal-broiled

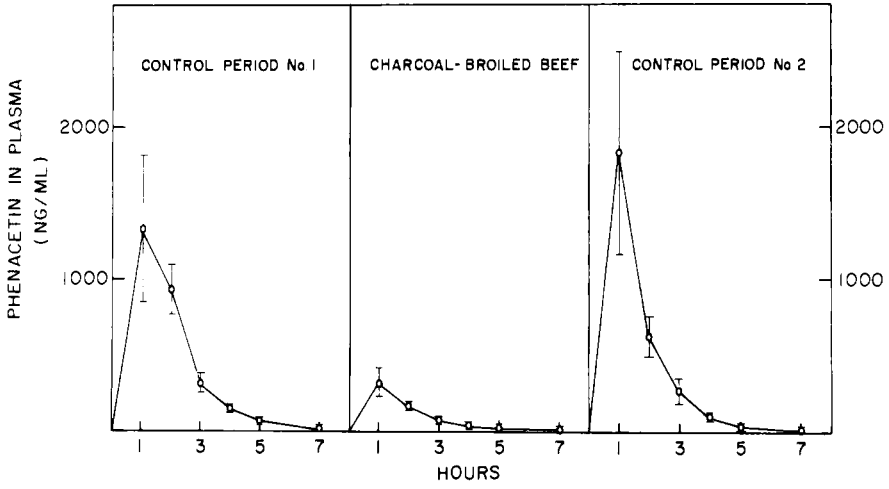

FIG. 1. Effect of a diet containing charcoal-broiled beef on the plasma concentration of phenacetin in humans. Nine subjects were given 900 mg of phenacetin after 7 days on control diet, 4 days on charcoal-broiled beef diet and 7 days on the control diet a second time. Each value represents the mean ± S.E. Taken from data by Conney et al (1976).

beef stimulates the metabolism of phenacetin in the gastrointestinal tract and/or during its first pass through the liver.

Marked individual variations occurred in the plasma concentrations of phenacetin among the nine subjects who had been fed the control diet, and there were also individual variations in their responsiveness to charcoal-broiled beef. Switching from control to charcoal-broiled beef diet resulted in a decreased area under the curve for plasma concentration of phenacetin against time in seven of the nine subjects. The two subjects who did not show this had very low plasma concentrations of phenacetin while on the control diet. The reason(s) for these low concentrations throughout the study is unknown, but a low concentration of phenacetin in plasma may have resulted from genetic and/or environmental factors. One of the two subjects worked as a carpenter and may therefore have been exposed to volatile oil inducers of drug metabolism that are present in certain soft woods.

The effects of a charcoal-broiled beef diet on the metabolism of antipyrine and theophylline are summarized in Table 2. In this study, subjects were given antipyrine (1.8 mg/kg) and theophylline (5 mg/kg) respectively on (1) days 4 and 5 of a seven-day control diet period, (2) days 4 and 5 of a five-day period on a diet containing charcoal-broiled beef and (3) days 6 and 7 of a second period on the control diet. The concentrations of antipyrine or theophylline in plasma at various times after the administration of these drugs were determined in each subject. Further details of this study are given elsewhere (Kappas et al 1978). Feeding the charcoal-broiled beef diet to the volunteers for three days decreased the mean plasma half-life of antipyrine by 22% and increased its apparent metabolic clearance rate by 38% (Table 2); there was little or no effect on the apparent volume of distribution of an-

TABLE 2

Effect of a charcoal-broiled beef diet on antipyrine and theophylline metabolism in humans

	Antipyrine		*Theophylline*	
Dietary regimen	*Half-life (h)*	*Metabolic clearance rate (ml/min)*	*Half-life (h)*	*Metabolic clearance rate (ml/min)*
Control (1st time)	13.7 ± 1.3	45.8 ± 4.2	6.0 ± 0.5	69.8 ± 6.3
Charcoal-broiled beef	10.7 ± 1.2	63.2 ± 6.4	4.7 ± 0.4	90.9 ± 9.3
Control (2nd time)	13.3 ± 1.1	47.5 ± 4.5	6.4 ± 0.7	69.1 ± 6.6

Eight subjects were given antipyrine or theophylline as described in the text. Each value represents the mean ± S.E. Taken from studies by Kappas et al (1978).

tipyrine. Similar data were obtained when theophylline was administered. Feeding the charcoal-broiled beef diet to the subjects for four days decreased the theophylline half-life by 22% and increased its apparent metabolic clearance rate by 30% (Table 2); no change was observed in the apparent volume of distribution of theophylline. The half-lives and apparent metabolic clearance rates returned to control values when the subjects were again fed the control diet.

EFFECT OF FEEDING CABBAGE AND BRUSSELS SPROUTS ON HUMAN DRUG METABOLISM

Wattenberg (1971) showed that feeding rats a diet containing brussels sprouts, cabbage, cauliflower, or certain other vegetables markedly stimulated the activity of benzo[*a*]pyrene hydroxylase (EC 1.14.14.1) in the intestine. It was subsequently found that indole-3-acetonitrile, indole-3-carbinol, and 3,3′-diindolylmethane are present in brussels sprouts and cabbage and that these compounds are strong inducers of benzo[*a*]pyrene hydroxylase activity in the rat (Loub et al 1975). We found that feeding brussels sprouts or cabbage to rats stimulated the intestinal metabolism of phenacetin, 7-ethoxycoumarin and hexobarbitone, and similar results were obtained after oral administration of indole-3-acetonitrile, indole-3-carbinol and 3,3′-diindolylmethane (Pantuck et al 1976b). We also studied the effects of cabbage and brussels sprouts on the metabolism of antipyrine and phenacetin in 10 normal volunteers aged 21–32 years. They were non-smokers and were not heavy drinkers of alcohol, coffee or tea. They took no drugs during the experiment, except an occasional aspirin. Antipyrine and phenacetin metabolism were studied after the subjects had been fed (1) a control diet, (2) a diet containing cabbage and brussels sprouts and (3) the control diet a second time. Each diet contained 12% protein, 28% fat, 60% carbohydrate and 2600 kcal/day (1.088×10^4 kJ/day). The first control diet was fed for 10 days, with antipyrine being administered on day 7 and phenacetin on day 10. The control diet was continued for an additional three days and the test diet was fed for the next seven days, with antipyrine being administered on day 4 and phenacetin on day 7 of the test period. The second control diet followed immediately and lasted for 10 days, with antipyrine being administered on day 7 and phenacetin on day 10. The control and test diets were identical except for the vegetable content. The control diet contained a lettuce–cucumber salad and 150 g of green peas at lunch and 100 g of green beans and 150 g of green peas at dinner. These vegetables have little or no enzyme-inducing properties. In the test diet, the above vegetables were replaced by 150 g of brussels sprouts

TABLE 3

Effect of a diet containing brussels sprouts and cabbage on the plasma concentration of phenacetin in humans

Diet	*Plasma concentration of phenacetin (ng/ml)*						
	0.5 hr	*1 hr*	*2 hr*	*3 hr*	*4 hr*	*5 hr*	*7 hr*
Control (1st time)	2316 ± 1150	2396 ± 960	1430 ± 473	497 ± 137	202 ± 56	111 ± 29	32 ± 10
Brussels sprouts and cabbage	982 ± 402	1576 ± 663	662 ± 170	228 ± 49	90 ± 20	36 ± 8	11 ± 3
Control (2nd time)	1570 ± 946	1843 ± 891	975 ± 280	776 ± 304	277 ± 97	113 ± 7	25 ± 8

Phenacetin (900 mg) was administered on day 10 of the first control diet, on day 7 of the diet containing cabbage and brussels sprouts, and on day 10 of the second control diet. Each value represents the mean ± S.E. for 10 subjects. Taken from data by Pantuck et al (1979).

and 100 g of cabbage for both lunch and dinner. The brussels sprouts, cabbage, green beans and green peas were prepared by steaming them lightly. The doses of phenacetin (900 mg) or antipyrine (1.8 mg/kg) were administered orally, two hours before breakfast. Further details of this study are given elsewhere (Pantuck et al 1979). The diets containing cabbage and brussels sprouts reduced the mean plasma half-life of antipyrine by 13% in 10 subjects and increased the apparent metabolic clearance rate of antipyrine by 11%. Returning the subjects to the control diet increased by 17% the plasma half-life of antipyrine and decreased by 13% its apparent metabolic clearance rate. Although these effects of feeding cabbage and brussels sprouts on antipyrine metabolism are small, they are statistically significant.

The mean plasma concentrations of phenacetin at various times after its oral administration were reduced by 34–67% after six days on the diet of cabbage and brussels sprouts (Table 3). The plasma concentrations of total *N*-acetyl-*p*-aminophenol were slightly increased at the early time intervals, 0.5–3 h, and were slightly lower at the late time intervals, 5–7 h, after phenacetin administration. The ratios of the mean plasma concentrations of total *N*-acetyl-*p*-aminophenol to the mean plasma concentrations of phenacetin were increased when the subjects were fed the cabbage and brussels sprouts, and these ratios returned towards control values when the subjects were again fed the control diet. The results suggest that a diet containing cabbage and brussels sprouts stimulates the metabolism of phenacetin in the gastrointestinal tract and/or during the first pass of phenacetin through the liver. In addition, the ratios of the plasma concentrations of conjugated to unconjugated *N*-acetyl-*p*-aminophenol at the various times after phenacetin administration were increased by 40–50% after the test diet. These results suggest that ingestion of cabbage and brussels sprouts had enhanced the conjugation of *N*-acetyl-*p*-aminophenol.

EFFECT OF METHYLXANTHINES ON HUMAN DRUG METABOLISM

Relatively large amounts of caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) and/or theophylline (1,3-dimethylxanthine) are found in certain foods and beverages such as coffee, tea, cocoa, cola drinks and chocolate. Recent studies have suggested that ingestion of these methylxanthines inhibits human drug metabolism. Treatment of human subjects with 6 mg theobromine/kg body weight once daily for five days lengthened the plasma half-life and decreased the metabolic clearance rate of theobromine (Drouillard et al 1978). Dietary abstention from methylxanthines for two weeks shortened the plasma half-life of theobromine from 9 h

during a normal diet to 6.1 h and increased its metabolic clearance rate from 72 ml/min to 114 ml/min (Drouillard et al 1978). Similar results were obtained when theophylline metabolism was measured (Monks et al 1979).

A recent report suggested that the chewing of cola nuts, which contain high concentrations of caffeine and smaller amounts of theobromine, inhibited the metabolism of antipyrine in West African villagers (Fraser et al 1976). In a second study, however, the chewing of cola nuts by Caucasian subjects in South Central Pennsylvania failed to influence antipyrine metabolism (Vesell et al 1979).

EFFECT OF ETHANOL ON HUMAN DRUG METABOLISM

Alcoholics, when sober, are tolerant to several drugs but, when inebriated, are highly sensitive to many drugs. Indeed, deaths have occurred after inebriated individuals have taken sedatives or hypnotic drugs. The tolerance to drugs that is observed in alcoholics may be explained by the increase in activity of certain monooxygenases in human liver after chronic ingestion of ethanol (Rubin & Lieber 1968) and by the enhanced rate of drug metabolism measured *in vivo* in alcoholics (Misra et al 1971, Iber 1977). In contrast to the stimulatory effect of chronic ingestion of alcohol on drug metabolism, acute pretreatment with large amounts of ethanol just before administration of meprobamate or pentobarbitone increased the plasma half-lives of these two drugs by twofold to fourfold (Rubin et al 1970). This inhibitory effect of acute ethanol administration on human drug metabolism *in vivo* can explain the dangerous and synergistic central depression that is observed when ethanol and a sedative or hypnotic drug are ingested together.

EFFECT OF STARVATION AND MALNUTRITION ON HUMAN DRUG METABOLISM

The fasting of obese subjects for 7–10 days does not influence the metabolism of antipyrine or tolbutamide (Reidenberg & Vesell 1975). In addition, malnourished individuals do not have a markedly abnormal half-life of antipyrine in the plasma (Krishnaswamy & Naidu 1977). These results indicate that severe caloric restriction has little or no effect on drug metabolism in humans.

EFFECT OF FLAVONOIDS ON THE ACTIVITY OF MONOOXYGENASES IN HUMAN LIVER

We have studied the effects of flavone, tangeretin, nobiletin, 7,8-benzoflavone, quercetin, kaempferol, morin and chrysin on monooxygenase activi-

ty in microsomes obtained from surgical biopsy samples of human liver. The structures of these compounds are shown in Fig. 2. Except for the synthetic flavonoid, 7,8-benzoflavone, all of the compounds shown in Fig. 2 are normal constituents of edible plants. The addition of 100 μM quercetin, kaempferol, morin or chrysin (hydroxylated flavonoids) to human liver microsomes inhibited benzo[*a*]pyrene hydroxylation by 82, 72, 36 and 32% respectively (Fig. 3). In contrast to these results, the addition of 7,8-benzoflavone, flavone, tangeretin or nobiletin (non-hydroxylated flavonoids) to human liver

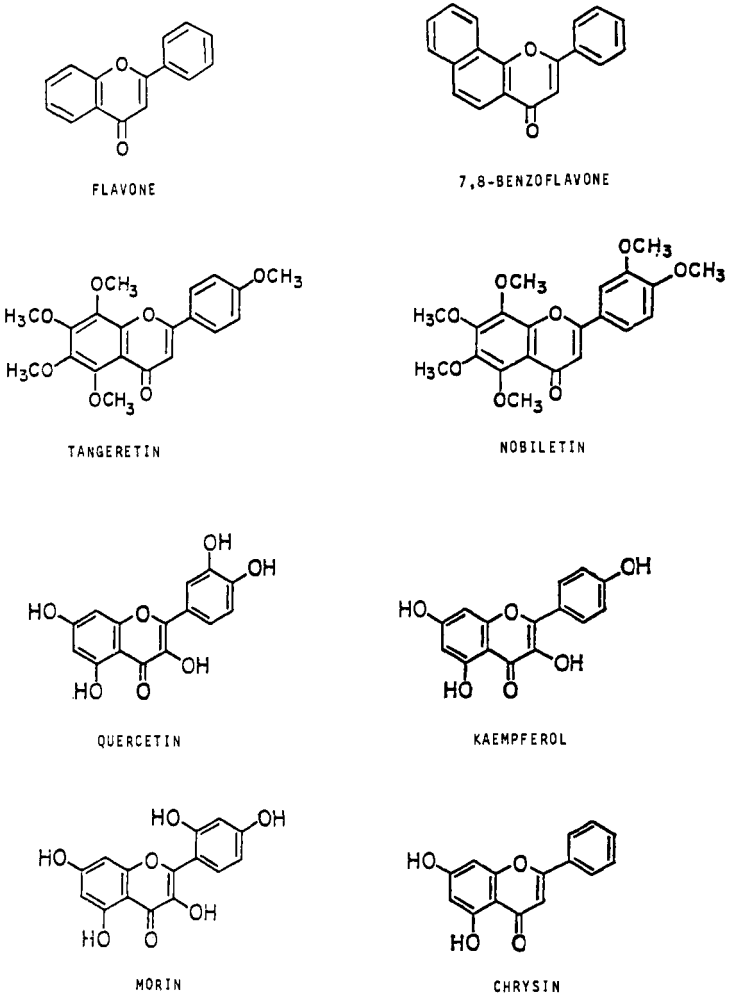

FIG. 2. Structures of flavonoids.

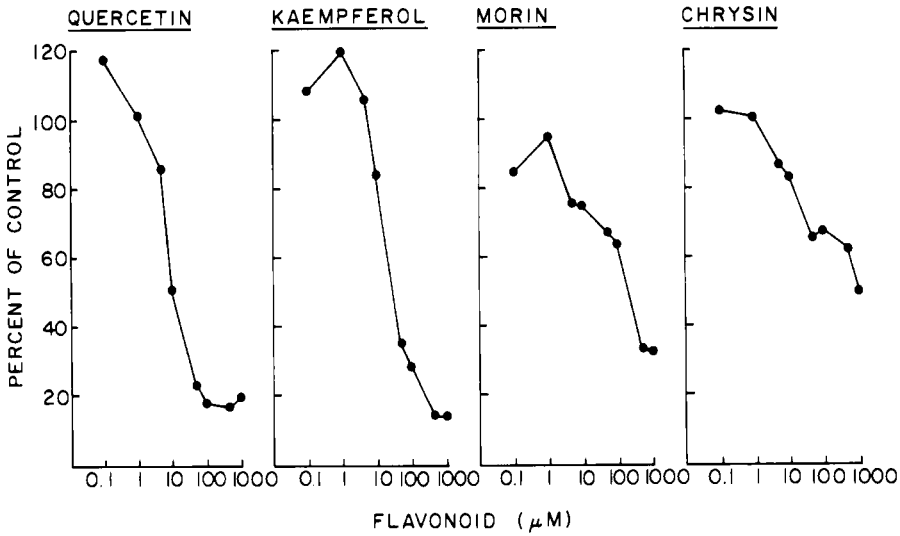

FIG. 3. Inhibitory effect of quercetin, kaempferol, morin and chrysin on the hydroxylation of benzo[*a*]pyrene by human liver microsomes.

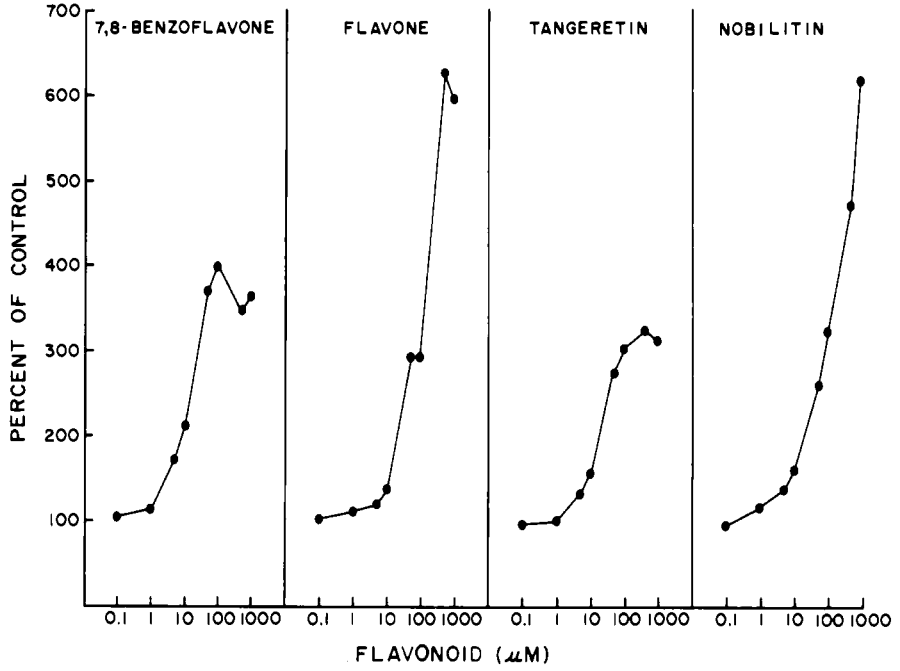

FIG. 4. Stimulatory effect of 7,8-benzoflavone, flavone, tangeretin and nobiletin on the hydroxylation of benzo[*a*]pyrene by human liver microsomes.

microsomes caused a many-fold activation in the hydroxylation of benzo[*a*]pyrene (Fig. 4). The same four compounds also increased the metabolism of aflatoxin B_1 to mutagens (Fig. 5). In other studies with human liver microsomes, it was found that 7,8-benzoflavone also activated the hydroxylation of zoxazolamine and antipyrine, but the oxidative metabolism of hexobarbitone, coumarin and 7-ethoxycoumarin was not appreciably influenced (Kapitulnik et al 1977). The specificity of the activating effect of 7,8-benzoflavone provides evidence for multiple hydroxylating enzymes in human liver microsomes. It is of interest that the activation of benzo[*a*]pyrene hydroxylation by flavonoids is specific to certain species. 7,8-Benzoflavone causes little or no increase in the hydroxylation of benzo[*a*]pyrene or in the metabolism of aflatoxin B_1 to mutagens when rat liver microsomes are used as the source of monooxygenase. However, a many-fold activation occurs with both human and rabbit liver microsomes (Kapitulnik et al 1977, Buening et al 1978, Thorgeirsson et al 1979). Recent work by Dr M.T. Huang in our laboratory (unpublished results) indicates that 7,8-benzoflavone stimulates the NADPH-dependent reduction of cytochrome *P*-450 in rabbit liver microsomes, and this effect may play a role in the activation of monooxygenases. The effects of naturally occurring flavonoids on the hydroxylation of benzo[*a*]pyrene and on the metabolism of aflatoxin B_1 to

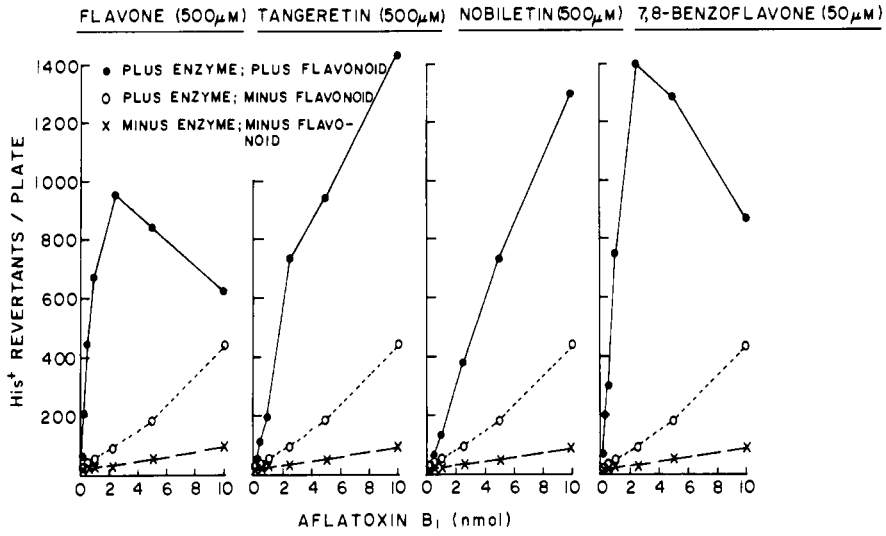

FIG. 5. Stimulatory effect of flavone, tangeretin, nobiletin and 7,8-benzoflavone on the metabolism of aflatoxin B_1 to mutagens by human liver microsomes.

mutagens by human liver *in vitro* suggests a need to determine whether flavonoids can alter the metabolism of drugs and carcinogens *in vivo*. If such changes occurred, they would be highly significant since they could immediately alter the rates of metabolism of drugs, carcinogens and other lipid-soluble chemicals that are present in the human environment.

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Woolhouse NM, Andoh B, Mahgoub A, Sloan TP, Idle JR, Smith RL 1979 Debrisoquin hydroxylation polymorphism among Ghanaians and Caucasians. Clin Pharmacol Ther 26:584-591

Discussion

J. W. Bridges: Dr Conney, have you looked at activation *in vivo* or in intact cells, liver slices or perfused liver?

Conney: We have only used microsomes and whole homogenate.

J. W. Bridges: We found that betamethasone produced activation in whole cells and it would be very interesting to know whether the compounds you have used behave in the same way (D. Benford & J.W. Bridges, unpublished results).

Connors: Is it possible that the increased mutagenicity that you observed need not be due to increased metabolism but to an alteration in the pathways of metabolism?

Conney: Although it is possible that flavonoids alter pathways of metabolism of chemicals by mechanisms that are not dependent on activation, our work with human liver indicates that 7,8-benzoflavone stimulates the overall rate of metabolism of benzo[*a*]pyrene and benzo[*e*]pyrene to multiple products under conditions in which the cytochrome *P*-450 system is saturated with substrate.

Gillette: What fraction of the dose of the compound or mutagen is metabolized during the *in vitro* tests? If most of the compound is metabolized during the mutagenic test, changes in the rates of metabolism along alternative pathways could affect the response; if only a small proportion of it were metabolized, then changes in the rates of metabolism by the alternative pathways would have no effect.

Conney: Only a small percentage of the substrate was metabolized under the conditions of our experiments.

Garner: Compounds such as harman and norharman increase the

mutagenicity of other chemicals when the two are added to a *Salmonella typhimurium*–microsome assay. The mechanism is not understood but metabolism may be involved. The difficulty with mutagenicity assays is that the processes that lead to the end point of mutation may be complex. For example, some compounds may be mutagens in their own right, whereas others may affect DNA repair in the bacteria. We should not, therefore, put all our emphasis on metabolism.

Conney: We believe that the effect of flavonoids to enhance the mutagenicity of chemicals in the presence of hepatic metabolizing enzymes is an effect on the metabolism of the chemical, because the effect of the flavonoid depends on the source of liver enzyme used. 7,8-Benzoflavone enhances the mutagenicity of aflatoxin B_1 in the presence of human liver, but little or no effect is observed when rat liver is used.

Connors: In the work you just mentioned on betamethasone, Professor (J.W.) Bridges, were you using human cells?

J.W. Bridges: We have done some work on human microsomes but our cell and *in vivo* work is only in rats. In each situation we observed activation. So far the discussions at this meeting have concentrated on induction as a means of increasing enzyme activity; this is a relatively slow process. The important thing about activation of drug-metabolizing enzymes is that it represents a very rapid response of an enzyme to a chemical, and it would therefore be interesting to know if it has any physiological significance.

Davies: In collaboration with Professor (J.W.) Bridges, we have examined the effect of betamethasone on oxidation of antipyrine by human liver microsomes (Kahn et al 1980). Addition of betamethasone to liver microsomes *in vitro* increased up to two-fold the 4-hydroxylation of antipyrine, but it had no effect on 3-hydroxylations. These results suggest that different forms of cytochrome *P*-450 catalyse these hydroxylations. However, activation was not observed with all the liver samples, which suggests that inter-individual differences occur in response to betamethasone.

McLean: Dr Davies, could the quantities of steroids that you used be termed physiological doses, and have you examined people being treated with steroids, to see if activation occurs in them?

Breckenridge: Several years ago, Dr Davies, Dr Michael Orme and I infused hydrocortisone into volunteers and we found that the rate of antipyrine metabolism was very rapidly changed. This has subsequently been confirmed by measurements of the excretion of metabolites of antipyrine (Breckenridge et al 1973, M.R. Bending & D.S. Davies, unpublished results). We were surprised at the results, which have proved to be controversial; many people have tried to repeat them, and failed. It is encouraging that biochemical phar-

macologists are now providing some rational explanations for our data.

J.W. Bridges: In rats, the natural corticosteroids have the same enzyme-activating action as betamethasone but they are not as potent.

Davies: In the work discussed by Professor Breckenridge one of the subjects showed no change in half-life or in rate of production of 4-hydroxyantipyrine. However, the sample size was small, as is often the case in this type of study. Further work using a larger group of subjects is needed.

Hunter: In relation to the possible effect of environmental chemicals such as polychlorinated biphenyls and pesticides on drug metabolism in humans, most of our exposure to these chemicals is in the fat that we eat. However, the fat intake of some of your volunteers, Dr Conney, was increased from 10% to 70% without any effect on their drug metabolism. This suggests that these chemicals are therefore not very important in increasing human drug metabolism at our *present* levels of exposure.

Farber: Perhaps the body fat in these subjects was already saturated with the chemicals.

Conney: Fat has an enormous capacity for halogenated pesticides. In an earlier study of factory workers exposed to large amounts of DDT, we observed only moderate changes in their rates of drug metabolism; e.g. phenylbutazone half-lives in the plasma decreased by about 20%, and 6β-hydroxycortisol excretion in the urine was increased by about 60%. The plasma concentrations of DDT in these factory workers were 20–30 times higher than those present in the normal population (Poland et al 1970).

Hunter: But presumably there is an additive effect of other compounds to which we are all exposed, e.g. dieldrin, aldrin, endrin and polyhalogenated biphenyls, in addition to DDT. It may therefore be misleading to study the plasma concentrations of one chemical in isolation from the rest.

Amos: Is the pattern of enzyme induction measured during dietary alterations related to that produced by phenobarbitone or to that produced by 3-methylcholanthrene?

Conney: Enzyme induction by charcoal-broiled beef feeding is probably associated with polycyclic aromatic hydrocarbons. Enzyme induction by cabbage and brussels sprouts is probably caused by certain indole inducers that they contain.

Amos: Is there any evidence of extrahepatic enzyme induction (e.g. in the gastrointestinal tract) occurring as a result of dietary modifications?

Conney: Feeding cabbage, brussels sprouts or charcoal-broiled beef to rats stimulates the intestinal metabolism of many chemicals.

Breckenridge: Dr Conney, you have reported some data on intraindividual variations in rates of drug oxidation which reveal that people living in a

relatively stable environment exhibited two- or three-fold differences in their rates of metabolism of several drugs (Alvares et al 1979). Therefore, how significant are the changes that you have described today, with respect to the magnitude of 'background noise' that can occur?

Conney: Our results indicate that carefully controlled changes in the human diet cause significant changes in drug metabolism. The question of intraindividual differences in drug metabolism is an important one. Repeated tests on the same individual in an uncontrolled environment provide a means of assessing the effects of changes in the environment on human drug metabolism (Alvares et al 1979). We found that intraindividual variations were greater after phenacetin administration than after antipyrine or phenylbutazone. In addition, some individuals exhibited very little intraindividual variations in drug metabolism while others showed a large variation.

Smith: You have shown some striking data on plasma concentrations of phenacetin in people whose enzymes were induced by consumption of charcoal-broiled steak. Did you see any change in the qualitative or quantitative aspects of disposition of phenacetin? For example, were other metabolic pathways such as those leading to the formation of 2-hydroxyphenetidine affected? It would be interesting to know how the two main pathways of phenacetin metabolism, namely *O*-dealkylation and aromatic hydroxylation, are affected by induction.

Conney: We have no detailed information on the profile of metabolites after administration of phenacetin but preliminary data suggest that feeding charcoal-broiled beef decreases the amount of 2-hydroxyphenetidine in the urine.

Garner: In relation to your studies of aflatoxin, does activation occur in the rat through phenobarbitone-type cytochrome *P*-450 and in the human through a *P*-448-type of cytochrome? If the rat, which is extremely sensitive to aflatoxin as a carcinogen, metabolizes the chemical through a completely different cytochrome system to that used by humans, then it would be difficult to assess the usefulness of rat studies for predicting the risks of aflatoxin to man.

Conney: The only way to answer that definitively would be to isolate all of the different cytochrome *P*-450s in pure form from human and rat liver, and to study the metabolism of aflatoxin B_1 by the different purified enzymes.

Garner: But since carcinogen metabolism obviously differs between species, how can we assess the relevance of rodent carcinogenicity to man?

Conney: We can study the comparative metabolism of carcinogens by homogenates and microsomes of rat and human liver, to determine if human liver metabolizes the carcinogen in the same way as rat liver does. Liver from

both rats and humans metabolizes aflatoxin B_1 to mutagens and to DNA-bound adducts.

Gillette: You suggested, Dr Conney, that we should purify the enzyme system in order to separate the activity of different components of that system. I don't think that is necessary because what we need to know is the fraction of the dose that is converted to the reactive metabolite (or to a series of reactive metabolites) rather than what contribution each cytochrome makes to the formation of those particular metabolites.

Garner: Would you suggest the use of a whole cell system for this?

Gillette: Yes.

Connors: And would you need to measure binding to specific molecules?

Gillette: The problem that arises when there are so many chemically reactive metabolites is the identification of which metabolite and which nucleophile are the most important. If we knew what the target really was, then measurement of the binding to the target would be important. The trouble is we seldom know this.

Higginson: Recently Reitz et al (1979) stated that when they examined the incidence of angiosarcoma due to vinyl chloride in a group of men, and provided that they took into account the differences in metabolic activation between rat and human liver, the number of cases observed corresponded to the estimates made from *in vitro* models. This relationship had not been found in estimates made by other workers.

Selikoff: But we must take into account the fact that until the 1950s and 1960s when the industry began to expand, there were few people employed in it (Selikoff & Hammond 1975). Most workers in vinyl chloride polymerization factories have therefore been exposed to the chemicals for less than 15–20 years, and many for even less time, which may not be long enough to provide conclusive information on incidence of carcinogenesis. It is well known that most cancer appears more than 20 years after the onset of exposure to carcinogens and often 30 or 40 years later.

J. W. Bridges: There is also the point that man may not respond quite like *Salmonella* in the Ames test!

McLean: Do we have any idea if the vinyl chloride effect is like that of cigarette smoking, in which cessation of exposure greatly reduces the risk? (Doll 1978).

Selikoff: The vinyl chloride industry reduced exposure of its workers to the chemical only within the last five years, so a comparison with cigarette smoking should be possible soon. In our studies the concentrations of vinyl chloride to which workers had previously been exposed were not known, but the workers we questioned could detect the smell of the chemical, which sug-

gests that the concentrations must have reached at least 500 p.p.m. This concentration is not enough to produce cerebral symptoms. Some of them, however, must have been exposed to around 3000 p.p.m. because they experienced unconsciousness during their working lifetimes.

Connors: How many years were they exposed to those high doses?

Selikoff: The first plant to produce vinyl chloride in the USA began production in 1938. The industry was able to reduce the concentration to 1 p.p.m. within a year of setting up control standards in 1974. This may sharply reduce the incidence of cancer, but we must wait for the data to confirm that.

J. W. Bridges: Did you record whether the people you studied were taking sleeping tablets or other inducing drugs?

Selikoff: Very few of them were. We intend to examine this in more detail, and to determine also if the results are correlated with alcohol consumption.

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DAVID EVERED and GERALYN LAWRENSON

Influence of foreign compounds on formation and disposition of reactive metabolites

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Abstract Many toxic compounds are unreactive and need biotransformation in order to exert their toxic effects. Several enzymes control the formation or disposition of reactive metabolites. Especially well studied is the group of enzymes responsible for the control of reactive epoxides. Such epoxides may bind spontaneously to DNA, RNA and protein. These alterations of critical cellular macromolecules may disturb the normal biochemistry of the cell and lead to cytotoxic, allergenic, mutagenic and carcinogenic effects. Whether these effects will be manifested depends on the chemical reactivity as well as on other properties (geometry, lipophilicity) of the epoxide in question. Enzymes controlling the concentration of epoxides are another important contributing factor. Several microsomal monooxygenases exist. Some monooxygenases preferentially attack large substrates at single sites, specific for each enzyme. Some of these steps produce reactive metabolites; others are detoxification pathways. Enzymes that metabolize the epoxides represent a further determining factor. These enzymes include epoxide hydrolase (EC 3.3.2.3) and glutathione transferases (EC 2.5.1.18), which do not play a purely inactivating role but can, in some cases, act also as coactivating enzymes.

Some of these enzymes have been shown to be influenced by foreign compounds. Acute effects by activation and inhibition of the enzymes as well as long-term effects by induction and repression have been observed. Since different foreign compounds differentially influence various enzymes, they can produce changes not only in overall metabolic activity but also changes in metabolite pattern and in selective toxicities.

The major task of the mammalian enzymes that metabolize foreign, often lipophilic, compounds is to convert them into excretable hydrophilic metabolites. Since these enzymes have to handle compounds with different chemical structures, a very flexible metabolic system is needed. Thus, a set of monooxygenases has evolved which possesses broad and overlapping substrate selectivities. The chemical reaction that this system catalyses will

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have to be of a very general nature applicable to the great diversity of chemical structures of foreign compounds. In fact the system generates a reactive oxygen species near a lipophilic region of the enzymes, where the substrates are bound. A disadvantage of this flexibility is that not all reactions catalysed by it produce an ideal metabolite. Thus, inert precursor compounds can be transformed into carcinogens, mutagens and frequently, also, into cytotoxins and allergens by enzymic conversion to electrophilic metabolites (for references see Miller & Miller 1974). The nature of the reactive metabolite(s) produced varies with the structural components of the compound in question. The discussion in this paper is restricted to aromatic and olefinic moieties which occur very widely in foreign compounds and which are possibly the most thoroughly studied structural elements in regard to metabolic activation and inactivation.

ENZYMES THAT CONTROL REACTIVE METABOLITES OF AROMATIC AND OLEFINIC HYDROCARBONS

Olefinic and aromatic structural elements can be transformed by microsomal monooxygenases to epoxides. Due to their electrophilic reactivity the epoxides can bind covalently to nucleophilic sites in DNA, RNA and proteins. Such alterations of critical cellular macromolecules can lead to fundamental disturbances of the normal biochemistry of a cell and can produce cytotoxic, mutagenic or carcinogenic effects. Several further steps of epoxide metabolism compete with the reaction between epoxides and nucleophilic sites in tissue macromolecules: (a) rearrangement to phenols, in the case of arene oxides, and to aldehydes or ketones, in the case of alkene oxides; (b) hydration by the action of epoxide hydrolase (EC 3.3.2.3); and (c) addition of glutathione with or without the aid of glutathione transferase (EC 2.5.1.18) (Oesch 1973, Jerina & Daly 1974, Sims & Grover 1974, Heidelberger 1975, Nebert et al 1975, Yang et al 1978). All these enzymes are subject to modulation by foreign compounds.

INHIBITION

The enzymes that control reactive metabolites of foreign compounds possess broad and overlapping specificities. Many foreign compounds will therefore influence the metabolism of other foreign compounds by competition for the same enzyme(s), i.e. by mutual competitive inhibition of the two substrates. In some cases, competitive inhibition is also observed with compounds that are not substrates but which still bind to the catalytically active

site of the enzyme(s) in question. Many monooxygenase inhibitors and many drug–drug interactions of this type are known (for references see Gillette et al 1972, Netter 1973).

Several distinct monooxygenases exist. Despite the broadness of their substrate specificities, the individual forms possess distinct substrate preferences (Lu et al 1973). Accordingly, monooxygenase inhibitors exist which possess marked preferences for certain monooxygenase forms: metyrapone preferentially inhibits those monooxygenase forms that are induced by phenobarbitone (Goujon et al 1972); α-naphthoflavone specifically inhibits those induced by 3-methylcholanthrene (Wiebel et al 1971); and tetrahydrofuran preferentially inhibits those induced by ethanol (Ullrich et al 1975). The various monooxygenase forms preferentially attack specific sites when they oxidize large molecules (Holder et al 1974, Rasmussen & Wang 1974, Wiebel et al 1975) and thereby produce different patterns of metabolites, which also differ in biological activities. Specific inhibitors of these various monooxygenase forms therefore will change these metabolite patterns and the corresponding toxicities. On the other hand, there are monooxygenase inhibitors that potently inhibit all monooxygenase forms investigated (Lesca et al 1978). Foreign compounds belonging to this class of inhibitor are expected to change the relative accumulation of reactive metabolites only by altering the overall rate of their formation and without altering the pattern of metabolites.

The microsomal epoxide hydrolase is also inhibited by a large variety of foreign compounds. More than 90 epoxides of widely varying structures were tested with epoxide hydrolase partially purified from the liver microsomal fraction of guinea pigs (Oesch et al 1971), rats and humans (Oesch 1974), with styrene oxide as substrate. Monosubstituted oxiranes with small substituents such as methyl, ethyl, or vinyl did not significantly inhibit the hydration of styrene oxide, but those with larger and lipophilic substituents, such as *tert*-butyl, *n*-hexyl, phenyl or benzyl, did (Table 1). This inhibition by the latter group of compounds probably reflects their affinity to lipophilic binding sites near the catalytically active site of the enzyme (Oesch et al 1971). Conversely, the presence of hydrophilic groups in the neighbourhood of an oxirane ring seems to prevent its access to the catalytically active site of the enzyme. Whilst 4-vinyl-1-cyclohexene dioxide potently inhibited hydration of safrole epoxide by microsomal epoxide hydrolase from rabbit liver, the corresponding dihydroxylated metabolites 1,2-dihydroxy-4-vinylcyclohexane oxide and 4-(1′,2′-dihydroxyethyl)-1-cyclohexene oxide were inactive (Watabe & Sawahata 1976). Similarly, dihydrodiolepoxides derived from polycyclic aromatic hydrocarbons appear to be only poor substrates, if at all, of epoxide

TABLE 1

Monosubstituted oxiranes that inhibit hydration of [^{3}H]styrene oxide[a] by epoxide hydrolase

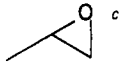
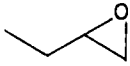
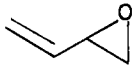
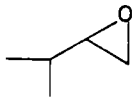
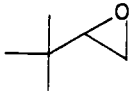
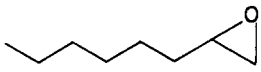
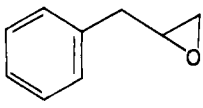
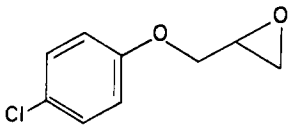
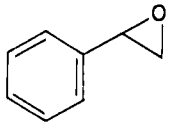
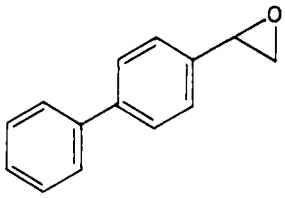
Structure of inhibitor	*% Inhibition[b] of epoxide hydrolase purified from liver microsomes of*		
	Humans	*Guinea pig*	*Rat*
 [c]	ns	ns	ns
	ns	ns	ns
	ns	ns	ns
	12 ± 1.2	9 ± 2.3	17 ± 2.1
	37 ± 5.2	39 ± 2.1	43 ± 6.0
	59 ± 4.4	50 ± 3.1	82 ± 3.2
	56 ± 2.3	28 ± 1.3	40 ± 2.1
	37 ± 5.4	38 ± 6.2	28 ± 2.7
	51 ± 2.1	49 ± 1.5	52 ± 2.2

TABLE 1, *continued*

Structure of inhibitor	*% Inhibition[b] of epoxide hydrolase purified from liver microsomes of*		
	Humans	*Guinea pig*	*Rat*
	68 ± 4.3	74 ± 9.2	79 ± 2.3

[a] Substrate ([^{3}H]styrene oxide) and inhibitor concentrations 2 mM. The inhibitors were added in 20 μl acetonitrile at zero time with no preincubation. The same amount of acetonitrile was added to the controls. Fifty μl of purified epoxide hydrolase preparation were used per assay.

[b] Values represent means ± S.E.M. of triplicate incubations at the two protein concentrations. Significant effects, $P<0.001$ (ns = not significant).

[c] Incubation in sealed tubes at 22 °C.

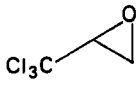
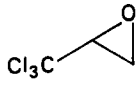
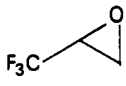
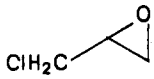
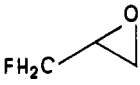
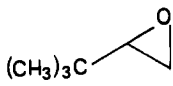
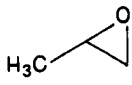
From Oesch (1974), reprinted by permission of The Biochemical Society, London.

hydrolase (Wood et al 1976, Bentley et al 1977).

The potent epoxide hydrolase inhibitor, 1,1,1-trichloropropene oxide (Oesch et al 1971) also belongs to the group of monosubstituted oxiranes. This compound, at one fifth the concentration of substrate, completely inhibited the hydration of styrene oxide. Both the bulk and the strongly electron-withdrawing properties of the trichloromethyl group appear to be essential for its unusual potency in inhibition of epoxide hydrolase (Oesch et al 1971). If the trichloromethyl substituent was replaced either by the strongly electron-withdrawing but less bulky trifluoromethyl group, or by the bulky but not electron-withdrawing *tert*-butyl group, the potency was drastically reduced (Table 2). Monochloromethyl-, monofluoromethyl- and methyl-substituted oxiranes were inactive, or almost inactive (Table 2). The oxirane ring was also essential for inhibitory activity. A variety of analogous compounds with a trichloromethyl but without an adjacent oxirane group did not significantly inhibit the hydration of styrene oxide (Oesch et al 1973). The compounds tested included 1,1,1-trichloroacetone, 1,1,1-trichloro-2-hydroxypropane, hexachloroacetone, chloral, trichloroacetonitrile and 1,1,1-trichloroethane. The fact that 1,1,1-trichloropropene oxide reacts effectively with glutathione and will, therefore, after administration to animals, reduce their glutathione levels (Oesch & Daly 1972) should also be mentioned.

TABLE 2

Effect of electronegativity and bulk of substituents on the potency of propene oxides as inhibitors of epoxide hydrolase[a]

Inhibitor		*% Inhibition[b] of epoxide hydrolase purified from liver microsomes of*		
Structure	*Concentration*	*Humans*	*Guinea pig*	*Rat*
	2 mM	99 ± 0.9	99 ± 0.6	98 ± 1.4
	0.4 mM	95 ± 1.2	97 ± 1.5	90 ± 2.1
	2 mM[c]	30 ± 5.4	60 ± 5.1	32 ± 4.8
	2 mM	14 ± 2.3	14 ± 1.9	24 ± 3.7
	2 mM	ns	ns	ns
	2 mM	37 ± 5.2	39 ± 2.1	43 ± 6.0
	2 mM[c]	ns	ns	ns

[a] Substrate ([^{3}H]styrene oxide) concentration 2 mM. Incubation conditions see Table 1.

[b] Significant effects, $P<0.001$ (ns = not significant). Values represented as in Table 1.

[c] Incubation in sealed tubes at 22 °C.

From Oesch (1974), reprinted by permission of The Biochemical Society, London.

Introduction of more than one substituent directly at the oxirane ring proved to have interesting effects that depended on the stereochemistry around the oxirane ring. Such polysubstituted oxiranes were again tested with epoxide hydrolase that was partially purified from the liver of guinea pigs (Oesch et al 1971), rats and humans (Oesch 1974), and with styrene oxide as substrate.

TABLE 3

Effect of the substitution pattern of the oxirane ring on the inhibitory potency of various styrene oxides on hydration of [^{3}H]styrene oxide[a]

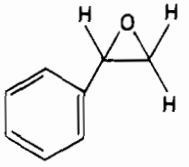
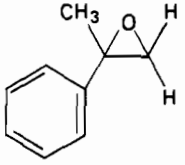
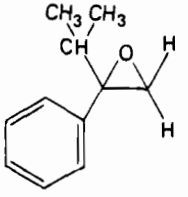
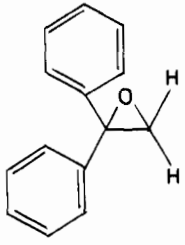
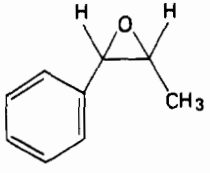
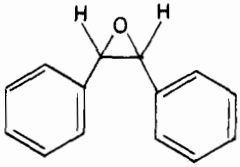
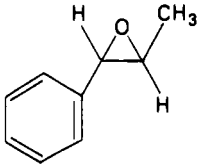
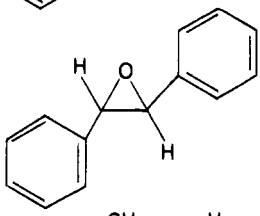
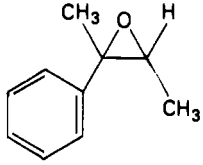
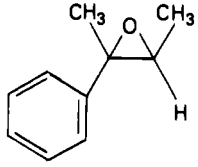
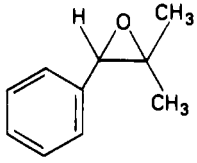
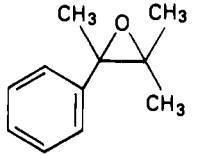
Inhibitor	*% Inhibition[b] of epoxide hydrolase purified from liver microsomes of*		
	Humans	*Guinea pig*	*Rat*
	51 ± 2.1	49 ± 1.5	52 ± 2.2
	32 ± 4.1	30 ± 3.1	40 ± 4.3
	24 ± 3.8	22 ± 3.4	29 ± 5.0
	19 ± 2.7	18 ± 2.1	16 ± 3.2
	46 ± 3.0	74 ± 6.1	73 ± 4.2
	21 ± 8.1	16 ± 3.2	14 ± 2.3

table continued p. 176

TABLE 3, *continued*

Effect of the substitution pattern of the oxirane ring on the inhibitory potency of various styrene oxides on hydration of [^{3}H]styrene oxide[a]

Inhibitor	*% Inhibition[b] of epoxide hydrolase purified from liver microsomes of*		
	Humans	*Guinea pig*	*Rat*
	ns	ns	ns
	ns	ns	ns
	ns	ns	ns
	ns	ns	ns
	ns	ns	ns
	ns	ns	ns

[a] Substrate ([^{3}H]styrene oxide) and inhibitor concentration 2mM. Incubation conditions see Table 1.

[b] Significant effects with $P<0.001$ (ns = not significant). Values represented as in Table 1. From Oesch (1974), reprinted by permission of The Biochemical Society, London.

1,1-Disubstituted oxiranes were less potent inhibitors (Table 3) than their monosubstituted counterparts (Table 1). Also, the potency decreased with increasing bulk of the additional substituent, unless the first substituent did not have sufficient size for inhibitory activity. Thus, 1,1-diethyl oxirane is a weak but active inhibitor whereas the monoethyl analogue had no significant activity (Table 1). An interesting dependency on geometrical isomerism was noted for 1,2-disubstituted oxiranes with respect to their activity as epoxide hydrolase inhibitors. If the second substituent was *cis* with respect to the first (of adequate lipophilicity and size, e.g. phenyl) the corresponding derivatives proved to be active epoxide hydrolase inhibitors, but not if the geometry was *trans* (Table 3). This may be caused by steric interference of the *trans*-substituent with access to the catalytically active site of epoxide hydrolase. Tri- and tetrasubstituted oxiranes were inactive or very weakly active as either inhibitors (Table 3) or substrates of epoxide hydrolase in several studies (Maynert et al 1970, Oesch et al 1971, Oesch 1974). No systematic study is available on preferential inhibitors of the various glutathione transferase forms and their structure–activity relationships.

