ADVANCES IN DRUG RESEARCH

Volume 28

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ADVANCES IN

DRUG RESEARCH

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PREFACE: CIRCLES IN DRUG RESEARCH

The two previous volumes of *Advances in Drug Research* were rather special in their layout, one (Volume 26) featuring only general subjects, and the other (Volume 27 on antidiabetic agents) being dedicated in its entirety to a single therapeutic class. The present volume of *Advances in Drug Research* rediscovers the more traditional style of the series, with a blend of general topics and specific therapeutic classes. Neither style is intrinsically preferable, the importance in the Editors' view being a tolerable degree of variety.

The book opens with a general chapter by Michel Eichelbaum and Annette Gross on the stereochemical aspects of drug action and disposition. This is of course a topic of constant interest in drug research, and one that is treated here with a masterly intertwining of clinical and biochemical levels. Drawing from their great experience at both levels, the authors illustrate the pharmacokinetic and pharmacodynamic aspects of chirality with convincing arguments and examples.

The second chapter is one of great current interest, namely that of pharmaceutical antioxidants being used as drugs or evaluated as drug candidates. Editors and readers alike must be grateful to Thomas Högberg and his colleagues for succeeding in the preparation of a timely and comprehensive review. At first glance, the chapter appears as a large compilation of data and chemical structures – which it is at the superficial level. To careful readers, however, this chapter will reveal its essence, namely that of a critical and stimulating work where delusions are exposed and solid scientific facts prevail.

The same urge for clarification and rationalization is obvious in the chapter on multidrug resistance by Susan Kane. Here, we stand at the cutting edge of methodology, with molecular and cellular biology being dedicated to solving the mysterious mechanisms by which cancer cells can acquire the ability to survive and thrive when exposed to highly poisonous agents. The problem is of course a principal one in the treatment of cancer patients, but it is also a fascinating example of natural selection operating at the cellular time scale and open to our investigative curiosity.

The last three chapters have something important in common – they all discuss "old" classes of drugs (in the sense that their first members have been in therapeutic use for a number of decades) which in recent years have witnessed a profound rejuvenation and an exponential growth in interest. Here as in a number of other therapeutic classes, progress in molecular pharmacology and biology has deepened our knowledge about ourselves and has opened the road to the discovery of new generations of drugs.

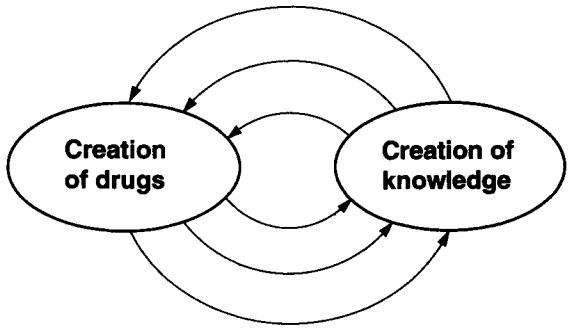
The fourth chapter by Henning Schröder discusses organic nitrates and

other nitrovasodilators. Many of these agents have been prescribed by generations of medical doctors, yet only in recent years have their molecular mechanisms of action begun to be understood with the discovery of nitric oxide, NO synthases, and the innumerable factors, physiological and/or pathological, that act to regulate or alter their effects. As a result of such progress, new drug candidates have been designed which should lead to better drugs and new fundamental advances.

The story of the vitamin D endocrine system is an interesting and instructive one, as convincingly told by Ruth Ettinger and Hector DeLuca. A number of significant breakthroughs have been made in recent years, several of which were contributed by DeLuca and his team. At the fundamental, biological level, a coherent image of the vitamin D endocrine system is beginning to emerge. Simultaneously, the search for and discovery of synthetic vitamin D analogues have opened new therapeutic opportunities. But most impressive of all in our minds is the manner in which the progress along these two axes has reinforced each other. Such a synergy is one of the most admirable characteristics of drug research, as discussed below.

The sixth and last chapter deals with the structure and function of dopamine receptors and is again written by one of the key players in the field. Here, Philip Strange explains with clarity and inspiration how molecular biology and novel dopaminergic ligands have opened promising horizons in the search for new and selective drugs, while at the same time unravelling neurobiochemical mechanisms of singular fundamental value.

Taken as a whole, the six chapters in this volume, together with many chapters in previous volumes, illustrate a single plot played by different actors under different circumstances. This plot is build around the two basic objectives of drug research, namely the creation of drugs and the creation of knowledge. (Whether the word "discovery" is more adequate than "creation" will not be debated here.) Drug researchers are indeed in the unique position that the fruits of their efforts can serve two fundamental aspirations of humankind, that of well-being and health, and that of knowledge and understanding.



But these two objectives cannot be pursued independently from each other. This is the essential and forceful message of so many chapters in *Advances in Drug Research*, namely the undissociable nature of the two objectives and indeed their admirable synergy (Testa, 1995). Advances in our understanding create the conditions for the discovery of new drugs, which in turn are used to probe biological systems and gain new understanding, which in turn . . .

Such are the unending circles of drug research.

BERNARD TESTA
URS A. MEYER

Reference

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Stereochemical Aspects of Drug Action and Disposition

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1 Introduction

Ever since Louis Pasteur discovered the optical isomerism of tartaric acid over 130 years ago (Drayer, 1993), the significance of stereoisomerism in relation to biological activity has been a topic of much interest to biochemists, chemists and pharmacologists. Assessing the activity, pharmacodynamics, pharmacokinetics, side-effects and toxicity of the individual stereoisomers of chiral drugs has become a most active area of research in experimental and clinical pharmacology and toxicology. These studies have revealed the composite nature of many widely used drugs and have demonstrated that the two enantiomers contained in a racemate have to be considered as two distinct drugs. This in turn has initiated a lively discussion as to whether the chiral drugs to be introduced in the future should be stereochemically pure and whether the racemic drugs presently used should be reformulated to contain only the stereoisomers responsible for therapeutic efficacy.

The stereochemistry of the drugs available to prescribing physicians has been surveyed in recent years (Roth and Kleemann, 1982; Simonyi, 1984; Ariëns *et al.*, 1988; Millership and Fitzpatrick, 1993). All surveys concur that the vast majority of drugs derived from natural sources (22% of those available) are chiral and that more than 98% are used as enantiomers. This is a consequence of the stereospecificity of biological syntheses. The majority of synthetic drugs do not contain a chiral centre (60% in the most recent survey). In contrast to the natural chiral drugs, of the 40% of drugs with a chiral centre produced by chemical synthesis, more than 85% are used as racemates, since chemical synthesis usually leads to a racemic compound unless stereospecific synthesis is employed (Fig. 1). Approximately 20% of all available drugs are sold as mixtures of stereoisomers (Millership and Fitzpatrick, 1993). These compounds include many important medicines introduced into therapeutic practice since the 1960s. The most recent survey based on the data available in *Therapeutic Drugs* (Dollery, 1991) indicates that over the past decade the proportion of synthetic chiral drugs available as single enantiomers has increased from 12% in 1982 to 44% in 1991 (Millership and Fitzpatrick, 1993). Differences in the range of drugs sold in the different countries surveyed and their classification will account for some of this discrepancy; however, a trend to an increasing use of stereochemically pure synthetic drugs is revealed. The latest survey also indicates that the proportion of all drugs available which are used as single enantiomers has increased from 31% in 1982 to 34% in 1991. Drug regulatory authorities

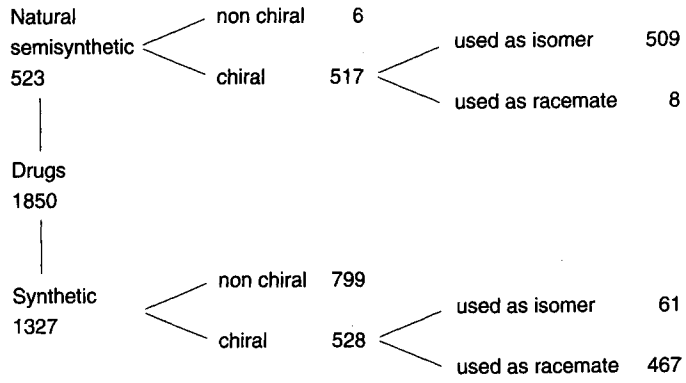

FIG. 1. Number of drugs used as isomers and racemates. Values are based on surveys carried out by Roth and Kleemann (1982), and Ariëns *et al.* (1988).

worldwide have recently developed guidelines for the approval of drugs with chiral centres for both new chemical entities or drugs developed as pure enantiomers from racemic drugs already in clinical use (Cayen, 1991, 1993; De Camp, 1993; Nation, 1994).

In view of the widespread clinical use of racemates it is the purpose of this article to provide background information on the implications of stereochemistry for drug action and disposition. It is not intended to cover all the literature published on this topic but we will rather focus on the principles involved and use illustrative examples to demonstrate the implications for the therapeutic use of chiral drugs. Monographs and review articles also addressing this topic include Ariëns *et al.* (1983), Simonyi (1984), Williams and Lee (1985), Ariëns *et al.* (1988), Lam (1988), Testa and Mayer (1988), Wainer and Drayer (1988), Coutts and Baker (1989), Testa (1989), Testa and Trager (1990), Tucker and Lennard (1990), Wainer (1993a) and Mayer and Testa (1994).

2 Stereoselectivity in Drug Action

Binding of a drug molecule to a receptor, enzyme or other protein depends on certain structural features. The effective drug molecule has several points of attachment to corresponding areas at the receptor site and, in addition for drugs with an asymmetric carbon atom, a certain spatial configuration of the functional groups is required for optimal binding to the receptor. For instance in the case of directly acting adrenergic agonists, the Easson–Stedman hypothesis proposes a three-point attachment of the active enantiomer (−)-(*R*)-noradrenaline to the adrenergic receptor as opposed to only a two-point attachment for (+)-(*S*)-noradrenaline which has a lower affinity

for the receptor (Easson and Stedman, 1933; Patil *et al.*, 1975). For this enantiomeric pair of molecules the orientation of the benzylic β-hydroxyl group of the β-carbon atom is critical for interaction with the adrenergic receptor. The fact that a certain spatial orientation of the functional groups of a drug molecule is required results in the biological activity of chiral drugs being associated partly or almost exclusively with one enantiomer. The more points of attachment that are required at the site of action to elicit a response, the higher the stereoselectivity that ensues. Therefore, if only one or two points of attachment are involved, stereoselectivity in activity will not be observed.

Depending on the mode of interaction of chiral drug molecules with the macromolecules involved in eliciting certain pharmacological effects, different degrees of stereoselectivity can be observed for the same drug for different effects. Thus it is not unusual for enantiomers to exhibit varying stereoselectivity for different pharmacological actions. For example, the decrease in heart rate mediated by β-adrenoceptor antagonists is highly stereoselective for the (*S*)-enantiomers, whereas no enantioselectivity is observed for the local anaesthetic effects. For each pharmacological action observed, the more potent enantiomer is termed the eutomer and the less potent enantiomer the distomer.

In evaluating information on the relative pharmacological actions of the stereoisomers of chiral drugs, it is pivotal to consider the optical purity of the isomers used in assessing the potency ratio for a given pharmacological effect. If the optical purity of a distomer is 99.5% (e.g. it contains 0.5% of the eutomer) and the eutomer is 1000 times more potent, the activity observed will be attributed to the distomer, whereas in fact it is due to the small quantity of the eutomer present (Patil *et al.*, 1975). For example, when the activity of the enantiomers of isoproterenol was originally assessed, a relative potency ratio of 11.8 was observed. However, following repeated fractional crystallization to increase the optical purity of the stereoisomers tested, the potency ratio increased to 1000 (Lands *et al.*, 1954).

The situation that both enantiomers are equipotent is rather seldomly encountered. In most cases differences in activity in either qualitative or quantitative terms are observed between enantiomers. No generalizations can be made concerning whether and to what extent either in qualitative or quantitative terms the activity of the stereoisomers of a chiral drug will differ. In this section several scenarios for the relative activity of the stereoisomers of therapeutic compounds will be discussed, focusing attention on the pharmacological effects held responsible for efficacy.

2.1 STEREOISOMERS ARE EQUIPOTENT

In various experiments, including (a) animal models of arrhythmias, (b) the binding affinity to receptors associated with cardiac sodium channels and (c)

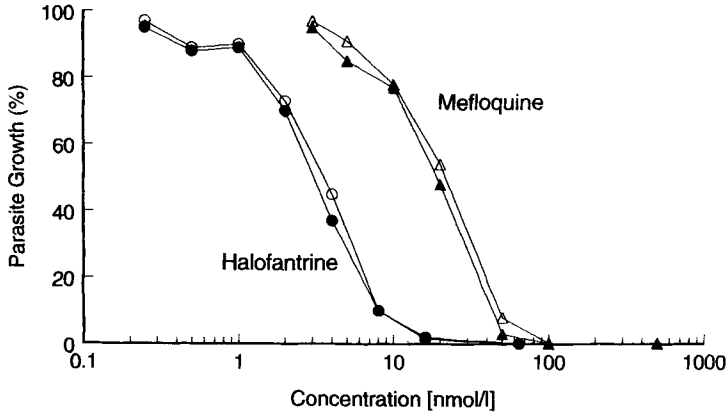

FIG. 2. *In vitro* activity of the enantiomers of mefloquine ((+), △; (−), ▲) and halofantrine ((+), ○; (−) ●) against the chloroquine-resistant strain FCM29 of *Plasmodium falciparum*. Each point represents the mean of three to five replicate tests. No difference in the activity of the enantiomers is observed in this *in vitro* model. (Adapted from Basco *et al.* (1992), with permission of Blackwell Science, London.)

the electrophysiological activity in isolated Purkinje fibres, small or no differences between the enantiomers of the class 1 antiarrhythmic drugs flecainide, mexiletine, tocainide and propafenone have been observed (Banitt *et al.*, 1986; Hill *et al.*, 1988; Kroemer *et al.*, 1989; Lie-A-Huen *et al.*, 1989). For tocainide a twofold to threefold greater antiarrhythmic potency of the (*R*)- than (*S*)-enantiomer was observed in the chloroform mouse model (Block *et al.*, 1988) and smaller differences were noted in the coronary ligated dog (Uprichard *et al.*, 1988). Whether these differences are due to a greater potency of (*R*)-tocainide or reflect species differences in stereoselective metabolism remains to be determined.

The enantiomers of the racemic antimalarials mefloquine, halofantrine and enpiroline are also equipotent against chloroquine-resistant *Plasmodium falciparum* (Basco *et al.*, 1992) (Fig. 2). If in the intact animal stereoselective drug disposition occurs after administration of the racemate, systemic exposure to the two enantiomers may differ between species. To attain the same systemic concentrations of the eutomer in different species, varying doses of the racemate and thus the active stereoisomer may have to be administered. To date the relative efficacy of the enantiomers of chiral drugs has in general been compared in animal studies on the basis of the dose administered and not the concentrations achieved *in vivo* at the time at which the effects were observed. Therefore the relative efficacy may not have been measured at equivalent concentrations and the relative potency measured may be misleading.

TABLE 1

Potency ratio of (*S*)- and (*R*)-verapamil for different pharmacological effects

Pharmacological effect	*S*/*R* potency ratio	Reference
Increase in coronary blood flow	2	Satoh *et al.* (1980)
Blood pressure reduction	4	Curtis and Walker (1986)
Binding to calcium channel of human cardiac muscle	5	Ferry *et al.* (1985)
Negative inotropic effect	7.5	Ferry *et al.* (1985)
	10–20	Curtis and Walker (1986)
	10	Satoh *et al.* (1980)
Negative dromotropic effect on atrioventricular conduction	7.5	Satoh *et al.* (1980)
	10	Echizen *et al.* (1985a)
	20	Echizen *et al.* (1988)
Reversal of P170-mediated multidrug resistance	1	Eichelbaum (1988b)
	1	Gruber *et al.* (1988)
	1	Plumb *et al.* (1990)
	1	Häußermann *et al.* (1991)

2.2 STEREOISOMERS HAVE THE SAME QUALITY OF ACTION BUT DIFFER IN POTENCY

Calcium antagonists are a chemically heterogeneous group of drugs with phenylalkylamine, dihydropyridine and benzothiazepine structure. With the exception of nifedipine, which is an achiral dihydropyridine derivative, and the benzothiazepine derivative diltiazem, which is marketed as a *cis* (+)-stereoisomer, all currently used calcium antagonists are racemic mixtures. The enantiomers of these drugs display quantitative differences in potency, rather than in the spectrum of pharmacological effects elicited. For example, the calcium channel blocker verapamil exerts negative chronotropic, dromotropic and inotropic, as well as vasodilating effects. With regard to these properties there are only quantitative differences in the potency of the (*R*)- and (*S*)-enantiomers, (*S*)-verapamil being more active (Table 1). The most pronounced stereoselectivity is observed for negative dromotropic effect where (*S*)-verapamil is at least 20 times more potent than the (*R*)-enantiomer (Fig. 3). However, for blood pressure lowering only a four-fold difference is observed and in the case of modulation of P170-mediated multidrug resistance both enantiomers are equipotent. The initial observation by Bayer *et al.* (1975) that there are also qualitative differences in activity between the enantiomers, namely that (*S*)-verapamil is mainly a calcium channel blocker whereas (*R*)-verapamil reduces sodium channel conductance, could not be confirmed (Nawrath *et al.*, 1981).

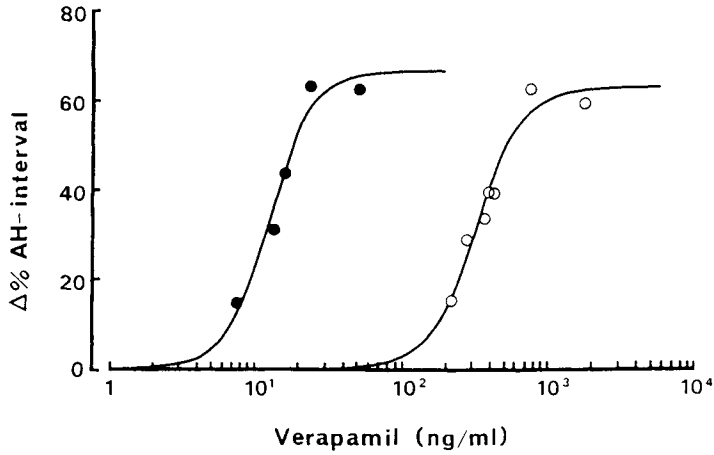

FIG. 3. Relationship between (*R*)-verapamil (○) and (*S*)-verapamil (●) plasma concentrations and percentage AH-interval prolongation. Based on the plasma concentration that elicits 50% of a maximal dromotropic effect, (*S*)-verapamil (EC_{50} 10.6 ng ml^{-1}) is 20 times more potent than the (*R*)-enantiomer (EC_{50} 258.7 ng ml^{-1}). (Adapted from Echizen *et al.* (1988), with permission of the authors and Raven Press, New York.)

2.3 ACTIVITY IS MAINLY ASSOCIATED WITH ONE STEREOISOMER

All β-adrenoceptor antagonists contain at least one chiral centre and, with the exception of timolol and penbutolol, are administered as racemates. The β-blocking activity of the (*S*)-enantiomers of the various β-adrenoceptor antagonists is at least one to two orders of magnitude greater than that of the (*R*)-enantiomers, and this enantiomer is thus responsible for the blood-pressure-lowering effects of the β-adrenergic antagonists. In contrast to the high degree of stereoselectivity noted for β-blockade, comparable potencies for the (*S*)- and (*R*)-enantiomers are observed for other actions such as membrane-stabilizing effects (Jaillon *et al.*, 1980), the effects on the conversion of thyroxine to tri-iodothyronine (Heyma *et al.*, 1980; Eber *et al.*, 1990; Stoschitzky *et al.*, 1992) and on intraocular pressure (Alkondon *et al.*, 1986).

The increase in plasma renin activity after administration of the racemic dihydropyridine calcium antagonist is also stereoselective in humans (Mikus *et al.*, 1995). As shown in Fig. 4, the increases in plasma renin activity after administration of the racemate are related to the concentration of (*S*)- and not (*R*)-nitrendipine. Other examples where the therapeutic activity of a chiral compound is associated predominantly with one enantiomer are listed in Table 2.

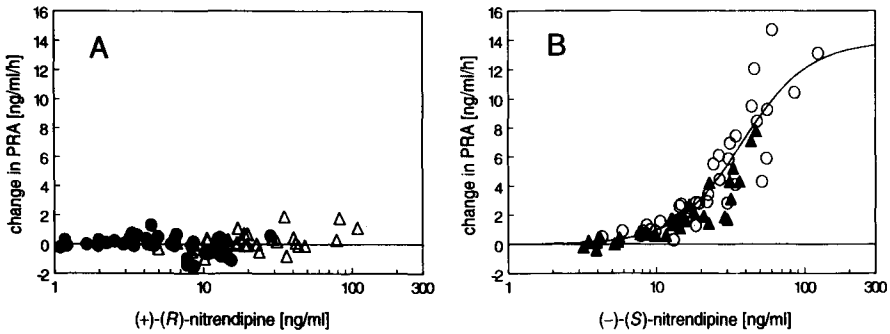

FIG. 4. (A), Relationship between change in plasma renin activity (PRA) and serum (*R*)-nitrendipine concentration after administration of 20 mg (*R*)-nitrendipine (●) and 80 mg (*R*)-nitrendipine (△). (B) Relationship between change in PRA and serum (*S*)-nitrendipine concentration after administration of 20 mg (*S*)-nitrendipine (○) and 20 mg (*R*)/(*S*)-nitrendipine (▲). The fitted line corresponds only to the data after 20 mg (*S*)-nitrendipine. Data from all six subjects are included. (Adapted from Mikus *et al.* (1995), with permission of the authors and Mosby Year Book, St Louis, Missouri.)

2.4 BOTH STEREOISOMERS ARE ACTIVE BUT HAVE QUALITATIVELY DIFFERENT ACTIONS

2.4.1 Agonists at Different Receptors

The enantiomers of dobutamine are both active, although they have qualitatively different actions as the enantiomers are agonists at different receptors. (+)-Dobutamine has β_1- and β_2-adrenoceptor agonist activities which result in peripheral vasodilatation in addition to positive inotropic and chronotropic effects. (−)-Dobutamine, however, has α_1-adrenoceptor agonist activity, which produces a positive inotropic effect with concomitant vasoconstriction (Ruffolo and Messick, 1985).

2.4.2 Antagonists at Different Receptors

Labetalol is a unique β-adrenoceptor antagonist because, in addition to its β-blocking activity, it exhibits α-adrenergic antagonistic effects. In contrast to the majority of stereoisomeric drugs, which are 50:50 mixtures of two enantiomers, labetalol has two chiral centres and therefore is composed of equal proportions of four stereoisomers. The (*R*,*R*)-diastereoisomer is predominantly responsible for the β-blocking activity, whereas the α-

blocking activity resides mainly with the (*S*,*R*)-diastereoisomer (Baum *et al.*, 1981; Brittain *et al.*, 1982). The two other compounds contained in commercially available preparations, namely the (*S*,*S*)- and (*R*,*S*)-diastereoisomers, seem to contribute minimally to the overall activity of labetalol.

2.4.3 Agonist and Antagonist at the Same Receptor

It is unusual for the two isomers of a chiral drug to elicit opposite effects, that is one enantiomer is an agonist and the other is an antagonist at the same receptor. This scenario has, however, been observed for a number of investigational 1,4-dihydropyridines, including Bay K 8644 and 202-791. These compounds exhibit opposite effects at calcium channels where one enantiomer behaves as a calcium channel antagonist and the other as a calcium channel agonist (Franckowiak *et al.*, 1985; Hof *et al.*, 1985).

2.5 ACTIVE CHIRAL METABOLITES

Since biotransformation of chiral drugs is often stereoselective, preferential metabolism of one enantiomer to a chiral metabolite may occur. If the metabolite is pharmacologically active it may be anticipated that the same difference in potency between the stereoisomers of the parent compound will apply to the stereoisomers of the chiral metabolite. Hence the contribution of the metabolite to the overall therapeutic efficacy of the drug will depend on the configuration of the metabolite formed. Usually the activity of chiral metabolites is assessed with a racemic mixture and, as stereoselective metabolism is common, the 50:50 proportion of enantiomers in the racemate will not reflect the proportion of the metabolite isomers present *in vivo*. Thus the contribution of a chiral metabolite to the therapeutic effect *in vivo* cannot be extrapolated from *in vitro* data where equal concentrations of the two enantiomers have been studied. For example, the chiral metabolite of verapamil, norverapamil, has 5–10% of the electrophysiological effects of the parent compound. By analogy with verapamil, (*S*)-norverapamil is expected to be at least ten times more active than (*R*)-norverapamil. After verapamil administration two-thirds of the norverapamil in plasma has (*R*)-configuration (Mikus *et al.*, 1990). Comparable unresolved (*R*+*S*) concentrations of verapamil and norverapamil are observed *in vivo* after verapamil administration. However, as two-thirds of plasma concentrations consist of the less active (*R*)-norverapamil, the contribution of norverapamil to the pharmacological effects after administration of verapamil is less than that predicted on the basis of the total metabolite plasma concentration.

TABLE 2

Chiral drugs where the activity responsible for therapeutic efficacy is predominantly elicited by one stereoisomer; some relative potencies for other pharmacological actions are also given

Drug	Activity	Relative potency[a]	Reference
Aminoglutethimide	Antisteroidigenic activity	$(+) \gg (-)$	Salhanick (1982)
Atenolol	β-adrenergic antagonist	$S \gg R$	Stoschitzky *et al.* (1993)
	Central hypotensive effect	$S \gg R$	Pearson *et al.* (1989)
Baclofen	Antinociception	$(-) \gg (+)$	Sawynok and Dickson (1985)
	Myotonolytic activity	$(-) \gg (+)$	Olpe *et al.* (1978)
Bicalutamide	Antiandrogen activity	$R \gg S$	McKillop *et al.* (1993)
Bupivacaine	Nerve block effect	$(-) = (+)$	Aps and Reynolds (1978)
	Vasoconstriction	$(-) > (+)$	
Cromakalim	Potassium channel activation	$(-) \gg (+)$	Hof *et al.* (1988)
	Antihypertensive/vasodilator	$(-) \gg (+)$	Clapham *et al.* (1991)
Disopyramide	Antiarrhythmic effect	$S \gg R$	Lima *et al.* (1985, 1990)
	Negative inotropic effect	$S = R$	Kidwell *et al.* (1989)
	Anticholinergic effect	$S > R$	Giacomini *et al.* (1980)
Felodipine	Calcium antagonist	$S \gg R$	Soons *et al.* (1993)
Gossypol	Antifertility effect	$(-) \gg (+)$	Wu *et al.* (1986)
Isoflurane	Anaesthetic sleep time	$(+) > (-)$	Harris *et al.* (1992)
Ketamine	Analgesic, hypnotic	$S > R$	White *et al.* (1985)

Methadone	Miosis, respiratory depression	$(-) > (+)$	Olsen *et al.* (1977)
Threo-methylphenidate	Inhibits dopamine and noradrenaline	$(+) > (-)$	Patrick *et al.* (1987)
	Induces locomotor activity	$(+) > (-)$	
Metoprolol	β-adrenergic antagonist	$S \gg R$	Walle *et al.* (1988)
	Ocular β_2-adrenoceptor antagonist	$S > R$	Nathanson (1988)
Mianserin	Serotonin antagonist, antidepressant	$S \gg R$	Pinder and van Delft (1983)
	Histamine H_1 antagonist, sedative	$S = R$	
Nitrendipine	Vasodilatation, hypotensive effect	$S > R$	Mikus *et al.* (1995)
	Stimulates plasma renin activity	$S > R$	
Ofloxacin	Prevents multiplication of Gram-positive and Gram-negative bacteria	$S \gg R$	Okazaki *et al.* (1989)
Oxazepam	Affinity to benzodiazepine receptor	$S \gg R$	Vree *et al.* (1991)
Pazinaclone	Anxiolytic effect	$S \gg R$	Hussein *et al.* (1993)
Pentobarbital	Duration of sedation produced	$S > R$	Cook *et al.* (1987)
Phenprocoumon	Anticoagulant effect	$S > R$	Jähnchen *et al.* (1976)
Propranolol	β_2-adrenoceptor antagonist	$S \gg R$	Barrett and Cullum (1968)
	Local anaesthetic activity	$S = R$	
Rolipram	Inhibition of phosphodiesterase	$R \gg S$	Krause *et al.* (1990)
Sotalol	β-adrenergic antagonist	$R \gg S$	Fiset *et al.* (1993)
	Class 3 antiarrhythmic potency	$R = S$	
Terbutaline	β_2-adrenoceptor agonist	$R \gg S$	Borgström *et al.* (1989)
	Affects airway responsiveness to histamine	$S > R$	Chapman *et al.* (1992)
Vigabatrin	γ-aminobutyric acid transaminase inhibitor	$S \gg R$	Haegele and Schechter (1986)
Warfarin	Inhibition of vitamin K_1 2,3-epoxide reductase	$S > R$	Choonara *et al.* (1986b)

[a] >, Up to tenfold difference in potency; ≫, greater than tenfold difference in potency.

TABLE 3

Pharmacokinetic parameters for the enantiomers of chiral drugs in humans

Drug	Dosage[a]	Route[b]	Clearance[c]		Volume of distribution (V_d)		Half-life		Reference
Acenocoumarol	5 mg rac	Oral	*R*: 35 ± 8 ml min^{-1}	*S*: 496 ± 142 ml min^{-1}	*R*: 32.4 ± 6[e] litres	*S*: 37.8 ± 19.5[e] litres	*R*: 11.5 h	*S*: 1.1 h	Thijssen *et al.* (1986)
Bupivacaine	30 mg rac	i.v.	*R*: 0.40 ± 0.08 l min^{-1}	*S*: 0.32 ± 0.07 l min^{-1}	*R*: 117 ± 47 litres	*S*: 71 ± 34 litres	*R*: 210 ± 95 min	*S*: 157 ± 77 min	Burm *et al.* (1994)
Carvedilol	20 mg rac	Oral	*R*: 872 ± 250 ml min^{-1}	*S*: 1263 ± 169 ml min^{-1}	*R*: 302 ± 149 litres	*S*: 487 ± 296 litres	*R*: 5.3 ± 1.9 h	*S*: 5.1 ± 1.8 h	Fujimaki *et al.* (1990)
Etodolac	200 mg rac	Oral	*R*: 22 ml h^{-1} kg^{-1}	*S*: 288 ml h^{-1} kg^{-1}	*R*: 0.211 kg^{-1}	*S*: 1.6 l kg^{-1}	*R*: 6.6 h	*S*: 4.3 h	Brocks *et al.* (1992)
Flurbiprofen	100 mg rac	Oral	*R*: 1.47 ± 0.50 l h^{-1}	*S*: 1.23 ± 0.34 l h^{-1}	*R*: 8.4 ± 3.0 litres	*S*: 4.2 ± 1.2 litres	*R*: 4.2 ± 1.3 h	*S*: 4.2 ± 1.2 h	Jamali *et al.* (1988)
Hexobarbital	500 mg rac	Oral	*R*: 15.6 ± 16.4 ml min^{-1} kg^{-1}	*S*: 1.9 ± 0.3 ml min^{-1} kg^{-1}	—	—	*R*: 2.1 ± 0.9 h	*S*: 6.0 ± 2.0 h	Smith *et al.* (1991)
Hexobarbital	200 mg rac	Oral	*R*: 136 ± 113 l h^{-1}	*S*: 21 ± 13 l h^{-1}	—	—	*R*: 6.7 ± 3.6 h	*S*: 2.8 ± 3.2 h	Adedoyin *et al.* (1994)
Ibuprofen	800 mg rac	Oral	*R*: 87.6 ± 26.9 ml min^{-1}	*S*: Cl	*R*: 11.5 ± 2.3[e] litres	*S*: Cl	*R*: 1.7 ± 0.4 h	*S*: 2.5 ± 0.2 h	Lee *et al.* (1985)
Ibuprofen	600 mg rac 400 mg *S*	Oral	*R*: 84.4 ± 30.6 ml min^{-1}	*S*: 90.3 ± 21.2 ml min^{-1}	—	—	*R*: 2.0 ± 0.7 h	*S*: 2.1 ± 0.7 h	Geisslinger *et al.* (1993a)
Ifosfamide	4 g rac	i.v.	*R*: 0.061 ± 0.013 l h^{-1} kg^{-1}	*S*: 0.072 ± 0.014 l h^{-1} kg^{-1}	*R*: 0.62 ± 0.17 l kg^{-1}	*S*: 0.63 ± 0.14 l kg^{-1}	*R*: 7.3 ± 1.9 h	*S*: 6.2 ± 1.5 h	Corlett and Chrystyn (1994)
Indobufen	200 mg rac	Oral	*R*: 1.21 ± 0.06 l h^{-1}	*S*: 2.26 ± 0.09 l h^{-1}	*R*: 15.1 ± 0.8 litres	*S*: 20.2 ± 1.0 litres	*R*: 8.7 ± 0.2 h	*S*: 6.2 ± 0.3 h	Benedetti *et al.* (1992)
Indoprofen	400 mg rac	i.v.	*R*: 48.8 ± 15.5 ml min^{-1}	*S*: 32.2 ± 11.1 ml min^{-1}	*R*: 12.6 ± 4.2 litres	*S*: 9.0 ± 2.2 litres	*R*: 187 ± 62 min	*S*: 201 ± 43 min	Björkman (1985)
Ketamine	2 mg kg^{-1} rac	i.v.	*R*: 16.5 ± 4.8 ml min^{-1} kg^{-1}	*S*: 19.1 ± 7.2 ml min^{-1} kg^{-1}	*R*: 3.0 ± 1.2[e] l kg^{-1}	*S*: 3.3 ± 1.3[e] l kg^{-1}	*R*: 2.6 ± 1.0 h	*S*: 2.5 ± 0.9 h	Geisslinger *et al.* (1993b)
Ketorolac	30 mg rac	i.m.	*R*: 19.0 ± 5.0 ml h^{-1} kg^{-1}	*S*: 45.9 ± 10.1 ml h^{-1} kg^{-1}	*R*: 0.075 ± 0.014[e] l kg^{-1}	*S*: 0.135 ± 0.022[e] l kg^{-1}	*R*: 3.6 ± 0.8 h	*S*: 2.4 ± 0.2 h	Hayball *et al.* (1994)
Mephobarbital	400 mg rac	Oral	*R*: 170 ± 55 l h^{-1}	*S*: 1.5 ± 0.3 l h^{-1}	*R*: 716 ± 448[d] litres	*S*: 105 ± 46[d] litres	*R*: 3.1 ± 1.7 h	*S*: 50.5 ± 20.1 h	Hooper and Qing (1990)
Methylphenobarbital	200 mg rac	Oral	*R*: 0.47 ± 0.18 l h^{-1} kg^{-1}	*S*: 0.02 ± 0.001 l h^{-1} kg^{-1}	*R*: 5.32 ± 3.53 l kg^{-1}	*S*: 1.73 ± 0.31 l kg^{-1}	*R*: 7.5 ± 1.7 h	*S*: 69.8 ± 14.8 h	Lim and Hooper (1989)
Metoprolol	100 mg rac	Oral ss	*R*: 1.7 l h^{-1} kg^{-1}	*S*: 1.2 l h^{-1} kg^{-1}	*R*: 7.6 ± 4.7[d] l kg^{-1}	*S*: 5.5 ± 2.3[d] l kg^{-1}	*R*: 2.7 h	*S*: 3.0 h	Toon *et al.* (1988)

Mexiletine	300 mg rac	Oral	*R*: 8.60 ± 2.30 ml min^{-1} kg^{-1}	*S*: 8.10 ± 2.50 ml min^{-1} kg^{-1}	*R*: 6.6 ± 2.6 l kg^{-1}	*S*: 7.3 ± 2.4 l kg^{-1}	*R*: 9.1 ± 2.9 h	*S*: 11.0 ± 3.8 h	Igwemezie *et al.* (1989)
Nicoumalone	20 mg rac	Oral	*R*: 1.28 ± 0.16 l h^{-1}	*S*: 17.5 ± 2.2 l h^{-1}	*R*: 12.5 ± 1.4 litres	*S*: 22.6 ± 3.5 litres	*R*: 6.8 ± 0.4 h	*S*: 0.9 ± 0.2 h	Gill *et al.* (1988)
Nitrendipine	20 mg rac	Oral	*R*: 6.6 ± 2.0 l min^{-1}	*S*: 3.1 ± 1.3 l min^{-1}	—	—	*R*: 7.5 h	*S*: 7.7 h	Mast *et al.* (1992)
Nivaldipine	4 mg rac	Oral	*R*: 110 ± 13 ml min^{-1} kg^{-1}	*S*: 39.5 ± 1.3 ml min^{-1} kg^{-1}	—	—	*R*: 3.2 ± 0.7 h	*S*: 3.4 ± 0.7 h	Tokuma *et al.* (1987)
Penbutolol	40 mg sep	Oral	*R*: 43.7 ± 8.1 ml min^{-1} kg^{-1}	*S*: 15.9 ± 2.6 ml min^{-1} kg^{-1}	—	—	*R*: 2.1 ± 0.2 h	*S*: 1.5 ± 0.1 h	Ochs *et al.* (1986)
Pentobarbital	100 mg rac	Oral	*R*: 2.58 l h^{-1}	*S*: 1.96 l h^{-1}	*R*: 1.24 ± 0.55 l kg^{-1}	*S*: 1.11 ± 0.55 l kg^{-1}	*R*: 20.6 h	*S*: 23.4 h	Cook *et al.* (1987)
Prenylamine	200 mg rac	Oral	*R*: 4.0 ± 1.5 l min^{-1}	*S*: 20.5 ± 17.3 l min^{-1}	—	—	*R*: 8.2 ± 3.1 h	*S*: 24 ± 26 h	Gietl *et al.* (1990)
Propafenone	150 mg rac	Oral ss	*R*: 1460 ± 480 ml min^{-1}	*S*: 920 ± 300 ml min^{-1}	—	—	*R*: 3.3 ± 1.9 h	*S*: 4.6 ± 1.9 h	Kroemer *et al.* (1989)
Propranolol	0.1 mg kg^{-1} rac	i.v.	*R*: 1.21 ± 0.15 l min^{-1}	*S*: 1.03 ± 0.12 l min^{-1}	*R*: 4.82 ± 0.34 l kg^{-1}	*S*: 4.08 ± 0.33 l kg^{-1}	*R*: 3.5 ± 0.2 h	*S*: 3.6 ± 0.3 h	Olanoff *et al.* (1984)
Propranolol	80 mg rac	Oral	*R*: 6.93 ± 0.60 l min^{-1}	*S*: 4.55 ± 0.37 l min^{-1}	—	—	*R*: 4.3 ± 0.5 h	*S*: 4.8 ± 0.5 h	Zhou *et al.* (1992)
Propranolol	80 mg rac	Oral	*R*: 30.3 ± 9.6 ml min^{-1} kg^{-1}	*S*: 22.8 ± 6.7 ml min^{-1} kg^{-1}	—	—	*R*: 3.9 ± 0.7 h	*S*: 3.8 ± 0.6 h	Lalonde *et al.* (1990)
Terbutaline	0.125 mg sep	i.v.	(*R*): 0.19 ± 0.04 l h^{-1} kg^{-1}	(*S*): 0.13 ± 0.03 l h^{-1} kg^{-1}	*R*: 1.90 ± 0.16[e] l kg^{-1}	*S*: 1.76 ± 0.42[e] l kg^{-1}	*R*: 12.7 h	*S*: 15.3 h	Borgström *et al.* (1989)
Tranylcypromine	20 mg rac	Oral	(+): 13.8 ± 18.6 l min^{-1}	(−): 0.59 ± 0.22 l min^{-1}	—	—	(+): 1.2 ± 0.6 h	(−): 1.7 ± 0.3 h	Weber-Grandke *et al.* (1993)
Warfarin	25 mg rac	Oral	*R*: 234 ± 43 ml h^{-1}	*S*: 333 ± 85 ml h^{-1}	*R*: 13.3 ± 2.7 litres	*S*: 13.9 ± 3.7 litres	*R*: 39.6 ± 6.8 h	*S*: 29.5 ± 6.0 h	Toon *et al.* (1987b)
Warfarin	0.75 mg kg^{-1} rac	Oral	*R*: 2.36 ± 0.24 ml h^{-1} kg^{-1}	*S*: 3.10 ± 0.50 ml h^{-1} kg^{-1}	*R*: 144 ± 10[d] ml kg^{-1}	*S*: 140 ± 13[d] ml kg^{-1}	*R*: 43.3 ± 3.5 h	*S*: 35.7 ± 5.5 h	O'Reilly *et al.* (1980b)
Zopiclone	150 mg rac	Oral	(+): 196 ± 65 ml min^{-1}	(−): 660 ± 242 ml min^{-1}	(+): 98.6 ± 19.4 litres	(−): 192.8 ± 89.8 litres	(+):399 ± 161 h	(−):226 ± 179 h	Fernandez *et al.* (1993)

Values are mean ± SD or SEM.

[a]rac, racemate; sep, separate administration of the enantiomers.

[b]i.v., intravenous administration; i.m., intramuscular administration; ss, steady-state administration.

[c]Apparent oral clearance after oral administration; total clearance after i.v. or i.m. administration; CI, chiral inversion of $S \rightarrow R$.

[d]V_{area}.

[e]V_{ss}.

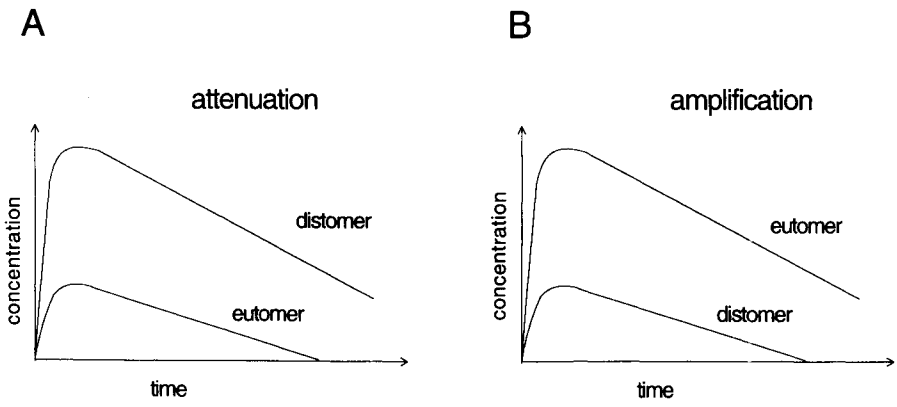

FIG. 5. Consequences of stereoselective metabolism for *in vivo* compared with *in vitro* drug potency of a racemate where the eutomer is 20 times more potent than the distomer. The eutomer/distomer ratio in the drug preparation is 1. If drug metabolism affects mainly the eutomer, the eutomer/distomer ratio of total drug concentration will be less than 1. Therefore drug effects are attenuated (A). If drug metabolism affects mainly the distomer, the eutomer/distomer ratio of total drug concentration will be greater than 1 and the drug effect is amplified (B). (Adapted from Kroemer *et al.* (1994b), with permission from the authors and John Wiley, Chichester.)