Several compounds affect more than one system. 1,1,1-Trichloropropene oxide potently inhibits epoxide hydrolase at low concentrations, but higher concentrations also inhibit monooxygenase (Yang & Strickhart 1975). Thus, the actual effect of many foreign compounds on reactive metabolites will depend on their concentration.

ACTIVATION

Many compounds are known to activate microsomal monooxygenases (for references see Anders 1973, Cinti 1978). These include commonly used solvents such as acetone, clinically used drugs such as metyrapone, environmental carcinogens such as safrole and benzo[*a*]pyrene, foreign compounds used for therapeutic purposes and endogenous compounds such as corticosteroids. Many of these agents have marked preferences for some monooxygenase forms and a dual effect, inhibition or activation, dependent on the concentration of the agent (Leibman 1969, Wiebel et al 1971).

Also, many compounds are known to activate liver microsomal epoxide hydrolase from guinea pig (Oesch et al 1971), rat (Levin et al 1978) and humans (Oesch 1974). Since intermediate arene and alkene oxides which are formed by the action of microsomal monooxygenases potentially produce disturbance of normal cell function, owing to their alkylating ability, and since epoxide hydrolase transforms the epoxides that are substrates of the enzyme to much less reactive diols (see references in Oesch 1973, Jerina &

Daly 1974 and Sims & Grover 1974), activators of epoxide hydrolase(s) appear to be of special interest. Such activators could serve as tools to provide insight into the mechanism of carcinogenesis, mutagenesis and cytotoxicity produced by aromatic olefinic compounds. The action of each compound would be even more pronounced if the compound were at the same time an inhibitor of microsomal monooxygenases; it would thus counteract the accumulation of intermediate epoxides by inhibiting their formation and facilitating their further metabolism. On the other hand, in the series of angular polycyclic hydrocarbons, dihydrodiol bay-region epoxides have a special reactivity (Jerina et al 1977) and epoxide hydrolase is needed for their biosynthesis. Several monooxygenase inhibitors, namely two imidazole compounds and metyrapone, are activators of human hepatic epoxide hydrolase *in vitro.* Aminogluthetimide which, with respect to monooxygenases, has several properties in common with metyrapone has no effect on epoxide hydrolase. Ethanol, which has been reported to induce monooxygenases, enhanced at high concentrations (2.7 M) the activity of epoxide hydrolase *in vitro,* whereas acetone, which stimulates activity of monooxygenases *in vitro* had no effect on epoxide hydrolase. Changes produced by these compounds in the activity of epoxide hydrolase *in vitro* are qualitatively similar for microsomal epoxide hydrolase purified from the liver of humans, guinea pig or rat (Oesch 1974).

INDUCTION

Many foreign compounds are capable of inducing the very enzymes that metabolize them, and some compounds can act as inducers without being metabolized by the enzymes in question at an observable rate (gratuitous inducers). Sometimes (e.g. Schmassmann & Oesch 1978), but not always, increases in enzyme activities after pretreatment of animals with potential inducers were accompanied by higher levels of enzyme protein and were dependent on active protein synthesis. Therefore, the term induction will be used in this section to denote an increase in enzyme activity after treatment of animals with potential inducers, regardless of the underlying mechanism.

There exist several classes of foreign compounds that induce different monooxygenase forms (see Lu et al 1972 for references). Interestingly, pretreatment of adult male rats with monooxygenase inducers of three distinctly different classes – (a) phenobarbitone and 'related' drugs, (b) the steroid pregnenolone 16α-carbonitrile, and (c) polycyclic aromatic hydrocarbons – leads always to an induction of both monooxygenase and microsomal epoxide hydrolase (Oesch 1976). However, since the onset of the inducibility of some enzymes is known to take place at different stages of fetal development, and

since transplacental inducibility of monooxygenase has been demonstrated (Nebert & Gelboin 1969), an attempt was made to dissociate the induction of monooxygenase and epoxide hydrolase by transplacental treatment with potential inducers.

Treatment of pregnant rats with phenobarbitone led to measurable increases of benzo[*a*]pyrene monooxygenase (EC 1.14.14.1) and microsomal epoxide hydrolase activities in the fetal livers only when massive doses were used (80 mg/kg daily for three days). These marginal increases, just statistically significant at the $P < 0.05$ level, were of similar magnitude and occurred over a similar time course for both monooxygenase and epoxide hydrolase. Transplacental treatment with the steroid pregnenolone 16α-carbonitrile, an inducing agent with properties distinctly different from either the phenobarbitone or the 3-methylcholanthrene group of inducers (Lu et al 1972), also led to an increase in activity of both monooxygenase and epoxide hydrolase in the fetal liver. The increase in monooxygenase activity was very marked, while epoxide hydrolase activity was only slightly (but at the $P < 0.05$ level statistically significantly) increased. A complete dissociation of the induction of benzo[*a*]pyrene monooxygenase and epoxide hydrolase in the fetal liver was achieved by treatment of pregnant rats with two polycyclic aromatic hydrocarbons, 3-methylcholanthrene and benzo[*a*]pyrene. A single dose of 20 mg/kg of 3-methylcholanthrene transplacentally led to marked increases in hepatic monooxygenase activity within 24 h, without influencing epoxide hydrolase activity. The basal levels of both enzymes increased with the fetal development, and so did the levels of the induced monooxygenase. Monooxygenase activity was increased by about 35- or 8-fold, respectively, depending on whether it was based on 3-hydroxybenzo[*a*]pyrene concentration or on determination of the displacement of tritium from any of the positions of the benzo[*a*]pyrene molecule. The activity of epoxide hydrolase, however, was not measurably increased under these conditions, even though the available biological material and the sensitivity and reproducibility of the assay were such that an increase of about 30% would easily have been demonstrable. Similarly, monooxygenase activity in fetal liver was greatly increased at one day after injection of pregnant rats with a single dose of 60 mg/kg of benzo[*a*]pyrene. Observation of enzyme activities for several days showed that the increased levels of monooxygenase were fairly short-lived. Epoxide hydrolase activity was not significantly increased at any time; this indicates that the observed lack of correlation in the response of monooxygenase and epoxide hydrolase to potential transplacental inducers is not due to different time courses of induction but rather to the occurrence and non-occurrence of induction, respectively, of each enzyme (Oesch 1976). Thus,

although several observations had indicated that the biosynthesis of monooxygenase and epoxide hydrolase might be under common control, transplacental treatment with some agents (e.g. polycyclic aromatic hydrocarbons) but not with others (e.g. phenobarbitone) leads to marked increases in benzo[*a*]pyrene monooxygenase activity while epoxide hydrolase activity remains unchanged.

BIOLOGICAL CONSEQUENCES OF CHANGES IN THE CONTROL OF REACTIVE METABOLITES

Recently, we discovered that *trans*-stilbene oxide influences the enzymes that control reactive metabolites derived from aromatic and olefinic compounds in a very interesting manner. It potently induces microsomal epoxide hydrolase (Schmassmann & Oesch 1978) and some monooxygenase forms. This induction substantially shifts the metabolism of benzo[*a*]pyrene (BP) from the bay to the K-region (Bücker et al 1979). The quantity of metabolites oxidized at the benzo ring (7,8-dihydroxy-7,8-dihydro-BP; 9,10-dihydroxy-9,10-dihydro-BP; 9-hydroxy-BP) was greatly reduced, and far more K-region metabolites (4,5-dihydroxy-4,5-dihydro-BP; BP 4,5-oxide) were formed. Compared to results from control microsomes, the ratio between 7,8-dihydroxy-7,8-dihydro-BP and 4,5-dihydroxy-4,5-dihydro-BP was more than 20 times lower with microsomes induced by *trans*-stilbene oxide. As expected from the induction of epoxide hydrolase, a much higher percentage of the metabolically produced benzo[*a*]pyrene 4,5-oxide was converted to the corresponding dihydrodiol by microsomes induced by *trans*-stilbene oxide than by control microsomes.

It was of special interest to investigate how the shift of metabolism which is caused by *trans*-stilbene oxide would affect the mutagenicity of benzo[*a*]pyrene, since this compound can be activated both at the benzo ring and at the 4,5-K-region to highly mutagenic metabolites; activation at the benzo ring mainly produces dihydrodiolepoxides, while that at the K-region produces the 4,5-oxide (Glatt et al 1975, Wood et al 1976, Bentley et al 1977). For this purpose, various doses of benzo[*a*]pyrene were activated by microsomal or postmitochondrial fractions to mutagens that were detected by the reversion of various *his*$^-$ *Salmonella typhimurium* strains. Induction by *trans*-stilbene oxide decreased the mutagenicity of benzo[*a*]pyrene in all instances. Some differences were observed between the two tissue fractions and the different bacterial strains. The greatest reduction of the mutagenic effect (by more than 90%) was obtained when the postmitochondrial fraction and the strain TA 100 were used.

Different *his*$^-$ strains vary in their susceptibility to reversion by different

benzo[*a*]pyrene metabolites (Wood et al 1976, Bentley et al 1977). TA 100 and TA 98 strains are easily reverted by benzo[*a*]pyrene 7,8-dihydrodiol 9,10-oxides and by benzo[*a*]pyrene 4,5-oxide. The strain TA 1537 is less sensitive towards the 7,8-dihydrodiol 9,10-oxides but is highly sensitive to the 4,5-oxide (Bentley et al 1977). The greater decrease in mutagenicity produced by *trans*-stilbene oxide induction in the strains sensitive to 7,8-dihydrodiol 9,10-oxides (TA 100, TA 98) than in a strain that is relatively insensitive towards these dihydrodiolepoxides (TA 1537) suggests that the reduction of the mutagenicity is caused to a significant extent by the decreased oxidation of benzo[*a*]pyrene at the benzo ring. The decreased benzo ring metabolism is accompanied by increased K-region metabolism, leading to the mutagenic benzo[*a*]pyrene 4,5-oxide, but this epoxide is a good substrate of epoxide hydrolase (Wood et al 1976, Bentley et al 1976), which is induced by *trans*-stilbene oxide. Thus, the two effects of *trans*-stilbene oxide – the shift of the site of metabolic oxidation and the induction of epoxide hydrolase – synergistically provide protection against the mutagenic effects of benzo[*a*]pyrene.

These remarkable changes in the pattern of benzo[*a*]pyrene metabolites and the consequent profound changes in mutagenic effects show that modulations by foreign compounds of enzymes that are responsible for the control of reactive metabolites can lead to significant changes in the toxicity of other foreign compounds.

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Discussion

Orrenius: Wouldn't you expect to see a *decrease* in mutagenicity as you *increase* the substrate concentration, since benzo[*a*]pyrene at high concentration may compete with the further activation of the primary metabolites by the cytochrome *P*-450 system?

Oesch: When we use a strain of *Salmonella* that is very sensitive to benzo[*a*]pyrene 7,8-dihydrodiol-9,10-oxides, in principle we would expect a decrease in mutagenicity since inhibition of the second monooxygenase step would occur in the presence of a large excess of the substrate (benzo[*a*]pyrene) but this was not observed under the conditions of our experiments. There was no inhibition of activation of benzo[*a*]pyrene 7,8-dihydrodiol, but our substrate concentrations may not have been large enough.

Orrenius: When we measure DNA-binding we observe an increase in binding at concentrations of benzo[*a*]pyrene up to about 40 μM and at higher concentrations there is a decrease. We interpret this to mean that formation of binding products is inhibited by the high concentration of benzo[*a*]pyrene.

Oesch: The numerical value 96 μM given for the benzo[*a*]pyrene concentration may be misleading because this is a plate incorporation assay. We don't know how much of the benzo[*a*]pyrene goes into the ground agar and how much stays in the top agar; neither do we know the concentration at the enzyme active site. But when we use liver cells, the medium is liquid and it is easier to estimate concentrations of substrate, at least in the medium.

McLean: But if you are using a very lipophilic substrate like benzo[*a*]pyrene, the concentration at the microsome or in the cell will vary with the ratio of volume of medium to volume of cell or to amount of added enzyme. I would expect a high proportion of the benzo[*a*]pyrene to be accumulated in the lipid phase.

Oesch: Yes, it is expected that selective accumulation would occur within the lipid membranes of the microsomes.

McLean: Perhaps Jim Bridges might know what the concentration of benzo[*a*]pyrene would be in the medium, after incubation?

J. W. Bridges: There are very low concentrations (10^{-8}–10^{-9} M) – most of the substrate is in the cells, or on the walls of the apparatus!

Oesch: The concentrations I have given are operational.

Orrenius: Absolutely. In no case do we know the exact concentration at the cellular level.

Gillette: It's possible that inhibition does not occur because the two reactions are catalysed by two different enzymes. The question then arises as to whether a concentration of the polycyclic hydrocarbons is reached that is above the Michaelis constant of the substrate for one of the forms of cytochrome *P*-450 but below the K_m for another form.

Oesch: Well, then, a straight-line relationship between degree of mutagenicity and concentration of premutagen would not be expected. In order to solve this problem, one would need to isolate the individual enzymes to find out their respective K_m values.

Gillette: Ideally yes. But you may be able to obtain reasonable estimates of the Michaelis constants from the non-linear plots. If the Michaelis constants were nearly the same, of course, the plots would appear to be linear. It surprises me that you still obtain linear plots at micromolar concentrations of the substrate, because the concentrations of the cytochrome *P*-450 in the assay are frequently about the same order of magnitude as the apparent Michaelis constant.

Bend: What is the activity of the dihydrodiol dehydrogenase you described, and is it an important enzyme in diol metabolism by the liver?

Oesch: I showed a reduction of mutagenicity of metabolically activated benzo[*a*]pyrene in the situation where epoxide hydrolase (EC 3.3.2.3) doesn't

reduce the mutagenicity substantially. In order to obtain maximal reduction in this situation we need an excess of about 15-fold of pure dihydrodiol dehydrogenase over that which would have been present in a whole homogenate (Glatt et al 1979). What is present in the cell is probably poorly effective for the benzo[*a*]pyrene substrate. We cannot exclude the possibility that 15-fold inductions would occur, as has been observed for monooxygenases, but it is not very likely. However, it *is* likely that the dihydrodiol dehydrogenase is more important for other substrates. We are studying this question at the moment.

Farber: What is the product of this dehydrogenase?

Oesch: A catechol, which is unstable and is usually converted to the quinone.

Bend: Our results (Smith & Bend 1979, Smith et al 1980) from studies of the metabolism of benzo[*a*]pyrene 4,5-oxide, and the benzo[*a*]pyrene 4,5-dihydrodiol formed in perfused livers and lungs, were consistent with the dihydrodiol dehydrogenase being a minor pathway since we did not detect any catechol or quinone products.

J. W. Bridges: Is there any coupling of epoxide hydrolase and cytochrome *P*-450 in the endoplasmic reticulum, or do these enzymes act quite independently so that the reactive epoxides are required to traverse the membrane in order to become hydrated?

Oesch: We originally obtained data which suggested that there was a close relationship between some monooxygenase forms and epoxide hydrolase. Recently, we have made subfractions of the microsomal vesicles by treating them with detergents and separating them according to density. Then we examined whether the populations of vesicles that were rich in epoxide hydrolase were also rich in the components of monooxygenase. They were not. There was a population that was rich in cytochrome b_5, cytochrome b_5 reductase (EC 1.6.2.2) and epoxide hydrolase, and there was another population that was rich in cytochrome *P*-450 and in NADPH cytochrome *c*-reductase (EC 1.6.2.5). This is not conclusive evidence for the *absence* of a close structural and functional relationship between the monooxygenase and epoxide hydrolase, but it is not possible for them to form a stable and tight complex.

J. W. Bridges: So does your data suggest that all the cytochrome *P*-450s have a similar relationship with epoxide hydrolase or are some of them more likely to be associated with it than others?

Oesch: We have excluded the possibility that the bulk of *P*-450 is firmly associated with the bulk of epoxide hydrolase. The evidence would suggest that 3-methylcholanthrene-induced monooxygenase form(s) may exist in complexes with epoxide hydrolase.

Meyer: There is evidence for the presence of cytoplasmic and nuclear epoxide hydrolases. Could you tell us something about their relevance?

Oesch: All the evidence suggests that there is no substantial difference between the epoxide hydrolase in the *nuclear* membrane and that in the microsomes or endoplasmic reticulum (see Mukhtar et al 1979). This is in line with the assumption of a common biogenesis for these membranes. We have produced antibodies against epoxide hydrolase that was isolated from the microsomes, and we labelled it with ferritin. When we looked electron-microscopically at the nuclei fraction, we could see labelling by these antibodies, so the nuclear epoxide hydrolase does exist, and is not a contamination artifact. Its specific activity, however, is about 1/20th of that in the microsomal membrane.

The first information on cytoplasmic epoxide hydrolase came from Gill et al (1972). They showed that this enzyme was active towards a juvenile hormone, a terpenoid epoxide. Later work (Gill et al 1974) again reported activity towards the same group of substrates. However, very recent work by Gill & Hammock (1979) suggests a broader substrate specificity for this enzyme. A major difference between the *cytoplasmic* and *microsomal* epoxide hydrolases is that the substrate that we use routinely, styrene oxide, does not serve as substrate for the cytoplasmic enzyme. In addition *trans*-disubstituted oxides which do not serve as substrates for the microsomal enzymes are active substrates for the cytoplasmic enzymes.

Meyer: Can we assume that epoxides normally dealt with by microsomal enzymes could equally well be dealt with by the cytoplasmic epoxide hydrolase?

Oesch: Yes. Even if the substrate is very lipophilic there is always an equilibrium so that although the substrate is predominantly in the membrane, some of it is also in contact with the cytoplasmic enzymes.

J. W. Bridges: Your argument would be supported by the fact that benzo[*a*]pyrene is metabolized on the endoplasmic reticulum to 3-hydroxybenzo[*a*]pyrene which, although still highly lipophilic, readily interacts with cytoplasmic sulphatransferase(s) to form 3-hydroxybenzo[*a*]pyrene sulphate (Cohen et al 1977).

De Matteis: You described, Professor Oesch, that some inhibitors of the enzyme have to fit on the active site, and are therefore competitive. Can you identify features, either chemical or structural, that make a substrate a *poor* substrate?

Oesch: When we studied inhibition of hydration of styrene oxide by solubilized and purified microsomal epoxide hydrolase, we found that the oxygen atom of the oxirane ring can be replaced by sulphur in a competitive in-

hibitor without loss of potency of the inhibitor. This replacement in a non-competitive inhibitor did produce loss of potency. Oxaziridines, which contain both an oxygen and a nitrogen atom in a three-membered ring, are good inhibitors; aziridines, which have the oxygen atom of the ring replaced by a nitrogen atom, generally are not. Cyclopropanes are also poor inhibitors: the oxygen in the oxirane ring cannot be replaced by a CH_2 group.

An effective inhibitor requires a lipophilic substituent. If a methyl group is introduced at the same carbon atom, there is no difference in inhibitory potency, but if this second group is large, then the potency of inhibition decreases somewhat. Epoxides having the oxirane ring *trans*-disubstituted are poor inhibitors; those that are *cis*-disubstituted are good. These requirements are qualitatively similar for rodents and for humans.

De Matteis: Does the inhibitor therefore have a great affinity for the active centre?

Oesch: Well I wouldn't say *great*. These inhibitors were used at concentrations equimolar to styrene oxide as substrate and they produced 30–90% inhibition.

De Matteis: Why were the inhibitors not hydrolysed?

Oesch: They were hydrolysed. When they were preincubated, inhibition was reduced. But the inhibitors can be quite effectively studied when they are added together with the substrate and if the incubation is done for a short enough time. This is *alternative substrate inhibition.*

Connors: Are the members of this class similar in their chemical reactivity and in the nucleophiles that they alkylate?

Oesch: They are rather similar, because none of them have very pronounced electron-withdrawing groups.

Hunter: As a physician, I wonder how you apply this work to the intact animal. Is there any indirect way of assessing epoxide hydrolase activity? Could you, for example, give a precursor *in vivo* and measure quinones or diols in the urine to see how active the enzyme is in the whole organism?

Oesch: That may be possible. We have only done *in vitro* studies.

Conney: The *in vivo* approach is important, and we are trying to develop a chemical that can be given safely, so that we can assess epoxide hydrolase activity *in vivo.*

Farber: What is needed is a biological endpoint. We are now using our assay (for initiation) in co-operation with Professor Oesch, and these studies should provide a clearer indication of the activity of these substances *in vivo* in relation to carcinogenesis.

Hunter: I know that you have shown, Professor Oesch, that there is more epoxide hydrolase in human liver than in mouse liver. Have you looked at

variations of the enzyme during disease states?

Oesch: We are just doing that. So far we have obtained only about two-fold changes in the mean concentrations of the enzymes in populations with different diseases or under different drug treatments. But the total range of individual activities was 17-fold, which is much larger than the range in an apparently healthy population. In addition, some contraceptive treatments markedly increase epoxide hydrolase activity, while many other drugs have no effect (H.R. Glatt, K. Vogel, P. Bentley and F. Oesch, unpublished observations).

Davies: We have examined biopsies of human liver that had a preserved (i.e. normal) hepatic architecture. We found a five-fold variation in epoxide hydrolase activity in these apparently normal livers, which were from males. It is important to take this range of activity into account before discussing changes observed in disease states.

Conney: It's worth pointing out that bay-region diolepoxides (ultimate carcinogens) of polycyclic aromatic hydrocarbons are very poor substrates for epoxide hydrolase. On the other hand, K-region arene oxides of polycyclic aromatic hydrocarbons, which have little or no carcinogenic activity, are good substrates for this enzyme. If the hydroxy groups of bay-region diolepoxides are removed, the resulting tetrahydroepoxides are also good substrates for epoxide hydrolase.

Oesch: Exactly. This is similar to the structure–activity relationship that I discussed and which showed that a lipophilic substituent near the oxirane ring is required before competition with substrate can occur.

Farber: Would the purified and microsomal epoxide hydrolase have the same substrate specificity as the cytosolic enzyme?

Conney: Yes.

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Environmental Chemicals, Enzyme Function and Human Disease
DAVID EVERED and GERALYN LAWRENSON

Pharmacokinetic factors governing the steady-state concentrations of foreign chemicals and their metabolites

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Abstract It is well known that various environmental chemicals can either increase or decrease the activities of enzymes that metabolize foreign compounds and their metabolites. In addition, changes in the activities of these enzymes can alter the concentration of the foreign compounds and metabolites in tissues. However, the theoretical basis by which we can quantitatively relate a change in the activity of a given enzyme to a change in the concentrations of these compounds remains in its infancy. In some instances, the change in concentration of a foreign compound or metabolite will be inversely proportional to the change in the enzyme activity. But in other instances, marked changes in enzyme activity may result in only trivial changes in the concentration of the compounds and their metabolites at sites of action. Pharmacokinetics provide a means for testing the validity of our concepts of how the concentrations of foreign compounds and their metabolites depend on enzyme activities, rates of excretion by the kidneys and lung, reversible binding to tissue components, rates of diffusion across cellular membranes, blood flow rates and the sites of administration. The present paper presents mathematical equations that show the effects of the route of administration on the steady-state concentrations of foreign compounds and their metabolites in tissues when the substances are metabolized by the liver and excreted by the kidney. These equations are especially relevant to studies of chemically reactive metabolites.

Many of the toxicological actions of drugs *in vivo* may be caused partly by drug metabolites, and the effect of these metabolites may either closely resemble or differ markedly from that of the parent compound. Occasionally, the parent compound is pharmacologically and toxicologically inert and its effects *in vivo* are mediated solely through its active metabolites.

Pharmacologists use several strategies to elucidate whether a response to a drug is mediated by metabolites. When a response depends on the reversible binding of the drug or metabolite to receptor sites, and when it appears soon after administration of the drug, the intensity and duration of response frequently depend on the drug concentration in the blood. The relationship bet-

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ween the duration of action of a drug and its concentration in the blood, however, will be altered when the response is caused partly by a metabolite and also when the rate constant of elimination of the metabolite is greater than that of the parent drug. Under these conditions, the half-life of the metabolite during the terminal phase of metabolism will appear to be the same as that of the drug and thus the duration of action may appear to be approximately related to the concentration of the parent drug, even when the action is caused solely by the metabolite. A better way to elucidate the toxicological effects of drug metabolites is to isolate and identify the metabolites, to synthesize them, and to test their activities. Even this strategy, however, frequently fails when the toxic metabolite is chemically reactive and cannot easily be isolated or synthesized; even if it could be synthesized, and were administered by the usual routes, it might not reach the site of action that it reaches after administration of the parent compound. Clearly, a different strategy is needed to distinguish the chemically reactive metabolites that cause the toxic effects from those that are innocuous.

Several years ago my colleagues and I devised a strategy to determine whether a given type of toxicity is caused by a chemically reactive metabolite. In developing the approach (Gillette 1974 a, b, c), we considered that reactive metabolites could cause toxic reactions, such as cellular necrosis, by several different mechanisms (Fig. 1).

Conceivably, the target of a reactive metabolite could be an intracellular enzyme (or its substrates) that is (are) required for the function of cells: it could

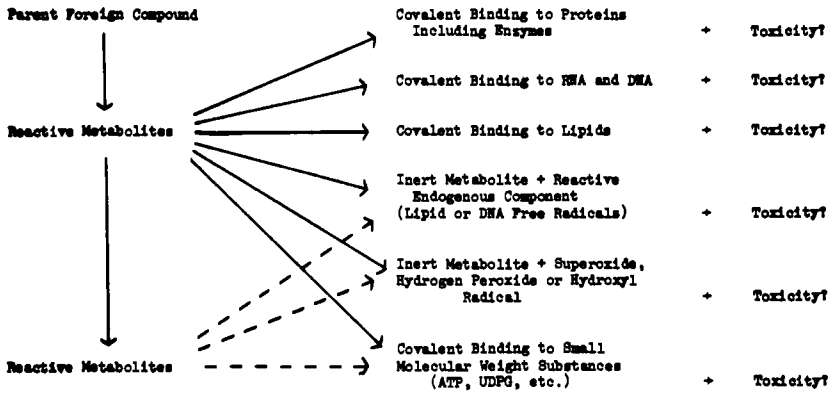

FIG. 1. Possible mechanisms of toxicity caused by chemically reactive metabolites of a foreign parent compound.

be a phospholipid in cellular membranes, which control the compartmentalization of intracellular componcnts; it could be part of the protein synthesis machinery required for the normal replacement of intracellular enzymes, or it could be the DNA that is required for cellular replication. It also occurred to us that the toxicity might not be manifest unless several of these targets were impaired simultaneously.

Although a reactive metabolite might alter the target substances by combining covalently with them, it is also plausible that the toxic response might depend on mechanisms in which the reactive metabolite is not covalently bound to the target substance. The reactive metabolite might react with a lipid or with DNA and thereby cause the toxicity; for example, the reaction of the free radical, trichloromethyl, with lipid to form chloroform and free-radical lipids has been suggested as an initial step in the liver necrosis caused by carbon tetrachloride (Recknegal 1967). A reactive metabolite can react with oxygen to form superoxide, hydrogen peroxide or hydroxyl free radicals which in turn cause the toxicity (Bus et al 1974).

When we developed our approach, we were profoundly influenced by the many studies that indicated relationships between carcinogenesis and the formation of the reactive metabolites of foreign compounds (Miller & Miller 1966, Miller 1970, Heidelberger & Moldenhauer 1956, Magee & Barnes 1967). It was evident that most reactive metabolites do not react with a single kind of macromolecule, but instead react with many tissue components including proteins, lipid, nucleic acids, glycogen and micromolecular substances, such as ATP, NADPH, NADH and UDPG. In addition, the rates of reaction of a given metabolite with the various nucleophiles in cells depend on several factors. For example, the rates of reaction with thiol groups differ markedly from those with amino groups of proteins or with oxygen or nitrogen groups of the nucleic acids. Hence, a multiplicity of different reaction products may occur. On the other hand, stable reactive metabolites may combine reversibly with certain sites of proteins before the complex rearranges to form covalently bound material. In this case, the reactive metabolite in low concentrations may combine with relatively few kinds of macromolecules. Indeed, the inhibition of pseudocholinesterase (EC 3.1.1.8) by phosphorus insecticides is an example of this kind of mechanism. Therefore, a reactive metabolite may combine preferentially with certain cellular proteins either because the proteins contain an unusually large number of nucleophilic groups on their surfaces or because they have a high affinity for the reactive metabolite.

Since different reactive metabolites react with various tissue nucleophiles at relatively different rates, it seemed likely to us that measurements of the total covalent binding of reactive metabolites to proteins would not provide a

reliable estimate of the relative toxicity of the metabolites. Indeed it seemed entirely possible that a reactive metabolite could react extensively with protein and still be non-toxic. It also seemed possible that a toxic agent might be converted to a reactive metabolite that combined with protein, even though the toxicity is caused directly by the parent substance.

It occurred to us, however, that we might be able to determine whether toxicity was caused by reactive metabolites if we studied the effects of various inducers and inhibitors on the metabolism of the toxic agent. According to our view, the covalent binding of the reactive metabolite to protein would be approximately proportional to the area under the curve of the cellular concentration of the reactive metabolite plotted against time. The amount of covalent binding would therefore be an indirect measure of the amount of reactive metabolite in contact with the target component of the cells. Thus, any treatment that changed the area under the curve would cause parallel changes both in the covalent binding of the metabolite to protein and in the severity of the toxicity, when the toxicity is caused by the reactive metabolite or by another metabolite derived from it. Moreover, the correlation should have occurred even when the toxicity did not result from covalent binding to any intracellular component.

This approach may be expressed mathematically. The amount of metabolite that combines with a target substance may be expressed as the product of the dose, the proportion of the dose that is converted to a reactive metabolite (Ratio A) and the proportion of the reactive metabolite that becomes covalently bound to the target substance (Ratio B).

$$\textit{metabolite-target complex} = \textit{dose} \cdot A \cdot B \tag{1}$$

Similarly the amount of metabolite that ultimately binds covalently to protein in the target tissue may be expressed as a fraction of the dose of the toxic agent that binds to protein. This fraction may be expressed as the product of Ratio A and the proportion of the reactive metabolite that binds covalently to protein (Ratio B'). Thus:

$$\textit{metabolite-protein complex} = \textit{dose} \cdot A \cdot B' \tag{2}$$

It follows, therefore, that any treatment that changes Ratio A, or both Ratio B and Ratio B', without substantially changing the relative rates of reaction of the metabolites with protein and with the target substance will result in parallel changes in the severity of toxicity and in the covalent binding to protein, even when the target substance itself is not a protein. Thus, alterations in

the toxicity caused by various treatments that are known to alter the metabolism of the toxic agent or the inactivation of its metabolites can be useful in determining the type of toxicity that is mediated by a reactive metabolite.

The concept may also be extended to cover situations in which the metabolite that reacts with protein is not toxic but instead is converted to another metabolite that causes toxicity. In this case, any treatment that changes the fraction of the dose that is converted to the reactive metabolite (ratio A) will cause parallel changes in both the covalent binding of the metabolite to protein and the severity of the toxicity. However, a treatment that preferentially alters the conversion of the reactive metabolite to the toxic metabolite will cause inversely related changes in both the magnitude of the covalent binding and the severity of the toxicity.

In addition to various treatments that alter enzyme activities, a change in the dose of the toxic agent may also change ratios A, B and B'. At low doses, both the rates of conversion of the parent compound to its various metabolites (including the reactive metabolite) and the rates of disposition of the reactive metabolite will be first order. Under these conditions the values of the ratios A, B and B' will be independent of the dose. But as the dose is increased, the maximum concentration of the parent compound reached in the organs of elimination may be sufficient to saturate one or more of the enzymes that catalyse its metabolism, and thereby ratio A may be changed. If the enzyme that has the lowest Michaelis constant (K_m) catalyses the formation of the reactive metabolite, ratio A will be decreased. But if the enzyme having the lowest K_m catalyses the formation of an innocuous metabolite, ratio A will be increased. Moreover, increases in the dose of the parent compound will lead to increases in the amount of reactive metabolite formed, and in turn may lead to the depletion of intracellular nucleophiles, such as glutathione; thus, an increase in the dose may lead to an increase in ratios B and B'.

Ratio A in the target tissue may also be changed by an alteration in the activity of an enzyme in a tissue other than the initial target tissue and also in some cases by a change in the route of administration. An understanding of the kinetics of these effects is especially important, because they can account in part for a shift of the toxicity from one organ to another (see Boyd 1980).

In order to understand the conditions under which (i) changes in the activity of an enzyme in a non-target tissue and (ii) changes in the route of administration of the toxic agent might alter the severity of the toxicity, it is useful to consider several pharmacokinetic models, in which all clearances of the toxic agent and its metabolites remain constant from the time that the toxic agent is administered to an animal until all of the toxic agent and its metabolites are

eliminated from the body. In such models it is also assumed that the toxic agent and its metabolites are not metabolized in the blood. Under these conditions, several basic equations may be written to illustrate the relationships between clearance and the area under the curve of the arterial concentration of the toxic agent plotted against time when the agent is administered directly into the left ventricle of the heart. (Rowland et al 1973, Gibaldi & Perrier 1975).

When a single dose of the parent toxic agent (P) is injected instantaneously into the left ventricle of the heart, the equation for the area under the curve for the concentration of P in arterial blood ($AUC_{(arterial)P}$) may be described as follows:

$$AUC_{(arterial)P} = \frac{dose_{P(left\ ventricle)}}{Cl_{t,P}} \tag{3}$$

where $Cl_{t,P}$ is the total body clearance of the parent toxic agent.

When a given dose of toxic agent is repeatedly injected into the left ventricle of the heart at constant time intervals (τ) until a *steady state (ss)* is reached, the areas under the curves of the toxic agent and its metabolites per time interval will remain constant. The equation for the area under the curve for the concentration of P in arterial blood at steady state then will be:

$$AUC_{ss(arterial)P} = \frac{dose\ per\ \tau_{P(left\ ventricle)}}{Cl_{t,P}} \tag{4}$$

The mean concentration of the toxic agent in arterial blood, $\hat{C}_{ss(arterial)P}$, will be

$$\hat{C}_{ss(arterial)P} = \frac{AUC_{ss(arterial)P}}{\tau} = \frac{dose\ per\ \tau_{P(left\ ventricle)}}{\tau\ Cl_{t,P}} \tag{5}$$

When the toxic agent is constantly infused, at a constant rate equal to $k_{0\ P(left\ ventricle)}$, into the left ventricle of the heart until a steady state is reached, after which time the concentrations of the toxic agent and its metabolites remain constant, the equation for the steady-state concentration of the toxic agent in arterial blood, $C_{ss(arterial)P}$ will be:

$$C_{ss(arterial)P} = \frac{k_{0P(left\ ventricle)}}{Cl_{t,P}} \tag{6}$$

The total body clearances in each of these equations is the same and thus the following relationships are equivalent (Gillette & Pang 1977):

$$Cl_{t,P} = \frac{k_{0P(left\ ventricle)}}{C_{ss(arterial)P}} = \frac{dose\ per\ \tau_{P(left\ ventricle)}}{AUC_{ss(arterial)P}} = \frac{dose_{P(left\ ventricle)}}{AUC_{(arterial)P}} \quad (7)$$

Since the total body clearance, as estimated during constant infusion, is the same as the clearance measured by the area under the curve after a single dose, it follows that the factors that will affect the clearance will also affect both $C_{ss(arterial)P}$ and $AUC_{ss(arterial)P}$. In order to distinguish the factors that affect clearance from those that don't, however, it is sometimes easier to visualize how a given factor will affect a constant infusion system. In a multicompartment system, at the steady state achieved after constant infusion, there will be no net exchange of the drug between the blood and the non-elimination tissues (tissues that do not contain mechanisms for the elimination of the drug from the body); thus it may be seen why $AUC_{(arterial)P}$ is independent of the number and the apparent volumes of the non-elimination compartments. In other words, the relationships shown in equation 7 are valid, regardless of the number of non-elimination compartments present in the pharmacokinetic model. It also follows from these considerations that the area under the curve for the concentration of toxic agent in venous blood, draining a tissue that does not contain mechanisms for the elimination of the toxic agent, will be the same as the area under the curve for toxic agent in arterial blood, even though the concentrations in the venous and arterial blood at any given time are usually not identical.

When a toxic agent is cleared from the body by only one organ, the kinetic equations relating blood flow and clearance are conceptually rather simple. According to the classical view of clearance (Rowland et al 1973):

$$Cl = Q(C_{in} - C_{out})/C_{in} = Q(1 - F) = Q \cdot E \quad (8)$$

in which Q is the blood flow rate through the organ; C_{in} and C_{out} are the total concentrations of toxic agent in blood entering and leaving the organ respectively; F is the available fraction (C_{out}/C_{in}) and E is the extraction ratio.

In order to estimate clearance by the AUC method, one should measure the concentration of the toxic agent in blood entering the organ rather than in that leaving it. However, the fact that this is not always done becomes apparent when the constantly infused system is considered (Fig. 2). When the toxic agent is constantly infused directly into the left ventricle of the heart and is eliminated by only one organ in the body, the steady-state concentration of

the toxic agent in the arterial blood is the same as that in the blood entering the organ of elimination, regardless of what that organ might be. However, when the toxic agent is administered by constant infusion intravenously (i.v.) or by inhalation, its steady-state concentration in arterial blood represents the concentration in blood leaving the lung, but entering the other organs of the body. When the toxic agent is administered orally (p.o.), and is completely absorbed unchanged in the intestine, the steady-state concentration of the toxic agent in arterial blood is the concentration leaving the intestinal mucosa, liver or lung and entering the other organs of the body. As indicated above, however, the ratio of the steady-state concentrations of the toxic agent in blood leaving or entering the organ of elimination is the available fraction, F. Thus the area under the curve for the toxic agent in arterial blood depends on the route of administration, the location of the organ of elimination and the extraction ratio of the organ. When the toxic agent is eliminated from the body only by the liver, the following equations apply:

$$\mathrm{AUC}_{(arterial)\mathrm{P\ i.v.}} = \frac{dose_{\mathrm{P}(intravenous\ or\ inhalation)}}{Cl_{H,\mathrm{P}}} \tag{9}$$

$$\mathrm{AUC}_{(arterial)\mathrm{P\ p.o.}} = \frac{F_{H,\mathrm{P}} \cdot dose_{\mathrm{P}(\mathrm{oral})}}{Cl_{H,\mathrm{P}}} \tag{10}$$

in which $Cl_{H,\mathrm{P}}$ is the hepatic clearance of the toxic agent and $F_{H,\mathrm{P}}$ is the fraction of the toxic agent escaping elimination by the liver. It should be emphasized that in these equations it is assumed that the toxic agent is completely absorbed unchanged from the intestine after oral administration. When the value of F_H is significantly less than unity, the toxic agent is said to undergo a *first-pass effect* (Harris & Riegelman 1969) or a *significant presystemic clearance* (Routledge & Shand 1979).

Fig. 2 reveals, however, that the route of administration of the toxic agent does not change its steady-state concentration in the blood entering the organ of elimination, provided that there is only one such organ. Indeed, this has to be true for a first-order system, because in a *steady state,* by definition, the rate of elimination must equal the rate of infusion. Thus:

$$(rate\ of\ \mathrm{P}\ metabolism)_{ss} = \frac{k_{0\mathrm{P}} \cdot Cl_{H,\mathrm{P}}}{Cl_{H,\mathrm{P}}} = k_{0\mathrm{P}} \tag{11}$$

We may arrive at similar conclusions with single doses of the toxic agent.

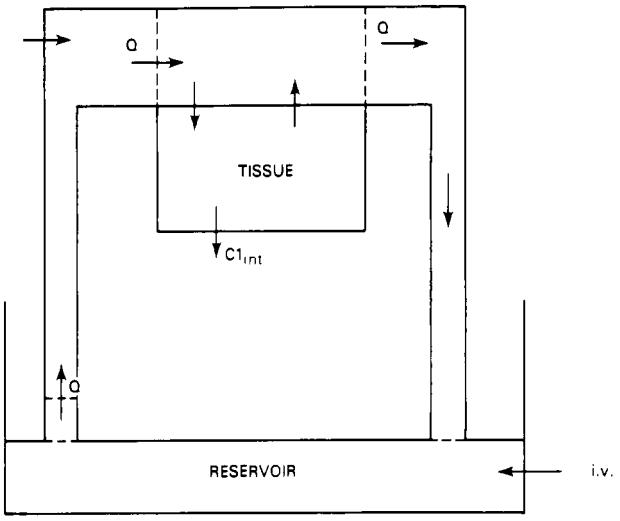

FIG. 2. Model illustrating the perfusion of the liver. The model also may be used to illustrate the perfusion of any other organ of elimination. Symbols: Q, blood flow rate through the organ; Cl_{int}, intrinsic clearance; i.v., intravenous administration.

After intravenous administration, all of the dose ultimately leaves the body. By rearrangement of equation 9, we obtain:

$$(\textit{amount of P metabolized})_{\text{i.v.}} = \textit{dose}_{\text{P i.v.}} = \text{AUC}_{\text{P i.v.}} \cdot Cl_{H,\text{P}} \qquad (12)$$

Similarly, after the oral administration of the toxic agent, the amount may be visualized as occurring in two parts: (1) the amount of the toxic agent that is metabolized before it reaches the systemic circulation ($\textit{dose}_{\text{P,p.o.}} \cdot E_{H,\text{P}}$) and the amount of the toxic agent that is metabolized after it reaches the systemic circulation ($\textit{dose}_{\text{P,p.o.}} \cdot F_{H,\text{P}}$). The sum of these amounts is the dose. We may write these two amounts as:

$$(\textit{amount of P metabolized})_{\text{p.o.}} = \textit{dose}_{\text{P, p.o.}}(E_{H,\text{P}} + F_{H,\text{P}}) \qquad (13)$$

By solving equation 10 for $F_{H,\text{P}} \cdot \textit{dose}_{\text{P,p.o.}}$, and substituting the result into equation 13, we obtain:

$$(\textit{amount of P metabolized})_{\text{p.o.}} = (\textit{dose}_{\text{P, p.o.}} \cdot E_{H,\text{P}}) + (\text{AUC}_{\text{P, p.o.}} \cdot Cl_{H,\text{P}}) \qquad (14)$$

Both the rate of formation of a given metabolite from the toxic agent, after

continuous infusion of the toxic agent, and the amount of the metabolite (M) formed after the administration of a single dose of the toxic agent, may be expressed as a fraction, $f_{(P-M)}$, of the total metabolism of the toxic agent. Thus, regardless of the route of administration of the toxic agent,

$$(\textit{rate of } \mathrm{M} \textit{ formation})_{ss} = k_{0P} \cdot f_{(P-M)} \tag{15}$$

and

$$(\textit{amount of } \mathrm{M} \textit{ formed}) = dose_P \cdot f_{(P-M)} \tag{16}$$

It may be concluded, therefore, that if a toxic agent is eliminated by only one tissue by first-order processes (i.e. the total body clearance Cl_t is independent of the concentration of the toxic agent in blood and tissues), the fraction of the dose that is converted to a given metabolite is independent of the route of administration of the toxic agent. It also follows that if metabolite M is chemically reactive and does not escape from the cells in which it is formed, the amount of metabolite M that reacts with the target organ will be independent of the route of administration of the toxic agent, provided that all the reactions by which metabolite M is eliminated from the cells also follow first order kinetics.

A toxic agent, however, is frequently eliminated from the body by more than one organ. When this occurs, the equation for the total body clearance of the agent can be complicated. If the toxic agent is eliminated from the body by the gut (bioavailability < 1.0) or by enzymes in the gastrointestinal mucosa, the diffusion of the toxic agent from blood into the intestinal contents and its excretion into the bile will affect the total body clearances (Gillette & Pang 1977). In order to gain an insight into the interrelationships among the clearances by the various organs in the body, let us assume that the toxic agent is completely absorbed unchanged from the gastrointestinal tract and does not pass back into the tract either by diffusion from mucosal blood or by excretion into bile. Under these conditions, the total body clearance may be viewed as the sum of the *effective clearances* by the various organs of elimination. In order to understand the term *effective clearance,* it is useful to visualize the constant infusion of the toxic agent into the left ventricle of the heart until a steady state is reached. Since the clearance of a substance by an organ may be defined as the rate of elimination of the substance divided by the steady-state concentration of the substance in arterial blood (Riggs 1963) we may express the rate of elimination of the substance by an organ as the

product of the rate at which the substance is delivered to the organ and the extraction ratio. Thus:

$$\textit{rate of elimination by organ} = C_{(arterial)ss} \bullet Q_{organ} \bullet E_{organ} \tag{17}$$

If the blood has previously passed through an organ that also eliminates the substance from the body, the steady-state concentration of the substance entering the organ is the product of the arterial concentration and the available fraction escaping the previous organ ($F_{pre.}$). Thus:

$$\textit{rate of elimination by organ} = C_{(arterial)ss} \bullet F_{pre} \bullet Q_{organ} \bullet E_{organ} \tag{18}$$

We may now write the following equations for the *effective clearances* ($Cl_{effective}$) in terms of extraction ratios (E values) and available fractions (F values). The effective renal clearance is simply:

$$Cl_{(effective)R} = \frac{rate_R}{C_{(arterial)ss}} = (Q_{LR} \bullet E_{LR}) + (Q_{RR} \bullet E_{RR}) \tag{19}$$

where $rate_R$ is the rate of renal elimination of the substance at a given steady-state concentration in arterial blood; Q_{LR} and E_{LR} are the blood flow rate and the extraction ratio, respectively, of the left kidney and Q_{RR} and E_{RR} are the blood flow rate and the extraction ratio of the right kidney. Similar equations may be written for any other organ that is perfused directly by arterial blood.

Only a part of the intestinal blood perfuses the intestinal mucosa. If the elimination of the substance by the intestine is restricted to the mucosa, therefore, we may express the effective mucosal clearance as the fraction of the intestinal blood flow (f_M) that perfuses the mucosa.

$$Cl_{(effective)M} = \frac{rate_M}{C_{(arterial)ss}} = Q_I \bullet f_M \bullet E_M \tag{20}$$

Q_I is the blood flow rate in the intestine and E_M is the extraction ratio of the mucosa. Shunting of blood through non-elimination parts of tissues may also be expressed in this way.

The elimination of a substance by the liver is complicated because only a part of the blood entering the liver is arterial. Another part is portal blood that has previously passed through the mucosa, and thus the hepatic concentration of a substance may be lower than that in systemic arterial blood. The effective hepatic clearance may thus be expressed in terms of three blood

flows: that which passes through the intestinal mucosa, $(Q_I f_M)$, that which passes through intestinal muscle, $Q_I(1-f_M)$, and that which enters by the hepatic artery, Q_{HA}. Thus:

$$Cl_{(effective)H} = \frac{Rate_H}{C_{(arterial)ss}}$$
$$= (Q_I \cdot f_M \cdot F_M \cdot E_H) + (Q_I \cdot (1-f_M) \cdot E_H) + (Q_{HA} \cdot E_H) \quad (21)$$

The effective clearance by the lung is even more complicated because all the blood must pass through various tissues before it is returned to the arterial circulation. Hence, the effective clearance of a substance by the lung may be expressed as follows:

$$Cl_{(effective)L} = \frac{Rate_L}{C_{(arterial)ss}}$$
$$= (Q_{LR} \cdot F_{LR} + Q_{RR} \cdot F_{RR})E_L + (Q_I \cdot f_M \cdot F_M \cdot F_H \cdot E_L) + \quad (22)$$
$$(Q_I(1-f_M)F_H \cdot E_L) + (Q_{HA} \cdot F_H \cdot E_L) + (Q_{other} \cdot E_L)$$

If a toxic agent were eliminated by the intestinal mucosa, liver, lung and kidney, the total body clearance would be the sum of equations 19–22. It is evident, therefore, that we may construct rather complicated models from the simple relationships illustrated by equations 17 and 18.

Equations 19–22 also provide an insight into the fraction of a dose of a toxic agent that may be converted into a chemically reactive metabolite in a given tissue. In the interests of simplicity, let us assume that the parent compound is eliminated from the body solely by the liver and the kidney, by first order reactions (Fig. 3).

If the parent compound is constantly infused intravenously until a steady-state concentration is reached, in arterial blood the equation would be:

$$P_{(arterial)ss,\,i.v.} = \frac{k_{0P,\,i.v.}}{Cl_{H,P} + Cl_{R,P}} \quad (23)$$

in which $Cl_{H,P}$ and $Cl_{R,P}$ are the effective clearances of the parent compound by the liver and kidneys respectively. The steady-state rate of elimination by the kidneys would thus be:

$$rate_{R,P\ i.v.} = P_{(arterial)ss,i.v.} \cdot Cl_{R,P} = \frac{k_{0P\ i.v.} \cdot Cl_{R,P}}{Cl_{H,P} + Cl_{R,P}} \quad (24)$$

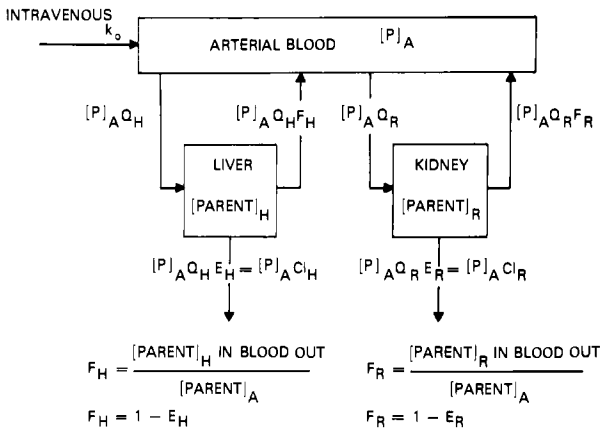

FIG. 3. Model illustrating the perfusion of the liver and kidney. The model also may be used to illustrate the perfusion of any system in which two organs of elimination are perfused directly by arterial blood. Symbols: k_o, constant rate of infusion of a foreign substance (P); [P], steady-state arterial concentrations of substance P. Q, F, E and Cl are the blood flow rate, available fraction, extraction ratio and clearance, respectively. Subscript H refers to the liver and subscript R refers to the kidney.

The rate of formation of a chemically reactive metabolite by the kidney may be expressed as a fraction ($f_{R(P-M)}$) of the rate of elimination of the parent compound by the kidneys. Thus:

$$rate_{R(P-M)i.v.} = \frac{k_{0P\ i.v.} \cdot Cl_{R,P} \cdot f_{R(P-M)}}{Cl_{H,P} + Cl_{R,P}} \tag{25}$$

It is evident, therefore, that the rate of formation of a reactive metabolite in the kidneys depends on the relative clearances of the parent by the liver and the kidneys, $(Cl_{R,P}/(Cl_{H,P} + Cl_{R,P}))$, as well as on the fraction of the parent substance, eliminated by the kidneys, that is converted to the reactive metabolite. Similarly, we may derive an equation for the rate of formation of a reactive metabolite by the liver:

$$rate_{H(P-M)i.v.} = \frac{k_{0P,i.v.} \cdot Cl_{H,P} \cdot f_{H(P-M)}}{Cl_{H,P} + Cl_{R,P}} \tag{26}$$

Thus, the rate of formation of a chemically reactive metabolite in the liver depends on the relative clearances of the parent toxic agent by the liver and the kidneys, $(Cl_{H,P}/(Cl_{H,P} + Cl_{R,P}))$, as well as on the fraction of the parent,

cleared by the liver, that is converted to the reactive metabolite.

Equations 25 and 26 reveal why inducers of microsomal enzymes in liver may decrease toxicity in kidney and either increase or diminish the toxicity of the parent in the liver. An increase in hepatic clearance will decrease the value of $(Cl_{R,P}/(Cl_{H,P} + Cl_{R,P}))$, without affecting the value of $f_{R(P-M)}$. But an increase in the activity of the enzyme that catalyses the formation of the reactive metabolite in liver will increase the value of $(Cl_{H,P}/(Cl_{H,P} + Cl_{R,P}))$ and frequently will increase the value of $f_{H(P-M)}$. If the inducer led to a selective increase in the activity of an enzyme that catalyses the conversion of the parent to a chemically inert metabolite, however, the value of $f_{H(P-M)}$ may be decreased to an extent that would reduce the rate of formation of the chemically reactive metabolite in the liver.

The route of administration of the parent may also affect the rate of formation of reactive metabolites in liver and kidneys. If the parent compound were constantly infused intraportally (or, in this model, orally) until a steady state is reached, the steady-state concentration of the toxic agent in arterial blood would be:

$$P_{(arterial)ss,p.o.} = \frac{k_{0P,p.o.} \cdot F_{H,P}}{Cl_{H,P} + Cl_{R,P}} \tag{27}$$

The rate of elimination of the parent by the kidneys would thus be:

$$rate_{R,P,p.o.} = P_{(arterial)ss,p.o.} \cdot Cl_{R,P} = \frac{k_{0P,p.o.} \cdot F_{H,P} \cdot Cl_{R,P}}{Cl_{H,P} + Cl_{R,P}} \tag{28}$$

The rate of formation of a reactive metabolite by the kidneys would be:

$$rate_{R(P-M)p.o.} = \frac{k_{0P,p.o.} \cdot F_{H,P} \cdot Cl_{R,P} \cdot f_{R(P-M)}}{Cl_{H,P} + Cl_{R,P}} \tag{29}$$

By contrast, the rate of elimination of the parent compound by the liver may be expressed as the sum of two eliminations: its elimination as it passes from the site of administration through the liver to the systemic circulation, and its elimination in the systemic circulation that is recycled through the liver. Thus:

$$rate_{H,P,p.o.} = \left[\frac{k_{0P,p.o.}}{Q_H} + P_{(arterial)ss,p.o.}\right] Cl_{H,P} \tag{30}$$

in which $Q_H = Q_I + Q_{HA}$.

On substitution of equation 27 into equation 30 we obtain:

$$rate_{H,\mathrm{P,p.o.}} = \left[\frac{k_{0\mathrm{P,p.o.}}}{Q_H} + \frac{k_{0\mathrm{P,p.o.}} \cdot F_{H,\mathrm{P}}}{Cl_{H,\mathrm{P}} + Cl_{R,\mathrm{P}}}\right] Cl_{H,\mathrm{P}} \tag{31}$$

On expansion of equation 31, and since $Cl_{H,\mathrm{P}} = (Q_I + Q_{HA})\, E_H = Q_H E_H$, we obtain

$$rate_{H,\mathrm{P\ p.o.}} = k_{0\mathrm{P,p.o.}} \cdot Cl_{H,\mathrm{P}} \left[\frac{E_{H,\mathrm{P}} + F_{H,\mathrm{P}} + (Cl_{R,\mathrm{P}}/Q_H)}{Cl_{H,\mathrm{P}} + Cl_{R,P}}\right] \tag{32}$$

But $E_{H,\mathrm{P}} + F_{H,\mathrm{P}} = 1$ and thus:

$$rate_{H,\mathrm{P,\ p.o.}} = k_{0\mathrm{P,p.o.}} \cdot Cl_{H,\mathrm{P}} \left[\frac{1 + (Cl_{R,\mathrm{P}}/Q_H)}{Cl_{H,\mathrm{P}} + Cl_{R,\mathrm{P}}}\right] \tag{33}$$

The rate of formation of a chemically reactive metabolite by the liver in this system would be:

$$rate_{H(\mathrm{P-M})\mathrm{p.o.}} = k_{0\mathrm{P,p.o.}} \cdot Cl_{H,\mathrm{P}} \cdot f_{H(\mathrm{P-M})} \left[\frac{1 + (Cl_{R,\mathrm{P}}/Q_H}{Cl_{H,\mathrm{P}} + Cl_{R,\mathrm{P}}}\right] \tag{34}$$

Equations 25 and 29 reveal that the ratio of the rates of formation of a chemically reactive metabolite during the constant infusion of the same dose of parent compound intravenously and orally will depend on the available fraction escaping the liver, $F_{H,\mathrm{P}}$. Thus, as long as all processes follow first order kinetics:

$$\frac{rate_{R(\mathrm{P-M})\mathrm{p.o.}}}{rate_{R(\mathrm{P-M})\mathrm{i.v.}}} = F_{H,\mathrm{P}} \tag{35}$$

By contrast, equations 26 and 34 show that the ratio of the rates of formation of reactive metabolite by the liver under the same conditions depends on the clearance of the toxic agent by the kidneys and on the blood flow rate through the liver. Thus, as long as all processes follow first order kinetics:

$$\frac{rate_{H(\mathrm{P-M})\mathrm{p.o.}}}{rate_{H(\mathrm{P-M})\mathrm{i.v.}}} = 1 + (Cl_{R,\mathrm{P}}/Q_H) \tag{36}$$

It may be concluded, therefore, that differences in the route of administration

of a toxic agent will affect the formation of a reactive metabolite in a target organ only when the toxic agent is cleared in significant amounts by other organs in the body. If the value of $F_{H,P}$ were nearly 1.0 in equation 35, and the clearance of the toxic agent by the kidney were small compared with the hepatic blood flow rate, the route of administration of the parent toxic agent would be irrelevant. Under these conditions equation 29 changes to equation 25 and equation 34 changes to equation 26.

When $F_{H,P}$ nearly equals 1.0 and $Cl_{R,P}$ is much less than Q_H, however, we may express the effective organ clearance in terms of both the intrinsic clearances of the enzymes and the other processes that lead to the elimination of the toxic agent, regardless of the model we choose to represent the elimination of the toxic agent.

Occasionally, a drug is metabolized so rapidly in an organ of elimination that its clearance approaches the blood flow rate through the organ. When this occurs the rate of metabolism depends on the activities of the drug-metabolizing enzymes within the organ, the degree of reversible binding of the drug to blood components and the blood flow rate through the organ. Unfortunately, there is no universally accepted model that describes the relationship between clearance and these factors. In the simplest model, referred to as the 'well-stirred' model, the unbound concentration of the drug is assumed to be the same in all of the cells of the organ, and to be the same as that in blood leaving the organ. For this model, we express the clearance as:

$$Cl_{organ} = \frac{Q_{organ} \cdot f_B \cdot Clint_{(organ)}}{Q_{organ} + f_B \cdot Clint_{(organ)}} \tag{37}$$

in which f_B is the fraction of the toxic agent in blood existing in its unbound form, and $Clint_{(organ)}$ is the intrinsic clearance of the processes that lead to the elimination of the toxic agent. In turn, the intrinsic clearance is the sum of the individual intrinsic clearances of each of the processes. For an enzymic reaction, the intrinsic clearance of a reaction by an individual enzyme may be expressed as the Michaelis-Menten equation divided by the unbound concentration of the substrate:

$$Clint_{(individual\ enzyme)} = V_{max}/(K_m + P_{free}), \tag{38}$$

which approaches a constant when P_{free} is much less than K_m. Thus, the value of $Clint_{(organ)} \cdot f_{organ(P-M)}$ may be rewritten as $Clint_{(organ)(P-M)}$. In this context, equation 25 may be rewritten:

$$rate_{R(P-M)i.v.} = \frac{k_{0P,i.v.} \cdot Clint_{R(P-M)}}{Clint_{H,P} + Clint_{R,P}} \quad (39)$$

and equation 26 may be rewritten as:

$$rate_{H(P-M)i.v.} = \frac{k_{0P,i.v.} \cdot Clint_{H(P-M)}}{Clint_{H,P} + Clint_{R,P}} \quad (40)$$

In the light of this discussion, we may come to the following conclusions:

(1) If a drug is eliminated from the body by flow-independent first-order processes, the fraction of the dose that is converted to a highly unstable metabolite in a tissue depends on (i) the intrinsic clearance of the enzyme that forms the metabolite and (ii) the sum of the intrinsic clearances of the elimination processes in both tissues.

(2) If a drug is eliminated from the body by flow-dependent first-order reactions in two tissues, the fraction of the dose that is converted to a highly reactive metabolite may depend on the route of administration of the drug when its administration by one route necessitates passage through one of the organs before the drug reaches the systemic circulation.

When the drug is eliminated by both the liver and the kidneys, the ratio of the fractions converted to a highly unstable metabolite in the kidney after oral and intravenous administration of the drug will depend on the hepatic availability (F_H). By contrast, the ratios of the fractions converted to an unstable metabolite in the liver after oral and intravenous administration of the drug will depend on the relation, $1 + (Cl_R/Q_H)$. Under these conditions, the percentage change in the fraction of the dose converted to an unstable metabolite in liver, caused by a change in the activity of a hepatic enzyme, will be independent of the route of administration of the drug.

The approach I have used in this paper may be extended to other pairs of organs or even to the elimination of toxic agents by several organs simultaneously, but I shall not derive the equations describing such systems here. It should be emphasized, however, that in the derivation of the equations I have assumed that all processes of elimination follow first-order kinetics. If an enzyme catalysing the elimination of the toxic agent becomes saturated with its substrate, or if the clearance of the toxic agent is changed during the course of the experiment, the equations will be invalid. I hope, nevertheless, that my paper helps to clarify the interrelationships between various organs that contribute to the excretion and the metabolism of toxic agents; and also that it provides a clearer concept of the role of first-pass effects in the formation of reactive metabolites in various tissues.

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Discussion

Connors: Is it possible to obtain any useful information from humans, given the limited measurements that can be made?

Gillette: No, the information we obtain by measuring the concentrations of the foreign compound and its metabolites in blood is frequently too limited to be assessed in this way. Some studies cite only the half-life of what the investigator believes to be the terminal phase of elimination (in a two-pool system this is referred to as the β-phase). From such measurements, however, it is not possible to determine where in the body the foreign compound is metabolized. In order to do that we would have to consider the individual

contributions of each organ of elimination. Our approach has been to derive equations for the complex first-order systems in order to illustrate how various changes in the activities of different enzymes should affect not only the concentration of foreign compounds and their metabolites in blood, but also their concentrations in the tissues when the foreign compound is eliminated by more than one organ and is limited by the blood flow rates of the organ. Such equations show the investigator ways of designing experiments that will provide the maximum amount of information that can be obtained from measurements of the foreign compound and its metabolites in blood. Just as importantly, however, the equations show when such data cannot differentiate between various plausible mechanisms. The equations further provide predictions that can be used to test the validity and completeness of our concepts. All too frequently, data are said to support certain conclusions when in reality they do not.

McLean: Dr Gillette, you have not taken into account repair or recovery; you have assumed that the rate of formation of reactive metabolite is not of consequence, and that what matters is the ultimate quantity of metabolite that is produced. The proportion of metabolites produced in different organs is influenced by induction, as you have pointed out, because when one alters the clearance in the liver there are more metabolites available for the kidney. But a second point is that if the rate of delivery of metabolites is sufficiently slow, repair or recovery may occur, and therefore the damaging effects will be altered even though the total quantity of metabolite produced is the same, partly because it is not dependent on induction and partly because there are no alternative pathways. I believe this is true for dimethylnitrosamine.

Gillette: I agree that the kinetics of toxic responses are very important. I believe that in many instances, however, the rates of repair are slow compared with the rates of metabolism of the foreign compound and its metabolites. Of course there are times when the rates of drug metabolism by various pathways can affect the fraction of the dose that reacts with target substances in cells. That will occur when one or more of the reactions no longer follows first-order kinetics. For example, the concentration of glutathione in a tissue depends on the rate at which a chemically reactive metabolite is formed, and reacts with glutathione in tissue cells, relative to the rate of synthesis of glutathione by the cells. Indeed, we have used this argument to account for the increase in toxicity of bromobenzene caused by pretreating rats with phenobarbitone (Gillette 1977). As you may recall, in untreated rats bromobenzene is converted almost entirely to bromobenzene-3,4-epoxide. Thus, induction of the enzyme that catalyses the formation of the epoxide cannot increase the fraction of the dose that is converted to the epoxide. The

pretreatment of the animals with phenobarbitone should not have increased the toxicity unless the treatment also altered the fraction of the epoxide that reacted with the target substance. If glutathione were not depleted, the pretreatment should have decreased the toxicity by inducing the synthesis of the hydrolase or the glutathione transferase in liver.

J. W. Bridges: Although I agree that small amounts of toxic dietary constituents that are metabolized to reactive metabolite will tend to accumulate, is this really comparable to the situation in which several large doses of the material are given over a much shorter period? Surely the repair of damage incurred is likely to be much more effective in the former instance?

Gillette: When toxic material is continuously ingested in the diet, a *quasi*-steady-state relationship between the rate of formation of toxic metabolite and its elimination is established. In addition, the rate of reaction of the toxic metabolite with the target approaches a constant. Whether the incidence or the severity appears to increase with time depends on the presence and activity of the repair mechanisms. If the rate constant of repair is very small, the incidence and severity will appear to increase with time until the rate of alteration of the target substance equals the rate of repair. Of course there are many other factors to consider. Perhaps the steady-state amount of impaired target substance is too small for any toxicity to be manifested; perhaps the homeostatic mechanisms of the cell are adequate to cope with small amounts of the impaired target and the toxicity is never manifested; perhaps the mechanisms by which the toxicity is manifested, after the reaction of the reactive metabolite with the target substance, are changed and the cells become either tolerant to the insult or more sensitive to the insult. Indeed, we might define toxicity as an inability to cope adequately or appropriately with an insult caused by foreign compounds. In the past, I have expressed our ignorance of the various processes by a probability term that relates the impaired target substance to the incidence of toxicity.

De Matteis: In practice, when you are faced with inhibition and you want to study the half-life of a drug, how do you determine the distribution volume?

Gillette: In order to determine the effect of an inhibitor, what we should really estimate is the intrinsic clearance, and not the half-life. If the clearance is not limited by the blood flow through the organs of elimination, one should theoretically be able to calculate the average intrinsic clearance of the substance from the area under the curve for concentration of the substance after intravenous administration. Once the clearance is calculated, the volume of distribution (*Vd*) may be calculated from the clearance and from the half-life of the terminal phase by the relationship:

$$Vd_{(\beta)} = t_{-\frac{1}{2}} \times clearance/\ln 2$$

However, several problems arise in these calculations. Since the inhibitor is also eliminated during the experiment, the intrinsic clearance may also change. Indeed, if the inhibitor is rapidly eliminated the half-life of the drug during the terminal phase may appear to be unaffected by the inhibitor. Moreover, we should also recognize that the value of the volume of distribution will depend to a certain extent on the terminal half-life of the substance.

A change in the clearance can change the apparent volume of distribution of the substance during the terminal phase even when the inducer does not alter the volume of distribution of the substance at equilibrium. This difference between the effects of inducers on the two kinds of volume of distribution occurs with substances that are highly bound in the peripheral pharmacokinetic compartment and are rapidly eliminated from the central compartment. The reason for the difference becomes clear when one considers what has happened after the (so-called) *distributional phases* have been completed and the substance begins to leave the peripheral compartment. For this to occur, a concentration gradient between the two compartments is created. The more rapid the metabolism relative to the rate constant for the passage of the substance from the peripheral compartment to the central compartment, the greater the concentration gradient becomes. Since the apparent volume of distribution is calculated as the total amount of substance in the body divided by the concentration of the substance in the central pool (i.e. the concentration in blood) the value of $Vd_{(\beta)}$ will increase as the clearance of the substance is increased. Thus, changes in the intrinsic clearance may not affect the half-life of the substance as much as might be supposed. It is also possible, of course, that the inducer or inhibitor can alter the equilibrium volume of distribution (Vd_{eq}) by displacing the substance from reversible binding sites in blood and tissues as well as by changing the intrinsic clearance. In order to evaluate this possibility, one must assume a model, calculate the various rate constants and substitute the constants into the appropriate equation, usually:

$$Vd_{eq} = (V_c) \times (1 + (k_{12}/k_{21})),$$

where V_c = volume of the central compartment.

Changes in the volume of distribution can be serious because they frequently mask a change in the clearance. For example, the usual doses of quinidine in patients apparently decrease both the clearance and the volume of distribution of digoxin by about 50% (Hager et al 1979). Thus, quinidine does not significantly affect the biological half-life of digoxin even though it increases the steady-state concentration of digoxin by two-fold.

Boyd: The validity of your pharmacokinetic treatment depends in part upon the validity of your initial fundamental assumptions. In particular, I find it difficult to accept your assumption that the rate at which the metabolite interacts with the cell irreversibly is inconsequential. (This question was also raised by Dr McLean.) Biologically, it doesn't make a lot of sense to me. Your assumption rests on the view that although the critical biochemical interactions (e.g. the covalent binding of the metabolite) occur relatively early, the biological consequences (e.g. cellular necrosis) occur much later. In my opinion this is too simplistic a view; it depends upon the particular biological endpoint that you select, and upon the particular criteria and methods with which you define and measure the response.

Gillette: Whether the incidence rate or the severity of a toxicity is related to the maximum concentration of the chemically reactive metabolite, or to the area under the curve for concentration of the reactive metabolite within the target organ, depends on several interrelated factors. In an idealized model, in which all processes follow first-order kinetics and all apparent rate constants remain unchanged during the course of the treatment with the toxic agent, the relative importance of the two calculations depends on the magnitude of the rate constant for repair relative to that of the rate constant for elimination of the parent toxic agent. Let us assume, for example, that the elimination of the parent toxic agent may be described by a one-pool model. We may write an equation for the amount of damaged target at any given time as follows:

$$damaged\ target = dose_{i.v.} \left[\frac{k_{12}k_{23}e^{-at}}{(b-a)(c-a)} + \frac{k_{12}k_{23}e^{-bt}}{(a-b)(c-b)} + \frac{k_{12}k_{23}e^{-ct}}{(a-b)(c-b)} \right] \quad (I)$$

in which k_{12} is the rate constant for the formation of the chemically reactive metabolite and k_{23} is the rate constant for the damage of the target by the reactive metabolite; a is the rate constant of elimination of the parent compound, b is the rate constant of elimination of the reactive metabolite and c is the rate constant of repair of the damaged target. If the chemically reactive metabolite is inactivated so rapidly that it doesn't escape from the organ in which it is formed, b will be much greater than either a or c, and e^{-bt} will approach zero almost instantaneously. The equation then becomes:

$$damaged\ target = dose_{i.v.} \left[\frac{k_{12}k_{23}}{b(c-a)} \right] \left[e^{-at} - e^{-ct} \right] \quad (II)$$

From this equation, we may derive another one that predicts the maximum

amount of damaged target that would be expected:

$$(damaged\ target)_{max} = dose_{i.v.} \frac{k_{12}}{a} \frac{k_{23}}{b} \left[\frac{a}{b}\right]^{c/(c-a)} \quad \text{(III)}$$

This is an extension of the equations derived by Goldstein et al (1968) and by me (Gillette 1979). But (k_{12}/a) equals the 'A' and (k_{23}/b) equals the 'B' in equation (1) in my paper. Thus:

$$(damaged\ target)_{max} = dose_{i.v.} AB \left[\frac{a}{c}\right]^{c/(c-a)} \quad \text{(IV)}$$

By a similar line of reasoning, we may derive an equation for the maximum intracellular concentration of the chemically reactive metabolite:

$$[reactive\ metabolite]_{max} = \frac{dose_{i.v.} A}{V_{metabolite}} \left[\frac{a}{b}\right]^{b/(b-a)} \quad \text{(V)}$$

If we assume that a is considerably smaller than either b or c, then equations (I), (IV) and (V) degenerate to:

$$(damaged\ target) = dose\ B\ (k_{12}/c)e^{-at} \quad \text{(VI)}$$

$$(damaged\ target)_{max} = dose\ (k_{23}/b)(k_{12}/c) \quad \text{(VII)}$$

and

$$[reactive\ metabolite]_{max} = \frac{dose_{i.v.}(k_{12}/b)}{V_{metabolite}} \quad \text{(VIII)}$$

If we substitute equation (VIII) into equation (VII) we obtain:

$$(damaged\ target)_{max} = V_{metabolite} \cdot [reactive\ metabolite]_{max} \cdot (k_{233}/c) \quad \text{(IX)}$$

Equations (VI), (VII) and (IX) indicate that when repair occurs very rapidly, the maximum amount of damage will be proportional to the maximum concentration of the reactive metabolite, both of which will occur almost immediately after intravenous administration of the parent toxic agent. The equations also predict that under these conditions, any side reactions by which the parent compound is eliminated from the body would have little effect on the severity of the toxicity. A similarly derived equation, however, shows that such side reactions can be important when the toxic agent is slowly absorbed

after oral or intramuscular administration of the parent toxic agent. Thus:

$$(damaged\ target)_{max} = dose\ B\ (k_{12}/c)\ \left[\frac{k_a}{a}\right]^{a/(a-k_a)}, \qquad (X)$$

in which k_a is the rate constant of absorption.
By contrast, if the rate constant of elimination of the parent toxic agent is much larger than the rate constant of repair, equation (IV) degenerates to:

$$(damaged\ target)_{max} = dose\ AB \qquad (XI)$$

which is equation 1 in my paper. Thus, I have assumed that the rate constant of repair is small. To put into perspective what I mean by small, however, I should like to point out that when the rate constants for the elimination of the parent toxic agent and the repair of the damaged target are equal, equation IV becomes:

$$(damaged\ target)_{max} = dose_{i.v.} AB/e \qquad (XII)$$

where e is the base of the Napierian logarithms. Thus, we may conclude that the rate constant for the repair of the damaged target must be at least as large as the rate constant for the elimination of the parent toxic agent before the maximum concentration of the reactive metabolite becomes a better estimate of target damage than does the fraction of the dose that reacts with the target. I agree that the 'ratio concept' is probably over-simplistic, especially with chemically reactive metabolites that cause cellular necrosis. I think most of us would agree that there must be a critical amount of damaged target above which the homeostatic mechanisms in the target cells can no longer cope, and below which the target cells recover. Nevertheless, equations (XI) and (XII) provide us with a general concept of what the rate constant of the repair of damaged target would have to be before induction of the enzyme that catalyses the formation of the reactive metabolite would be expected to change markedly the maximum amount of damaged target. Let us assume for example, that initially the rate constants for the elimination of the parent toxic agent and for the repair of the damaged target were equal. Let us assume, further, that after induction of the enzyme the one-compartment model is still valid, and that ratio A remains substantially unchanged. Division of equation (XI) by equation (XII) reveals that the $(damaged\ target)_{max}$ can be increased at most by only e, or about 2.7-fold.

When a toxicity appears only after multiple dosing of the toxic agent, I

think that one would have to assume that the rate constant of repair is small. So the 'ratio concept' is probably more valid with toxic agents that have to be given repeatedly before the toxicity appears than it is with those that produce their toxicities after single doses. But unfortunately, with most toxicities, we know very little about the kinetics of repair. Indeed, we seldom know even what the target substance is.

Boyd: So, given the same amount of reactive metabolite that interacts with the cell (or a number of cells), if we were to measure necrosis as a suitable endpoint, would your assumption be that the *rate* at which the total amount of metabolite was reacting would be irrelevant?

Gillette: Yes, especially if we are talking about environmental chemicals to which we are constantly exposed.

Boyd: To what extent is your assumption related to the sensitivity with which you are measuring the biological endpoint?

Gillette: Cellular necrosis, as revealed by light microscopy after the administration of paracetamol to mice, reaches a maximum at 24–48 h, which is long after all of the paracetamol has been eliminated from the body (Mitchell et al 1973). These observations suggest to me that the rate constant for the repair of the damaged target substance, whatever it may be, must be smaller than the rate constant for elimination of paracetamol. If you tell me that all the cells actually died while most of the paracetamol was still in the body then I obviously can't use this argument to estimate the relative sizes of the rate constants. If the rate constant for repair of the target were considerably larger than the rate constant for the elimination of the drug, however, I would find it difficult to understand why cysteamine and *N*-acetylcysteine can still serve as effective antidotes for up to 18 h after ingestion of the drug (Prescott et al 1974, Peterson & Rumack 1977).

Farber: Are you saying that you can't use this formulation with a single dose of a chemical?

Gillette: No, I'm not saying that! Measurement of clearance of the parent drug (calculated from the area under the curve, and from estimates of the fraction of the dose that is converted to the chemically reactive metabolite from the inactive products excreted into urine) can frequently be useful in estimating the intrinsic clearance of the enzyme that catalyses the formation of the chemically reactive metabolite.

Breckenridge: When a drug with a high first-pass effect is given chronically, do your conclusions about its first-pass effect still apply?

Gillette: Yes. If the compound is eliminated solely by a single organ, the route of administration is irrelevant to the amount of drug metabolites which will be formed. When there is a first-pass effect by the liver, the route of ad-

ministration of the toxic agent has more influence on the formation of active metabolites in other organs than it does on the formation of the active metabolites in the liver, because under these conditions the equations for multi-organ systems would apply.

McLean: But normally multiple metabolic pathways and multiple tissues are able to metabolize drugs. Induction is still likely to influence the outcome of exposure because it is unusual for induction to affect all the factors by the same amount. For example, if every enzymic pathway increases its activity by half as much again, then you may get no alteration in distribution of the drug.

Gillette: Particularly when there is no first-pass effect. Equations 29 and 37 in my paper really sum up what you're saying, because they take into account the fraction of the dose that is eliminated by a particular tissue, the fraction of the reactive metabolite that reacts with the target within the tissue and the other factors that determine the incidence or severity of the toxicity.

McLean: Therefore we can't make the assumption, for any toxic material, that induction will or will not have any effect on toxicity.

Selikoff: Does a given concentration of polychlorinated biphenyl, that has been in tissues for one hour, have the same metabolic effect as that which has been present as long as one month, or a year?

Gillette: Since polychlorinated biphenyls are inducers one might expect that the changes in activities of the enzyme systems might change the fraction of the dose that is converted to a given metabolite of a toxic agent.

Selikoff: When we note a concentration of 1 p.p.m. of polychlorinated biphenyl in the blood, what does that mean for the individual, and is the duration of exposure to that concentration relevant?

Gillette: Well the approach that I have used is one way of detecting that. I have outlined the theoretical basis by which one can compare the data. The equations show what might be expected to happen when there is a first-pass effect and when there are no changes in the activities of the enzyme systems. If induction or saturation of an enzyme system occurs then there will be a change in the average concentration of the parent toxic agent, or its metabolites, or both, in blood. In this way, the equations can be used as tools in order to help us decide if something has changed and if so, what has changed.

Higginson: You mentioned saturation of an enzyme system. Theoretically, it is possible that the effects of a carcinogen could be qualitatively different if it were given above rather than below the saturation levels.

Gillette: Yes, saturation of the enzyme will change the intrinsic clearance of the substance by the enzyme and therefore will alter the fraction of the dose of the substance that is converted to a given metabolite.

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Toxicological implications of polymorphic drug metabolism

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Abstract The occurrence of genetic polymorphisms of drug metabolism means that populations contain subgroups (phenotypes) that differ sharply in their abilities to effect a number of metabolic reactions. Because of this, major inter-phenotype differences occur in responsiveness to drugs and toxic substances. The well established genetic polymorphisms of acetylation and hydrolysis illustrate the important association that exists between phenotype and propensity to develop toxic and exaggerated responses to some substances.

Recently, for metabolic oxidation, a new genetic polymorphism of drug metabolism has been described and it promises to provide a better understanding of inter-individual variability in the metabolic handling of, and responsiveness to, drugs and toxic substances.

The following effects of the polymorphism are described here:

(a) its influence in determining variable presystemic metabolism and hence systemic drug availability;

(b) its role in determining alternative toxic pathways of metabolism in individuals who have a genetically determined impairment of oxidative capacity and

(c) its influence on the development of agranulocytosis associated with metiamide administration.

The natural phenomenon of genetic polymorphism, which gives rise to varying amounts or variant structures of proteins, is well known. Biologists view the phenomenon as an organizational arrangement by which variability can be incorporated into the population. This inbuilt variability allows for selection and a better chance of survival, should changes occur in the environment. Ford (1940) has described genetic polymorphism as a 'type of variation in which individuals with sharply distinct qualities co-exist as normal members of a population.'

Genetic polymorphisms arise because of the occurrence in the population of mutant alleles that can influence the structure or amounts of protein syn-

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thesized. Which of these occurs depends upon whether the mutation affects the regulator or the structural gene. The simplest genetic polymorphism can arise in the population from the occurrence of two different alleles which can control synthesis of a particular polypeptide or protein. These two alleles may be paired in three different ways, in either of two homozygous forms or in a heterozygote, and they may then determine the synthesis of variant structures or amounts of the protein.

Some indication of the prevalence of genetic polymorphism is shown by the large number of human blood proteins that exist in variant forms (Table 1). Harris (1970) has estimated that about 30% of human gene products exhibit polymorphic variation. There is obviously enormous scope for polymorphic variation among the large number of cellular proteins that exist. Genetic polymorphisms and variant protein structure could be important in responsiveness to toxic substances in two ways. First, they could influence dispositional events such as absorption, distribution, metabolism and excretion. Second, they could also affect receptor events. (For reviews of these aspects see Atlas & Nebert 1977, Price-Evans 1977, and Boobis 1979.) This paper is concerned with genetic polymorphisms of drug metabolism and their toxicological implications, and in particular with metabolic oxidation.

GENETIC POLYMORPHISMS OF DRUG METABOLISM

Variable ability to metabolize drugs and toxic substances is commonly encountered in the human population and is due to variations in the nature and amounts of the enzymes that metabolize drugs. These enzymes are controlled by a complex interplay of genetic and environmental factors. The two types

TABLE 1

Some human blood proteins which exist in variant forms

Protein	*No. of variant forms*
Catalase	3
Caeruloplasmin	3
Amylase	4
Pseudocholinesterase	5
Acid phosphatase	5
Carbonic anhydrase	5
6-Phosphate gluconate dehydrogenase	7
Haemoglobin: α-chain	26
β-chain	46

(From Cavalli-Sforza & Bodmer 1971)

TABLE 2

Types of genetic control of drug metabolism in humans

Type of control	*Population distribution of the metabolic reaction*	*Reaction example*
Polygenic	unimodal	Glycine and glucuronic acid conjugation of salicylic acid
Monogenic	polymodal	Acetylation of isoniazid Hydrolysis of succinylcholine Hydroxylation of debrisoquine

of genetic control of metabolic reactions in humans are shown in Table 2. In the first, the frequency distribution histogram that relates incidence to metabolic response shows a continuous (normal) distribution. This is because the reaction is controlled and influenced by a number of genetic and environmental factors; hence the control is referred to as polygenic and multifactorial, e.g. the glycine conjugation of salicylic acid, which shows a normal distribution within the population but which nevertheless encompasses within that distribution 12-fold variations in individual ability to perform the reaction (Caldwell et al 1980). The second type of genetic control of metabolism occurs when there is a discontinuity of metabolic response, reflected in a bimodal or trimodal distribution curve. This distribution indicates monogenic control of the reaction, and the individual modes correspond to different allelic pairings. Appropriate studies of families and individuals can be used to determine whether a metabolic reaction is under monogenic control and exhibits genetic polymorphism.

The detection of genetic polymorphisms depends upon the differences in expression of the various alleles and upon the methods used. If the product is an enzyme, functional differences of variant forms may be detected and characterized by appropriate substrate, inhibitor and kinetic studies. It may be, however, that although variant forms exist, these functional differences are not detectable. Indeed, the so-called normal distribution may mask a whole series of polymorphic variants, the differences between which are not readily detectable by the methods used.

Table 3 shows several metabolic reactions that exhibit genetic polymorphism and have been studied in human populations, families and individuals. The best known polymorphic reactions are acetylation of various sulphonamide drugs and hydrolysis of succinylcholine. Their discovery over 20 years ago was important for many reasons, including their identification as sources of inter-individual variation in toxic responses to drugs. Indeed the

TABLE 3

Polymorphic drug metabolism in humans

Metabolic reaction exhibiting polymorphism	*Examples of substrates*
Acetylation	sulphonamides (sulphamethazine), arylamines (dapsone), hydrazines (isoniazid)
Hydrolysis	succinylcholine, paraoxon
Hydroxylation of carbon centres	debrisoquine, guanoxan, phenacetin
Oxidation at nitrogen centres	sparteine
Glucuronidation	paracetamol (acetaminophen)

respective phenotypes constitute sharply distinct subpopulations that have markedly different abilities to metabolize drugs and that exhibit different responses to drugs metabolized by these polymorphic pathways. Table 4 shows the alleles that are responsible for three genetic polymorphisms of drug metabolism: acetylation, hydrolysis and hydroxylation. For two reasons, it is highly likely that other, as yet undiscovered, genetic polymorphisms of drug metabolism exist in humans. First, recent work on the enzymology of drug metabolism has consistently demonstrated that many of the enzymes that metabolize drugs exist in multiple forms. Recent work has demonstrated, for example, at least two forms of glucuronyltransferase (Burchell 1977) and of the amino acid acyl transferases (Webster et al 1976), as well as multiple forms of sulphotransferase (Sekura & Jakoby 1979) and glutathione *S*-transferase (Jakoby et al 1976). The possibility that some of these are molecular variant forms of the same enzyme protein is quite high. The second reason is that species and strain differences exist. Species defects in certain metabolic

TABLE 4

Alleles responsible for genetic polymorphisms of drug metabolism

Metabolic reaction	*Alleles controlling the reaction*	*Effect*
Acetylation	slow	slow acetylation;
	rapid	rapid acetylation
Hydrolysis	various alleles at E_1 locus	impaired hydrolysis of succinylcholine
Hydroxylation	D^H	extensive hydroxylation of debrisoquine;
	D^L	impaired hydroxylation of debrisoquine

pathways indicate differences among mammalian species in the nature of the gene pool controlling these reactions. The main problem in detection of polymorphisms of drug metabolism in humans is one of organization of studies on large numbers of individuals under controlled conditions. It is also difficult to develop analytical methods and strategies that are capable of discerning inter-phenotype differences.

TOXICOLOGICAL IMPLICATIONS OF POLYMORPHIC DRUG METABOLISM

The important relationship between the metabolism of a chemical and its toxicity has been pointed out by several speakers at this symposium. Therefore variations in metabolism can be a major influence on inter-individual differences in responsiveness to toxic substances. This influence is evident if we consider the acetylation polymorphism and cholinesterase variants in terms of toxic responses and susceptible phenotypes (Table 5). Indeed, both these polymorphisms archetypically indicate the influence of phenotype and genotype on toxic responses. Thus, in the case of the acetylation polymorphism, people who are slow acetylators are more likely to develop peripheral neuritis (Hughes et al 1954) and the systemic lupus

TABLE 5

Genetic polymorphisms of metabolism and drug toxicity

(a) Acetylation polymorphism		
Drug	*Toxic effect*	*Susceptible phenotype*
Isoniazid	peripheral neuritis	slow acetylator
	SLE[a] syndrome	slow acetylator
	hepatitis	rapid acetylator
Hydralazine	SLE[a] syndrome	slow acetylator
Salicylazosulphapyridine	cyanosis and haemolysis	slow acetylator

(b) Plasma cholinesterase variants		
Type of enzyme	*Genotype (E_1 locus)*	*Response to normal doses of succinylcholine*
Normal	$E_1^u\ E_1^u$	rapid hydrolysis
Atypical	$E_1^u\ E_1^a$	prolonged apnoea
Silent gene	$E_1^s\ E_1^s$	prolonged apnoea
Fluoride-resistant	$E_1^u\ E_1^f$	rapid hydrolysis
	$E_1^f\ E_1^f$	prolonged apnoea

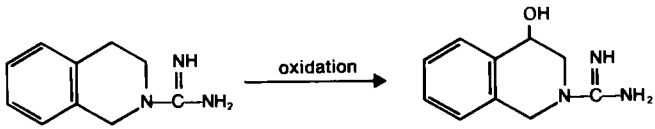
[a]SLE, systemic lupus erythematosus

erythematosus (SLE) syndrome (Zingale et al 1963), as toxic reactions to isoniazid, than are those who are rapid acetylators. Conversely, in isoniazid-induced hepatitis, rapid acetylators constitute the susceptible phenotype (Mitchell et al 1975). In the hydralazine-induced SLE syndrome (Perry 1973) and in salicylazosulphapyridine toxicity (Das et al 1973), slow acetylators appear to be more at risk than are rapid acetylators. Similarly, atypical and prolonged apnoeic reactions may occur after normal doses of the muscle relaxant, succinylcholine, according to the type of genetically determined cholinesterase variant present. Some allele pairings at the E_1 locus produce forms of plasma cholinesterase that can rapidly hydrolyse the drug, whereas other combinations (eg. $E_1^uE_1^a$ and $E_1^fE_1^f$) result in variant forms with low affinity for the succinylcholine substrate (Harris et al 1963, Lehman & Liddell 1964).

GENETIC POLYMORPHISM OF METABOLIC OXIDATION

The previous discussion has focused on the nature and types of metabolic reactions that display genetic polymorphism in the population. The acetylation and pseudocholinesterase variant polymorphisms were described in order to illustrate how the phenotype influences inter-individual differences in toxic responses to chemicals.

In 1977, a new genetic polymorphism of drug metabolism was discovered. This finding opened up new understanding of inter-individual differences in metabolic disposition of, and responsiveness to, drugs and toxic substances. This genetic polymorphism influences metabolic oxidation at various types of carbon centre (Mahgoub et al 1977, Sloan et al 1978b). The compound that most reveals these genetically determined differences is the guanidine-based antihypertensive, debrisoquine. In humans, the drug is metabolized to 4-hydroxydebrisoquine by alicyclic hydroxylation, and variable amounts of both parent drug and metabolite are eliminated in the urine of different subjects. The simple quotient, % dose eliminated as debrisoquine/% dose eliminated as 4-hydroxydebrisoquine in urine after a single 10 mg oral dose (the metabolic ratio), is bimodally distributed in the population as a consequence of the polymorphic 4-hydroxylation. Studies with individuals, populations and families show that the oxidative metabolism of debrisoquine is under the control of a single autosomal gene locus (Evans et al 1980).



Debrisoquine 4-Hydroxydebrisoquine

Two phenotypes are seen in the population. The first and major phenotype in British Caucasian subjects (91% of population) is characterized by an ability to effect rapid and extensive 4-hydroxylation of debrisoquine with a metabolic ratio of 0.01–10; (this is the extensive metabolizer phenotype, EM). The second phenotype (9% British Caucasians) is characterized by a defective ability to hydroxylate debrisoquine. Such individuals excrete less than 2% of a dose as metabolites and have metabolic ratios in the range of 18–200; (these form the poor metabolizer phenotype, PM). The striking feature of this polymorphism is that the measured variable of drug oxidation (the metabolic ratio) reveals a variation in rates of hydroxylation up to 20 000-fold. The PM trait is recessive, and family and population studies show that the extent of dominance of the allele that controls rapid oxidation (D^H) over the variant allele (D^L) is approximately 30%. It has also been shown that the D^H and D^L alleles influence other oxidative reactions of drugs. They influence the aromatic hydroxylation of debrisoquine and guanoxan (Sloan et al 1978b) and that of phenytoin (Idle et al 1979a), the *O*-dealkylation of phenacetin (Sloan et al 1978b) and that of 4-methoxyamphetamine (Kitchen et al 1979) and the alicyclic hydroxylation of debrisoquine (Mahgoub et al 1977). Metabolic oxidation reactions that are not influenced by the debrisoquine genetic polymorphism include the aromatic hydroxylation of acetanilide (Wakile et al 1979) and the *N*-dealkylation of antipyrine (J.C. Ritchie, T.P. Sloan, J.R. Idle and R.L. Smith, unpublished observations). There is thus some specificity in the types of oxidative reactions that are influenced by the debrisoquine hydroxylation alleles, and this requires further investigation.

The uncovering of a genetic polymorphism of metabolic oxidation raises many questions including its toxicological implications. The studies described here were performed to clarify the significance of the polymorphism: (a) in the variability in presystemic metabolism and linked events such as systemic drug availability and responsiveness to drugs, (b) in the alternative pathways of metabolism that lead to formation of toxic metabolites in individuals who exhibit defective oxidation and (c) in metiamide-induced agranulocytosis.

PRESYSTEMIC METABOLISM OF DRUGS AND GENETIC POLYMORPHISM OF OXIDATION

Many drugs undergo extensive presystemic metabolism (first-pass loss) when they are administered orally (Blaschke & Rubin 1979). This loss can affect both systemic availability of a drug and linked events such as responsiveness to the drug. For many drugs, the major factor responsible for first-pass metabolism is hepatic oxidation. Since drug oxidation can exhibit genetic

polymorphism it follows that pre-systemic metabolism, systemic availability and response could also be influenced by the polymorphism. This aspect of polymorphic drug oxidation can be illustrated by the adrenergic neurone blocking drug, debrisoquine.

The alicyclic 4-hydroxylation of debrisoquine displays genetic polymorphism (Mahgoub et al 1977). As described earlier, this reaction is controlled by two alleles that act at the same locus and some 2–10% of the population, according to ethnic group (Idle & Smith 1979), are homozygous for the allele that determines impaired hydroxylation. This is manifest in a much reduced ability to perform the alicyclic hydroxylation reaction. Studies on the urinary elimination of debrisoquine and its major inactive metabolite, 4-hydroxydebrisoquine have established that the oxidation step occurs presystemically (Sloan et al 1978a). It was therefore of interest to investigate to what extent

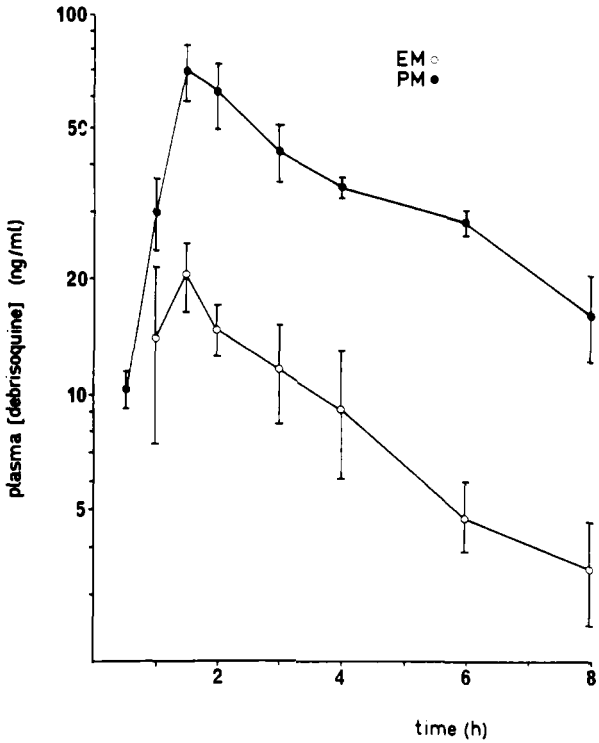


FIG. 1. Polymorphic oxidation and presystemic metabolism. Plasma levels (systemic availability) of debrisoquine in two oxidation phenotypes after a single oral dose of the drug. EM extensive metabolizing phenotype. PM poor metabolizing phenotype.

the polymorphic hydroxylation of debrisoquine affected both the systemic availability of the parent drug and responsiveness in terms of hypotensive effects.

For this purpose selected individuals of known oxidation phenotype (EM, $n = 4$; PM, $n = 3$) were each given a 10 mg oral dose of debrisoquine and serial blood and urine samples were collected for eight hours after the dose and were analysed for their debrisoquine content.

The plasma concentrations of debrisoquine attained in the two phenotypes are shown in Fig. 1. The PM subjects showed significantly higher concentrations of debrisoquine at all times from one hour after the dose. The measurements derived from these data are shown in Table 6. It was considered more appropriate to compare the phenotypes in terms of peak and mean plasma concentrations of the parent drug and in terms of areas under the time curves for plasma concentration, rather than in terms of measurements associated with postsystemic elimination mechanisms such as plasma clearance and half-life.

Fig. 1 shows that PM subjects were exposed to 3–4 times the amount of parent drug compared to EM subjects, irrespective of whether the inter-phenotype difference is expressed in terms of peak or mean plasma concentration or in terms of area under the curves. No significant inter-phenotype differences were observed in either elimination half-life or renal clearance.

These findings have both specific and general implications. First, the demonstration that oxidation phenotype decisively influences systemic availability of the drug almost certainly explains why subjects of the PM phenotype are much more susceptible to the hypotensive effects of small oral doses of the drug than are subjects of the EM phenotype (Idle et al 1978). Indeed, single oral doses of 20 mg of debrisoquine can elicit postural hypotension in PM but not in EM subjects, who may require many times this dose to evoke this response.

TABLE 6

Oxidation phenotype and presystemic metabolism of debrisoquine

Oxidation phenotype	*Peak plasma concentration (ng/ml)*	*Mean plasma concentration (ng/ml, 0–8 h)*	*AUC (ng/ml · min)*
EM($n = 4$)	21.9 ± 2.9	11.5 ± 0.7	105.6 ± 7.0
PM($n = 3$)	72.7 ± 11.8	37.7 ± 4.2	371.4 ± 22.4

Dose of debrisoquine: 10 mg orally
Results are mean values ± SD
AUC, area under the curve

In general the findings with debrisoquine suggest that genetically determined defective metabolic oxidation at the first-pass stage, with no alternative metabolic pathway available, can profoundly influence systemic availability of, and responsiveness to, drugs.

OXIDATION PHENOTYPE AND ALTERNATIVE (TOXIC) PATHWAY METABOLISM

When the oxidative metabolism of a drug is subject to genetic polymorphism while, nevertheless, alternative pathways of metabolic disposal of the drug also exist, the situation is more complex. An example of such a drug is phenacetin, which can undergo oxidative *O*-deethylation, deacetylation, aromatic hydroxylation and a number of other minor reactions (Brodie & Axelrod 1949, Büch et al 1967, Nery 1971). The metabolism of phenacetin is of considerable interest in view of the toxicity of certain of its metabolites (Goldberg et al 1971, Jaffe 1972). One metabolite, 2-hydroxyphenetidine, has been a focus of some interest since it is a potent producer of methaemoglobin. Some authors have speculated that the haemolysis and renal toxicity associated in some circumstances with phenacetin may be attributable to the formation of *N*- or 2-hydroxyphenetidine.

Not only does phenacetin undergo metabolism along multiple pathways but the relative importance of these pathways is modulated by many factors including the classical inducers (Conney et al 1966) and inhibitors (Welch et al 1966) of microsomal enzymes, dose size (Raaflaub & Dubach 1969), genetic factors (Shahidi 1967) and the concurrent ingestion of other drugs such as aspirin, caffeine and codeine (Thomas et al 1972). Production of the toxic 2-hydroxyphenetidine is decreased by smoking (Welch et al 1972) and increased by other analgesics (Welch et al 1966) and its formation is dose-dependent (Raaflaub & Dubach 1969). Its formation is also greatly increased in siblings who have an impaired ability to effect *O*-deethylation, probably because of a genetic defect (Shahidi 1967). The latter observations raise a question about the extent to which the metabolism of phenacetin is under genetic control and, in particular, about whether or not it exhibits polymorphism. The results of the studies described here showed that the *O*-dealkylation of phenacetin is under the same allelic control as that which determines the 4-hydroxylation of debrisoquine. Subjects who have a defective ability to 4-hydroxylate debrisoquine also show an impaired ability to *O*-dealkylate phenacetin. Furthermore, in subjects with this 4-hydroxylation impairment there is a propensity to generate more of the toxic metabolite, 2-hydroxyphenetidine, because of the existence of an alternative pathway which is not controlled by the same gene locus.