3 Stereoselectivity in Drug Disposition

Whereas differences in activity between stereoisomers have been known for many years, only recently has the importance of stereoselectivity in drug disposition been realized and the possible therapeutic consequences of this phenomenon appreciated. This development has been brought about by rapid progress in analytical chemistry which now allows for the sensitive and specific determination of the stereoisomers of numerous drugs in biological fluids (Gal, 1993; König, 1993; Wainer, 1993b; Wright and Jamali, 1993). There can be no doubt that the application of stereospecific analysis to the study of the disposition of racemic drugs has been very helpful in clarifying some apparently paradoxical pharmacological phenomena (Eichelbaum, 1988a; Evans *et al.*, 1988).

Since the processes of drug absorption, distribution, metabolism and excretion all involve an interaction between chiral drug molecules and chiral biological macromolecules, stereoselectivity of drug absorption, distribution, metabolism and excretion is to be expected. As a consequence drug disposition is stereoselective. Some examples of enantioselective pharmacokinetics for chiral drugs used in humans are given in Table 3. It is often overlooked that the relative activity of pairs of enantiomers as determined

in vitro cannot be extrapolated to the *in vivo* situation since stereoselective drug disposition can lead to an enantiomer ratio in the organism which differs substantially from that in the dosage form administered.

Stereoselectivity in drug disposition seems to be the rule rather than the exception and, depending on whether the more or less active enantiomer is preferentially affected, an amplification or attenuation of the *in vivo* drug potency as compared to the *in vitro* systems will be observed (Fig. 5).

3.1 ABSORPTION

Drug absorption is usually a passive process which depends on properties such as lipophilicity and pK_a, and therefore the rate and extent of absorption do not differ for enantiomers as they have the same physicochemical properties. In the case of the few drugs which are actively absorbed, enantioselectivity has been demonstrated. For example, the L-forms of methotrexate and dopa are actively absorbed and hence have a greater bioavailability than the D-forms, which are only passively absorbed. As the compounds are highly polar only limited passive absorption occurs, resulting in stereoselective absorption of the actively transported enantiomer (Wade *et al.*, 1973; Hendel and Brodthagen, 1984).

Stereoselective absorption of passively absorbed drugs can occur, however, if the dosage form of a racemic drug contains chiral excipients. Preferential intermolecular interaction of chiral excipients, such as cellulose derivatives with one of the enantiomers of a racemate within the drug formulation, may occur and the rate of dissolution of the drug from the dosage form may consequently be stereoselective. The rate of absorption of the individual stereoisomers may therefore differ. For example, the release of propranolol from a formulation containing hydroxypropylcellulose is stereoselective (Duddu *et al.*, 1993) and verapamil dissolution from modified release dosage forms can favour the (*R*)-enantiomer (Carr *et al.*, 1993).

3.2 DISTRIBUTION

3.2.1 Plasma Protein Binding

The drug in plasma not bound to proteins is responsible for the pharmacological effects since it is the unbound concentration that is able to traverse biological membranes and is in equilibrium with receptors at the site of action. Hence, if the plasma protein binding of enantiomers is different at the same total concentration in plasma, the active concentration at the site of action will differ. Differences in the binding of stereoisomers to plasma proteins have been reported for acidic drugs which bind stereoselectively to

the benzodiazepine or warfarin binding sites on the albumin molecule and basic drugs which bind to α_1-acid glycoprotein (Müller, 1988; Noctor, 1993) (Table 4). Several recent studies have shown that the plasma concentration of α_1-acid glycoprotein is lower in individuals from Chinese than Caucasian populations (Kalow and Bertilsson, 1994). Ethnic differences in the enantioselective plasma protein binding of basic drugs have consequently been reported. For example, the proportion of the pharmacologically important free (*S*)-propranolol in plasma is higher in Chinese than Caucasian subjects (Zhou *et al.*, 1991), which may in part account for the higher sensitivity of Chinese patients to propranolol (Zhou *et al.*, 1989).

Competition of enantiomers for the same protein binding sites can result in higher free fractions when both enantiomers are present than when the single enantiomers are studied individually (Evans *et al.*, 1989). The plasma protein binding of disopyramide is enantioselective and concentration dependent (Fig. 6) (Takahashi *et al.*, 1991). Furthermore the enantiomers compete for plasma protein binding sites. When the racemate is given the altered plasma protein binding of the enantiomers of disopyramide, this leads to changes in the disposition of (*R*)- and (*S*)-disopyramide compared with separate administration of the enantiomers (Giacomini *et al.*, 1986). The plasma protein binding of any chiral metabolites produced *in vivo* can also be stereoselective and should be considered when the relative activity of the enantiomers of chiral metabolites is assessed. For example, the albumin binding of the hydroxylated and reduced metabolites of warfarin is high and stereoselective (Chan *et al.*, 1993).

The plasma protein binding of a drug in turn influences its renal clearance as renal glomerular filtration is a product of the free fraction in plasma and the glomerular filtration rate. If stereoisomers differ in the extent of protein binding, the glomerular filtration of the enantiomers will differ, and stereoselective total renal clearance results. Enantioselective differences in plasma protein binding can also affect the metabolic clearance of low-clearance drugs, since only the free drug has access to intracellular drug-metabolizing enzymes. Consequently enantiomers with a higher free fraction will have a higher metabolic clearance, as has been demonstrated for warfarin enantiomers (Toon *et al.*, 1986a).

3.2.2 Tissue Binding

Enantioselective tissue binding, which is in part a consequence of enantioselective plasma protein binding, has been reported. For example, the transport of the arylpropionic non-steroidal anti-inflammatory drug (NSAID) ibuprofen into both synovial and blister fluids is preferential for (*S*)-ibuprofen owing to the higher free fraction of this enantiomer in plasma (Seideman *et al.*, 1994). In addition the affinity of stereoisomers for binding sites in

TABLE 4

Stereoselective plasma or serum protein binding of chiral drugs in humans

Drug	Concentration	Free fraction of each enantiomer (%)		Reference
Bupivacaine	600 $\mu g\,l^{-1}$	*R*: 6.6 ± 3.0	*S*: 4.5 ± 2.1	Burm *et al.* (1994)
Carvedilol	1 $mg\,l^{-1}$	*R*: 0.45 ± 0.20	*S*: 0.63 ± 0.7	Fujimaki *et al.* (1990)
Chloroquine	200 $\mu g\,l^{-1}$	*R*: 57.3	*S*: 33.3	Augustijns and Verbeke (1993)
	100 $\mu g\,l^{-1}$	*R*: 51.5	*S*: 33.3	Ofori-Adjei *et al.* (1986a)
Disopyramide[a]	~1 $mg\,l^{-1}$	*R*: 12.5	*S*: 7.5	Le Corre *et al.* (1988)
	~2.2 $mg\,l^{-1}$	*R*: 33.8 ± 21.4	*S*: 20.7 ± 11.9	Lima *et al.* (1990)
E-10-OH-nortriptyline	—	(+): 69	(−): 54	Dahl-Puustinen *et al.* (1989)
Etodolac	10 $mg\,l^{-1}$	*R*: 0.47	*S*: 0.85	Brocks and Jamali (1994)
Flurbiprofen[a]	<26 $mg\,l^{-1}$	*R*: 0.082 ± 0.017	*S*: 0.048 ± 0.007	Knadler *et al.* (1989)
Gallopamil	~20 $\mu g\,l^{-1}$	*R*: 4.0	*S*: 5.7	Gross *et al.* (1993)
Homochlorcyclizine	~50 $mg\,l^{-1}$	(+): 2.6	(−): 1.4	Nishikata *et al.* (1992)
Ibuprofen[a]	0.5–30 $\mu g\,l^{-1}$	*R*: 0.42 ± 0.05	*S*: 0.64 ± 0.09	Evans *et al.* (1989)
Mephobarbital	*R*: 0.4 $mg\,l^{-1}$	*R*: 36.5	*S*: 43.9	O'Shea and Hooper (1990)
	S: 0.9 $mg\,l^{-1}$			
Methadone	~500 $\mu g\,l^{-1}$	(+): 9.2 ± 1.6	(−): 12.4 ± 1.5	Romach *et al.* (1981)
Mexiletine	0.2–2 $mg\,l^{-1}$	*R*: 19.8 ± 1.5	*S*: 28.3 ± 1.5	McErlane *et al.* (1987)
Pentobarbital	300 $\mu g\,l^{-1}$	*R*: 37	*S*: 26	Cook *et al.* (1987)
Propafenone	5 $mg\,l^{-1}$	*R*: 3.9 ± 0.6	*S*: 2.5 ± 0.3	Mehvar (1991)
Propranolol	125 $\mu g\,l^{-1}$	*R*: 20.3 ± 0.8	*S*: 17.6 ± 0.7	Olanoff *et al.* (1984)
	400 $\mu mol\,l^{-1}$	*R*: 12.2 ± 1.9	*S*: 10.9 ± 2.0	Albani *et al.* (1984)
	6 $\mu g\,l^{-1}$	*R*: 10.8 ± 1.8	*S*: 9.2 ± 1.8	Walle *et al.* (1994)
Quinine (QN) and quinidine (QD)	~4 $mg\,l^{-1}$	QN: 9.4	QD: 22.8	Karbwang *et al.* (1993)
	1 $mg\,l^{-1}$	QN: 7.5 ± 2.2	QD: 12.3 ± 2.3	Mihaly *et al.* (1987)
Sotalol	—	*R*: 65 ± 9	*S*: 62 ± 9	Fiset *et al.* (1993)
Verapamil[b]	200 $\mu g\,l^{-1}$	*R*: 13 ± 2	*S*: 23 ± 3	Gross *et al.* (1988)
Warfarin	0.2–5 $mg\,l^{-1}$	*R*: 0.56	*S*: 0.43	Banfield *et al.* (1983)

[a]Plasma protein binding is concentration dependent and the enantiomers compete for binding sites *in vivo*.
[b]After oral administration.

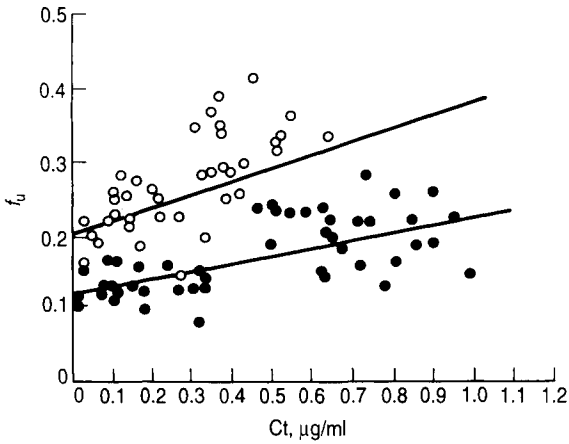

FIG. 6. Enantioselective and concentration-dependent plasma protein binding of disopyramide. The unbound fraction (f_u) *versus* total plasma concentrations (Ct) of disopyramide enantiomers after oral administration of a 100-mg dose in eight subjects. •, (*S*)-(+)-disopyramide; ○, (*R*)-(−)-disopyramide. (Adapted from Takahashi *et al.* (1991), with permission from the American Pharmaceutical Association, Washington, DC.)

specific tissues may also differ and contribute to stereoselective tissue binding. For example, the preferential binding of the (*S*)-enantiomers to synovial proteins may also contribute to the stereoselective distribution of NSAIDs into synovial fluid (Evans, 1992). By contrast the uptake of ibuprofen into lipids is also stereoselective but favours (*R*)-ibuprofen (Williams *et al.*, 1986). A further example is racemic leucovorin, used to prevent 5-fluoruracil central nervous system toxicity in cancer chemotherapy. The naturally occurring (*S*)-stereoisomer accumulates in tumour cells *in vitro* to a greater extent than the (*R*)-stereoisomer (Mader *et al.*, 1994).

3.3 RENAL ELIMINATION

The renal elimination of many chiral drugs by glomerular filtration and tubular secretion is stereoselective. Whether or not stereoselectivity of these elimination processes has an impact on the disposition of the enantiomers will depend on how much of a dose is renally excreted as parent drug. If renal excretion of the unchanged drug accounts for not more than 20–30% of the total elimination process, stereoselective renal excretion will not influence to a large extent the stereoselectivity of the overall drug disposition. Stereoselective renal elimination has been demonstrated for an increasing number of drugs where it has been examined (Table 5).

TABLE 5

Stereoselective renal clearance of chiral drugs in humans

Drug		Renal clearance		Units	Reference
Chloroquine	T	(+): 276 ± 47	(−): 267 ± 108	$ml\ min^{-1}$	Ofari-Adjei *et al.* (1986b)
	U	824 ± 140	519 ± 209		
Diacetolol	T	*R*: 70 ± 36	*S*: 53 ± 30	$ml\ min^{-1}$	Piquette-Miller *et al.* (1991)
Disopyramide	T	*R*: 64.6 ± 13.0	*S*: 36.5 ± 7.6	$ml\ min^{-1}$	Giacomini *et al.* (1986)
	U	*R*: 1.97 ± 0.26	*S*: 4.11 ± 1.08	$ml\ min^{-1}\ kg^{-1}$	Le Corre *et al.* (1988)
	U	*R*: 182	*S*: 338	$ml\ min^{-1}$	Lima *et al.* (1985)
E-10-hydroxynortriptyline	U	(+): 0.44 ± 0.14	(−): 0.57 ± 0.16	$l\ kg^{-1}\ h^{-1}$	Dahl-Puustinen *et al.* (1989)
Flurbiprofen	T	*R*: 0.25 ± 0.08	*S*: 0.18 ± 0.07	$ml\ min^{-1}$	Knadler *et al.* (1992)
Hexobarbital	T	*R*: 1.47 ± 2.43	*S*: 0.13 ± 0.12	$l\ h^{-1}$	Adedoyin *et al.* (1994)
Hydroxychloroquine	T	*R*: 199 ± 290	*S*: 277 ± 349	$ml\ min^{-1}$	McLachlan *et al.* (1993)
	U	*R*: 316 ± 460	*S*: 771 ± 972	$ml\ min^{-1}$	
Metoprolol	T	*R*: 74.8 ± 22.3	*S*: 69.7 ± 22.2	$ml\ min^{-1}$	Lennard *et al.* (1984)
Mexiletine	T	*R*: 0.61 ± 0.20	*S*: 0.72 ± 0.26	$ml\ min^{-1}\ kg^{-1}$	Igwemezie *et al.* (1989)
Ofloxacin	T	*R*: 7.53 ± 1.28	*S*: 7.14 ± 1.37	$l\ h^{-1}\ 1.73\ m^{-2}$	Okazaki *et al.* (1991)
Pindolol	T	*R*: 204 ± 61	*S*: 176 ± 55	$ml\ min^{-1}$	Hsyu and Giacomini (1985)
	S	*R*: 157 ± 48	*S*: 196 ± 47	$ml\ min^{-1}$	
Prenylamine	T	*R*: 1.3 ± 1.4	*S*: 4.0 ± 5.2	$ml\ min^{-1}$	Gietl *et al.* (1990)
Quinine (QN) and quinidine (QD)	T	QN: 24.7 ± 8.5	QD: 99 ± 31	$ml\ min^{-1}$	Notterman *et al.* (1986)
Sotalol	T	*R*: 96 ± 42	*S*: 89 ± 39	$ml\ min^{-1}$	Fiset *et al.* (1993)
Terbutaline	T	(+): 0.148 ± 0.024	(−): 0.112 ± 0.015	$l\ h^{-1}\ kg^{-1}$	Borgström *et al.* (1989)
Tranylcypromine	T	(+): 24.9 ± 17.9	(−): 15.3 ± 9.0	$l\ min^{-1}$	Weber-Grandke *et al.* (1993)

T, renal clearance of total (bound plus free) drug; U, renal clearance of free (unbound) drug; S, secretion clearance.

In the case of drugs filtered by the glomerulus, stereoselectivity in plasma protein binding results in stereoselective renal elimination, since renal clearance is the product of glomerular filtration rate and the fraction unbound. For those drugs, however, where active renal tubular secretion and reabsorption contribute substantially to the renal clearance of the compound, and where a substantial fraction of the dose is excreted as parent drug, stereoselective renal transport will have an impact on the pharmacokinetics of the stereoisomers. Net stereoselective renal tubular secretion or reabsorption is a consequence of a stereoselective interaction with the anion or cation transport proteins in the basolateral or brush border membranes of renal tubular epithelial cells (Ott and Giacomini, 1993; Higaki *et al.*, 1994). Enantioselectivity has been observed when the chiral centre of the drug is adjacent to the site of interaction with the transport protein (Gross and Somogyi, 1994). As these transporters have a finite capacity, the enantiomers of racemic compounds can compete at these sites (Higaki *et al.*, 1992), potentially altering the disposition of each enantiomer when the racemate is given. For example, in the monkey the clearance of (*S*)-ofloxacin is reduced when (*R*)-ofloxacin is co-administered as the (*R*)-enantiomer inhibits the renal tubular secretion of (*S*)-ofloxacin (Okazaki *et al.*, 1992). Furthermore, co-administered drugs may preferentially inhibit the renal excretion of one enantiomer, resulting in stereoselective drug interactions. For example, in the rat the renal secretion of (−)-sultopride is inhibited by probenecid whereas that of (+)-sultopride is unaffected (Kamizono *et al.*, 1993).

The renal tubular secretion of chiral metabolites can also be stereoselective. For example, the higher renal clearances of (*S*)-verapamil and its metabolite (*S*)-norverapamil in comparison to the (*R*)-enantiomer are explained by their higher glomerular filtration rates due to their lower protein binding. However, the renal clearance of the verapamil metabolite D617 (which has *N*-methylamine structure) is higher for the (*S*)-configurated metabolite, although the protein binding of (*R*)- and (*S*)-D617 are the same. Since renal clearances of both enantiomers of D617 are greater than their glomerular filtration rates, renal tubular secretion occurs which, in addition, is stereoselective favouring the (*S*)-metabolite (Mikus *et al.*, 1990).

3.4 METABOLISM

Biotransformation by the drug-metabolizing enzymes exhibits the greatest degree of stereoselectivity of all the processes contributing to drug disposition. Numerous pathways of drug metabolism, including both phase I and II reactions, display stereoselectivity (Jenner and Testa, 1973; Trager and Jones, 1987).

3.4.1 Substrate and Product Stereoselectivity

Stereoselective metabolism may refer to either substrate or product stereoselectivity. In the case of substrate stereoselectivity the two enantiomers are metabolized either at different rates or by different routes leading to the preferential metabolism of one enantiomer. For most drugs a twofold to fivefold difference in net metabolic clearance between stereoisomers is observed. This modest degree of stereoselectivity is a consequence of the fact that more than one enzyme is in most cases responsible for the metabolism of each enantiomer and that the different pathways catalysed by different enzymes exhibit varying degrees of substrate stereoselectivity. For example, one pathway may exhibit stereoselectivity for one enantiomer, whereas another metabolic reaction may preferentially metabolize the other enantiomer or not display stereoselectivity. For example, the glucuronidation (Silber *et al.*, 1982) and ring oxidation of propranolol are stereoselective whereas side-chain oxidation is non-stereoselective (Walle *et al.*, 1984).

By contrast product stereoselectivity, which implies the differential formation of stereoisomeric metabolites from a single substrate containing one or several centres of prochirality, is often characterized by a high degree of stereoselectivity leading to the almost exclusive formation of only one of two possible enantiomeric metabolites (Trager and Jones, 1987; Eichelbaum, 1988a; Testa, 1988). This is attributable due to the metabolite being produced via one enzyme alone.

3.4.1.1 Different Rates and Different Routes. Differences in both the rates and routes of metabolism of the stereoisomers of chiral drugs have been observed. The different spatial orientation of the functional groups of the two enantiomers may lead to only one stereoisomer interacting with the active site of the enzyme, and therefore only one enantiomer will be metabolized along a given pathway. If one enantiomer has a more optimal orientation of functional groups, the rate of metabolism along that pathway to the enantiomers of the metabolite will be enantioselective. If the centre of chirality is remote from the functional groups interacting with the catalytic site of an enzyme, that metabolic pathway is not anticipated to be stereoselective (Ariëns, 1993).

Differences in the rate of metabolism of the enantiomers of chiral drugs have been observed for many chiral drugs both *in vivo* and *in vitro*. For example, the enantioselectivity of the metabolism of verapamil to norverapamil, D617, D703 and D702, has been compared *in vivo* in humans after oral administration and *in vitro* using human liver microsomes (Kroemer *et al.*, 1992). Different enantioselectivity is observed for each pathway of metabolism. The rank order of the metabolic clearance to each metabolite for both enantiomers *in vivo* can be predicted from the intrinsic clearance

measured *in vitro*. Differences in the enantioselectivity for each enantiomer are attributable to the fact that different enzymes catalyse the various pathways of verapamil metabolism: the formation of noverapamil is catalysed by CYP3A4 whereas the formation of D617 is catalysed by both CYP3A4 and CYP1A2 (Kroemer *et al.*, 1993). Both enzymes show a modest stereoselectivity for (*S*)-verapamil. The *O*-demethylated metabolites 703 and 702 are formed by enzymes of the 2C subfamily, e.g. 2C8, 2C9 and 2C18 (Busse *et al.*, 1995). Since it is this pathway which shows a high degree of stereoselectivity for the (*S*)-enantiomer, enzymes of the CYP2C subfamily are responsible for the preferential metabolism of (*S*)-verapamil.

For many chiral drugs the routes of metabolism of each enantiomer can differ. For example, the metabolism of the racemic anticonvulsant mephenytoin is enantioselective (Küpfer *et al.*, 1981). The (*S*)-enantiomer is metabolized by oxidation to 4-hydroxymephenytoin, a reaction catalysed by the polymorphic enzyme CYP2C19 (de Morais *et al.*, 1994; Goldstein *et al.*, 1994). (*R*)-mephenytoin is metabolized principally by *N*-dealkylation to phenylethylhydantoin. Oxidation of (*S*)-mephenytoin is far more rapid than *N*-demethylation of (*R*)-mephenytoin and thus large differences in the pharmacokinetics of (*R*)- and (*S*)-mephenytoin result (Wedlund *et al.*, 1985).

Both enantioselectivity and regioselectivity in the metabolism of the enantiomers of the chiral anticoagulant warfarin have been reported (Hermans and Thijssen, 1993). (*S*)-warfarin is metabolized principally via 6- and 7-hydroxylation which is catalysed by the enzyme CYP2C9 (Rettie *et al.*, 1992). (*R*)-warfarin is metabolized principally to reduced alcohol metabolites (Fig. 7). Smaller quantities of a number of hydroxylated metabolites of (*R*)-warfarin are recovered in urine; however, (*R*)-warfarin is not a substrate for CYP2C9 (Rettie *et al.*, 1992) and these metabolites are produced by different enzymes. CYP1A2 catalyses (*R*)-warfarin 6-hydroxylation and CYP3A4 catalyses (*R*)-warfarin 10-hydroxylation. As (*S*)-warfarin is responsible for the primary pharmacological effect of racemic warfarin, the anticoagulant activity of warfarin *in vivo* is modulated by the activity of CYP2C9, the enzyme catalysing (*S*)- and not (*R*)-warfarin metabolism.

The chirality of both the parent drug and metabolite is to be considered when stereoselective metabolism is discussed. Five scenarios are possible and are discussed below.

3.4.1.2 Achiral–Chiral. Metabolism of an achiral compound introduces a centre of chirality and a chiral metabolite is produced. As a consequence of the stereospecificity of biological synthesis, generally only one of the possible metabolite stereoisomers is produced *in vivo*. For example, the neuroleptic haloperidol is achiral. The major pathway of haloperidol metabolism is reduction of the ketone to a secondary alcohol, reduced haloperidol, which creates a chiral centre. This reaction is catalysed by

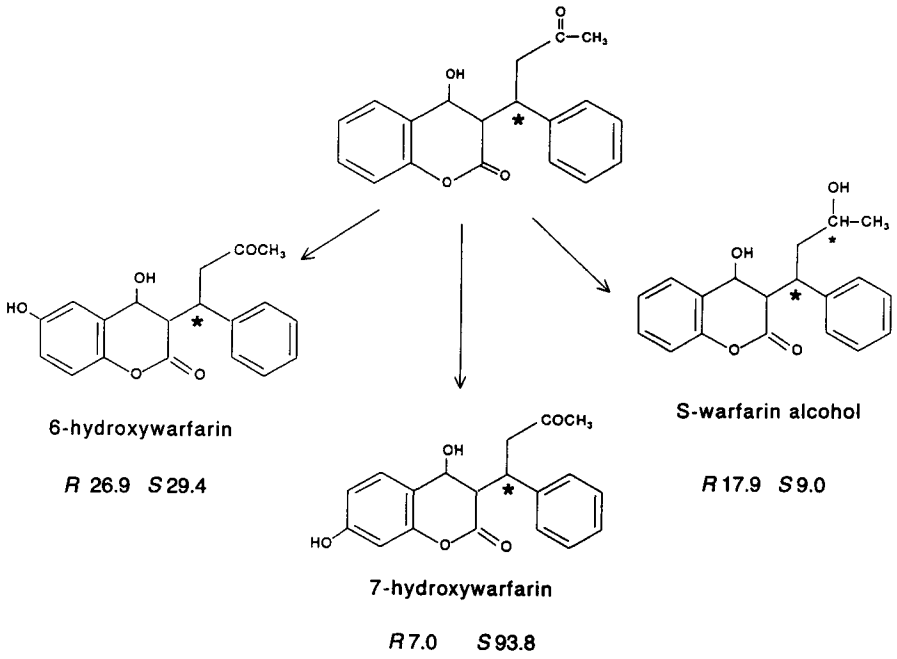

FIG. 7. Metabolic pathways of warfarin. The formation clearances (ml min^{-1}) from (*R*)- and (*S*)-warfarin for each metabolite in healthy volunteers are given (Niopas *et al.*, 1991). The formation of 7-hydroxywarfarin is the major pathway of metabolism for (*S*)-warfarin, whereas reduction to the diastereoisomeric alcohol is the major pathway of metabolism for (*R*)-warfarin. Clearance to 6-hydroxywarfarin is the same for both enantiomers.

cytosolic ketone reductases. In human blood, liver and brain the reduction of haloperidol is stereospecific, producing only the (*S*)-enantiomer (Eyles and Pond, 1992).

Another example is the 4-hydroxylation of the antihypertensive debrisoquine which is catalysed by cytochrome P450 2D6. This enzyme exhibits a genetic polymorphism (Mahgoub *et al.*, 1977; Eichelbaum *et al.*, 1979) and in extensive metabolizers more than 99% of the 4-hydroxydebrisoquine metabolite has (*S*)-configuration. In contrast, poor metabolizers of debrisoquine not only have a severely impaired capacity to form 4-hydroxydebrisoquine, but also show less stereoselectivity, with 5–36% of the 4-hydroxydebrisoquine possessing (*R*)-configuration (Eichelbaum *et al.*, 1988).

3.4.1.3 Chiral–Chiral. Biotransformation of the parent chiral drug does not occur at the chiral atom and the metabolite formed retains the chiral centre. If the order of priority of the functional groups remains unaltered,

the absolute configuration of the parent drug and metabolite will be the same. For example, (*S*)-chloroquine is metabolized to (*S*)-de-ethylchloroquine, and (*R*)-chloroquine is metabolized to (*R*)-de-ethyl-chloroquine (Augustijns and Verbeke, 1993). If the chiral drug is administered as a racemic mixture (enantiomeric ratio = 1) the rate of biotransformation to each enantiomer of the chiral metabolite can differ and the enantiomeric ratio of the chiral metabolite *in vivo* will not be unity.

Retention of the stereochemistry of the parent compound by the metabolite can be used to identify the stereochemical form of the parent compound ingested. For example, in the USA, workplace urine drug screening to detect abuse of amphetamine and related compounds is common. Methamphetamine is a chiral sympathomimetic and both enantiomers are available as stereochemically pure medicinals: (*S*)-methamphetamine on prescription for narcolepsy and (*R*)-methamphetamine in an over-the-counter cold remedy. Illicit synthesis of methamphetamine for abuse produces the racemate. Both enantiomers are metabolized to amphetamine, with retention of absolute configuration; however, the rate of metabolism of (*S*)-methamphetamine is far more rapid than that of (*R*)-methamphetamine, and after ingestion of the racemate late urine samples contain only (*R*)-methamphetamine. Enantiospecific analysis of the chiral metabolite amphetamine is therefore critical to establish whether any methamphetamine detected in urine samples has been used legitimately, as indicated by a single enantiomer of amphetamine, or illicitly, as indicated by identifying a substantial proportion of both the enantiomers of amphetamine (Cody and Schwarzhoff, 1993; Cooke, 1994).

3.4.1.4 Chiral–Diastereoisomer. Both phase I and phase II metabolism can introduce a second chiral centre into a chiral drug molecule. The metabolite will therefore be a diastereoisomer and two stereoisomers can be produced from each enantiomer. For example, conjugation of drugs possessing a single chiral centre with β-D-glucuronic acid or L-glutathione produces diastereoisomers (Trager and Jones, 1987), and the oxidation of the β-adrenergic antagonists such as bufuralol (Dayer *et al.*, 1986) and metoprolol (Murthy *et al.*, 1990) also produces diastereoisomers. The stereochemistry of the α-hydroxy metabolite of metoprolol has been investigated in human liver microsomes *in vitro* and following administration of pseudoracemic metoprolol *in vivo* (Murthy *et al.*, 1990). All four possible diastereoisomers of α-hydroxymetoprolol were identified. Formation of the diastereoisomers was stereoselective: 75% was of the (1′*R*)-configuration and 25% of the (1′*S*)-configuration. This stereoselectivity was comparable for the α-hydroxymetabolite produced from both (*R*)- and (*S*)-metoprolol. Therefore, the propanolamine side-chain of metoprolol, containing the chiral carbon atom, is remote from the catalytic site for α-hydroxylation and substrate stereoselectivity does not occur.

3.4.1.5 Chiral–Achiral. Metabolism can result in loss of the chiral centre. Such a chiral–achiral transformation occurs in the case of all chiral calcium antagonists with dihydropyridine structure where oxidation of the dihydropyridine ring to the corresponding pyridine analogue leads to the loss of chirality. The high degree of stereoselectivity in first-pass metabolism observed, for instance, for nitrendipine is not due to the stereoselective formation of this pyridine metabolite. It is the cleavage of the ester bonds of the substituents at the dihydropyridine ring which is responsible for the stereoselective metabolism (Soons and Breimer, 1991; Mast *et al.*, 1992; Soons *et al.*, 1993).

3.4.2 Chiral Inversion

The non-steroidal anti-inflammatory 2-arylpropionic acid derivatives are a group of drugs for which metabolism of one enantiomer to its optical antipode, a phenomenon termed chiral inversion, has been demonstrated. For some NSAIDs chiral inversion of the less potent (*R*)-enantiomers to the potent (*S*)-enantiomers explains why the activity of the (*R*)- and (*S*)-enantiomers *in vivo* can appear to be comparable. With the exception of naproxen, which is administered as the (*S*)-enantiomer, all 2-arylpropionic acids are used as racemates (Caldwell *et al.*, 1988; Evans, 1992). *In vitro* the (*S*)-enantiomers of these drugs are between 10 and 800 times more potent in inhibiting prostaglandin synthesis than the (*R*)-enantiomers. However, this degree of stereoselectivity in drug action is not always observed *in vivo* (Williams, 1990). For example, (*S*)-ibuprofen is 160 times more potent than (*R*)-ibuprofen *in vitro*, but only 1.4 times more potent *in vivo*. This decrease in relative potency *in vivo* is due to the unique unidirectional metabolic inversion of the chiral centre from the inactive (*R*)-enantiomer to the active (*S*)-enantiomer. This chiral inversion has been demonstrated to occur in humans for ibuprofen (Kaiser *et al.*, 1976; Lee *et al.*, 1985), benoxaprofen (Bopp *et al.*, 1979; Simmonds *et al.*, 1980), cicloprofen (Hutt and Caldwell, 1983), fenoprofen (Rubin *et al.*, 1985) and thioxaprofen (Diekmann *et al.*, 1979) but is not a characteristic of all 2-arylpropionic acid derivatives (Geisslinger *et al.*, 1994).

Chiral inversion appears to be a consequence of the formation of an acyl coenzyme A (CoA) thioester of the (*R*)-enantiomers (Knights *et al.*, 1988). A proportion of acyl CoA–(*R*)-ibuprofen is racemized by a CoA racemase to an enolate intermediate which is then hydrolysed by CoA hydrolase, thereby liberating the (*S*)-enantiomer. Since the (*S*)-enantiomer is not a substrate for CoA, the acyl-CoA thioester of this enantiomer is not formed and the chiral inversion is unidirectional from the (*R*)- to the (*S*)-enantiomer (Hall and Xiaotao, 1994). Experiments with deuterated (*R*)-ibuprofen

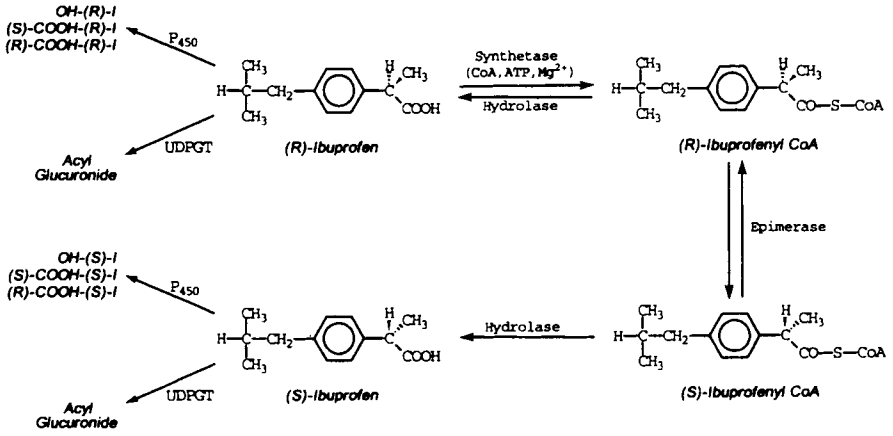

FIG. 8. Schematic representation of ibuprofen metabolism by inversion and non-inversion pathways; OH-I, 2-[4-(2-hydroxy-2-methylpropyl)phenyl] propionic acid; COOH-I, 2-[4-(2-carboxypropyl)phenyl] propionic acid; UDPGT, uridine diphosphate glucuronosyl-transferase. (Adapted from Hall and Xiaotao (1994), with permission of Elsevier Science Ireland.)

indicate that the proposed enolate intermediate will also revert to the parent (*R*)-enantiomer. The mechanism proposed for the chiral inversion is shown in Fig. 8 (Caldwell, 1992). The CoA thioesters formed during chiral inversion of the (*R*)-enantiomers can be incorporated into triglycerides and deposited in lipid stores. When incorporated into the phospholipids of cell membranes these hybrid triglycerides may perturb cell function (Williams, 1990). The toxicity of both the thioesters and the hybrid lipids formed remains to be determined (Hall and Xiaotao, 1994). The proportion of the dose of (*R*)-ibuprofen converted to the (*S*)-enantiomer in humans *in vivo* has been reported to vary from 28% to 90%, depending on the calculation method used (Geisslinger *et al.*, 1993a). The degree of inversion appears to be independent of dose (Evans, 1992).

3.4.3 Enantiomer–Enantiomer Interaction

If the enantiomers of chiral drugs are metabolized by or bind to the same enzymes, the disposition of the enantiomers when administered separately may differ to that observed when the racemate is given. As a consequence of such a metabolic interaction the pharmacological profile for the enantiomers and the racemate differs. There are three possible scenarios for metabolic interactions between the enantiomers of chiral drugs.

(1) Both enantiomers are metabolized by the same enzyme at different rates and they compete for metabolism at the catalytic site. Mutual competitive inhibition occurs and the disposition of both enantiomers is altered when the racemate is taken.
(2) The metabolism of both enantiomers is catalysed by the same enzyme; however, only one enantiomer is a competitive inhibitor. In this case, when the racemate is used the metabolism of only one enantiomer is impaired.
(3) Only one enantiomer is a substrate for the enzyme; however, the optical antipode can inhibit this enzyme and thus the metabolism of the enantiomer.

Such metabolic enantiomer–enantiomer interactions have been observed in humans *in vivo* for propafenone (Kroemer *et al.*, 1994a), nitrendipine (Mast *et al.*, 1992) and propranolol (Lindner *et al.*, 1989). Enantiomer–enantiomer interactions have also been observed in animal models or *in vitro* for amphetamine (Gal *et al.*, 1976), propoxyphene (Murphy *et al.*, 1976), propranolol (Masubuchi *et al.*, 1993) and warfarin (Kunze *et al.*, 1991). These studies demonstrate that the disposition and potency of enantiomers will be different depending on whether the enantiomers or the racemate are administered. This has been shown for the class I antiarrhythmic propafenone which is used as racemate. (*R*)- and (*S*)-propafenone are equally potent sodium channel blockers; however, only the (*S*)-enantiomer has modest β-adrenoceptor blocking properties (Kroemer *et al.*, 1989). The concentration of (*S*)-propafenone associated with β-blockade is $>1000\,\text{ng ml}^{-1}$. Following oral administration of standard doses of (*S*)-propafenone, these levels are not reached in the majority of subjects and hence no β-adrenoceptor blockade is expected. However, in the clinical setting where the racemate is administered, β-blockade is observed quite frequently. This clinical observation can be explained by an interaction between the enantiomers of propafenone which occurs when the racemate is administered (Kroemer *et al.*, 1994a).

Propafenone is eliminated mainly as 5-hydroxypropafenone (Kroemer *et al.*, 1989). Experiments with human liver microsomes *in vitro* have demonstrated that (*R*)-propafenone is a competitive inhibitor of the cytochrome P450 2D6 catalysed 5-hydroxylation of (*S*)-propafenone (Kroemer *et al.*, 1991). In agreement with these *in vitro* data are studies in healthy volunteers where the pharmacokinetics, β-blockade and electrophysiological effects during multiple dosing (4×150 mg) of (*R*)-, (*S*)- and racemic propafenone were studied. The propafenone plasma concentrations measured are shown in Fig. 9. The pharmacokinetic parameters of (*R*)-propafenone are similar after administration of the enantiomer or the racemate, considering the different doses of the enantiomer administered. However, when the racemate is given, the clearance of (*S*)-propafenone is

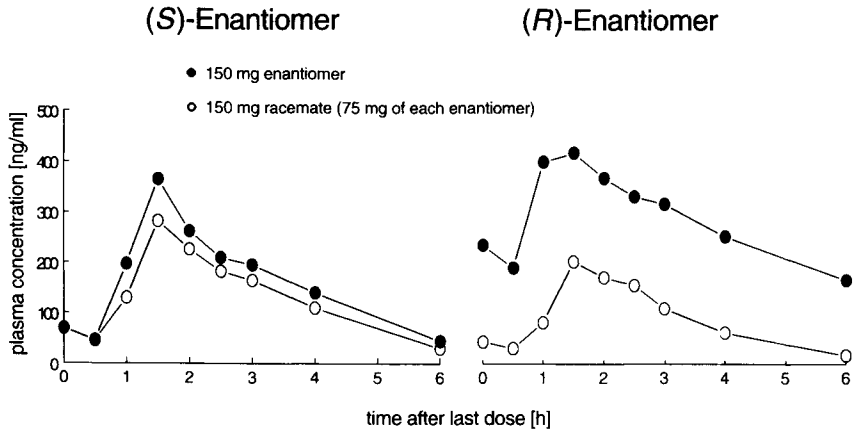

FIG. 9. Steady-state concentration *versus* time profile of (*S*)-propafenone (left) and (*R*)-propafenone (right) in one volunteer after administration of the enantiomers (150 mg (*S*)- or (*R*)-propafenone every 6 h for 4 days) on two different occasions or the racemate (150 mg (*R*)/(*S*)-propafenone every 6 h for 4 days). (Adapted from Kroemer *et al.* (1994a), with permission of the authors and the American Heart Association.)

reduced substantially and plasma levels of (*S*)-propafenone are similar to those observed after double the dose is given as the separate enantiomer. The degree of interaction can be appreciated if the *S*/*R* ratio of the mean apparent oral clearances of (*S*)- and (*R*)-propafenone are considered. When the enantiomers are administered this ratio is 1.97, which agrees well with that previously reported in healthy volunteers (2.4) (Brode *et al.*, 1988). The *S*/*R* ratio is reduced threefold when the racemate is administered (*S*/*R* = 0.63), which agrees with a previous study in patients (*S*/*R* 0.58) (Kroemer *et al.*, 1989).

The QRS interval prolongation was similar after (*R*)-, (*S*)- and racemic propafenone, consistent with previous reports that the (*R*)- and (*S*)-enantiomer are equipotent with respect to the sodium channel blocking effect of propafenone. In contrast, β-blockade assessed as a decrease in exercise-induced change in heart rate occurred only after (*S*)-propafenone and the racemate. Although only 50% of (*S*)-propafenone was administered when the racemate was given compared with (*S*)-enantiomer administration, β-blockade was the same. Therefore (*R*)-propafenone inhibits the metabolism of (*S*)-propafenone, which is eliminated principally via 5-hydroxylation, whereas the (*S*)-enantiomer does not impair the metabolism of (*R*)-propafenone. Thus the β-blockade observed in patients receiving racemic propafenone is due to the increased (*S*)-propafenone levels resulting from a metabolic enantiomer–enantiomer interaction *in vivo* when the racemate is given (Kroemer *et al.*, 1994a).

3.4.4 Consequences of Stereoselective Metabolism for Drug Action

3.4.4.1 In vivo *and* in vitro *Potency*. The consequences of stereoselective drug metabolism for the *in vivo* compared with the *in vitro* activity of racemic drugs depend on whether differences in potency and toxicity exist between the enantiomers. If the two enantiomers of a racemic drug have the same potency and toxicity then it is of no consequence if one stereoisomer is preferentially metabolized. However, if the stereoisomers differ in potency, stereoselective metabolism leads to a greater or smaller potency *in vivo* depending on whether the eutomer or distomer is preferentially metabolized. The pharmacodynamic profiles of the enantiomers *in vivo* may consequently contrast with those measured *in vitro* using equal amounts of the enantiomers. For example, metabolism of the oral anticoagulant acenocoumarol displays the greatest degree of stereoselectivity in total clearance so far reported for coumarin anticoagulants (Thijssen *et al.*, 1986; Hermans and Thijssen, 1993). The metabolic clearance of (*S*)-acenocoumarol *in vivo* is 14 times greater than that of (*R*)-acenocoumarol. As a consequence of the extremely rapid elimination of the (*S*)-enantiomer, which *in vitro* is three to five times more potent than the (*R*)-enantiomer, far higher concentrations of the (*R*)-enantiomer are observed *in vivo*. Therefore, based on equimolar doses, the (*R*)-enantiomer is ten times more potent than the (*S*)-enantiomer in healthy volunteers.

3.4.4.2 First-Pass Metabolism. Drugs with a low oral bioavailability due to extensive presystemic first-pass metabolism merit discussion. For these drugs, the total drug concentration required to elicit a certain pharmacological effect may differ depending on the route of administration. Pronounced amplification or attenuation of the response can be observed after oral compared with intravenous administration, depending on whether the distomer or eutomer is preferentially metabolized.

Stereoselective first-pass metabolism has been clearly demonstrated for a number of drugs including calcium channel blockers and β-adrenoceptor antagonists. The negative dromotropic effect of the (*S*)-enantiomer of verapamil on AV-node conduction is at least an order of magnitude greater than that of (*R*)-verapamil (Table 1) (Echizen *et al.*, 1985a, 1988). A much higher dose of verapamil (80–160 mg) is required after oral than after intravenous administration (5–10 mg) to elicit a defined PR-interval prolongation, the pharmacological effect held responsible for therapeutic efficacy in the treatment of supraventricular tachyarrhythmias. This has been attributed to the low bioavailability of verapamil due to extensive first-pass metabolism. Since the bioavailability of verapamil is 20–40%, the effective oral dose should be 25–50 mg, but in practice doses exceeding those predicted are required to elicit an antiarrhythmic effect comparable to 5–10 mg intravenously. Measurement of plasma concentrations, however, revealed that

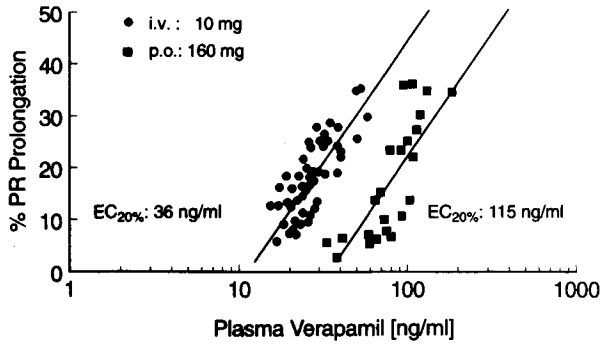

FIG. 10. Relationship between total verapamil plasma concentration and percentage PR-interval prolongation in the surface electrocardiogram following intravenous (•) and oral (p.o.) administration (■). Based on the same total plasma concentration, verapamil appears to be at least three times more potent after intravenous (i.v.) administration. $EC_{20\%}$ is the verapamil concentration which elicits a 20% prolongation of PR interval. (Adapted from Eichelbaum (1993), with permission of the author and Springer, Berlin.)

three times higher concentrations of verapamil are required after oral than after intravenous administration to elicit comparable effects on AV-node conduction (Fig. 10). For example, in healthy volunteers the EC_{50}, or concentration eliciting a 50% prolongation of PR-interval, after intravenous administration of 10 mg is 40 ng ml^{-1} and after oral administration of 160 mg the EC_{50} is 136 ng ml^{-1} (Eichelbaum *et al.*, 1980; Echizen *et al.*, 1985b). This observation at first appears to contradict the principle that, irrespective of the route of administration, the same drug concentration should elicit the same response.

To explain this paradox it was postulated that following oral administration stereoselective first-pass metabolism affecting preferentially the more potent (*S*)-enantiomer occurs. As a consequence of stereoselective presystemic metabolism at the same total verapamil plasma concentration less (*S*)-verapamil is present after oral than after intravenous administration (Eichelbaum *et al.*, 1980). Indeed, whereas after intravenous administration plasma concentrations of the less active (*R*)-enantiomer were two times higher than those of the more active (*S*)-enantiomer (*R*/*S* ratio 2), following oral administration the plasma concentration of the less active (*R*)-enantiomer was five times greater than that of the more active (*S*)-enantiomer (*R*/*S* ratio 5). This is due to enantioselective first-pass metabolism, where preferential metabolism of the (*S*)-enantiomer leads to lower bioavailability of this enantiomer (bioavailability of (*R*)-verapamil 50%, bioavailability of (*S*)-verapamil 20%). Therefore, after oral dosing the active (*S*)-enantiomer accounts for a smaller proportion of the total verapamil plasma concentration

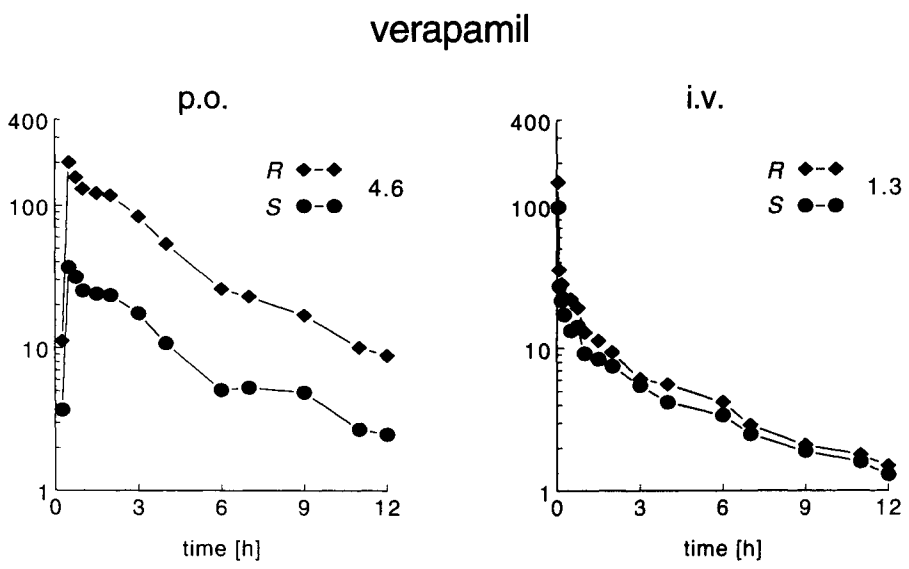

FIG. 11. Plasma concentration–time curve of (*R*)-verapamil (◆) and (*S*)-verapamil (●) following intravenous (i.v.) administration of 10 mg and oral (p.o.) administration of 160 mg racemic verapamil in a healthy volunteer. The *R/S* ratio after intravenous administration is 1.3. After oral administration pronounced differences are observed. Due to stereoselective first-pass metabolism the plasma concentration of the more potent (*S*)-enantiomer is substantially lower than that of the (*R*)-enantiomer and the *R/S* ratio is 4.6. (Adapted from Kroemer *et al.* (1994b), with permission of the authors and John Wiley, Chichester.)

than after intravenous administration (Vogelgesang *et al.*, 1984) (Fig. 11). Substantially higher total plasma concentrations of verapamil are therefore required after oral therapy than after intravenous administration to attain comparable concentrations of the active (*S*)-enantiomer and produce the pharmacological effect desired. If one relates the PR-interval prolongation to the plasma concentration of (*S*)-verapamil, which is the enantiomer mainly responsible for antiarrhythmic efficacy, then there is no longer a difference in verapamil potency between oral and intravenous administration (Echizen *et al.*, 1985b).

The reverse situation, however, is encountered in the case of the β-adrenoceptor antagonist propranolol. Based on total propranolol plasma levels, the concentration–effect curve is shifted to the left following oral compared with intravenous administration (Fig. 12) (Coltart and Shand, 1970). Based on total drug concentrations a greater degree of β-blockade is achieved after oral administration. This has been attributed to higher plasma concentrations of the active metabolite 4-hydroxypropranolol after

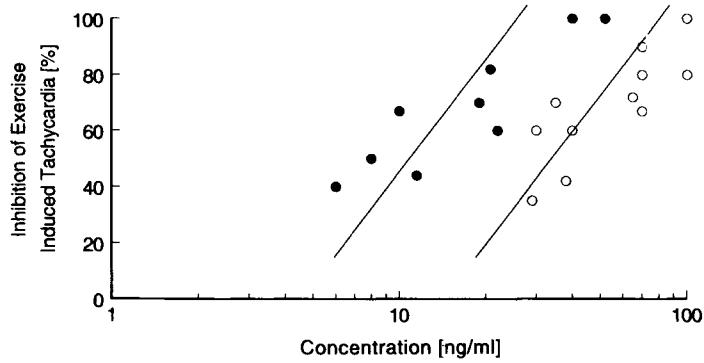

FIG. 12. Relationship between total propranolol plasma concentration and percentage inhibition of exercise-induced tachycardia after intravenous (○) and oral (●) administration. Based on the same total plasma concentration, propranolol appears to be at least twice as potent after oral administration. (Adapted from Coltart and Shand (1970), with permission of the BMJ Publishing Group, London.)

oral compared with intravenous administration. In view of recent studies, however (Silber and Riegelman, 1980; von Bahr *et al.*, 1982; Olanoff *et al.*, 1984; Lindner *et al.*, 1989), it is more likely that the shift to the left of the concentration–effect curve is a consequence of stereoselective first-pass metabolism. The bioavailability and hence plasma concentration of (*S*)-propranolol, which is the enantiomer responsible for β-blockade, are on average 1.5 times (range 0.9–2.8) higher than those of the (*R*)-enantiomer (Lindner *et al.*, 1989). In support of this contention are observations that the 4-hydroxylation of propranolol co-segregates with that of debrisoquine. Poor metabolizers of debrisoquine and sparteine do not form significant amounts of 4-hydroxypropranolol. If 4-hydroxypropranolol contributes to β-blockade, then at comparable doses a greater effect should be observed in extensive metabolizers relative to poor metabolizers. However, at equivalent concentrations of propranolol, comparable β-adrenoceptor blockade is observed in poor and extensive metabolizers (Lennard *et al.*, 1984; Raghuram *et al.*, 1984), demonstrating that this metabolite does not contribute to the β-adrenoreceptor antagonistic effect of the drug. Thus the enhanced response is due to relatively higher concentrations of the more potent (*S*)-enantiomer at the same total plasma concentration in plasma after oral compared with intravenous administration. Further examples of stereoselective first-pass metabolism of chiral drugs are listed in Table 6.

3.4.4.3 Impact of Diseases. There are almost no data showing whether diseases can alter the stereoselectivity of drug action and disposition. In view of the central role of the liver in drug metabolism, the most pronounced

TABLE 6

Further examples of stereoselective first-pass metabolism in humans

Drug	Bioavailability		Reference
Carvedilol	*R*: 0.32 ± 0.12	*S*: 0.18 ± 0.07	Fujimaki *et al.* (1990)
Threo-methylphenidate	(+): 0.22 ± 0.08	(−): 0.05 ± 0.03	Srinivas *et al.* (1991)
Nicoumalone	*R*: 0.97	*S*: 0.71	Gill *et al.* (1988)
Nimodipine	*R*: 0.22	*S*: 0.035	Sporkmann and Eichelbaum (1991)
Nitrendipine	*R*: 0.08 ± 0.04	*S*: 0.13 ± 0.06	Soons and Breimer (1991)
	R: 0.21 ± 0.11	*S*: 0.47 ± 0.24	Mast *et al.* (1992)
Terbutaline	(+): 0.08	(−): 0.15	Borgström *et al.* (1989)

Stereoselectivity has also been reported for acenocoumarol (Thijssen *et al.*, 1986), acebutolol (Piquette-Miller *et al.*, 1991), bicalutamide (McKillop *et al.*, 1993), penbutolol (Ochs *et al.*, 1986) and prilocaine (Tucker *et al.*, 1990).

consequences can be expected in liver cirrhosis. In cirrhosis there are several significant changes in liver function.

(1) Reduction in the functional liver cell mass. The capacity of the liver to metabolize drugs is therefore reduced. Drug extraction ratios are decreased and bioavailabilities are increased in proportion to the fall in extraction ratio.
(2) Decrease in liver blood flow which results in reduced clearance of highly cleared drugs.
(3) Portal hypertension leads to intrahepatic and extrahepatic shunting of the portal blood supply and, as a consequence, a significant proportion of the oral dose absorbed will bypass the liver, thereby escaping first-pass metabolism. This part of the dose will be completely bioavailable.

In patients with cirrhosis administered a racemic drug, the fraction of the dose delivered to the liver will undergo first-pass metabolism and the stereoselectivity of this process will be maintained. The fraction of the dose bypassing the liver will be completely bioavailable and not subject to stereoselective first-pass metabolism (Fig. 13). As a consequence there will be a loss of stereoselective metabolism. In the case of low-clearance drugs the decrease in functional liver cell mass, and thus decrease in metabolism, does not alter stereoselective disposition. For high-clearance drugs with low oral bioavailability, the situation is somewhat different and a potentiated therapeutic effect may be anticipated. If first-pass metabolism of the eutomer is significant in patients with normal liver function, an amplification of the drug effect may be observed in those with cirrhosis, far in excess of that predicted on the basis of total concentrations of the drug (Fig. 13).

For example, the calcium antagonist nimodipine is used as a racemate in

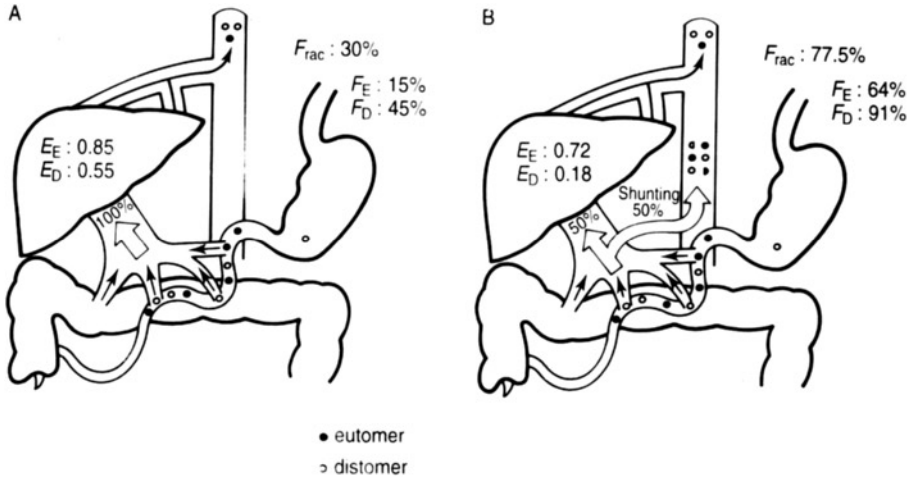

FIG. 13. Stereoselective first-pass metabolism. E_E/E_D, extraction ratio of the eutomer/distomer; F, bioavailability: $F = 1 - E$. (A) Normal liver function. The eutomer is preferentially metabolized. (B) Liver cirrhosis. Only 50% of the portal blood passes through the liver; 50% of the portal blood flow is shunted past the liver and this drug is totally bioavailable. Compared with individuals with healthy livers, the bioavailability of the racemate is increased by a factor of 2.5, whereas that of the eutomer is increased by a factor of 4.3. (Adapted from Eichelbaum (1993), with permission of the author and Springer, Berlin.)

the treatment of subarachnoid haemorrhage and stroke (Langley and Sorkin, 1989). With respect to its cardiovascular properties the (*S*)-enantiomer is at least ten times more potent than the (*R*)-enantiomer. Following oral administration in healthy volunteers the bioavailability of the drug based on total plasma concentrations is 10–15% (Sporkmann and Eichelbaum, 1991). However, due to stereoselective presystemic metabolism, pronounced differences in the bioavailability of the enantiomers occur, being 3–4% for (*S*)-nimodipine and 20–25% for (*R*)-nimodipine. In patients with liver cirrhosis, presystemic metabolism of nimodipine enantiomers is affected quite differently. The diminished first-pass metabolism in patients with cirrhosis leads to a threefold to fourfold increase in bioavailability of the racemate, to 50–60%. A quite different picture emerges, however, when total plasma concentrations are resolved for (*S*)- and (*R*)-nimodipine. The bioavailability of the (*S*)-enantiomer shows a 17-fold increase to 40–45%, whereas the bioavailability of the (*R*)-enantiomer increases only threefold to fourfold, to 60–65% (Sporkmann, 1992). It is quite apparent that these aspects must be taken into account when recommending dose adjustments in patients with liver cirrhosis.

3.4.4.4 Drug Interactions. Stereoselective drug metabolism can have important consequences in the case of drug interactions if differences in potency exist between enantiomers and the biotransformation of the enantiomers is differently affected by the causative drug.

Inhibition: If the enantiomers of a chiral drug are metabolized by different enzymes, a co-administered drug may inhibit only the metabolism of the eutomer and accelerate or not affect the metabolism of the distomer. Therefore, despite an unchanged or even decreased total plasma concentration, an increase in activity can be observed. On the other hand, if the interacting drug affects only the metabolism of the distomer and not the eutomer, the resulting higher total concentration has no or only minor consequences for therapeutic efficacy. The coumarin anticoagulant warfarin provides an excellent example of this scenario (Serlin and Breckenridge, 1983; Park, 1988). Until the elegant studies by Lewis *et al.* (1974), O'Reilly *et al.* (1980a) and Banfield *et al.* (1983) unravelled the stereochemical basis of the interactions of warfarin with drugs such as phenylbutazone, several theories including displacement from plasma protein binding sites were proposed to explain the interactions observed. The interaction of sulphinpyrazone with warfarin illustrates the stereochemical basis of warfarin interactions.

During co-administration of warfarin and sulphinpyrazone, the hypoprothrombinaemic effect of warfarin is enhanced, although warfarin plasma concentrations remain unchanged or even decrease. Toon *et al.* (1986b) resolved this paradox by measuring the fate of the individual warfarin enantiomers. In all subjects it was found that sulphinpyrazone reduced the clearance of the more active (*S*)-enantiomer by inhibiting its metabolism. The effect of sulphinpyrazone on the disposition of the less active (*R*)-enantiomer differed. The metabolism of (*R*)-warfarin was not inhibited to the same degree as that of the (*S*)-enantiomer. In addition sulphinpyrazone stereoselectively displaced the (*R*)-enantiomer from plasma protein binding sites. As a consequence of the raised free fraction of the (*R*)-enantiomer, the clearance of (*R*)-warfarin was enhanced during sulphinpyrazone administration. Whether in an individual subject the total clearance of racemic warfarin was increased or decreased by the co-administration of sulphinpyrazone depended on the relative magnitude of the independent effects of sulphinpyrazone on the metabolism and protein binding of (*R*)- and (*S*)-warfarin. Only by monitoring the plasma concentrations of the active (*S*)-enantiomer could the increased pharmacodynamic response be explained (Fig. 14). By contrast, stereoselective interactions of warfarin which influence the (*R*)-enantiomer rather than (*S*)-warfarin have also been observed. For example, cimetidine and enoxacin increase racemic warfarin concentrations; however, this is of no therapeutic consequence because the interacting drug affects the disposition of only the (*R*)-enantiomer (Fig. 13). Little net change in the pharmacodynamic effect therefore occurs. The drug interactions with

each enantiomer of warfarin that have been reported are listed in Table 7.

The interaction of verapamil and cimetidine demonstrates inhibition of both stereoselective metabolism and renal elimination (Mikus *et al.*, 1990). Cimetidine inhibits the metabolism of verapamil leading to a 20% increase in the total area under the plasma concentration–time curve of verapamil. The negative dromotropic effect of verapamil on AV-node conduction is also enhanced, but to a greater extent than predicted from the increase in total drug concentration. Enantioselective analysis demonstrates that there is a 40–50% increase in the area under the curve of (*S*)-verapamil. As this enantiomer has a 20-fold greater negative dromotropic effect on AV-node conduction than (*R*)-verapamil (Table 1), the increased pharmacological effect is anticipated. The same study also observed that the renal elimination of the chiral verapamil metabolite D617 is inhibited in a stereoselective manner by the achiral compound cimetidine. Renal tubular secretion of (*R*)-D617 remained unchanged whereas that of (*S*)-D617 was diminished. These data suggest that cimetidine displacement of racemic drugs from the renal cation transporter can be stereoselective. Such an interaction could be of clinical relevance in the case of chiral drugs where renal secretion is a major route of elimination and differences in potency exist between enantiomers.

Induction: For racemic drugs where each enantiomer is metabolized by different enzymes, induction of specific P450 isoforms can result in changes in stereoselective metabolism. The interaction of hexobarbital and rifampicin clearly demonstrates stereoselective induction of metabolism (Smith *et al.*, 1991). In young healthy volunteers the oral clearance of (*S*)-hexobarbital is increased sixfold from 1.91 to 11.9 $ml\,min^{-1}\,kg^{-1}$ by rifampicin whereas the clearance of (*R*)-hexobarbital is increased 73-fold from 15.6 to

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FIG. 14. Stereoselective inhibition of warfarin metabolism and the consequences for its anticoagulant effect. CL_{met} is the clearance of warfarin via metabolism.(A) Without inhibitor. (*S*)-warfarin, which is approximately five times more potent than the (*R*)-enantiomer, is mainly responsible for the hypoprothrombinaemic effect as measured by the prothromin time (PTT). *S/R* (CL_{met}) ratio in plasma is only 0.5 compared with 1 in the drug preparation since the metabolic clearance of the (*S*)-enantiomer is two times greater than that of the (*R*)-enantiomer. (B) Inhibitor affects the metabolism only of the (*S*)-enantiomer and as a consequence the *S/R* ratio increases to 2. Although total drug concentration remains unchanged, the hypoprothrombinaemic effect is markedly increased due to doubling of the (*S*)-warfarin concentration. (C) Inhibitor affects only the (*R*)-enantiomer. Although the interaction causes a doubling of total warfarin plasma concentration, this interaction has no pharmacodynamic consequences because (*S*)-warfarin concentration remains unchanged.

1146.7 ml min^{-1} kg^{-1}. Clearly preferential induction of (*R*)-hexobarbital occurs, and when rifampicin is co-administered total plasma concentrations of hexobarbital are enriched in (*S*)-hexobarbital, the distomer.

The clinical consequences of stereoselective induction will depend on the relative activity of the enantiomers and on whether the metabolism of the

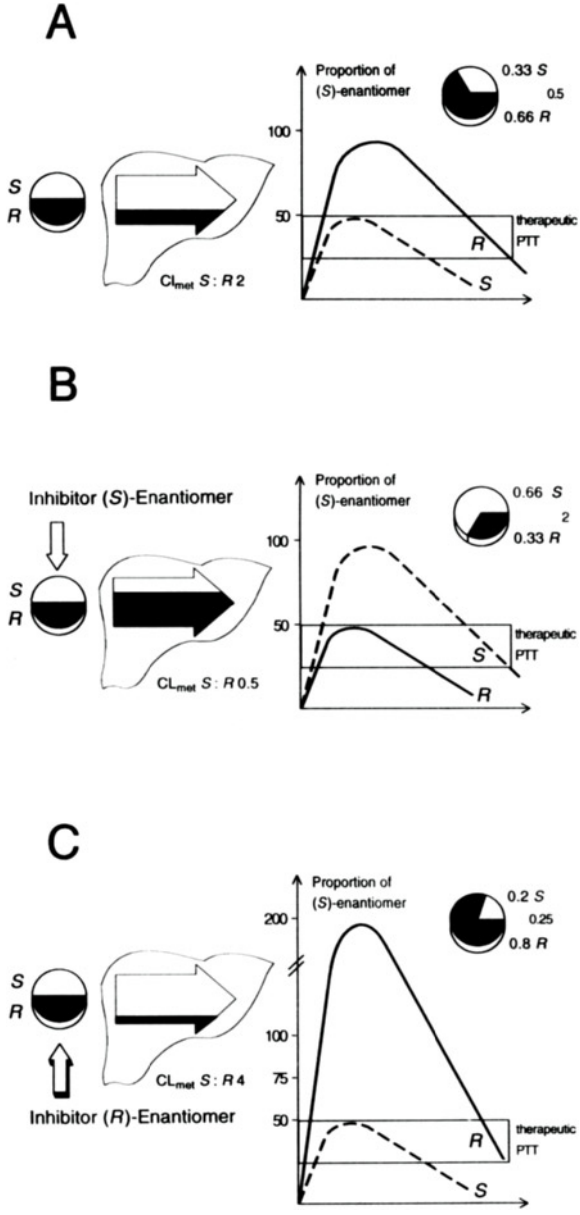

TABLE 7

Effect of co-administered drugs on the apparent oral clearances of (*R*)- and (*S*)-warfarin and the prolongation of prothrombin time

Interacting drug	Change in clearance		Change in effect	Reference
	R	*S*		
Amiodarone	↓	↓	↑	Heimark *et al.* (1992)
	↓	↓	↑	O'Reilly *et al.* (1987)
Cimetidine	↓	↔	↔	Toon *et al.* (1986b)
	↓	↔	↔	Choonara *et al.* (1986a)
	↓	↔	↔	Toon *et al.* (1987b)
Cotrimoxazole	↔	↓	↑	O'Reilly (1980)
Enoxacin	↓	↔	↔	Toon *et al.* (1987a)
Metronidazole	↔	↓	↑	O'Reilly (1976)
Miconazole	↓	↓	↑	O'Reilly *et al.* (1992)
Omeprazole	↓	↔	↔	Sutfin *et al.* (1989)
	↓	↔	↔	Unge *et al.* (1992)
Phenylbutazone	↑	↓	↑	Lewis *et al.* (1974)
	↑	↓	↑	O'Reilly *et al.* (1980a)
	↑	↓	—	Banfield *et al.* (1983)
Rifampicin	↑	↑	↓	Heimark *et al.* (1987)
Secobarbital	↑	↑	↓	O'Reilly *et al.* (1980b)
Sulphinpyrazone	↑	↓	↑	O'Reilly (1982a)
	↑	↓	↑	Toon *et al.* (1986b)
Ticrynafen	↔	↓	↑	O'Reilly (1982b)

Increases (↑), decreases (↓) or no significant change (↔) in clearance or effect are indicated. No change in the clearance of either (*R*)- or (*S*)-warfarin or prothrombin time has been reported with co-administered ketorolac (Toon *et al.*, 1990), ranitidine (Toon *et al.*, 1987b), roxithromycin (Paulsen *et al.*, 1988) and terodiline (Höglund *et al.*, 1989).

eutomer or distomer is preferentially induced. We have recently observed a patient receiving verapamil for the treatment of supraventricular tachycardia who was also prescribed the antiepileptic phenytoin. In this patient the *R*/*S* verapamil plasma concentration ratio was 10, twice that usually observed in patients and, despite a high verapamil dose of 720 mg, no antiarrhythmic effect was observed. It is known that phenytoin induces P450s of the 2C subfamily. Since the *O*-demethylation of verapamil is catalysed mainly by CYP2C9 (Busse *et al.*, 1995) and is preferential for (*S*)-verapamil (Mikus *et al.*, 1990), the data indicate that the lack of antiarrhythmic efficacy in this patient is due to selective induction of (*S*)-verapamil metabolism by phenytoin (unpublished results).

3.4.4.5 Genetics

Genetic polymorphisms in drug metabolism: Genetic polymorphisms in drug-metabolizing enzymes have been widely studied over the past 15 years. A number of chiral substrates of the polymorphic enzymes CYP2C19 (mephenytoin) and CYP2D6 (sparteine/debrisoquine) have been identified. For these compounds stereoselective metabolism, and thus disposition, differs in individuals possessing these enzymes (extensive metabolizers) and in poor metabolizers who lack these enzymes.

As discussed previously the enantiomers of the anticonvulsant mephenytoin are metabolized via different pathways. CYP2C19 catalyses (*S*)-mephenytoin 4-hydroxylation and is a polymorphic enzyme: 3% of Caucasians are poor metabolizers for (*S*)-mephenytoin due to a mutation in exon 5 which creates an aberrant splice site. As a consequence, a truncated non-functional protein is synthesized (de Morais *et al.*, 1994; Goldstein *et al.*, 1994). The metabolism of (*S*)-mephenytoin is impaired in poor metabolizers whereas the metabolism of (*R*)-mephenytoin is unaffected. The stereoselective disposition of mephenytoin thus differs in extensive and poor metabolizers, with a loss of stereoselective metabolism of the (*S*)-enantiomer in poor metabolizers. Large phenotype differences in the stereoselective disposition of nirvanol (Küpfer *et al.*, 1984), hexobarbital (Adedoyin *et al.*, 1994) and mephobarbital (Küpfer and Branch, 1985) have also been reported (Fig. 15).

The polymorphic enzyme CYP2D6 also displays differences in substrate and product stereoselectivity between extensive and poor metabolizers. Chiral CYP2D6 substrates include encainide (Turgeon *et al.*, 1991), flecainide (Gross *et al.*, 1989), mexiletine (Abolfathi *et al.*, 1993; Vandamme *et al.*, 1993), metoprolol (Lennard *et al.*, 1983), mianserin (Dahl *et al.*, 1994) and propafenone (Kroemer *et al.*, 1989). Impaired metabolism of one enantiomer in poor metabolizers who are deficient in CYP2D6 results in altered enantioselective pharmacokinetics or pharmacodynamics in poor and extensive metabolizers.

Ethnic differences: Ethnic differences in the activity of drug-metabolizing enzymes can result in differences in the metabolism of the stereoisomers of chiral drugs in individuals from different populations. For example, a series of elegant studies investigating the disposition of propranolol in Chinese relative to Caucasian subjects has been published by Zhou and colleagues. After single-dose oral administration, the clearance of both propranolol enantiomers is higher in Chinese than in Caucasian volunteers (Fig. 16) (Zhou and Wood, 1990). The enantiomeric ratio of propranolol clearance (*S*/*R*) is comparable in Chinese (1.5) and Caucasians (1.6). The metabolism of both enantiomers is therefore enhanced to the same degree and no ethnic difference in enantioselectivity is observed.

The proportion of poor metabolizers of sparteine/debrisoquine and (*S*)-mephenytoin varies in different ethnic groups (Kalow and Bertilsson,

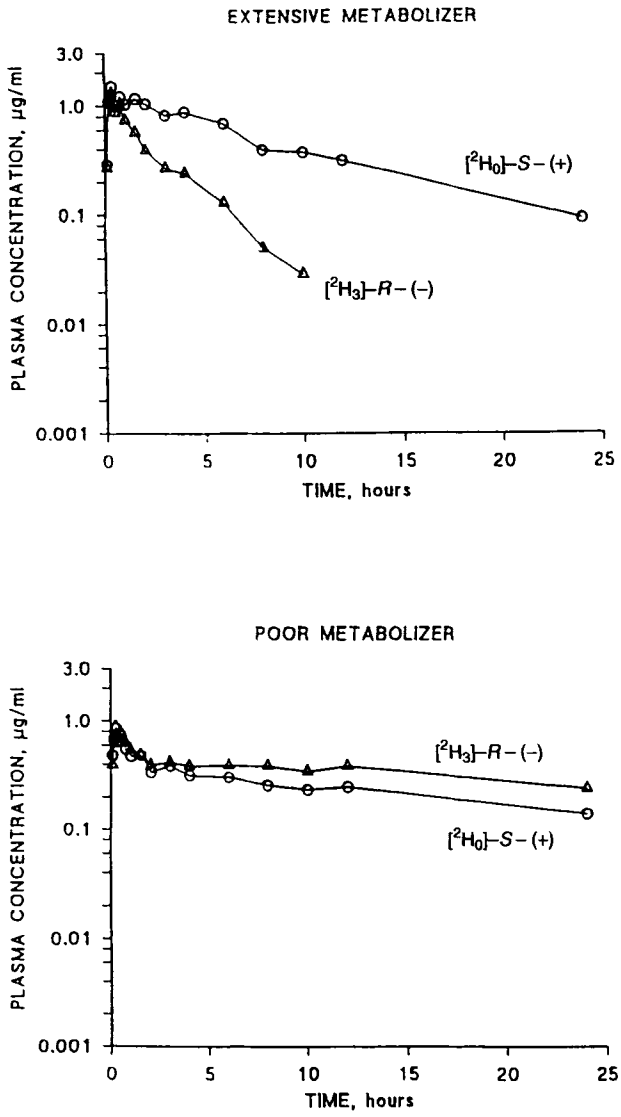

FIG. 15. Differences in enantioselective hexobarbital pharmacokinetics in extensive and poor metabolizers of mephenytoin. Plasma concentration–time profiles of the individual enantiomers of hexobarbital (△, (*R*)-hexobarbital; ○, (*S*)-hexobarbital) after oral administration of 200 mg pseudoracemic hexobarbital in an extensive metabolizer (upper panel) and a poor metabolizer (lower panel). (Adapted from Adedoyin *et al.* (1994), with permission of Chapman and Hall, London.)

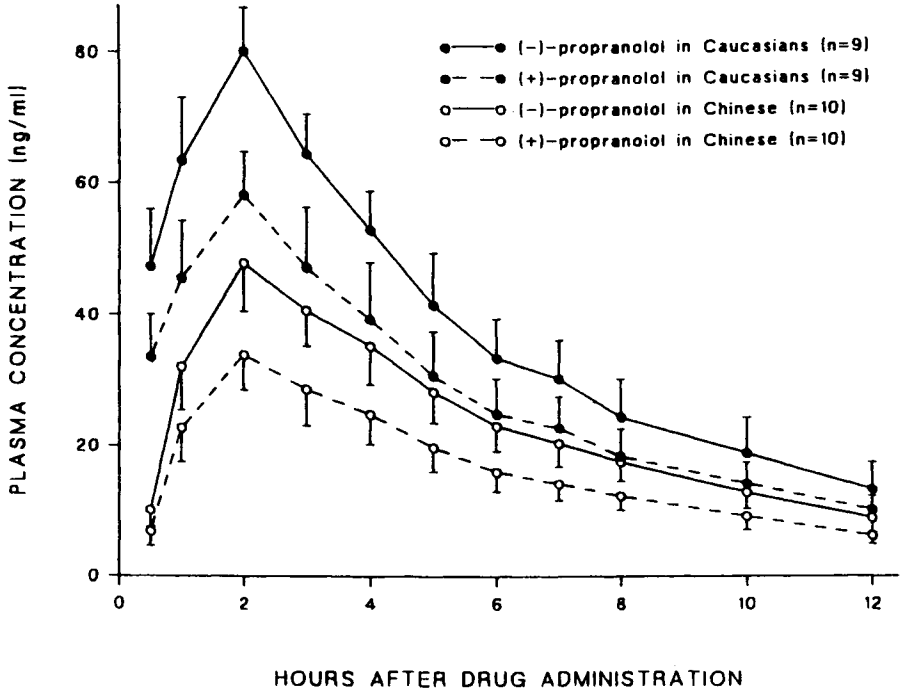

FIG. 16. Ethnic differences in the disposition of chiral drugs. Plasma concentrations of (*S*)- and (*R*)-propranolol in Caucasian ($n = 9$) and Chinese ($n = 10$) subjects after an oral dose of 80 mg propranolol. (Adapted from Zhou and Wood (1990), with permission of Mosby Year Book, St Louis, Missouri.)

1994). The distribution of altered stereoselective metabolism of chiral substrates of the polymorphic enzymes will therefore also vary, and may account for different average doses of some drugs in different populations. As ethnic differences in the disposition of many widely used drugs continue to be investigated (Kalow and Bertilsson, 1994), additional examples of altered disposition will no doubt be described.

3.4.4.6 Changes in Stereoselective Metabolism with Age. The activity of drug-metabolizing enzymes can diminish with age. If the enantiomers of a racemic drug are metabolized by different enzymes which are affected by ageing to a different degree, stereoselective metabolism and hence disposition can exhibit age dependency. This scenario was originally described for the chiral barbiturate hexobarbital (Chandler *et al.*, 1988). The oral clearance of (*R*)-hexobarbital was similar in young and elderly healthy volunteers, whereas the oral clearance of (*S*)-hexobarbital was twofold greater in the young than in the elderly. Although total clearance of hexobarbital was decreased in the

elderly, no change in the pharmacological response was observed since the clearance of the active (*R*)-hexobarbital was not altered.

For mephobarbital the change in stereoselective metabolism and hence disposition with age is sex specific (Hooper and Qing, 1990). The disposition of (*S*)-mephobarbital is similar in the young and elderly, men and women. The apparent total body clearance of (*R*)-mephobarbital is also similar in young and elderly women. By contrast (*R*)-mephobarbital clearance is greater in young men and diminishes with age to levels comparable to those of women. The stereoselective disposition of mephobarbital is therefore dependent on both age and sex. A decrease in the metabolism of both (*R*)- and (*S*)-verapamil also occurs with age and may be preferential for the (*S*)-enantiomer (Abernethy *et al.*, 1993; Sasaki *et al.*, 1993; Schwartz *et al.*, 1994). The disposition of the enantiomers of propranolol has also been compared in the young and the elderly. Overall, no change in enantioselectivity was observed; however, different studies report that clearance is independent of age (Lalonde *et al.*, 1990; Colangelo *et al.*, 1992; Zhou *et al.*, 1992) or diminished in the elderly (Gilmore *et al.*, 1992).

3.4.5 Stereoselective Biliary Excretion

Transport into bile and thus biliary elimination can also be stereoselective. For example, disopyramide is eliminated by metabolism to mono-*N*-dealkyldisopyramide which is excreted principally in the urine. Le Corre *et al.* (1992) administered single oral doses of (*R*)- and (*S*)-disopyramide to three patients undergoing cholecystectomy. Despite the fact that these compounds have relatively low molecular weights, stereoselective biliary recovery of both the parent drug and metabolite was observed, albeit accounting for only a small proportion of the dose administered. The authors suggested that, as the bile-to-plasma concentration ratio was high, transport into bile was an active process. Interestingly biliary excretion favoured (*R*)-disopyramide, whereas all other chiral interactions *in vivo*, including protein binding, renal excretion and hepatic metabolism, favour (*S*)-disopyramide.

4 Stereoselectivity and Toxicity

In view of the fact that drug toxicity, mutagenicity and teratogenicity can be mediated by reactive intermediates and that metabolic pathways are stereoselective leading to the preferential formation of one stereoisomer of the metabolites, it is quite likely that only one enantiomer may generate toxic chiral metabolites. If the side-effects and toxicity are related to a chiral reactive intermediate resulting from the less active enantiomer, then side-

effects and toxicity not related to the parent compound could be minimized by administering an enantiomerically pure compound. There is, however, relatively little information published concerning the relative toxicity of stereoisomers (Vermeulen, 1989; Vermeulen and te Koppele, 1993).

As the stereoselective aspects of drug disposition and action are being increasingly studied, additional examples of medicines where one enantiomer may make a disproportionate contribution to toxicity are being described. For example, the central nervous system toxicity of the anticancer drug ifosfamide, used as a racemate, may be related to stereoselective dechloroethylation (Wainer *et al.*, 1994). The stereoselective urinary recovery of the enantiomers of ifosfamide and its major metabolites was studied in seven patients, one of whom had severe neurotoxic symptoms: somnolence, dizziness, lethargy, tremors and hallucinations. The urinary recovery of the (*R*)-enantiomers of the two dechloroethylated metabolites in the patient experiencing toxicity was over twice that observed in the other patients. The (*R*)-dechloroethylated metabolites may thus be neurotoxic and enhanced stereoselective metabolism via this pathway may be a risk factor for this severe adverse effect.

5 Implications and Consequences

The examples discussed above clearly demonstrate that, in view of the pronounced differences in activity and disposition which exist between enantiomers, two drugs are in fact being administered when a racemic drug is prescribed. As a result of the knowledge gained over recent years one can ask whether the use of racemic drugs should be continued or whether in the future only pure stereoisomers should be used. For many racemic drugs an educated decision is difficult to make because data on the activity, toxicity and pharmacokinetics of the individual enantiomers are not complete. However, even when data have been published, frequently only the pharmacological effect held responsible for therapeutic efficacy has been evaluated.

The decision whether to use a single enantiomer or the racemate should be made on a case-by-case basis, and various scenarios can be envisaged concerning the relative merits of developing stereochemically pure drugs or continuing to use the racemate.

5.1 ENANTIOMERS HAVE THE SAME POTENCY

The (*R*)- and (*S*)-enantiomers of flecainide are equipotent in respect to antiarrhythmic activity (Banitt *et al.*, 1986) and their effect on cardiac sodium channels (Hill *et al.*, 1988; Lie-A-Huen *et al.*, 1989). In addition the

disposition of flecainide exhibits no pronounced stereoselectivity (Gross *et al.*, 1989). Therefore no data are available that indicate that one enantiomer is superior to the other with regard to potency, although information on the toxicity of the enantiomers is not complete. Thus on scientific grounds it would be impossible to select one enantiomer for development of an enantiomerically pure dosage form. Furthermore it may be questioned whether there would be any benefit from using a single enantiomer rather than the racemate. Since both enantiomers are effective, the argument that one enantiomer is isomeric ballast is not valid for flecainide.

5.2 ENANTIOMERS HAVE THE SAME ACTIVITY BUT DIFFER IN POTENCY

It has been proposed that the use of individual verapamil enantiomers instead of the racemate might offer certain advantages in special clinical settings (Powell *et al.*, 1988). (*S*)-verapamil is only 1.5–2 times more potent than (*R*)-verapamil in increasing coronary blood flow and decreasing coronary resistance. However, with regard to negative chronotropic, dromotropic and inotropic, as well as its blood-pressure-lowering, effects much greater differences in potency have been noted (Table 1). Based on these data it had been proposed that (*S*)-verapamil might be better suited for the treatment of supraventricular tachyarrhythmias whereas (*R*)-verapamil would be the better drug for the treatment of angina pectoris (Powell *et al.*, 1988). Since, with the exception of electrophysiological effects, the other activities of (*S*)- and (*R*)-verapamil have not been studied in humans, such recommendations are premature.

Whereas the cardiovascular effects of verapamil are stereoselective, pharmacological effects not mediated via calcium channel blockade are not enantioselective. Racemic verapamil has been shown both *in vitro* and *in vivo* to reverse P170 glycoprotein-mediated multidrug resistance (MDR) (Tsuruo *et al.*, 1981). Several clinical trials were performed using racemic verapamil in combination with chemotherapy in patients with cancer to reverse MDR. Initially the results of these trials were not encouraging. Although the addition of verapamil to conventional chemotherapy elicited partial response in some patients with previously refractory tumours, verapamil concentrations of 2–10 μmol l^{-1}, which are effective *in vitro*, could not be attained. The negative chronotropic, dromotropic and inotropic effects of (*S*)-verapamil precluded any further increase in dose and limited the total concentration of verapamil that could be attained (Miller *et al.*, 1991; Pennock *et al.*, 1991). (*R*)-verapamil is as effective as (*S*)- and racemic verapamil in reversing MDR in human tumour cell lines (Eichelbaum, 1988a; Gruber *et al.*, 1988; Plumb *et al.*, 1990; Häußermann *et al.*, 1991). Therefore, (*R*)-verapamil might be superior to racemic verapamil for reversing MDR. Because of its lesser cardiovascular effects, (*R*)-verapamil can be adminis-

tered at the much higher doses that are required to achieve effective *in vivo* concentrations. In a recently completed phase I clinical trial with (*R*)-verapamil, the lesser cardiotoxicity of the (*R*)-enantiomer enabled doses up to 2400 mg day^{-1} to be administered. At these doses (*R*)-verapamil plasma concentrations as high as 12 μmol l^{-1} were attained. Thus (*R*)-verapamil may be more appropriate than racemic verapamil for reversing MDR in patients (Schumacher *et al.*, 1992).