For the purpose of this study twelve volunteers (EM, $n = 5$; PM, $n = 7$) of known oxidation phenotype each took phenacetin (1 g orally). Serial urine samples were collected for up to 8 h after the dose, for estimation of paracetamol and 2-hydroxyphenetidine concentrations. From these data the rate constants describing metabolic *O*-deethylation and aromatic hydroxylation were calculated (Table 7). Marked interphenotype differences occur in the metabolism of phenacetin. Subjects of the PM phenotype displayed rates of paracetamol production 3–4 times slower than those of the EM subjects. In contrast, the reactions leading to the formation of the toxic 2-hydroxyphenetidine were apparently 2–3 times faster in the PM subjects than in the EM subjects. However, the apparent first-order rate constants describing aromatic hydroxylation in the two phenotypes indicate that PM subjects do not necessarily possess a preferential or more extensive ability, *per se*, to perform 2-hydroxylation. Rather, both phenotypes have a similar ability, but different body-loads of phenacetin substrate are available for aromatic hydroxylation because of the difference between the phenotypes in their capacity to perform *O*-deethylation. In other words, PM subjects have received a higher effective dose of phenacetin by virtue of their limited *O*-deethylation capacity and they have the unusually high concentrations of

TABLE 7

Oxidation phenotype and alternative pathways of toxic metabolite formation

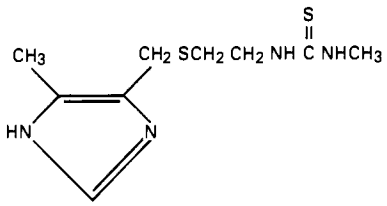
Metabolic measurement	*Oxidation phenotype*		
	Extensive (EM)	*Poor (PM)*	*Significance level*
	% of dose excreted in 0–8 h as each metabolite		*(2-tailed t-test)*
Paracetamol	58.1 ± 3.8	34.7 ± 2.5	S ($2P < 0.001$)
2-Hydroxyphenetidine	3.8 ± 1.7	9.3 ± 3.7	S ($2P < 0.01$)
	rate constants (h^{-1})		
$K_{f\ \text{paracetamol}}$	0.251 ± 0.088	0.092 ± 0.015	S ($2P < 0.005$)
$K_{f\ \text{2-hydroxyphenetidine}}$	0.015 ± 0.009	0.017 ± 0.006	NS
	ratio		
O-Deethylation: 2-hydroxylation	18.1 ± 10.6	4.2 ± 1.5	S ($2P < 0.01$)

Each subject received 1 g phenacetin orally. Serial urine samples were analysed for concentrations of paracetamol and 2-hydroxyphenetidine. Values are means ± SD (EM, $n = 5$; PM, $n = 7$). S, significant; NS, not significant; $K_{f\ \text{paracetamol}}$, apparent first-order rate constant describing paracetamol formation by *O*-deethylation; $K_{f\ \text{2-hydroxyphenetidine}}$, apparent first-order rate constant describing formation of 2-hydroxylation products by aromatic hydroxylation.

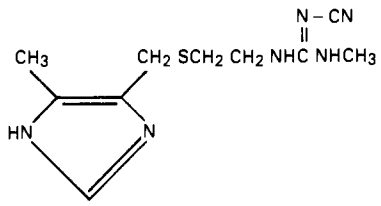
2-hydroxylated products that high doses of phenacetin are known to produce. This interpretation of the data is consistent with the finding that the aromatic hydroxylation of acetanilide to form paracetamol (acetaminophen) is not governed by the alleles that control debrisoquine hydroxylation and phenacetin *O*-dealkylation (Wakile et al 1979). Thus, subjects of the PM phenotype generate more of the toxic metabolite 2-hydroxyphenetidine than do subjects of the EM phenotype. Therefore, subjects of the PM status could be more susceptible to the toxic effects of phenacetin (eg. the propensity to develop methaemoglobin) that are attributable to the formation of 2-hydroxyphenetidine.

Nevertheless, the findings indicate what may be an important principle: when a genetically determined impairment of a particular metabolic route occurs in certain individuals, then the relative importance of secondary pathways may increase, with associated pharmacological and toxicological implications. This principle is, of course, already recognized in inborn errors of metabolism such as phenylketonuria. Impairment of the normal major pathway (aromatic hydroxylation) of phenylalanine is associated with a compensatory increase in the importance of otherwise relatively minor pathways of degradation of the phenylalanine side-chain.

OXIDATION PHENOTYPE AND METIAMIDE-INDUCED AGRANULOCYTOSIS



Metiamide



Cimetidine

Metiamide, a synthetic histamine H_2-receptor antagonist, first underwent extensive clinical trials in 1973 (Wood & Simkins 1973). During the course of these trials, however, it was found that the use of the drug was associated with the occurrence in a few individuals of reversible agranulocytosis (Burland et al 1975, Feldman & Isenberg 1976, Fleischer & Samloff 1977). Out of the 700–800 patients who received the drug, nine were reported to have developed agranulocytosis, and in one of these the condition was fatal. This naturally halted development of the drug. Metiamide well illustrates a nightmare in development of new drugs: after massive expenditure and extensive preclinical safety evaluation, the future development of the drug becomes

confounded at a late stage, by the unpredicted appearance within a small susceptible group of an unacceptable side-effect. Bone marrow biopsies taken from four of these patients showed that erythrocyte precursors were active and normal in appearance but that there was a marked reduction or absence of segmented neutrophils and metamyelocytes, which is consistent with some forms of agranulocytosis. The agranulocytosis was therefore thought to be due to a suppression in the formation of granulocyte precursor cells rather than to the peripheral destruction of mature granulocytes.

Metiamide was subsequently withdrawn from clinical use and replaced by cimetidine (Brimblecombe et al 1975). No cases of agranulocytosis attributable to cimetidine have been described, to date. Structurally, cimetidine differs from metiamide only in that a cyanoguanidine side-chain has replaced the thiourea moiety. The latter function was therefore implicated in the toxic mechanism and this was consistent with the discovery that several thiourea derivatives, particularly antithyroid drugs like propylthiouracil and carbimazole, can precipitate the development of agranulocytosis in sensitive patients (McGavack & Chevalley 1954). Although it is difficult to establish an animal model for drug-induced agranulocytosis in humans, it is noteworthy that haematological changes had been observed in beagle dogs that were given metiamide chronically (Brimblecombe et al 1974). After three months of the treatment, these changes occurred in at least five out of 68 dogs; two of the susceptible dogs were litter mates. These human and dog studies therefore suggested that metiamide-induced blood dyscrasia may depend on an hereditable factor that may produce either an inborn susceptibility of the haemopoietic tissues to metiamide, or a genetically determined variation in the metabolism of the drug.

Recent biochemical studies have indicated the significance for toxicity of metabolic oxidation of the sulphur centre of thioureas (Ziegler 1978). Oxidation of the thione group of thioureas can produce reactive metabolites of sulphenic acid and sulphinic acid, which can interact covalently with tissue macromolecules. It therefore appears that metabolic oxidation could be a key factor in the toxicity of thiourea derivatives. We therefore advanced an hypothesis that the toxicity of metiamide was associated, at least partly, with its metabolic oxidation. Thus, a subject with a genetically determined high oxidative capacity might be more likely to develop a toxic reaction to the drug than would a person with a low oxidative capacity. In other words it is possible that the genetic polymorphism of metabolic oxidation described earlier might influence susceptibility to the development of metiamide-induced agranulocytosis.

As a first step in evaluation of this hypothesis, we studied the metabolism of

metiamide in panels of volunteers whose oxidation phenotype (EM or PM) had previously been established (Idle et al 1979b). Four EM subjects and four PM subjects were each given orally a single low dose (one-tenth of the therapeutic dose) of metiamide (50 mg; 10 μCi). Urine samples over the first 24 h were analysed for parent drug, its glucuronic acid conjugate, and the oxidation products metiamide sulphoxide and hydroxymethyl metiamide (Table 8). These data revealed a small but significant inter-phenotype difference in the metabolic handling of metiamide. The EM subjects excreted a greater proportion of the drug as oxidation products, while the PM subjects excreted larger amounts of conjugation products, probably because of their lower capacity for metabolic oxidation. At higher doses the inter-phenotype differences may be larger because of the greater ease of saturation of the oxidation capacity of the PM subjects.

In order to investigate whether these inter-phenotype differences in metiamide metabolism were related to the development of blood dyscrasic reactions to the drug, we determined the oxidation phenotypes of patients who had experienced metiamide-induced agranulocytosis (Table 9).

All four patients who had developed agranulocytosis proved to be homozygous extensive metabolizers (i.e. genotypically $D^H \times D^H$). It is interesting that the patient with the highest metabolic activity (ratio 0.2) had developed agranulocytosis about 60 days after the start of metiamide treatment (800 mg/day), while for a second patient (ratio 0.6) the reaction was not observed until 100 days after treatment had begun.

TABLE 8

Oxidation phenotype and the metabolic disposition of metiamide

Metabolic measurement	*Oxidation phenotype*	
	% [^{14}C] excreted as each metabolite	
	EM	*PM*
Metiamide	58.0 ± 6.9	49.9 ± 6.3
Metiamide sulphoxide Hydroxymethylmetiamide	19.2 ± 4.6	15.4 ± 0.8
Conjugated metabolites[a]	23.1 ± 5.3	33.4 ± 5.2
Ratio $\frac{\text{conjugated metabolites}}{\text{oxidation products}}$	1.2	2.2

[a]mainly metiamide *N*-glucuronide

8 subjects (4 EM & 4 PM) each received 50 mg of [^{14}C]metiamide orally (10 μCi) and the 0–24 h urine was analysed for parent drug and metabolites.

Results are mean values ± S.D.

TABLE 9

Oxidation phenotypes of patients who have developed agranulocytosis associated with metiamide administration

Patient	*% of dose (in debrisoquine equivalents) excreted as each metabolite over first 8 h*						*Total % of dose excreted*	*Metabolic ratio*	*Phenotype*	*Genotype*
	D	*4-OHD*	*5-OHD*	*6-OHD*	*7-OHD*	*8-OHD*				
1	10.0	15.0	0.4	2.6	0.8	n.d.	28.8	0.5	EM	$D^H \times D^H$
2	6.4	26.8	4.3	2.7	4.8	1.7	46.7	0.2	EM	$D^H \times D^H$
3	24.7	20.6	3.8	3.7	4.5	1.2	58.5	0.7	EM	$D^H \times D^H$
4	10.1	14.4	n.d.	n.d.	2.4	0.3	27.2	0.6	EM	$D^H \times D^H$

Metabolic ratios were calculated from the quotient: % dose excreted as debrisoquine/% dose excreted as total oxidation products; D^H = allele governing extensive hydroxylation of debrisoquine.
(It was previously confirmed that the patients had agranulocytosis associated with metiamide administration by both blood counts and bone marrow examination.) Each patient took 10 mg debrisoquine (D) orally and the subsequent 0–8 h urine was collected and analysed for the products of oxidative metabolism, namely, 4-, 5-, 6-, 7- and 8-hydroxydebrisoquine.
n.d. = not detected.

These findings indicate that oxidation phenotype may control the propensity of some individuals to develop agranulocytosis on exposure to metiamide while others do not. About 20% of the Caucasian population exhibit metabolic ratios of 0.5 or less and are homozygous for the D^H allele. We may speculate that these individuals could be more at risk; their greater oxidation capacity may cause more extensive metabolic activation of sulphur centres in compounds such as metiamide, with the consequent formation of reactive and toxic metabolites.

Metiamide-induced agranulocytosis is a complex condition that may reflect many other variables, including dose, duration of exposure, other environmental factors and sensitivity of the haemopoietic tissue. Nevertheless, these findings suggest that a genetically determined metabolic factor controls interindividual variations in the propensity to develop agranulocytosis associated with metiamide administration.

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Discussion

Amos: What you have discussed, Professor Smith, is a classical Type II reaction; a drug is incorporated into a haemopoietic cell membrane, and then antibodies are developed against the membrane-fixed drug. The Ag/Ab complex fixes complement and the cell lyses.

Idle: Professor Smith and I have recently analysed some samples from a population in Nigeria. Debrisoquine hydroxylation is a good marker for several types of drug oxidation, so we studied this reaction in the Nigerian population in an attempt to determine if there was any variation within the population in the ability to oxidize the fungal toxic contaminant aflatoxin B_1 to a putative proximate carcinogen. We examined a population of 54 patients, aged 20–60, who presented with primary carcinomas that one might attribute to aflatoxin in the diet – hepatoma, and primary cancer of the stomach, colon and rectum. The patients were not receiving any other drugs at the time of the investigation. We also examined 130 healthy Nigerians in the same district, Ibadan. The control individuals were ranked, with respect to their ability to hydroxylate debrisoquine and, by association, several other compounds, and they were compared with the ranked distribution of the individuals presenting with carcinoma. If genetically determined differences in oxidation of aflatoxin had nothing to do with the aetiology of carcinoma, we should expect to find that the patients with carcinoma were distributed about the median in the same way as the control population. However, we found that 39 of the 54 patients with cancer had faster hydroxylating ability than the median person from the control population. The rank-sum tests that we have done account for the distributions of hydroxylating ability either side of the median value. We have found a statistically significant association between the genetically determined ability to hydroxylate debrisoquine rapidly and the presentation of certain carcinomas. There are also studies underway in this country on carcinoma of the bronchus, colon and bladder, in which activation of environmental carcinogens via oxidation has often been implicated. The premise in this case is that substances in cigarette tar, like benzo[*a*]pyrene, may undergo microsomal oxidation to carcinogenic metabolites at rates that vary between individuals in the population. We have applied the same analysis to these results, to find out whether genetically determined oxidation plays any part in these cancers or not. The U.K. data are, to date, inconclusive due to their paucity. Nevertheless, the 60 bladder, colon and bronchial carcinoma patients so far studied show a trend towards rapid hydroxylation, when compared with the control population of 350 individuals. Obviously, these studies should be extended to larger numbers of cancer patients.

We do not yet have enough data to enable us, for example, to attribute these carcinomas to cigarette smoking, although all the bladder and lung cancer patients were smokers or ex-smokers. Furthermore, in addition to genetic factors, one must also consider the *dose* of, and duration of exposure to, the carcinogen or environmental agent so that a combination of dose, duration and genetic make-up might explain individual susceptibility to these carcinomas.

B.A. Bridges: I would like some more information on the debrisoquine oxidation. You have said, Dr Idle, that the rate of the reaction reflects a genetic polymorphism. Is it caused by an autosomal recessive gene?

Smith: Dr Idle and I have studied 11 families and our evidence shows that impaired oxidative status is a recessive trait determined by an autosomal gene.

B.A. Bridges: Can you identify a single enzyme or co-enzyme that is deficient during this recessive condition and which could therefore explain the (seemingly) pleiotropic effects that you observe?

Smith: I can suggest that two alleles occurring in the population govern the metabolic oxidation of a number of drugs and influence the synthesis of a particular protein involved with the microsomal oxidation enzymes.

B.A. Bridges: Is there any evidence that all these different oxidations are mediated by the same oxidase? This seems to be unlikely.

Conney: The available evidence suggests that hepatic drug oxidations are not mediated by one enzyme but by multiple monooxygenases present in each individual (Kapitulnik et al 1977a, 1977b).

B.A. Bridges: There is an analogy here with *xeroderma pigmentosum* in which patients apparently exhibit a deficiency in one stage of DNA repair, which might be attributed to a missing incision enzyme. On closer examination, they prove to have the enzyme but they are deficient in this and a number of other repair systems. They have for example, lower concentrations of vestigial photoreactivating enzyme, and some of them have a defective apurinic endonuclease. This implies a genetic control over differences in the pattern of repair of DNA. Could these differences in pleiotropic effects within a population be related in any way to the spatial arrangement of the enzymes within the microsomal membrane?

Davies: I am not sure if this will answer your question Professor Bridges, but Professor Smith suggested that the deficiency that he observed in phenacetin oxidation might be one of capacity (i.e. the absence of a low-affinity, high-capacity enzyme). We have found that the oxidation of phenacetin by human liver depends on a high-affinity, low-capacity and a low-affinity, high-capacity component (Boobis et al 1980).

α-Naphthoflavone inhibited the high-affinity but not the low-affinity enzyme. This suggests that two forms of cytochrome *P*-450 take part in this oxidation. One explanation of Professor Smith's results would be that the subjects who are poor metabolizers of high doses of phenacetin or debrisoquine do not have the low-affinity, high-capacity enzyme; but with low doses, when the two groups show similar results, only the high-affinity form would be involved.

B.A. Bridges: Would you expect the single form of the enzyme to affect the

other metabolic steps one after another?

Davies: Yes.

Smith: Dr Idle and I have some data on animal models for the polymorphically controlled metabolism of debrisoquine in humans.

Idle: We duplicated a typical Nebert mouse induction experiment but we did it *in vivo*. We chose 10 strains of mice, all of which had been studied by Nebert (see Nebert 1979), and we gave them debrisoquine and established their ability to perform 4-hydroxylation of debrisoquine. Initially, all strains metabolized debrisoquine like the poor-metabolizing human phenotype, and none of them excreted more than 2% of the dose as 4-hydroxydebrisoquine. Then we gave them 3-methylcholanthrene and we were able to show that only in the responsive mice described by Nebert was the level of 4-hydroxylation increased (from 2% to 6 or 8%). So these results correlate with the inducibility of about 20 enzyme activities described by Nebert. These metabolic conversions seem to be under the control of a single gene locus, as far as induction is concerned.

Breckenridge: Professor Smith, have you shown genetic polymorphism in only one of the metabolic routes of antipyrine?

Smith: We cannot be sure of that. Some of the data suggest that one of the reactions of antipyrine metabolism, the 4-hydroxylation reaction, is controlled by a single gene locus and displays a genetic polymorphism parallel to that of debrisoquine. More studies are required, however, to clarify this.

Breckenridge: In the light of your findings, then, how do you interpret the results from studies of antipyrine metabolism in twins? Are the two findings compatible?

Smith: The studies on antipyrine have demonstrated to us that when a drug is metabolized along multiple pathways, it is difficult to discern significant genetic effects on a reaction. Antipyrine is metabolized by several oxidative reactions: hydroxylation of the methyl group; further oxidation to the carboxylic acid; 4-hydroxylation of the heterocyclic ring and oxidative *N*-dealkylation. Each of these reactions may be under separate genetic control as well as being variably influenced by environmental factors. Therefore it can be difficult to identify a polymorphic reaction, against this background of competing metabolic options. This is probably not uncommon, as many drugs are metabolized by competing pathways; our present approach is perhaps inadequate to discern significant genetic effects.

McLean: Professor Smith, you have carefully described a bimodal distribution. You have explained that you are essentially dealing with a single gene and that the cut-off point allows you to infer the genotype. But in your work with Dr Idle on the analysis of the enzyme activity in cancer patients, you have

disregarded the previous results and used a rank-order analysis.

Smith: The results we have obtained simply suggest an association between genetically determined oxidation status (e.g. the ability to activate certain chemical carcinogens by metabolism) and the occurrence of primary hepatoma and gastrointestinal carcinoma among Nigerians.

Idle: It is important to record the actual numerical value of an individual's metabolic ratio (Mahgoub et al 1977), instead of recording only the *mean* of each of the two phenotypes. For example, if you estimate the number of Nigerians who need to be phenotyped before a poor metabolizer is found, this does not allow for the fact that the first hundred examined may have very low metabolic ratios. The metabolic ratio is not normally distributed in the population and therefore non-parametric tests must be used. In this way you can take into account the full spectrum of variability in the population and not just the variability within one phenotype.

McLean: But these are not genotypes; you have not divided them into the two genotypes.

Idle: We could have plotted the actual numerical values of metabolic ratio instead of rank data and you would see that these aggregate towards rapid oxidation in the cancer group.

McLean: You have said that the actual values of the metabolic ratios do not express the genotype, but that the metabolic ratio indicates where there is a cut-off point (i.e. that the enzyme activity is bimodal and there is practically no overlap). Now, if that first point is true, then the subsequent analysis assumes that within a group with the same genotype what matters is the position of an individual's enzyme activity within that genotype, and here enzyme activity would be under environmental influence.

Idle: Physiological, pathological and secondary genetic factors may also give rise to variation within a given phenotype.

J. W. Bridges: In your study, Dr Idle, did you use any drugs for which there were no large genetically determined differences in metabolism in order to ascertain whether the drug metabolism in your subjects was generally depressed?

Idle: One of the problems that arises is that the presence of a large hepatoma might interfere with the phenotyping test, which might give an apparently impaired hydroxylation of debrisoquine.

J. W. Bridges: If the metabolism of a drug that is largely immune from genetic variations is examined in the same subjects wouldn't this help with phenotyping?

Smith: But our experience shows that people with hepatoma are ranked amongst the faster hydroxylators.

Idle: Yes; so the presence of hepatoma, which is known to reduce oxidative metabolism by the total hepatic tissue, may give rise to artifactually increased metabolic ratios in these patients.

Smith: In answer to your other question, Dr McLean, the data from Dr Idle's 58 subjects are not normally distributed but skewed to the left of the distribution, i.e. towards the faster oxidation status.

Hunter: One of the factors that is associated with the development of hepatocellular carcinoma in Africa is persistence of hepatitis B antigen (Sherlock 1975). Do you know how many of your patients had a positive hepatitis B antigen, and do you consider that such an association is significant in your results?

Idle: Before we started the Nigerian study we found evidence that high plasma bilirubins interfered with phenotyping because they depressed metabolism, possibly by competitive inhibition. We therefore used patients who had bilirubin concentrations of less than 15 mg/100 ml serum. We did not investigate specifically the hepatitis-associated antigen, but we eliminated people who had hepatitis, jaundice or elevated bilirubin concentrations, in case these conditions interfered with our phenotyping by causing apparently high values of metabolic ratio.

Hunter: But a patient may have persistent hepatitis B antigen and yet have a completely normal bilirubin concentration in plasma.

Idle: Yes, but amongst the 58 patients was a sub-group of 10 people with cancer of the stomach but without tumours; all of them had metabolic ratios of 0.7 or less, i.e. they were below the population median value of 1.0.

McLean: There is good experimental evidence about suppression of drug metabolism by transplanted subcutaneous tumours. This is similar to the suppression of hepatic enzyme activity which can be produced by inflammatory reactions in the model of arthritis based on inflammation in the rat paw (Villa et al 1978).

Smith: We should remember that it is difficult, for various reasons, to do sophisticated field work under the conditions that can pertain in West Africa. The patients we investigated were young people, aged between 20 and 30 years, with primary hepatoma. Because of economic pressures at the time, virtually no drugs were available for treatment, and the patients died within 3–4 months of admission.

McLean: Yes, I am sure that we ought to emphasize that. It is of vital importance that you are performing biochemical epidemiology of this type, even though ideal controlled conditions may not prove possible.

Conney: We have been trying to find drugs that can predict the rates of metabolism of specific carcinogens in samples of livers from different in-

dividuals (Kapitulnik et al 1977a). We have done comparative studies on the metabolism of aflatoxin B_1 and other drugs, using liver microsomes from biopsy samples in humans. These samples exhibited a many-fold range in activity for the metabolism of aflatoxin B_1 to mutagens. This metabolic activation of aflatoxin B_1 to mutagens by the various liver samples was highly correlated ($r = 0.92$) with metabolism of hexobarbitone but was poorly correlated with metabolism of zoxazolamine. It would be of interest to do similar studies with debrisoquine.

McLean: The level of the activation in your system, Dr Conney, correlates negatively with our results on tumour production in the whole animal, in which phenobarbitone protects against the toxicity of aflatoxin (McLean & Marshall 1971).

Idle: I wish to add something about the metabolism of aflatoxin. In a previous discussion, Dr Garner (p 64-65) mentioned that the mouse exhibits little binding of aflatoxin to DNA. We have found that mice (compared, for example, to rats), are poor hydroxylators of debrisoquine. In the absence of a good species difference in the results on aflatoxin B_1 metabolism, I would like to propose that mice have a poor ability to form the 2,3-epoxide of aflatoxin B_1; hence the observation of low aflatoxin binding to DNA in mice.

Garner: That's the point. Mouse liver microsomes are good metabolizers of aflatoxin, but *in vivo* mice appear to activate only a small proportion to the epoxide and most of the aflatoxin is converted to detoxification products.

Idle: In all other respects mice behave like the human phenotype that has a poor metabolizing ability. Another example of a correlation with your work, Dr Garner, is between our cancer data and yours on aflatoxin binding to mouse DNA. Rats, on the other hand, which behave like the human extensive-metabolizing phenotype with respect to debrisoquine metabolism, readily bind aflatoxin to their DNA. It is extensive metabolism in Nigerians that we are saying is associated with hepatoma development.

Oesch: The different monooxygenase forms do not show a preference for the types of chemical reactions that they will catalyse.

Higginson: To return to the Nigerian studies, we know that the offspring of mothers who are carriers of hepatitis B have a higher risk of liver cancer. The livers of Africans who develop hepatomas show histological abnormalities, suggestive of hepatitis and cellular death. It might be useful to compare groups of high- and low-risk children several years before the age at which hepatoma develops.

Farber: Human carriers of hepatitis B virus often have cells that are similar to those found in the rat, in hyperplastic nodules that precede cancer; these cells have abundant smooth endoplasmic reticulum which is high in epoxide

hydrolase but low in monooxygenases (Cameron et al 1976, Levin et al 1978).

Idle: Some groups in Nigeria do not intermarry for tribal reasons, and their offspring therefore exhibit similar phenotypes (i.e. similar oxidative activity). For example, in the Bendell state, 25% of people were poor metabolizers, compared to 8% of the general population. By coincidence we found very few tumours associated with those people. In contrast, in the Oyo State, where all the people we've examined are good metabolizers, a large number of people presented with these tumours. These are small numbers, but the connection may be more than coincidental.

Farber: How does the incidence of hepatitis B vary between the two states?

Idle: We don't yet know that, but the data should be available in Nigeria.

J. W. Bridges: In your work on metiamide, Professor Smith, you attributed the genetic abnormalities to cytochrome *P*-450 abnormalities. However, in the light of Ziegler's work on sulphur oxidation, the oxidation of metiamide may depend on a flavin-dependent monooxygenase enzyme (Ziegler 1978).

Smith: Oxidation of thio (S – H) and thione (C = S) groups can be accomplished both by Ziegler's enzyme and by microsomal cytochrome *P*-450.

De Matteis: Was the thiourea moiety of metiamide desulphurated to give the corresponding urea?

Smith: Yes, a urea analogue is formed in humans (from about 4 or 5% of the dose), by the metabolic desulphuration of metiamide. However, estimation of the amount of urea analogue is not a good index for total desulphuration because other products are also formed. The metabolic desulphuration of thioureas is complex, generating S-oxides, sulphenic acids and cyanamides, as well as urea analogues.

De Matteis: Could a reactive form of sulphur be produced?

Smith: Yes.

Gillette: Professor Smith, do you have any evidence that the acetylated form of 2-hydroxyphenacetin is formed from phenacetin in humans?

Smith: Yes, a small amount of 2-hydroxyphenacetin is excreted (0.1–0.2% of the oral dose of phenacetin). The major 2-hydroxylation product, however, is the sulphate conjugate of 2-hydroxyphenetidine.

Conney: One approach to assessing the role of environment in drug metabolism is to measure the plasma concentrations of the drug after repeated doses to healthy individuals who are permitted to pursue their normal life styles. We gave phenacetin to seven individuals at 5–6 week intervals. The details of these studies are described elsewhere (Alvares et al 1979). For one individual who received phenacetin on five occasions, we found that the peak plasma concentration of phenacetin was 1500 ng/ml on one occasion and 200 ng/ml on another occasion. This was due to differences in the first-pass

metabolism of phenacetin when it was given on several occasions. The plasma concentrations of the metabolite, *N*-acetyl-*p*-aminophenol, were not very different when phenacetin was given on several occasions. The areas under the curves for plasma concentration of phenacetin plotted against time gave estimates for the first-pass metabolism of phenacetin. For one individual, there was a 127% difference in the areas under the curves after administration of phenacetin on different occasions. However, for another subject, the intraindividual variability was 759%. In other studies we have shown that charcoal-broiled beef or cigarette smoking markedly lowered the area under the curves for phenacetin (Pantuck et al 1972, 1974, 1976, Conney et al 1976). The marked effects of diet and cigarette smoking on plasma concentrations of phenacetin indicate that environmental factors can have a marked effect on phenacetin metabolism.

Smith: I am perplexed by these findings, even though *we* are measuring rates of formation of metabolites and *you* are measuring rates of disappearance.

Conney: I believe that we are determining the effects of environmental factors on the first-pass metabolism of phenacetin by the gastrointestinal tract or by the liver, or both. In our studies on cigarette smoking (Pantuck et al 1972, 1974) we measured the rate of urinary excretion of *N*-acetyl-*p*-aminophenol and found no difference between smokers and non-smokers, even though there was, as I mentioned, a large difference in their plasma concentrations of phenacetin.

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Environmental Chemicals, Enzyme Function and Human Disease
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Immunologically mediated toxicity

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Abstract A major consideration which precedes the release of a new chemical into the environment of human beings is the possibility of its producing an undesirable or toxic effect. Toxicity is the clinical end-point of a mechanism which results in tissue damage. One such mechanism is mediated via the immunological system.

The effect of some chemicals on immunologically competent cells has been to induce perturbations of immunological competence. This is currently under debate as a toxic hazard, and the concept is explored here, particularly in relation to the action of alkyl tins.

Hypersensitivity, which is tissue damage resulting directly from an allergic reaction, depends on a substance acting as an antigen. Since many compounds of toxicological interest have a molecular weight less than 5000, the ability to induce an allergic state is governed in part by factors that influence antigen formation, covalent interactions with macromolecules and metabolism. These factors, together with an analysis of 'immunological specificity', are illustrated by experimental work on practolol, which demonstrates that an antibody present in human sera reacts with a product of first-phase metabolism.

The concept of immunologically mediated toxicity has recently achieved recognition among toxicologists, but the role played by a specifically activated immunological system needs clarification.

To date, two well defined roles have been assigned to areas of immunological–toxicological interaction; one concerns the toxic action of compounds on cells taking part in the allergic response and the other concerns compounds that act as antigens and induce tissue damage by hypersensitivity reaction.

A possible third interaction may exist, i.e. an allergic state may modify the expression of toxic damage, but this needs further development.

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1. COMPOUNDS EXERTING A TOXIC EFFECT ON CELLS TAKING PART IN THE IMMUNE RESPONSE

The generation of antibodies or specifically allergized cells is the end-point of an inductive process in which macrophages and lymphocytes are the chief cellular components.

Lymphocytes are a functionally heterogeneous population with two major sub-groups, T cells and B cells. T cells are lymphocytes that have been processed during ontogeny by the thymus gland and have various roles ranging from antigen recognition to a cell-killer function. The role of B cells is to make antibody in response to a coded message received from antigen-activated T cells.

Macrophage play a dual role in the development of an allergic response, in that they process antigen into a form that T cells can recognize and they act as intermediary cells in T and B cell cooperation. This subject is well reviewed by Asherson (1976).

If a compound is toxic to lymphocytes or macrophage the manifestation of toxicity may indeed be complex. In the simplest form of toxicity, the individual will be immunosuppressed, but if the toxicity is selective for a particular functional cell-type, the balance of the allergic response might be destroyed. This loss of balance might destroy both immunological tolerance and the development of auto-allergic disease; more speculatively, it might destroy immune surveillance and result in neoplastic growth. It must be emphasized that auto-allergic disease and neoplasia have not been shown unequivocally to result from an immunosuppresed state; nevertheless, such a mechanism is theoretically possible.

The induction by drugs of immunosuppression for clinical purposes has been studied for some time by immunologists and clinicians, but the attention of toxicologists was drawn relatively recently by the work of Seinen & Willems (1976) and by a review by Vos (1977).

Seinen demonstrated in a series of papers (Seinen & Willems 1976, Seinen et al 1977a,b), that various organo-tin compounds, when fed to rats, reduced thymus weights. These workers subsequently showed that treated animals were immunosuppressed and that there was a species variation in the response. These findings caused consternation among regulatory authorities, and the inevitable consequence now is that a mandatory requirement for testing the toxicity of drugs on the immune system will be made at some stage in the future. Whether sufficient time will be permitted for the evaluation of minor degrees of immunosuppression as toxic hazards before regulatory action is taken remains to be seen.

2. COMPOUNDS THAT ACT AS ANTIGENS AND INDUCE HYPERSENSITIVITY

Compounds which, by means of their antigenicity, produce hypersensitivity and tissue damage are the most difficult to pick out in any primary toxicology screen. This is due to constraints imposed on the system. These constraints include compound factors, which influence antigenicity; host factors, which govern development of the allergic state; and unknown factors, which push the allergic response into the realm of tissue damage.

Compound factors

Most chemicals of toxicological interest are compounds of small molecular weight which will not induce an allergic state unless they are firmly coupled to a macromolecule. Landsteiner & Jacobs (1936), using a halogenated nitrobenzene model system, showed that only those analogues which could bind covalently to proteins were capable of stimulating specific *hapten* antibodies. This requirement for covalent binding, however, is probably not absolute; electrostatic interactions will also be sufficient to form a complete antigen, i.e. a hapten–protein complex, provided that the binding energy approximates to that of a covalent bond. The evidence to support this hypothesis is based on experiments which demonstrate that antibodies can be formed against compounds of small molecular weight which are structurally incapable of binding covalently to proteins (Plescia & Palczuk 1964).

Host factors: genetic control

The overall immunological response to a xenobiotic of small molecular weight depends not only on genetic factors, which control the metabolic processes that generate immunologically reactive species, but also on the expression of specific structural genes that code for antigenic specificity.

(a) *Genetic control of metabolism:* At a meeting of this type, devoted to drug metabolism, it would be superfluous to discuss evidence for genetic regulation of the microsomal drug-metabolizing enzymes. It is sufficient to point out that variations in metabolic pathways can result in the formation of idiotypic or allotypic metabolites which have immunogenic potential.

(b) *Genetic control of the immune response:* The ability of an individual to respond immunologically to a specific antigenic stimulus is primarily governed by the expression of immune response (Ir) genes.

Much of the mapping of the Ir gene complex has been with synthetic antigens of restricted specificity (McDevitt & Chinitz 1969). It appears that Ir genes are inherited as autosomal dominants and are closely linked to major histocompatibility complexes. Recombinational events can take place within the Ir gene region indicating that there are at least two Ir loci, but further dissection of the gene systems is beginning to demonstrate considerable complexity, which will need considerable time to be worked out (Liberman & Humphrey 1972).

The absence of structural genes for a particular antigenic determinant can be taken to mean that the individual will not recognize that determinant as foreign. The corollary to this, i.e. that failure to respond to an antigen means that the specific structural gene is lacking, is not necessarily true; other genetic constraints that operate at a higher level, e.g. on the processing of the antigen by macrophage, can also influence the overall development of the immune response (Biozzi et al 1972).

Host factors: reactive chemical species

The requirement for chemicals of small molecular weight to bind to macromolecules before they can activate the immunological system highlights the mechanism for generation of reactive species.

Xenobiotics are metabolized mainly in the liver by the mixed function oxygenases located in microsomes.

By sophisticated biochemical analyses, it is possible to isolate and identify the more stable metabolic products, but short-lived, highly reactive intermediates are more difficult to elucidate. In terms of antigen formation, these reactive intermediates might be of considerable importance, since they could theoretically bind to albumin, secreted *de novo* by the hepatocyte, and they could leach into the circulation as hapten–protein complexes. This mechanism is at present speculative; it is, however, open to experimentation and there is evidence, from work on practolol, that reactive intermediates can induce antibody formation.

Practolol, a cardioselective β-blocking drug, was withdrawn from use because it produced severe and adverse clinical symptoms that became known as *the oculomucocutaneous syndrome.* Extensive epidemiological studies failed to demonstrate any common factors that indicated an underlying mechanism behind these symptoms (Nicholls 1978), but the studies showed that the syndrome was unique to practolol, even though other β-blocking drugs were structurally homologous. In addition, the studies clearly demonstrated that the response was not dose-related. These two findings sug-

gested that the adverse effect was idiosyncratic and that an allergic reaction might explain it.

Fig. 1 shows the structure of practolol. By chemical manipulation of the compound *in vitro,* it was possible to form two different immunogens. One was made by synthesis of the desisopropyl derivative and coupling of its side-chain to human serum albumin; the other was made by coupling at the *para*-position with desacetylpractolol. These immunogens readily stimulated specific antibodies in animals and it was reasonable to expect that they would react with any antibodies that were present in human sera and that were specific either for the side chain or for the *N*-acetyl function. But using these model determinants in sensitive radio-coprecipitation assays, H. Gregory (personal communication) failed to demonstrate any antibody activity.

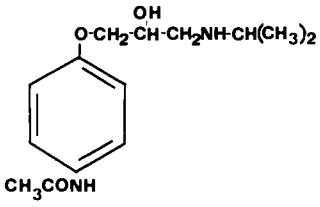

FIG. 1. The structural formula of practolol.

Antibodies could be demonstrated, however, if the drug was first added *in vitro* to a microsomal system that generated mixed function oxygenases (Amos et al 1977). The difficulty was in the extraction of the metabolites in a form that could be used in an assay. We achieved this by adding either an affinity-purified antibody to human 'O' cells or an iodine-labelled human serum albumin to the system. These compounds acted as scavenger molecules and provided the macromolecular moiety for the hapten (metabolite)–protein complex. The corresponding immunological assays that we used were a red cell-linked antigen–antiglobulin system for the rabbit antibody (Coombs et al 1953), and a modified Farr radio-precipitation technique for the labelled antigen (Amos et al 1978). The results demonstrated that antibody activity was associated mainly with an IgM antibody, detected in the haemagglutination system, and that the antibody was capable of specifically precipitating the hapten that was coupled to an homologous carrier protein.

The question posed by these results – i.e. what was the metabolic reactive species that stimulated antibody formation – required a two-phase experiment to provide an answer.

First, we altered the conditions governing the metabolism of practolol to see if the antigen was generated and secondly, we tested model compounds, which were homologous to the parent molecule, in an inhibition system, to see if they blocked specific interaction of the antibody with metabolic antigen.

Alteration of the conditions of the mixed function oxidases

(a) *Animal species.* Orton et al (1977) had previously shown that hamster liver microsomes bound practolol metabolites at a high rate and that the binding correlated with the high mixed function oxygenase activity known to be associated with this species (Litterst et al 1975). Therefore, we substituted hamster liver microsomes for rat microsomes in an attempt to increase the amount of metabolites formed.

(b) *Role of cytochrome* P-*450.* Cytochrome *P*-450 mixed function oxidases were shown to be involved in antigen formation by the demonstration that antigen production was NADPH-dependent. The definitive experiment, i.e. replacement of the oxygen atmosphere with nitrogen, was not performed and therefore, there is no absolute proof that formation of the antigenic metabolite(s) required the activation of cytochrome *P*-450; nevertheless such a possibility is likely to occur.

(c) *Effect of enzyme inducer.* Hamsters that donated the liver microsomes were pretreated with phenobarbitone or 3-methylcholanthrene (3-MC), to see if the active species could be further stimulated. We argued that if more antigen was generated under fixed conditions as a consequence of pretreatment of the animals with enzyme inducers, this should be detectable by the demonstration of antibody binding at a higher dilution of antigen. The results obtained (Table 1) were somewhat unexpected: phenobarbitone did not increase, and methylcholanthrene considerably inhibited, the formation of antigen. It is interesting that Orton et al (1977) failed to demonstrate any increased binding of ring-labelled practolol to hamster liver microsomes in phenobarbitone-treated animals and also that covalent binding was decreased in their 3-MC-treated group.

(d) *Addition of tricyclopropene oxide (TCPO).* TCPO, an inhibitor of epoxide hydrolase (EC 3.3.2.3), had no effect on generation of the antigen. Although this result does not disprove the formation of an epoxide intermediate, it suggests that such an intermediate is not part of the antigenic determinant.

TABLE 1

Induction of the microsomal enzyme system

Code for source of serum	*Animal pretreatment*	μg HSA[a] bound/ml original serum
MA44/75	no treatment	6.5
DO26/75		14.0
AD22/75		11.8
NHS		1.5
MA44/75	sodium phenobarbitone	8.2
DO26/75	100 mg/kg × 5 injections	12.0
AD22/75		12.8
NHS		1.8
MA44/75	3-methylcholanthrene	2.25
DO26/75	20 mg/kg × 5 injections	1.6
AD22/75		3.5
NHS		1.4

All sera were used at 1:50 dilution
[a]HSA, human serum albumin

(e) *Addition of glutathione.* Reduction in the amount of available antigen by the presence of glutathione suggested that the antigenic metabolite was an electrophilic species.

(f) *Addition of sodium fluoride.* The results obtained when sodium fluoride was added to the system were difficult to interpret; the response was variable and it was impossible to determine a trend. This finding agreed with the studies of Orton et al (1977), who showed a similar variable response in covalent binding, provided that the microsomes were prepared from pooled livers. Using individual liver preparations, they were able to discern a variable but consistent stimulation of microsomal binding.

Since hamsters can *N*-hydroxylate many *N*-acetyl aryl amines rapidly (Weisburger & Weisburger 1971) and since *N*-hydroxylation is increased by sodium fluoride (Hinson et al 1975), the data focused attention on the *N*-acetyl amino function of practolol as a possible site for metabolic conversion of practolol to the antigen.

Effect of model compounds

(a) *Compounds added to the generating system.* Practolol was compared with desacetylpractolol (1-(4-aminophenoxy)-3-isopropylaminopropan-2-ol),

methylpractolol (1-(4-*N*-methylacylamidophenoxy)-3-isopropylaminopropanol), ethanolamine, the tertiary amine and a contaminant of commercial practolol, and paracetamol for its ability to generate the antigenic metabolite.

Methylpractolol was the only tested compound which, other than practolol, formed material capable of binding the antibody. The data are given in Table 2. At first sight the finding appeared to refute the idea that metabolic activation of the *N*-acetyl amino site is central to antigenic formation, since methylpractolol is incapable of *N*-hydroxylation. T.C. Orton (personal communication 1977) pointed out that methylpractolol is unstable, and is a substrate for microsomal *N*-demethylase. Thus it is likely that the positive results we obtained with the two sera 28/77 and 32/77 (Table 2), were due to further metabolism of practolol that was formed during the incubation, by the action of demethylating enzymes.

TABLE 2

Interaction of antisera and control sera with practolol antigenic metabolite and with methylpractolol

	Code for source of serum	*Practolol antigenic metabolite*	*Methylpractolol*
Test	44/75	32	6.5
	26/77	77	6.9
	28/77	67	15.1
	32/77	60	15.0
	99/75	58	1.5
	117/75	70	2.6
Control	1	8.0	2.6
	2	13.5	4.3
	3	5.6	6.0
	4	1.0	2.0

Figures represent the percent of radioactivity in the precipitated antigen–antibody complexes

(b) *Model compounds added to immunological assay systems.* A number of model compounds were synthesized and kindly supplied by I.C.I. Pharmaceuticals for inhibition studies. The rationale behind these experiments was that if a model compound resembled the specific antigen structurally, it would compete for antibody, and reduce the amount available for interaction with the antigen. A summary of the results is given in Fig. 2.

Compounds in which the isopropylaminopropan side-chain was modified failed to inhibit antibody binding, as did the major terminal metabolite of practolol, 3-hydroxypractolol. Partial inhibition was achieved with desacetyl-

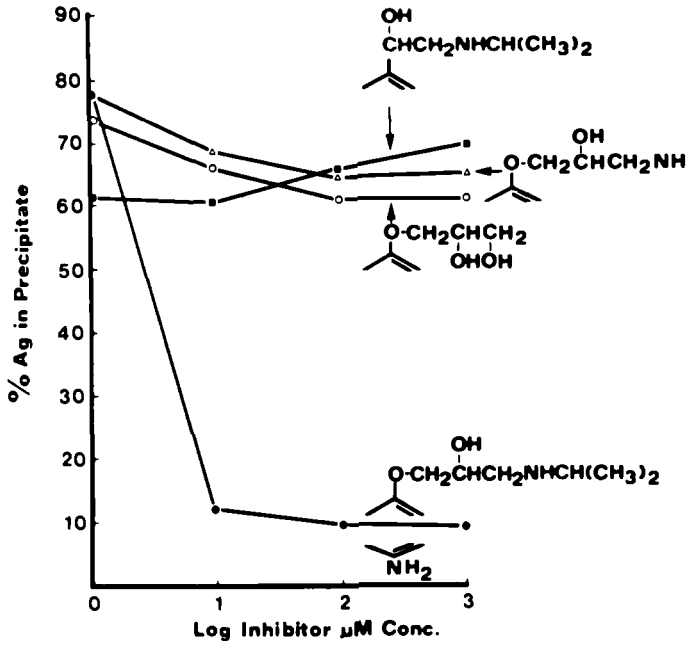

FIG. 2. Inhibition of antigen–antibody interaction by model compounds. Ag, antigen; ■ phenoxy-derivative; △ *des*isopropyl practolol; ○ 1-2 propane diol derivative; ● *des*acetyl practolol.

practolol, a finding which is difficult to explain. It was known that substituting desacetylpractolol for practolol in the incubation system did not generate material that would interact with antibody, although desacetylpractolol would avidly bind covalently to microsomes. One possible explanation would be that the compound was contaminated with practolol, but clearly more work is needed for investigation of a possible heterogeneous antibody response.

Implication of the study for toxicology

The model currently under development for determining the nature of the practolol antigen might also provide an alternative approach for studying the role of reactive intermediates of both drugs and industrial chemicals. There is an increasing awareness that reactive electrophilic species may be important in many toxicological reactions, including carcinogenesis, and that conventional biochemical techniques may lack the sophistication to identify short-lived

metabolites which interact covalently with macromolecules. An immunological approach could identify such structures, particularly if there is a good understanding and a willingness to collaborate between immunologists, chemists and biochemists.

3. EFFECT OF IMMUNOLOGICAL ACTIVATION ON THE EXPRESSION OF TOXIC TISSUE DAMAGE

In contrast to the other areas in which immunological methods would be useful, this third area is more speculative. The contention is that many toxic substances also act as antigens and will activate the immunological system. Thus, pathogenesis of any tissue damage must reflect both the specific biochemical lesions induced by the toxic action of the compound and the modifying influences exerted by the immunological system.

This concept has not yet been tested experimentally, but there is evidence to indicate that such a mechanism might operate. An investigation by Reeves & Krivanek (1974) of experimentally induced berylliosis showed that beryllium administered to guinea pigs by inhalation produced toxic effects in the lungs similar to the effects of berylliosis in humans. These workers also demonstrated that guinea pigs in which beryllium had failed to induce cutaneous sensitization were more prone to the toxic lung lesion; the existence of an allergic state to beryllium seemed to protect against the toxicity.

Since the immunological system is one of the body's basic defence mechanisms, it is possible that the extent of a pathological lesion initiated by a limited and toxic biochemical event might be modified by the constraints of a specific allergic reaction.

We are currently using beryllium in a number of model systems (H.E. Amos & D. Skilleter, unpublished) with the objective of exploring this concept and determining its significance for toxicology.

CONCLUSIONS

The three immunological models discussed here are by no means exhaustive, but they represent areas in which immunological reactions are causing concern for toxicologists. A practical approach is urgently required both for the evaluation of xenobiotics as antigens capable of inducing hypersensitivity and for realistic testing of compounds for their toxic effects on immunologically competent cells. At the same time, some original and imaginative ways of using the allergic response in toxicological research are necessary. We also need to integrate the various inter-relating forces that eventually produce a pathological response – the toxic reaction.

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Discussion

Orrenius: Is it probable that any reactive metabolite of this kind could interact with proteins to produce an immunological response, and that this may potentiate the toxic interaction of the metabolite? The immediate response to the accumulation of massive amounts of reactive metabolite may distract us from consideration of a more long-term effect.

Amos: Yes, that is possible. We haven't examined the toxicological significance of an antigen–antibody interaction, but many of these small molecular weight compounds of toxicological interest must combine with a macromolecule. Metabolites of such compounds are highly reactive and could combine with macromolecules to form immunogenic complexes which give rise to an activated immunological state, and so alter the expression of toxic damage. When we look at toxic damage, we may see a balanced interaction between the effect of a compound that produces a biochemical lesion, and the effect of the immunological system acting to prevent secondary pathological events which would make the lesion worse.

Garner: Conversely, it could be argued that the system 'mops up' compounds as they enter the body; do you think that this could be used as a protective mechanism?

Amos: Yes, it *is* used as a protective mechanism. When an antigen–antibody complex forms, phagocytosis increases and the antigen is removed; that is the principle of opsonification.

Smith: You pointed out, Dr Amos, that practolol produces the oculomucocutaneous syndrome in some individuals while other drugs damage the haemopoietic system and produce mast cell breakdown. Are there any theories about what determines the target tissue for the immune-based toxic response to a drug?

Amos: There is no simple answer to that. When the mast cell is involved, a particular antibody is formed, the IgE antibody, and one of its biological properties is that the end that is not attached to the antigen has a 'receptor' which binds it to mast cells and basophils. Thus, tissue damage is likely to be confined to sites where antigen interacts with the passively sensitized cells, e.g. in the lungs.

In another type of reaction, antigen is incorporated, by various mechanisms, into cell membrane components. On contact with antibody, and possibly with complement, the cell becomes damaged. This type of reaction is mainly confined to the formed elements of the blood.

I think the overall principle is that tissue damage usually occurs where there is an accumulation of antigen.

De Matteis: But not everybody who came into contact with the drug developed the oculomucocutaneous syndrome.

Amos: That's right. If we return to Professor Smith's results, there is very good evidence that only a certain proportion of the population generates antigenic structures which are able to react biologically with tissues.

De Matteis: Is the idiosyncratic nature of the response due to the generation of this immunologically effective species, or is it due to an individual variation in the immunological response? In other words, could it be that many people form these species, but their immune systems fail to respond to them?

Amos: Probably both. There are immune-response genes that recognize determinant structures, which are about the size of 20 amino acids. A determinant is capable of stimulating an antibody that will exactly fit its antibody-combining site. A large protein molecule could therefore react with many antibodies which all have different determinant specificities. So the immune-response genes control whether a response occurs to a particular (foreign) chemical structure. Other genetic systems operating at a higher level of complexity are probably also involved: if the body recognizes an antigen as foreign, it must possess the required immune response genes but the antigen must then be processed by macrophages, to emerge as 'superantigen' which will react with T cells. There is evidence to show that populations can be subdivided into high and low responders in relation to this antigen, and that the genetic control is at the level of macrophage handling (Biozzi et al 1972).