5.3 ENANTIOMERS HAVE THE SAME POTENCY BUT ONLY ONE ENANTIOMER HAS AN ADDITIONAL EFFECT WHICH CONTRIBUTES TO EFFICACY

As discussed previously, the (*S*)-enantiomer of propafenone has β-blocking effects whereas both enantiomers have similar effects on sodium channels. The use of an enantiomerically pure dosage form containing only (*R*)-propafenone may diminish the incidence of side-effects, especially in those patients at risk of β-adrenoceptor mediated side-effects (Stoschitzky *et al.*, 1990). Conversely therapy with the (*S*)-enantiomer may produce greater arrhythmia suppression in patients in whom a combined sodium channel and β-blocking effect is desirable (Kroemer *et al.*, 1989).

5.4 ENANTIOMERS DIFFER GREATLY IN POTENCY FOR THE MAJOR THERAPEUTIC EFFECT BUT HAVE SIMILAR POTENCY FOR DIFFERENT INDICATIONS

The β-adrenergic antagonists produce a large number of adverse effects which are seldom life threatening. Since the therapeutic effect as well as most of the adverse reactions to these compounds are due to their antagonism of noradrenaline at β-adrenoceptors, it is doubtful that removal of the less active isomer would improve the safety and reduce the incidence and severity of side-effects associated with the use of these β-adrenoceptor antagonists in the treatment of coronary heart disease and hypertension. For example, the profile and incidence of adverse drug reactions for timolol, which is used as a pure enantiomer, are similar to those of racemic β-adrenergic antagonists.

There is, however, an indication for which the use of the distomer of timolol could offer a therapeutic advantage. (*S*)-timolol is devoid of local anaesthetic activity and therefore, in order to lower intraocular pressure in the treatment of wide-angle glaucoma, timolol is superior to other classes of compounds and also other β-adrenergic antagonists used as racemates, such as propranolol. (*S*)-timolol applied topically to the eye not only lowers intraocular pressure but also elicits systemic β-blockade and therefore should not be used in patients for whom β-adrenergic antagonists are contraindicated (Leier *et al.*, 1986). However, negligence of this fact has caused a substantial number of severe cardiovascular, pulmonary and central nervous system

adverse drug reactions which, according to a survey of 450 case reports, has resulted in the death of at least 32 patients (Nelson *et al.*, 1986). Since (*R*)-timolol has one-quarter the activity of (*S*)-timolol with regard to lowering ocular pressure but only 1/50th to 1/90th the systemic β-blockade, (*R*)-timolol may be expected to be superior to (*S*)-timolol for the treatment of wide-angle glaucoma (Share *et al.*, 1984). Therefore, by using (*R*)- instead of (*S*)-timolol it may be postulated that serious cardiovascular, pulmonary and central nervous system side-effects could be avoided and the drug might even be able to be used in patients with wide-angle glaucoma and concomitant diseases which contraindicate the use of (*S*)-timolol (Keates and Stone, 1984). However, in patients with asthma the dose of (*R*)-timolol required to lower intraocular pressure to the same extent as (*S*)-timolol produces an increase in airway resistance (Richards and Tattersfield, 1987). Thus, in patients with asthma (*R*)-timolol may not be superior to (*S*)-timolol for the treatment of wide-angle glaucoma as had been predicted on the basis of data obtained in various animal models and healthy volunteers.

5.5 STEREOISOMERS DIFFER IN POTENCY, AND SIDE-EFFECTS ARE PREDOMINANTLY ASSOCIATED WITH THE DISTOMER

There are several interesting examples where one enantiomer contributes little to therapeutic efficacy but is mainly responsible for undesirable side-effects. Baclofen, a chiral synthetic derivative of γ-aminobutyric acid, is a skeletal muscle relaxant used to relieve spasticity and is also effective in preventing the painful paroxysms of trigeminal neuralgia. Baclofen is administered as the racemate and its pharmacological effects are stereoselective. (*R*)-baclofen is up to 20 times more potent at spinal reflexes and five times more potent as an antinociceptive agent than (*S*)-baclofen. In a double-blind cross-over study in patients with trigeminal neuralgia, Fromm and Terrence (1987) administered equianalgesic doses of (*R*)-baclofen (2 mg) and racemic baclofen (10 mg). The lower dose of (*R*)-baclofen was more effective than the racemate and, in addition, the racemate produced substantially more sedation, confusion, tremors, nausea and vomiting than (*R*)-baclofen. The efficacy of the active (*R*)-enantiomer was thus diminished when the racemate was given. These *in vivo* data concur with the results of animal and *in vitro* studies which demonstrate that (*S*)-baclofen antagonizes the action of (*R*)-baclofen (Olpe *et al.*, 1978; Sawynok and Dickson, 1985). Baclofen efficacy and patient compliance might, therefore, improve if (*R*)-baclofen rather than the racemate were to be administered.

The intravenous anaesthetic ketamine is administered as a racemic mixture and its use has been associated with distressing emergence reactions. The disposition of ketamine is stereoselective and the clearance of (*S*)-ketamine is greater than that of its optical antipode (Geisslinger *et al.*,

1993b). Equianaesthetic doses of (*S*)-ketamine (1 mg kg^{-1}), (*R*)-ketamine (3 mg kg^{-1}) and racemic ketamine (2 mg kg^{-1}) have been compared in patients undergoing elective surgery. Both quantitative and qualitative differences between the enantiomers were noted. (*S*)-ketamine produced less agitated behaviour, less perioperative pain (White *et al.*, 1980), a more rapid recovery of psychomotor skills (White *et al.*, 1985) and was more acceptable to patients than (*R*)- or racemic ketamine. (*R*)-ketamine did not produce electroencephalographic slowing comparable to that of (*S*)-ketamine or the racemic mixture, and thus it would be more difficult to achieve an adequate depth of anaesthesia with (*R*)-ketamine (White *et al.*, 1985). Administration of an enantiomerically pure dosage form containing (*S*)-ketamine may, therefore, improve the margin of safety and patient acceptance of ketamine anaesthesia.

5.6 ENANTIOMERS HAVE DIFFERENT ACTIVITIES

The enantiomers of α-propoxyphene have different pharmacological actions. The (+)-enantiomer of α-propoxyphene is a potent analgesic drug whereas the (−)-enantiomer is a potent antitussive agent devoid of analgesic activity. Recognition some decades ago of the different spectrum of pharmacological effects of the enantiomers of α-dextropropoxyphene resulted in the development of two separate stereochemically pure drugs: α-dextropropoxyphene (Darvon), an analgesic, and α-levopropoxyphene (Novrad), an antitussive. The generic names of the enantiomers indicate the stereochemical nature of each drug and in addition the trade names are, quite appropriately, mirror images of each other.

The enantiomers of dobutamine also have different actions and in this case administration of the racemate has a therapeutic advantage over administration of the single stereoisomer. Racemic dobutamine stimulates both the α_1- and β_1-adrenoceptors in the myocardium, resulting in a greater inotropic response than after administration of the individual enantiomers. When the racemate is given, this therapeutic effect is accompanied by little net change in blood pressure, since the α-adrenoceptor-mediated vasoconstriction of the (−)-enantiomer is counteracted by the β_2-adrenoceptor-mediated vasodilatation caused by the (+)-enantiomer. Therefore, both enantiomers contribute to the therapeutic effect and the racemate is superior in therapeutic efficacy to administration of a single enantiomer. The racemate may also be superior to the single enantiomers for the β-adrenoceptor antagonist carvediol (Bartsch *et al.*, 1990). (*S*)-Carvedilol initiates β-blockade, whereas (*S*)- and (*R*)-carvedilol are equipotent α-adrenoceptor antagonists. As a result of stereoselective metabolism, threefold higher plasma concentrations of (*R*)- than (*S*)-cavedilol are observed in plasma. Consequently administration of the racemate and the enhanced α-blockade that occurs may be of therapeutic

benefit relative to administration of the single β-blocking (*S*)-enantiomer (Bartsch *et al.*, 1990).

5.7 ACTIVITY RESIDES WITH ONE ENANTIOMER; THE INACTIVE ENANTIOMER IS RESPONSIBLE FOR TOXICITY

L-dopa and D-penicillamine are drugs that were originally investigated as racemic mixtures. Because of severe adverse drug reactions caused by the enantiomers which did not contribute to therapeutic efficacy, the racemates were replaced by enantiomerically pure drug preparations. By removing the distomer from the racemic dosage form, drug toxicity could be avoided or drastically reduced. D-penicillamine is used in the treatment of Wilson's disease, cystinuria and rheumatoid arthritis. Severe adverse reactions such as nephritis are attributed to the L-enantiomer and, therefore, only the D-enantiomer is used. The mechanism underlying the toxicity is assumed to result from the incorporation of L-penicillamine into proteins (Williams, 1990). D-penicillamine cannot undergo such a reaction as mammalian proteins consist of L-amino acids (Man and Bada, 1987).

Recognition that Parkinson's disease was associated with diminished brain L-dopamine concentrations resulted in a trial of oral dopa for the treatment of Parkinson's disease. It was hypothesized that L-dopa would be metabolized to L-dopamine *in vivo* and would thus be active. However, as costs associated with production of the pure enantiomer L-dopa were high, initial studies in humans were performed using the racemate. Racemic dopa was efficacious, but the incidence of side-effects, in particular granulocytopenia, was unacceptably high (Cotzias *et al.*, 1967). By administering L-dopa alone efficacy was maintained while the incidence of side-effects decreased (Cotzias *et al.*, 1969).

5.8 CHIRAL INVERSION

In situations where pronounced differences in potency exist and the less or inactive enantiomer is metabolically converted to the active enantiomer, as in the case of some 2-arylpropionic acid derivatives, the use of the active enantiomer instead of the racemate might be advantageous. The administration of the active (*S*)-enantiomer of 2-arylpropionic acid derivatives would circumvent interindividual variability in the metabolic conversion of the inactive to the active enantiomer. In addition potential drug interactions affecting the chiral inversion as well as the formation of hybrid triglycerides with the (*R*)-enantiomer, the toxicological significance of which has not been established, would be avoided (Brune *et al.*, 1992; Hall and Xiaotao, 1994).

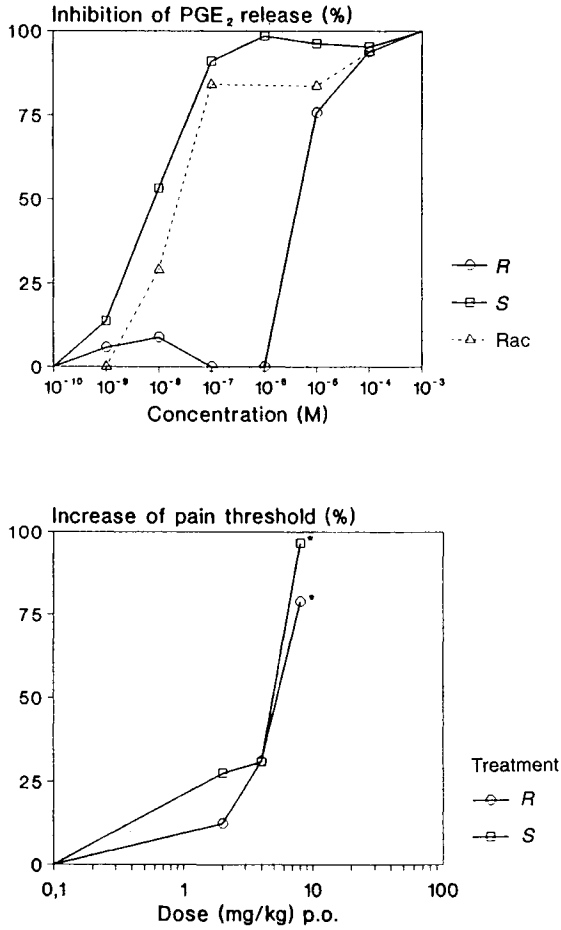

FIG. 17. Pharmacodynamic effects of (*R*)- and (*S*)-flurbiprofen. *Upper panel*: Inhibition of prostaglandin (PGE_2) synthesis by mouse peritoneal macrophages is stereoselective. (*S*)-flurbiprofen (IC_{50} 8.08[−IgM]) is more than 500 times more potent than (*R*)-flurbiprofen (IC_{50} 5.34). The anti-inflammatory activity of each enantiomer is related to its potency as a prostaglandin synthesis inhibitor *in vitro*. *Lower panel*: By contrast, (*S*)- and (*R*)-flurbiprofen are of almost identical potency as antinociceptive drugs in the rat Randall–Seliio assay following the injection of interleukin 1. (Adapted from Brune *et al.* (1991), with permission of Birkhäuser, Basel.)

Initial investigations have compared the efficacy and tolerability of racemic ibuprofen with an equivalent dose of the (*S*)-enantiomer in patients with rheumatoid arthritis (Brune *et al.*, 1992). The side-effect profile and anti-inflammatory actions of the two dosage forms were comparable, consistent with the administration of equivalent doses of the prostaglandin

inhibitor (*S*)-ibuprofen. Interestingly a greater analgesic effect was reported after administration of the racemate. These clinical observations support the recent results of animal studies demonstrating that the (*R*)- and (*S*)-enantiomers of flurbiprofen have equivalent analgesic effects in some pain models (Fig. 17) (Brune *et al.*, 1991). Therefore at low doses the anti-inflammatory and analgesic effects of flurbiprofen are independent. Reformulation of racemic NSAIDs to remove the (*R*)-enantiomer would therefore also remove an active analgesic agent. Alternatively (*R*)-flurbiprofen alone, a NSAID which does not undergo chiral inversion, may be a mild analgesic without anti-inflammatory effects, and with possibly less gastrointestinal toxicity than the racemate (Brune *et al.*, 1991). Recent animal studies suggest that the gastrointestinal side-effects of the NSAID flurbiprofen may be attributable to the (*S*)-enantiomer alone (Brune *et al.*, 1991), and that (*R*)-flurbiprofen is not ulcerogenic (Wechter *et al.*, 1993). These hypotheses await testing with stereochemically pure dosage forms of NSAIDs not subject to chiral inversion in humans, such as flurbiprofen. Prescribing enantiomerically pure dosage forms of NSAIDs would simplify therapeutics and could result in drugs with wider therapeutic windows and which would therefore be preferable to the racemates currently prescribed (Caldwell *et al.*, 1988).

5.9 OPTICAL PURITY

The critical importance of optical purity for evaluating the safety and efficacy of an enantiomerically pure drug can be illustrated with D-thyroxine. D-thyroxine was used in the late 1960s to treat patients with hyperlipidaemia. L-thyroxine, which is the naturally occurring stereoisomer of this thyroid hormone, is at least 15–40 times more potent than D-thyroxine. Compared with L-thyroxine, D-thyroxine induces a greater decrease in serum cholesterol concentration per increase in oxygen consumption and heart rate, indicating that D-thyroxine has a preferential effect on cholesterol metabolism. However, a double-blind trial of D-thyroxine (6 mg) in patients with coronary heart disease and previous myocardial infarction was halted prematurely as the mortality rate associated with D-thyroxine was greater than that of placebo (Coronary Drug Project Research Group, 1972). It had been assumed that the D-thyroxine tablets used contained negligible amounts of L-thyroxine. In 1984 Young *et al.* reported that the available D-thyroxine dosage forms contained between 0.5 and 2.3% L-thyroxine. This contamination is apparently small. However, at D-thyroxine doses of 8 mg day^{-1} up to 180 μg per day of L-thyroxine will be administered, which is equivalent to an average replacement dose for adequate treatment of hypothyroidism. Treatment of athyrotic patients with these D-thyroxine tablets at a dose of 8 mg day^{-1} produced physiological L-thyroxine levels which were proportional to the

degree of L-thyroxine contamination in the D-thyroxine tablets. Therefore, it is quite likely that the D-thyroxine action observed and side-effects, including adverse cardiac effects, were due to the contaminant L-thyroxine (Young *et al.*, 1984). Recently the pharmacological effects of D-thyroxine have been re-evaluated using a dosage form that contains only 0.05% contamination with L-thyroxine. This study has demonstrated that (*R*)-thyroxine does have cholesterol-lowering properties and is indeed a potent thyromimetic agent (Hamon *et al.*, 1993).

6 Drug Development

Although only a limited number of the many racemic drugs in therapeutic use have been investigated with regard to stereoselectivity of action and disposition, the results clearly demonstrate that it is not valid to consider these drugs as single compounds. In view of the differences in activity and disposition that can exist between enantiomers, two drugs are in fact being administered when a racemic medicine is prescribed. Should we, therefore, continue to administer racemic medicines or use only stereochemically pure drugs? The very existence of stereoselectivity in drug action and disposition does not necessarily mean that in the future only stereochemically pure compounds rather than racemates should be used.

In assessing the merits and benefits of employing enantiomerically pure drugs two cases must be considered. Firstly, for those drugs with a chiral centre which are presently at an early stage of development, it is prudent to take stereochemical considerations into account and to explore whether a stereochemically pure drug instead of a racemic mixture should be developed. For new drugs the development of a racemate will be justified if: (a) both enantiomers are equipotent in terms of efficacy and toxicity; (b) the racemate is superior to the enantiomer; (c) racemization occurs to yield fixed ratios of enantiomers in the body; or (d) separation of the enantiomers is technically not feasible. Recent surveys indicate that pharmaceutical companies are indeed introducing an increasing number of their new drug entities as single enantiomers or achiral molecules, thereby avoiding racemic drugs and the implications of their composite nature (Cayen, 1991; Shindo and Caldwell, 1991; Millership and Fitzpatrick, 1993).

Secondly, the many racemic drugs already in therapeutic use or about to be introduced must be considered, as this situation is somewhat different. At the time of development of these drugs there was little concern about stereoselectivity in drug action and disposition, and the technology for the synthesis and analysis of enantiomers was at a rudimentary stage.

Several arguments favour the use of individual enantiomers instead of racemates. There can be no doubt that the use of stereochemically pure drugs will result in a less complex concentration–effect relationship, less complex

pharmacokinetics, less complex drug interactions and, in some instances, there will be an improved pharmacological profile. Although this information is of interest, it does not necessarily translate into improved therapeutic efficacy and safety of a single enantiomer compared with a racemic mixture and, therefore, does not alone justify the development of a stereochemically pure drug.

An existing racemate can be considered to be a combination preparation containing two drugs that have been tested and evaluated for efficacy, safety and toxicity as a fixed-dose combination. In evaluating the benefits of developing a stereochemically pure drug from an existing racemate, the same criteria that apply to drug combinations should thus be used. In the case of drug combinations, both components should contribute to therapeutic efficacy. If one drug of such a combination is mainly responsible for side-effects and toxicity and does not contribute to therapeutic efficacy, then a fixed combination would not be approved. However, the view that only one enantiomer is active and the other is *inactive*, and that the *inactive* enantiomer is responsible for toxicity (Ariëns, 1984), is a simplified view of an often complex situation. For racemic drugs presently in therapeutic use there are few convincing examples where one enantiomer is active and the other is therapeutically inactive but makes the major contribution to side-effects and toxicity. For these compounds the introduction of a stereochemically pure drug should be considered in order to improve the safety and efficacy of the drug and to remove an unnecessary burden of xenobiotic material on the body, especially during long-term therapy (Ariëns *et al.*, 1988). Indeed the inactive enantiomer *can* be considered to be a 50% impurity in the dosage form. Good manufacturing practice requires that the content of structurally unrelated impurities be minimal and therefore this 50% impurity should not be tolerated (Ariëns, 1983; Ariëns *et al.*, 1988). In this context, however, the term *inactive* applies only to efficacy. Even if the inactive enantiomer does not contribute to toxicity, no drug is totally devoid of all pharmacological activity and the term inactive can therefore be misleading.

For the majority of racemic drugs straightforward scenarios that clearly indicate whether to develop a stereochemically pure drug do not exist. In most instances both enantiomers contribute to therapeutic efficacy and it is often not possible to discern whether one enantiomer makes a disproportionate contribution to side-effects and toxicity. Although there can be a high degree of stereoselectivity for one action, other pharmacological effects that contribute to therapeutic efficacy may exhibit only a modest degree or no stereoselectivity. Since most side-effects are an extension of the desired pharmacological actions, they are a consequence of the inappropriate use of the drug rather than an issue of stereoselectivity. It is doubtful, therefore, that the safety of such compounds would be improved if stereochemically pure drugs instead of a racemate were to be given. For example, the

(*R*)-enantiomers of propranolol and metoprolol are equipotent to the racemate in the prevention of migraine. If these drugs were to be reformulated to contain a single enantiomer, for example (*S*)-propranolol, twice the dose of this enantiomer would have to be given in order to achieve equivalent propranolol levels for the prophylaxis of migraine. The β-blocking and adverse effects caused would increase in proportion to the increase in dose of (*S*)-propranolol. The safety and efficacy of migraine prophylaxis would therefore not be improved by using the single enantiomer rather than the racemate.

Only a few racemic drugs in therapeutic use today have been studied in adequate detail to indicate that their safety and efficacy could be improved by using a stereochemically pure drug. This may be attributable to the fact that drugs for which the distomer may be responsible for toxicity were withdrawn from the market or were not further developed. Thus it might be worthwhile to re-evaluate older, promising, racemic drugs whose development was stopped because of side-effects and toxicity to establish whether the problems observed may be related to stereochemical aspects. At present a number of racemic drugs are being investigated as stereochemically pure medicines (Ariëns, 1993), no doubt stimulated in part by the ability to patent the single enantiomers of drugs presently used as racemates. Dexfenfluramine, developed from the racemate fenfluramine, has been available for a number of years, and compounds being investigated include (*S*)-atenolol, (*S*)-flurbiprofen, (*R*)-fluoxetine, (*S*)-nitrendipine, (*R*)-salbutamol, (*S*)-terfenadine and (*R*)-verapamil (Ariëns, 1993).

Consideration of the optical purity of a stereochemically pure drug adds a further dimension to the discussion of chirality and drug development. Chemical synthesis to produce an enantiomer to a high degree of stereochemical purity (up to 99.5%) can be exorbitantly expensive. Strict guidelines as to the proportion of the distomer that may be present in a dosage form of the eutomer are presently being adopted by the regulatory authorities. However, the chiral contamination that can be tolerated varies for each chiral drug and therefore a more flexible position should be adopted by the authorities, with each drug assessed individually. When testing the activity and relative potency of individual enantiomers, the highest possible purity that is technically feasible is required. For stereochemically pure drugs in therapeutic use there are, however, a number of scenarios.

If the distomer is responsible for toxicity, the highest technically feasible optical purity of the eutomer should be employed to maximize safety. For example, the toxicity observed with D-penicillamine formulations can be traced to contamination with L-penicillamine in the dosage form used. An alternative scenario applies if one uses an eutomer that is 10–20 times more potent than the distomer and the distomer and the eutomer exhibit the same spectrum of adverse effects. In this case a high proportion of stereoisomeric contamination of the distomer, for example up to 5%, may be tolerable.

However, if the distomer exerts unwanted effects, then a much more stringent stereochemical purity may be required. If the distomer is used for an alternative indication to that of the eutomer, the scenario is again different. The tolerated impurity will depend on the differences in potency and even 2–4% contamination by the eutomer may be acceptable. This principle can be illustrated with (*R*)-verapamil, used in clinical trials to modulate multidrug resistance in patients with cancer (Schumacher *et al.*, 1992). The (*R*)-verapamil synthesized for this study contained 4% of the (*S*)-enantiomer and, as a result of the very high doses of (*R*)-verapamil administered (up to 2400 mg daily), pharmacologically active (*S*)-verapamil doses (up to 94 mg daily) are co-administered, and hence (*S*)-verapamil concentrations could be attained that cause AV-node conduction disturbances.

However, in contrast to trials with racemic verapamil, where severe rhythm disturbances and negative inotropy prevented dose escalation, in this clinical trial (*R*)-verapamil caused substantial falls in blood pressure which prevented further increases in dose. The modest changes in AV-node conduction produced by the contaminant (*S*)-verapamil was not dose limiting. Rather the highest tolerated dose was determined by the profound blood-pressure-lowering effect of (*R*)-verapamil. Therefore reducing the proportion of (*S*)-verapamil to 0.5% would not improve the safety and efficacy of the (*R*)-verapamil formulation but only substantially increase the production costs. The cost of manufacturing the eutomer to a high degree of stereochemical purity, for example less than 0.5%, may presently be prohibitive for the development of promising compounds where such a high degree of stereochemical purity may not be required. Rapid advances in synthetic chemistry and the introduction of biosynthetic techniques are, however, reducing the costs of synthesizing the large quantities of stereochemically pure compounds required (Cayen, 1993).

7 Conclusion

For racemic drugs in widespread use or to be introduced in the near future, data on stereoselectivity in action, disposition and toxicity should be available. Once this information is known each drug should be evaluated on a case-by-case basis to determine whether a single enantiomer may offer a therapeutic advantage to the racemate. Although one cannot expect practising physicians to know of all the details associated with the stereochemistry of the racemic drugs they prescribe, they should be aware of the phenomena. Until recently stereochemical aspects of drug action and disposition have not been mentioned in drug product information, pharmacology textbooks and many scientific publications. In the future, drug companies and the authors of scientific reports should clearly state the composite nature of the racemic drugs that are used.

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Advances in the Development of Pharmaceutical Antioxidants

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ASA	Aminosalicylic acid
BHA	Butylated hydroxyanisole, i.e. 2-*tert*-butyl-4-methoxyphenol
BHT	Butylated hydroxytoluene, i.e. 2,6-di-*tert*-butyl-4-methylphenol
CIBD	Chronic inflammatory bowel disease
DHBA	2,3-Dihydroxybenzoic acid
DMPO	5,5-Dimethyl-1-pyrroline *N*-oxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl radical
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediamine tetra-acetic acid
fMLP	*N*-formylmethionyl-leucyl-phenylalanine
GSHpx	Glutathione peroxidase
LDL	Low-density lipoprotein
MDA	Malondialdehyde
MPO	Myeloperoxidase
NDGA	Nordihydroguaiaretic acid
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory agent
PMA	Phorbol myristate acetate
PMNL	Polymorphonuclear leukocytes
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SARD	Slow-acting antirheumatic drug
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reactive substance
TNBS	Trinitrobenzene sulfonic acid
UC	Ulcerative colitis

1 Introduction

1.1 INTRODUCTORY REMARKS AND SCOPE OF THE CHAPTER

The area of free radical biology and medicine has undergone an explosive development during the past decade. Along with the necessary refinement of experimental techniques to detect the biological footprints of short-lived chemical entities, evidence for the involvement of oxidative tissue injury in a growing number of diseases seems to accumulate. However, the elusive nature and chemical reactivity of the free radicals involved have left direct observations scarce, and therefore certain aspects of the area remain somewhat controversial.

Many aspects of free radical pathology and the toxicity of oxygen metabolites have been reviewed excellently by others. The reader is referred to, for example, the books by Halliwell and Gutteridge (1989), Rice-Evans and Dormandy (1988), Sies (1991, 1985), Grisham (1992), Halliwell and Aruoma (1993) and Cheeseman and Slater (1993) for comprehensive accounts of primarily biological aspects. A very thorough and up-to-date overview written by recognized experts in the field is provided in two recent volumes of *Methods in Enzymology*, edited by Packer (1994). Very useful review articles covering all aspects of free radical biology can be found by consulting the volume indices of the journal *Free Radical Biology and Medicine*. Biologically relevant free radical chemistry has been covered, for instance, in the books by Vigo-Pelfrey (1990), Lazar *et al.* (1989) and the somewhat older series edited by Pryor (1976). Compilations of laboratory techniques and standards assays employed in free radical biology have appeared fairly recently (Halliwell, 1990; Rice-Evans *et al.*, 1991).

However, there is an unfortunate lack of reviews treating the field from a medicinal chemical point of view. Thus, no extensive compilations of structural formulae and treatises addressing the structural characteristics of biologically relevant antioxidants with an emphasis on chemistry are available. The present review represents an attempt to narrow the gap where antioxidant pharmacology meets with organic chemistry. This chapter is intended to serve as a reference to substances that have been described, designed or developed as therapeutically relevant antioxidants. It is obvious that the present contribution cannot cover more than a limited, but hopefully representative, number of examples of antioxidants from the large variety of structural classes which have been studied as possible drug candidates. For this purpose we judged it most fruitful to cover compounds that have emerged from *in vitro* experimentation and structure–activity studies. We have therefore deliberately omitted studies concerning the application of simple alcohols, polyols or thiols and other standard chemicals, such as dimethylsulfoxide, inositol and dimethylthiourea. We have also elected to

leave out the majority of the references concerning crude extracts from plants and marine organisms. Although such studies are of great value, and often serve to provide the pharmaceutical companies and academic researchers with completely novel leads and avenues of research, we find it easier to discuss antioxidant effects in molecular and structural terms.

In this chapter, the synthetic compounds are classified chemically, rather than by therapeutic area, since the latter, in most instances, is a matter of the preference of the researchers rather than inherent properties of the substances. Some ranges regarding dose or concentration are included to indicate the approximate potencies of compounds, especially when standard *in vitro* assays have been utilized. However, since data on IC_{50} and related parameters are often difficult to reproduce, and readers would need to go to the original literature anyway, we have tried to refer them to the relative potencies whenever the authors have provided data on reference compounds.

The biological significance and pharmacological effects of members of the endogenous defence against oxidative insults have been reviewed excellently by others, and will not be discussed at length. Thus, the importance of the vitamins E and C as well as the ubiquinols and carotenoids in disease prevention has become widely recognized and is supported by a host of clinical and epidemiological studies. It should be pointed out, though, that the beneficial effects of antioxidant vitamins on, for example, carcinogenesis, atherosclerosis and coronary heart disease suggested by epidemiological studies have still to be confirmed conclusively in controlled trials (Barber and Harris, 1994; Burr, 1994; Elinder and Walldius, 1994; Rautalahti and Huttunen, 1994; van Poppel *et al.*, 1994). The enzyme systems that are devoted to detoxification of reactive and potentially pathological oxygen metabolites (superoxide dismutase, glutathione peroxidase, catalase, etc.) have also been thoroughly treated by others, and will be mentioned here just to provide a basis for the discussion of synthetic enzyme mimics.

We have chosen not to treat enzyme inhibitors that serve to block the generation of reactive oxygen metabolites (superoxide, hydrogen peroxide, hypohalites) or the signal transduction preceding this production. This is not to suggest that such enzyme or signal transduction inhibitors are not useful for treating conditions associated with oxidative stress or membrane lipid peroxidation, but rather to limit the scope of this contribution to a reasonable size.

Hardly surprisingly, the first line of synthetic compounds, and a still very important class of biologically relevant antioxidants, have been analogues of the naturally occurring antioxidant vitamins E and C. Another class of important and promising substances has emerged through studies of non-mammalian antioxidants, or compounds ascribed a possible antioxidant capacity. For example, several interesting phenolic and amine-containing substances have been isolated from plants, including the flavonoids, and

suggested as potential antioxidant remedies. In several instances, the development of new and safe food preservatives, for instance from spice extracts, has served to provide the pharmaceutical community with interesting leads.

The study of the antioxidant capacity of existing drugs, both those where the mechanism of action is considered to be well elucidated and those where there is considerable uncertainty in that respect, has been an area of intense activity. However, as the field of free radical biology has matured, the number of publications presenting only *in vitro* studies of antioxidant effects recorded using established drugs has started to drop in favour of mechanistic investigations. It appears that there are still certain cases where the concentrations of drug obtained *in vivo* may give some relevance to the effects described in the *in vitro* situations, where micromolar levels of the compound under study are often necessary to establish these effects. In recent years, in particular, much work has been devoted to the study of the possible antioxidant capacity of aminosalicylic acids and their metabolites and prodrugs.

Agents mimicking the activity of the endogenously occurring antioxidant enzymes are of course very attractive targets in drug discovery. The concept of catalytically acting antioxidant compounds for pharmaceutical use seems on the whole to have gained popularity over the past few years. However, specific, low-molecular-weight enzyme mimics are even more difficult to develop than antioxidants regenerable *in vivo*. The most significant progress in the field has been made with mimics of glutathione peroxidase and superoxide dismutase. In the case of the former agents, virtually all synthetic compounds utilize the redox chemistry of organic selenium and tellurium compounds. The latter class of agents have in most cases been transition metal complexes, some of which have been judged to be of limited applicability due to possible toxic effects from leakage of the metals *in vivo*.

It seems that the pharmaceutical industry has been unusually slow in establishing forceful programmes to take advantage of the development of knowledge in the free radical biology field. This is possibly partly related to the difficulty in determining that any pharmacodynamic effect is a direct function of antioxidant activity alone. Nevertheless, an increasing number of patents and journal articles has appeared, describing efforts to develop biologically relevant antioxidants through synthesis screening programmes. We hope that the reader will find that the scope of this review is sufficient to serve as a useful guide to the structural diversity that has emerged from the development of free radical biology and medicine.

A short introduction follows, which attempts to present the various conceivable approaches that are at hand for attenuating oxidative tissue injury and the harmful effects that are ascribed to (primarily oxygen-centred) free radicals. Only very short introductory sections have been included on

biological and pathological considerations. These might serve as a brief introduction to readers who are not familiar with the field, whereas those who are will probably not benefit from reading them.

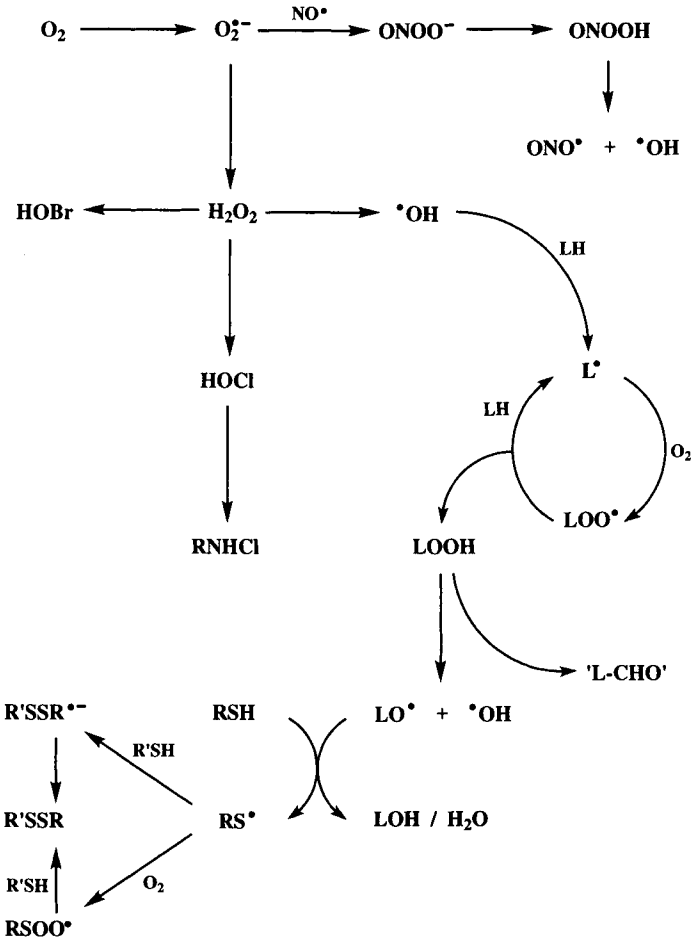
1.2 FREE RADICALS IN BIOLOGY

Living organisms are continuously exposed to oxidants from external sources, e.g. air pollution, ultraviolet (UV) radiation, natural radioactive gases and dietary constituents. The production of free radicals and various reactive oxygen species (ROS) is also an inherent phenomenon of the body's normal defence systems and metabolic reactions.

There are a number of radicals, primarily oxygen centred, which are considered biologically relevant. A very simplified overview of their interrelations is provided in Scheme 1. There is an elaborate endogenous defence system to control the production and level of these species (Cotgreave *et al.*, 1988). Excessive formation of ROS, however, may lead to degradation or inactivation of essential biomolecules. This has been suggested as a primary or secondary factor in an increasing number of pathophysiological conditions (see Section 1.3). Several excellent textbooks (see, for example, Sies, 1985, 1991; Halliwell and Gutteridge, 1989; Lazar *et al.*, 1989; Vigo-Pelfrey, 1990; Aruoma and Halliwell, 1991; Grisham, 1992; Cheeseman and Slater, 1993; Halliwell and Aruoma, 1992) and recent reviews (Chow, 1991; van der Vliet and Bast, 1992; Gougerot-Pocidalo and Revillard, 1993; Jackson *et al.*, 1993; Salvemini and Botting, 1993; Stefanovic-Radic *et al.*, 1993; Testa, 1994) discussing the occurrence and reactivity of biologically relevant free radicals are available. The reader is referred to these sources for detailed discussions. Only for the sake of completeness is a very brief summary of the most relevant properties of common ROS given below.

Superoxide anion: Superoxide anion $O_2^{\cdot -}$ is in equilibrium with its conjugate acid hydroperoxyl radical ($HOO^{\cdot}$) in aqueous solution (pK_a 4.7). Whereas at normal physiological pH the equilibrium is strongly in favour of the deprotonated form, the undissociated acid may become relevant at lower pH, for instance in phagolysosomes or in ischaemic or inflamed tissues. Superoxide anion is not a very strong oxidant and is generally not regarded as a particularly toxic ROS, but it rapidly dismutates to produce oxygen and hydrogen peroxide. It is probable that the so-formed hydrogen peroxide and its secondary products, together with hydroperoxyl radical, are responsible for the toxic effects of superoxide. The reductive capacity of superoxide towards ferric iron is well documented and may be of pathophysiological importance, particularly via Fenton and Haber–Weiss chemistry (see below). Superoxide anion is formed as a byproduct in mitochondrial respiration and from various oxidases and peroxidases as well as by the phagocyte reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.

Scheme 1 Biologically important transformations involving reactive oxygen species



Hydrogen peroxide: Hydrogen peroxide (H_2O_2) is an important ROS, although it is not a radical. It is a weak oxidant, capable of oxidizing protein thiol groups to disulfides. The principal toxic effects of hydrogen peroxide, though, arise via its reaction with transition metals to produce hydroxyl radical. The comparatively high lipid solubility and stability of hydrogen peroxide makes this species capable of diffusing through membranes and thus acting as a transporter of oxidative stress.

Hydroxyl radical: Hydroxyl radical ($HO^{\cdot}$) is an extremely reactive free radical, which attacks the majority of molecules present in the cell. As mentioned above, hydroxyl radical is generally thought to be responsible for

the damaging effects that are recorded after exposure of cells or tissues to superoxide or hydrogen peroxide. It can be formed from the latter, mainly in the presence of transition metals such as iron or copper. Combining the Fenton reaction with superoxide-mediated reduction of ferric to ferrous iron leads to the iron-driven Haber–Weiss reaction:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^- + HO^{\cdot} \quad \text{(Fenton reaction)}$$
$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$$
$$\text{Net: } H_2O_2 + O_2^{\cdot -} \rightarrow O_2 + HO^- + HO^{\cdot} \quad \text{(Haber–Weiss reaction)}$$

Biologically relevant transition metal complexes which have been suggested to take part in such redox reactions include adenosine diphosphate (ADP), citrate and amino acid complexes of iron. Iron bound to transport and storage proteins does not participate in the Fenton reaction, but can become released upon exposure to superoxide. Hydroxyl radical has also been suggested to result from the decomposition of peroxynitrous acid (ONOOH), the protonated form of peroxynitrite (see below). Another potential source of this radical may be the interaction of superoxide and hypochlorous acid, generated by neutrophil myeloperoxidase.

Hydroxyl radical reacts with biomolecules by hydrogen abstraction, addition to multiple bonds or electron transfer reactions. In many instances, the reaction rates are diffusion controlled; thus, the radical will react with the first molecule encountered. The former reaction may for instance initiate lipid peroxidation through the abstraction of a hydrogen atom from a polyunsaturated fatty acid residue. Addition to aromatic residues in proteins may lead to hydroxylated products. The measurement of other hydroxylated products is sometimes used to diagnose DNA damage.

Singlet Oxygen: Although oxygen is a di-radical species, its ground state (triplet) electron configuration is not very reactive. However, excitation to the singlet state enhances the reactivity considerably. For instance, singlet oxygen readily undergoes addition to conjugated dienes and alkenes, a reaction of importance in lipid peroxidation. It also reacts with phenols, cholesterol, amino acids and NADPH. Singlet oxygen may be produced from the illumination of certain photosensitizing chemicals, notably porphyrins, bilirubin, riboflavin and retinal, or from the spontaneous dismutation of superoxide anion.

Hypohalites: The hypohalites (HOCl, HOBr) are formed through peroxidase-catalysed reactions in neutrophils, monocytes (myeloperoxidase) and eosinophils (eosinophil peroxidase). These enzymes utilize hydrogen peroxide and halide ions as substrates. HOCl has a pK_a of 7.5, and is therefore dissociated to approximately 50% at physiological pH. The hypohalites are halogenating agents of considerable reactivity, which interact with a variety of biological molecules such as thiols, unsaturated lipids and aromatic amino acids. The chlorination of amines by hypochlorous acid is

of particular interest, since the resulting chloroamines are cytotoxic and readily diffuse through biological membranes.

Thiyl and peroxysulfenyl radicals ($RS^{\cdot}$, $RSOO^{\cdot}$). Since the pK_a values of most thiols are around 9–10, significant concentrations of thiolate ion appear at physiological pH. These thiolates are efficient electron donors which can become oxidized to thiyl radicals, which are generally less aggressive than the oxygen counterparts. Secondary reactions of thiyl radicals include combination with thiols, to form disulfide radical anions, or oxygen, yielding peroxysulfenyl radicals. Despite the presence of high concentrations of both low-molecular and protein thiols *in vivo*, the role of radicals derived from them has received relatively little attention. The involvement of free radical species in thiol toxicity has been reviewed (Munday, 1989).

Nitric oxide (NO) and peroxynitrite ($ONOO^-$): Nitric oxide (endothelium-derived relaxing factor, EDRF) is a comparatively stable free radical (cf. di-oxygen) with a key role in blood pressure control, blood clotting and neurotransmission via activation of guanylate cyclase (Palmer *et al.*, 1987; Radomski *et al.*, 1987; Ignarro, 1991; Moncada *et al.*, 1991; Bredt and Snyder, 1992). There is a growing body of evidence for the involvement of NO in immune regulation and inflammation (Kolb and Kolb-Bachofer, 1992; Stefanovic-Radic *et al.*, 1993). Nitric oxide is exceedingly unstable under physiological conditions and is usually transported as *S*-nitrosothiol adducts of, for example, albumin, cysteine or glutathione. The propensity of NO to react with free radicals, and to form secondary radical products, has given this molecule an increasingly important role in free radical biology.

Nitric oxide synthase, oxidatively forming NO and citrulline from L-arginine, is either constitutive (cNOS) and calcium dependent or inducible (iNOS) and independent of calcium (Stefanovic-Radic *et al.*, 1993). Nitric oxide can scavenge the superoxide anion to produce peroxynitrite in a reaction that initially was regarded as a protective role for NO. However, upon protonation, peroxynitrite decomposes to the substantially more toxic hydroxyl radical and nitrogen dioxide radical (Beckman *et al.*, 1990; Henry *et al.*, 1993). Thus, macrophages with iNOS induced by endotoxins or cytokines (Nathan and Hibbs, 1991; Henry *et al.*, 1993) would lead to the formation of both NO and superoxide anion.

$$O_2^{\cdot -} + NO^{\cdot} \rightarrow ONOO^-$$
$$ONOO^- + H^+ \rightarrow HO^{\cdot} + NO_2^{\cdot}$$

Both pro- and anti-inflammatory properties have been ascribed to NO; it increases vascular leakage in several tissues and exacerbates tissue injury in inflammation, but it also inhibits adhesion of neutrophils to endothelium, suppresses the production of superoxide by neutrophils and inhibits rat osteoclast function (Stefanovic-Radic *et al.*, 1993). These discrepancies may to some extent be attributed to concentration and time effects, i.e. cNOS

produces transient and low levels of NO, whereas iNOS produces sustained and high levels of NO.

Lipid peroxidation: Polyunsaturated fatty acids (PUFAs) are especially sensitive to attack under oxidative stress, which will lead to deterioration of biomembranes and malfunction of several cellular and extracellular events that need to be compartmentalized (Vigo-Pelfrey, 1990). The chain reaction can be divided into three steps ($L^{\cdot}$ = PUFA radical):

Initiation:	formation of $L^{\cdot}$
Propagation:	$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$
	$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$
Termination:	$LOO^{\cdot} + LOO^{\cdot} \rightarrow$ non-radical products

The first step is an initiation reaction involving, for instance, hydroxyl radical as initiator, forming a conjugatively stabilized carbon radical, $L^{\cdot}$. This then reacts extremely rapidly with oxygen to form the peroxyl radical $LOO^{\cdot}$, which in its turn abstracts a *bis*-allylic hydrogen in a slower reaction to form lipid hydroperoxides (LOOH) and a new $L^{\cdot}$ radical until the chain reaction is terminated (Cheeseman, 1993). Apart from the fact that membrane lipid peroxidation is destructive *per se*, the hydroperoxides formed during the process are potentially toxic since they can act as initiators of further oxidative insult through metal-catalysed decomposition into hydroxyl or alkoxyl radicals. A further family of products from peroxidation are the lipid-derived aldehydes ("LCHO"). These substances, in particular 4-hydroxyalkenals, have been shown to possess various biological activities and suggested to be second toxic messengers.

An increase in lipid peroxidation, which can be measured as free radical-induced formation of conjugated dienes or malondialdehyde (MDA), is especially notable during ischaemia followed by reperfusion (Massey and Burton, 1988; Esterbauer *et al.*, 1991; Campo *et al.*, 1994). MDA, which reacts with DNA, is the major mutagenic and carcinogenic product generated by lipid peroxidation. It has recently been observed, though, that MDA is an abundant product of lipid peroxidation in disease-free human liver tissue (Chaudhary *et al.*, 1994). The investigators found 5400 MDA–deoxyguanosine adducts per cell, a frequency similar to that obtained during exposure to exogenous carcinogens. This study emphasizes the importance of endogenous antioxidants and the necessity to establish baseline levels of DNA damage in drug-related investigations.

1.3 FREE RADICAL PATHOLOGY

A role for reactive oxygen species and other free radicals in human disease states is being proposed with increasing frequency. Reports on the detection

of footprints of oxidative stress in a variety of animal models are available to corroborate these suggestions in several cases, although surprisingly few model studies have been performed in which the severity of disease has been correlated to depletion and subsequent supplementation, with natural or synthetic antioxidants, of the endogenous antioxidant defence. The question of the precise time-course of ROS production and tissue damage is much more difficult to address experimentally. As a consequence, little is known in detail concerning the role of oxidants, which may be causative or merely secondary to the primary disease process. In fact, the latter alternative is probably true for many human diseases.

Rather than reiterate free radical pathology at length in this contribution, we included this paragraph simply to allow us to list a selection of review articles available. Apart from consultation of the general texts cited in section 1.1, the references provided below should suffice to guide the reader into the wealth of literature covering free radical pathology that is now at hand.

For general discussions of the current status of antioxidant pharmacotherapy, the review articles of Rice-Evans and Diplock (1993), Bast (1994) and Halliwell (1991), for example, should provide good starting points. A *Scrip* report on "Reactive Oxygen and Oxidative Stress" was released during the finalization of the present manuscript. Potential sources of free radicals in tissues have been discussed, for instance, by McCord and Omar (1993). The most important indications and physiological systems where ROS are being considered as mediators are covered in monographs such as those edited by Cheeseman and Slater (1993) and Das and Essman (1990). These monographs include chapters devoted to free radical considerations in relation to indications such as atherosclerosis, carcinogenesis, inflammation, myocardial reperfusion, brain ischaemia, lung disease and liver damage, as well as the use of antioxidants in organ preservation.

The role of ROS as regulators of signal transduction pathways is subject to a growing interest (e.g. Schreck and Baeuerle, 1991; Schreck *et al.*, 1992; Meyer *et al.*, 1993; Schenk *et al.*, 1994). NF-κB is a ubiquitous transcription factor with a pivotal function in the early pathogen response and activation of the immune system by initiation of the transcription of a number of genes encoding immunologically relevant proteins such as cell adhesion molecules, cytokines, acute-phase proteins, immunoreceptors and haematopoietic growth factors (Baeuerle and Henkel, 1994). The effect of several different inducers can be abrogated by various antioxidants, e.g. NDGA, ebselen, vitamin C, BHA, vitamin E derivatives and *N*-acetylcysteine, which has led to the assumption that ROS can serve as common intracellular messengers for NF-κB activation (Schreck and Baeuerle, 1991; Schreck *et al.*, 1992; Meyer *et al.*, 1993; Manning and Anderson, 1994; Suzuki *et al.*, 1994). Intracellular redox levels regulate the NF-κB signal transduction pathway by a common protein tyrosine phosphorylation step (Anderson *et*

al., 1994). On the other hand, antioxidants do not block the activation of NF-κB triggered by inhibitors of serine–threonine protein phosphatases (Suzuki *et al.*, 1994).

The areas of free radical pathology that have evolved into reasonable maturity have of course been the subject of numerous reviews. The tabulation below provides a few recent accounts of ROS-mediated effects in the indicated areas.

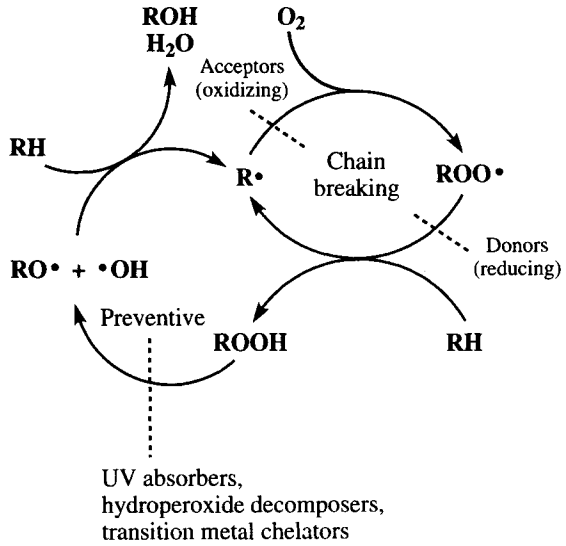
• Intestinal diseases	van der Vliet and Bast (1992), Onori *et al.* (1993), Grisham (1994)
• Atherosclerosis	Ferns *et al.* (1993), Jackson *et al.* (1993), Witztum (1994)
• Rheumatic disorders	Parnham and Blake (1993)
• Reperfusion injuries	Valen and Vaage (1993), Bulkley (1994), Janero (1995), Panetta and McCall (1995)
• Neurodegeneration	Jenner (1994), Olanow and Arendash (1994)
• Respiratory tract disorders	Cross *et al.* (1994), Winklhofer-Roob (1994)
• Cardiac diseases	Kaul *et al.* (1993), Schimke and Haberland (1993)
• Cancer	Cerutti (1994), Guyton and Kensler (1993)
• Ageing	Ames *et al.* (1993), Poeggeler *et al.* (1993)
• Transplantation	Keith (1993)
• Inflammation	Witko-Sarsat and Descamps-Latscha (1994)
• Diabetes	Tesfamariam (1994)

1.4 STRATEGIES FOR ANTIOXIDANT THERAPY

In the broadest possible definition, an antioxidant is any chemical that attenuates the rate of oxidative degradation of the material or substrate under study. Halliwell and Gutteridge (1989) defined an antioxidant as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate". However, when considering pharmaceutically relevant antioxidants, one may also need to include compounds that do not behave exclusively as peroxidation inhibitors. Thus, for the present discussion, an antioxidant of biological relevance may be defined as a chemical that inhibits the pathological consequences of oxidative insult by reacting with biologically relevant oxidants or radicals.

The termination of radical chain reactions has been an important issue within areas such as polymer stabilization and food preservation for a very long time. The necessity to control oxidative degradation of synthetic

Scheme 2 Antioxidant mechanisms



polymers and lubricants has emerged similarly to the field of anticorrosion for metal preservation. There are hypothetically several approaches available mechanistically, when attempting to intercept oxidative degradation (Scheme 2). Thus, various approaches are at hand to impede initiation reactions (UV absorbers, hydroperoxide decomposers, transition metal chelators) as well as terminating radical chain reactions (reviewed by Scott, 1988). Chain-terminating agents can be envisaged to act by either reducing the peroxyl radicals (which are easily reduced) or oxidizing carbon-centred species. When considering the destructive peroxidation of mammalian tissues, the peroxyl radical is the species of primary importance. Thus, in the *in vivo* situation, where oxygen is abundantly distributed, only the donating chain-breakers have been studied to any appreciable extent.

In the pharmacological context, antioxidants are commonly divided into two groups depending on their principal mode of action. Agents that are thought to act primarily through attenuating the initiation step of peroxidation are called *preventive* antioxidants. It is important to note that such agents, perhaps especially peroxide-decomposing enzyme mimics, may well have pharmacodynamic properties that result from effects on the redox status (or "hydroperoxide tone") of the cells rather than from blocking indiscriminate membrane peroxidation. For example, the involvement of oxidative signalling in immune regulation has become increasingly recognized (Gougerot-Pocadilo and Revillard, 1993; Meyer *et al.*, 1993). Compounds that act through terminating free radical chain reactions are referred to

as *chain-breaking* antioxidants. This distinction has been made also in the following sections, although many substances could belong to both categories.

A collection of the most commonly occurring strategies for antioxidant therapy must include the following:

(1) Enhancement of tissue endogenous antioxidant levels (e.g. through administration of antioxidant vitamins or glutathione precursors).
(2) Prevention of initiation events by scavenging (commonly discussed for hydroxyl radical scavengers).
(3) Chelation of metal ions (by introducing multidentate complexing agents which prevent transition metals from decomposing hydroperoxides into radical products).
(4) Decomposition of peroxides and superoxide into non-radical products (using antioxidant enzymes or synthetic superoxide dismutase or glutathione peroxidase mimics).
(5) Termination of free radical peroxidation (through administration of generally lipophilic chain-breaking antioxidants).

2 Endogenous Defence Systems

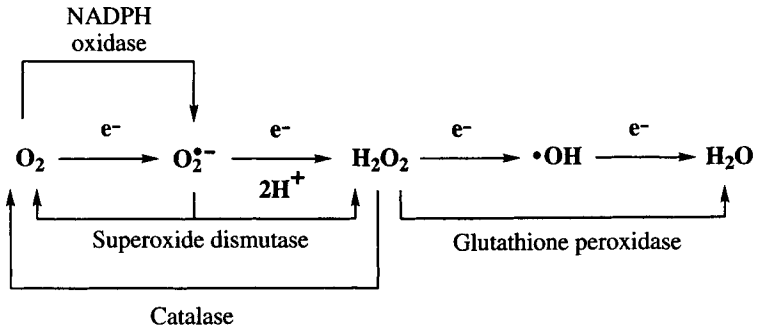
Any over-production of ROS is normally controlled by different endogenous enzymatic and non-enzymatic antioxidants (Halliwell and Gutteridge, 1989, Sies, 1991; Salvemini and Botting, 1993). These include preventive antioxidants diminishing the rate of chain initiation as well as scavenging or chain-breaking agents interfering with propagation steps (Stocker and Frei, 1991). The oxidative stress on an organ is a function of the amount of ROS produced and the antioxidant capacity. Thus, oxidative damage can be a result of excessive oxidative stress or an insufficient antioxidant level.

2.1 ENZYMATIC ANTIOXIDANTS

The enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHpx) are the primary antioxidants within the cells (Halliwell and Gutteridge, 1989). SOD acts as a scavenger of superoxide anions by catalysing their dismutation into hydrogen peroxide. Catalase and glutathione peroxidase reduce hydrogen peroxide as well as alkyl hydroperoxides into water and alcohols, respectively (Scheme 3). Both enzymes use the endogenous tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) as the sacrificial reducing agent.

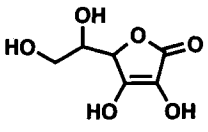
The importance of iron chelators, capable of forming bidentate complexes to inhibit iron-induced free radical damage, has been established in several studies (e.g. Kontoghiorghes *et al.*, 1986).

Scheme 3 Enzymatic antioxidants

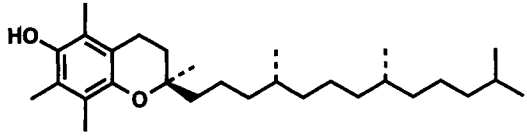


2.2 NON-ENZYMATIC ANTIOXIDANTS

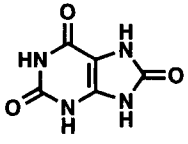
The vitamins C (ascorbic acid, **200**) and E (mainly (*R*,*R*,*R*)-α-tocopherol, **201**) are believed to be the major antioxidants in human plasma (Bendich *et al.*, 1986; Halliwell and Gutteridge, 1989; Chow, 1991). Other extracellular defences in human plasma are transferrin (binds iron), lactoferrin (binds iron), ferritin (binds iron), caeruloplasmin (oxidizes Fe^{II} to Fe^{III}), haptoglobin (binds free haemoglobin), haemopexin (binds free haem), albumin (binds copper, traps HOCl), uric acid (**202**, binds iron and copper, scavenger), bilirubin (scavenger) and glucose ($HO^{\bullet}$ scavenger). Several of the antioxidant proteins are acute-phase proteins related to inflammatory conditions. Further lipid-soluble antioxidants are β-carotene (**203**), bilirubin



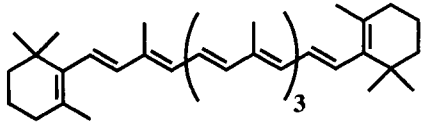
200
ascorbic acid



201
(*R*,*R*,*R*)-α-tocopherol



202
uric acid



203
β-carotene

and ubiquinol-10 (Stocker *et al.*, 1991). The antioxidant properties of analogues of vitamins E and C, sulfhydryl compounds, coenzyme Q, carotenoids and flavonoids are well documented in biological systems (references in Chen and Tappel, 1994). The effect of antioxidant nutrients on the oxidative damage of rat liver measured as oxidized haem proteins has been studied by Chen and Tappel (1994). They found a strong protection of vitamin E and selenium and a synergistic effect by increasing the diversity and quantity of antioxidants in the diet.

The 11 conjugated double bonds in β-carotene (**203**) makes it the most effective scavenger of singlet oxygen known (Rousseau *et al.*, 1990). β-Carotene protects against UVB radiation and diminishes genotoxic markers in tobacco-chewing individuals. There is convincing epidemiological evidence that vitamins C and E and β-carotene protect against cancer of the lung and other epithelial tissues by the involvement of a variety of mechanisms conveyed by their antioxidant and radical-scavenging properties (Perera *et al.*, 1993).

2.2.1 α-Tocopherol

The lipophilic α-tocopherol (**201**) is transported in the outer phospholipid layer of plasma lipoproteins. One particle of low-density lipoprotein (LDL) contains about six molecules of vitamin E and 1000 molecules of polyunsaturated fatty acids (PUFAs). α-Tocopherol provides the most important line of defence against lipid peroxidation of cell membranes. A high dietary intake of PUFA increases the daily requirement for vitamin E (Ferns *et al.*, 1993). The naturally occurring tocopherols found in vegetable and fish oils have also long been known for their beneficial antiatherogenic properties (Janero, 1991a, b; Ferns *et al.*, 1993; Jackson *et al.*, 1993). Vitamin E has been implicated in controlling various disease states that are believed to involve free radicals and in particular lipid peroxidation-mediated pathways, i.e. ageing, cancer, arthritis, atherosclerosis, ischaemia–reperfusion, cataracts, excessive exercise and air pollution (Chow, 1991; Rose and Bode, 1993; Janero, 1995).

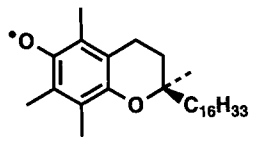
Some of the cellular effects of α-tocopherol may be mediated via a specific and saturable binding site on cell membranes (Kitabchi and Wimalasena, 1982). It also reduces platelet cyclooxygenase activity (Violi *et al.*, 1990) and inhibits phospholipase A_2 activity (Douglas *et al.*, 1986). The immunosuppressive effects of vitamin E may possibly be mediated by reducing prostaglandin synthesis (Ferns *et al.*, 1993). Plasma vitamin E levels appear to be inversely related to mortality from coronary heart disease and the risk of angina (Riemersma *et al.*, 1989, 1991).

2.2.1.1 Chemical aspects. Vitamin E is the major, and perhaps only, lipid-soluble chain-breaking antioxidant that prevents lipid peroxidation and

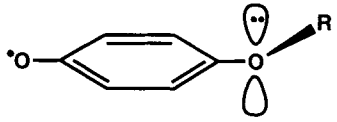
modulates the arachidonic acid cascade. As well as ascorbic acid, glutathione (GSH) present in the cytosol can regenerate vitamin E. The interaction between vitamins C and E during free radical-mediated oxidative stress has been studied by electron spin resonance (ESR) (Sharma and Buettner, 1993). Only after virtual disappearance of the initially formed ascorbyl radical could the tocopheroxyl radical be detected, corroborating the role of ascorbate as the terminal small-molecule antioxidant in biological systems. The pecking order, i.e. hierarchy, for free radical reactions of biorelevant molecules has been reviewed by Buettner (1993). The relative one-electron reduction potentials are in agreement with biological experimental data on the role of various antioxidants.

The antioxidant potencies for a number of natural antioxidants and representative analogues of 2-hydroxytetronic acid, α-tocopherol and ascorbic acid have been studied in an anionic dodecyl sulfate micellar model system by Pryor *et al.* (1993). The compounds could be grouped into three potency classes in relation to α-tocopherol. The induction periods due to α-tocopherol and ascorbic acid were found to be additive and not synergistic in this system. The results corroborate that peroxyl radicals react virtually exclusively with tocopherol and that the reducing equivalents of ascorbate are used to re-reduce the tocopheroxyl radical. A surprisingly low activity was observed for β-carotene and vitamin K_1-chromenol **304** (see below) in this model system. A word of caution was also put forward regarding the use of antioxidant data for charged antioxidants obtained in anionic or cationic micellar systems.

In membranes, α-tocopherol (α-TOH) inhibits lipid peroxidation by quenching the peroxyl radicals ($LOO^{\cdot}$), which are the chain carriers, to form the fairly stable tocopheroxyl radical (**204**, α-$TO^{\cdot}$) and lipid hydroperoxides



204
tocopheroxyl radical

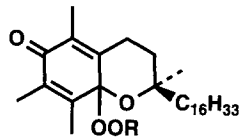


205

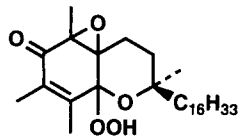
(LOOH) (Liebler, 1993). The stability of tocopheroxyl results from a combination of steric and conjugative effects, as indicated in **205** (see section 3.1).

$$LOO^{\cdot} + \alpha\text{-TOH} \rightarrow LOOH + \alpha\text{-}TO^{\cdot}$$
$$\alpha\text{-}TO^{\cdot} + LOO^{\cdot} \rightarrow \text{non-radical products}$$
$$\alpha\text{-}TO^{\cdot} + \text{Red-XH} \rightarrow \text{Red-X}^{\cdot} + \alpha\text{-TOH}$$

The trapping reaction initiated by hydrogen abstraction from the phenolic group in α-tocopherol is several orders of magnitude faster than the propagation reaction (Burton and Ingold, 1981, 1986). The subsequent reactions have been investigated in homogeneous systems and bilayer model systems (Liebler, 1993). In a homogeneous *tert*-butylperoxyl radical generating system, the fate of α-TO$^{\cdot}$ was found to depend on the amounts of hydroperoxides and oxygen radicals in the medium. The possibility for regeneration to α-tocopherol by glutathione or ascorbic acid as reducing agents is greater when the concentrations of oxygen radicals and hence of α-TO$^{\cdot}$ increase (Matsuo *et al.*, 1989). Liebler (1993) found two competing reactions of the tocopheroxyl radical (**204**) with peroxyl radicals in both homogeneous systems and bilayer systems to produce 8a-(alkyldioxy)tocopherones (**206**) and epoxytocopherones (**207**). The first pathway results in radical trapping, but the second, epoxide-forming, reaction may cause autoxidation of α-tocopherol.



206
8a-(alkyldioxy)tocopherone



207
epoxytocopherone

Sterically hindered phenolic antioxidants react with nitric oxide first to produce phenoxyl radicals and subsequently to form NO adducts from which NO can dissociate (Janzen *et al.*, 1993). This dissociation is more facile for the more stable phenoxyl radicals such as α-tocopheroxyl, which has been proposed to be a NO carrier in biological systems (Janzen *et al.*, 1993). The other established transport mode for NO *in vivo* is via *S*-nitrosothiols derived from molecules such as albumin and glutathione (e.g. Moncada *et al.*, 1991).

2.2.2 *Ascorbic Acid*

Ascorbic acid (**200**) is present in the extracellular and intracellular aqueous phase. It has been shown to scavenge a number of different ROS ($O_2^{\cdot -}$, H_2O_2, HOCl, HO$^{\cdot}$, peroxyl radicals, singlet oxygen) (Bendich *et al.*, 1986; Stocker and Frei, 1991). Initiation of lipid peroxidation by aqueous peroxyl radicals in plasma can be totally prevented by ascorbic acid but only retarded by protein thiols, uric acid and α-tocopherol (Frei *et al.*, 1989). Once ascorbate has been consumed, the peroxyl radicals diffuse into the plasma lipids and

initiate peroxidation. The propagation is then effectively inhibited by α-tocopherol, which is essentially the only and last defence against membrane peroxidation (see below). Thus, α-tocopherol must also be protected *in vivo* in order to ensure its role. A plausible primary physiological role for ascorbic acid is to serve as an antioxidant reservoir to regenerate vitamin E by reducing oxidized forms of α-tocopherol (Bendich *et al.*, 1986).

Most animals and plants synthesize ascorbic acid from glucose, but humans, primates, flying mammals and guinea-pigs lack L-gulonolactone oxidase, the terminal enzyme in the biosynthesis, which makes dietary intake essential (Chatterjee, 1973). In the guinea-pig, chronic marginal ascorbic acid deficiency produces lipid peroxidation in all organs including liver, kidney, lung, adrenal gland and testes (Chakraborty *et al.*, 1994). Notably, the effect was not due to lack of other antioxidants such as vitamin E, glutathione, protein thiols, catalase or glutathione peroxidase. In an *in vitro* model of superoxide-induced lipid peroxidation using guinea-pig liver microsomal membranes, ascorbic acid provided a complete prevention in contrast to α-tocopherol, glutathione, uric acid or catalase (Chakraborty *et al.*, 1994).

Ascorbic acid is found in high concentrations in many human tissues and may play a role in protecting lung tissue from radical-induced damage caused, for example, by cigarette smoke, ozone and inflammatory cells (Bendich *et al.*, 1986). Dietary intake of ascorbic acid protects against the development of chronic respiratory symptoms (Schwartz and Weiss, 1990) and it has been positively correlated with the pulmonary function (forced expiratory volume in one second; FEV_1) in a population of asthmatics and normal subjects (Schwartz and Weiss, 1994). Asthmatics have chronically lowered plasma levels of ascorbic acid (Olusi *et al.*, 1979; Aderele *et al.*, 1985). Anderson *et al.* (1983) found that supplementation with ascorbic acid to asthmatic children improved cellular immune functions such as neutrophil chemotaxis and phagocytosis. There is also a negative correlation between vitamin C intake and vascular death rates (Acheson and Williams, 1983). Dietary deficiencies of vitamin C and/or E have been stated to be associated with defects in phagocytic functions, e.g. chemotaxis and autoxidation (Bendich, 1992).

Ascorbic acid also reduces Fe^{III} to Fe^{II} *in vivo*, thus stimulating lipid peroxidation and formation of hydroxyl radicals, which during conditions of iron overload may lead to increased tissue damage (Aruoma, 1991). In normal plasma, with transition metal ions tightly bound, however, no switch from antioxidant to pro-oxidant properties has been observed (Frei *et al.*, 1989). The ability of ascorbic acid to intensify hydroxyl radical formation by a chemical ($Fe_{(II)}$/EDTA/H_2O_2), an enzymatic (xanthine/xanthine oxidase/$Fe_{(II)}$/EDTA) and a cellular system with PMA-activated human polymorphonuclear leukocytes (PMNLs) or murine macrophages was compared (Nowak *et al.*, 1991). In agreement with previous studies, ascorbic acid strongly enhanced hydroxyl radical formation by chemical and enzymatic

systems, but in the cell systems only very small effects in the concentration range 25–500 μM were observed.

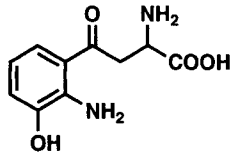
2.2.3 *Uric Acid*

Xanthine oxidoreductase is the only enzyme capable of producing uric acid (**202**) from adenine- and guanine-based purines. The physiological role of uric acid has recently been reviewed by Becker (1993). This ubiquitous compound is a selective antioxidant, which reacts especially with hydroxyl radicals and hypochlorous acid by conversion into harmless products such as urea, glyoxylate, allantoate and allantoin. These processes have been shown to take place in isolated organs and in the human lung *in vivo*. The reaction with hydroxyl radicals leads to the relatively stable urate radical, which can readily react with ascorbate, in analogy with the situation for α-tocopheroxyl radical (Simic and Jovanovic, 1989). In the absence of redox partners, which is an unlikely situation *in vivo*, the urate radical can be further oxidized to allantoin (Kaur and Halliwell, 1990) or act as a fairly potent oxidant towards, for example, α_1-antiproteinase (Aruoma and Halliwell, 1989). Several other endogenous antioxidants react more readily with hypochlorous acid than urate does, but the latter has the capacity to react with three moles of the oxidant (Winterbourn, 1985; Becker, 1993).

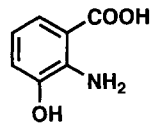
It has been proposed by Ames *et al.* (1981) that uric acid in plasma has taken over the role of ascorbic acid during human evolution and the loss of ability to synthesize ascorbic acid. The plasma levels of urate are considerably higher in humans than in most other mammals, which primarily excrete allantoin and urea from purine catabolism (Becker, 1993). Microvascular endothelium is a major site of production of urate, and a net release from the human myocardium *in vivo* has been observed. Urate protects against reperfusion damage induced by activated granulocytes which produce the ROS. Urate also prevents endothelial enzymes such as cyclooxygenase and angiotensin-converting enzyme (ACE) from oxidative degradation. Thus, urate may contribute to the ability of the vascular endothelium to relax during oxidative stress, since urate does not react with the radical nitric oxide (Becker, 1993). Urate and ascorbate both retard oxidation of a phospholipid bilayer initiated in the aqueous phase, but not oxidation initiated in the lipid phase (Burton and Ingold, 1986). However, as mentioned above, urate is less effective in preventing the initiation.

2.2.4 *Kynurenines*

The antioxidant properties of certain tryptophan metabolites of the kynurenine pathway have been investigated (Christen *et al.*, 1990, 1992). In



208
3-hydroxykynurenine



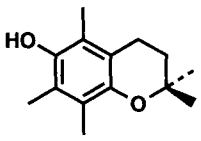
209
3-hydroxyanthranilic acid

particular, 3-hydroxykynurenine (**208**) and 3-hydroxyanthranilic acid (**209**) inhibited peroxyl radical-mediated oxidation of B-phycoerythrin more effectively than ascorbate or the hydrophilic vitamin E analogue trolox (**312**). 3-Hydroxykyurenine may be of particular physiological relevance, since its concentration in the lung is increased threefold during viral pneumonia accompanied by oxidative stress (Christen *et al.*, 1990).

3 Semisynthetic Analogues of Endogenous Antioxidants

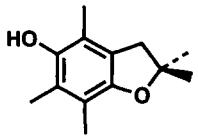
3.1 TOCOPHEROL ANALOGUES

Several efforts have been made to develop tocopherol analogues by taking advantage of their inherent favourable antioxidant properties. The structure–activity relationships and antioxidant actions of tocopherols have been extensively investigated (Nilsson *et al.*, 1968, 1970; Nakamura and Kijima, 1971, 1972; Scott *et al.*, 1974, 1976; Burton *et al.*, 1983; Mukai *et al.*, 1984, 1989a, b; Burton and Ingold, 1986). The relative antioxidant capacities of tocopherols in homogeneous solutions are independent of the nature of the oxyradicals used. The hydrogen transfer is the rate-controlling step (Burton *et al.*, 1985), but model studies indicate that the effect of steric hindrance is small (Mukai *et al.*, 1987). The high reactivities of α-tocopherol and the truncated analogue **300** have been attributed to the stereoelectronic effects conferred by the fused six-membered heterocyclic ring (see structure **205**). The fused ring ensures that the orientation of the 2p-type lone-pair orbital of the ring oxygen becomes almost perpendicular (approximately 73°) to the aromatic ring, thus optimizing stabilization of the semioccupied molecular

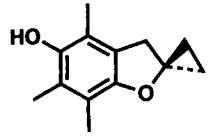


300

orbital in the developing phenoxyl radical (Burton *et al.*, 1980, 1981; Burton and Ingold, 1986). An even better overlap (approximately 84°) is provided in the corresponding dihydrobenzofuran **301**, which also is a slightly better antioxidant compared with the α-tocopherol system **300** (Burton and Ingold, 1986).



301

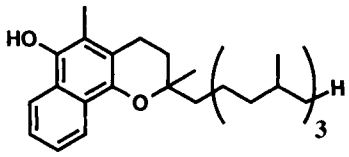


302

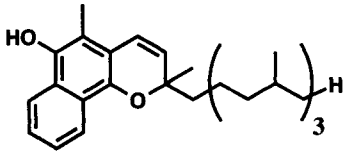
A cyclopropane spiro-annelated dihydrobensofuran, **302**, has been developed to enhance further the stereoelectronic properties of the system (Gilbert and Pinto, 1992). However, the spirocyclopropyl group did not enhance the antioxidant activity in relation to the gem-dimethyl analogue **301**. AM1 calculations could correctly rank the relative antioxidant abilities.

3.2 LIPOPHILIC ANALOGUES

Few of the first-line tocopherol analogues have the lipophilic phytyl side-chain in the 2-position required for phospholipid penetration, and accordingly their *in vivo* activity could be expected to be inferior to that of the vitamin (Mukai *et al.*, 1989a). Thus, phytyl side-chain analogues **303** and **304**, with modifications in the aromatic annelated system, have been made to produce compounds with higher antioxidant activities than α-tocopherol (Mukai *et al.*, 1989a, b). The absolute reactivities of the tocopherols to oxyradicals increase with the number of electron-donating alkyl groups in the aromatic ring. The vitamin K_1-chromanol (**303**) and K-chromenol (**304**) were found to be five- to sevenfold more active than α-tocopherol itself.

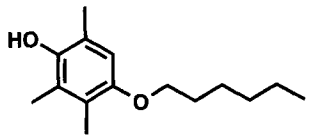


303
vitamin K_1-chromanol



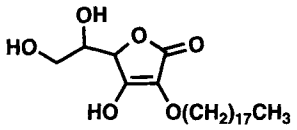
304
vitamin K_1-chromenol

The lipophilic analogue 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ, **305**) has been developed as an antioxidant with antimutagenic and anticarcinogenic activity against most carcinogenic heterocyclic amines by blocking their metabolic activation (Hirose *et al.*, 1994). HTHQ possessed stronger antioxidant activity than BHT (butylated hydroxytoluene, i.e. 2,6-di-*tert*-butyl-4-methylphenol), propyl gallate (cf. **435**) and α-tocopherol in lipid peroxidation assays utilizing rat liver microsomes.

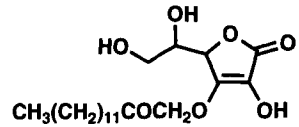


305
HTHQ

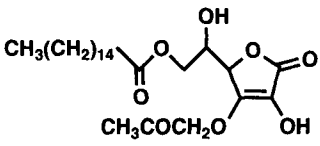
By similar reasoning, ascorbic acid has been converted into more lipophilic derivatives (**306–308**) by acylation or alkylation at the 2-, 3- or 6-position (Tanaka and Yamamoto, 1966; Kato *et al.*, 1988; Nihro *et al.*, 1991a, b; 1992). Alternatively, the 5- and 6-hydroxy groups have been acetalized with lipophilic aldehydes such as dodecanal (**309**; Bharucha *et al.* 1980). 2-*O*-octadecylascorbic acid (**306**, CV-3611) reduced myocardial lesions induced by ischaemia and reperfusion in rats (Kato *et al.*, 1988). A series of 3-*O*-alkylascorbic acids, such as 3-*O*-[(dodecylcarbonyl)methyl]-ascorbic acid (**307**, HX-0112), were found to be good chain-breaking antioxidants with high affinity for biomembranes (Nihro *et al.*, 1991a, b). Accordingly, HX-0112 was protective against reperfusion injury without influencing haemodynamics (Nihro *et al.*, 1992). CV-3611 and the 5,6-*O*-dodecylideneascorbic acid (**309**)



306
CV-3611



307
HX-0112



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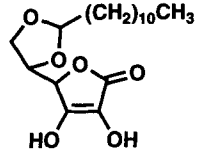
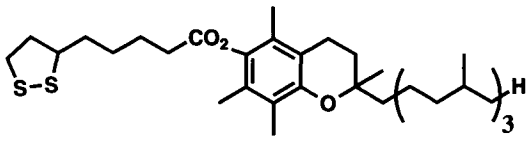


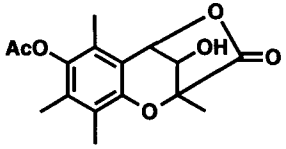
309

also showed pronounced effects on reperfusion injury, but reduced arterial blood pressure and heart rate in rats.

A series of potential anti-inflammatory antioxidants (e.g. **310**) have been made by esterification of lipoic acid with α-tocopherol (Sada and Wada, 1991). The compound is an antioxidant with cytoprotective activity against free radical-induced toxicity associated with decreased levels of glutathione (Sumathi *et al.*, 1993). The combined antioxidant **310** inhibited the oxidation of linoleic acid slightly better than α-tocopherol. It also blocked carrageenan-induced rat paw oedema after oral administration (Sada and Wada, 1991).



310
α-tocopheryl lipoate

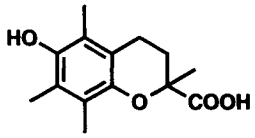


311

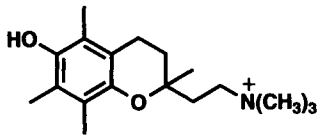
A series of derivatives containing acetylated and lactonized hydroxy groups, as exemplified by **311**, has been developed as prodrugs, probably with improved distribution, for crisis treatment of reperfusion injury in connection with stroke, coronary infarction and surgical interventions, or for treatment of inflammatory bowel disease (Grisar *et al.*, 1993a).

3.3 HYDROPHILIC ANALOGUES

Water-soluble tocopherol analogues have also been made in order to utilize their efficient scavenging properties, but alter their distribution, especially in connection with treatment of oxidative stress during myocardial infarction. Trolox (**312**), the most well-known example, has a distribution coefficient (log D) of −1.02 (octanol/water, pH 7.4). It has been found to reduce infarct size in dogs subjected to myocardial ischaemia followed by reperfusion (Mickle *et al.*, 1989). The hydrophilic (log P = −0.60) quaternary ammonium analogue MDL 73404 (**313**) has been found to have cardioselective distribu-

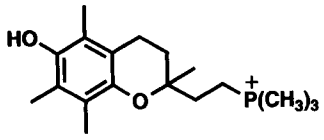


312
trolox



313
MDL 73404

tion and to be active in a myocardial reperfusion infarct rat model (Grisar *et al.*, 1991). The same laboratory has extended this approach to claim the corresponding dimethylamino (Robinson and Heineke, 1992), phosphonium (**314**) and sulfonium (**315**) analogues (Grisar *et al.*, 1993b, c) and quaternary hydrazides **316** (Grisar *et al.*, 1993d) for the treatment of especially inflammatory bowel disease and reperfusion damage.



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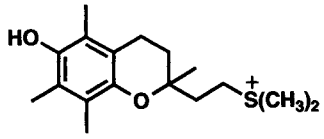

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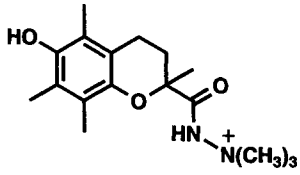
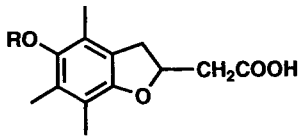


316

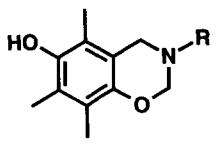
The tocopherol analogue IRFI-005 (**317**) was designed with the same intention as the above hydrophilic derivatives (Campo *et al.*, 1994). However, IRFI-005 was found to be unstable to light and heat and was therefore converted into the prodrugs IRF-016 (**318**) and IRFI-048 (**319**). The acetate IRFI-016 prevented lipid peroxidation and enzyme release induced *in vitro*



	R	
IRFI-005	H	**317**
IRFI-016	CH_3CO	**318**
IRFI-048	CH_3	**319**

by oxidant injury in human bronchial epithelial cells and pulmonary macrophages. It also showed cardioprotective effects in the rat. The methyl ether IRFI-048 has a longer half-life than the acetate and was found to reduce significantly the myocardial infarct size in an ischaemia–reperfusion rat model in a dose-dependent manner. A pronounced reduction in neutrophil infiltration was noted both in the area of risk and the necrotic area. IRFI-048 is metabolized in the cells of intestinal villi and the active metabolite IRFI-005 can rapidly reach the circulation.

A Japanese group has made isosteric dihydrobenzoxazine derivatives such as **320** by reacting trimethylhydroquinone with paraformaldehyde and amines (exemplified with R = $PhCH_2$), which were claimed as antioxidants, ulcer inhibitors and anti-ischaemics (Sakane *et al.*, 1989). A related series (**321**) of compounds containing a 1,3-benzoxathiole (R = H, methyl or propyl) potently inhibited lipid peroxidation *in vitro* but showed only a small hypolipidaemic effect *in vivo* (Yoshioka *et al.*, 1989). The investigators extended their work by incorporating 1,3-benzoxathiole or chroman moieties into known hypolipidaemic and hypoglycaemic agents, and found that CS-045 (**322**) also reduced alloxan-induced lipid peroxidation *in vivo*.



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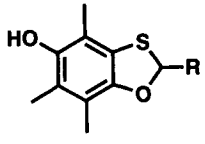

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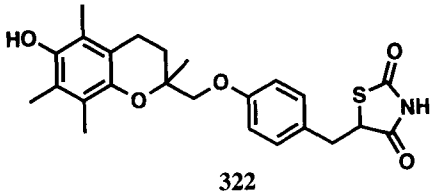
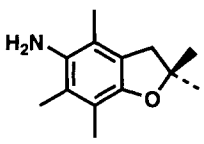


322
CS-045

A large number of 5-amino substituted 2,3-dihydrobenzofuran derivatives, such as **323**, the direct analogue of **301**, have been developed (Aono *et al.*, 1992b). A series of amide analogues of trolox, e.g. **324**, were designed as inhibitors of lipid peroxidation and prostanoid biosynthesis (Le Baut *et al.*, 1992a, b). The corresponding thiocarboxamides were studied by the same investigators with respect to inhibition of lipid peroxidation and leukotriene B_4 synthesis. For example, **325** was reported to be a potent inhibitor of spontaneous (MDA) and induced (iron/ascorbate) lipid peroxidation (Le Baut *et al.*, 1994).



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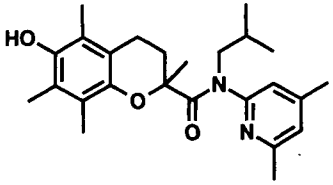

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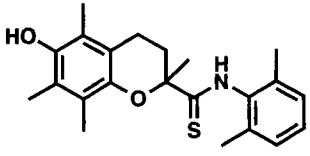
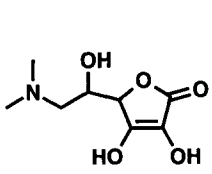


325

A series of quaternary and tertiary amine derivatives of ascorbic acid, e.g. **326**, were claimed to be useful as free radical scavengers in the treatment of rheumatoid arthritis (Marciniak and Grisar, 1993) and cancer (Suskovic *et al.*, 1994). The ascorbic acid α-tocopheryl phosphate ester **327** inhibited lipid peroxidation in rat brain homogenates to a slightly higher degree than α-tocopherol and reduced the size of the myocardial infarct in reperfused



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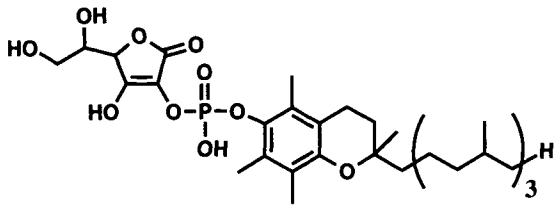
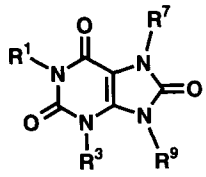


327

rat heart to about 50% at 5 mg per kg intravenously (Shimamoto and Ogata, 1989).

3.4 URIC ACID ANALOGUES

Uric acid and 5,6-diaminouracil have been *N*-alkylated with long-chain alkyl groups to produce a series of derivatives such as **328** and **329**, having a wide range of log P values (Fraisse *et al.*, 1993). The compounds were investigated for antioxidant properties (TBARS formation in the presence of mitochondrial membranes) and the ability to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) as well as lipid radicals in homogeneous or liposomal systems. The diaminouracils (**329**) may be mono-*N*-alkylated without loss of reactivity towards DPPH, but the 5-amino group seems necessary. The presence of two protons are essential in the urates (**328**), one on *N*-7 and one on *N*-3 or *N*-9, for maximal reducing capacity. Maximal protection of lipid peroxidation in mitochondrial membranes required urates (**328**) with log P > 3 (dodecyl and hexadecyl derivatives) and diaminouracils (**329**) with log P = 3–6 (decyl and dodecyl (**330**) derivatives). The antioxidant properties appear to be highly related to radical-scavenging activity, but 7-*N*-dodecyluric acid is an antioxidant despite low chain-breaking activity. The influence of other mechanisms, such as metal ion chelation or redox reactions, cannot be excluded. Dodecylurates and decyl- and dodecyldiaminouracils were chosen for extended investigations and they have also been subject to a patent application (Fraisse *et al.*, 1991).