Davies: Since the hamster apparently produces reactive metabolites of practolol, are you surprised that despite all efforts you have been unable to produce any lesions in laboratory animals?

Amos: Yes. This drug exerts some of the most serious adverse effects that are known, and which had not previously been seen after drug treatment, and yet these diverse responses were not detected in animal studies. We do not understand the mechanisms responsible for these reactions, and we have no suitable animal model. Consequently, no further advances with practolol are likely to be made which would enable us to improve our system of safety evaluation.

J. W. Bridges: Studies in which antisera to chemicals are deliberately raised by the process of binding them onto albumin have suggested several possible causes of the variability in antibody response between individuals to administered drugs. First, more than one drug molecule per carrier macromolecule is normally required to initiate antibody production. The optimum ratio for raising antisera appears to be between 5 and 50, i.e. the protein must be heavily loaded with the drug. Second, antigenicity will depend on

the nature and position of the bonding of the drug to the carrier (Marks et al 1980). It is difficult to envisage how a carrier molecule could become labelled with sufficient intensity unless it was in very close proximity to cytochrome *P*-450; could the nascent protein (albumin), as it comes off the endoplasmic reticulum, be the target? Do you know of any studies in which nascent protein has been examined for covalently bound drug?

Amos: No. But theoretically, it would be possible to do that with an isolated hepatocyte culture into which a drug is injected. Small quantities of nascent albumin, probably coupled to the drug, could then be removed and used *in vitro* to search for a primary immune response. That is perfectly feasible immunologically.

Conney: Since there are cytochrome *P*-450s in many tissues, is it possible that you have not observed toxicity in the animal model because the tissue of interest does not form the reactive metabolite? We may need to study metabolism of the chemical in a human tissue which is sensitive.

Amos: Yes, that's a possibility. We know that practolol localizes in certain tissues, but to my knowledge nobody has looked at the cytochrome *P*-450 species or at the metabolic species of the drug in those tissues.

Breckenridge: How extensively has the metabolism of practolol been studied in those patients who developed this toxic reaction?

Amos: The two metabolites identified in rats and hamsters, the 3-hydroxy- and the *des*acetyl-derivative, have not been identified and neither has any other metabolite been found in humans. However, some metabolic process must occur because there is a certain amount of material which is unaccounted for by classical metabolic studies. Reeves et al (1978) have shown that the deacetylation can occur in humans but the *des*acetyl product has not been identified.

Smith: Dr Amos, you discussed the multiple genetic control of metabolism, recognition and gene expression, and this seems to be the essence of adverse responses to drugs. A specific combination of genetic polymorphisms and environmental factors may be necessary before a certain adverse response occurs in an individual. This requirement may explain the rarity of some adverse reactions. The problem with studying the reactions to practolol is identification of all the predisposing genetic and environmental factors that are operating. This is why it is difficult to develop an animal model for this situation.

Amos: It is very important that we should be able to provide positive evidence to support that hypothesis, rather than to reach it by negative evidence.

Gillette: To return to the things that prevent binding to the antibody, have

you ever tested an antigen containing the side-chain itself, without the ring configuration?

I ask this because there is a peculiar 3,4-epoxidation mechanism by which the phenacetin can be activated simultaneously to two chemically reactive metabolites (Nelson et al 1979). The ethyl group comes off as a carbonium ion, and can react with glutathione; in addition, the epoxidation product can react with glutathione to form a complex. I wondered if the antibody could recognize the side-chain itself, even though activation may occur at the ring?

Amos: We haven't looked specifically at the side-chain in isolation, but it is common in a number of other β-blockers and there appears to be no cross-reactivity. If the side-chain was an integral part of the antigenic determinant I would have expected some degree of cross-recognition.

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Environmental Chemicals, Enzyme Function and Human Disease
DAVID EVERED and GERALYN LAWRENSON

Toxicological significance of liver hypertrophy produced by inducers of drug-metabolizing enzymes

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Abstract Changes in enzyme activity due to induction by chemicals is an important property that can determine the type of response seen in tissues exposed to environmental chemicals. Two major types of response, acute irreversible liver cell injury or death (necrosis) and long-term cancer induction, are discussed in terms of their modulation by enzyme induction. Most commonly, enzyme induction leads to a more severe toxic response by the liver, and to more cell death. However, inducers may have a protective effect, especially in carcinogenesis, when they most frequently protect against cancer induction if used early in the process. There is a discrepancy between this observation and the increase in mutagenic activity of liver preparations observed after induction. However, when enzyme induction occurs at a later stage, after initiation, it often accelerates or promotes cancer induction. Also, new cell populations constantly observed during liver carcinogenesis are composed of very hypertrophic hepatocytes containing a large amount of smooth endoplasmic reticulum. This is associated with a radical change in enzyme activities in the reticulum, which may account in part for the characteristic resistance exhibited by initiated cells to hepatotoxins and carcinogens. The resistance is considered to be an important property that may play a key role in the development of cancer under some circumstances.

It is now eminently clear that the response patterns of cells and tissues to some toxic hazards are intimately dependent upon the physiological state of those tissues during the times of exposure. Major quantitative and qualitative differences in the way cells respond can be seen as functions of many physiological and environmental influences including the phase of development of the organism, both prenatal and postnatal, and its age, nutrition, past and present exposure to drugs or environmental chemicals and the presence and nature of pre-existing disease.

Although patterns and mechanisms of enzyme induction are extremely worthy of study in themselves, modulations in enzyme induction are significant

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for both short-term and long-term function and survival of an organism. This broader approach necessitates bridging the gap between quite different levels of organization in biological systems. We are only now beginning to explore the relationships between molecular or biochemical processes on the one hand and the function of cells and organs on the other. Our successes to date have been few and we have yet to define complex physiological or pathological processes in detailed molecular terms. Yet the possibilities for this are increasing as we unravel the chemical anatomy and physiology of cells.

One important challenge is in the relation between a quantifiable chemical or biological change and the biological response to such a change. Let me illustrate by a simple example (Fig. 1). The exposure of an animal to a single dose of dimethylnitrosamine leads to methylation of DNA, RNA, protein and presumably other cell constituents and to death of liver cells. The degree of methylation is continuous and proportional to the dose. The appearance of cell death is discontinuous i.e. there is no detectable lethal response below a dose of about 13 mg/kg but increasing necrosis occurs at higher doses (Farber et al 1976). As yet we cannot explain this phenomenon. Does the discontinui-

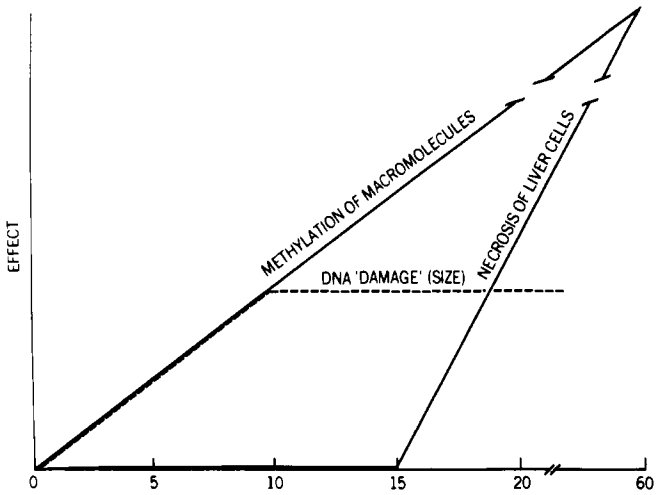

FIG. 1. Schematic representation of the dose responses of 3 different effects of DMN in rat liver: methylation of a macromolecule, induction of DNA damage as measured by strand breaks in alkali (DNA size), and the induction of liver cell necrosis. Note especially the sharp onset of necrosis when the dose is increased above 15 mg/kg. Even a small decrease in the generation of the active moiety or moieties of DMN by the liver could result in a striking loss of a new qualitative effect, necrosis. (Reproduced with permission from Cancer Research, Farber et al 1976.) DMN, dimethylnitrosamine; abscissa: dose (mg/kg).

ty in cell response reflect a similar discontinuity at the molecular level or is the relationship more subtle?

In this presentation, I plan to select examples of both short-term (acute) and long-term (chronic) response patterns and relate these to our current understanding of enzyme induction and cell hypertrophy. Even though most cells or tissues may exhibit enzyme induction and cell hypertrophy in response to environmental chemicals, our knowledge about responses of the liver is more advanced and I shall restrict myself to consideration of this organ. Perhaps a detailed understanding of mechanisms within the liver will help us eventually to understand the responses of other important organs or tissues.

Although the subject assigned to me was liver hypertrophy produced by drug-metabolizing enzyme inducers, I shall concentrate largely on the commonest result of enzyme induction by many chemicals, that associated with proliferation of the endoplasmic reticulum (ER). However, in liver cell hypertrophy induced by some chemicals, most notably among hypolipidaemic agents such as clofibrate and nafenopin, 'proliferation' of other organelles sometimes occurs. These compounds induce a striking proliferation of peroxisomes which leads to significant enlargement of the liver cell cytoplasm (Leighton et al 1975, Reddy & Krishnakantha 1975, Kolde et al 1976, Moody & Reddy 1976). The relationship between the increased number of peroxisomes and the toxicological effects is poorly understood.

CELL DEATH

The induction of irreversible damage that leads to death of cells appears to be a multi-step process involving more than one cell organelle and also including the cell membrane as an important target in a later step (Farber 1971, Farber et al 1971, Trump & Arstilla 1975, Popp et al 1978, Farber 1979). The multi-step process appears to be present both in toxic injury induced by chemicals and in ischaemic necrosis (Chien & Farber 1977, Chien et al 1977, 1978).

Most chemicals that are hepatotoxic require preliminary metabolic conversion to active derivatives (Slater 1978) which appear to initiate the series of events leading to hepatocyte death. Since the liver is probably the most active site for metabolic conversion of xenobiotic or foreign agents, it is also a common target organ. In addition, since most environmental chemicals are metabolized by the endoplasmic reticulum, this organelle is most closely linked to toxic injury of the liver (Schulte-Hermann 1979). This link involves not only the generation of active moieties by these membranes but also the attack on the membranes themselves by the active compounds. Thus, endoplasmic

reticulum of hepatocytes is one of the most vulnerable targets in both reversible and irreversible cell injury (Smuckler & Arcosay 1969, Shinozuka 1971, Reynolds & Ree 1971, Trump & Arstilla 1975).

Predictably, a subsequent increase in the amount of the ER, coupled with enzyme induction, intensifies the cellular damage induced by several environmental chemicals. The first clear-cut example of this was presented by McLean & McLean (1966) who showed that the administration of phenobarbitone or DDT would reverse the initial inhibition of hepatotoxicity produced by carbon tetrachloride (CCl_4) administration to rats on a protein-free diet. This group subsequently reported the rapid induction of liver cirrhosis when phenobarbitone was administered with CCl_4 (McLean et al 1969, McLean 1975).

Similar enhancement of cytotoxicity, including cell death, has been reported for carbon disulphide (CS_2) (Bond & De Matteis 1969, Bond et al 1969), halogenated aromatic hydrocarbons (Reid et al 1973) and thioacetamide (Hunter et al 1977). Also, several drugs such as halothane, paracetamol (acetaminophen) and furosemide used in clinical medicine, can be made considerably more toxic by pretreatment of the organism with enzyme inducers (Gillette et al 1974, Rao 1977).

However, the phenomenon is by no means clear-cut. Some inducers not only fail to increase the hepatotoxicity of some chemicals but even protect against it. For example, pregnenolone-16α-carbonitrile and 3-methylcholanthrene have been reported to protect against the hepatotoxicity of CCl_4 (Schulte-Hermann 1974, Tuchweber et al 1974). This is not surprising because of the complexity of the structure and function of the ER (DePierre & Ernster 1977) and the differences in the patterns of enzyme induction produced by different xenobiotic agents. In this context, although it has been known for many years that different chemicals induce the activity of different cytochromes and other enzymes in the ER, our work has indicated that this is more complex than was previously thought. For example, xenobiotics produce a minimum of 12 different patterns of induction of microsomal proteins in the molecular weight range of 40 000–60 000, including various cytochromes and epoxide hydrolase (EC 3.3.2.3) (Cameron et al 1979a,b, Sharma et al 1979).

The various biological consequences of the generation of reactive molecules from environmental chemicals depend on the fate of those molecules. It has been established that the concentrations of possible trapping agents, such as glutathione and other competing reagents, can influence in a major way the effects produced by administration of some chemicals, e.g. paracetamol and some carcinogens. Some additional factors that might be important in deter-

mining the toxicity of some specific compounds, such as phenacetin and paracetamol, have been discussed recently in detail by Gillette (1979) in an interesting and provocative article.

Thus, there is a complex relationship between hypertrophy of ER in liver cells and the acute biological effects of some environmental chemicals. Yet, some of the key elements that are involved in this relationship have been recognized. These include the competition between enzymes and cellular constituents for interaction with the active derivatives generated from foreign chemicals. This should lead in time to the development of a more rational basis for predicting acute toxicity of environmental chemicals.

It should be emphasized, however, that no studies to my knowledge have been made on the possible influence of cell hypertrophy and enzyme induction on steps in the necrogenic process subsequent to the initial activation of the administered compound. It is certainly reasonable to assume that membranes and other target sites in cells may have different potentials for irreversible damage depending upon the physiological state of the cell. For example, the protein-synthetic apparatus of the liver cell varies considerably in its response patterns to toxic derivatives of dimethylnitrosamine, CCl_4 and other chemicals. These patterns depend upon the equilibrium state of the ribosomes (Farber et al 1971, Farber 1972). Carbon tetrachloride has no deleterious effects on the protein-synthetic apparatus when the ribosomes are 'frozen' on polysomes. However, one or more components of the ribosome are very susceptible to irreversible damage by CCl_4 when the ribosomes are in their free or unbound form.

CARCINOGENESIS

There are at least two important aspects of the relationship between cell hypertrophy resulting from enzyme inducers, and chronic toxic responses such as carcinogenesis: (a) the effects of reversible enzyme induction *per se* on one or more steps in the process and (b) the effects in new cell populations of cell hypertrophy originally induced by environmental chemicals but no longer dependent upon their presence.

(a) Reversible enzyme induction – initiation and promotion

In general some of the principles discussed above in relation to acute toxicity apply to at least one chronic response pattern, cancer induction, but some major discrepancies requiring rationalization are evident even in the present state of knowledge.

It is now well established that the majority of carcinogens are not active *per se* but, like acute hepatotoxic agents, require preliminary metabolic conversion to reactive derivatives in order to initiate development of cancer. Although the similarity in activation in acute and chronic hepatotoxicity is striking, there are also some differences.

As discussed above, cell hypertrophy produced by enzyme inducers increases lethal toxicity of many chemicals towards liver cells. If the generation of cell injury and the initiation of carcinogenesis are similar in principle, then enzyme induction should promote cancer initiation and this in turn should be associated with an equal or even increased incidence of cancer compared with that in tissue in which enzyme induction has not occurred. Also, if the active initiating species is, or is related to, mutagenic species, then in conditions of enzyme induction there should be a parallel increase in the generation of mutagens and in the initiating ability of a chemical.

The opposite results are observed in the laboratory. When phenobarbitone or 3-methylcholanthrene is administered simultaneously with an active procarcinogen such as 2-acetylaminofluorene, diethylnitrosamine or 4-dimethylaminoazobenzene or a derivative of one of these, induction of liver cancer is prevented or considerably delayed. Similar findings were more recently reported for α-hexachlorocyclohexane and polychlorinated biphenyls. Thus, when enzyme inducers are given early they can prevent rather than enhance the cancer formation produced by chemicals (Farber & Cameron 1980).

In addition, homogenate fractions (S9) from rat liver which has been subjected to induction by polychlorinated biphenyls, are often used for conversion of many procarcinogens into mutagenic agents in the Ames test, or in other short term genotoxic tests. However, the inducers used for such preparations are often effective inhibitors of carcinogenesis.

These discrepancies and contradictions are difficult to explain at present. Presumably different, activated, forms of carcinogens are involved in the different end-points and the genesis of these is modulated in different ways by xenobiotic agents.

Fewer problems seem to be apparent in reconciling different results on the process of carcinogenesis that follows initiation. Once initiation has occurred, by brief exposure to a carcinogen coupled with a round of cell proliferation (Cayama et al 1978), several enzyme inducers facilitate or promote the carcinogenic process. Phenobarbitone, DDT and butylated hydroxytoluene (Peraino et al 1978, Pitot et al 1978), polychlorinated biphenyls (Kimura et al 1976), alpha-hexachlorocyclohexane and cyproterone acetate (Schulte-Hermann 1978, Ohde et al 1979a,b) have all been shown to accelerate or facilitate the carcinogenic process. Since many enzyme inducers also act as

mitogens in the liver, the relative contribution of enzyme induction and mitogenesis to promotion of liver tumours remains to be clarified. In addition, the step or steps influenced by these agents in the carcinogenic process must be determined.

Using a new model designed to allow the study of carcinogenesis in liver sequentially (Solt & Farber 1976, Solt et al 1977), we have found that phenobarbitone or 3-methylcholanthrene prevents the selection by 2-acetylaminofluorene of initiated cells induced by diethylnitrosamine. This model, which uses 'differential inhibition' to encourage the growth of initiated cells, is influenced differently by enzyme inducers than are the models of Peraino, Schulte-Hermann and others who use 'differential stimulation' for selection or promotion.

This emphasizes that the effects of enzyme inducers on the course of chemical carcinogenesis in the liver are very much a function of the conditions and circumstances under which these agents are used. Either protection or enhancement can be observed reproducibly, depending upon the time at which the inducers are administered and the particular step or steps under study. This is not a new principle, but it must be emphasized in any discussion of the way cell hypertrophy due to enzyme inducers influences cellular toxicologic responses.

(b) Cell hypertrophy in new cell populations

An important aspect of cell hypertrophy in carcinogenesis is the presence of abundant smooth ER in focal hyperplastic lesions that as a group are known to be precursors for the genesis of liver cancer (Farber 1973, 1976). When the number of initiated cells is increased, by selection or promotion, the cells soon acquire increasing amounts of smooth ER (Ogawa et al 1979) which eventually dominates the topography of the cells. Such hypertrophied hepatocytes, or 'ground glass hepatocytes' are an important hallmark of the late preneoplastic stages of liver carcinogenesis (Farber et al 1979).

Associated with the increasing hypertrophy due to the abundant ER are characteristic changes in the pattern of enzyme activity. There is a large decrease in the activity of cytochrome *P*-450, aryl hydrocarbon hydroxylase (EC 1.14.14.1) and other components of the monooxygenase system in the ER (Gravela et al 1975, Cameron et al 1976, Okita et al 1976), while epoxide hydrolase shows a characteristic elevation in activity (Cameron et al 1979a, b, Kizer et al 1979).

This pattern of enzyme activity might well account in part for the resistance to hepatotoxic agents demonstrated by the hepatocytes of the nodules (Farber

et al 1976). This resistance appears early in new populations induced by several carcinogens and is believed to be the first biologically significant change associated with initiation of carcinogenesis. In fact, it is attractive to consider that the rare resistant cell may be the initial progenitor from which cancer ultimately evolves under conditions of prolonged exposure to a carcinogen. Under such circumstances, it is conceivable that differential inhibition is the major mechanism for selection and promotion of precursor cells for cancer development.

In this context, it is noteworthy that hepatitis B virus infection is associated with the appearance of hepatocytes that resemble closely the ground glass hepatocytes in chemical carcinogenesis. The ground glass hepatocytes in patients exposed to hepatitis B virus are usually positive for one or more of the viral antigens.

If this similarity between the ground glass cells seen in hepatitis and in chemical carcinogenesis has any biological significance, it may be that two suspected causes for cancer of the liver, a virus and chemicals, may induce a common kind of cell, conceivably by a synergistic action.

In summary, it is clear that cell hypertrophy resulting from enzyme induction plays an important role in several facets of the response of the liver to environmental carcinogens, including acute injury responses and the induction of liver cancer.

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Discussion

Oesch: The polycyclic hydrocarbons usually do not lead to a proliferation of endoplasmic reticulum, or to liver cancer. If you treat animals under conditions in which polycyclic hydrocarbons do produce cancer, e.g. after partial hepatectomy, do you then see a proliferation of endoplasmic reticulum?

Farber: We do not yet know. All we know is that with diethylnitrosamine cancer develops in this system.

Conney: Administration of several carcinogens to rodents increases hepatic epoxide hydrolase and DT-diaphorase (EC 1.6.99.2) activities. Are these responses part of the cell's defence mechanism?

Farber: I don't know. A significant amount of the genome is probably related to how the cell behaves in a hostile environment. Planet Earth has always been hazardous for living organisms, because of ultraviolet light and other environmental influences and we have had to evolve survival mechanisms! There are variations not only in the *amount* of death produced by activated toxic agents but also in the response patterns in different cells.

Conney: Do you have any ideas about how epoxide hydrolase and DT-diaphorase help the cell?

Farber: It is hard to say. DT-diaphorase is ubiquitous in the cytoplasm. We have an hypothesis that the preneoplastic phase of carcinogenesis is protective and has survival value (Farber & Cameron 1980). Normally, exposure of an animal to a severe toxic agent may eventually kill it; but if the animal has previously been exposed to a carcinogen its liver has cells that are more resistant. Eventually, if the toxic agent is removed the liver cell population returns to normal and regular cycles of growth and maturation return.

Higginson: I want to ask about hepatitis. When you say 'all' carriers, do you mean carriers with both antigen and antibody present, or those with antigen alone? Do you find these changes invariably in such carriers?

Farber: This has not been studied carefully. The ground glass cells are seen frequently in hepatitis B carriers as well as in those that have chronic hepatitis.

Orrenius: I would like to come back to the benzo[*a*]pyrene–acetylaminofluorene (AAF) system. Does AAF act entirely as a promoter in that system?

Farber: I don't know yet. Under those circumstances AAF does not initiate cancer development (Tsuda et al 1980) because it is inhibitory to cell proliferation. However, when rats are given a single dose after partial hepatectomy, AAF will initiate liver carcinogenesis. Peraino et al (1971) used weanling rats and found initiation in the livers. Conceivably, the amount of activation in the young is not as high. In this age group, the AAF probably does not inhibit cell proliferation to the same degree as it does in the adult. Most chemicals, when used in a wide dose range, are both initiators and promoters.

Gillette: Does interferon produce the same effect as hepatitis B virus?

Farber: Interferon has a protective effect.

Gillette: Interferon also has an effect on cytochrome *P*-450, and I wonder if this leads to a proliferation of endoplasmic reticulum?

Farber: I don't know.

B.A. Bridges: Professor Farber, in some published figures you have plotted responses on a linear scale against dose on a logarithmic scale, and this can artificially create a threshold. I believe that if the same results were plotted on a linear–linear scale, the error bars would be so great that it would not be possible to detect a significant threshold. Would you like to comment on that?

Farber: Yes; the threshold is low and is within the background range, but a threshold is visible (i.e. the line does have a bend) even on a linear–linear scale (shown diagrammatically in Fig. 1).

Garner: In the mouse skin system, initiation and promotion can be separated by a considerable time. Can you do the same in your system?

Farber: We normally do assays for initiation at two weeks after the dose of carcinogen. If we wait 44 weeks, the results are the same. So, presumably, once initiated, the process proceeds at the same rate.

Garner: The agents you have used for promotion require metabolism themselves. You are suggesting that the pre-malignant cells are low in drug-metabolizing enzymes. What happens if you use a direct-acting agent?

Farber: I don't know. We are studying that at present.

McLean: There is a difference between the system of Peraino et al (1971) and your system in that phenobarbitone does not promote tumour growth in your system.

Farber: It prevents promotion, when mixed with AAF.

McLean: There is a discrepancy here. In Peraino's experiments, there was a pure proliferative stimulus, without inhibition, and yet promotion occurred.

Farber: It is a question of the different ways in which promotion is operating in the two systems.

McLean: Could that kind of stimulus also have its effects in your system? If you found the right stimulus, could you get tumours from the nitrosamine-treated livers, without giving a second carcinogen?

Farber: The phenobarbitone system is not good for sequential analysis because there is total lack of synchrony in the development of the various putative preneoplastic lesions in the hepatocytes. Histological examination of the livers shows focal proliferative lesions of many different sizes, making quantitation very difficult, if not impossible (Peraino et al 1971).

Higginson: Effects may depend on whether hepatectomy is performed before or after administration of the carcinogen, as shown by the different results observed by Glinos et al (1951), with dimethylaminoazobenzene, and Laws (1959), with 2-acetylaminofluorene. Hepatectomy is one of the simplest ways of causing cellular proliferation, as distinct from chemically induced hyperplasia, and the differences between different experiments are not surprising. Another point is that the terms 'initiation' and 'promotion' are constantly used in two-stage carcinogenesis, thus implying a threshold for initiation or for a non-effective dose of a carcinogen.

J. W. Bridges: Can we define any special properties of a cell that undergoes hypertrophy in the presence of phenobarbitone, other than to say that it contains more metabolizing enzymes and can therefore create larger amounts of active metabolites?

Farber: That is an interesting point, which cannot be answered at present. Methylnitrosourea extensively methylates cellular DNA and other cell constituents but does not produce any obvious cell damage. It would be a simple task to test it after induction as well to eliminate the activation phenomena.

J. W. Bridges: Do you observe an increase in nuclear ploidy during hypertrophy, or is nuclear ploidy totally unrelated to hypertrophy?

Farber: The hepatocytes in the hyperplastic nodules appear to be diploid (Inui et al 1971). It is possible that ony the diploid cells are at risk in carcinogenesis.

Amos: What is the evidence that an initiator acts also as a promoter? If an initiator induces cancer, how do we know that it is also promoting? Does it promote *another* initiator? How do we find out?

Farber: One would need an assay for initiated cells like the one we have developed for the rat liver. It may be that there are different kinds of initiators and different 'promoting environments' which is probably a better term than 'promoters'.

Amos: I agree.

Farber: The different environments may select different populations depending on mechanisms of action. The liver could have thousands of 'in-

itiated' altered cells that would be selected only under specific environmental conditions.

Breckenridge: I would like you to clarify precisely how phenobarbitone and 3-methylcholanthrene can act as negative promoters (i.e. exert a protective effect).

Farber: The acetylaminofluorene presumably has to be activated before it will inhibit cell proliferation. Phenobarbitone and 3-methylcholanthrene prevent the inactivation of AAF and thus prevent the inhibition of cell proliferation. Perhaps 'channelling along a new path' would be a better term than 'inhibition' to describe the early effects of phenobarbitone and 3-methylcholanthrene.

J. W. Bridges: Have you checked that phenobarbitone and 3-methylcholanthrene are inducing in the normal way?

Farber: Presumably they are, but we have not checked.

Garner: Presumably if you initiate with aflatoxin and then promote with AAF, the latter could not influence the metabolism of the former.

Farber: That's right. Resistant cells could grow more readily.

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Environmental Chemicals, Enzyme Function and Human Disease
DAVID EVERED and GERALYN LAWRENSON

The influence of nutrition and inducers on mechanisms of toxicity in humans and animals

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Abstract A high proportion of toxic and carcinogenic effects of chemicals develop through the pathway of lethal synthesis. A part of this pathway is along the inducible cytochrome *P*-450-linked enzyme system. It has previously been suggested that the variations in disease patterns between individuals and between national groups may be due to differences in nutritional intake which in turn act by altering the pathways controlled by cytochrome *P*-450. However, patients with epilepsy, who are taking large amounts of inducing anticonvulsants, and who are known to have increased cytochrome *P*-450-linked enzyme activity, fail to show clear-cut changes in their patterns of mortality. It is possible that the reactions in the cytochrome *P*-450 pathways are not usually rate-limiting steps in toxicity in humans, and that we must look elsewhere, beyond the activation step, for the cause of variability in human responses to toxic materials in the environment.

There are in current circulation three major theories of acute cell injury by chemicals. Using isolated liver cells and slices we conclude that none of them offers a reasonable explanation of the events of cell injury and necrosis in their usual simple form. Lipid peroxidation and calcium entry into cells can be blocked by antioxidants and calcium-free media, without interference with cell injury. Covalent binding of *p*-aminophenol to liver is far greater than binding of paracetamol, but it does not cause cell injury. The reversibility of some cell injuries implicates the existence of specific metabolic blocks by reactive metabolites rather than any generalized attack by insertion of metabolite groups.

The ability of a chemical to cause injury to humans is familiar to us because of acute poisonings of people at home, at work or through clinical and epidemiological experience. In addition we have evidence from animal experiments which should enable us to avoid harm to humans. But all substances are toxic if the dose is great enough, and an infinite variety of model systems can be built to demonstrate the various kinds of harmful effects. We are left with the need to verify our experimental models, to see if they function in the real world.

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International comparisons of mortality from cancer and other diseases suggest that environmental factors have a powerful influence on the incidence of disease (Doll & Vodopija 1973). It is now generally recognized that lethal synthesis (Peters 1963) is one of the most common underlying mechanisms of chemical toxicity and that reactive metabolites of oxidative drug metabolism account for many toxic effects, to liver, lung, kidney and bone marrow. The cytochrome *P*-450 systems are central to the activation of many toxins.

A question which worries at least some members of the committees who regulate the use of chemicals in food and industry is whether the process of induction of drug-metabolizing enzymes by foreign compounds leads to illness and disease or whether it is, on balance, a succesful protective mechanism. Many natural and man-made substances in food are inducers, and there is no clear threshold, or 'no-effect' dose (McLean & Driver 1977).

We have known for some years that induction of the cytochrome *P*-450 systems, together with their associated transferases for sulphate, glucuronate, glutathione and other groups, can lead to increased sensitivity to some toxic materials, such as carbon tetrachloride, or paracetamol (acetaminophen) (McLean & McLean 1966, Mitchell et al 1973). In contrast, induction before exposure to aflatoxin protects against both the toxic and the carcinogenic effects of this substance, and of several other carcinogens.

Some confusion is sometimes brought about by the observations that massive doses of inducers, such as phenobarbitone or DDT, cause liver cell growth and proliferation, and that such treatment given after initiating doses of carcinogens increases the number of tumours produced (McLean & Marshall 1971, Peraino et al 1977, Pitot 1977). Phenobarbitone administered on its own produces liver tumours in some strains of mice.

The balance of all these possible effects on humans can only be determined by epidemiology, and then for only one environment at a time. Clemmesen and his colleagues (1974) have reviewed these factors and studied cancer incidence in a group of patients with epilepsy. We have examined the mortality of some 2000 people with severe epilepsy to look for evidence of any alteration in incidence of fatal disease that might be linked to the known induction effects produced by anticonvulsant therapy (White et al 1979).

Table 1 shows that overall mortality was far greater in the study population than would be expected for 2000 people of the same age randomly drawn from the population of England and Wales. However, this mortality is mostly from epilepsy and though there is more mortality than expected for many causes of death among people with epilepsy the increase in cancer is small, and would probably become non-significant if correction for social class were possible. For liver cancer, of which there is no reported case in the 636 deaths, we can

TABLE 1

Deaths in patients with epilepsy in 1951–1977

Cause	*Number of observed deaths (O)*	*Number of expected deaths (E)*	*O/E ratio and (95% confidence limits)*
All malignant neoplasms	78	51.5	1.5 (1.2– 1.9)
[Liver and gall bladder[a]	1	0.6	1.5 (0.0– 8.5)]
[CNS neoplasms	4	0.3	12.5 (3.4–32.0)]
Circulatory diseases	143	95.4	1.5 (1.3– 1.8)
Respiratory diseases	99	25.2	3.9 (3.2– 4.8)
Accidents and suicide	64	14.6	4.4 (3.3– 5.6)
All causes	636	208.7	3.0 (2.8– 3.3)

[a]One cancer of the gall bladder (from White et al 1979)

say that if anticonvulsants have any effect in either reducing or increasing the incidence of liver cancer then the effect must be small. Because the disease is rare, any decrease in incidence would have little impact on the overall mortality. Any increase in incidence must be so small that it does not show up in the study of over 600 deaths.

The general picture that emerges is that an increase of drug-metabolizing activity in the liver caused by inducers of the phenobarbitone type is unlikely to have major effects on public health. In consequence, the inducing activity on the liver of natural foods, antioxidants and other food additives and contaminants need not worry us. However, this may well not apply to substances that induce enzymes in the lung and gastrointestinal tract. We are left with a number of questions about enzyme induction and health.

1. Is there a threshold of effect, or does the intake of all natural and synthetic inducing molecules add up to give an overall load of inducing pressure in the separate tissues, and in individuals within a population? (McLean & Driver 1977.)

2. Are the *man-made* substances such as drugs, industrial chemicals and food additives significant factors in determining the activity of the mixed function oxidases and their associated enzymes, or are the *natural* inducers in food and environment predominant? (Wattenberg 1975.)

3. Are genetic factors of overriding importance so that inducers fade into insignificance?

4. Does the level of induction affect health of individuals or populations

both in the incidence of cancers and in the prevalence of other, perhaps non-fatal, diseases?

The steps between exposure to a disease-producing environment, and the development of the disease are largely unknown. Even massive restructuring of the environment for health reasons may be unproductive if we do not understand the steps of pathogenesis in proper detail. For instance, the general decline in cancer of the stomach in Western Europe and America is unexplained (Doll 1979) and similarly we have little knowledge of what to do about colon cancer. Only for lung cancer and bronchitis can we suggest a programme which would certainly reduce incidence, and even there we may be unsuccessful because the population is unwilling to act on this advice.

It may well be that in natural populations, as distinct from colonies of rats, the determining factor in toxicity is not at the activation stage. The question is: what mechanisms cause cell injury after the reactive metabolite has been formed, and how could these steps vary with the nutritional, environmental and genetic make-up of the affected organism?

MECHANISMS OF CELL INJURY

We have extensive descriptions of what happens in tissues after the administration of carbon tetrachloride, dimethylnitrosamine (*N*-nitrosodimethylamine), paracetamol, and several other materials that have acute toxic effects. Many events are described. For carbon tetrachloride (CCl_4) the pretoxin, the CCl_4 molecule, is distributed about the whole body, but only in the target cells does it find and bind to cytochrome *P*-450. It is rapidly metabolized, the metabolites bind covalently to cell components, lipid peroxidation takes place, and calcium content of the cell rises. Later on protein synthesis is inhibited and the endoplasmic reticulum is disrupted. Each of these early events has been thought to account for the subsequent process of cell injury leading to cell deaths (Recknagel 1967, Castro & Gomez 1972, Judah 1969, Gallagher et al 1956, Dawkins et al 1959, Judah et al 1970).

The problem is that each of the early events undoubtedly takes place during the development of toxicity but the events can also take place in situations not followed by cell death; e.g. in carbon disulphide or ethionine poisoning. It seems unlikely that any one of these steps provides a sufficient explanation for what follows in the course of cell death (Bond & De Matteis 1969, Farber et al 1964).

Paracetamol injury to the liver gives a practical example of the problem we face (Mitchell et al 1973). In rats the concentration of paracetamol found in liver or plasma after an intraperitoneal injection drops exponentially to

negligible values after about six hours. Reactive metabolites have been generated and at first react with glutathione. When glutathione levels fall the metabolites form covalent links to protein and other cell components. All this takes place in the first six hours, but after modest doses cell death, rise of serum enzyme levels and histological changes begin only at about seven hours and reach a maximum at about 18 hours in the rat.

In humans these events are separated by one or two days. When patients come to the hospital with a history of paracetamol overdose we can treat them in the first 15 hours by giving them SH donors or glutathione precursors such as methionine or acetylcysteine (McLean 1974, McLean & Day 1975, McLean 1975, Prescott et al 1979, Vale et al 1979). But after 15–24 hours, the paracetamol has been metabolized and we are left waiting for evidence of liver cell death in terms of rising concentrations of serum enzymes, jaundice, coma and death. In the long latent interval between drug metabolism and death of liver cells we understand so little of the mechanisms of cell necrosis that we can do nothing but wait and hope that the liver injury will not be severe.

Cell death is not a discrete event but a process which is set in motion by the initial interaction between toxic material and cell components. For some substances we know the site of initial interaction. There is no toxic material for which the subsequent steps are known in any detail, and not even the basic mechanisms of cell necrosis are understood. If we consider what happens after the reactive metabolites are generated, we find that the main mechanisms proposed are lipid peroxidation, irreversible calcium entry, and covalent binding of reactive metabolites to protein. These are most commonly invoked as explanations for cell death (Fig. 1) (McMurtry et al 1978, Mitchell et al 1973,

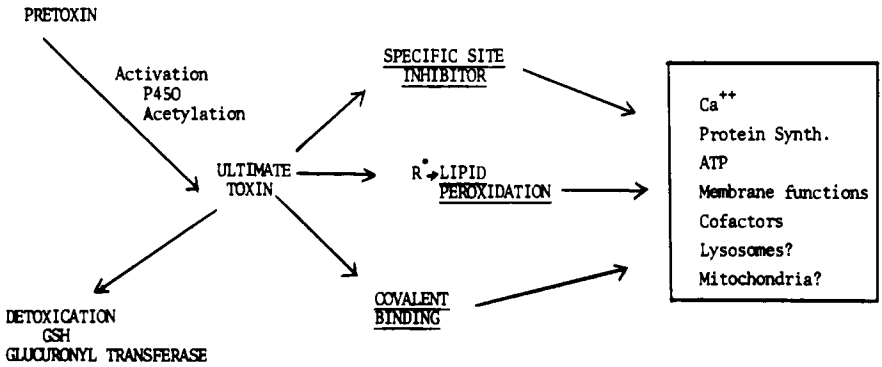

FIG. 1. Pathways of cell injury by chemicals. P450, cytochrome *P*-450; R•, free radical; GSH, glutathione.

Recknagel 1967, Judah 1969, Castro & Gomez 1972, Gallagher et al 1956, Judah et al 1966a,b).

The weakness of the argument for calcium entry as an essential step in cell necrosis is shown in Table 2. Liver slices incubated with paracetamol for two hours show loss of potassium, tenfold increase in leakage of enzymes, and histological appearance of cell necrosis after four hours further incubation (McLean & Nuttall 1978). Slices incubated in the absence of calcium or with ethylenediamine tetracetic acid (edetic acid, EDTA), added to chelate any residual calcium, still show the usual decline of K^+ content and the usual enzyme leakage after paracetamol treatment. Similar results are found for isolated liver cells. Both *in vivo,* and in the slice experiments when calcium is present, the paracetamol-injured cells do accumulate calcium. So while calcium entry certainly takes place during cell injury, it is not an essential link in the series of events leading to cell death.

Lipid peroxidation is commonly invoked as an explanatory event in carbon tetrachloride (CCl_4) injury. However, vitamin E deficiency and vitamin E supplementation do not alter the injurious effects of CCl_4 in properly conducted experiments (McLean 1967). Recent experiments showing inhibition

TABLE 2

Effect of calcium on enzyme leak from control and paracetamol-treated rat liver slices.

Additions to incubation medium (Hepes Ringer)	*Enzymes leak % of total at 6 hours (isocitrate dehydrogenase)*	*Ca^{2+} content of slices at 6 hours* μmol/g dry wt
+ Ca^{2+} 1 mM	1.9 ± 0.7	5.0 ± 0.8
+ Ca^{2+} 0.1 mM	3.4 ± 1.6	1.9 ± 0.3
+ No Ca^{2+}	6.3 ± 3.2	0.9 ± 0.2
+ Na^+ EDTA 0.1 mM	4.9 ± 2.4	0.7 ± 0.3
Hepes Ringer + 8 mM paracetamol 1st two hours		
+ Ca^{2+} 1 mM	42 ± 17	9.0 ± 4.2
+ Ca^{2+} 0.1 mM	55 ± 2	2.0 ± 0.1
+ No Ca	58 ± 7	1.3 ± 0.7
+ Na^+ EDTA 0.1 mM	61 ± 3	1.4 ± 0.1

Results are mean ± 1SD values from a typical experiment repeated three times. Slices were cut from livers from phenobarbitone-treated rats and incubated for two hours with or without 8 mM paracetamol as described by McLean & Nuttall (1978) but using Hepes buffered Ringer solution with varying calcium concentration.

After 2 hours the slices were transferred to fresh paracetamol-free media with varying calcium content and enzyme leakage into the medium was measured 4 hours later, together with ion content of the residual slices.

of drug metabolism after massive intramuscular doses of vitamin E explain some previous results (Dashman & Kamm 1979). Perhaps the following experiment with paracetamol will cast light on the relation between peroxidation of cell lipids and cell injury.

Dosage with vitamin E does not alter the injury caused by paracetamol to rats fed stock diets, and lipid peroxidation is not found in liver cells treated with paracetamol. However, if rats are fed a diet containing 10% herring oil, which is deficient in vitamin E and rich in long chain polyunsaturated fatty acids, then the rats become more sensitive to injury by paracetamol. This effect is reversed by administration of vitamin E. These observations suggest that lipid peroxidation is not a necessary part of paracetamol injury, but if the cell is injured it becomes liable to peroxidative attack, which then makes the injury worse (Table 3). It is likely that a similar relationship will be found for many of the events of cell injury and necrosis. We need to know whether an event is a necessary step in the sequence, whether it is sufficient, or contributory, or whether it is an epiphenomenon, that is, something that happens but is of no importance to the sequence which we are investigating.

Covalent binding of reactive metabolites to liver cell proteins (Table 4) takes place after rats are dosed with paracetamol or with *p*-aminophenol (PAP). Rather more PAP gets bound than paracetamol. However, liver necrosis does not take place after PAP binding, although it does after paracetamol binding. It is likely that covalent binding is a sign of the generation of reactive molecules in cells, but whether it leads to cell injury depends on the size and shape of the reactive molecule, and on the particular cell macromolecule to which it binds. We also have evidence of reversibility of cell injury after the covalent binding stage (McLean & Nuttall 1978).

We already have evidence that some DNA sites are more important than

TABLE 3

Effect of Vitamin E on paracetamol-induced liver injury in rats fed herring oil.

Diet	*Number in group*	*Deaths*	*Plasma isocitrate dehydrogenase activity, nMol ml^{-1} min^{-1} geometric mean ± 1SD range*	
41B stock pellets	10	0	8.5	(2–39)
41B + 15% herring oil	11	4	248	(17–3,600)
41B + 15% herring oil + 100 mg α-tocopherol (oral)	9	0	9.4	(1–80)

All rats were given phenobarbitone (1 mg/ml) in drinking water for seven days before dosing with paracetamol (400 mg/kg) i.p. (A.E.M. McLean & D. Tame, unpublished).

TABLE 4

Liver injury and covalent binding of *p*-aminophenol (PAP) and paracetamol to rat liver protein *in vivo*.

Time after dosage	*Binding nMol/mg protein*	
	PAP	*Paracetamol*
2 h	0.78	–
4 h	0.82	0.25
6 h	–	0.68
Injury at 24 h	No liver necrosis	Extensive necrosis

PAP data from Greaves (1979). Paracetamol data from McMurtry et al (1978).
–, not measured.

others in the process of carcinogenesis. It is probably just as true in the processes of protein binding and cell necrosis.

IMPLICATIONS

If we wish to assess the public-health consequences of the exposure of a population to a new chemical then we need to know what happens to the chemical in the body and how the body reacts.

In order to interpret our biochemical findings and the reaction of animals or model systems, such as cells or bacteria, to a new chemical we need a deeper understanding of the ways in which cells behave in adverse environments, and in response to chemical attack. Only then will we be able to predict with any reasonable reliability what will happen to human beings when they are exposed to a new chemical (McLean 1979).

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Discussion

Breckenridge: Dr McLean, I am interested in your data on mortality among people with epilepsy. It is widely accepted that there are agents which act either as promoters or as initiators of carcinogenesis in mouse liver systems. According to your data, this concept put forward by Henry Pitot (1977) and many others, including Professor Farber, would not seem to be valid for humans. Is this because we do not come into contact with the initiators and promoters, or is it that the model is irrelevant to human cancer?

McLean: Clemmesen's (1974) work from Denmark also implies that hepatic cancer is not an important consequence of induction by drugs. The doses of phenobarbitone that are used in the promotion experiments in rats cause liver cell proliferation. (Augenlicht & Argyris 1975, Kunz et al 1966.) For humans, the dose of phenobarbitone may not be large enough to cause cell proliferation. Chronic hepatitis or a sudden increase in food consumption are more likely to give rise to liver cell proliferation in humans. An interesting aspect of the promotion experiments described by Professor Farber and others is the way they suggest that aflatoxin and hepatitis might interact to produce liver cancer in humans.

Farber: In several models of carcinogenesis, only a brief exposure to the carcinogen is used. So we must be cautious about extrapolating from these models to humans. Occasionally, in humans, cancer develops after only a brief or limited exposure to a carcinogen.

Idle: Dr McLean, you said that genetic differences can be modulated by the environment. Professor Smith and I have acted as subjects in a comparative study of oxidation of several substrates, including debrisoquine, guanoxan, phenacetin and phenytoin, and we represent extreme ends of the range between poor-metabolizing phenotypes and extensive-metabolizing phenotypes. We found a 300-fold difference between our rates of hydroxylation of debrisoquine and guanoxan (measured from the first-order rate constants for hydroxylation). This was also the maximum range within the whole group. How significant do you think environmental modification can be amongst people like us, who are from genetically different backgrounds?

McLean: Let us consider a population with a genetically determined mean enzyme activity, in the uninduced state. The distribution may be unimodal or bimodal, and many genes will contribute to the spread of values around the mean. Now we add a spot of environment! There are inducers, inhibitors and so on, in the diet, and the means and distributions of the values of enzyme activity shift. When you warm up a gas the mean velocity of the molecules rises, a higher proportion of the molecules has the necessary activation energy for

any chemical reaction to take place, and a 10°C rise in temperature doubles reaction rates. In the same way, a small shift in the mean enzyme activity could produce a large shift in the number of individuals whose enzyme activity was above some critical value. In fact, an increase in standard deviation without any shift in the mean value could generate such an effect. We have to explain the large variations in disease patterns between countries, which affect people when they move from one country to another.

I am interested in the effect that public policy on nutrition, pesticides and drugs can have on disease states in populations. I am not disputing that large genetic variations exist but, after all, public policy or emigration will not influence that.

Idle: But the order of magnitude of changes induced by environmental influences, e.g. smoking, is minute compared with those that exist because of genetic differences within a population.

McLean: Smoking is not likely to alter the rates of metabolism of debrisoquine but it affects the pattern of diseases.

Higginson: I do not understand how a 2% shift in the population as a result of an environmental factor could be significant in the face of 300-fold individual variations which, you say, are of a genetic origin. It may be significant in the tail ends of the population, but how can genetic factors be very important when the total populations are compared?

McLean: We know that environmental factors massively alter disease patterns – so what is the mechanism of action of the environmental factors? There are large genetic variations within a population in the ability to metabolize drugs. If the environment of the whole population is altered, then the whole population will be shifted.

De Matteis: It would be simpler to state that if 300-fold differences can exist, the distribution is not likely to be unimodal, but is more likely to be at least bimodal.

Smith: We should refer to different 'modes' rather than to the 'spread' within the human population. Different genotypic and phenotypic modes can respond differently to toxic environmental agents. For example, there are two phenotypes with different susceptibility to the toxic effects of drugs that undergo polymorphically controlled acetylation. In addition, people with the $E_1^U E^q$ genotype for succinylcholine hydrolysis are in no doubt about their reactions to a normal therapeutic dose of the drug!

McLean: We are looking at one particular drug-metabolizing system, and there are many other systems. In all probability the overall result which would take into account all the different metabolizing enzymes is likely to produce a unimodal distribution. Essentially, genetic and environmental factors cannot

be separated because all individuals are exposed to both. If the population were to be placed in a uniform environment, then only genetic factors would matter. Only environmental differences should make differences between identical twins. What actually happens is that different individuals inside a society respond differently to drugs and to toxins. It is very likely that those inter-individual differences have a considerable genetic origin. To know about these genetic differences is useful if we wish to examine individuals to sort out who should be exposed to this or to that drug or chemical. The genetic factors cannot be used when we are deciding about exposure of populations to chemicals, since we have no control over the genetic factors.

Idle: Only with a cloned population would we be able to isolate environmental factors totally from genetic factors. When we discuss induction and the influence of dietary or social factors we should refer to a baseline. And in our work, when the people in one phenotype, which comprised 10% of the white population in the UK, have a 300-fold difference in their rates of hydroxylation of some substrates, there is a radically different baseline against which to compare the environmental influences on induction within that phenotype.

Conney: I don't think we can say that the 300-fold differences in hydroxylation rates of drugs in different people are caused entirely by genetic factors. Interactions between genes and environment make it difficult to rule out a role of environment in your studies, Dr Idle.

McLean: Do you think that this range in metabolizing ability has any biological significance, Dr Idle?

Idle: It may have a toxicological significance, as Professor Smith mentioned in his paper.

Selikoff: We still do not know whether enzyme induction predisposes towards cancer. Dr McLean noted that the therapy given to people with epilepsy did not produce increased incidence of cancer. But unless enzyme induction leads to different latency periods compared to cancer in general (i.e. different periods between exposure to a carcinogen and the appearance of the cancer), only those cancers that occur 20 or more years after onset of exposure could be related to ingestion of the drug. Deaths occurring *before* 20 years may be totally unrelated to the effects of the chemical.

McLean: Most of the results on people with epilepsy were compiled from records and they followed people who had well over 20 years of exposure before the study started.

Selikoff: Percivall Pott's first description of scrotal cancer was in 1775 (Pott 1775). Benzo[*a*]pyrene has now been recognized as an enzyme inducer that might have been responsible for this type of cancer amongst chimney

sweeps over the age of 30. But the sweeps started to clean chimneys when they were only six or seven years old, so this provides some of the earliest evidence that there is a long latent period between initial exposure and development of cancer. Our own recent work on benzo[*a*]pyrene has been done on roofers exposed to coal tar pitch in the United States. Some of the materials contained as much as 1% benzo[*a*]pyrene. The average inhalation of benzo[*a*]pyrene during a seven-hour working day was around 16 μg, equivalent to about 700 cigarettes/day. In 1960 we began to study 6000 men who had been working in the roofing industry for at least 10 years (Hammond et al 1976). Those exposed for less than 30 years showed no increased incidence of cancer when compared to the death rates in groups matched for age, year and sex in the general population of the USA. Those exposed for longer than 30 years, however, had a significantly greater incidence of cancers of the lung, bladder and oropharynx. But there was no greater incidence of liver cancer. Out of almost 2500 deaths we saw only two that were presumably due to cancer of the liver, and we did not have the opportunity to verify those histologically.

Higginson: Here we are dealing with a well-recognized initiator, and the 20-year latent period is well known.

However, the enzyme-inducing effects of benzo[*a*]pyrene have yet to be shown to be important, i.e. its relevance in terms of increasing individual susceptibility to other carcinogens during the latent period. The important question here is whether the enzyme-inducing properties of phenobarbitone in Dr McLean's studies have had an enhancing or promoting effect on carcinogenicity. Thus far, this does not seem to have occurred, at least over 20 years; phenobarbitone cannot be regarded as an initiator in humans. We must therefore distinguish between the action of initiators and non-initiators when we analyse such studies.

Garner: We do not know the average age at death of subjects in the phenobarbitone study and surely that should be taken into account; if they all died at the age of 35, then it would be unlikely that they could develop cancer, because of the long latency period.

McLean: That is not so, because for each 5-year period the population is divided, by age groups, and is compared with the number of cancers expected both in that age group and in that 5-year period, in the *general* population of England and Wales. Therefore, the number of people who died from other causes doesn't affect the analysis. One is calculating the expected deaths for the number of people of different ages who are alive, and at risk, year by year.

Garner: Should you not compare it with another *hospital* population rather than with the general population?

McLean: Yes, we would have done that if such a control group had been

available. Our present work no longer deals with hospital populations, which are abnormal; we are examining free-living people with epilepsy.

Connors: Has anyone shown that there was induction of specific enzymes in those people at intervals over the 20-year period?

McLean: There have been a number of studies of people with epilepsy which show that the rate of disappearance of antipyrine from the plasma is shifted so that the half-lives are 20–50% shorter than would be expected.

Davies: Dr McLean, how much induction of liver enzymes did you expect to see in the Chalfont epileptic patients?

McLean: We did not monitor that but it has been done by Dr Cheryl Padgham (1976), with Dr A. Richens.

Davies: There are limitations to the information that can be obtained about induction in human liver. We have measured (unpublished results) drug metabolism in a number of biopsies of human liver with preserved hepatic architecture. We had 20 control patients who were not receiving known inducers, and two other patients, one receiving primidone (450 mg) and phenobarbitone (30 mg) and the other receiving phenytoin (300 mg). The changes observed were not large: benzo[*a*]pyrene hydroxylase activity was increased by 2-fold; ethoxycoumarin *o*-deethylase activity was increased by less than 2-fold, and the cytochrome *P*-450 content was increased by only 35%. The large variation in hydroxylating activity which Dr Idle mentioned earlier contrasts sharply with these very modest changes that are due to enzyme induction.

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Environmental Chemicals, Enzyme Function and Human Disease
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Influence of environmental chemicals on drug therapy in humans: studies with contraceptive steroids

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Abstract The effects have been studied of various environmental factors on the variability in response to oral contraceptive steroid therapy in women. Ten- to thirty-fold variations in plasma concentrations of norethisterone, L-norgestrel and ethinyloestradiol have been shown in samples taken 12 h after administration of oral contraceptives in mid-menstrual cycle. Factors shown to be responsible for this variation include passage into the enterohepatic circulation, a variable first-pass effect, and changes in metabolism in the gut wall or liver due to diet, disease, smoking or administration of drugs. Phenobarbitone and the antibiotic rifampicin increase both oestrogen and progestogen metabolism in women and in experimental animals by increasing hepatic and gut wall metabolism. In animals, other antibiotics (ampicillin, neomycin and lincomycin) suppress the gut flora that normally hydrolyse steroid conjugates excreted in bile; enterohepatic circulation of oral contraceptive steroids is thus reduced and their plasma concentrations lowered by up to 90%. In the human, ampicillin has a variable but less dramatic effect on elimination of oral contraceptives. Samples of gut wall mucosa obtained from patients with coeliac disease are defective in their ability to metabolize oral contraceptives. Cigarette smokers eliminate ethinyloestradiol more rapidly than non-smokers; an increased production of reactive steroid metabolites may thus be a cause of vascular disease in women who smoke and take contraceptive steroids.

It has been estimated that over 50 million women use oral contraceptive steroids. In spite of this large number, and the fact that these agents have been widely available for more than 15 years, it is only recently that detailed studies have been made of the clinical pharmacology of these drugs. Two facts are frequently cited to defend this paucity of information. First, as oral contraceptive preparations are taken mainly by healthy women rather than being prescribed to women with disease, these drugs represent a unique group in therapeutics. Second, until recently, contraceptive steroids have been given in supratherapeutic doses, thus minimizing any problems arising from inter-individual variation in their elimination. Neither of these positions can now

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be defended. The fact that healthy women take the drug makes any manifestation of toxicity a matter of widespread concern. Further, the recent reductions in both oestrogenic and progestogenic components of oral contraceptive preparations that have been made in attempts to minimize toxicity may expose some women to a dose incompatible with contraceptive protection.

The first preparation to undergo large scale clinical trials was Enovid®, which contained 10 mg of a progestogen (norethynodrel) and 150 μg of an oestrogen (mestranol). Most contraceptive steroid preparations now contain 1 mg or less of progestogen and 20, 30 or 50 μg of oestrogen. Thus, any factor that reduces the bioavailability of the newer steroid preparations may become important. Further, certain drugs in common use may interact with contraceptive steroids, thereby reducing their efficacy. Since contraceptives are frequently taken for periods of many years, women taking them are virtually certain to be prescribed other drugs during this time. Other environmental and constitutional factors such as smoking, variations in diet and concurrent disease may alter the bioavailability of contraceptive steroids and modify their effects correspondingly.

Over the last few years we have been examining both the basic and the clinical pharmacology of the variations found in responses to contraceptive steroids. This paper will highlight several aspects of the programme which are related to the possible effect of environmental chemicals.

VARIATIONS IN PLASMA CONCENTRATIONS OF CONTRACEPTIVE STEROIDS

We have developed radioimmunoassay methods for the measurement of norethisterone, L-norgestrel and ethinyloestradiol (EE2) (Back et al 1978c). Fig. 1 shows plasma concentrations of these steroids measured in blood samples taken 12 h after drug administration from groups of women in mid-menstrual cycle. These large inter-subject variations are in part due to variations in rates of metabolic elimination. The time curve for plasma concentration for each steroid given as a single oral dose is best described as a biexponential decay. In groups of six subjects the half-life for norethisterone during the terminal phase ranged from 6.1 to 12.3 h; for L-norgestrel, it was 7.8 to 19.7 h, and for EE2, 3.1 to 11.7 h. These steroids are well absorbed, and plasma concentrations reach a peak within 2 h of drug administration. The steroids are extensively protein-bound, both to albumin, a low-affinity, high-capacity system, and to sex hormone binding globulin (SHBG), a high-affinity, low-capacity system (Back et al 1978c). Administration of both EE2 and inducing agents such as phenobarbitone increases SHBG capacity; fur-

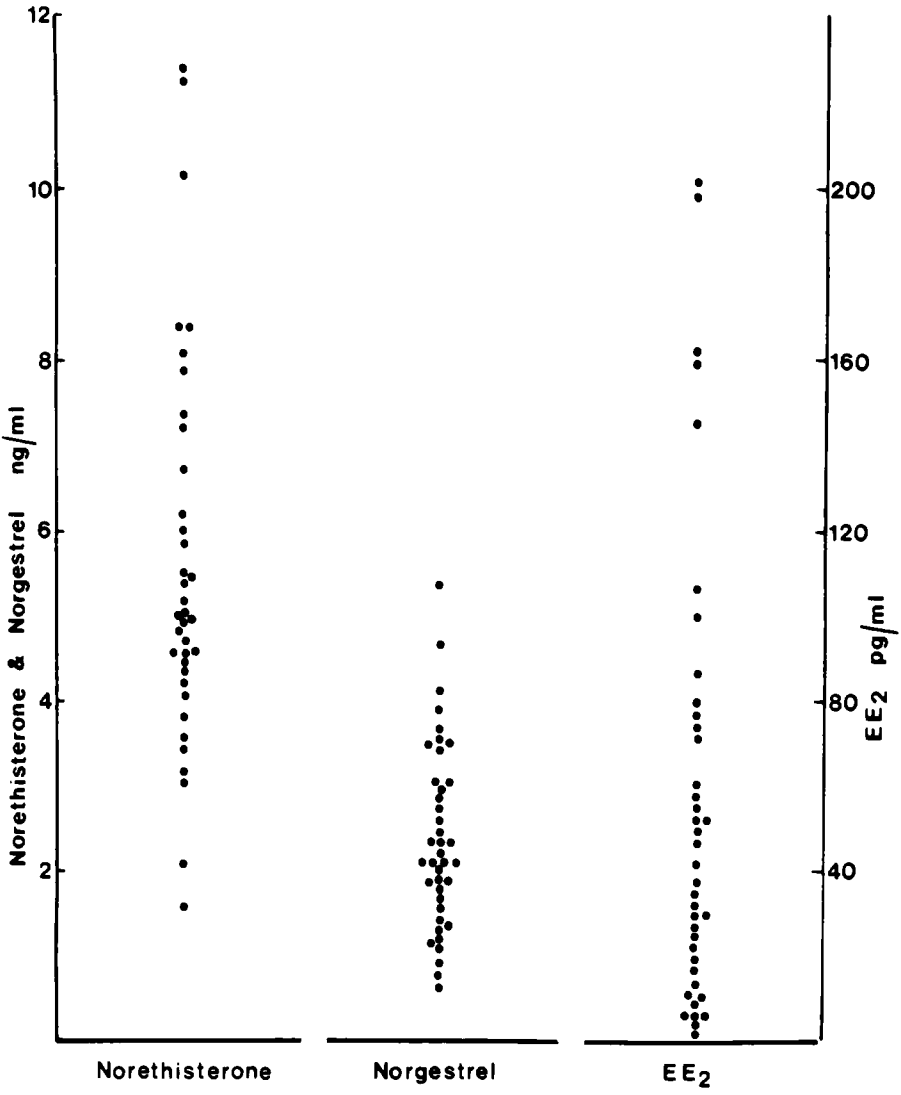

FIG. 1. Plasma steroid concentrations in blood samples taken from women approximately 12 h after administration of preparations containing norethisterone acetate (1 mg), L-norgestrel (250 μg) or ethinyloestradiol (EE2 50 μg).

ther, there is a close correlation between SHBG capacity in plasma and plasma norethisterone or L-norgestrel concentrations (Back et al 1980a).

Contraceptive steroids are subject to a first-pass effect, that is, their systemic bioavailability after oral administration is incomplete. Fig. 2 shows

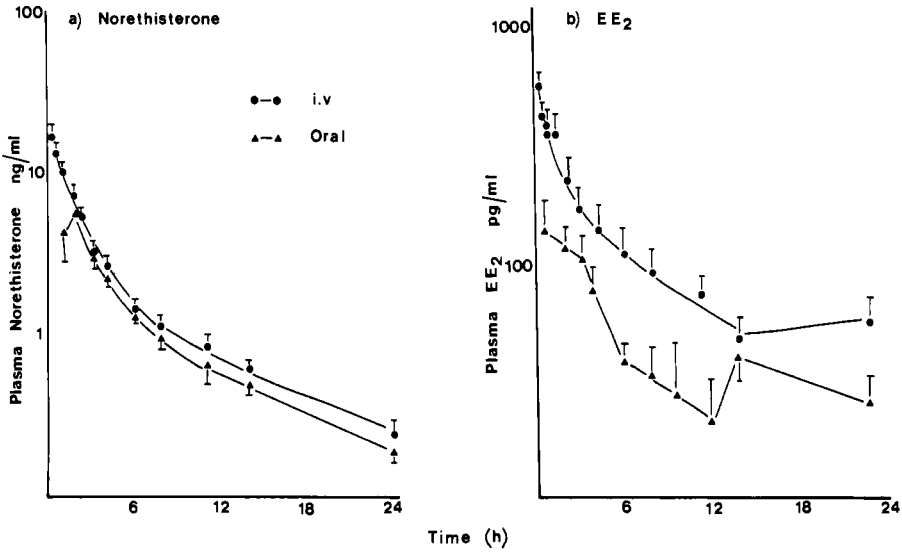

FIG. 2. Plasma concentration of (a) norethisterone and (b) EE2 in women after intravenous (i.v.) or oral administration of preparations containing norethisterone (1 mg) and EE2 (50 μg). Each curve represents data ± S.E.M. from 5 women.

time curves for plasma concentration for norethisterone and EE2 after both oral and intravenous administration. The systemic bioavailability for norethisterone is 64% (36% first-pass effect) and for EE2, 42% (58% first-pass effect). Both liver and gut wall may contribute to the first-pass effect, and a series of animal and clinical studies have helped to delineate their relative importance.

We gave norethisterone to anaesthetized rats by mouth, by systemic venous injection and into the hepatic portal vein (Back et al 1978b, Back et al 1978d). As shown in Fig. 3, there was both a hepatic and a gut component to the first-pass effect. After portal venous administration of the drug, the area under the curve for plasma concentration plotted against time was 32% of that after systemic intravenous administration, whereas after oral dosing it was only 13%. In studies on everted gut sacs from rat intestine, both norethisterone and EE2 were found to be extensively metabolized (Back et al 1978b). Further, when norethisterone was given intraduodenally to rats, and plasma was obtained from carotid artery and hepatic portal vein, the concentration of metabolites was found to be higher in the portal vein than in the carotid artery.

In humans, we did *in vitro* studies of the metabolism of EE2 using samples of jejunal mucosa obtained, by means of a Quinton Hydraulic Biopsy

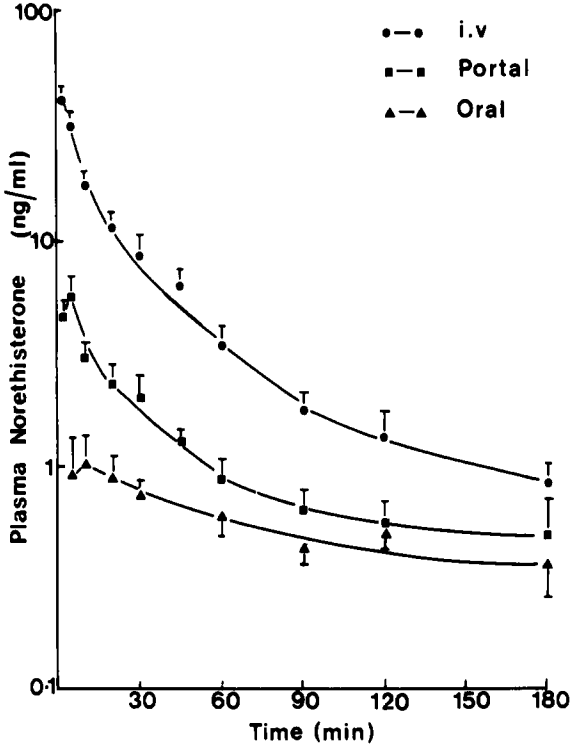

FIG. 3. Plasma concentrations of norethisterone after i.v., hepatic portal and oral administration of the steroid (50 μg/kg) to rats. Each curve is mean ± S.E.M. of data from at least 5 experiments.

Sampler, from healthy relatives of patients with coeliac disease. These studies (Table 1) show that large amounts of EE2 were metabolized in the gut wall, and that the major metabolite formed is EE2-sulphate. This differs from results obtained with two other oestrogens, oestradiol and oestriol, in which a glucuronide is formed. Similar studies of biopsy material obtained from patients with coeliac disease show some reduction, by comparison with normal controls, in their ability to form conjugates. Despite treatment of these patients with a gluten-free diet, the mucosa, although becoming histologically more normal, does not regain its ability to conjugate EE2 normally (Back et al, unpublished observations).

The gut is normally exposed to a large number of exogenous agents which could alter its ability to metabolize drugs; thus the effects of long-term therapy on metabolism in the gastrointestinal wall may be of importance. For

TABLE 1

Percentage of ethinyloestradiol conjugated in test and control tubes by human jejunal mucosa

	Test	*Control*
	% (mean of pair)	*% (single measurements)*
1.	46.9	3.9
2.	43.6	3.2
3.	39.3	4.3
4.	44.1	5.6
5.	26.4	1.5
6.	29.3	1.5
7.	44.3	1.0
8.	32.3	2.8
Mean	38.3	3.0
S.E.M.	± 2.6	± 0.5
	$n = 8$	$n = 8$

$P < 0.001$ (1-tailed t-test).

'Test' samples were from relatives of patients with coeliac disease. Control tubes also contained sodium thiomersal, a metabolic inhibitor.

example, we found that administration of vitamin C altered the pharmacokinetics of EE2 in healthy volunteers. Six volunteers were given Ovranette® (Wyeth, 30 μg EE2, 250 μg L-norgestrel) and the area under the curve for plasma concentration of EE2 plotted against time was measured. In a second study, one hour before the dose of Ovranette® (1 g), effervescent ascorbic acid was given to the same six volunteers. The mean area under the curve for EE2 increased dramatically, suggesting that the vitamin C, which itself forms a sulphate conjugate, had thus allowed a greater bioavailability of the steroid (Back et al, unpublished observations).

DRUG INTERACTIONS WITH CONTRACEPTIVE STEROIDS

As shown above, vitamin C may increase the bioavailability of ethinyloestradiol; but most concern is naturally expressed about drug interactions with contraceptive steroids that result in diminished contraceptive efficacy. Three groups of drugs have been shown to react adversely with contraceptives.

1. Rifampicin

The first reports of interactions of drugs with contraceptive steroids ap-

peared in 1971 when Reimers & Jezek (1971) found that of 51 women who had tuberculosis and were taking contraceptive steroids, 30 had either intermenstrual or breakthrough bleeding when given rifampicin. To date (October 1979) at least 18 pregnancies have been reported in women taking rifampicin in conjunction with contraceptive steroids. Rifampicin, of course, has been shown to shorten the plasma half-life of tolbutamide, warfarin and hexobarbitone mainly through its ability to stimulate hepatic microsomal enzyme activity. There is now good evidence in humans that rifampicin increases the rate of metabolism of both the oestrogenic and progestogenic components of oral contraceptives. Bolt et al (1975) compared the hydroxylation rates of EE2 in an *in vitro* system of liver biopsies from patients treated with rifampicin and from control subjects and found a four-fold increase in the rate of steroid metabolism. The same group also demonstrated that rifampicin caused an increase in the rate of elimination of radioactive EE2 from plasma. Administration of rifampicin caused a reduction from 3.3 ± 0.9 h to 1.5 ± 1.7 h in the half-life of the elimination phase in five volunteers.

We have investigated the pharmacokinetics of both EE2 and norethisterone in eight women during and five weeks after rifampicin treatment (Back et al 1979). None of these women were taking oral contraceptives regularly, and each was given a single tablet of Minovlar® (50 μg EE2 and 1 mg norethisterone) on the two occasions, for the purpose of this study. Tables 2 and 3 show marked changes in area under the curve and the half-life of elimination of both steroids during rifampicin treatment. Antipyrine clearance, urinary 6β-hydroxycortisol excretion and plasma γ-glutamyltrans-

TABLE 2

Pharmacokinetics of norethisterone during and after treatment with rifampicin

Patient	*A.U.C.*[a] *(ng/ml × h)*		*Plasma half life (h)*	
	During	*After*	*During*	*After*
H.A.	22.3	32.8	3.3	5.2
G.B.	32.3	64.0	2.6	4.9
I.C.	19.7	25.9	4.0	5.5
J.N.	21.2	25.9	5.1	7.7
A.O'C.	22.5	35.4	3.5	6.0
K.P.	15.1	41.0	2.4	4.2
A.S.	14.9	48.4	2.8	6.3
B.S.	27.6	28.8	1.9	8.6
Mean	21.9	37.8	3.2	6.2
± S.D.	± 5.9	± 13.1	± 1.0	± 1.7
Significance	$P < 0.01$		$P < 0.0025$	
	$n = 8$		$n = 8$	

[a]A.U.C. = area under the curve

TABLE 3

Pharmacokinetics of ethinyloestradiol during and after treatment with rifampicin

Patient	*A.U.C.*[a] *(ng/ml × h)*		*Plasma half-life (h)*	
	During	*After*	*During*	*After*
H.A.	437	1952	2.3	4.3
G.B.	2021	2328	not done	7.8
I.C.	472	1704	3.1	4.1
J.N.	1678	2111	5.6	11.6
A.O'C.	249	1191	1.9	5.8
K.P.	245	760	0.4	3.1
B.S.	2005	2195	4.2	9.1
Mean	1015	1749	2.9	6.5
± S.D.	± 778	± 534	± 1.7	± 2.9
Significance	$P < 0.05$		$P < 0.01$	
	$n = 7$		$n = 7$	

[a]A.U.C. = area under the curve

TABLE 4

Plasma antipyrine half-life and clearance, urinary excretion of 6β-hydroxycortisol and plasma γ-glutamyltranspeptidase activity during and after rifampicin therapy

	During rifampicin (mean ± S.D.)	*After rifampicin (mean ± S.D.)*	*P*	*n*
Plasma antipyrine half-life (h)	7.3 ± 2.1	9.8 ± 1.7	< 0.05	7
Plasma antipyrine clearance (ml h^{-1} kg^{-1})	72.4 ± 24.3	42.6 ± 3.9	< 0.02	7
6β-hydroxycortisol excretion (μg/day)	1177 ± 313	341 ± 65	< 0.001	7
γ-glutamyltranspeptidase activity i.u.	29.6 ± 20.0	18.0 ± 6.6	< 0.05	7

ferase (EC 2.3.2.2) activity also increased during rifampicin therapy (Table 4). These findings are compatible with enzyme induction being the underlying mechanism of the interaction. As with many other examples of enzyme induction, the inter-individual variation is important. The decrease caused by rifampicin in the area under the curve obtained for norethisterone ranged from 4.2 to 69.4% and that for EE2 ranged from 13.2 to 77.6%.

2. *Anticonvulsant drugs*

There is a plethora of data in both humans and experimental animals which demonstrates the enzyme-inducing properties of barbiturates. We have recently studied the effect of phenobarbitone on plasma concentrations of EE2 in four epileptic women who were on long-term contraceptive steroid

therapy. Blood samples were taken for five days during a control cycle and then for a similar period during a cycle when the women were taking, in addition, 60 mg phenobarbitone. When phenobarbitone was given, concentrations of EE2 fell dramatically in two women and less markedly in the other two; these results underline the variability in enzyme induction referred to above. Data from one patient are shown in Table 5. In another study we measured the plasma concentrations of EE2 and L-norgestrel in a group of epileptic women taking contraceptive steroids. The anticonvulsants they were taking included diphenylhydantoin, carbamazepine, folic acid, ethosuximide, sodium valproate and phenobarbitone. The mean plasma concentrations of EE2 in six women in mid-cycle, 12 h after they took a pill containing 50 μg EE2, was 14.2 ± 6.5 (S.E.M.) pg/ml; the measurement for a similar group of non-epileptic women was 35.4 ± 4.2 (S.E.M.) pg/ml. The mean L-norgestrel concentration in seven epileptic women taking 250 μg of the steroid was 0.80 ± 0.14 (S.E.M.) ng/ml and in seven controls 2.26 ± 0.23 (S.E.M.) ng/ml. This drug interaction is now well recognized and practitioners should advise patients to use either an oral contraceptive preparation with a higher steroid content or alternative contraceptive methods.

We have demonstrated from animal studies that phenobarbitone can increase steroid metabolism in both liver and gut wall. Phenobarbitone was given in drinking water (1 g/l for 5 days) to rabbits or by gavage (80 mg/kg for 5 days) to rats. In neither species did phenobarbitone have any effect on the pharmacokinetics of norethisterone given intravenously (Fig. 4a), but it significantly reduced the area under the curve after oral administration of the steroid (Fig. 4b). Since norethisterone is highly extracted by the liver, enzyme induction causes little change in the i.v. profile of the drug, but it produces a

TABLE 5

Concentrations of plasma EE2 (pg/ml) before and during treatment with phenobarbitone (patient L.M.)

Day of cycle	*Cycle 1 (control)*	*Cycle 2 (phenobarbitone)*
11	106	27
14	210	57
16	131	27
18	116	43
21	65	20
Mean ± S.E.M.	125 ± 24	35 ± 7
n	5	5

EE2, ethinyloestradiol

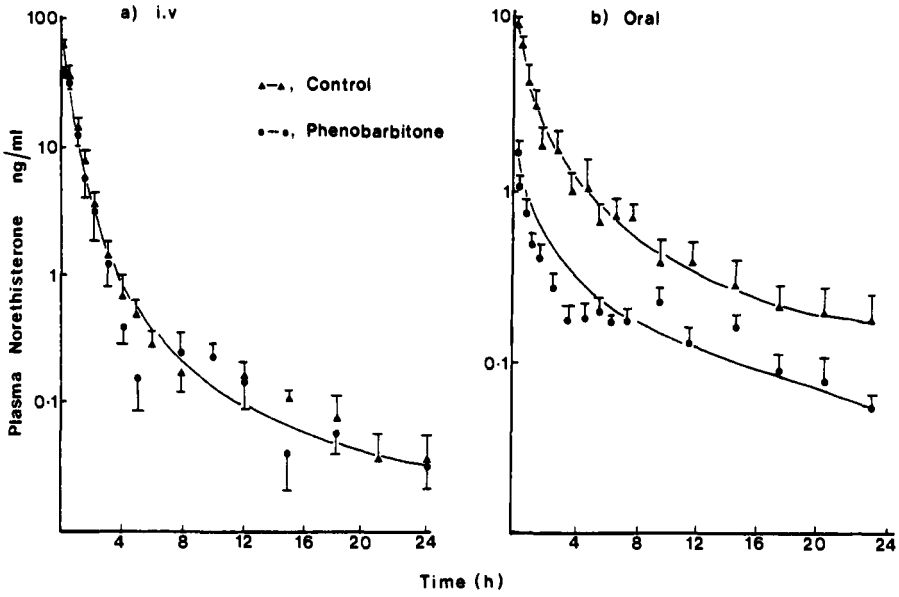

FIG. 4. Plasma norethisterone concentrations in control and phenobarbitone-treated rabbits after (a) i.v. and (b) oral administration of norethisterone (85 μg/kg). Each curve is the mean ± S.E.M. of data from at least 4 experiments.

smaller peak height and an identical half-life in the profile after oral administration.

In order to determine whether the liver or gut wall was responsible for these changes, we infused norethisterone into the hepatic portal vein of control and phenobarbitone-pretreated rats for 5 min. In the latter group, the area under the curve was significantly reduced compared to that in controls, although the plasma half life was unchanged, indicating an increased hepatic first-pass effect (Fig. 5). Further, when labelled norethisterone was administered into the duodenum of control and phenobarbitone-treated rats, and blood samples were obtained simultaneously from portal vein and carotid artery, there was a significant increase in steroid conjugates in portal venous blood but not in the carotid artery (Back et al 1980b). It seems likely, therefore, that phenobarbitone increases norethisterone metabolism in the gut wall, and this has been confirmed by the *in vitro* studies on gut sacs described above.

3. Antibiotics

There have been sporadic reports of women becoming pregnant while taking contraceptive steroids concurrently with a variety of antibiotics. The

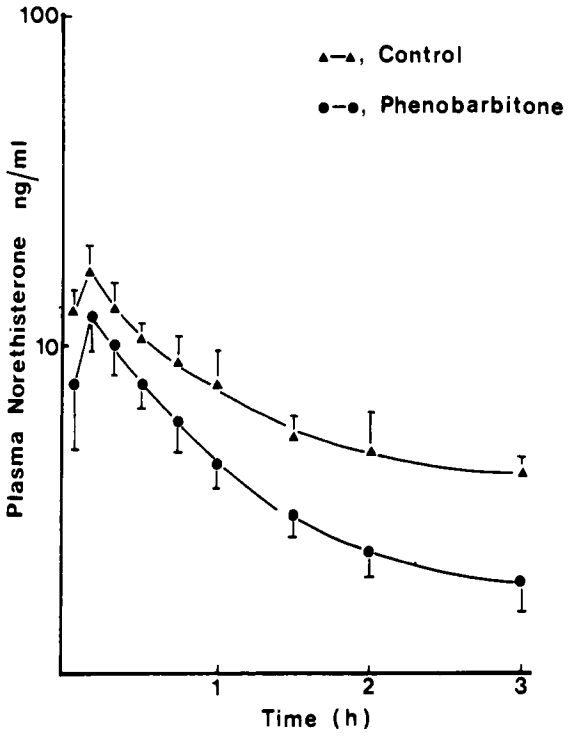

FIG. 5. Plasma norethisterone concentrations in control and phenobarbitone pre-treated rats after infusion (over 5 min) of norethisterone (500 μg/kg) into the hepatic portal vein. Each curve is the mean ± S.E.M. of data from at least 4 experiments.

number of these instances appears small, but this may be due to under-reporting of cases, or to a contraceptive failure being attributed to non-compliance by the patient. Interaction with antibiotics is potentially the most common of those discussed here. Current controlled studies in our laboratory suggest that ampicillin may cause only a small alteration in steroid pharmacokinetics and pharmacodynamics (i.e. for follicle-stimulating hormone and progesterone) but yet again there appears to be marked inter-individual variation in the interaction.

We have investigated the possible mechanism of the interaction between antibiotics and contraceptive steroids in animal models. Synthetic oestrogens and progestogens are extensively excreted as glucuronide or sulphate conjugates in bile (Smith 1974) in both human beings and laboratory animals. These conjugates subsequently pass into the enterohepatic circulation since they can be split by hydrolytic enzymes in the gut microflora, thus liberating

intact drug which may be reabsorbed (Back et al 1978f). Oral antibiotic therapy may interfere with this hydrolytic process and reduce or eliminate any steroid reabsorption from the gastrointestinal tract. We therefore isolated tritiated conjugates of norethisterone or EE2 from the bile of rats previously injected with parent steroids and infused these conjugates into the caecum of 'recipient' rats whose bile was subsequently collected for some 6 h (Back et al 1978e). Radioactivity appearing in the bile of 'recipient' rats is a measure of the extent of deconjugation in the gastrointestinal tract, since only unconjugated steroid can be reabsorbed across the gastrointestinal mucosa. We then pretreated recipient rats with a variety of antibiotics (Table 6) and studied the effect on EE2 metabolism. As shown in Fig. 6, ampicillin, neomycin + lincomycin, and cefoxitin each decreased the biliary excretion of radioactivity associated with EE2, and there was a good correlation between the suppression of the enterohepatic circulation of the steroid and the change in gut flora (Table 6).

TABLE 6

Effect of chronic antibiotic treatment on the enterohepatic circulation of EE2 and on the gut microflora

Treatment	*% excretion of EE2 in bile*	*Caecal flora*[a]	
		LFC	*m.an.*
Control	32.6 ± 2.3	+ + +	+ + +
Ampicillin (200 mg kg^{-1} day^{-1} for 4 days)	8.1[b] ± 2.6	+	±
Neomycin + lincomycin (100 + 100 mg kg^{-1} day^{-1} for 4 days)	6.9[b] ± 1.7	0	±
Cefoxitin (100 mg kg^{-1} day^{-1} for 4 days)	6.2[b] ± 1.3	not done	not done

[a]LFC, lactose fermenting coliforms (e.g. *E. coli; Streptococcus faecalis*); m.an., mixed anaerobes (e.g. *Clostridium* spp., *Bacteroides* spp.); ±, 10^3/ml; +, 10^3–10^5 ml; + + +, 10^7–10^{10}/ml.
[b]Significantly different ($P < 0.001$) from controls
EE2, ethinyloestradiol

In the above experiments, antibiotics abolish the secondary peak of steroid concentration in the plasma, providing further evidence that antibiotics can alter the enterohepatic circulation of steroids. Whether the same is true in humans remains to be seen.

OTHER ENVIRONMENTAL FACTORS

Smoking has been shown to increase the rate of metabolism of drugs such as phenacetin, antipyrine and theophylline, but not diazepam, phenytoin or

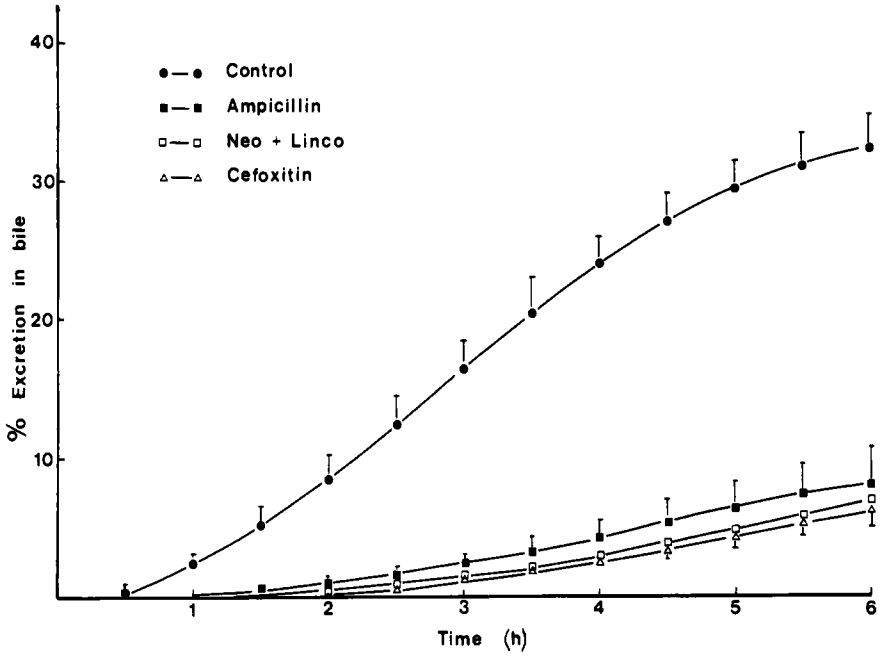

FIG. 6. Percentage cumulative excretion of radioactivity in bile after intracaecal administration of [^{3}H]EE2 conjugates to control and antibiotic treated rats. Each point is the mean ± S.E.M. of data from 4 experiments.

warfarin. Preliminary data from our laboratory (Back et al, unpublished observations) suggest that plasma concentrations of EE are lower amongst cigarette smokers than in non-smoking controls. This may be important in the toxicity of contraceptive steroids, since these smokers may have higher plasma concentrations of reactive steroid metabolites. These metabolites have been postulated to be the cause of the excess vascular disease found in women who smoke and take contraceptive steroids.

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Discussion

Connors: Could the great number of reports of drug interactions with contraceptive steroids nowadays be due to there now being less steroid in the contraceptive preparations?

Breckenridge: Yes; as the dose of steroid is reduced, these drug–drug interactions become more important. An epileptic patient on anticonvulsants who requires oral contraceptive therapy should be advised to take a contraceptive with a higher steroid content.

Amos: You mentioned a difference between the control and coeliac groups in relation to the metabolism of contraceptive steroids *in vivo*. Presumably, in the coeliac group, the mucosa is atrophied. Could the difference be a quantitative effect?

Breckenridge: In some of the coeliac patients studied (by Dr MacIver & Dr Orme) in our group the gut mucosa had returned to normal; in others it had not. Irrespective of whether these patients had responded to a gluten-free

diet, there was a difference in their ability to metabolize the steroids.

Hunter: Was the jejunal mucosa of the patients on the gluten-free diet histologically normal?

Breckenridge: Not always. But Peters has shown that not all enzymes in the gut wall of patients with coeliac disease return to normal, even if the histological appearance does (Peters et al 1978). It may be that the enzymes concerned with metabolizing contraceptive steroids fall into the same category.

Hunter: As regards the enterohepatic circulation, some Finnish workers studying oestrogens claimed that the 16-glucuronide was formed in the gut and excreted in the urine (Adlercreutz 1974). How could that be applied to your work?

Breckenridge: From the work done in our group by Drs MacIver and Rowe, the predominant metabolite from the gut appears to be the sulphate, and from the liver, the glucuronide and the sulphate. I was therefore interested to hear the statement in a previous discussion that sulphate conjugation cannot be induced. We would have to analyse our data further to see if we have any direct evidence of this.

Davies: Should we therefore advise women to take salicylamide with their contraceptive pills?

Breckenridge: We would need to collect more data before we could advise that.

Gillette: Were the bile ducts cannulated all the time in your experiment on the effect of antibiotics on the biliary secretion? If so, how could there be an enterohepatic circulation?

Breckenridge: These studies were carried out by Dr Back in our group. He cannulated the bile duct of both donor and recipient animals. The bile cannulae for the donor rats were implanted into the gastrointestinal tracts of the recipient animals. The bile from their cannulae was, in turn, collected. Thus, enterohepatic circulation took place, if in a slightly modified manner.

Gillette: In addition to undergoing enterohepatic circulation compounds or their conjugates can diffuse from the blood back into the intestinal tract and this may be important.

J.W. Bridges: There is a general assumption that compounds diffuse straight across from the lumen into the blood. We have some results from guinea pigs and rats that indicate that sulphate and glucuronic acid conjugates of some phenols are formed in the intestinal wall and then pass partially into the lumen (Dawson & Bridges 1980).

Orrenius: My question relates to your first set of experiments, Professor Breckenridge, in which you attempted to evaluate the contribution of the gut

to overall metabolism. How can you use your system to detect which conjugates were produced in the gut wall?

Breckenridge: By analysing plasma from the portal vein, we found a conjugate which was predominantly sulphate.

Orrenius: But then you would have to make single-pass perfusions, otherwise you would also pick up conjugates formed in the liver at the same time.

Breckenridge: Yes.

Orrenius: Conjugates formed in the liver or the gut are secreted with the bile and most of them are hydrolysed in the gut lumen and then reabsorbed by the gut so they come back into the portal vein. Is this correct?

Breckenridge: We take blood samples 10 minutes after administration of the steroid and we find low concentrations of conjugates in peripheral venous blood, presumably reflecting arterial blood passing to the gut. We find higher concentrations of conjugates in portal venous blood i.e. that passing from the gut.

De Matteis: Koskelo et al (1966) have reported increased urinary excretion of 5-aminolaevulinate in healthy women receiving contraceptive steroids that contain ethinyl substituents. This would be compatible with the idea that steroids may increase the turnover of cytochrome *P*-450-haem. Have you observed this?

Breckenridge: We do not have any data that would either confirm or refute this.

Idle: Do you have good evidence that the production of sex hormone binding globulin (SHBG) was increased in the presence of inducing agents? If so, could that single observation explain the change in plasma concentrations observed in your phenobarbitone-treated volunteers?

Breckenridge: Yes, SHBG concentration is increased in the presence of inducing agents. We have no evidence, however, that SHBG plays any role in the kinetics of ethinyloestradiol. It is of importance with respect to progestogens. So your suggestion may be partly correct.

Idle: Are you talking about an induction of conjugation or of a cytochrome *P*-450 mediated reaction?

Breckenridge: Norethisterone undergoes both phase I (i.e. cytochrome *P*-450 mediated) and phase II metabolism. Ethinyloestradiol predominantly undergoes conjugation.

Smith: This question of whether or not significant enterohepatic circulation of drugs occurs in humans is interesting. There are a few examples of drugs that recycle in humans e.g. cardiac glycosides, contraceptive steroids and iophenoxic acid. If it occurs for the contraceptive steroids can you estimate how much it might contribute to the bioavailability of these drugs?

Breckenridge: This is very difficult. How can we calculate the bioavailability of a drug that has a marked enterohepatic circulation simply from the plasma concentrations of the drug? I don't know.

Gillette: Kinetically that is extraordinarily complicated. If a drug is neither metabolized in the mucosa nor eliminated by the gut itself, biliary secretion cannot affect bioavailability.

Connors: Why do you conclude that the alteration in slope of the graph (after 16 h) implies the presence of an enterohepatic circulation? (Fig. 2).

Breckenridge: It is only an hypothesis; we have no firm evidence to support it, but that shape is absolutely reproducible.

McLean: Does anyone have an alternative explanation for the shape of the curve being caused by enterohepatic circulation?

Gillette: In kinetic terms, the shape of the graph implies the presence of a hold-up pool. The biliary secretion into the intestine could be a plausible mechanism for such a pool.

Smith: The hold-up may be related to gall bladder function. A bolus of the drug can be stored in the gall bladder for many hours and then released in response to a dietary stimulus. All the intestinal hydrolysis factors then come into play. The time of appearance of secondary humps in the plasma curves for both rabbit and humans would support this.

J.W. Bridges: Biphasic profiles of drug concentrations can be observed for other drugs in rats that do not have a gall bladder!

Gillette: In our original studies with imipramine (J. Dingell & J.R. Gillette, unpublished results), we occasionally found a small increase in the plasma concentration about twenty minutes after the intravenous administration of the compound. The only explanation we could put forward was that some material was accumulated in the lymph and that then there was a time delay for the lymph to pass to the thoracic duct.

J.W. Bridges: But only a very small percentage of a drug and/or its metabolites would be expected to travel in the lymph, surely?

Gillette: Well this drug is highly bound in the periphery, and thus has a large volume of distribution, so even relatively small amounts of highly concentrated material that initially pass into the hepatic circulation can be enough to produce the small increase in concentration in blood plasma after the so called α-phase is complete.

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General discussion II

Connors: The usefulness of the measurement of covalent binding has been mentioned on several occasions during this symposium. However, many of the participants have pointed out that electrophilic reactants do not discriminate well between different nucleophilic centres; they may bind to sulphur, nitrogen and oxygen in a variety of important and non-essential molecules. Since the majority of covalent binding that occurs may not represent toxic events, can gross measurement of such binding tell us anything about the toxicity of a chemical? For many agents that are metabolized to reactive species, binding to DNA is the important reaction, but this is small compared to the total binding that occurs. Segerbäck et al (1978) claimed that measurement of binding to haemoglobin can be used as an index of binding to DNA in tissues and hence as a means of monitoring the potential toxicity of reactive species. I would like to know whether gross measurement of covalent binding can tell us something about the potential hazard of a chemical.

Conney: Total covalent binding of potential carcinogens is not as useful as the binding of carcinogens to *specific* sites on DNA. Much more work is needed to identify the biologically important binding of carcinogens and drugs. (+)-7β,8α-Dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene ((+)-diolepoxide 2) and (−)-7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene ((−)-diolepoxide 2) are chemically reactive, optically active enantiomers. Although both enantiomers bind to DNA, the (+)-diolepoxide 2 binds 20 times more than the (−)-diolepoxide 2 to the 2-amino group of guanine in double-stranded DNA. This specificity of binding of the enantiomers does not occur, however, at other sites on the DNA (Meehan & Straub 1979). This high degree of specificity of binding of the (+)-diolepoxide 2 to the 2-amino group of guanine is paralleled by uniquely high mutagenic activity in Chinese hamster V79 cells and by uniquely high car-

cinogenic activity relative to the (−)-diolepoxide 2 (Wood et al 1977, Buening et al 1978).

B.A. Bridges: It is a question of degree: you cannot say that the non-carcinogenic ones are not at all mutagenic.

Conney: In Chinese hamster V79 cells, the (+)-diolepoxide 2 is about 20 times more mutagenic than the (−)-diolepoxide 2, but only twice as mutagenic in *Salmonella typhimurium* strains.

Garner: The difference between mutagenicity in *Salmonella* and in V79 cells could be enzymic. Perhaps there are enzymes in V79 cells that inactivate one isomer and not the other.

McLean: The compound *para*-aminophenol binds easily to liver. It binds more than paracetamol (acetaminophen) and does not produce necrosis in the liver. This means that covalent binding is a good index for exposure but it is a poor predictor of the effects of a new compound. In other words, if we want to know the overall biological effect of a compound administered for a long time, then the observation that covalent binding occurs does not solve the problem of prediction of whether it will be necrogenic or carcinogenic or not. But if the compound is known to be a carcinogen then the extent of binding provides a useful estimate of the exposure under those conditions.

Farber: But exposure is not necessarily equivalent to carcinogenicity.

B.A. Bridges: Ehrenburg might say it is (Calleman et al 1978), but many others would disagree.

Farber: Let us consider dimethylnitrosamine (*N*-nitrosodimethylamine) and diethylnitrosamine (*N*-nitrosodiethylamine). Dimethylnitrosamine methylates ten or a hundred times more avidly than diethylnitrosamine, and yet diethylnitrosamine is the better carcinogen.

B.A. Bridges: I would add a word of caution! If we measure binding, e.g. to haemoglobin, in order to relate this to binding to DNA, there is a danger that we may assume that all DNA is the same, whether it is inside the cell or in a test tube. We have demonstrated a photochemical interaction with 8-methoxypsoralen (methoxsalen) and u.v. A. light. We have shown that one strain of *E. coli* is 8 times more resistant to killing by that treatment than another strain (Bridges & Mottershead 1979). This effect is unrelated to DNA repair because it occurs in strains in which essentially all DNA dark repair has been blocked. The intracellular concentration of 8-methoxypsoralen is comparable in each strain and when phage-T3 is injected into the bacterial cytoplasm, it is killed in the same proportion by each strain. However, the sensitivity of the bacterial chromosomes in the two strains differs by a factor of 8. Preliminary data with a number of other intercalating agents suggests that they show a similar difference. Thus even within one species, DNA can

be organized in such a way that it is more or less accessible to intercalating agents. It is too early to estimate the importance of this and to determine how widespread it is, but over the next few years we may begin to understand how different configurations of DNA (e.g. degree of superhelicity) may affect the extent of DNA damage.

Farber: It is now clear that interaction of chemicals with DNA is not necessarily random. There may be some steric configuration that determines which interactions occur. Sarma et al (1979) have shown that if spermine or distamycin A (an antibiotic that binds to adenine–thymine-rich regions) is added to double-stranded DNA without histones, methylation is inhibited with methylnitrosourea but not with methane sulphonate or dimethyl sulphate (Sarma et al 1979). The problem with this work is that the DNA fractionation techniques for eukaryotic cells are still crude.

B.A. Bridges: This is nevertheless an exciting development. There is always a risk that when DNA is taken out from a cell, it may lose the property responsible for its behaviour inside the cell, whether that property is superhelicity or the presence of bound polyamines or proteins.

Garner: We must not assume that all carcinogens act in the same way. For example, administration of 2-acetylaminofluorene produces a lot of protein binding and much less nucleic acid binding in rat liver (Miller 1970). On the other hand the protein-bound species induced by the hydrocarbons may arise in a different way from the nucleic acid-bound species. When the carcinogen used is aflatoxin, protein binding in the liver is often the same in different species that have different susceptibilities. The protein binding induced by aflatoxin B_1 and that induced by aflatoxin G_1 are the same (Garner et al 1979) but the amount of nucleic acid binding varies with the susceptibility or resistance of the species, or with the particular aflatoxin studied. Therefore, with the aflatoxins, total DNA binding may be a measure of the biologically active adducts, but this may not be the case for many other carcinogens.

Oesch: There are many ways of studying covalent binding with respect to risk evaluation. Some groups propose that a binding index be obtained by measurements of total binding to DNA per dose (Lutz 1979). The binding index is proposed as a means of predicting the potential toxicity of a compound. The danger is that this obscures the fact that binding to different specific places in the DNA has different consequences, and the extent of binding of a compound is not necessarily an indication of its potential toxicity.

Orrenius: I think the use of a binding index is a question of concentration and rate of formation of metabolite. Total covalent binding provides a measure of the rate of formation of a reactive metabolite. In many cases, however, so much metabolite is produced that we overload the system so that

binding takes place not only to critical targets but to a variety of other nucleophiles as well. This excessive binding may obscure the toxicologically significant binding. As we lower the concentrations of the reactive metabolite, we may be able to see selectivity in the interaction with cellular nucleophilic sites.

J. W. Bridges: Are you suggesting that determination of covalent binding could be used as a general toxicity test?

Orrenius: I am suggesting that 'total' covalent binding is only a reflection of the formation of electrophilic metabolite(s) which, however, probably 'contain' the toxicologically significant interaction.

Gillette: The concept that a chemically reactive metabolite initially reacts only with DNA implies that DNA has a high affinity for the reactive metabolite. Essentially the kinetics would be like affinity labelling. When DNA has a high affinity for the metabolite, reduction of the dose may result in preferential binding to DNA. But if the chemically reactive metabolite reacts with protein, with DNA and with other macromolecules rather indiscriminately, then lower doses should not change the relative proportions of material covalently bound to the various kinds of macromolecules.

Farber: What is the result of this selectivity at low concentrations of reactive metabolite?

Orrenius: Selectivity in interaction occurs, both with unspecific nucleophiles and with specific nucleophilic sites in DNA or protein.

Gillette: I think it is a mistake to make the assumption that covalent binding is a necessity for carcinogenesis. A free radical of the toxic agent may abstract a hydrogen atom from the DNA molecule, and the free radical of the DNA may produce toxicity. Thus there would be no net covalent binding.

Orrenius: I would like to expand on my previous point. During metabolism of benzo[*a*]pyrene, two reactive products are formed which interact with DNA; one is a metabolite of 9-hydroxybenzo[*a*]pyrene and the other one is benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide. When DNA binding is studied in a system containing benzo[*a*]pyrene, rat liver microsomes and purified DNA from calf thymus, the contribution of activated 9-hydroxybenzo[*a*]pyrene to DNA binding predominates over that of the diolepoxide. If isolated liver nuclei replace the microsomes and purified DNA in this system, the reverse is true. Further, the addition to the latter system of a relatively weak nucleophile like albumin lowers the contribution of activated 9-hydroxybenzo[*a*]pyrene to DNA binding even further, whereas binding of the diolepoxide to nuclear DNA is much more difficult to affect, and appears to require that both glutathione and glutathione transferase are included in the system.