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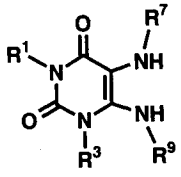

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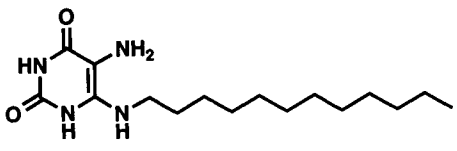


330

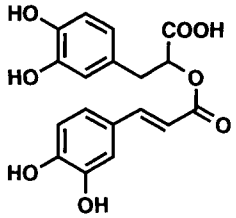
4 Natural Products

Many anti-inflammatory agents have been found in plants and marine sources (summarized by Lewis, 1989). Several antioxidants exist in plants, with the flavonoids being the major group. In this chapter, only some representative examples of natural products from different classes associated with radical scavenging or antioxidant properties are discussed. Interestingly, Oriental cooking by prolonged heating at 80–95°C, subsequent brewing and final lipophilization by emulsification in oil liberates identifiable low-molecular-weight antioxidants, originally incorporated in high-molecular-weight compounds (Niwa *et al.*, 1988).

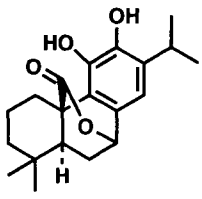
4.1 PHENOLIC NATURAL PRODUCTS

Most natural products with antioxidant activity isolated from plants, e.g. curcumin, catechin, vitamin E, myricetin, quercetin, kaemferol, vanillin, gallic acid and caffeic acid, contain phenolic groups. Usually, at least two oxygen functions are present per aromatic moiety to provide sufficient stabilization of the formed phenoxyl radical. Löliger (1991) has provided a short review of potential soures of natural antioxidants. However, many investigations used crude extracts rather than isolated substances, which makes conclusions regarding mechanisms and efficiencies difficult.

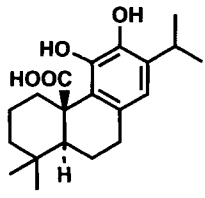
Potent synthetic antioxidants, such as BHA (butylated hydroxyanisole, i.e. 2-*tert*-butyl-4-methoxyphenol), BHT and propyl gallate, and natural antioxidants (e.g. eugenol, vanillin, nordihydroguaiaretic acid (NDGA), ascorbic acid and α-tocopherol) are widely used to preserve food from oxidative degradation of the polyunsaturated fatty acids of the lipids (Löliger, 1991). There is a current trend to promote and use natural rather than synthetic antioxidants (such as BHT and BHA). Rosemary (*Rosmarinus officinalis* L.) contains several active antioxidant compounds (**400–404**), i.e. rosmarinic acid, carnosol, carnosic acid, carsolic acid and rosmaridiphenol (Löliger,



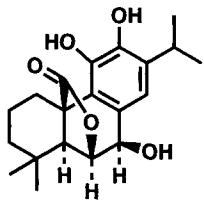
400
rosmarinic acid



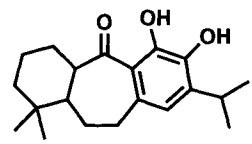
401
carnosol



402
carnosic acid



403
carsolic acid

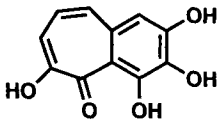


404
rosmaridiphenol

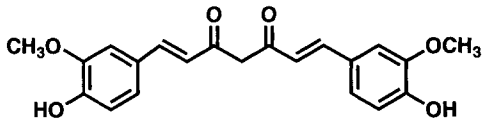
1991). Crude and refined extracts are commercially available as antioxidants and flavouring ingredients for foods. It is not surprising that attention has also been focused on these agents as potential therapeutic drugs.

Rosmarinic acid (**400**) does not influence chemotaxis, phagocytosis or oxygen consumption by human PMNLs but decreases cytotoxicity and scavenges hydrogen peroxide (van Kessel *et al.*, 1985). Rosmarinic acid also inhibits pulmonary injury, mimicking early adult respiratory distress syndrome (ARDS), induced by infusion of zymosan-activated plasma to rabbits under hypoxia (Nuytinck *et al.*, 1985). It is likely that generation of ROS plays an important role in the model and the anti-inflammatory effect was ascribed to antioxidant properties.

Purpurogallin (**405**), a well-known iron chelator (Kontoghiorghes *et al.*, 1986), and its mono- and diglucosides have been claimed as cytoprotective agents during ischaemia–reperfusion injury (Wu, 1992). Purpurogallin was a more effective inhibitor (IC_{50} 0.12 mM) than trolox (IC_{50} 0.74 mM) of radical-induced haemolysis. An intravenous dose of about 9 μmol kg^{-1} purpurogallin provided 75% organ salvage in a rabbit heart ischaemia–reperfusion model. Purpurogallin prolonged the survival of rat hepatocytes subjected to oxidative stress (hypoxanthine/xanthine oxidase) to a greater extent than did ascorbate, mannitol, trolox, SOD or catalase (Wu *et al.*, 1991). The *in vitro* effect was confirmed in a clinically more relevant rat model of hepatic ischaemia–reperfusion.



405
purpurogallin

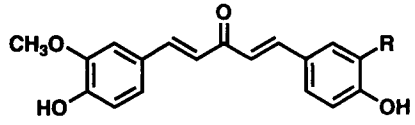


406
curcumin

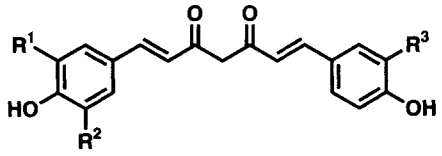
Curcumin (**406**) is a bright yellow pigment from the rhizome of the perennial herb *Curcuma longa*, responsible for the colour of curry. It has been found to be a potent anti-inflammatory agent and a good scavenger of hydroxyl radicals at high concentrations (Srimal, 1987; Lewis, 1989) but to

generate hydroxyl radicals through the Fenton reaction by reducing Fe^{III} to Fe^{II} at low concentrations (Kunchandy and Rao, 1989). Perhaps of greater importance for the anti-inflammatory action, curcumin was a potent scavenger of superoxide radicals generated by the alkaline dimethyl sulfoxide method (Kunchandy and Rao, 1990). Curcumin has also been found to inhibit phorbol ester-induced lipid peroxidation and formation of 8-hydroxydeoxyguanosine from deoxyguanosine in mouse fibroblast cells (Shih and Lin, 1993). These data indicate that curcumin inhibits phorbol ester-induced tumour promotion by acting as a hydroxyl radical scavenger.

The rhizomes of *Curcuma domestica* (Zingiberaceae) have been used as a yellow colouring agent and a remedy in traditional Asian medicine. Their antioxidant and anti-inflammatory effects have been attributed to the main constituent curcumin (**406**) (Srimal, 1987). Two new phenolics, **407** and **408**, related to curcumin and the three curcuminoids **409–411** have recently been reported to inhibit autoxidation of linoleic acid (Masuda *et al.*, 1993a). The two phenolics **407** and **408** were more active antioxidants than curcumin (**406**). All compounds were effective inhibitors of phorbol ester-induced oedema in the mouse ear. Surprisingly, the bisdemethoxycurcumin (**410**) had the strongest activity of the derivatives investigated (**406–411**) in the latter test.

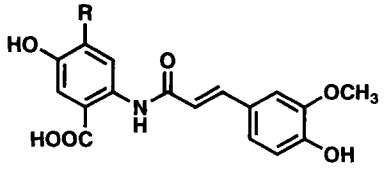


	R
407	OMe
408	H



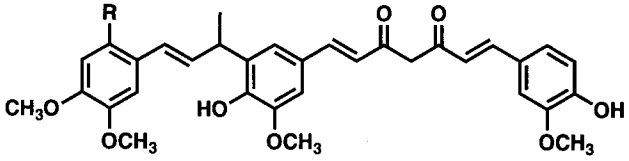
	R^1	R^2	R^3
406	OMe	H	OMe
409	OMe	H	H
410	H	H	H
411	OMe	OMe	OMe

Oat grains are rich in lipids, which are protected by antioxidants such as tocopherols. Recently, two new antioxidants were isolated from oat grains, i.e. avenanthramide 1 and 2 (**412** and **413**), which inhibit oxygen consumption in a linoleic acid system (Häll Dimberg, 1993). The avenanthramide 2 was the most active, having 57% of the activity of α-tocopherol.

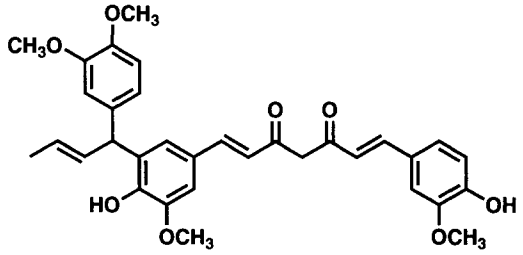


	R	
avenanthramide 1	H	**412**
avenanthramide 2	OMe	**413**

Rhizomes of *Zingiber cassumunar* (Zingiberaceae) have also been used in tropical traditional medicine as an anti-inflammatory agent (Masuda *et al.*, 1993b). Recently, three novel antioxidants, cassumunin A, B and C (**414–416**) were isolated and characterized (Masuda *et al.*, 1993b). The inhibition of

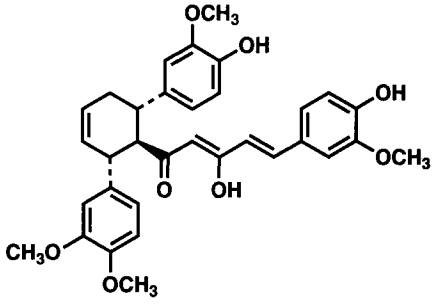


	R	
cassumunin A	H	**414**
cassumunin B	OMe	**415**

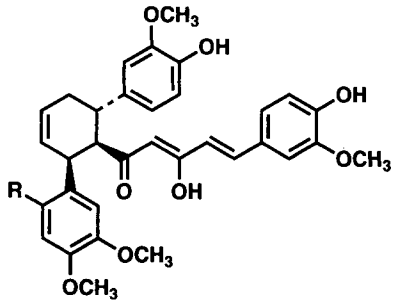


416
cassumunin C

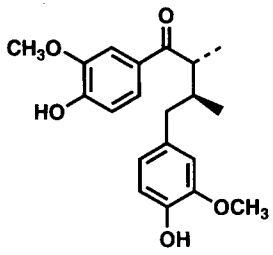
autoxidation of linoleic acid by cassumunin A, B and C was stronger or equal to that by curcumin (**406**), the diarylheptanoid moiety of the cassumunins. They also inhibited phorbol ester-induced oedema in the mouse ear. From the same source Jitoe *et al.* (1994) also isolated the novel antioxidants cassumunarin A, B and C (**417–419**). The new derivatives inhibited autoxidation of linoleic acid slightly more potently than curcumin, i.e. 93–95% for **417–419** compared with 78% for **406** at 135 μM.



417
cassumunarin A



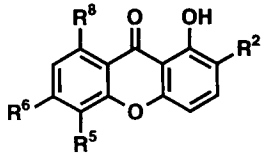
	R	
cassumunarin B	H	**418**
cassumunarin C	OMe	**419**



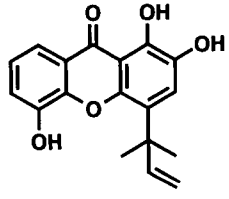
420
cinnamophilin

The lignan cinnamophilin (**420**) and three known compounds were isolated from the roots of *Cinnamomum philippinense* by a bioassay-directed fractionation (Wu *et al.*, 1994). The antioxidant activity of cinnamophilin was comparable to that of α-tocopherol.

Several xanthones documented from the *Garciania* species exhibit a wide range of biological activities such as cytotoxic and anti-inflammatory effects. From the woods of *Garcinia subelliptica* four new xanthones and a new benzophenone have been isolated and evaluated for their antioxidant activities (Minami *et al.*, 1994). The catechol derivatives **421**, **422** and **424**,

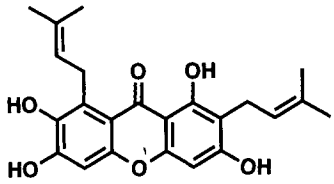


	R^2	R^5	R^6	R^8
421	OH	OH	H	H
422	OH	OMe	OMe	H
423	H	H	OMe	OH



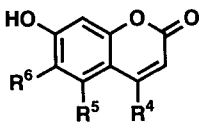
424

and the 8-hydroxylated analogue **423**, were active at 5–10 $\mu g\ ml^{-1}$ in the three assays used, *viz.* inhibition of lipid peroxidation, scavenging of the DPPH radical and scavenging of superoxide anion radicals. Some closely related 1,4-hydroquinone derivatives were inactive even at 100 $\mu g\ ml^{-1}$ (Minami *et al.*, 1994). The related γ-mangostin (**425**) has been isolated from *Garcinia mangostana* and reported to be a more potent antioxidant than BHA or α-tocopherol as assessed by the ferric thiocyanate method (Yoshikawa *et al.*, 1994).



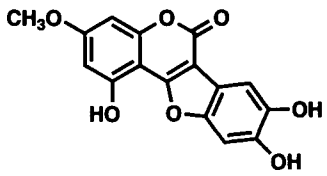
425
γ-mangostin

Coumarins are able to prevent lipid peroxidation in membranes and to scavenge superoxide anion radicals, hypochlorous acid, hydroxyl radicals ($k = 2–7 \times 10^9\ M^{-1}\ s^{-1}$) and peroxyl radicals to varying degrees depending on the substitution patterns (Payá *et al.*, 1992a, b). The *meta*-substituted 5,7-dihydroxy-4-methylcoumarin (**426**) was an effective antioxidant in this respect and it was also found to be a potent inhibitor of cyclooxygenase (Hoult *et al.*, 1994). The 6,7-dihydroxy analogues esculetin (**427**) and 4-methylesculetin (**428**) were selective 5-lipoxygenase inhibitors, probably due to their iron-chelating and reducing capabilities. The latter two 6,7-dihydroxy compounds, as well as 7,8-dihydroxycoumarin (daphnetin), inhibited rat liver microsomal lipid peroxidation and peroxyl radical-induced inactivation of lysozyme to the same extent as **426**, but they also acted as weak pro-oxidants in the $Fe_{(III)}/EDTA/H_2O_2$ system in contrast to the meta-dihydroxycoumarin **426** (Payá *et al.*, 1992a, b). 7-Hydroxycoumarin (umbelliferone) and 7-hydroxy-6-methoxycoumarin (scopoletin) do not inhibit lipid peroxidation or reduce cytochrome *c*, but they are potent hydroxyl and superoxide anion radical scavengers. The structural requirements for antiperoxidative activity of coumarins are similar to those reported for flavonoids (Payá *et al.*, 1992a).

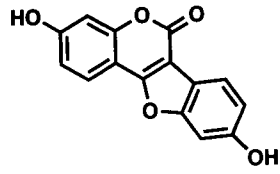


	R^4	R^5	R^6
426	Me	OH	–
427	–	–	OH
428	Me	–	OH

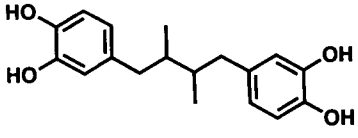
The coumestan derivative wedelolactone (**429**) has been isolated from *Eclipta alba* (Asteraceae) and *Wedelia calendulacea* (Asteraceae), used in India for treating liver diseases (Wagner and Fessler, 1986; Lewis, 1989). Wedelolactone was a potent inhibitor (IC_{50} 2.5 μM) of 5-lipoxygenase in porcine leukocytes with no effect on cyclooxygenase. The activity was comparable to that of NDGA (**431**) (IC_{50} 1.5 μM), but considerably higher than that for the related coumestrol (**430**) (IC_{50} 100 μM) lacking the catechol grouping. The effect on 5-lipoxygenase was ascribed to the radical-scavenging effect seen in PMNLs stimulated by phorbol ester.



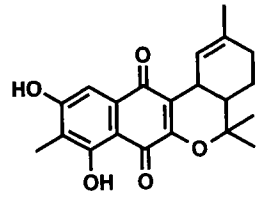
429
wedelolactone



430
coumestrol



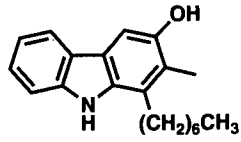
431
NDGA



432
CL190Y2

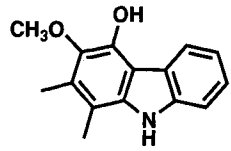
The quinone CL190Y2 (**432**) has been reported as an antioxidant from *Streptomyces aeriouvifer* (Seto *et al.*, 1991). It inhibited lipid peroxidation in rat liver microsomes (IC_{50} 5.3 $\mu g\,ml^{-1}$) more effectively than vitamin E (IC_{50} 9.4 $\mu g\,ml^{-1}$).

An interesting carbazole, carazostatin (**433**), has been isolated from the microorganism *Streptomyces chromofuscus* (Kato *et al.*, 1989). The heptyl



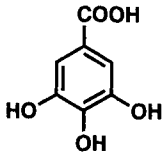
433
carazostatin

side-chain in combination with the two *para*-oriented heteroatoms makes it an efficient antioxidant. Carazostatin inhibits lipid peroxidation induced by free radicals (iron/ascorbate) in rat brain homogenates with a low IC_{50} value of 0.17 μM. In the same model, BHT had an IC_{50} value of 4.9 μM. Other phenolic carbazoles, e.g carbazomycin B (**434**), have been reported as antibiotics (Sakano and Nakamura, 1980) and lipoxygenase inhibitors (Hook *et al.*, 1990).

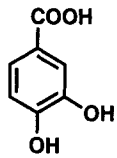


434
carbazomycin B

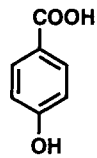
Gallic acid (**435**) and protocatechuic acid (**436**) were tenfold more active as inhibitors of luminol-dependent chemiluminescence generated by zymosan-stimulated PMNLs compared with the non-catecholic analogues **437** and **438** (Kroes *et al.*, 1991). The scavenging activity of gallic acid for superoxide anion radicals was assessed in a cell-free system, suggesting that the inhibitory activity by **435** in the PMNL assay was mediated by the superoxide-scavenging effect.



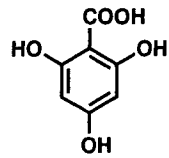
435
gallic acid



436
protocatechuic acid

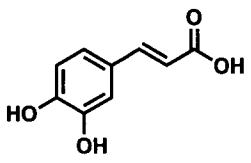


437

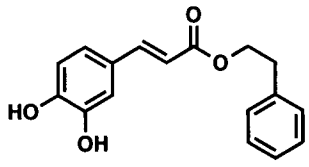


438

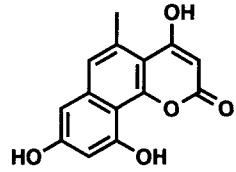
Caffeic acid phenethyl ester (**440**, CAPE) is an antioxidant component of propolis extracts (Grunberger *et al.*, 1988). CAPE inhibits 5-lipoxygenase by a completely uncompetitive mechanism and it decreases chemiluminescence of phorbol ester-stimulated neutrophils and of a cell-free superoxide-generating (xanthine–xanthine oxidase) system (Sudina *et al.*, 1993). The effectiveness of caffeic acid (**439**) and CAPE as lipoxygenase inhibitors is related to their redox properties.



439
caffeic acid



440
CAPE



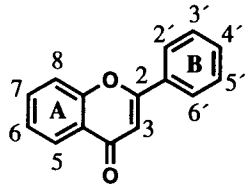
441
Q-2819

The compound Q-2819 (**441**) has been isolated from a bacterium of the genus *Chrysosporium* (Imai *et al.*, 1991). Q-2819 scavenged superoxide anion radicals and showed an antioxidant capacity comparable with that of α-tocopherol in the DDPH assay. It was also found to inhibit ferric-induced lipid peroxidation with an IC_{50} value of 50 μM.

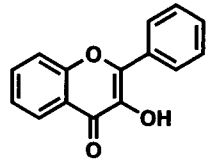
4.2 FLAVONOIDS

Flavonoids are present at high levels (>1 mM) in a variety of health foods, fruits and vegetables. Some examples of the various classes of flavonoids are shown in Scheme 4. The flavonoids are one of the most interesting groups of natural products from a medicinal chemistry standpoint, since they are

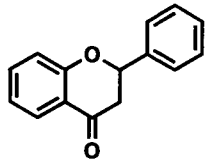
Scheme 4 Common flavonoids



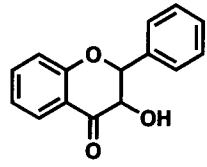
flavone



flavonol

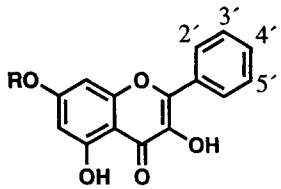


flavanone



anthocyanidine

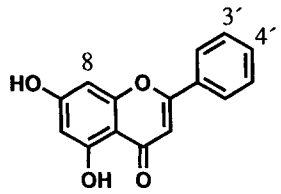
associated with an array of biological activities, e.g. anti-inflammatory, antioxidant, antihepatotoxic, antitumour, antihypertensive, antibacterial, antiviral and antiallergic activities (reviewed by Pathak *et al.*, 1991). Flavonoids and other plant phenolics are claimed to inhibit, for example, lipoxygenase, lipid peroxidation, NADPH oxidase and xanthine oxidase, as reviewed by Cody *et al.* (1988), Larson (1988), Lewis (1989), Aruoma (1991) and Löliger (1991). The flavonols myricetin (**442**) and quercetin (**443**) act as efficient chain-breaking inhibitors of iron-dependent lipid peroxidation in rat liver microsomes. However, these flavonols and other food antioxidants can behave as pro-oxidants in non-lipid model systems containing $Fe_{(III)}$/EDTA/H_2O_2 by reducing the iron and stimulating OH· production (Laughton *et al.*, 1989; Aruoma, 1991).



flavonols

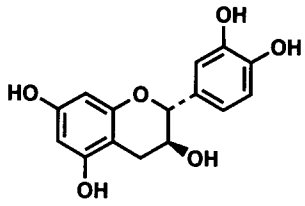
		2'	3'	4'	5'	R
442	myricetin	H	OH	OH	OH	H
443	quercetin	H	OH	OH	H	H
444	kaempferol	H	H	OH	H	H
445	galangin	H	H	H	H	H
446	morin	OH	H	OH	H	H
447	rhamnetin	H	OH	OH	H	Me

The oxygen radical-scavening properties have been investigated for a number of flavonoids (Damon *et al.*, 1986). A 2,3-double bond, a 4-keto function, a 3-hydroxy group, two *ortho* hydroxyls in the B-ring, and two *meta* hydroxyls in the A-ring increase the scavenging activity, whereas glycosylation at the 3- or 7-position abolishes activity. In another study, the capacity of flavonoids to scavenge hydroxyl radicals generated from UV photolysis of hydrogen peroxide was investigated (Husain *et al.*, 1987). The activity increased with the number of hydroxyl groups in the B-ring, i.e. myricetin (**442**) > quercetin (**443**) > rhamnetin (**447**) > morin (**446**) > diosmetin (**449**) > apigenin (**448**) > catechin (**451**) > kaempferol (**444**) > flavone. However, no positive contribution could be attributed to a 3-hydroxy group (Pathak *et al.*, 1991).

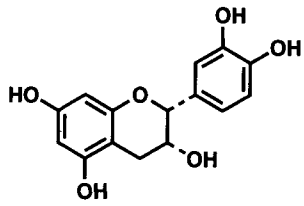


flavones

		3'	4'	8
448	apigenin	H	OH	H
449	diosmetin	OH	OMe	H
450	hypolaetin	OH	OH	OH



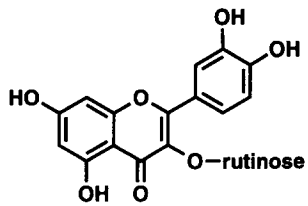
451
(+)-catechin



452
(-)-epicatechin

A series of flavonoid glycosides and the corresponding aglycones have been compared with respect to their ability to inhibit 5-lipoxygenase and cyclooxygenase (Moroney *et al.*, 1988). The aglycone hypolaetin (**450**) derived from the 8-glycoside from *Sideritis mugronensis*, which is used in Spanish traditional medicine, proved to be equipotent with quercetin (**443**) in inhibiting 5-lipoxygenase. The study confirmed the requirements for 3′- and 4′-hydroxy substituents in the B-ring (**443**, **450**), compatibility with methoxylation of the A-ring, lack of importance of a 3-hydroxy group (**450**) and the negative effects of glycosylation in the 3-, 7- or 8-positions for the free radical-scavenging effects that are likely to be involved in the inhibition of 5-lipoxygenase. Somewhat different structural requirements were found for cyclooxygenase inhibition (Moroney *et al.*, 1988).

The controversy regarding the superoxide radical-scavenging ability of flavonoids has been investigated in detail by Jovanovic *et al.* (1994). The reactivity of flavonoids toward superoxide anion depends on their redox properties, which are dictated primarily by the B-ring substituents. The highest reactivities were found for the 3′,4′-dihydroxy derivatives quercetin (**443**) and rutin (**453**) with rate constants of 4.7×10^4 and $5.1 \times 10^4\ \text{M}^{-1}\text{s}^{-1}$, respectively, for the reduction of superoxide anion. The *meta*-substituted derivative morin (**446**) is 30-fold less reactive than **443**. A very low reactivity ($k = 8.8 \times 10^2\ \text{M}^{-1}\text{s}^{-1}$) was found for galangin (**445**), which is unsubstituted in the B-ring. The electrostatic repulsion between the superoxide anion and deprotonated flavonoids decreases the reaction rate by a factor of 2 for each

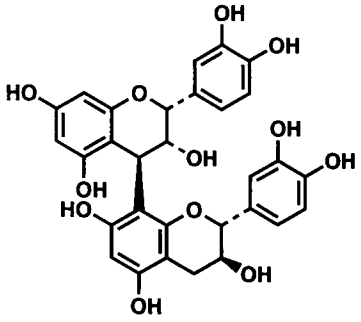


453
rutin
(quercetin-3-O-rutinose)

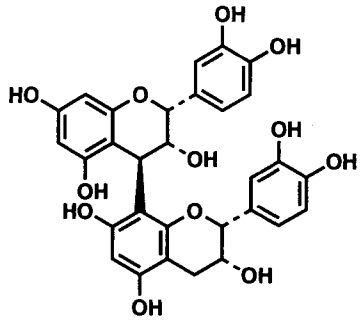
additional negative charge. Because of the relatively low pK_a (4–5), radicals from 3′,4′-dihydroxyflavonoids are negatively charged at physiological pH, which would retard their passage through membranes. The reduction potentials of flavonoid radicals (0.5–0.7 V) are lower than those of alkylperoxyl radicals (1.05 V) and superoxide anion (0.94 V) at pH 7, which means that flavonoids have the capacity to inactivate these radicals (Jovanovic *et al.*, 1994).

The anti-inflammatory effects of flavonoids have been reviewed by Pathak *et al.* (1991). In particular, quercetin (**443**) has been extensively investigated in various inflammation models, e.g. inhibition of croton oil-induced oedema, mast cell histamine secretion, release of lysosomal enzymes, oxygen consumption, free radical generation and chemotaxis. The anti-inflammatory action of various species, e.g. *Achyrocline satureioides*, *Wrightia tinctoria* and *Delonix elata*, has also been related to quercetin and analogues. The cytoprotective effects of three flavonoids in iron-loaded rat hepatocytes with respect to lipid peroxidation and intracellular enzyme release was studied by Morel *et al.* (1993). The hepatoprotective potency ranking order – catechin (**451**) > quercetin (**443**) > diosmetin (**449**) – follows the iron-chelating capacity but only partly the free radical-scavenging potency reported in the literature. Quercetin (**443**) and rutin (**453**) prevent cytotoxicity resulting from oxidation of LDL, either by inhibiting lipid peroxidation at high concentrations (IC_{50} 10–20 μM) or by blocking the cytotoxicity directly at the cellular level at lower concentrations (Nègre-Salvayre and Salvayre, 1992a). Thus, cells preincubated with the flavonoids were protected against the cytotoxic effects of previously oxidized LDL (IC_{50} 0.1–3 μM). It was suggested that dietary flavonoids could be involved in the prevention of atherosclerosis, not only by inhibition of LDL oxidation but also by increasing the cellular resistance to the damage inflicted by oxidized LDL.

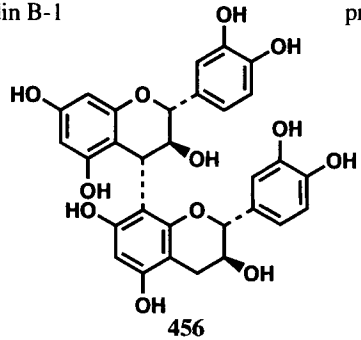
Procyanidins, i.e. (+)-catechin (**451**) and (−)-epicatechin (**452**) oligomers linked by C4→C8 or C4→C6 bonds and sometimes esterified by gallic acid (**435**) are essential grape constituents (Cheynier *et al.*, 1992). Besides contributing to the colour, flavour and ageing of wine, the procyanidines have attracted interest due to their free radical-scavenging properties (Uchida *et al.*, 1987; Ariga and Hamano, 1990; da Silva *et al.*, 1991). 3-*O*-gallate esters of epicatechin and various procyanidins (B-2, B-5 and C-1) were potent radical scavengers of DPPH, hydroxyl radical, hydrogen superoxide and superoxide anion (Uchida *et al.*, 1987). The procyanidins B-1 (**454**) and B-3 (**456**) from azuki beans were found to be very effective scavengers, especially of hydrophilic peroxyl radicals (Ariga and Hamano, 1990). The dimeric procyanidins **454** and **456** could trap eight peroxyl radicals, whereas ascorbic acid, α-tocopherol and (+)-catechin could trap only one, two and four peroxyl radicals, respectively. The induction periods (measured as oxygen uptake) were considerably longer for the procyanidins compared with ascorbic acid, (+)-catechin (**451**) and α-tocopherol.



454
procyanidin B-1



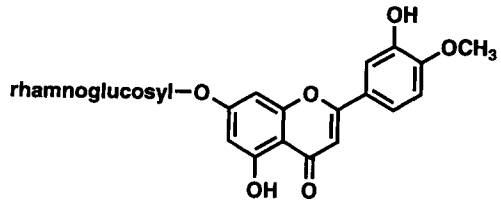
455
procyanidin B-2



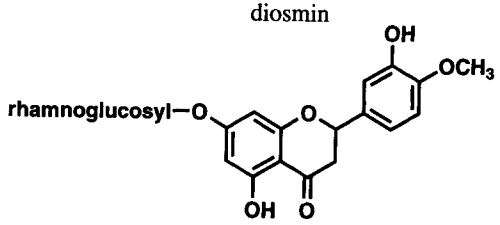
456
procyanidin B-3

Epicatechin 3-*O*-gallate and various procyanidins obtained from grape seeds were tested as scavengers of superoxide anion (compared with trolox) and of hydroxyl radical (compared with ethanol and mannitol) (da Silva *et al.*, 1991). The site of esterification by gallic acid influences the scavenging ability. The dimeric procyanidins were more effective than the monomers as observed earlier. Dimers with C4→C8 linkages were more active than procyanidins with C4→C6 bonds (B-5). Thus, the procyanidin B-2 3′-*O*-gallate was the most potent scavenger of superoxide anion radicals, i.e. twice as effective as trolox. The 3-*O*-esterified procyanidin B-2 and the aglycon procyanidin B-2 ((−)-epicatechin-(4β→8)-(−)-epicatechin, **455**) were equipotent with trolox, whereas catechin (**451**) and epicatechin (**452**) were considerably less effective scavengers of superoxide anion radicals. However, the activity of the oligomers and monomers was almost the same when expressed as monomer equivalents. Procyanidin B-2 3′-*O*-gallate was also the most potent hydroxyl radical scavenger, but the difference from cathechin and epicatechin was marginal, albeit higher than for ethanol and mannitol (da Silva *et al.*, 1991).

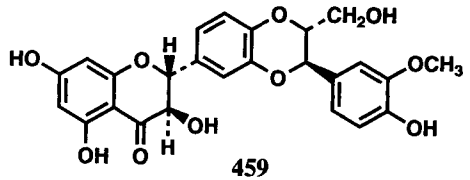
Daflon (Laboratories Servier) is a purified flavonoid fraction, S 5682, containing 90% diosmin (**457**) and 10% hesperidin (**458**) (Lonchampt *et al.*, 1989). The S 5682 fraction showed a dose-dependent protection against



457
diosmin



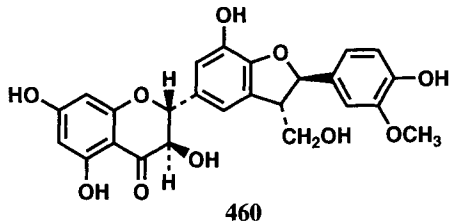
458
hesperidin



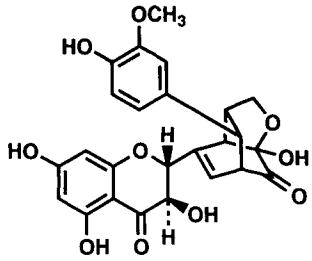
459
silybin

oxygen radicals during alloxan-induced diabetes in the rat. S 5682 also inhibited chemiluminescence (IC_{50} 16 μM) induced by generation of ROS during PMNL phagocytosis (Lonchampt *et al.*, 1989). The scavenging effects are suggested to explain anti-inflammatory and anti-oedematous effects observed in inflammatory granulomas in the rat.

The chemotactic peptide fMLP (*N*-formylmethionyl-leucyl-phenylalanine) can induce formation of superoxide anions and hydrogen peroxide from cytochalasin B-treated human neutrophils (Fantozzi *et al.*, 1983). The FMLP-activated human neutrophils evoke histamine release from mast cells in parallel with the formation of oxygen radicals (Fantozzi *et al.*, 1986). The neutrophil-mediated histamine release could be inhibited in a dose-dependent manner by silymarin, which is the active flavonoid principle of milk thistle, consisting of the three isomers, silybin (**459**), silychristin (**460**) and silydianin (**461**) (Lorenz *et al.*, 1984; Fantozzi *et al.*, 1986; Valenzuela *et al.*, 1986). Silymarin was also shown to have a direct scavenging effect on oxygen radical production by a cell-free xanthine oxidase–hypoxanthine system and to inhibit the production of superoxide anion radicals and hydrogen peroxide from fMLP-stimulated neutrophils (Fantozzi *et al.*, 1986).



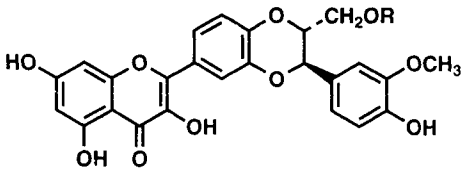
460
silychristin



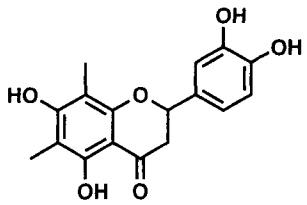
461
silydianin

The inhibitory effect of silymarin on lipoxygenase, prostaglandin synthetase, plant peroxidases and lipid peroxidation has been reported (Koch and Löffler, 1985). The three main constituents in silymarin, i.e. silybin, silychristin and silydianin (**459–461**), proved to be the most potent inhibitors of *N*-ethyl maleimide-induced lipid peroxidation in human platelets in a study of several representative flavonoids, cathechines and phenolic acids (Koch and Löffler, 1985). Only rutin (**453**) and caffeic acid (**439**) were devoid of inhibitory effects. Silychristin (**460**) was the most active substance (IC_{50} 0.05 mM), followed by silydianin and silybin. The closely related quercetin (**443**), aglycon of the glycoside rutin (**453**), was significantly less active (IC_{50} 1.8 mM). Catechin (**451**) and epicatechin (**452**) were tenfold less active than the most active silymarin constituent, **460**, but equipotent with the reference α-tocopherol (IC_{50} 0.44 mM) in this test. Surprisingly, the 4′-hydroxylated kaempferol (**444**) was equipotent with the 3′,4′-dihydroxylated analogue **443**.

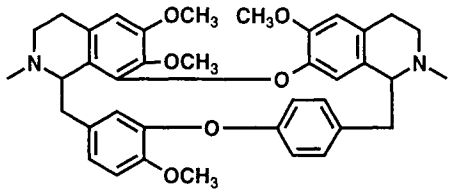
The radical-scavenging (DPPH) and antioxidant (MDA) properties of silybin (**459**) have been improved by one order of magnitude by introduction of a double bond to give 2,3-dehydrosilybin (**462**) (Biffi *et al.*, 1993). The poor solubility was improved by making salts of esters of dicarboxylic acids, such as the sodium hemisuccinate (**463**) or hemiphthalate. The DPPH-scavenging activity and inhibition of lipid peroxidation was comparable for **462**, **463** and **451**, but the toxicity was reduced for **463**.



462 R = H (2,3-dehydrosilybin)
463 R = $COCH_2CH_2COONa$



464
IdB 1031



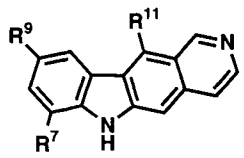
465
tetrandrine

The flavone 3′-hydroxyfarrerol (IdB 1031, **464**) was equipotent with quercetin (**443**) in scavenging DPPH and in inhibiting microsomal lipid peroxidation induced by carbon tetrachloride or iron (Ursini *et al.*, 1994). The antioxidant activity could be related to the chain-breaking activity of **464**. When tested on human neutrophils activated by fMLP, IdB 1031 inhibited the respiratory burst, probably through inhibition of protein kinase C.

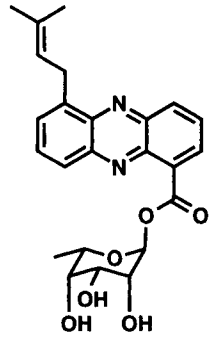
4.3 ALKALOIDS

The benzylisoquinoline tetrandrine (**465**), isolated from "hanfangji", the root of *Stephania tetrandra*, which is used in traditional Chinese medicine, has been found to have beneficial effects in the treatment of silicosis (Seow *et al.*, 1988). Tetrandrine has a suppressing effect on random movement, chemotaxis and phagocytosis of human neutrophils at a concentration of $10\,\mu g\,ml^{-1}$, but it inhibits superoxide generation from neutrophils at a considerably lower dose of $0.1\,\mu g\,ml^{-1}$. This effect was related to the ability of **465** to scavenge superoxide radicals.

Structure–activity relationships in a series of ellipticine analogues (**466**) were investigated with respect to antioxidant activity (autoxidation of linolenate) and ability to reduce DPPH radicals (Rousseau-Richard *et al.*,



	R^7	R^9	R^{11}
466	H	H	Me
467	H	OH	Me
468	OH	H	Me
469	H	OH	H

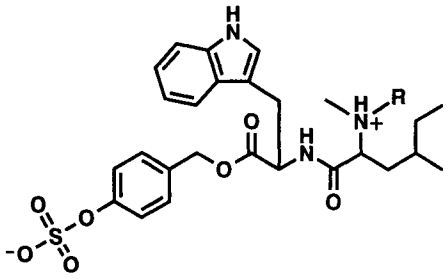


470
phenazoviridin

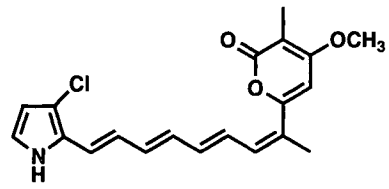
1990). Both activities required the presence of a hydroxy group. The antioxidant activity decreased in the order **467** > **469** = **468** > **466**, which shows the importance of the position of the hydroxyl group and the beneficial influence of the 11-methyl group. The activity of the 9-hydroxy derivative **467** was comparable to that of α-tocopherol and propyl gallate. A good correlation was observed between cytotoxic effects and antioxidant properties, but not between cytotoxic effects and the capability to reduce DPPH in this series of ellipticine analogues (Rousseau-Richard *et al.*, 1990). Thus, the presence of the 9-hydroxy group enables these agents to become bioactivated by formation of oxy-radicals and electrophilic quinoneimines with ability to interact with topoisomerase II, i.e. features likely to be important for the antitumour effects (Auclair and Paoletti, 1981; Auclair, 1987).

Phenazoviridin (**470**) has been isolated from a *Streptomyces* species and found to have strong inhibitory activity (IC_{50} 15 μM) against iron/ascorbate-induced lipid peroxidation (MDA) in rat brain homogenates (Kato *et al.*, 1993). In this test, α-tocopherol was less effective ($IC_{50} > 100\ \mu$M). Phenazoviridin also showed a pronounced protective activity against cyanide-induced acute hypoxia in mice when given 100 mg kg^{-1} intraperitoneally.

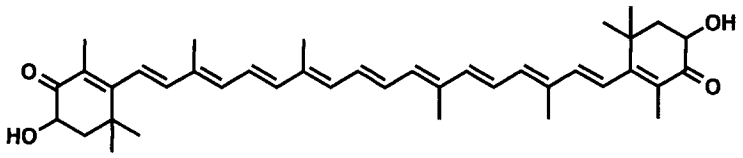
Three unusual indole derivatives (denticin A, B and C), isolated from the seaweed *Martensia denticulata*, had inhibitory activity towards UVB-induced lipid peroxidation of erythrocyte membrane ghosts with IC_{50} values of 160–300 μg ml^{-1} (cf. α-tocopherol, IC_{50} 60 μg ml^{-1}) (Murakami *et al.*, 1994).



471 R = H denticin B
472 R = Me denticin C



473
rumbrin



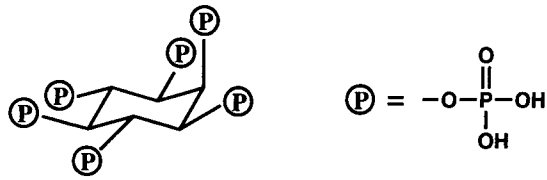
474
astaxanthin

The most potent members were denticin B (**471**) and C (**472**). Rumbrin (**473**) has been isolated from the fungus *Auxarthron umbrinum* in a screening programme for cytoprotective agents (Yamagishi *et al.*, 1993). Rumbrin prevented cell death induced by calcium and inhibited lipid peroxidation in rat brain homogenates with an IC_{50} value of 0.47 $\mu g\,ml^{-1}$.

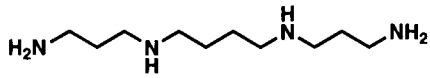
4.4 MISCELLANEOUS

Astaxanthin (**474**) is a carotenoid pigment found in many plants and animals, and the compound is easily available from the yeast *Phaffia rhodozyma* (Kurashige *et al.*, 1990). The antioxidant effects of **474** have been assessed both *in vitro* and *in vivo*. Thus, astaxanthin inhibits Fe^{II}-induced oxygen consumption and oxidative phosphorylation of mitochondria from vitamin E-deficient rats, inhibits lipid peroxidation of erythrocyte membranes (TBARS) from vitamin E-deficient rats upon supplementation in the diet, and also inhibits Fe^{II}-induced lipid peroxidation of mitochondria obtained from vitamin E-deficient rats 100–500 times more strongly than vitamin E (Kurashige *et al.*, 1990).