Farber: How do these results compare with results obtained after different doses of benzo[*a*]pyrene *in vivo*?

Orrenius: They compare quite well with results from intact liver cells, where we find that the diolepoxide always predominates in DNA binding at low concentrations of benzo[*a*]pyrene.

J.W. Bridges: How can you eliminate the influence of selective binding of metabolites to albumin and cytosol constituents in your experiments?

Orrenius: We cannot eliminate that. Our working hypothesis is that DNA-binding metabolites are formed by recycling primary metabolites through the cytochrome *P*-450 system, and that the endoplasmic reticulum is responsible for generation of most of the primary metabolites, whereas the nuclear system may contribute to the final activation. Part of our evidence is based on this effect of cytosolic nucleophiles, which suggests that electrophilic metabolites generated in close proximity to nuclear DNA may have a greater chance of reaching this target.

Bend: What is your evidence that the glutathione transferases are involved enzymically in the reaction between benzo[*a*]pyrene 7,8-dihydrodiol-9,10-epoxide and glutathione?

Orrenius: Our evidence is not conclusive but relies on preliminary experiments with different cytosol fractions.

Higginson: Does cellular DNA always have the same binding for a carcinogen *in vitro,* irrespective of whether the DNA is from a fibroblast, liver or epidermal cell? There are, apparently, differences *in vivo;* do such differences represent purely a target dose effect, or are they a function of selectivity of DNA or of repair in different cells?

Garner: There is some evidence that benzo[*a*]pyrene diolepoxide 1 will bind to particular base sequences rather than to specific bases (Kakefuda & Yamamoto 1979, Jahn & Litman 1979).

Boyd: As we emphasized previously, the simple observation that a compound is bound covalently to a significant extent *in vivo* does not in itself predict the potential toxicity and/or carcinogenicity of the compound. However, if we can establish additionally that (a) the toxicity of a given compound is due to a highly reactive metabolite and not to the parent compound itself, and that (b) the tissue covalent binding of radioactive products *in vivo* after the administration of the radiolabelled compound reflects the formation of the toxic reactive metabolite, then the measurement of covalent binding may predict either the tissue selectivity and/or the relative potency of the compound. This is illustrated by some of our recent work on species (Dutcher & Boyd 1979, Buckpitt & Boyd 1978), age (Jones et al 1979), and sex differences (Longo & Boyd 1980) in the covalent binding and toxicity of 4-ipomeanol.

As I described previously in my paper (p 43-60), the concentrations of covalently bound 4-ipomeanol in rats were higher in the lungs than in the liver or kidneys. 4-Ipomeanol was also covalently bound preferentially to the lungs of rabbits, guinea pigs, adult female mice, and immature male or female mice. In contrast, in adult male mice, the levels of not only pulmonary alkylation but also renal alkylation were high. Another unique pattern of tissue specificity was seen in birds; both the pulmonary and the renal alkylation levels were very low in comparison to the high levels of covalent binding of 4-ipomeanol observed in the liver.

In sharp contrast to these species differences, the patterns of tissue alkylation within different strains of a given species were highly consistent. For example: three strains of rats (Sprague–Dawley, Lewis, and Fisher 344) showed the pattern of organ-specific covalent binding of 4-ipomeanol typical for rats; six strains of mice (Swiss, C57BL/6J, DBA/2J, A/J, C3H/HeJ, and BALB/cJ) showed the pattern typical of adult male mice; and two kinds of birds (quail and chickens) gave tissue alkylation patterns that were similar to each other.

The major target organs for 4-ipomeanol toxicity *in vivo* in different species reflected the respective organ specificities of covalent binding of the compound *in vivo*. Thus, in rats, rabbits, guinea pigs, adult female mice, and immature male or female mice, the lungs were the primary site of damage by 4-ipomeanol; in adult male mice, however, both the lung and the kidneys were consistently damaged; and in chickens and quail, the liver was the major site of damage.

Thus, these studies showed that the particular pattern of organ specificity for covalent binding of 4-ipomeanol would have been a good predictor of the organ specificity for toxicity of the compound in animals of a particular species, age and sex.

In addition, especially among different strains of a given species, the actual magnitude of the covalent binding in target organs appeared to predict the acute lethality of 4-ipomeanol and/or its potency in causing tissue damage. For example, the Lewis rat was the most resistant strain of rat and had the significantly lowest covalent binding in the lung *in vivo*. Similarly, the three mouse strains that were least resistant to 4-ipomeanol had significantly higher covalent binding in the lungs and kidneys *in vivo* than the more resistant mouse strains.

The correlation between covalent binding and potency of 4-ipomeanol was less consistent in inter-species comparisons, but was occasionally apparent. For example, the hamster species was highly resistant to 4-ipomeanol, and the covalent binding in hamster lung was invariably lower than in other species.

Therefore, within certain limitations that must be clearly defined for any specific compound, the covalent binding of a toxic compound *in vivo* may predict its potential tissue selectivity or its relative potency, or both.

Farber: I agree. We have studied methylnitrosourea which is a direct-acting agent that alkylates DNA and RNA of liver efficiently. But it does not induce necrosis or tumours in the liver unless the conditions are manipulated (Cayama et al 1978). Therefore caution is necessary.

Boyd: Yes; these effects are extraordinarily dependent on the compounds used. Once a strong correlation between the covalent binding and the pathological effects is established for any compound, covalent binding can be a very useful experimental tool, without necessarily implying that it means anything biologically. It may help us to elucidate the critical biochemical interactions that lead to cell death, or to neoplastic transformation.

Davies: In summary it seems that covalent binding, in the absence of toxicity *in vivo,* is of little significance.

Farber: In that case, neither can we conclude that covalent binding of protein *per se* is the cause of necrosis.

Gillette: We have no idea what the mechanism of necrosis is!

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Vitamin D metabolism in patients treated with phenytoin and phenobarbitone

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Abstract There are many reports of abnormalities in calcium metabolism and even of frank osteomalacia in patients taking anticonvulsant drugs. Although enzyme induction has been suggested to be the cause of increased requirements for vitamin D leading to these changes, this has still to be proved. We have studied the metabolism of [^{3}H]cholecalciferol and 25-hydroxycholecalciferol in subjects taking anticonvulsant drugs. The rate of removal of cholecalciferol from the plasma was no different from that in control subjects and no abnormality was detected in the production or disappearance of 25-hydroxycholecalciferol. However, plasma concentrations of 25-hydroxycholecalciferol fell more rapidly during the winter in epileptic subjects than in control subjects. Our results do not confirm that these changes are due to enzyme induction.

VITAMIN D METABOLISM

Vitamin D is obtained largely from the action of sunlight on the skin, dietary vitamin D being of minor importance (Poskitt et al 1979, Lawson & Davie 1980).

The vitamin is inactive and is converted to 25-hydroxyvitamin D (25-OHD) by a 25-hydroxylase enzyme occurring solely in the microsomal fraction of the liver (De Luca 1974).

25-OHD is the main circulating metabolite (Stamp 1975) but is probably inactive itself and is converted in the kidney to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (Fraser & Kodicek 1970) which is active in promoting intestinal calcium absorption and calcium resorption from bone (Kodicek et al 1970, Reynolds 1974). Other dihydroxymetabolites are found such as 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) (Boyle et al 1973) which may promote normal calcification of cartilage and bone (Ornoy et al 1979).

Metabolites of vitamin D are found in bile (Bell 1969). It has been claimed that 25-OHD appears in human bile (Arnaud et al 1975), but this was not

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found in rats (Bell 1969). The nature of these metabolites remains uncertain, but an enterohepatic circulation of vitamin D metabolites may exist and further metabolism may occur in the intestinal mucosal cell.

ABNORMALITIES OF CALCIUM, VITAMIN D, AND BONE METABOLISM AFTER ANTICONVULSANT TREATMENT

The association between osteomalacia and treatment with phenobarbitone or phenytoin was reported by Kruse (1968). Since then, there have been many reports of abnormalities in serum calcium, phosphate, alkaline phosphatase, and plasma 25-OHD together with a reduction in bone density (Christiansen et al 1972) in patients on anticonvulsant drugs. Serum calcium is low in many of these subjects (Richens & Rowe 1970), and correlates with the duration of treatment with anticonvulsants. Daily doses of 2000 i.u. vitamin D do not restore serum calcium to normal (Bouillon et al 1975) and larger daily doses of vitamin D are necessary to achieve calcium balance (Peterson et al 1976). The plasma concentration of 25-OHD is frequently low (Bouillon et al 1975) but in patients who were taking either phenytoin or phenobarbitone and who were surveyed during November, the plasma 25-OHD was found to correlate with the daily dose of phenytoin or phenobarbitone, expressed as mg/kg of body weight (M. Davie, C.E. Emberson, J.O. Barnes, G.E. Roberts & D.E.M. Lawson, unpublished observations). Plasma concentration of 25-OHD alone, however, is an unreliable index of osteomalacia (Davie et al 1978).

In addition to low plasma concentrations of 25-OHD, low levels of plasma $24,25(OH)_2D$ have also been found after anticonvulsant therapy (Weisman et al 1979). Plasma concentrations of $1,25(OH)_2D$ are, however, normal (Jubiz et al 1977).

There is increasing evidence that vitamin D may reduce the frequency of epileptic fits independently of any effect upon serum calcium or phosphate (Christiansen et al 1974, MacLaren & Lifshitz 1973, M. Davie, N. Barnes, G.E. Roberts & D.E.M. Lawson, unpublished observations). Ultraviolet light may be more effective in reducing the frequency of fits than vitamin D taken orally (M. Davie, N. Barnes, G.E. Roberts & D.E.M. Lawson, unpublished observations).

Since the abnormalities associated with anticonvulsant drugs are a function of the amount and duration of treatment, estimations of the incidence of abnormalities vary. Kruse (1968) found that 15% of patients examined radiologically had rickets or osteomalacia. Of fifty adult epileptics receiving outpatient treatment for ten years or more, 22% had low values for serum calcium and phosphate and raised alkaline phosphatase levels (EC 3.1.3.1)

while 8% showed histological osteomalacia (Ashworth & Horn 1977). Radiological evidence of rickets was found in 8% of children receiving anticonvulsants for longer than three months (Crosley et al 1975), but a higher incidence of 23% found by Tolman et al (1975) reflects these authors' use of criteria that may detect abnormality but may not reflect the presence of osteomalacia. Another survey of 15 000 epileptics failed to reveal a single case of rickets or osteomalacia (Livingstone 1973).

NATURE OF THE ABNORMALITY OF VITAMIN D METABOLISM

The epidemiological evidence pointing to an abnormality of vitamin D metabolism after anticonvulsants is supported both by the observed impairment in the healing of rickets in rats given phenobarbitone (Hunter 1976) and by the altered tissue distribution of radioactivity after intravenously administered [^{3}H]cholecalciferol (Silver et al 1974). The nature of the defect(s) is, however, still uncertain. Several studies have suggested that there is a greater requirement in epileptic patients for vitamin D either to heal osteomalacia or rickets or to restore calcium balance (Stamp et al 1972, Peterson et al 1976) although much smaller doses of 25-OHD appear to be necessary to achieve these effects (Stamp et al 1972, Lifshitz & MacLaren 1973). The relative resistance to vitamin D raised the possibility that the vitamin was being inappropriately metabolized. Silver found that excretion of vitamin D metabolites in bile was enhanced after administration of phenobarbitone to rats and he proposed that this might be the reason why phenobarbitone-treated rats retained less of an intravenous dose of [^{3}H]cholecalciferol than did control rats (Silver et al 1974).

The increased excretion of biliary metabolites of vitamin D was also taken as support for the theory of enzyme induction proposed by Dent et al (1970). This theory stated simply that osteomalacia in anticonvulsant-treated subjects was the result of enhanced vitamin D catabolism by non-specific microsomal hydroxylase enzymes induced by phenobarbitone or by phenytoin.

The administration of phenobarbitone leads to an increased activity of hepatic microsomal enzymes which can be measured in humans (Hunter & Chasseaud 1976) by the urinary excretion of D-glucaric acid (Hunter et al 1971a). In epileptic children, a negative correlation was found between serum calcium and excretion of D-glucaric acid (Hunter et al 1971b). In a subject with osteomalacia, who was taking glutethimide, evidence for enzyme induction was also suggested by a reduction of the half-life of antipyrine (Greenwood et al 1973).

However, there is no good evidence for a specific effect of enzyme induc-

tion on vitamin D metabolism. The reduction in half-life of radioactive vitamin D, reported by Hahn et al (1972) in human subjects taking anticonvulsants, failed to take into account the prevailing plasma concentration of 25-OHD. The best evidence for a specific effect is probably the enhanced excretion of biliary metabolites of vitamin D during phenobarbitone administration (Silver et al 1974). On the other hand, Sulimovici & Roginsky (1977) suggested that inhibition rather than induction of hepatic 25-hydroxylase may be responsible for the reduction in plasma concentrations of 25-OHD.

In view of the large number of substances capable of causing enzyme induction we have studied in detail the effect of phenytoin and phenobarbitone on vitamin D metabolism in humans.

STUDIES OF VITAMIN D AND 25-OHD IN SUBJECTS RECEIVING PHENYTOIN AND PHENOBARBITONE

Radioactive tracer studies

Tracer quantities of 1,2[^{3}H]cholecalciferol (vitamin D_3) or 26,27[^{3}H]25-hydroxycholecalciferol were injected intravenously and blood samples were then taken at regular intervals. [^{3}H]Cholecalciferol and [^{3}H]25-OHD were then separated by thin layer chromatography (Jung et al 1978). The half-life of [^{3}H]cholecalciferol was calculated during the period 4–48 hours after injection, and that of 26,27[^{3}H]25-hydroxycholecalciferol was calculated during the period 48–504 hours after injection.

The half-life of [^{3}H]cholecalciferol depended upon the plasma concentration of 25-OHD in normal subjects, and in 14 subjects receiving phenobarbitone, phenytoin, or both, no significant difference from normal was found (Fig. 1). Neither was the appearance of [^{3}H]25-OHD after injection of [^{3}H]cholecalciferol impaired (Fig. 2).

Investigations of the half-life of intravenously administered [^{3}H]25-OHD from plasma showed that the disappearance of [^{3}H]25-OHD is not dependent on the plasma concentration of 25-OHD and that subjects receiving phenobarbitone or phenytoin had a half-life similar to normal subjects (Fig. 3).

Studies of plasma concentrations of 25-OHD

Several surveys have shown that plasma concentrations of 25-OHD are low in subjects treated with anticonvulsant drugs. The response to controlled ultraviolet (u.v.) irradiation was compared in six non-epileptic and six epilep-

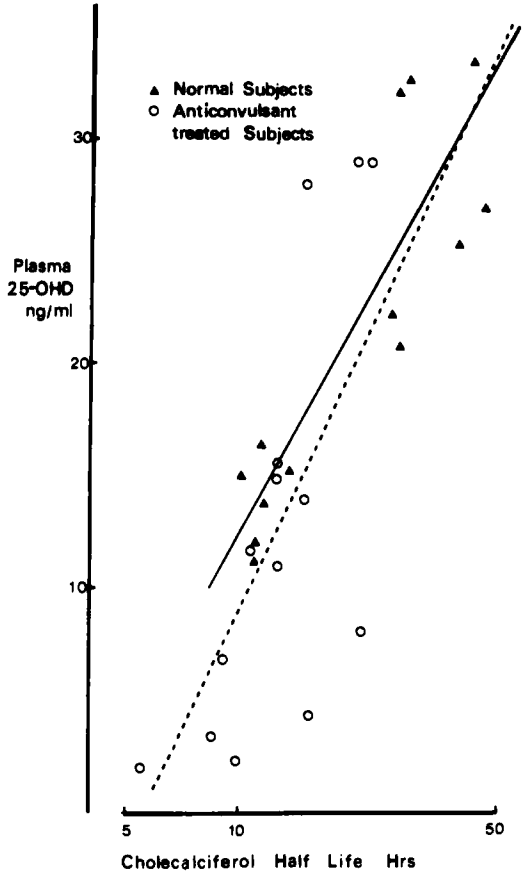

FIG. 1. [^{3}H]cholecalciferol half-life in normal and anticonvulsant-treated subjects. The half-life correlates with plasma concentrations of 25-OHD in both normal ($r = 0.82$; $P < 0.01$) and anticonvulsant-treated subjects ($r = 0.67$; $P < 0.05$). The solid line depicts the correlation for normal subjects and the interrupted line that for all subjects ($r = 0.80$; $P < 0.01$).

tic subjects matched for age, sex and weight. The epileptics were taking phenobarbitone (2.9 ± 1.3 (SD) mg kg^{-1} day^{-1}) and two of them were also taking phenytoin (3.7 ± 0.1 (SD) mg kg^{-1} day^{-1}). There was no significant difference between the two groups in the change in plasma concentration of 25-OHD after u.v. irradiation (Fig. 4) (Davie & Lawson 1979, Davie et al 1979).

The fall in plasma concentration of 25-OHD over the first 25 days after cessation of u.v. irradiation in subjects being treated with anticonvulsants was

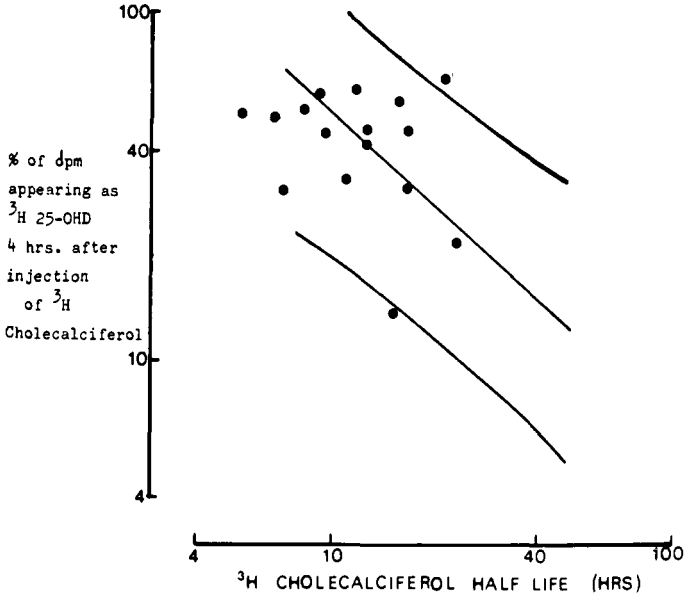

FIG. 2. The amount of [^{3}H]25-OHD$_3$, as a % of total disintegrations/min (dpm), appearing in plasma 4 hours after an intravenous injection of [^{3}H]cholecalciferol as a function of the half-life of [^{3}H]cholecalciferol. The lines show the correlation, with 95% confidence limits, for normal subjects. Results from subjects treated with phenobarbitone or phenytoin, shown by dots, fall within these limits.

also compared with that in normal subjects. The preliminary results (Fig. 5) suggest that there is a significant difference between the groups, with 25-OHD disappearing more rapidly in the anticonvulsant-treated subjects.

CONCLUSIONS

The studies with [^{3}H]cholecalciferol failed to demonstrate a difference in the half-life of [^{3}H]cholecalciferol in plasma when allowance was made for the plasma concentration of 25-OHD. Failure to make this allowance was the probable reason for the finding of a shorter half-life of cholecalciferol after anticonvulsant treatment in a previous study (Hahn et al 1972). In addition, we were not able to demonstrate any defect in the appearance of [^{3}H]25-OHD (from [^{3}H]cholecalciferol) in plasma. These observations suggest that intravenously administered vitamin D is removed and metabolized to 25-OHD at a rate appropriate to the prevailing vitamin D status.

The normal response of plasma 25-OHD to u.v. irradiation shows that in

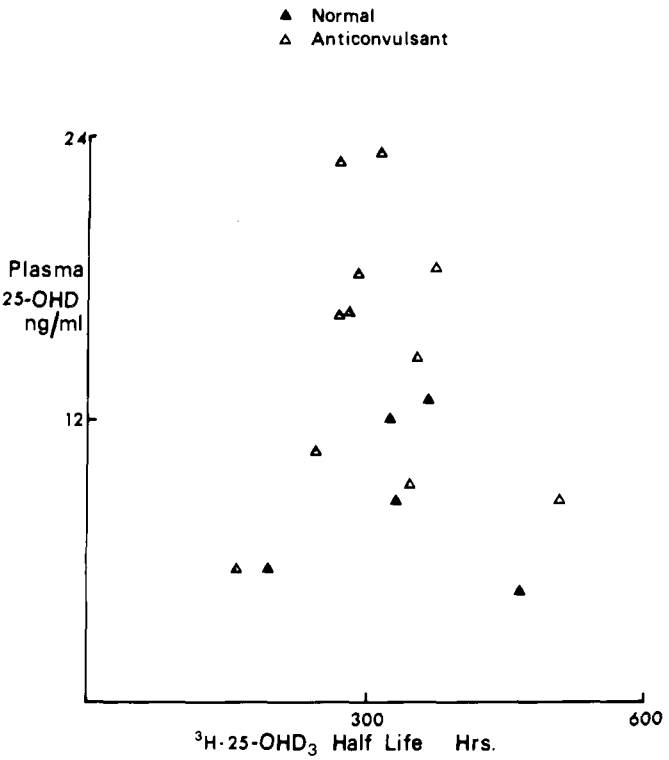

FIG. 3. The long [^{3}H]25-OHD$_3$ half-life does not correlate with the plasma 25-OHD concentration in either normal or anticonvulsant-treated subjects.

subjects treated with phenobarbitone the synthesis of vitamin D in the skin is not impaired and neither is its further metabolism to 25-OHD.

The disappearance of intravenously administered [^{3}H]25-OHD has not been extensively studied (Bec et al 1972). In normal subjects the long half-life (measured between 48–504 hours after injection of [^{3}H]25-OHD$_3$) does not appear to correlate with the plasma concentration of 25-OHD (P. Siklos, unpublished observations) and this was also a feature in anticonvulsant-treated subjects (Fig. 3).

The half-life derived from the fall in the plasma concentration of 25-OHD differs from that of [^{3}H]25-OHD in important respects. Not only is it longer but also there appears to be a difference between normal and anticonvulsant-treated subjects. The slower fall of plasma concentrations of 25-OHD suggests that some replenishment of plasma 25-OHD is occurring. This may

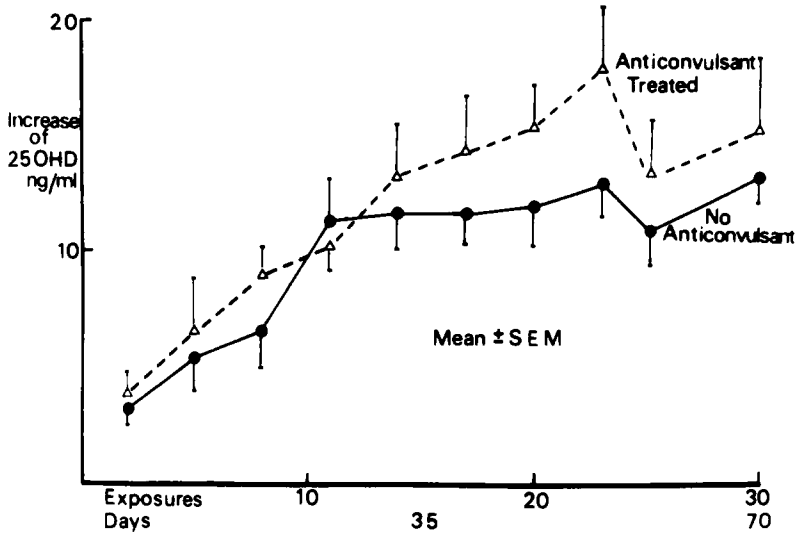

FIG. 4. Response of plasma concentration of 25-OHD to u.v. irradiation of 600 cm^2 of the dorsal skin in 6 normal and in 6 anticonvulsant-treated subjects. Change of plasma 25-OHD (ng/ml) is plotted against duration of irradiation. Plasma 25-OHD rises equally in both groups.

derive from precursors of 25-OHD in the metabolic pathway, since intravenously administered [^{3}H]25-OHD would be expected to equilibrate with extravascular stores of 25-OHD.

The distribution of vitamin D activity in the body is controversial. Many reports are concerned with the distribution of vitamin D activity after the administration of totally unphysiological amounts of the vitamin (Lawson & Davie 1980). Under normal circumstances plasma probably contains the highest concentration of vitamin D activity of any tissue (Quarterman et al 1964) but the vitamin is also found in muscle, fat, liver and kidney, and only small contributions from these sources would be sufficient to prolong the half-life of 25-OHD several times.

It is possible that the more rapid fall in plasma 25-OHD concentration observed in anticonvulsant-treated subjects, compared to controls, reflects an effect of these drugs on the storage of vitamin D in extravascular sites. This hypothesis would also account for the finding that phenobarbitone altered the tissue distribution of intravenous [^{3}H]cholecalciferol in rats (Silver 1974). Our work suggests that enhanced hepatic microsomal enzyme activity is not a major factor leading to the low plasma concentrations of 25-OHD observed in anticonvulsant-treated patients.

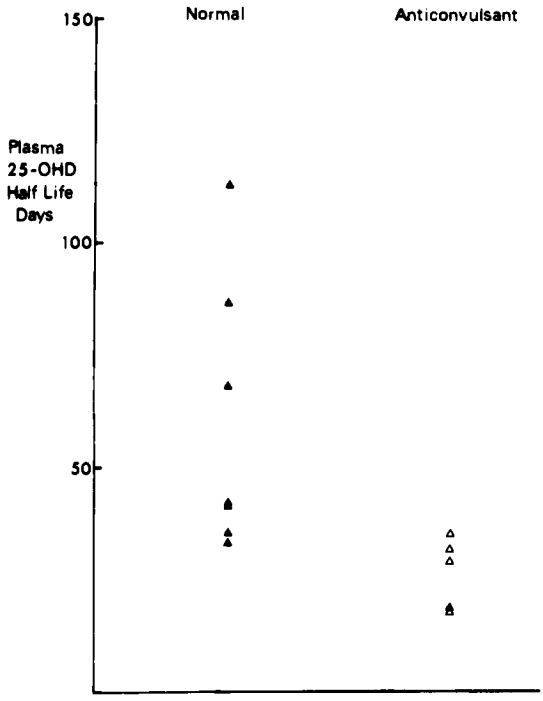

FIG. 5. Fall in plasma concentration of 25-OHD (expressed as a half-life). The plasma half-life of 25-OHD is slower than would be expected from the half-life of [^{3}H]25-hydroxycholecalciferol. In 7 normal subjects it was 59.7 ± 30.0 (SD) days. In 5 subjects treated with phenobarbitone ± phenytoin the half-life was 26.2 ± 7.9 (SD) days ($P < 0.02$).

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Discussion

Breckenridge: The work by Dr E. Smith and her colleagues in our group has shown that sex hormone binding globulin (SHBG) can be induced by phenobarbitone (Back et al 1980). Another explanation for your results, Dr Hunter, is that an increased amount of vitamin D binding globulin is present within the plasma after phenobarbitone administration and is avidly bound to the vitamin D. This would allow for the same conclusions that you have reached about a less available pool of vitamin D and its metabolites.

Hunter: Generally, saturation of the vitamin D-binding globulin by 25-hydroxyvitamin D (25-OHD) is very small. If it were bound tightly to the globulin in the circulation, the concentrations of 25-OHD in our patients would have remained high over the winter and would not have fallen faster than those in the control subjects.

De Matteis: Is the system responsible for the degradation of vitamin D self-regulated, i.e. can it be stimulated by providing more vitamin for degradation? Perhaps during the winter there is less production of vitamin D in the skin and its degradation is also slowed down to maintain the plasma concentration. That would be compatible with a prolonged half-life in the absence of inducers.

Hunter: We have not compared the half-lives in the summer and in the winter; measurements during the summer are hampered because the levels of 25-hydroxycholecalciferol rise as a result of exposure of the skin to sunlight, but we have drawn attention to the lack of correlation between plasma concentrations of 25-OHD and the [^{3}H]25-OHD$_3$ half-life.

McLean: Can we distinguish between the half-life of the *radioactive* drug in the plasma after a single dose, which seems to be dominated by exchange into tissues, and the half-life of the *unlabelled* drug whose concentration is falling because of lack of sunlight or vitamin D intake, this process being dominated by destructive pathways of metabolism?

De Matteis: If the degradation of vitamin D were to be slowed down in response to a decreased rate of synthesis, could an inducer then override this effect and produce the discrepancy?

Hunter: If that were true, I should have expected to have found a difference in the production of 25-OHD from cholecalciferol.

Gillette: Surely there cannot be a change in a binding site from 'on' to 'off' in a deep pool, without a change in half-life? If there is no change in half-life, but you wish to implicate a change in a deep binding site, then you also have to implicate a change in clearance, because the half-life = ln 2 × (volume of distribution/clearance).

Hunter: We did not find any change in clearance, so I must accept your interpretation.

McLean: Are the volumes of distribution reasonably similar in depleted and repleted subjects?

Hunter: If the clearance and the half-life are the same, then the volume of distribution is unchanged too.

Davies: De Luca's group (1979) has recently published evidence that the enzyme responsible for the 25-hydroxylation reaction is an hepatic microsomal cytochrome *P*-450.

Hunter: Well, the microsomes *are* responsible, but the reaction is not blocked by carbon monoxide.

De Matteis: What, then, is the actual evidence that the reaction is dependent on cytochrome *P*-450?

Hunter: De Luca (1974) states that the 25-hydroxylation of vitamin D oc-

curs predominantly in the liver microsomal fraction when it is supplemented with the cytoplasmic fraction. The reaction requires reduced pyridine nucleotide and molecular oxygen but is not inhibited by CO + O_2 mixtures, suggesting that it is not mediated by cytochrome *P*-450.

J.W. Bridges: Madhok & De Luca (1979) have recently provided evidence that cytochrome *P*-450 *is* involved in hepatic 25-hydroxylation of vitamin D.

Conney: Was a photochemical reactivation spectrum done on the carbon monoxide-inhibited reaction?

J.W. Bridges: Yes.

Gillette: If there is a very slow reduction rate of cytochrome *P*-450, it is quite possible for there to be little inhibition by the carbon monoxide. The degree of CO-inhibition may frequently depend not only on the affinity of reduced cytochrome *P*-450 for CO but also on the relative rates of reduction and oxidation of cytochrome *P*-450. A slight inhibition by CO may simply imply that most of the cytochrome *P*-450 is in the oxidized form under steady-state conditions. The hydroxylase could still be a unique form of cytochrome *P*-450. I believe that the cytochrome *P*-450 enzymes that catalyse the metabolism of vitamins and steroids to physiologically important metabolites are much more specific than those that are induced by phenobarbitone.

Amos: What is responsible for the further hydroxylation step in the kidney?

Hunter: This is a mitochondrial enzyme (the 1-hydroxylase) (Fraser & Kodicek 1970), and De Luca (1974, 1979) states that it is dependent on cytochrome *P*-450.

Connors: Would you comment on the incidence of osteomalacia between the various groups? There seems to be a considerable difference in the incidence in various studies.

Hunter: This is probably related to the amount of vitamin D in the diet; the American diet is highly supplemented with vitamin D, and this may be the reason for the low incidence of osteomalacia in the Johns Hopkins group. Large amounts of vitamin D in the diet *do* seem to be protective, and a number of workers have suggested that children and adults with epilepsy should receive supplementary doses.

Connors: Is the vitamin given prophylactically at the moment?

Hunter: No. The best procedure is to monitor Ca levels, and give vitamin D only if they start to fall. Prophylactic treatment may result in overdosage.

McLean: The Panel of Child Nutrition in the United Kingdom cut down the vitamin D intake of infants, because of idiopathic hypercalcaemia, which developed in several cases. Charles Dent believed that idiopathic hypercalcaemia could start to develop in sensitive children receiving 1000 i.u.

vitamin D per day. That suggests that a lot of children in the States may be receiving potentially toxic doses of vitamin D. Is that so? Do they develop idiopathic hypercalcaemia?

Davies: We have recently studied the effect of inducers on metabolism of vitamin D. We examined the effects of antipyrine on 25-OHD concentrations in Caucasians and in vegetarian Asians (Wilmana et al 1979). There was a dramatic fall in plasma concentrations of 25-OHD during intake of antipyrine over four weeks. The concentrations of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), measured only in the Caucasians, did not change. A dramatic fall in the concentration of 25-OHD was also observed by Brodie et al (1980) after rifampicin treatment, but there was no change in $1,25(OH)_2D$. These enzyme inducers reduce the concentration of circulating 25-OHD, but they appear to have no effect on the concentration of the $1,25(OH)_2D$.

Hunter: Those results are consistent with ours. However, I have never heard of a case of osteomalacia in association with either rifampicin or antipyrine.

Davies: The plasma concentration of 25-OHD is 500 to 1000 times greater than that of $1,25(OH)_2D$. Do the relative potencies of the two compounds differ by the same order of magnitude, Dr Hunter, and do you believe that the 25-OHD itself makes a contribution to the actions of the vitamin D metabolites?

Hunter: I think that the 25-OHD requires further conversion, to 24,25-dihydroxyvitamin D ($24,25(OH)_2D$), which is probably important in bone metabolism. Patients with anticonvulsant-induced osteomalacia respond to small doses of 25-OHD (50 i.u./day). There is also evidence that hypocalcaemia responds to even smaller doses of 1α-OHD. In patients with renal failure, 1α-OHD is far more effective and much more specific than 25-OHD in its effect on calcium levels and hyperparathyroidism.

Connors: You mentioned that unlike results from people on anticonvulsants there have been no cases of osteomalacia in patients treated with rifampicin. Is that because of the duration of treatment?

Hunter: Well, they are not treated for as long as epileptics who receive anticonvulsants. However, rifampicin may be taken for two years during treatment of tuberculosis, and that might be enough for abnormalities to be revealed. When we studied patients who were being treated with rifampicin at Papworth Chest Hospital (Cambridge), we examined enzyme induction, cortisone metabolism, calcium phosphate, and alkaline phosphatase concentrations in serum and found no differences in any of these from the measurements in control patients, who were in the same hospital but were not receiving rifampicin (Edwards et al 1974).

Connors: Did the patients in your study, Dr Davies, receive only one course of rifampicin treatment?

Davies: Yes, over a period of 2 weeks. It is true that there is no evidence of rifampicin-induced osteomalacia. Vegetarian Asians are the group of people most likely to develop this disease.

Hunter: Quite. Immigrants are also likely to get tuberculosis. I find it difficult to correlate your results, Dr Davies, with our own radioactive studies of [^{3}H]-cholecalciferol + 25-OHD, which appear to be completely normal. Possibly some of the vitamin is converted to a metabolite other than 25-OHD, which might explain the fall in plasma concentration. We have, however, searched extensively for evidence of other metabolites, and have found none.

Meyer: When you did the radioactive study, did you actually identify the different radioactive metabolites or did you just measure *total* radioactivity?

Hunter: We separated radioactive metabolites on thin layer chromatography. The position of 25-OHD was identified by simultaneous runs of the pure compound.

Davies: Another problem is that rifampicin and antipyrine can induce microsomal enzyme activity (Wilmana et al 1979, Brodie et al 1980). The 25-hydroxylase is a microsomal enzyme and yet the concentration of the 25-hydroxy-metabolite *falls* rather than rises. Is there any evidence that the 25-OHD is conjugated?

Hunter: Yes, there is. Bell (1968) studied the biliary excretion of various metabolites of vitamin D in the rat (and this is something that we now hope to continue in humans). He had difficulty because of the quenching produced by bile pigments.

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Environmental Chemicals, Enzyme Function and Human Disease
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Chemical disease in humans: problems in comparative toxicology

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Abstract Difficulties in assessment of the comparative pharmacology of drugs now apply to the effects on humans of foreign compounds from the environment (xenobiotics). Perhaps the most striking change in perspective has been the recognition that a latency of several decades may precede the development of toxic effects. Xenobiotics may persist and accumulate in tissue stores, and estimates of biologically active doses are sometimes complicated by the presence of highly toxic trace contaminants and homologues that have varying biological activity. The effects of chemical exposure on the tissues may be obscured by the absence of the pathognomonic symptoms normally associated with the structural changes of disease. Interactions between two or more xenobiotics or between xenobiotics and genetic and metabolic variables may explain the lack of coherence between carefully defined laboratory observations and the clinical effects of enzyme-inducing xenobiotics. Since we have considerable evidence that biological processes are strikingly similar in different mammalian species, the toxicological significance to humans of enzyme induction and related processes requires careful evaluation.

Biological processes responsible for molecular, cellular, tissue and organ functions are strikingly similar in different mammalian species. Distribution, metabolism and excretion of foreign compounds from the environment (xenobiotics) are also similar in laboratory animals and humans. This information has provided a useful framework for the comparison of actions of different drugs. Van der Kloot (1967) quotes Homer Smith: 'Biological patents carry no automatic expiration date...' and he concludes that comparative studies can give answers of general validity because evolution is profoundly conservative.

Nevertheless, despite the parallel evolution of different animal species, caution is required even in comparative pharmacology (Williams 1967). This uncertainty has been extended by recent investigations of the effect of

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xenobiotics, not only because of the use of different classes of chemicals in the environment but also as a consequence of different conditions of exposure to them and different disease responses.

We have been slow to direct attention to the disease potential of exogenous agents. Individual reports of disease are not hard to find, e.g. lead-poisoning over the last 100 years, scrotal cancer in the chimney sweeps observed by Percivall Pott, lung cancer in Schneeburg miners and pulmonary fibrosis from exposure to quartz for almost as long. Investigation of these diseases did not, however, prepare us for current problems. Harmful agents, e.g. solvents, were generally considered in the light of their short-term effects, the result of direct action on specific tissues in heavily exposed, comparatively small populations.

These problems remain and have been extended both by the larger number of chemicals that have been introduced and the reorientation of scientific concern to the study of long-term effects. U.S. production of synthetic organic chemicals in 1940 was 1.9 billion pounds but this had increased to 360 billion pounds in 1977. Research on carcinogens and mutagens has surely been stimulated by the risks from radiation exposure in this nuclear era, and by the unhappy incidence of cancer associated with cigarette smoking, unpredicted in the 1930s when the extraordinary rise in cigarette smoking began.

With these new agents and new concerns have developed a new spectrum of additional investigative approaches, each relevant to specific problems of human disease.

CLINICAL LATENCY

Perhaps the most striking change in perspectives has been the difference in time frames of reference between studies of disease 50 years ago, and now.

Nowhere is this more evident than in evaluation of carcinogenic potential of exogenous agents. When hepatic angiosarcoma was reported in vinyl chloride polymerization workers in 1974, almost the first question asked was 'When did these men begin work?' In the first 19 cases of the disease examined (by definition probably the earliest to occur), the average time from the onset of exposure to the chemical was 20.4 years (range, 11–30 years). The plant in which the first cases occurred (Louisville, Kentucky) opened in 1940, and the first haemangiosarcoma was reported in 1968. This was reminiscent of the haemangiosarcomas associated with Thorotrast, which was first used in diagnostic radiology in 1926; the first tumours were seen in 1947. The same latency is, of course, seen with cigarette smoking, and generally with bladder cancers caused by β-naphthylamine (2-naphthylamine) and benzidine. The

latency has been perhaps best documented for occupational exposure to asbestos (Selikoff et al 1979). Figs. 1 & 2 and Table 1 show that no deaths from mesothelioma and few from lung cancer occurred, in the groups studied, within 15 years from onset of exposure to asbestos. The greatest number of deaths from these causes was seen two or three decades later. Leukaemia after exposure to benzene or radiation is an exception; in Hiroshima and Nagasaki survivors, this disease reached a peak about four years after explosion of the bomb (Beebe et al 1978). It is in this light that concern about the long-term carcinogenic potential of polychlorinated biphenyls, dioxins and other halogenated aromatic hydrocarbons is best understood (Kimbrough 1979). The absence of evidence of increased risk of cancer at present is not entirely reassuring, because there is evidence of high concentrations of toxic chemicals in living tissues (tissue burdens) in many lands (Wasserman et al 1979), and there are reports of carcinogenicity in animals, and changes in metabolic activity reflected by enzyme induction.

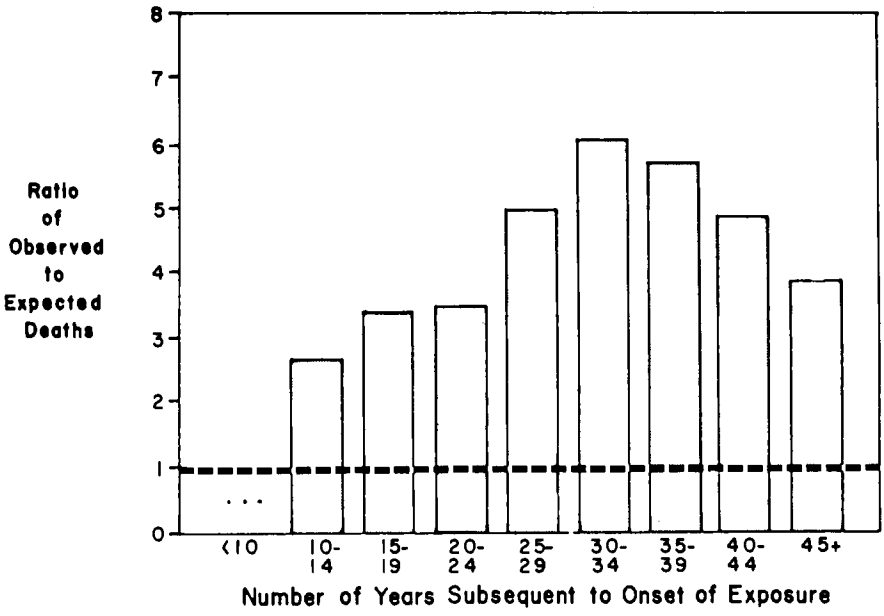

FIG. 1. Ratios of observed to expected deaths from lung cancer among 17 800 asbestos insulation workers in United States and Canada; prospective observations during 1967–1976 divided into 5 year periods after the start of employment in this trade. The decline in ratios after 30–35 years of exposure may be the result of a 'survivor-effect' because deaths associated with cigarette smoking (myocardial infarction, lung cancer, etc.) tend to change selectively the composition of the surviving group as time goes on, in relation to smoking status.

TABLE 1

Deaths among 17 800 asbestos insulation workers in United States and Canada, January 1, 1967–December 31, 1976. (Analysis by duration from onset of employment.)

Duration from onset (years)	*Number of men*	*Person–years of observation*	*Lung cancer*					*Pleural mesothelioma*			*Peritoneal mesothelioma*		
			Exp.[a]	*Observed*		*Ratio obs/exp*		*Number*		*No./1000 person–years*	*Number*		*No./1000 person–years*
				(BE)	*(DC)*	*(BE)*	*(DC)*	*(BE)*	*(DC)*	*(BE)*	*(BE)*	*(DC)*	*(BE)*
> 10	8 190	26 393	0.7	0	0	0	0	0	0	0	0	0	0
10–14	9 063	29 003	2.7	7	5	2.59	1.85	0	0	0	0	0	0
15–19	9 948	34 066	8.5	29	27	3.41	3.18	2	2	0.06	3	0	0.09
20–24	8 887	31 268	17.0	59	57	3.47	3.35	6	4	0.19	3	2	0.10
25–29	6 596	20 657	21.0	105	96	5.00	4.57	13	5	0.63	19	3	0.92
30–34	3 547	11 598	18.4	112	103	6.09	5.60	9	3	0.78	23	6	1.98
35–39	2 020	5 403	11.5	65	57	5.65	4.96	15	4	2.78	19	5	3.52
40–44	1 108	3 160	8.1	40	31	4.94	3.83	4	3	1.27	16	3	5.06
45 +	1 448	5 305	17.8	69	53	3.88	2.98	14	4	2.64	29	5	5.47

[a]Expected deaths are based upon age-specific U.S. death rates of white males, from the U.S. National Center for Health Statistics, 1967–1976; smoking habits not taken into account.

(BE): Best evidence. Number of deaths categorized after review of best available information (autopsy, surgical, clinical).

(DC): Number of deaths as recorded from death certificate information only.

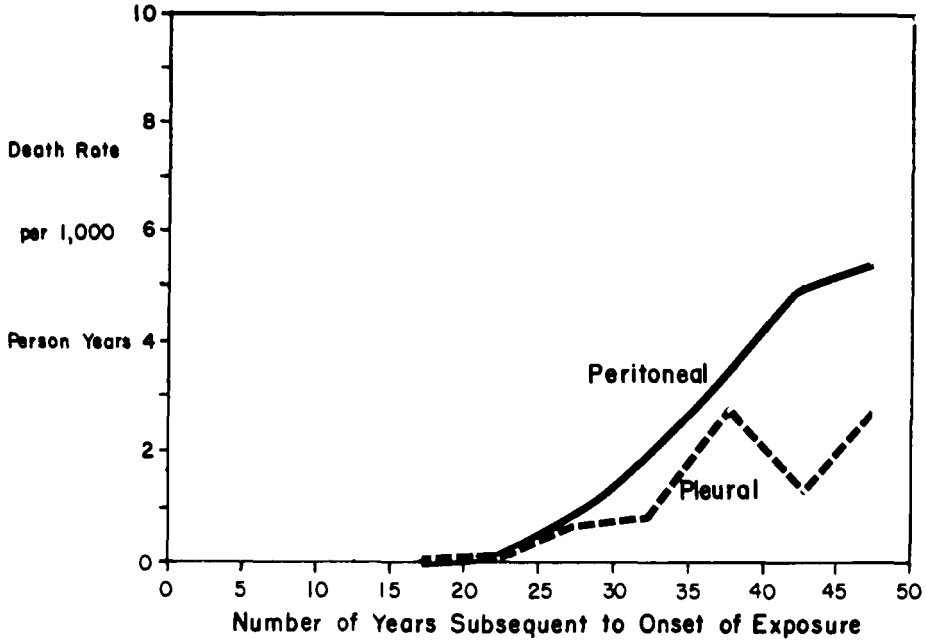

FIG. 2. Deaths from pleural and peritoneal mesothelioma per thousand person years among 17 800 asbestos insulation workers in United States and Canada during 1967–1976; observations divided into periods of five years duration from the onset of employment. Ratios between observed and expected deaths cannot be computed, since expected rates are not available for the general population. Up to 45 years from the onset of exposure, a decline in death rate was not noted. This is consistent with the finding that cigarette smoking did not influence the development of mesothelioma, although it was linked with lung cancer.

DOSE–RESPONSE

Straightforward analyses of physiological and metabolic responses in pharmacological studies are no longer adequate for evaluation of the effects of xenobiotics. Exposure and the effect of a given dose cannot always be equated. Multiple exposures to an agent, sequential and intermittent, are the rule and are often accompanied by accumulation of the agent in the tissues. Although this accumulation is familiar for lead, silica, and asbestos, it is disconcerting to find it true also for a number of xenobiotics. A current example of this is the case of polybrominated biphenyl (PBB) contamination of the food supply in Michigan. Contamination of a specific lot of dairy farm feed with PBB occurred in the spring and summer of 1973. The mistake was not clarified for more than nine months. By that time, contaminated meat, fowl, eggs, milk and other farm produce had entered the food supply of the

TABLE 2

Polybrominated biphenyl in serum and adipose tissue of Michigan residents in summer 1978.

Region	*Serum samples only (1651 residents)*				*Paired serum and adipose samples (393 residents)*[a]	
	No. of residents examined in each region	*% of residents in each region within each range of serum PBB (p.p.b.)*			*% of residents with low serum PBB (p.p.b.)*	*% of residents with no detectable PBB in adipose tissue*
		0–0.3	*0.31–3.0*	*> 3.0*	*0–0.3*	
Grand Rapids	320	11	61	28	13	2
Kalamazoo	181	25	65	10	21	0
Detroit	491	30	64	6	30	0
Bay City	246	26	66	8	41	4
Gaylord	231	16	70	15	9	2
Marquette	182	86	14	0	84	36
	1651	29	59	11	25.7	3.8
No. of cases:	(1651)	(486)	(976)	(189)	(101)	(15)

[a]Number analysed up to 1 October 1979
PBB, polybrominated biphenyls
p.p.b., parts per billion

state. Increasingly effective controls were subsequently instituted and by 1977 very little of the food supply contained polybrominated biphenyls. Nevertheless, analysis of serum and fat depots of more than 1600 Michigan residents, a carefully selected representative sample of the general population, in the summer of 1978, showed that a large majority (about 70%) of the residents had more than 0.3 p.p.b. (the minimum detectable amount) of the chemical in their serum (Table 2). Further, when depot fat (obtained by needle biopsy) was analysed in 393 residents, measurable amounts of PBB were present in the fat of over 90% of the subjects. The concentrations in adipose tissue were approximately 300 times greater than those in the serum, for a given individual. Unless we develop appropriate ways to accelerate excretion and metabolism of PBB, perhaps analogous to what has been done with cholestyramine resin and Kepone (chlordecone), *tissue burdens* (i.e. stores) may remain for the lifetime of the individuals.

Tissue-imprint

In some cases, the chemical may disappear, yet the cells retain a 'memory' or tissue-imprint of its effect; they are altered. In other instances it is difficult to measure persistent residual chemicals *(tissue burden)* and to draw conclusions from the measurements, because of problems of analytical sensitivity (e.g. with dioxins). The absence of measurable quantities of chemicals several decades after exposure is no guarantee of ultimate absence of effects from the chemical. This has been seen with β-naphthylamine-induced bladder cancer, which was not prevented simply by removal of the workers from the place of exposure; an increased incidence of the neoplasm still occurred two or three decades later. Similar observations have been made with vinyl chloride or bis(chloromethyl) ether (*sym*-dichloromethyl ether).