Phytic acid (**475**) is an iron chelator that assists in protecting seeds from oxidative damage by maintaining iron in the trivalent state, and thus obstructing the generation of hydroxyl radicals (Graf and Eaton, 1990). By the same mechanism phytic acid may protect against inflammatory bowel



475
phytic acid



476
spermine

disease and colonic cancer. Similarily, the polyamine spermine (**476**) is an antioxidant by virtue of its ability to form an unreactive complex with iron and to inhibit the generation or transport of superoxide anion from stimulated granulocytes.

5 Synthetic Antioxidants

From a medicinal chemical point of view, there are important differences between the two distinct approaches of preventive and chain-terminating antioxidant agents. Firstly, there is no question that the development of enzyme-mimicking low-molecular-weight compounds is a majestic challenge from a mechanistic perspective. It is always going to be difficult to produce substances that efficiently and catalytically reduce and detoxify reactive species such as the superoxide anion, hydrogen peroxide, hypohalites, singlet oxygen and the hydroxyl radical. Such substances, which so far have relied to a large extent on coordination compounds of transition metals, must also be stable enough under *in vivo* conditions to avoid toxicity problems. An additional difficulty to overcome is to provide a mechanism of action that guarantees that leakage of reactive byproducts or intermediates are minimized. The enzyme mimics, on the other hand, appear perhaps the most promising agents for antioxidant intervention in a multitude of disease states.

With regard to chain-terminating agents, pharmaceutical intervention of pathophysiological processes forceful enough to overwhelm the specialized defence provided by evolution requires very potent antioxidant compounds

endowed with physicochemical properties that provide a correct distribution in cells and tissues. One may identify a number of criteria, which should apply to a candidate for the application as a chain-terminating pharmaceutical antioxidant.

- A pharmaceutical antioxidant will have to be present in a high concentration in the target tissue, since the mode of action is to quench reactive species (such as alkylperoxyl radical).
- Local administration, or a good means of specifically delivering the antioxidant agent to the target tissue, may be required.
- A combination of preventive and chain-terminating modes of action may be needed to reach significant effects *in vivo* (these may reside in the same compound or be accomplished by combination therapy).
- A lead compound should be at least comparable to α-tocopherol or ascorbate with regard to intrinsic efficacy for chain termination.
- A chain-terminating antioxidant regenerable by endogenous sacrificial reducing agents will be far superior to a stoichiometric agent (this can be addressed experimentally).
- A high intrinsic antioxidant capacity in simple chemical or *in vitro* systems may produce false leads, since compounds that are too easily oxidizable may suffer from complications caused by pro-oxidant properties, redox cycling, rapid oxidative metabolism, formation of potentially toxic metabolites or insufficient stability towards autoxidation.

The following sections will provide a number of recent examples from the multitude of structurally diverse substances that have been considered for the application as preventive or chain-breaking therapeutic antioxidants.

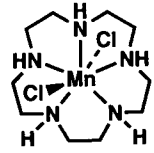
5.1 PREVENTIVE ANTIOXIDANTS

5.1.1 Agents with Superoxide Dismutase-like Activity

Since superoxide anion has been reported to be a mediator of tissue injury, particularly in ischaemia–reperfusion, but also in inflammation and vascular diseases (Hurst and Barrette, 1989; Rice-Evans and Diplock, 1993), efforts to mimic the capacity of the superoxide dismutase (SOD) enzymes is a logical, but difficult, approach to therapy. There is considerable support for the therapeutic potential of SOD-like catalysts to be found in numerous *in vivo* studies performed with purified enzyme preparations (see, for example, Salvemini and Botting, 1993). More recently, the supposed involvement of superoxide anion in the mediation of nitric oxide effects through the formation of peroxynitrite and related species (Fridovich, 1989) has given

research along these lines renewed relevance. Despite the promise represented by this class of antioxidant principles, progress in the field appears to have been limited (see Weiss *et al.* (1993) and references therein for an account), at least as judged by the number of patents and original articles that have appeared.

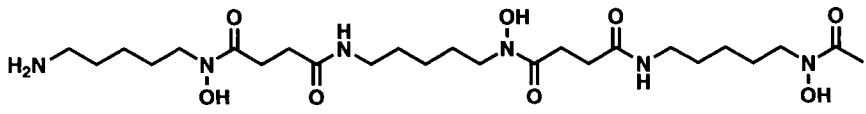
Interesting reports disclosing a novel, low-molecular-weight manganese complex possessing SOD-like properties have appeared recently (Riley and Weiss, 1994; Aston *et al.*, 1993). From a large number of analogues of different ring sizes and coordination geometries, only the complex **500** was found to possess significant SOD-like activity at physiological pH ($k_{cat} = 4 \times 10^7\ M^{-1}\ s^{-1}$). This figure compares favourably with that obtained for the manganese enzyme, which is only about tenfold more efficient. Importantly, the SOD-like activity was not decreased by the presence of excess chloride or other inorganic anions. The complex **500** was also reported to be efficient in reducing superoxide-mediated injury following ischaemia–reperfusion *in vivo*.



500

Covalently linked cofacial bisporphyrin systems have been claimed to mimic the activity of SOD as well as that of catalase, and were presented as potential anti-inflammatory agents (Bruice, 1992). In this instance, the presence of multiple bridging units was suggested to provide a selectivity to the systems, by permitting only small molecules to approach the active iron centre. Unfortunately, the patent application contains no experimental support for the claimed activity.

Much less complicated metal complexes showing SOD-like activity have also been reported in the literature. Fridovich and Darr (1988) described the water-soluble complex of manganese with the chelator desferrioxamine (**501**) and showed how this complex could protect against oxidative damage



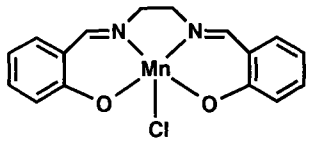
501

(measured as malondialdehyde production) of the rabbit lens *ex vivo*. The fact that the protective effect was accompanied by a normalization of glutathione levels compared with the untreated lens supports a mechanism of action involving removal of active oxygen. Similarly, a complex between copper and indomethacin has been claimed to possess SOD-like activity, as measured by ESR experiments in the presence of a spin trapping agent (Damerau and Wischnewsky, 1989). It appears that this type of complex might be comparatively labile, and perhaps less useful owing to toxicological complications; leaking copper ions would perhaps be expected to show more severe toxicity than manganese ions *in vivo*.

Further examples of metal complexes showing SOD-like activity include the earlier described copper diisopropylsalicylate complexes (reviewed by Reed and Madhu, 1987; Sorenson, 1989), salicylidene complexes containing glutamate, isothiocyanate and alaninate as additional ligands (Bergendi *et al.*, 1991), and copper and manganese complexes with peptides such as glycyl-histidyl-lysine or the corresponding esters (Pickart, 1991). It is important to consider, however, that SOD-like activity and reactions that would rather enhance the toxicity of the superoxide anion (such as hydroxyl radical formation) are both possible chemical outcomes from the interaction of transition metal complexes with superoxide (see, for example, Czapski and Goldstein, 1985). Further transition metal catalysts of the superoxide anion dismutation have been discussed by Sawyer and Tsang (1991), Goldstein *et al.* (1990) and Weinstein and Bielski (1980).

In a recent development of salen–metal complexes, a patent application disclosing bridged complexes of the type **502** claimed that significant protective effects towards ischaemia–reperfusion injury to the rat heart *ex vivo* could be achieved both structurally and functionally (Malfroy-Camine and Baudrey, 1994).

An interesting series of experiments by Bonne and co-workers, following a suggestion by Nilsson *et al.* (1989), compared the protective effect of SOD to that of the nitroxide radical TEMPO (2,2,6,6-tetramethylpiperidine-1-yloxy, **503**) *in vivo* and *in vitro* (Bonne *et al.*, 1991). Nitroxides have been shown to catalyse the dismutation of the superoxide anion via redox cycling with the corresponding hydroxyl amines, e.g. **504**. This compound was shown to be cytoprotective *in vitro* (with IC_{50} values in the micromolar range) and



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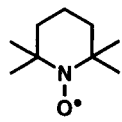

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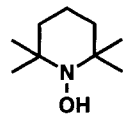


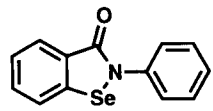
504

also to be effective *in vivo* when given together with catalase (ischaemia–reperfusion in the rabbit eye). The authors concluded that TEMPO constitutes a good model compound for the development of non metal-containing SOD mimics. A patent application claiming similar use of nitroxides has been filed (Mitchell *et al.*, 1990).

5.1.2 Agents with Glutathione Peroxidase-like Activity

The glutathione peroxidases are a family of enzymes which serve to detoxify alkyl hydroperoxides and hydrogen peroxide through reduction of these species to alcohols and water, respectively. Endogenous thiols, usually glutathione, serve as the stoichiometric reducing equivalents. The family comprises five distinct gene products, four of which are selenium-dependent (Flohé, 1989; Maiorino *et al.*, 1990). Since the comparatively stable hydroperoxides are potentially precursors to much more reactive species, such as the hydroxyl radical, antioxidant intervention through the use of glutathione peroxidase (GSHpx) mimics is an attractive strategy. There are today several classes of compounds that show GSHpx-like activity. Unfortunately, it has proven difficult to obtain synthetic molecules with sufficient efficiency in combination with good stability. In fact, the only example to date of a synthetic compound with a reaction rate approaching that of glutathione peroxidase is a semisynthetic selenoenzyme (see below). To our knowledge, the ability of organochalcogenides (selenides and tellurides) to undergo facile redox switching between the II and IV redox states has, without exception, provided the basis for synthetic catalysts.

5.1.2.1 Organoselenides. Ebselen (**505**) was the first compound suggested for hydroperoxide-inactivating therapy. The pharmacodynamic properties and mechanistic features of this agent have been reviewed recently (Sies,

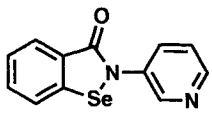


505

1993; see also Parnham *et al.*, 1988; Parnham and Graf, 1991). Although the earlier papers on the mechanism of action exerted by this compound discussed both glutathione peroxidase-like and chain-breaking properties, more recent reports seem to support the former as the principal function (Noguchi *et al.*, 1992). Subsequent studies have addressed the structure–activity relationships of ebselen analogues. These studies revealed that

somewhat more potent compounds can be obtained (e.g. **506**), particularly by introducing nitrogen substituents more electron-deficient than phenyl (Renson and Dereu, 1990).

The detailed mechanism of action of ebselen is still a matter of controversy, with some of the more recent contributions favouring an enzyme-like catalytic cycle (Morgenstern *et al.*, 1992). Briefly, the mechanism for hydroperoxide reduction involves the reaction of the corresponding selenol to form a selenenic acid intermediate (**507**), which reacts with thiol to form a selenosulfide (**508**). Reaction with a second molecule of thiol then releases the selenol and disulfide. In support of this mechanism, Haenen and



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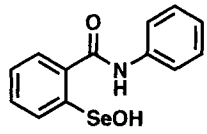

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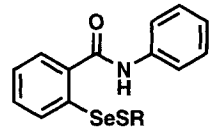
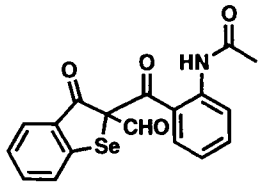


508

co-workers (1990) have reported that the dithiol dihydrolipoate is a better co-factor than glutathione. Other mechanisms have also been put forward, involving the diselenide derived from ebselen as an intermediate (Fischer and Dereu, 1987).

Ebselen has been found to be effective in a large number of disease models, and has also been given to humans (Sies, 1989; Parnham, 1990). It has recently been demonstrated that ebselen has only a low capacity to catalyse the potentially toxic thiol-mediated reduction of ferric cytochrome *c* (Engman *et al.*, 1994c). This is in contrast to GSHpx mimics which have the capacity to release benzeneselenolate ion (see below), which are efficient catalysts of that redox reaction. The substance appears to show a multitude of pharmacodynamic effects, some of which may be independent of its antioxidant properties, such as the modulation of interferon and tumour necrosis factor expression (Cembrzynska-Nowak and Inglot, 1992; Piasecki *et al.*, 1992). It is thus still too early to conclude that glutathione peroxidase-like activity is responsible for the multitude of pharmacological effects exerted by ebselen.

A series of compounds with structural resemblance to ebselen was prepared by Spector and co-workers (1989), who were interested primarily in anticataract properties. While compounds such as **509** and **510** showed peroxide-decomposing properties, they were more than tenfold less efficient than ebselen. This loss in catalytic activity may be a result of the poorer leaving group capacity of the carbonyl-stabilized analogues. However, in analysing the work of Reich and Jasperse (1987), who have utilized compounds such as **511** and **512** as model systems for studies on the



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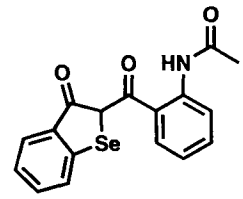

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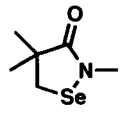

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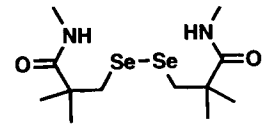

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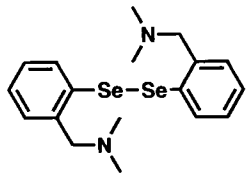

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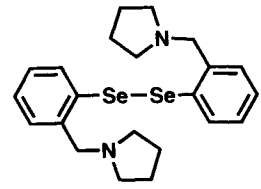
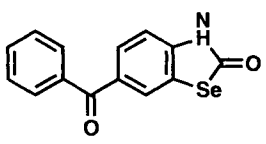


514

mechanism of glutathione peroxidase, the authors designed and synthesized the diselenides **513** and **514** (Wilson *et al.*, 1989). These substances afforded glutathione peroxidase-like activity about tenfold higher than that of ebselen. It was suggested that the amino substituents serve to assist in the nucleophilic attack on intermediate selenosulfides or the diselenide itself (Wilson *et al.*, 1989). The glutathione peroxidase-like activity of diphenyl diselenide was also reported to be about twofold higher than that of ebselen.

Although diselenides are probably too labile to be useful in more complex *in vivo* systems, it is interesting to note that such simple compounds can possess significant enzyme-mimicking properties. The compounds **513** and **514** have, in fact, subsequently been shown to behave very similarly to the enzyme (Iwaoka and Tomoda, 1994). It appears that the amino substituent both facilitates nucleophilic reactions at the selenosulfide intermediate, and also affects the nucleophilicity of the corresponding selenol and stabilizes an intermediate, selenenic acid.

In a recent publication, Galet *et al.* (1994) proposed the use of benzoselenazolinones **515** (as opposed to the benzoisoselenazolone structure of ebselen) as prodrugs to catalytically active diselenides such as **516**. This



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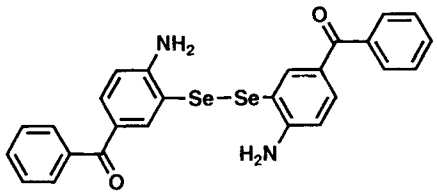
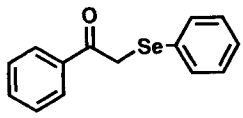


516

approach yielded a series of compounds with better catalytic efficiency than ebselen *in vitro*. However, *in vivo* (carrageenan-induced paw oedema) the potencies were similar. The authors also measured a relatively effective, and sometimes selective, inhibition of leukotriene B_4 and prostaglandin E_2 production in human granulocytes (virtually complete blocking at 10 μM concentrations). The mechanistic basis for this latter activity was not discussed by the authors, but it is not excluded that this is a secondary effect to the antioxidant properties. It would be interesting to see the performance of diselenide prodrugs with the ability to release the catalytically active diselenide functionality intramolecularly.

A series of α-(phenylselenenyl)acetophenone derivatives has been reported to possess GSHpx-like activity *in vitro*. The parent compound **517** accelerated the rate of oxidation of glutathione by a variety of peroxides,



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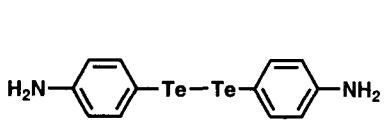
including hydrogen peroxide, approximately tenfold. In the assay using hydrogen peroxide, some derivatives were found to be more efficient (about twofold) than ebselen, with the 4-nitroacetophenone analogue most potent (Cotgreave *et al.*, 1992). Regarding structure–activity relationships in this series, the antioxidant capacity correlated with the stabilization of a putative acetophenone anion leaving group, which would indicate that benzeneselenol was the true catalyst. This mechanism is in agreement with the previously reported catalytic performance of diphenyl diselenide (see above) and was confirmed in subsequent experiments (Engman *et al.*, 1994a). It was shown that α-phenylselenenyl ketones, *S*-phenylselenenyl glutathione and diphenyl diselenide all yield phenylselenol upon treatment with excess glutathione. Further experiments using an alkylating agent (2,4-dinitrochlorobenzene) supported the importance of this selenol as an intermediate in the GSHpx-like activity of these compounds. In fact, α-phenylselenenyl ketones

and *S*-phenylselenosulfides can be regarded as prodrugs of diphenyl diselenide.

A comparison of the glutathione reductase-coupled standard assay for GSHpx-like activity with an assay based on measuring the peroxide content in the medium revealed a significant discrepancy between the two methods (Cotgreave *et al.*, 1992). A similar discrepancy was observed between the coupled assay and results obtained from measuring the removal of glutathione. In both cases the coupled assay appeared to produce an overestimate of the catalysis offered by the GSHpx mimics. A probable cause for the differences is that glutathione reductase can accept intermediates, such as the selenosulfides formed between catalysts and glutathione, as substrates. Thus, in some instances, results obtained by using the coupled assay should be treated with caution.

A quite different approach to a glutathione peroxidase mimic was taken by Wu and Hilvert (1990). These authors converted the serine protease subtilisin into the selenium analogue (selenosubtilisin), by changing the active site serine hydroxy group into a selenol. This semisynthetic enzyme was shown to be able to reduce *tert*-butyl hydroperoxide in the presence of an aromatic thiol. The rate of catalysis was found to be extremely fast, about 70 000 times faster than that of diphenyldiselenide in the model system used. The catalytic mechanism was reported to be similar to that of glutathione peroxidase.

5.1.2.2 Organotellurides. In analogy with diselenides, ditellurides have been shown to possess GSHpx-like activity *in vitro*. Whereas several diselenides and ditellurides were shown to be more potent than the reference ebselen in the reductase-coupled assay, only the ditellurides retained catalytic activity in a simple chemical system (Engman *et al.*, 1992). Thus, in an assay using hydrogen peroxide and thiols such as *N*-acetylcysteine, octyl mercaptan and *tert*-butyl mercaptan in methanol–water mixtures and assessing the oxidation of thiol to disulfide by nuclear magnetic resonance (NMR), diaryldditellurides such as **518** were reported to be very efficient GSHpx mimics. It was shown that the mechanism of catalysis involved the formation of tellurosulfides, which were sometimes isolable. The regeneration of the catalyst from the tellurosulfide was very slow using only thiol, and required the presence of both thiol and hydrogen peroxide, which was explained by assuming that a tellurinic acid thiol ester (e.g. **519**) was formed as an intermediate in the actual catalytic cycle. In contrast, selenosulfides derived from diselenides or ebselen were found to react only sluggishly with thiols in the presence of hydrogen peroxide. Structure activity considerations indicated that electron-releasing substituents in the *ortho* and *para* positions increased catalytic capacity, with the *para* position being most favourable, probably for steric reasons. Interestingly, ditellurides with amino groups in the *para* positions were comparable with the tellurium analogue of **513**. This



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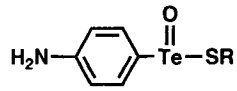

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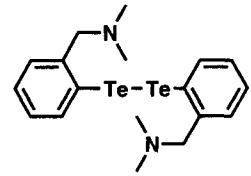

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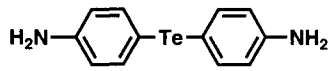

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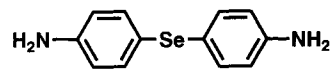

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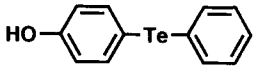
compound was without activity in the simple chemical assay, whereas the diselenide and ditelluride (**520**) were equipotent in the coupled assay. Dibutyl ditelluride was also a potent GSHpx mimic in all assays.

Sharply contrasting what has been found for selenium compounds, where diselenides but not the corresponding monoselenides showed useful GSHpx-like activity, diaryltellurides have been shown to be very effective GSHpx mimics. In fact, this property of organotellurides was suggested by some pioneering work made by Barton's group more than a decade ago (Ley *et al.*, 1981; Barton *et al.*, 1988).

A series of 4,4′-disubstituted diaryltellurides, such as **521**, showed potent catalysis of the reduction of hydrogen peroxide, *tert*-butyl hydroperoxide, cumene hydroperoxide and linoleic acid hydroperoxide by thiol (Andersson *et al.*, 1993). This substance was compared with ebselen in the reductase-coupled assay, and was four to nine times more efficient. The selenium analogue **522** was inactive. Electron-donating substituents in the *para* position were essential for good activity (amino and hydroxy analogues were particularly potent), whereas electron-withdrawing substituents prohibited the reaction (nitro, bromo and chloro analogues lacked activity). Importantly, the corresponding telluroxides showed approximately the same behaviour as the tellurides (see below). Compound **521** was found to be particularly effective in reducing the more lipophilic substrates, which might be useful in biological applications, although difficult to rationalize.

In an extended study (Engman *et al.*, 1994b), diaryltellurides as well as diheteroaryl tellurides, aryl alkyl tellurides and dialkyl tellurides were assessed as GSHpx mimics using an NMR method. Several of the compounds were highly efficient, for example **523**, **524** and **525**. For the diaryltellurides, a good correlation ($r = 0.92$) between catalytic activity and the Hammet σ_p^+



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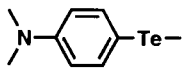

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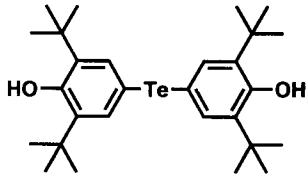

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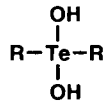
values of the aryl substituents was obtained using alkyl mercaptans, but not *N*-acetyl cysteine, as reducing agents. In general, electron-donating substituents were found to favour the reaction, and *para* substitution was preferred to *ortho* substitution. A two-step mechanism, involving hydrogen peroxide oxidation of the telluride to a tellurium (IV) dihydroxide (**526**) and reduction by thiol with disulfide formation, was suggested. The first step was shown to be rate determining by using the poorer oxidation agent *tert*-butyl hydroperoxide.

Another series of tellurium-containing compounds with peroxide-decomposing capacity are the tellurapyrylium dyes described by Detty and Gibson (1992). Although these compounds have not been developed primarily as GSHpx mimics, reports on their reactions with hydrogen peroxide as well as singlet oxygen make them interesting in this context. Thus, dihydroxytelluranes, such as **527**, which are readily formed from peroxide and the telluride **528** by an oxidative addition process, were reported to react smoothly with thiophenol to form the corresponding disulfide. The authors used an NMR assay. The sulfur and selenium analogues failed to catalyse the reaction between thiol and hydrogen peroxide, whereas the presence of **528** (0.1 mM) accelerated the rate from a half-life of about 1 day to complete conversion in 5 minutes. In disagreement with other reports (see above) the authors did not observe any catalysis when neutral tellurides (**529**) were employed. The capacity to scavenge singlet oxygen through a similar mechanism (Detty and Gibson, 1990, 1992) is interesting, and possibly also relevant for other tellurides.



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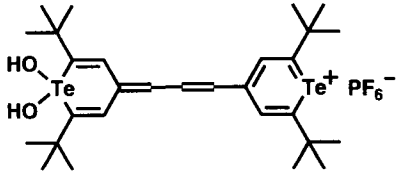

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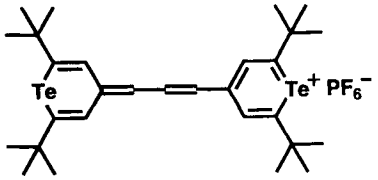

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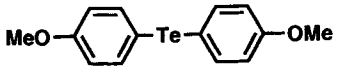

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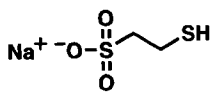
5.1.3 Chelators and Thiols

There have been numerous reports on the possibility that thiol-containing compounds exert protection against oxidative insult *in vivo*. The most well-documented examples are, of course, the drugs penicillamine (and its derivatives), *N*-acetylcysteine and even captopril (these are discussed further in Section 6.3). The endogenous cysteine-containing tripeptide glutathione holds a particularly prominent place in this respect. An altered balance between the reduced and oxidized forms of this peptide, as well as mixed disulfide formation, is often taken as evidence that the tissue has suffered oxidative stress (reviewed by Brigelius, 1985). It is not within the scope of this review to discuss the biological functions of endogenous thiols in detail. Other reviews are available (see, for example, Gillissen, 1993).

There appears to be some controversy regarding the basis for the antioxidant activity of low-molecular-weight thiols. Thus, direct scavenging of chain-carrying radicals as well as a preventive antioxidant action through the complexation of transition metals (iron, copper, etc.) could contribute to the antioxidant properties (see, for example, Halliwell and Gutteridge, 1989). We have chosen to discuss thiols here, not to suggest that the chelating capacity is a more probable mode of action, but merely because there would be great difficulty in dividing these compounds between the two classes of mechanisms.

N-acetylcysteine has been well studied as an antioxidant principle, particularly in lung injury (Bernard, 1991; see also Rice-Evans and Diplock, 1993). In a recent study, the antioxidant effects of this agent were compared with those of mesna (**530**), another mucolytic compound. As judged by the ability to reduce hydrogen peroxide, the two compounds were found equally



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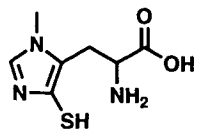
active, with IC_{50} values around 40 μM (Greisser *et al.*, 1993). Other simple thiols with chelating/antioxidant capacity include aminothiosulfonic acids, based on taurine, which have been claimed as effective in diseases caused by free radicals (Katsumata *et al.*, 1993)

Perhaps a more interesting approach is to use dithiols to attempt to scavenge active oxygen species. This approach appears to have some possibility to reduce such species more efficiently, avoiding the leakage of potentially reactive sulfur electrophiles, such as sulfenic acid derivatives. However, distinguishing between scavenger activity and chelating effects will pose obvious difficulty when applying such compounds to more complex assays. One might speculate that there is a possibility for a cooperative antioxidant capacity between these two modes of action, even if the relative potencies would be hard to optimize. This approach was taken by Pigiet and Millis, who disclosed that certain truncated thioredoxin peptides showed antioxidant activity in the rat liver microsome assay (malondialdehyde measurements). The patent claims that antioxidant activity, through complexation of iron, remains with derivatives as small as the hexapeptide **531**. The chelating ability of the compounds was further shown by high-performance liquid chromatography experiments, and the ability to impede the iron-catalysed formation of hydroxyl radical from superoxide anion (Pigiet and Millis, 1987). The design of suitable peptidomimetics of these substances should be an interesting development.

H–Trp–Cys–Gly–Pro–Cys–Lys–OH

531

Further examples of sulfur-containing substances which have been claimed to be biologically relevant antioxidants include thiohistidine derivatives (**532**) (Holler *et al.*, 1990) and triazoles (**533**). These agents might be suspected to coordinate transition metal ions in multidentate complexes. The latter were claimed to inhibit malondialdehyde formation more efficiently than α-tocopherol in rat cerebral homogenates (Chiba *et al.*, 1990), although the concentrations used were comparatively high (5–100 μM). The garlic-derived



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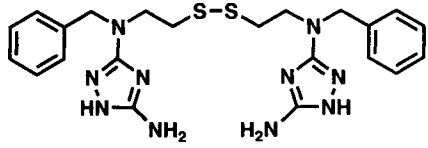

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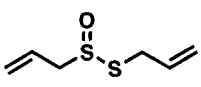
thiosulfinate allicin (**534**) has been attributed antioxidant capacity, although it does not appear to have chelating capability (Kourounakis and Rekka, 1991).



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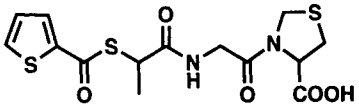

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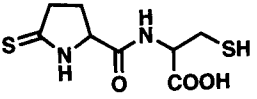

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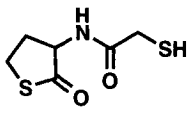
Among a multitude of other pharmacodynamic properties, antioxidant activity has been claimed for the thiazolidinecarboxylic acid **535**, which was protective in models of paracetamol toxicity, endotoxin-induced mortality and carbon tetrachloride intoxication in the rat. It was more effective than N-acetylcysteine in these models, and showed similar reactivity towards DPPH *in vitro* (Poli *et al.*, 1993b). Recently claimed thiol-containing compounds with antioxidant capacity include the cysteine derivative **536** (Poli *et al.*, 1993a) and **537** (Nicola *et al.*, 1993).



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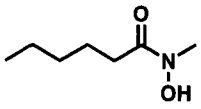

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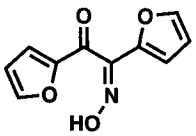


539

Davies and Rice-Evans (1992) have claimed that hydroxamic acids, such as **538**, can prevent reperfusion damage, particularly to the heart. This compound was reported to be more effective than desferrioxamine as an inhibitor of hydrogen peroxide-mediated ferrylmyoglobin generation from metmyoglobin. It was also shown to inhibit peroxidation *in vitro*.

Chelators of the type **539** have been claimed to be useful for regulating damage caused by exposure to UV irradiation (Bush *et al.*, 1991).

5.2 CHAIN-BREAKING ANTIOXIDANTS

We have chosen to discuss the synthetic chain-breaking antioxidants in relation to their chemical structural features, rather than therapeutic classifications based on the aims of the individual research groups. We felt that this was perhaps a more practical approach, especially since most promising, biologically relevant, antioxidant compounds are potentially

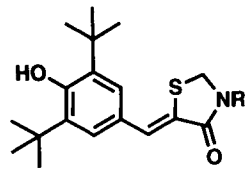
applicable within a multitude of pathological conditions. Structural classification is not at all straightforward and inconsistencies are certain to appear whatever system is used.

We have attempted to categorize the chain-breakers into phenols, amines, steroids, chalcogenides and miscellaneous compounds. This classification has been based on the principal functional groups present, and regarded as important for the antioxidant effects observed. In several cases, of course, substances carry multiple functionalities (amine and phenol, phenol and chalcogen, etc.). These have been classified as miscellaneous if it is not stated by the authors that a certain structural feature is essential for the antioxidant performance. Further, the labelling of hindered phenols as synthetic compounds or tocopherol analogues is arguably arbitrary in some instances. We have treated derivatives with the chroman moiety virtually unchanged as semisynthetic vitamin E derivatives (see Section 3).

5.2.1 Phenols and their Derivatives

A large number of papers have appeared reporting antioxidant capacity, often accompanied by lipoxygenase inhibition or other anti-inflammatory properties, of a plethora of sterically hindered phenols. In particular, 2,6-di-*tert*-butylphenols have been intensely studied. Several of these compounds are regarded to be redox-active inhibitors of the arachidonate metabolism, but it appears that the nature of the appendage (normally present as an aromatic 4-substituent) can be utilized to modulate the pharmacodynamic activity. This has indeed been taken advantage of by several groups. However, there are reports that anti-inflammatory activity and antioxidant capacity of sterically hindered phenols can be completely unrelated (Costantino *et al.*, 1993). It is not within the scope of this review to give a complete coverage of even the antioxidant properties of this class of compounds, but some recent literature will be cited.

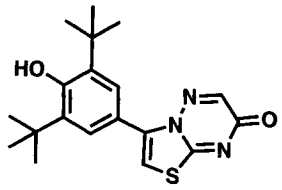
The Lilly compounds **540** (LY178002) and **541** (LY256548) are illustrative of the variety of pharmacological effects obtained with di-*tert*-butylphenols.



540 R = H
541 R = Me

These substances were found to be potent (IC_{50} 0.6 μM) inhibitors of iron-dependent lipid peroxidation of rabbit vesicular membrane lipids (TBARS measurements). These compounds have been shown to be orally active in models of arthritis, cerebral ischaemia and muscular dystrophy (Panetta *et al.*, 1991).

Further examples of dialkyl phenols are **542** (HWA-131), which has also been shown to modulate lymphocyte reactivity (Bartlett *et al.*, 1990) and related derivatives, such as **543** (BF-389; Bendele *et al.*, 1992), **544** (CI-986; Schrier *et al.*, 1994), **545** (PD-127443; Schrier *et al.*, 1989), **546** (E-5110; Shirota *et al.*, 1987) and **547** (BI-L-239XX; Noonan *et al.*, 1992), all of which



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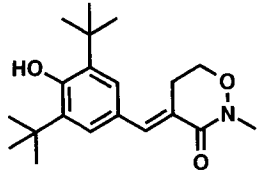

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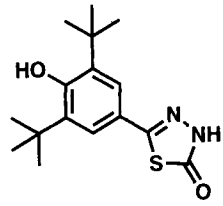

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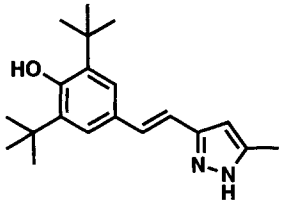

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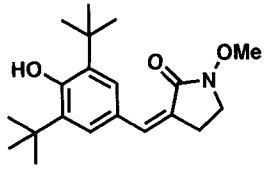

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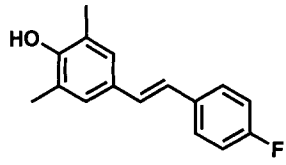

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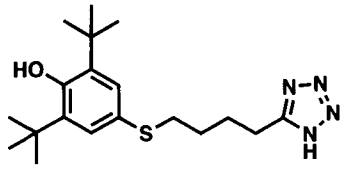
have been rather well characterized pharmacologically. The majority of the sterically hindered phenols have been developed as inhibitors of either cyclooxygenase or lipoxygenase (or both), and it has often been proposed that oxidation of the inhibitor by these enzymes is mechanistically important. It is interesting to note that the majority of these phenols have substituents in the *para* position which seem to stabilize a phenoxyl radical, either directly, as is the case in tocopherol, or vinylogously. Thus, the non-specific antioxidant capacity, as opposed to reduction of the enzyme iron, of such substances should be considered as an alternative mode of action.

The patent literature contains a number of further sterically hindered phenols, which have been claimed as antioxidants, including thioethers such

as **548** (Kita *et al.*, 1993), **549** (Mao and Parker, 1992), **550** (Doteuchi *et al.*, 1991) and, more recently, **551** (Dreckmann-Behrendt *et al.*, 1993). Oral anti-inflammatory activity (rat adjuvant arthritis) was claimed for the highly hydrophilic bisphosphonic acid **552**, whereas a reference lacking the *tert*-butyl substituents was inactive (Kawabe *et al.*, 1991).



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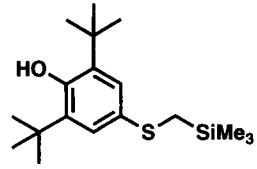

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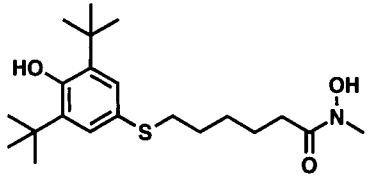

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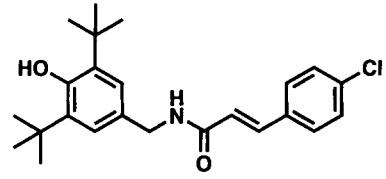

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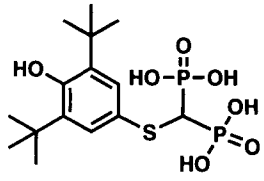

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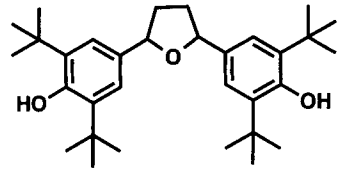
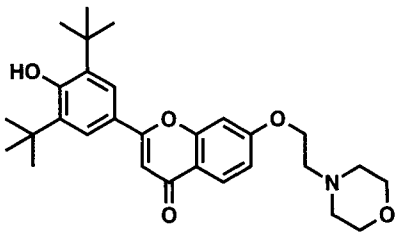


553

The incorporation of a potentially redox active phenolic substituent into a biologically active template molecule has been attempted by many groups. In a fairly recent application, Chugai researchers claimed tetrahydrofuran bisphenols such as compound **553** for treating, for example, atherosclerosis and inflammation. This substance is clearly related to the earlier described diaryltetrahydrofuran derivatives with platelet-activating factor antagonistic properties, and may represent an interesting development towards a new class of dually acting agents. The preparation of flavonoid di-*tert*-butylphenols such

as **554** has also been reported (Rolland *et al.*, 1993). IC_{50} values (1–3 μM), comparable to that of probucol (**636**), were observed with a number of analogues in a model of copper-mediated LDL peroxidation. With respect to inhibition of endothelial cell-initiated peroxidation of human LDL, the flavonoids were reported to be about tenfold more efficient than either probucol or vitamin E.



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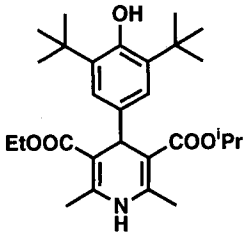
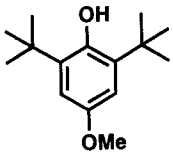


555

Submicromolar IC_{50} values (rat hepatic microsomal assay, TBARS measurements) were reported for a series of dihydropyridine derivatives, out of which **555** was the most potent antioxidant (IC_{50} 0.3 μM). The compounds, which did not show calcium antagonist activity, were claimed to be more effective than α-tocopherol (Galiano Ramos and Del Sol Moreno, 1992; see also Section 6.2.2). In other cases, it was of interest to combine these two activities. Thus, in an attempt to better counteract ischaemia–reperfusion injury with simultaneous blockade of calcium influx and peroxyl radical-scavenging effects, a series of synthetic and natural antioxidants has been investigated (Sgaragli *et al.*, 1993). Fifteen compounds, characterized by the presence of at least one hydroxyl group on the aromatic ring and a highly lipophilic moiety, showed both calcium antagonistic and antioxidant properties. The most interesting lead compound was the BHA analogue **556**. It certainly appears that interest in developing novel di-*tert*-butylphenols is continuing.

The two related phenols **557** (ONO-3144) and **558** (MK-447) were compared for their antioxidant capacity, since these compounds had been



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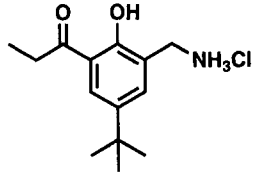

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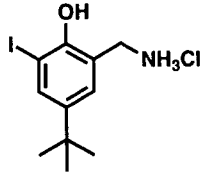
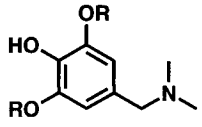


558

reported to be anti-inflammatory agents comparable to indomethacin (**601**), but with an obscure mechanism of action. In this study (Cheeseman and Forni, 1988), which yielded quantitative data on the reactivity towards reactive oxygen species, compound **558** was found to be the most potent antioxidant. Both phenols reacted readily with peroxyl radicals and hydroxyl radical but were inert to the superoxide anion and hydrogen superoxide. The immediate products obtained were the corresponding phenoxyl radical, which in the case of MK-447 was found to be regenerable with ascorbate. The results from pulse radiolysis studies correlated well with those from a number of *in vitro* microsomal lipid peroxidation assays, although the IC_{50} values reported seem fairly high.

An interesting set of structure–activity relationships was reported by Yu and co-workers (1993), who compared three series of phenols (**559**, **560** and **561**) as antioxidants. The compounds were assessed as peroxyl radical



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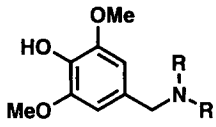

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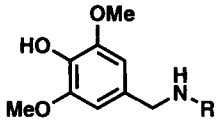
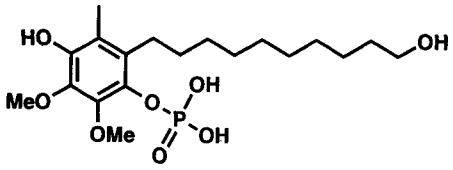


561

scavengers as well as inhibitors of iron-dependent lipid peroxidation (rabbit brain vesicular membrane lipids) and cytoprotective agents (rat hippocampal neurons) *in vitro*. The antioxidant capacity was discussed in terms of intrinsic efficiency, physicochemical properties and biomembrane stabilization. The investigation led to several impressive correlations between cytoprotective activity and physicochemical descriptors of substituent size. Importantly, efficient radical trapping in homogeneous solution did not correlate with inhibition of peroxidation in the lipid phase. In the former assay, smaller, less sterically demanding, substituents flanking the phenol function were favourable for kinetic reasons. On the other hand, optimal efficiency in the latter model was observed for compounds having one lipophilic and one small alkoxy substituent in the two *ortho* positions. With regard to substitution at the benzylic nitrogen, it was concluded that substituent length was more important than bulk, possibly due to a membrane-disordering effect with the more bulky analogues. For the compounds included in the study (up to *N*-nonyl and *N*,*N*-dipentyl) limiting lipophilicity was not reached, and linear relationships were always observed.

Hydroquinone derivatives carrying lipophilic substituents, e.g. compound **562**, were synthesized by Masuya and Yamaoka (1993). The principal mode of action within this class of compounds (which are structurally related to ubiquinol) was claimed to be inhibition of fatty acid peroxidation.



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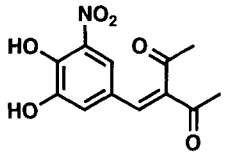


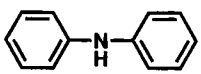
563

Nitecapone (**563**) was originally designed as a catechol-*O*-methyltransferase inhibitor (Nissinen *et al.*, 1988), but this compound and, more recently, a number of related catechols (Korkolainen *et al.*, 1991; Backstrom *et al.*, 1991) have also been claimed to be potent antioxidants. Apart from showing inhibition of xanthine oxidase, **563** has been reported to scavenge peroxyl radicals and superoxide anion as well as to inhibit lipid peroxidation. Interestingly, this nitro catechol was reported to recycle tocopherol in the presence of ascorbate (Suzuki *et al.*, 1992). Recently, it has been shown to attenuate damage caused by ischaemia–reperfusion in the rat heart, while simultaneously preventing the loss of sulfhydryl groups and tocopherol (Valenza *et al.*, 1993).

5.2.2 Anilines and Related Compounds

Aromatic amines have long been used as antioxidants in technical applications such as lubricants and polymers. The application of such substances in biological contexts has also been attempted, although the toxicity that can be expected from aniline derivatives suggests that simpler analogues are probably not very useful.