Compartments

There may be uncertainty about where to measure concentrations of a chemical in the body: should they be measured in the plasma, the brain, the adrenal glands, the liver, the bronchial mucosa, the kidney, the thymus or the bone marrow? The subtlety of this problem, which has been explored only in limited fashion in experimental animals and even less in humans, may be illustrated by our experiences with polybrominated biphenyls. We had seen, in 1977, alteration of lymphocyte function among Michigan dairy farmers exposed to PBB (Bekesi et al 1978). There was no clear relationship between the lymphocyte function and serum concentrations of PBB. We have since in-

TABLE 3

Distribution of polybrominated biphenyl in blood compartments and in different lymphocytes of Michigan chemical workers

Subject	*Concentration of PBB (ng/mg protein)*				*Ratio*				*Absolute numbers of lymphocytes*		
Code number	*Plasma*	*WBC*	*RBC*	*β-Lipo*	*Plasma*	*WBC*	*RBC*	*β-Lipo*	*T-Lymph*	*B-Lymph*	*Null*
7	10.2	32.0	0.63	282	1 :	3.2 :	0.06 :	28	1437	563	491
4	10.0	57.3	0.67	224	1 :	5.7 :	0.06 :	22	1405	516	683
9	0.23	1.8	0.03	–	1 :	7.5 :	0.13	–	1898	532	67
5	0.13	3.9	0.07	–	1 :	28.0 :	0.51	–	1825	573	172
Normal	0	0	0	–					1986	521	91

WBC, white blood cell; *RBC*, red blood cell; *β-Lipo*, β-lipoprotein fraction; *Lymph*, lymphocytes; *Null*, 'null cells'; –, not measured.

vestigated the distribution of PBB in blood compartments and in lymphocytes in four workers employed in the plant that manufactured PBB. Table 3 shows significant differentiation of distribution between the plasma, the β-lipoprotein fraction, red blood cells and lymphocytes (Roboz et al 1980). The concentrations of PBB, measured by mass spectrometry were significantly higher in lymphocytes than in red blood cells or in whole plasma; the highest concentration was in the β-lipoprotein fraction. There are various explanations for this differential compartmentalization of these lipophilic compounds, and one might be tempted to relate the differences to observed effects. It would be better, however, to encourage more cautious interpretation and more detailed investigations.

Trace contaminants

Despite measurable cumulative tissue burdens and measurable concentrations in various tissue compartments, it remains difficult to define the 'dose' associated with exposure to a particular agent. This is illustrated by the Yusho experience in 1968, where interpretation of the results of rice oil contamination by polychlorinated biphenyls (PCB) was beset by problems (Higuchi 1976). It was natural for the findings to be ascribed to the polychlorinated biphenyls, but recent data indicate that polychlorinated dibenzofurans had contaminated the PCB (Nagayama et al 1976). It is possible that some of the toxic effects were due to this far more toxic contaminant. Interest is at present directed towards the use of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in the United States and elsewhere, and to the complaints of Vietnam veterans believed to have been exposed to Agent Orange until 1970. In both instances, contamination of the herbicides by a highly toxic dioxin (TCDD) complicates the evaluation of effects on health. In the future there will be more interest in the control of exposure to solvents, many technical grades of which contain significant amounts of benzene.

Metabolites

The importance of enzyme induction introduces a further variable into the relationship between 'dose' and response (disease). The 'dose' could be the concentration of a metabolite, or the amount of enzymic alteration, or the concentration of the original xenobiotic. When 'dose' is expressed in terms of exposure to an agent, individual variability and duration of exposure may have to be taken into account.

Homologues

There is much evidence from animal studies that homologues of many xenobiotics have different biological effects and disease potential. Although these differences are well established for PCB, they have been less well studied for other agents. For example, does the presence of higher dioxins in pentachlorophenol presage different effects? The question is important, because of the wide use of this wood preservative.

Sex variations

Metabolic differences between males and females have long been known in drug metabolism. However, these differences have been incompletely explored for the xenobiotics, and identification of intrinsic physiological differences may be complicated by differential exposure. Exposure of rodents to vinylidine fluoride (1,1-difluoroethylene) provides an example of a metabolic difference: renal toxicity is seen at higher doses in males but not in females and renal neoplasms at lower doses are found in males (Maltoni & Tovoli 1979). Differences between males and females in bioassays for carcinogenicity of different agents are now commonplace, but not well understood. Differences in exposure may also be important: the PBB contamination in Michigan produced higher concentrations of the chemical in males than in females, presumably due to different amounts of food ingested. The sources of food are less likely to have been important since the PBB concentrations varied with different family groups. Other environmental differences may be critical, e.g. the prevalence in the past of cigarette smoking among men rather than among women.

Cumulative burdens

For agents with long tissue residence, definition of safe thresholds of exposure is difficult. Studies comparing tissue concentrations and biological changes are needed over the long term and are complicated by the effects of tissue compartments and trace contaminants already mentioned.

With all of these variables, how can we define a toxic dose? Dougherty and his colleagues (1980) have approached this thorny question by proposing a *level of concern* of 1 p.p.b. for chemicals that are significantly toxic. They calculated that this level would be equivalent to approximately 1000 molecules per cell, whereas 1 part per trillion (p.p.t.) might be equivalent to 2 molecules per cell (Dougherty et al 1980).

Dose-induction period

Recent data suggest that high doses increase not only the likelihood of disease, but also its speed of onset (Seidman et al 1979). This observation emphasizes the variables already considered for dose–responses. In addition, a person's age at first exposure to a chemical interacts to some extent with these variables. Few data are available, but our experience with asbestos workers suggests that individuals who are older at the onset of their employment may be more at risk of lung cancer than those who are younger; in older people, there is a greater incidence of neoplasms, which also develop sooner after the onset of exposure. Various explanations for this age difference may be offered, including less efficient DNA repair or deficient immunosurveillance with advancing age, or perhaps there is simply a longer time for older people to have been exposed also to other carcinogenic agents. However, further data are required to allow evaluation of these generalizations.

Persistence

In addition to their accumulation in the tissues, chemicals persist in the environment and individuals therefore are repeatedly exposed to them. Especially when the xenobiotics are retained in tissues, ingestion of contaminated creatures during steps in the ascending food chain may result in increasing concentrations of the xenobiotics (biomagnification), with the highest levels appearing in food eaten by humans. This is particularly true for aquatic species; predator fish may accumulate high concentrations of xenobiotics. They may not themselves be apparently affected, but when, in turn, they are ingested by birds or mammals, adverse effects can occur. Therefore evaluation of decay rates of xenobiotics in humans is difficult, especially for broadly disseminated xenobiotics such as hexachlorobenzene, polychlorinated biphenyls, and persistent pesticides.

ENVIRONMENTAL DISSEMINATION

Unlike the deliberate use of pharmacologically active agents, exposure to xenobiotics is generally unplanned. Their distribution and source is often identified long after the contamination has occurred. Food, air, water, consumer products and occupation may all be sources of contamination. Dissemination of environmental chemicals has been incompletely studied, particularly with regard to concomitant exposure of humans and the resulting *tissue burdens*. In addition there are, potentially, multiple sources of some

agents, e.g. polychlorinated biphenyls, metals and solvents. However, our knowledge of diseases due to environmental factors is becoming increasingly sophisticated. Studies of the area of contamination and the disease response at Seveso, Italy, promise to provide much useful data, since they are long-term. This work will supplement information gleaned from the more circumscribed but more serious accidents in the manufacture of trichlorophenol, leading to exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

We have studied the dissemination of polybrominated biphenyls since the accident in Michigan in 1973. In 1978, we examined a carefully selected group of more than 1600 residents, designed to represent the general population of Michigan, and to take into account geographical area, sex and age. The source of contamination was Western Michigan. Fig. 3 shows the distribution of serum levels and the centrifugal spread of this 'chemical epidemic'. When we compared histories of food intake with serum concentrations of PBB, only milk was significantly correlated with the concentrations (even though other foods were contaminated and undoubtedly contributed to the *tissue burdens* of the individuals concerned). Patterns of milk distribution in the state were consistent with these findings.

Evaluation of dissemination of chemicals may be further complicated by secondary sources of contamination. Breast feeding, for example, exposes infants not only to the large number of pharmaceutical agents found in breast milk, but also to the high levels of lipophilic xenobiotics which may be present. Breast-fed infants may thus have been exposed to these agents longer than those artificially fed, since the mother's body stores are only gradually depleted. We do not yet know whether this was a hazard in the Michigan population. Calves born to PBB-contaminated cows sometimes survived poorly, but there is no information about the possible significance of this observations for humans.

MULTIPLE FACTOR INTERACTIONS

In 1968, data confirmed that the predictions about interactions of multiple factors in animal studies applied also to humans (Selikoff et al 1968). The interaction between asbestos and cigarette smoking described at that time has since been amply confirmed (Selikoff & Hammond 1979). We made prospective observations on 12 051 asbestos workers from 1 January 1967 to 31 December 1976 (Table 4). All had been employed for at least 20 years (some for 40 years or more) and were therefore at an increased risk of death from lung cancer (see Table 1). Smoking habits were recorded in 1967. For comparison, the effects of smoking on 73 763 similar men in the general popula-

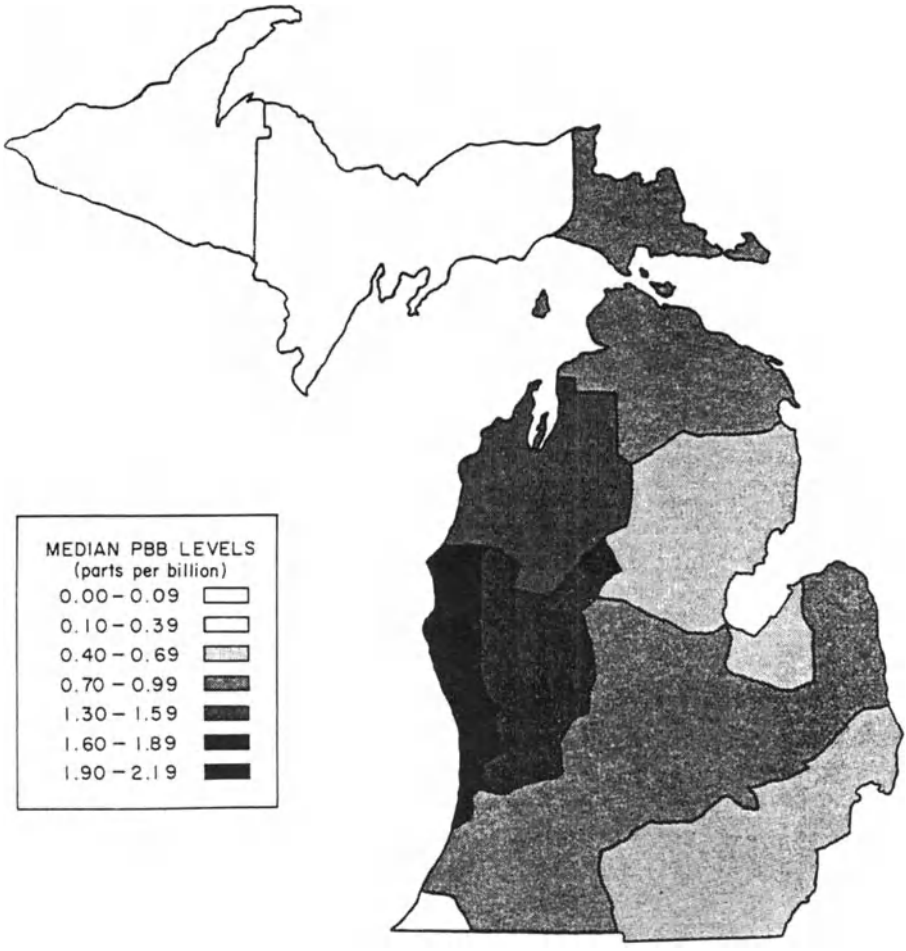

FIG. 3. Pattern of a 'chemical epidemic'. Serum concentrations of polybrominated biphenyls in a representative stratified sample of Michigan's general population during 1978. Contamination was centred in the western part of Michigan but it spread to the rest of the state in food, especially milk, derived from contaminated dairy farms.

tion were also analysed. Exposure to either asbestos or cigarette smoking alone significantly increased the risk of development of lung cancer, but when the two agents were combined, the resultant risk was greatly potentiated. The mechanism for this potentiation is not known. Additional interactions have been described between uranium and cigarette smoking in the development of lung cancer and between radiation and benzene in leukaemia. There are good theoretical reasons for believing that much human disease is multifactorial in

TABLE 4

Interaction between the effects of cigarette smoking and occupational exposure to asbestos on standardized rates of death from lung cancer

Group	*Exposure to asbestos?*	*History of cigarette smoking*	*Death rate*[a]	*Mortality difference from control non-smokers*	*Mortality ratio*
Control	No	No	11.3	0.0	1.00
Asbestos workers	Yes	No	58.4	+ 47.1	5.17
Control	No	Yes	122.6	+111.3	10.85
Asbestos workers	Yes	Yes	601.6	+590.3	53.24

[a]Rate per 100 000 man–years, standardized for age on the basis of the distribution of the man–years of 12 051 asbestos workers followed prospectively 1967–1976. Number of deaths from lung cancer based on death-certificate information.

origin. It is likely that this assertion applies not only to interaction of multiple xenobiotic agents but also to interaction of xenobiotics both with the immunological variations that they might induce and with genetic factors, as in juvenile diabetes.

PATHOGNOMONIC SYNDROMES

Characteristic syndromes produced by xenobiotics have been very poorly identified. Dost (1978) has reviewed the published work on TCDD effects and he concluded 'No pattern of pathology, tissue distribution, and biochemical or physiological change has emerged that is consistent enough to suggest a mechanism of lethal effect.' My own experience is consistent with this and I would extend it to the effects of polychlorinated biphenyls, polybrominated biphenyls and pentachlorophenols. There are some exceptions, e.g. certain clinical symptoms associated with styrene (Lorimer et al 1976), halogen acne occasionally produced by a variety of halogenated aromatic hydrocarbons, and the acro-osteolysis, Banti's syndrome and skin changes sometimes associated with vinyl chloride. However no changes that clearly predict angiosarcoma have yet been identified after exposure to vinyl chloride (Popper et al 1979) and even haematological changes observed after benzene exposure do not always predict the occurrence of leukaemia. A fuller understanding of physiological changes and their mechanisms after exposure to xenobiotics would improve our evaluation of premonitory events in the development of cancer. It is frustrating to anticipate approximately 100 000 new cases of cancer of the colon next year in the United States, with few

predictive markers available to us. So far, in this respect, we have had little guidance from current concepts of enzyme inducibility.

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Discussion

McLean: Do you have any evidence of asbestos-related disease in the communities of workers that you've studied?

Selikoff: No, but it might yet occur, because of the relatively low doses and therefore the longer induction period. However, we have had an example of a low dose that will give rise to chronic toxicity. When we were studying the Paterson asbestos workers (Anderson et al 1979) we also examined their wives and children who were living with them during 1941–1954 (the period of employment). They felt well and had no symptoms. However, X-ray revealed that one third had asbestosis. And among these households we have, so far, observed five deaths due to mesothelioma.

The concentrations of asbestos in the atmosphere were not measured 30 or 40 years ago. We have recently measured asbestos concentrations in households of asbestos miners in California and Newfoundland and we found a range of 100–1000 ng/m^3 (Nicholson et al 1979). We measured significantly lower asbestos concentrations of only 5–10 ng/m^3 in control households.

Amos: Professor Selikoff, you have mentioned some work on T & B lymphocytes which was unclear to me, because null cells are essentially uncommitted lymphocytes, and not lymphocyte precursors. It is possible that the reduced *number* of T cells that you found is responsible for the decreased effectiveness of the T cell response. We do not know whether the *function* of individual T cells is reduced. If this is a straightforward toxic reaction on a sensitive population of T cells, why should there be an increase in the number of null cells?

Selikoff: I cannot explain that either. We did these tests in 1976, three years after the period of exposure to polybrominated biphenyls (PBB). We could be seeing a late developing effect of PBB on the thymus or an effect on the circulating blood cells, especially since we have recently found that PBB is preferentially concentrated in the cellular fraction of the blood, with concentrations in the lymphocytes considerably higher than those in the red blood cells or plasma (Bekesi et al 1979, Roboz et al 1980). The life span of lymphocytes may be of interest since some, I believe, can survive ten years or more.

Amos: Only a small subsection of the lymphocyte population, the T memory cells, can live longer than 10 years; the majority of T lymphocytes are, in fact, short-lived.

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Environmental Chemicals, Enzyme Function and Human Disease
DAVID EVERED and GERALYN LAWRENSON

Implications for future studies in humans

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Abstract While an environmental link with cancer is widely accepted, its implications are not always understood. Because of the multistage nature of carcinogenesis, it is insufficient to study initiation only at the level of the 'target cell', since events before and after initiation may prove pivotal to neoplastic development. Estimates of the relative contributions of factors causing human cancer are briefly summarized. Tumours related to patterns of life style – including diet and behaviour – and individual susceptibility are discussed. It is in this group of tumours that metabolizing enzymes are probably most significant, especially for weak initiating stimuli, whether extrinsic or intrinsic. There are few epidemiological data available at present but future research can be suggested, to improve the definition in biochemical terms of individual susceptibility and effects of life style.

I have been asked to comment on the implication of this symposium to the design and interpretation of epidemiological studies in humans. Gardner (1979) emphasized that we should approach environmental toxicology very differently from in the past, when toxicologists predominantly predicted the effects of low doses by extrapolating from studies performed at high concentrations. Gardner also stressed the need for a wider multifactorial approach, including the study of enzyme induction: a view amply confirmed by earlier speakers at this symposium. Unfortunately, epidemiological studies in humans are not available to complement the extensive laboratory work and are limited largely to scattered observations in populations with certain genetic deficiencies (Omenn & Motulsky 1978).

My inclination and experience lead me to limit my comments to cancer, a disease in which the importance of a multidisciplinary approach has long been realized (Higginson 1969). I will attempt to draw attention here to some aspects of human cancer epidemiology which are most pertinent to individual

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susceptibility and 'life style'. My conclusions are necessarily both tentative and speculative since surprisingly little new data have become available since the meeting of the International Agency for Research on Cancer in Primošten (Doll & Vodopija 1973) where the possibilities of an epidemiological approach to host–environment interactions were extensively discussed. Between January 1977 and August 1979 cancer epidemiology was mentioned in only 10 of 1254 published papers on enzyme induction listed in CANCERLINE.*

NATURE OF ENVIRONMENTAL FACTORS AFFECTING HUMANS

In this paper, the term 'environment' is used to include not only defined chemical or physical carcinogenic stimuli, including chemical mixtures, e.g. cigarette smoking, but all exogenous influences or patterns of life style (dietary, cultural or behavioural) that are associated with changes in cancer incidence. In this context, it is desirable to distinguish between defined or discrete exogenous chemical or physical agents which are generally understood to be carcinogens and those factors which, while associated with an increased risk of cancer, cannot be defined as carcinogens in the usual sense. The latter risk factors include excess dietary fat, obesity, absence of fibre and behavioural patterns such as age at marriage. Carcinogenic 'risk factors' or 'risk indices' are convenient labels for these stimuli, without implying an understanding of the biological mechanisms involved. These indices are often included in the term 'life style' and they almost certainly reflect a wide variety of metabolic and biochemical reactions that govern the relationship between an individual and his intrinsic and extrinsic environment. Inferences and hypotheses from several sources allow cancers to be classified according to their suspected environmental origin (Higginson & Muir 1977, 1979).

Group I: Cancers for which there is good aetiological evidence

These are mostly epithelial neoplasms occurring in adults, and arising predominantly in the skin, upper respiratory and digestive tracts and genitourinary system. The majority are associated with personal habits such as smoking, excessive consumption of alcohol, sunbathing and betel quid chewing. An undetermined, but probably smaller, proportion is related to occupational or iatrogenic exposure.

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* An on-line cancer information system (International Cancer Research Data Bank of the National Cancer Institute in cooperation with National Library of Medicine, USA)

Group II: Cancers for which epidemiological data suggest a relationship to environment

This group includes neoplasms of the gastrointestinal tract (stomach, colon and rectum) and of the endocrine-dependent systems (prostate, breast, uterus and ovary). The aetiology of these tumours is imperfectly understood but I believe that the epidemiological data are more consistently related to life style (e.g. diet, behavioural and cultural patterns) than to exposure to multiple chemical carcinogens and mutagens in the ambient environment (Doll 1979, Higginson 1979).

Group III: Cancers for which epidemiological data do not suggest a relationship to environment

This group includes most childhood cancers and neoplasms of the haematopoietic system and soft tissues.

Fig. 1 (a,b) shows estimates of the proportion of cancers in these aetiological groups in a United Kingdom and an African population. The method of estimation is discussed elsewhere (Higginson & Muir 1979) and its imprecision should be recognized; assignment to a predominant cause, as in these estimates, does not imply that other contributing factors are excluded. A genetic component may modify individual susceptibility to the exogenous agent in some cases, but there is no evidence for this in humans except in certain skin cancers and rare syndromes. Studies of migrant populations indicate that genetic factors are less important than environmental factors.

The above epidemiological observations suggest that there are both qualitative and quantitative differences between the effects of modifying factors (including induced enzymes). Such factors may affect individual susceptibility by activation or inactivation of exogenous carcinogenic (genotoxic) stimuli (Group I), or they may have a pivotal role in cancers related (directly or indirectly) to life style or to intrinsic carcinogenic stimuli (Group II). These intrinsic stimuli include (i) the endogenous formation of carcinogens from non-carcinogenic precursors, e.g. nitrosamines, or steroid hormones, and (ii) endogenous factors that initiate or promote cancer. These factors may be difficult to identify objectively and measure since their contribution can be determined only indirectly in humans through various risk indices (e.g. age at first pregnancy; Miller 1978) of which none predominates and all are multiple (Higginson & Muir 1979). Studies of migrant populations and time changes show that although cancers attributed to life style are under exogenous influence, no common pattern of cancerous change is observed at different

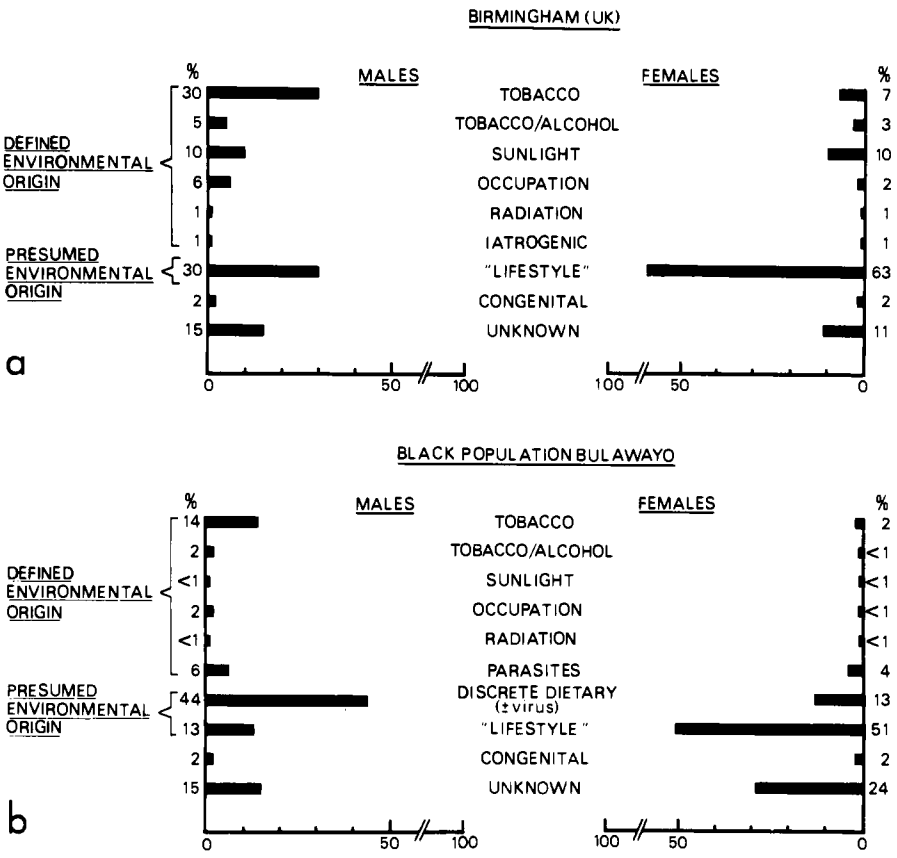

FIG. 1. Histograms indicating the proportion of cancers estimated to be due to defined or suspected environmental factors (a) in a United Kingdom and (b) in an African population. The methodology of such estimates is given elsewhere (Higginson & Muir 1979).

organ sites. Furthermore, some environmental factors have their greatest impact in childhood, and may require an accumulation of prenatal and postnatal events over more than one generation before they produce a visible effect. Alternatively, a slow change over several generations may be dependent on deeply rooted cultural habits that are not readily altered in a single generation.

FACTORS MODIFYING HUMAN CARCINOGENESIS: THEORETICAL CONSIDERATIONS

Previous speakers have described how various environmental factors affect drug-metabolizing enzymes; many of these factors are relevant to car-

cinogenesis. Previously, cancers of environmental origin have been generally considered only in terms of initiation, but today the concept of multistage and multifactorial carcinogenesis is widely accepted. Events before initiation, e.g. precarcinogen activation, were originally recognized as important by Boyland & Weigert (1947) and later by the Millers in a series of studies whose importance cannot be overestimated (Miller & Miller 1979). This recognition adds another dimension to the two-stage hypothesis of initiation and promotion that was put forward many years ago by Berenblum (see Berenblum 1979).

Fig. 2 is a simplified diagram of the possible nature of, and relationship between, the mechanisms at various phases of carcinogenesis. While the categorization into phases is probably unjustified owing to their overlap *in vivo*, it is a convenient description to use in referring to the design and evaluation of epidemiological investigations. It should be noted that pre-initiating events (Fig. 2 Phase 1) are not caused specifically by carcinogens, and may be

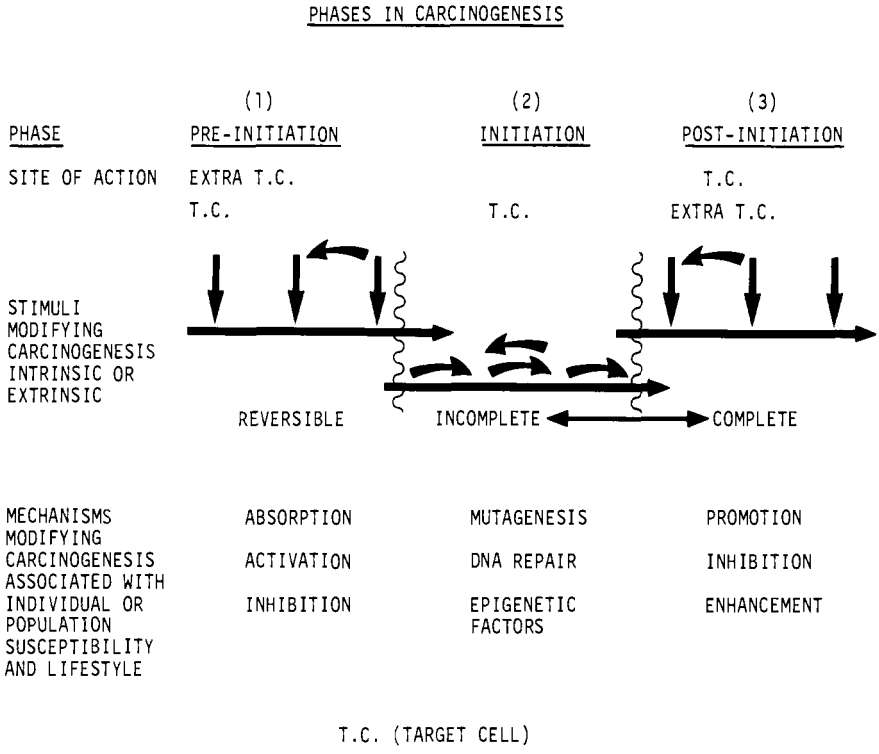

FIG. 2. Simplified diagram of the hypothetical phases in carcinogenesis which are believed to be affected by exogenous and endogenous stimuli. Enzyme induction is most probably important in phases I or III. Some of the mechanisms involved are shown.

reversible. Furthermore, the terms 'promotion', 'enhancement' and 'inhibition' are sometimes used to describe other toxicological phenomena and may be reversible.

Most evidence suggests that indices of individual susceptibility and carcinogenic risk (life style) will eventually be explicable in terms of reversible and irreversible biochemical variables and feedback mechanisms, involving all phases of carcinogenesis. Furthermore, the epidemiological data are consistent with this assumption, especially for tumours related to life style.

Pre-initiation

There has been extensive discussion here (see Fig. 2) of the importance both of activation and inactivation of precarcinogens and of the numerous enzyme systems involved (Miller & Miller 1979). Variations in these enzymes obviously could explain individual susceptibility, e.g. to lung cancer, in which only one in ten heavy smokers develops a neoplasm. Such variations in enzyme systems may modulate carcinogenesis related to intrinsic stimuli, e.g. endocrine-dependent tumours.

The protection against breast cancer for a woman who has her first pregnancy before the age of 20 years may be explained by subtle variations in her hormone metabolism (Cole et al 1976). Increased extra-glandular formation of oestrogen occurs largely in fat cells. In view of the relationship between oestrogens and endometrial cancer, this observation explains the association between over-eating, obesity and endometrial cancer as a result of

TABLE 1

Mechanisms of carcinogenesis (occurring in target cells in the liver or other organs), which could be investigated in whole populations

Activation, co-activation and inactivation
Selective metabolism
Conjugation and de-conjugation
Enhancement and inhibition
Promotion
Steroid hormone formation or inhibition
Hormone receptor induction
Increased or decreased carcinogen formation from extrinsic or endogenous precursors
Lipid oxidation
Genetic polymorphism

induction of aromatizing enzyme activity in fat tissue. Progesterone may induce endometrial oestradiol-17β-dehydrogenase (EC 1.1.1.62), an enzyme of possible significance in the inhibition of endometrial hyperplasia (Richardson & McLaughlin 1978). Moreover, carcinogens may be formed from non-carcinogenic precursors (e.g. nitrates, intestinal sterols, bacterial metabolites), and many of these reactions may be affected by enzyme induction.

Initiation

Initiation is the fundamental step for both genotoxic carcinogens and carcinogens or stimuli that do not operate as direct mutagens. The extent to which initiation is reversible is unknown at present, but DNA repair certainly operates. Induction of DNA repair *in vitro* has been discussed at this symposium by Professor B.A. Bridges (1980, this volume, p 67-79). That DNA repair may, however, be inducible *in vivo* and may modify the initial response to a carcinogen or a mutagen, or both, is suggested by the recent work of Montesano et al (1980). These observations are obviously relevant to humans, and notably to the exposure to carcinogens at very low doses. Further exposure to chemicals such as caffeine, an inhibitor of DNA repair, may be widespread, but present epidemiological data do not explain the significance of this and similar exposures.

Promotion and inhibition

There is now great interest in co-carcinogenesis – including promotion – and many mechanisms have been described (Slaga et al 1978). Enzyme induction has been observed after exposure to TPA (12-*O*-tetradecanoyl phorbol-13-acetate) and other promoters. Professor J.W. Bridges (1980, this volume p 5-18 has discussed the role of cytochrome oxidases in the conversion of cholesterol to steroid hormones and in the oxidation of fatty acids. These reactions may control tumour promotion, enhancement or inhibition as well as carcinogen activation and inactivation. Many other factors modify cancer development in the postinitiation phase, including dietary and other inhibitors (Wattenberg 1979) and immunological defences (Amos 1980, this volume p 245-256). However, the relative importance of initiation and promotion in cigarette-induced cancer is unknown, as is the role of immunosuppression in relation to general patterns of cancer in human populations.

Many of my comments above apply only to reactions in the target cell. Although the importance of carcinogen metabolism by organs other than the target cell has been demonstrated experimentally, e.g. for the liver, relatively

little epidemiological data are available to evaluate the significance of such mechanisms in humans. Despite its effects in enzyme metabolism, liver cancer in humans would appear to be an exceedingly rare occupational hazard. There is no evidence in humans that liver cirrhosis *per se* is associated with an increase in incidence of cancer in other organs, except when the latter are exposed to the same causative factor, as in the case of ingestion of excess alcohol in cancer of the oesophagus. On the other hand, hyperoestrogenaemia and gynaecomastia have been observed in cirrhotic patients.

This meeting has indicated the many potential mechanisms for induction of cancer in humans (Table 1). Several speakers have described the marked differences in induction of metabolizing enzymes between individuals. These differences may provide a measure of individual susceptibility either in relation to discrete exogenous causes or in relation to the less defined factors associated with life style. Moreover, the metabolizing enzymes are under environmental control and their activity may change rapidly in different environments (Conney et al 1980, this volume p 147-162). In contrast to the available information on specific parameters in individuals, evidence about the overall role of such factors in cancer patterns within populations is less satisfactory. Inconsistent effects on cancer patterns have been reported after human exposure to phenobarbitone (Clemmesen 1974, White et al 1979). Despite the number of epidemiological studies of diet and behaviour that have been performed, their relationship to enzyme induction and cancer incidence in humans remains ill-defined. Nonetheless, individual susceptibility and life style will be expressable to a greater degree in biochemical terms and some stimuli, whether mutagenic or non-mutagenic, may operate before and after initiation. While studies of migrant populations suggest that hereditary factors are less important, Ritchie et al (1980, this volume p 219-235) and Lower et al (1979) have pointed out that genetic polymorphism may influence individual susceptibility to the development of cancer. Furthermore, Sabadie et al (1980) have suggested that several systems of metabolizing enzymes for different chemical carcinogens may be genetically linked.

In conclusion, in future epidemiological research, the role of environmental factors should be considered not only in the modification of discrete carcinogens but also in various biochemical mechanisms which could modify the frequency of cancer directly or indirectly. Attention should therefore be concentrated not only on the immediate effects on the target cell but also on the pre- and postinitiation phases. Appropriate technology is already available for application to field studies on cancer which would allow evaluation of the possibilities in humans.

In view of the multiple variables and their possible overlap in each phase of

carcinogenesis it will be difficult to evaluate the rate-limiting effect of each variable. In some cases, however, experimental and epidemiological data may indicate that one effect predominates, e.g. the link between vinyl chloride and angiosarcoma (Selikoff 1980, this volume p 331-345). Theoretically, the importance of the pre- and postinitiating phases should be inversely proportional to the strength of the initiating stimulus. Thus, extrapolation from animals to humans is more likely to be practical for genotoxic initiators, e.g. alkylating agents, than for weak carcinogens, intrinsic factors or indices of life style which may act through non-mutagenic initiating mechanisms. Accordingly, dose–responses should be considered in the extrapolation of results from animals to humans, especially when several different mechanisms operate. Sugimura (1978) has also emphasized that detoxifying mechanisms in humans determine the importance of weak mutagens.

CONCLUSIONS

There is adequate epidemiological evidence to suggest that diet, cultural and behavioural patterns, life style, smoking, drugs and chemicals may be related to patterns of cancer in humans. Furthermore, the operation of many of these factors can be at least theoretically explained by biochemical reactions, including activity of metabolizing enzymes, activation or inactivation of carcinogens, and tumour promotion, enhancement or inhibition, some of which can be measured in humans. Future epidemiological studies should be directed notably to groups at a potentially high risk owing to their occupation, their intake of drugs or life style. Emphasis should be given also to the total effects on cancer incidence of patterns of diet and life style, the latter of which represents the summation of several complex mechanisms. Certain reactions may be pivotal and definable, and will thus be open to intervention. The fact that certain secular groups, e.g. Mormons, Seventh Day Adventists, African and Japanese populations (Higginson 1979) have considerably fewer cancers, especially of the endocrine-dependent systems, indicates that this approach may be effective.

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Environmental Chemicals, Enzyme Function and Human Disease
DAVID EVERED and GERALYN LAWRENSON

Final general discussion

Amos: Much effort goes into development of tests to identify initiators of carcinogenesis, and many decisions about which chemicals should be allowed into the environment are made as a result. In your summary paper, Dr Higginson, you considered this to be a relatively minor point in relation to epidemiology, and you emphasized that promotion, and the events that happen after initiation, are more important than initiation itself.

Higginson: When we are dealing with high levels of exposure such as those discussed by Professor Selikoff, the importance of initiation is obvious. However, in the study of the many other types of cancer that affect the general population, attempts to pinpoint the particular environmental factors that should be controlled are likely to be disappointing, because there are so many, and because there is no evidence that discrete initiators alone, in very low doses, are involved. I can reach no other conclusions from the epidemiological data.

Farber: What evidence do you have that all people are in the initiated state?

Higginson: That is difficult to answer because it is hard to define the number of initiated cells directly. Singer (1979) has demonstrated that a vast number of pyrimidine dimers formed by ultraviolet exposure are repaired and are thus without effect. Human skin is constantly being exposed to ultraviolet light and therefore, theoretically, initiation must be widespread in humans. However, if DNA repair is complete, cancer will not occur. Moreover, despite the wide variety of environmental chemicals to which humans are exposed by ingestion, liver cancer is rare except in individuals exposed to aflatoxin or excess alcohol. This suggests that initiation may be common in the liver, but is either incomplete or lethal.

Selikoff: I think that Dr Higginson's position is a conservative one. If it is true that we are all initiated, then our task is to prevent the development or promotion of cancer and not only to prevent initiation. We know that the risk

of cancer of the lung is reduced to near normal about ten years after cessation of smoking (Hammond 1966). We should therefore seek to identify other causes of cancer so that we can try to undo the effects of many years of exposure to them. It will be vitally important to observe the rates of angiosarcoma of the liver in vinyl chloride workers (Nicholson et al 1975) now that vinyl chloride exposure has been controlled since 1974–1975.

Connors: Do you feel that research effort should be directed mainly towards laboratory studies of other stages of carcinogenesis?

Higginson: Yes. I am convinced that we can no longer explain carcinogenesis by the simple formula that carcinogen type A leads to cancer type B, without considering the complexity of the many steps involved.

Farber: I agree. There is a low incidence of lung cancer among non-smokers and I therefore believe that the constituents of cigarettes are also promoting agents, and *not* just initiating agents.

Higginson: Initiating and promoting agents have been identified in cigarette smoke (Wynder et al 1978). We don't yet understand how smoking interacts with other environmental agents, e.g. asbestos, but we know that it increases the incidence of lung cancer. Moreover, the results of Sugimura et al (1976) on the discrepancy between the mutagenic activity of cigarette tar and the concentration of benzo[*a*]pyrene could be interpreted as implying the presence of a co-mutagen.

Selikoff: Richard Doll has noted that we cannot, at present, appropriately evaluate the importance of the age at which a person is exposed to a carcinogen (Doll 1971). The work of Case with rubber workers suggested an effect of age which he could not explain (Case & Hosker 1954, Case et al 1954). In our study of asbestos workers (Seidman et al 1979), we expected that those who were young at the time of their first exposure would have been much more likely to develop cancer. But the opposite occurred; it was the people who were older during initial exposure who developed cancer sooner. An explanation for this could be that the older workers had been exposed longer than the younger men to *other* environmental factors, or that their DNA repair or immunosurveillance systems may not have been as good. One priority within the next few decades should be to learn how to prevent or ameliorate the promoting effect.

B.A. Bridges: Burnet (1974) attributes all initiation to spontaneous mutation; he sees no place for environmental initiators. Although I think this approach is extreme, I would agree that the chemicals in the environment may not be the major initiators. The greater sensitivity of older people to environmental carcinogens is also discussed by Burnet (1974). At one time conventional wisdom directed that older people should be chosen for industrial

processes involving carcinogens. This, of course, was exactly the wrong thing to do, because the older person develops cancer sooner, and with greater probability, than the younger person. And, to return to Dr Amos's comments, I agree that we should attempt to screen out all carcinogens in occupational situations. Nevertheless where small populations may be subject to high doses, predictive tests would surely be justifiable. They would also be useful for studies of large populations exposed to high doses of drugs or food additives.

Amos: It would be interesting to see if, in 10–12 years time, cancer patterns will be different from present patterns as a result of our ability to detect and remove initiators.

J. W. Bridges: Should we be screening for promoters as well as for initiators of carcinogenesis?

B.A. Bridges: We all know that we should, but we cannot do that.

Selikoff: We have identified high-risk groups, i.e. people exposed to carcinogenic agents. It might be useful to group these people on a biochemical basis, for prospective observation. For example, in 1974, we wanted to extend our studies of chromosome aberrations in vinyl chloride workers (Ducatman et al 1975). We had hoped to characterize the chromosomes of all 1200 workers, so that within five years we might have been able to observe whether those with aberrant chromosomes were more likely to have developed cancer. We were unable to obtain support for the project.

Meyer: But what do you mean by 'biochemical characterization'?

Farber: There are so many variables that any such attempt would be unlikely to be useful in the absence of an hypothesis.

Smith: One approach would be that mentioned in a previous discussion by Dr Idle (p 235-243), i.e. to investigate whether there is a genetically determined factor, perhaps metabolic, associated with the propensity to develop cancer in certain environments. Many environmental chemicals require metabolic activation before they produce carcinogenicity and some individuals, for genetic reasons, may be better at this than others.

Selikoff: Yes, it could be done with haemoglobins, for example.

Connors: I think everybody is agreed that because there are so many variables, we must examine working hypotheses experimentally, regardless of whether they are necessarily relevant immediately to the human situation.

B.A. Bridges: Yes, there is a need to bring epidemiology and laboratory studies closer together, in this way. I am referring specifically to initiators of DNA-damaging agents. With certain groups of people exposed to alkylating agents (e.g. epichlorhydrin workers) it is possible to measure the amount of alkylation of haemoglobin and to characterize their chromosome aberrations.

It would be useful to examine other groups, e.g. cigarette smokers and vinyl chloride workers, to see if the extent of alkylation can be correlated with the amount of mutation in their lymphocytes. These observations would not be end-points in themselves but biological markers of the potential damage to DNA as a result of its covalent binding to the active metabolites.

Hunter: There can be difficult human problems in starting studies of that nature. For some years I have been trying to study pesticide workers, but companies are reluctant to allow tests that may promote fears or unrest among the workforce about the working conditions, without providing any useful results.

Higginson: There is a group of cancers in which I believe that intrinsic initiation is of major importance, i.e. cancers of the endocrine-dependent system and, possibly, of the gastrointestinal tract. The evidence suggests that apart from obviously iatrogenic causes, such as postmenopausal oestrogen therapy, intrinsic hormonal factors that are not necessarily mutagenic may be involved. This group forms the greatest proportion of cancers in females.

Amos: Did you say, Dr Higginson, that the debate about cigarette smoking rests on whether it initiates or promotes cancer?

Higginson: There must be an initiator in cigarette smoke, but we do not understand the relative importance of initiation versus promotion in the pathogenesis of lung cancer (Wynder et al 1978) or other cigarette-related tumours, e.g. oesophageal cancer.

Selikoff: People often consider the cigarette to be a powerful carcinogen but it could be argued that it is a comparatively weak one; it often has to be taken 20 times a day for 30 years before it produces cancer!

Higginson: After the 1950 Clean Air Act was introduced, the incidence of lung cancer in the United Kingdom continued to increase until recently (Waterhouse et al 1976). Therefore, the failure to observe a fall in lung cancer despite the fall in air pollutants suggests that the pollutants are not responsible for most lung cancers (Lawther 1976).

B.A. Bridges: That is an oversimplification of the results, because the real test of the effectiveness of the Act could only be determined 20 years after its introduction.

Higginson: Nearly all observed variations in incidence of lung cancer in different countries have been related to differences in cigarette smoking and not in air pollution (Cederlöf et al 1978). However, the variations between groups within any country are considerable, and therefore direct comparisons based on averages must be considered with prudence.

J.W. Bridges: Is the incidence of lung cancer falling in central London, where the Clean Air Act had a major impact?

Higginson: In London, lung cancer is falling amongst younger men, in whom the proportion of smokers is decreasing, but the fall is not occurring in older men. Changes in habits of different segments of the population may be masked if we consider only the overall trends; more information can be drawn from group data.

Smith: Dr Higginson, you mentioned that some heavy smokers do not develop cancer, despite their constant exposure to the carcinogen. What factors determine vulnerability to cancer under those circumstances?

Higginson: This is an interesting question. Kellerman's original report (1973) on benzo[*a*]pyrene hydroxylase (EC 1.14.14.1) apparently provided an explanation for increased susceptibility to cigarette smoking amongst certain people, but this has not been confirmed. However, it is true that some people may be more susceptible to cigarette-induced cancer, due to variations in their individual metabolizing enzymes.

McLean: One of my reservations about Kellerman's work (1973) is that the measurements were of *genetic* potential for benzo[*a*]pyrene hydroxylation after maximal induction. This may not have been related to the *actual* enzyme activity *in vivo*. Such an approach completely fails to take into account all the environmental factors which could be operative. Kellerman examined lymphocytes that had been stimulated initially by phytohaemagglutinin and then maximally by 1,2-benzanthracene, so he was testing the ability of the lymphocytes to respond to a maximal dose of polycyclic hydrocarbon; he was not measuring the rate of *metabolism* of polycyclic hydrocarbons *in vivo*. There could be an analogy here with the effect of cigarette smoke in the lung, but that is doubtful. Previously, we discussed the importance of detailed analysis of the *fate* of metabolites in both dangerous and non-dangerous pathways. We are a long way from that, in human studies, and we should recognize that rates of metabolism measured *in vitro* are not definitive.

B.A. Bridges: In addition to the potential genetic heterogeneity in metabolism, which has occupied much of our discussion, there is a genetic heterogeneity at the postinitiation level, and particularly in DNA repair. In three diseases that predispose people towards cancer – xeroderma pigmentosum, Fanconi's anaemia and ataxia telangiectasia – the evidence suggests that defects of DNA repair are present in the patients. Epidemiological studies suggest that relatives of those patients also have a greater risk of developing cancer than the general population. There may thus be a heterozygote effect. This is important because as many as 4% of the general population may be heterozygotes for those three syndromes. (We cannot give precise figures because we don't know how many loci are involved.) There may be other unidentified syndromes which fall into the same category.

Within the next 10 years, we should aim to sort out the heterozygote problem. At the cellular level, we cannot distinguish heterozygotes from wild-types, and yet the epidemiology studies suggest that there is a difference between them. If genetic diversity can operate at several levels of metabolism, and hence give rise to several independent ranges of sensitivity within a population, I would expect the overall effect to broaden the overall range of sensitivity. This would mean that those who were unlucky enough to be heterozygous for both a cancer-prone condition and also for the ability to activate certain carcinogens could be extremely susceptible to cancer. However, this argument could be criticized on the basis that the various effects would cancel each other out, so that the overall distribution of sensitivity would actually be narrower.

Smith: I would agree with the second interpretation. We should be considering combinations of genetic factors, particularly those affecting responses of receptors and disposition of toxic agents. I suspect that it is the combination of these, particularly in rarer phenotypes, that controls the differences in susceptibility to toxic substances. Some genetic effects 'switch on' later in life, and so this adds to the complexity.

Farber: I believe that the recognition of the heterozygotes within a population is less important than the recognition of those people who are already at risk and who are developing toxic reactions. We now have enough information to know that, in principle, markers are 'turned on' at an early stage in the carcinogenic process. They may not be specific for cancer, but they are indications that something is going wrong. This area should receive more attention in the hope that treatment could begin at an early stage. It is sad and frustrating to see a person developing lung cancer that may have taken 20 years to become apparent, and yet not to be able to do anything for him.

Selikoff: The success rate for cancer treatment is very poor indeed: little more than 5% of lung cancer victims are cured, and it has not been possible to cure patients with mesothelioma or angiosarcoma. In general, the disease is discovered only when it is in the final stages. The development of reliable predictive markers would be a real breakthrough.

J. W. Bridges: Yes. Such markers are badly needed for almost every area of chemically induced disease.

Higginson: There are a couple of final points worth mentioning. In Northern Iran, there is a high incidence of oesophageal cancer. One woman in five has a chance of dying from the disease if she reaches the age of 70 years. Oesophagoscopy indicates that over 80% of young individuals have precancerous lesions. Therefore, when we talk about susceptibility we ought to define both the term itself and the time-scale involved.

Secondly, female relatives of premenopausal women with bilateral cancer

have a very much higher risk of developing breast cancer, but the risk indices are only gradually being elucidated and enzyme imprinting, for example, cannot be excluded.

Connors: During the symposium we have outlined many problems and clarified future aims, and now the onus lies on us to design the experiments to provide some answers. We seem to have agreed that besides environmental factors, genetic factors may also be important in determining cancer incidence, although we have not fully discussed their relative importance. The major point that has emerged is that a bewildering range of toxic reactions are possible as the result of an initial interaction between chemicals and metabolizing enzymes. During the symposium we have defined toxicity as anything from alkylation of a molecule *in vitro* to induction of human cancer, but not even in the simplest case can we analyse the full sequence of events that take place between the reaction of a metabolite with a target molecule and the expression of gross toxicity. We have also learnt that some forms of toxicity are indirect, because a chemical may be effective by altering the metabolism of normal substrates or by interacting in a complex way with the immune system. It is particularly interesting that the latter type of toxicity would not have been uncovered by conventional toxicity testing alone. It is a disappointment that so few people are working in this field.

One clear conclusion of the meeting has been that we cannot yet formulate general rules from *in vitro* or animal studies that will enable us to predict responses in humans accurately. We cannot yet distinguish experimentally between truly dangerous chemicals, which should be removed from the environment, and chemicals whose risk of causing serious toxicity in humans is slight. It may well be that new concepts or fundamentally new discoveries are needed.

An appropriate concluding comment, attributed to Sir Douglas Black, might be that there is no area in biology so complicated that further research will not make it even more complicated!

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