Recently, a study of the biologically relevant antioxidant properties of the agent diphenylamine (**564**), and some additional amine-containing model compounds (e.g. **565** and **566**), raised several interesting mechanistic questions that may be relevant for other amines also. Thus, Sugihara and co-workers (1993) reported that **564**, which has been used as a diagnostic antioxidant in several studies, could actually promote the generation of hydroperoxides, while traditional TBARS measurements still indicated antioxidant capacity. It was proposed that redox cycling via the nitrogen-centred neutral radical, sufficiently reactive to initiate further lipid peroxidation, was responsible for this anomalous behaviour. Interception in this



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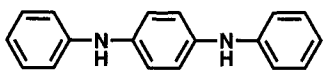

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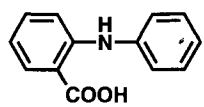
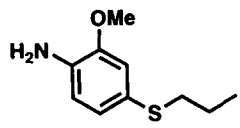


566

fashion of peroxyl radicals would suppress the formation of endoperoxides, which are necessary for malondialdehyde formation (Janero, 1990), but not peroxidation. This chain-branching effect was not shared by the other amines studied. The study was performed using *tert*-butyl hydroperoxide-stimulated peroxidation of red blood cell membranes. The fact that diphenylamine also promoted the prelytic leakage of potassium from red blood cells and abolished the formation of cyclooxygenase-dependent conversion of arachidonate in platelets further supported the suggested mechanism. The authors concluded that TBARS measurements can sometimes be unreliable and hence should be complemented with alternative methods.

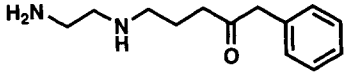
In another study involving the model antioxidant **565**, Sparrow and co-workers (1992) found it to be a relatively potent inhibitor of LDL peroxidation *in vitro*, and also to slow down the progression of atherosclerosis *in vivo* when given orally. In a limited structure–activity study using both copper- and endothelial cell-mediated oxidation of LDL, **565** was a better antioxidant than either probucol or BHT. Several *N*,*N*′-disubstituted *para*-phenylenediamines (phenyl, benzyl, methyl) were active antioxidants ($IC_{50} < 1\ \mu M$). Interestingly, *N*,*N*′-diphenyl *meta*-phenylenediamine and the dithio analogue of **565** were inactive. Since **565** lacks cholesterol-lowering activity, this study supports the hypothesis that antioxidants could show antiatherosclerotic activity. The antioxidant properties of probucol are further discussed in Section 6.3.

Komeshima and co-workers (1992) synthesized *para*-alkylthio-*ortho*-anisidine derivatives, such as **567**, possessing antioxidant capacity, and showed that these agents had anti-inflammatory activity in the Arthus reaction as well as in several experimental inflammatory oedemas. *In vitro* structure–activity studies (rat brain homogenate, TBARS measurements) revealed that the nature of the alkylthio substituent was not important, with ethyl through dodecyl virtually equipotent. *In vivo*, however, only the compounds carrying two to eight carbons in the alkylthio functionality were positive (lipopolysaccharide-shocked mice, plasma TBARS measurements), and in the Arthus reaction the propylthio analogue was preferred. With respect to nitrogen functionality, mono- or di-methylation was tolerated without a decrease in efficacy *in vivo*, whereas the introduction of higher alkyl substituents provided inactive compounds. Surprisingly, the sulfoxide, but not the sulfone corresponding to **567**, retained activity both *in vitro* and *in vivo*.



567

Ethylenediamine derivatives were synthesized and shown to possess anti-inflammatory activity in the Arthus reaction (Andreadou *et al.*, 1991). Compound **568** was found to be the most potent antioxidant in a limited structure–activity study (hepatic microsomal peroxidation, iron/ascorbate). The ethylenediamine moiety was essential for antioxidant activity, possibly because of interactions with membrane lipid phosphate groups. A correlation between *in vitro* potency and lipophilicity was reported using R_M values (these values are obtained by reversed-phase thin-layer chromatography, $R_M = \log[(1/R_f) - 1]$). In a subsequent study, the *in vitro* antioxidant capacity of eight compounds was correlated with anti-inflammatory effects in the carrageenan-induced rat paw oedema (Andreadou *et al.*, 1992). Unfortunately, the chelating capacity of this class of compounds has not been investigated.



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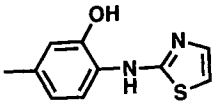
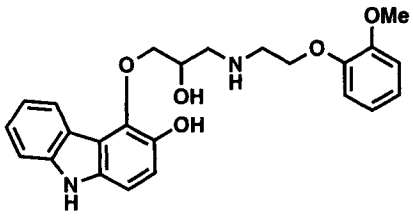


569

Bonne and co-workers (1989) developed the aminothiazole CBS-113A (**569**) as a dual inhibitor of lipoxygenase and cyclooxygenase with antioxidant activity. The compound was reported to be much more efficient than 5-ASA and indomethacin (see Section 6.1), and similar to α-tocopherol, with respect to inhibition of oxidation of phosphatidylcholine liposomes *in vitro*. It also quenched singlet oxygen with an efficiency similar to that of the vitamin. Inhibition of the leukocyte oxidative burst further added to the pharmacodynamic profile of the substance.

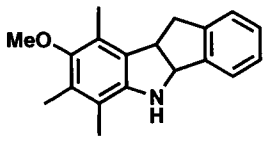
A recent contribution from Yue *et al.*, (1994) reported the antioxidant capacity of the carbazole SB 211475 (**570**). This compound, which is a metabolite of the antihypertensive agent carvedilol (carbazol hydroxylation), is interesting as it is clearly related to the naturally occurring antioxidant carazostatin (**433**, see Section 4.1). It inhibited the iron/ascorbate-initiated



570

peroxidation of rat brain homogenate as well as mouse macrophage-mediated oxidation of LDL more potently than probucol (TBARS measurements). Further evidence for the antioxidant capacity was obtained in several whole cell assays. It was suggested that the antioxidant activity of the metabolite may contribute to the therapeutic effects of carvedilol.

Tetrahydroindenoindoles have been claimed to be very efficient antioxidants and inhibitors of macrophage-induced LDL peroxidation (Sainsbury and Shertzer, 1992). In particular, permethylated analogues, such as **571**, were reported to have extremely low IC_{50} values (10 nM) in the iron/ascorbate-induced peroxidation of soy bean phospholipids. In an assay involving the peroxidation of human LDL mediated by mouse peritoneal macrophages, the compounds had similar IC_{50} values (TBARS measurements). These compounds, which might be regarded as hybrids between the established antioxidant ethoxyquin (**572**) and α-tocopherol, should be among the most potent antioxidants described in biological systems.



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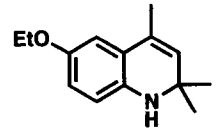

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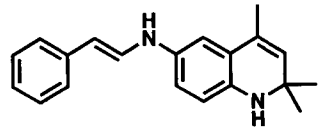
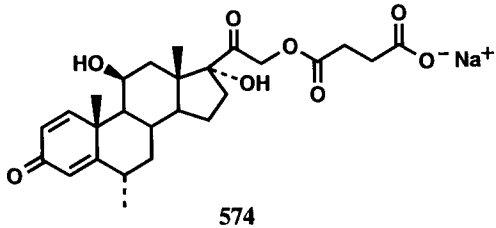


573

The dihydroquinoline **573** also seems to have been derived from ethoxyquin. This substance was claimed to be useful as an orally available therapeutic antioxidant, based on its inhibition of behavioural effects induced by the injection of ferric chloride into the spinal cord of mice (Aono *et al.*, 1992a).

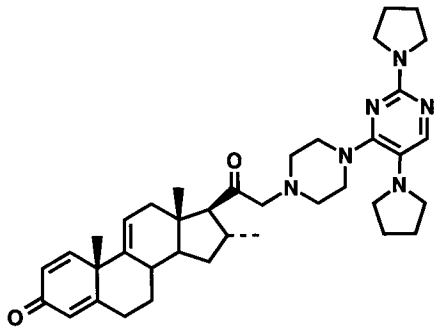
5.2.3 Steroid Derivatives

The "lazaroids" were developed at the Upjohn laboratories. These compounds are derived from methylprednisolone (e.g. **574**), having amino substituents in the 21-position, and were developed in an attempt to mimic the high-dose effects (Demopoulos *et al.*, 1982) of this material. The most prominent member of this family of compounds is tirilazad mesylate



574

(U74006F, **575**), which has reached clinical trials for the treatment of head injury. The 21-amino steroids have been shown to be potent inhibitors of lipid peroxidation *in vitro*, but essentially devoid of glucocorticoid and mineralocorticoid activities. The role of antioxidants in the therapy of ischaemia–reperfusion injury to the central nervous system has been reviewed recently (McCall and Panetta, 1992; Panetta and McCall, 1995).



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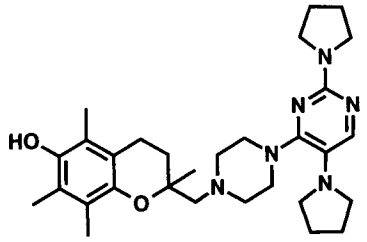
In particular, U74006F was found to be effective in inhibiting iron-dependent peroxidation in rat brain homogenates, and comparable to α-tocopherol in this respect. IC_{50} values as low as 3 μM have been reported. The compound has been shown to spare both ascorbate and α-tocopherol in reperfused gerbil brain (Sato and Hall, 1992). The antioxidant effect *in vitro* has been suggested to depend upon reduction of the peroxyl chain-carrying species, rather than complexation and inactivation of iron (Braughler *et al.*, 1988). On the other hand, assessing the capacity of U74006F to inhibit azo-initiated peroxidation of linoleic acid in homogeneous methanol solution, Braughler and Pregenzer (1989) found a much lower activity than expected based on the ability to inhibit iron-dependent lipid peroxidation. In this model system, the aminosteroid was clearly inferior to α-tocopherol. Additional antioxidant effects have been suggested to arise from effects on membrane fluidity, which were observed at micromolar concentrations (Jacobsen *et al.*, 1990). More recently, it has been hypothesized that this class

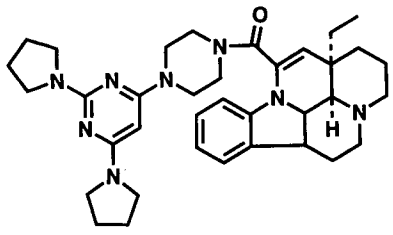
of antioxidants acts through controlling the redox state of iron in solution, and by that eliminating the initiation of oxidative reactions (Ryan and Petry, 1993). Although it appears that the mechanism of action of this class of antioxidants has still to be elucidated in detail, the 21-aminosteroids deserve attention since these substances will be among the first antioxidants to be evaluated clinically (the pharmacology of U74006F has been reviewed; see Braughler *et al.*, 1989). It should also be noted that methyl prednisolone itself, which essentially lacks antioxidant effects, has been claimed to be effective in the treatment of spinal cord injury in a controlled trial (Giannotta *et al.*, 1984; Prous *et al.*, 1993). Tirilazad has also been reported to be protective in models of hypoxia (Richards *et al.*, 1993) as well as endothelial injury (Pakala and Benedict, 1994).

An interesting lazaroid, although not a steroid, is the tocopherol-derived compound **576** (U78517F), which has been claimed to be a more potent antioxidant than the 21-aminosteroids. The *in vivo* cerebroprotective activity of this substance was, however, comparable to that of the traditional lazaroids (Puttfarcken *et al.*, 1993).



576

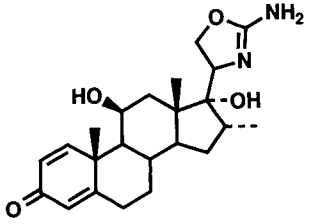
Another series of structurally related antioxidants was disclosed by Tuba *et al.* (1993), who claimed that eburnamenine derivatives such as compound **577** were efficient lipid peroxidation inhibitors, useful in ischaemic conditions. The compounds were evaluated using NADPH- as well as iron-induced peroxidation in rat brain homogenates. The eburnamenine derivatives were reported to be more potent than tirilazad mesylate, and comparable to



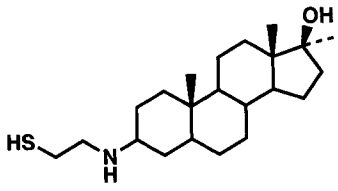
577

α-tocopherol in both *in vitro* assays. Kuno *et al.* (1992) have disclosed yet another series of cerebroprotective antioxidants containing piperazine and pyrimidine moieties.

A series of 17β-aminooxazolyl steroids (**578**) has been reported to possess local anti-inflammatory effects, which were attributed to an antioxidant capacity of the compounds (Rapi *et al.*, 1985). It was shown by the authors that the 2-aminooxazole moiety needed to be attached to a steroid nucleus in order to show this capacity, but since the antioxidant effect was supported only by reaction with hydrogen peroxide in the presence of iron, other mechanisms cannot be ruled out.



578

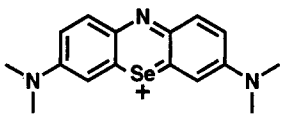


579

More recently, derivatives of androstanone substituted with amine-containing groups in the 3-position (e.g. **579**) were reported to possess significant antioxidant activity. The anti-inflammatory activity (carrageenan paw oedema) was found to be related to the antioxidant capacity, measured as microsomal peroxidation and reaction with DPPH (Spyriounis *et al.*, 1993). It must be noted, however, that millimolar concentrations were employed in the *in vitro* studies. It would be interesting to see the concept of using steroidal structures as carriers applied to more elaborate and potent antioxidant principles, such as hindered phenols or ascorbate analogues.

5.2.4 Organochalcogenides

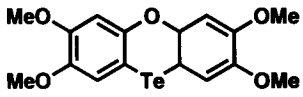
The selenium analogue (**580**) of methylene blue was reported to be threefold more potent than the parent compound, which is active (Chung and Nam,



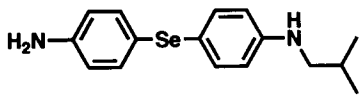
580

1975; Doroshow, 1986) in inhibiting iron-dependent lipid peroxidation of liver homogenates (Leyck *et al.*, 1993). This rank in potency was also seen *in vivo* in the rat paw oedema. However, unlike a number of organoselenides (see Section 5.1.2.1), selenomethylene blue did not possess thiol peroxidase activity. The authors speculated that the antioxidant capacity was related to inhibition of the initiation reaction, but this was not addressed experimentally.

Further tricyclic chalcogen-containing compounds have been reported to show antioxidant properties *in vitro*. Thus, the oxidation potentials of an extensive series of dibenzo[1,4]dichalcogenines (e.g. **581**) were found to correlate very well with their ability to inhibit iron-induced peroxidation of liver microsomes (Cotgreave *et al.*, 1991). A good correlation was reported between half-wave potentials, measured in methylene chloride, and the inhibitory performance in cell systems undergoing peroxidation. This class of organochalcogen compounds showed IC_{50} values in the submicromolar range in the microsome system, lower than those observed using BHT. The similarity in lipophilicity of the compounds allowed the conclusion that a redox potential lower than 0.9 V is essential for good antioxidant activity in the chosen assays. This observation seems to be in good agreement with the redox behaviour of the peroxyl radical–hydroperoxide couple (E_7 around 0.9 V) reported by Jovanovic *et al.* (1991).



581

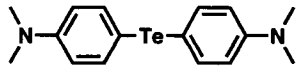


582

The selenoorganic compound **582** was found to be the most potent antioxidant within an extensive series of substituted diarylselenides (Andersson *et al.*, 1994b). Although several selenides were effective antioxidants, mono- or di-alkyl substituted diamino derivatives were preferred from lipophilicity considerations. Potent antioxidant activity was observed in microsomal as well as hepatocyte preparations (iron/ADP/ascorbate, *tert*-butyl hydroperoxide and diquat redox cycling were used as peroxidative challenge), with IC_{50} values in the low micromolar range. The substituent effects from a large number of *para*-substituted compounds indicated that mesomerically electron-releasing groups enhanced antioxidant activity. The unsymmetrical 4-nitro-4′-amino-analogue retained fairly high activity. The corresponding selenoxides were suggested as the ultimate degradation products of the antioxidants. Since selenoxides are readily reduced by thiols, catalytic chain-breaking antioxidant activity was considered relevant for this class of seleno-organic antioxidants. A qualitative correlation was observed

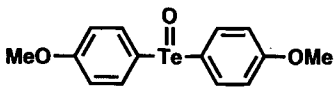
between oxidation peak potentials and antioxidant activity in the microsomal assay. In analogy with the study using dibenzo[1,4]dichalcogenines (see above), only compounds with an oxidation potential lower than 1 V were effective. Apart from their antioxidant properties, several compounds were found to be fairly potent inhibitors of leukotriene production. This effect was probably due to a direct interaction with 5-lipoxygenase, and did not correlate with antioxidant capacity or redox properties in the series.

A similar correlation between redox properties and antioxidant activity has been reported for diaryltellurides. These substances were much more potent than selenide analogues in the microsomal peroxidation assay (iron/ADP/ascorbate), showing IC_{50} values as low as 30–300 nM (Andersson *et al.*, 1994a). The most active in this series of *para*-substituted diaryltellurides was the *bis*(dimethylamino) derivative **583**, which also showed the lowest

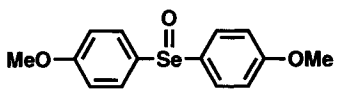


583

oxidation potential. This compound showed a dose-dependent delay of the onset of microsomal peroxidation. In a hepatocyte system (*tert*-butyl hydroperoxide initiation) all compounds showed similar protective activity, as measured by TBARS formation. The increased potency of the diaryltellurides, compared with their diarylselenide analogues, e.g. **522**, was interpreted by assuming a facile catalytic chain-terminating activity for the former. Alternatively, a combination of chain-breaking and GSHpx-like activity could produce the same effect (see Section 5.1.2.2). This assumption was corroborated by competitive experiments involving **584** and **585**, showing that the telluroxide was much more reactive towards thiol, thus regenerating the active tellurium$_{II}$ species readily. It was further found, in similar competitive experiments, that the diaryltelluride could decompose alkyl hydroperoxides, whereas the selenide was sluggish in this respect. The mechanism behind the chain-breaking antioxidant properties of diaryltellurides has been addressed in subsequent studies, which showed that these agents were efficient retarders of the peroxidation of linoleic acid in methanol. The most active compounds



584

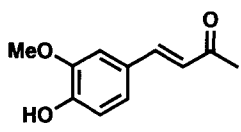


585

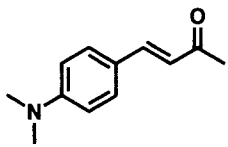
were equipotent with α-tocopherol in this system. However, diaryltellurides inhibited peroxidation for a long period of time, seemingly with an autocatalytic mechanism. The reactivity of 4,4′-diaminodiphenyltelluride (**521**) towards DPPH in methanol was found to be lower than that of α-tocopherol, but much higher than for the corresponding sulfur and selenium (**522**) analogues (Engman *et al.*, 1995).

5.2.5 *Miscellaneous*

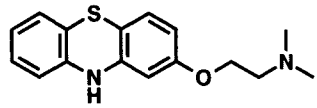
Several (*E*)-4-aryl-3-buten-2-ones were compared as scavengers of superoxide anion and hydroxyl radical (Saldanha *et al.*, 1990). Although several of the compounds, including dehydrozingerone (**586**), were active in both assays, only the former property correlated with *in vivo* anti-inflammatory activity (carrageenan-induced paw oedema). A correlation coefficient of 0.87 could be obtained if halogenated analogues were omitted in the regression analysis. It follows that there was no correlation between the values obtained in the two *in vitro* systems. Although the correlation was not too impressive, this is an interesting case where such a correlation of antioxidant capacity and an *in vivo* performance has been possible. However, the IC_{50} values reported were rather high throughout. This report also disclosed an analogue (**587**) of dehydrozingerone more potent than the natural product *in vitro* as well as *in vivo*.



586



587

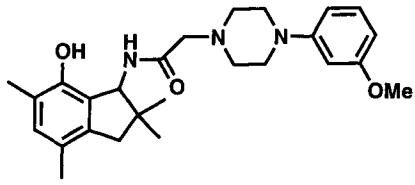


588

A series of phenothiazines was synthesized and evaluated for protective effects in iron-dependent lipid peroxidation of rabbit brain vesicular membrane lipids. The IC_{50} values for some members of the series were as low as 60–100 nM (Yu *et al.*, 1992). The most extensively studied compound (**588**) was more effective than probucol in blocking copper-induced oxidation of low-density lipoprotein *in vitro*. It also protected rat hippocampal neurons from hydrogen peroxide-induced damage, at a concentration of 5 μM. Structure–activity analysis within a series of analogues indicated a high tolerance towards structural modifications in the side-chain, whereas alkylation of the phenothiazine nitrogen diminished activity by two orders of magnitude, consistent with the higher redox potentials. The authors speculated that mechanisms other than antioxidant activity contributed to the

observed cytoprotective effect. Chlorpromazine (**650**, see Section 6.3) was earlier reported to be protective at high concentrations in similar models (Hoshi *et al.*, 1990).

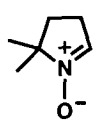
In a search for novel cerebroprotective drugs with CNS-stimulating activity, Oshiro *et al.* (1991) synthesized a series of 1-(acylamino)-7-hydroxyindane derivatives. Only compound **589** (OPC-14117), which appears somewhat



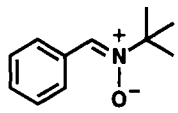
589

related to **576**, was active in both respects. This substance was also shown to be effective in protecting rat brain homogenates from peroxidation *in vitro* (TBARS measurements) more potently than α-tocopherol (IC_{50} values 10 versus 200 μM) but inferior to BHT (IC_{50} 2.5 μM). These *in vitro* results were in agreement with the observed *in vivo* antihypoxic effects. In terms of enhancing the survival of mice under hypoxic conditions, this substance was reported to be orally more efficient than α-tocopherol. The antihypoxic effect was found to be largely affected by structural modifications. Replacing the 7-hydroxy group with methoxy gave an inactive compound. More surprisingly, the presence of the 3-methoxy (or a chloro) functionality in the arylpiperazinyl appendage was critical for cerebroprotective activity. On the whole, a number of strict structural requirements in this series seem to suggest that there are contributions to the pharmacological effect observed in addition to antioxidant activity.

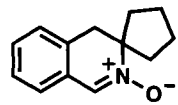
Spin traps have been used extensively as diagnostic agents in free radical biology *in vitro*. These agents act by combining with reactive carbon- or oxygen-centred free radicals to form more stable nitroxide radicals. These stable radicals can be isolated and studied by ESR spectroscopy (see, for instance, Britigan *et al.*, 1990), or even characterized by liquid chromatography and mass spectrometric techniques (Iwahashi *et al.*, 1990; Rao *et al.*, 1990). Interesting reports have also been made on the *in vivo* application (reviewed by Knecht and Mason, 1993), and even on therapeutic effects observed using such agents, for instance in ischaemia–reperfusion models (Bolli *et al.*, 1989). Most studies to date have employed traditional compounds such as 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, **590**) or *N-tert*-butyl-α-phenyl nitrone (**591**). The use of spin-trapping agents for therapy of a multitude of diseases has been patented (Carney and Floyd, 1992). A growing awareness of complicating metabolic and secondary



590



591



592

chemical reactions (see, for example, Samuni *et al.*, 1989) has stimulated the development of more efficient spin-traps for therapeutic use. For example, a series of nitrones such as **592** was recently claimed as dual spin-trapping and interleukin 1-inhibiting agents (Carr *et al.*, 1993).

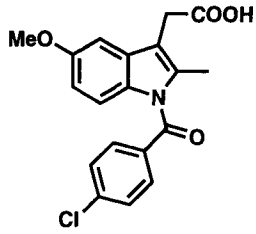
6 Clinical Agents

Since reactive oxygen metabolites may play important roles in a number of human disease states, it is not surprising that many of the drugs used in clinical practice have been evaluated with regard to their antioxidant capacity. These drugs, including not only non-steroidal anti-inflammatory agents (NSAIDs) but also calcium channel blockers, antagonists and agonists to adrenergic receptors, histamine H_2-receptor antagonists etc., have been examined in a variety of *in vitro* and *in vivo* systems. Unfortunately, adequate quantitative comparisons of the antioxidant capacities of these drugs and their roles in a potential oxidative burst process is difficult.

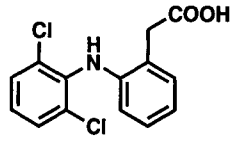
6.1 ANTI-INFLAMMATORY AND ANTIRHEUMATIC DRUGS

6.1.1 General

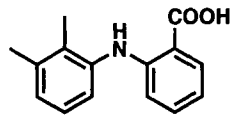
NSAIDs are believed to exert their anti-inflammatory action primarily by the inhibition of the biosynthesis of prostaglandins and related cyclooxygenase-derived products. NSAIDs can also interfere with free radical generation and by trapping reactive free radical species (Arrigoni-Martelli, 1985). Indomethacin (**601**), diclofenac (**602**), mefenamic acid (**603**), flufenamic acid (**604**), phenylbutazone (**605**), oxyphenbutazone (**606**) and acetylsalicylic acid (**607**) have been evaluated as inhibitors of the generation, or the activity, of oxygen free radicals in various assays. In one system where superoxide anion was generated by guinea-pig macrophages elicited by reaction with nicotinamide adenine nucleotide, diclofenac exhibited inhibition at 0.4 μM, acetylsalicylic acid at 100 μM and the other drugs in the range in between.



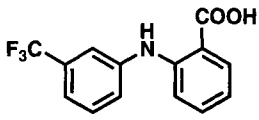
601
indomethacin



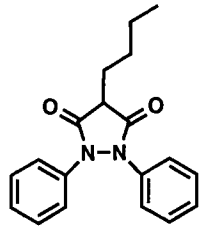
602
diclofenac



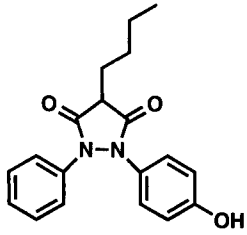
603
mefenamic acid



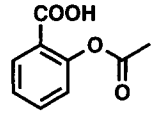
604
flufenamic acid



605
phenylbutazone

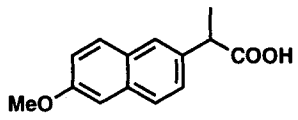


606
oxyphenbutazone

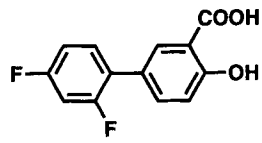


607
acetylsalicylic acid

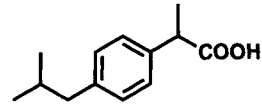
In another assay, superoxide anion was generated by hypoxanthine/xanthine oxidase. The activity was measured by the lactate dehydrogenase (LDH)-catalysed oxidation of NADH. Indomethacin, mefenamic acid and flufenamic acid exhibited an inhibitory effect (300 μM, 1 mM and 300 μM, respectively) while the other NSAIDs were inactive (Arrigoni-Martelli, 1985). Furthermore, the iron-binding and hydroxyl radical scavenging actions of 20



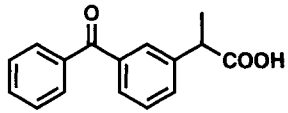
608
naproxen



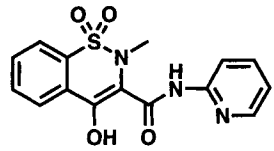
609
diflunisal



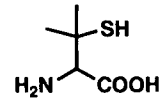
610
ibuprofen



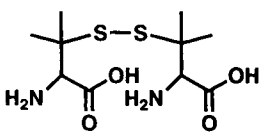
611
ketoprofen



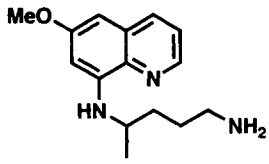
612
piroxicam



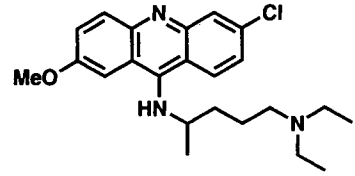
613
penicillamine



614
penicillamine disulphide

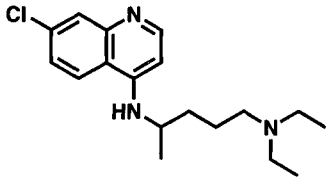


615
primaquine

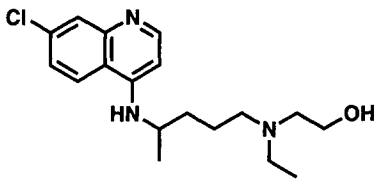


616
quinacrine

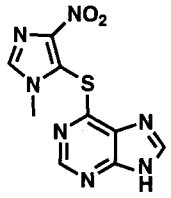
antiinflammatory drugs have been evaluated. These included naproxen (**608**), diflunisal (**609**), ibuprofen (**610**), indomethacin, ketoprofen (**611**), phenylbutazone, piroxicam (**612**), mefenamic acid, diclofenac, flufenamic acid, acetylsalicylic acid, DL-penicillamine (**613**), penicillamine disulfide (**614**), primaquine (**615**), quinacrine (**616**), chloroquine (**617**), hydroxychloroquine (**618**), azathioprine (**619**), phenobarbital (**620**) and acetaminophen (**621**). The ability to scavenge hydroxyl radicals and protect deoxyribose from radical degradation after addition of Fe^{III} was studied in a simple model system *in vitro*. All drugs had scavenging ability and also more or less pronounced iron-binding ability. The most effective drugs in both aspects were penicillamine, diclofenac, piroxicam, azathioprine, primaquine, chloroquine and



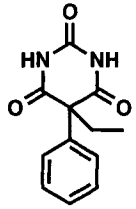
617
chloroquine



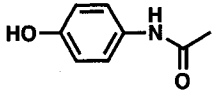
618
hydroxychloroquine



619
azathioprine



620
phenobarbital



621
acetaminophen

hydroxychloroquine. It was stated that the ability of an antiinflammatory drug to attenuate hydroxyl radical formation *in vivo* depends not only on its rate constant for reaction with the hydroxyl radical, but also on its metal-binding ability and on the geometry and redox potential of any metal complex formed (Aruoma and Halliwell, 1988).

It is well established from several *in vitro* studies that superoxide anion release is decreased by NSAIDs (van Dyke *et al.*, 1979; Abramson *et al.*, 1984a; Umeki, 1990; Colli *et al.*, 1991). For example, piroxicam diminishes superoxide production by activated phagocytes *in vitro*. The decreased oxidant generation demonstrated in cells isolated from patients taking piroxicam suggests that this effect is of biological relevance (Abramson *et al.*, 1984b; Kaplan *et al.*, 1984). Piroxicam interferes with the activation of NADPH oxidase, resulting in decreased superoxide secretion from isolated stimulated granulocytes from rheumatoid patients treated with the drug (Biemond *et al.*, 1986). In a related study, the production of superoxide by monocytes, stimulated with serum-treated zymosan and phorbol myristate acetate (PMA), was inhibited by diclofenac, 100 mg twice daily (Bell *et al.*, 1991). Diclofenac has also been reported to inhibit the chlorinating activity of myeloperoxidase (Zuurbier *et al.*, 1990).

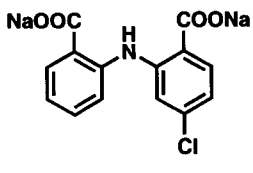
It has been suggested that indomethacin causes lipid peroxidation by a mechanism independent of the cyclooxygenase pathway, and that the lipid peroxides so formed inhibit prostaglandin synthetase and thereby cause

gastric ulcers (Puig-Parellada and Planas, 1985). It has further been proposed that oxygen free radicals interact with indomethacin to cause gastrointestinal injury (Del Soldato *et al.*, 1985) and that free radical products derived not only from indomethacin but also from acetylsalicylic acid are mediators of their gastric toxicity (Del Soldato *et al.*, 1987). In a recent study, the *in vivo* effects of indomethacin on the activity of antioxidant enzymes and on lipid peroxidation in erythrocytes, liver and small intestine of the rat were examined. The activity of the enzymes studied increased or remained unchanged, depending on the preparation and model used (treatment with therapeutic or ulcerogenic dose of indomethacin). Indomethacin inhibited lipid peroxidation in liver but not in erythrocytes. These results suggest that the stimulation of antioxidant enzymes, proposed to occur via metal complexes formed *in vivo*, is an alternative mechanism of the anti-inflammatory action of indomethacin (Kirkova *et al.*, 1992).

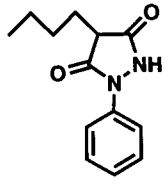
The inhibition of human polymorphonuclear leukocyte functions by ibuprofen has been examined. Pretreatment of neutrophils with ibuprofen resulted in an irreversible, concentration-dependent inhibition of superoxide anion generation after stimulation by the synthetic peptide fMLP or by C5a. In contrast to neutrophil responses mediated by fMLP and C5a, ibuprofen did not inhibit superoxide anion generation after stimulation with PMA. Ibuprofen did not function as a scavenger in a cell-free system, in which superoxide anion was generated by the aerobic action of xanthine oxidase on hypoxanthine (Nielsen and Webster, 1987).

Lobenzarit disodium (**622**), developed as an antirheumatic agent, appears to exert its therapeutic effect by modulating an immunological process (Yata *et al.*, 1982), although the precise mechanism of the antirheumatic action remains unknown. The drug showed a better quenching effect than mefenamic acid against hydroxyl radicals generated by the Fenton reaction. The effect of lobenzarit disodium on the autoxidation of linolenic acid and the UV irradiation of immunoglobulin G was examined. The results indicated that the compound had an antioxidant effect and protects against lipid and protein damage induced by ROS (Cynshi *et al.*, 1990).

Both phenylbutazone and mofebutazone (**623**) inhibit oxidative fragmentation of methionine derivatives induced by xanthine oxidase or by diaphorase-mediated hydroxyl radical production (Schneider and Elstner, 1993).



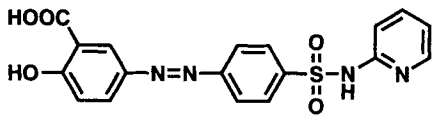
622
lobenzarit disodium



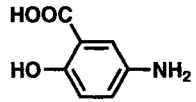
623
mofebutazone

6.1.2 Aminosalicylic Acids and Related Agents

Sulphasalazine (**624**) is effective in the treatment of ulcerative colitis (UC) and also gives benefit in rheumatoid arthritis. With regard to the effects on UC, 5-aminosalicylic acid (5-ASA, **625**), produced after reductive cleavage by bacteria in the human colon, is probably the active component. Inflammatory phagocytes may be activated by certain proinflammatory stimuli, and levels are known to be raised in UC, to release large amounts of reactive oxygen metabolites into the interstitial compartment (Sharon and Stenson, 1984; Wengrower *et al.*, 1987). It has been demonstrated that sulphasalazine and 5-aminosalicylic acid, used to attenuate the mucosal inflammation and injury associated with UC, are potent antioxidants (Hillier and Willson, 1983; Carlin *et al.*, 1985; Ahnfelt-Ronne and Nielsen, 1987; Aruoma *et al.*, 1987; Dull *et al.*, 1987; Miyachi *et al.*, 1987; Grisham, 1988; Ahnfelt-Ronne *et al.*, 1990; Greenfield *et al.*, 1991).

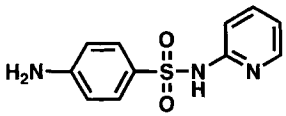


624
sulphasalazine

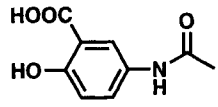


625
5-aminosalicylate
(5-ASA)

Sulphasalazine and also both its metabolites, sulphapyridine (**626**) and 5-aminosalicylic acid, were found to be powerful scavengers of hydroxyl radical as determined by pulse radiolysis and confirmed by assays based on deoxyribose degradation by hydroxyl radicals. 5-Aminosalicylic acid can also protect α_1-antiprotease against attack by the myeloperoxidase-derived oxidant hypochlorous acid. 5-ASA is a powerful scavenger of HOCl, whereas sulphasalazine is not (Aruoma *et al.*, 1987; Williams and Hallet, 1989a). Cytolytic activity in neutrophils, mediated by HOCl, was inhibited by 5-ASA at millimolar concentrations (Dallegri *et al.*, 1990). In another study (based on a spectrophotometric assay that utilized the stable free radical DPPH) 5-ASA, in contrast to sulphasalazine, sulphapyridine, *N*-acetyl-5-ASA (**627**)



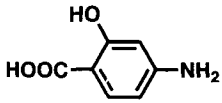
626
sulphapyridine



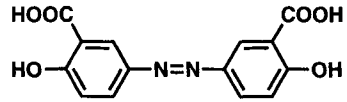
627
N-acetyl-5-ASA

and salicylates, shared with vitamins E and C the property of being a potent scavenger of free radicals (Ahnfelt-Ronne and Nielsen, 1987).

Also, 4-ASA (**628**), claimed to be beneficial in the topical treatment of UC, was found to be an excellent scavenger of HOCl (Halliwell *et al.*, 1988). The capacity of this drug to trap HOCl may well be of importance in the *in vivo* situation. 4-ASA exhibited no reactivity towards DPPH (Nielsen and Ahnfelt-Ronne, 1988). However, in an experiment where direct assessment of oxygen metabolites was made using ESR spectroscopy, 5-ASA and the new 5-ASA prodrug olsalazine (**629**), as well as 4-ASA, were found to possess superoxide-scavenging properties (IC_{50} 0.4, 0.4 and 1.0 mM, respectively).



628
4-aminosalicylate
(4-ASA)



629
olsalazine

Fatty acid radicals were not reactive toward the aminosalicylates (Allgayer *et al.*, 1992). Peroxidation of red blood cell membranes was inhibited dose-dependently by 5-ASA as deduced from experiments where malondialdehyde was used as a marker. Sulphasalazine was less effective (Greenfield *et al.*, 1991). In a related haemoglobin-catalysed lipid peroxidation experiment with phospholipids, 5-ASA exhibited antioxidant capacity, whereas sulphasalazine and olsalazine did not (Yamada *et al.*, 1991).

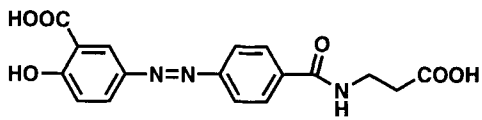
5-Aminosalicylic acid was less effective as a scavenger of the stable radical galvinoxyl than the corresponding 4-aminophenol as determined by ESR technique, demonstrating that presence of a carboxyl group in the *ortho* position diminishes the radical-scavenging capacity. Vitamins C and E exhibited comparable antioxidant capacity with 4-aminophenol in this experiment (Depew *et al.*, 1991).

The influence of 5-ASA, its *N*-acetylated metabolite, sulphasalazine and olsalazine in pharmacologically relevant concentrations (0.1–10 mM) were tested on PMNL superoxide production with either the receptor-specific peptide fMLP or phorbol ester. Inhibition of receptor-specific superoxide production occurred at 0.07, 0.32 and 0.63 mM (IC_{50} values) for 5-ASA, sulphasalazine and olsalazine, respectively. No inhibitory effects of sulphasalazine and olsalazine were observed when phorbol ester was applied as the stimulus (Nielsen *et al.*, 1993). In a related experiment, where superoxide anion radicals were generated after stimulation of neutrophils with phorbol ester, 5-ASA was also found to be more effective than the

azo-compounds as indicated by inhibiting luminol-dependent chemiluminescence (Gionchetti *et al.*, 1991).

The efficacies of sulphasalazine, 5-ASA and hydrocortisone were examined in a model of acetic acid-induced colitis in the rat. The ability of the antioxidant/5-lipoxygenase inhibitors gossypol and NDGA (**431**) and the cyclooxygenase inhibitor indomethacin to attenuate the macroscopic colonic damage and/or neutrophil influx (myeloperoxidase activity, MPO) associated with this model of colitis was tested (Fitzpatrick *et al.*, 1990). Oral pretreatment with either sulphasalazine, gossypol or NDGA significantly decreased colonic MPO activity induced by acetic acid. Intrarectal administration resulted in an even larger reduction of the colonic inflammation, with gossypol being the most potent compound. Corticosteroids also attenuated the parameters of acetic acid-induced colitis, while, in contrast, pretreatment with indomethacin was ineffective. The authors suggested that the drugs effective in this colitis model may act by scavenging of oxygen-derived free radicals.

A study of patients with chronic inflammatory bowel disease (CIBD) with respect to rectal mucosal lipid peroxidation (MDA method) has recently been performed, providing strong evidence in favour of a radical scavenger activity of 5-ASA *in vivo*. *In vitro* hydroxyl radical reaction with 5-ASA (Fenton reaction) revealed the formation of at least six metabolites. Three of these (unfortunately not identified structurally) were conclusively identified in six of seven randomly selected stool extracts from patients with CIBD treated with sulphasalazine. This study suggests that 5-ASA reacts with free radicals in the colon of patients with CIBD, and that free radicals are present in excess in the colon of such patients. A strong correlation was found to exist between the scavenging of free radicals in the colon (as determined by the level of lipid peroxides) and amelioration of the clinical symptoms (Nielsen and Ahnfelt-Ronne, 1991; Ahnfelt-Ronne *et al.*, 1990). 4-ASA is less effective than both 5-ASA and balsalazide (**630**) in inhibiting chemiluminescence in rectal biopsies from patients with active disease (Simmonds *et al.*, 1992). It is reasonable to assume that the antioxidant activity of 5-ASA could account for at least some of this drug's and the corresponding prodrugs' (sulphasalazine, olsalazine, balsalazide) anti-inflammatory effects in patients with UC. Yamada *et al.* (1990) have also proposed that the metal-binding



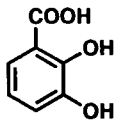
630
balsalazide

properties of 5-ASA provide an important mechanism by which it exerts anti-inflammatory activity *in vivo*.

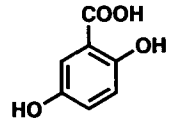
Inflammatory neutrophils may produce carcinogenic nitroso amines via the arginine-dependent formation of nitrogen oxides such as nitric oxide. 5-ASA, or N_γ-nitro-L-arginine methyl ester resulted in 80–85% inhibition of neutrophil-mediated nitroso amine formation. Inflammatory neutrophils may represent an important metabolic source of endogenous carcinogens during times of active inflammation (Grisham *et al.*, 1992), and thus antioxidants such as 5-ASA, olsalazine and sulphasalazine may prove useful in attenuating the formation of potentially carcinogenic agents *in vivo* (Grisham and Yamada, 1992; Grisham and Miles, 1994).

6.1.3 Salicylates

Salicylates are metabolized to hydroxylated compounds, including 2,3-dihydroxybenzoic acid (DHBA, **631**) and 2,5-dihydroxybenzoic acid (gentisic acid, **632**). These compounds are present in blood plasma and urine of



631
2,3-dihydroxybenzoic acid
(DHBA)



632
2,5-dihydroxybenzoic acid
(gentisic acid)

healthy human volunteers after aspirin ingestion, their identities having been confirmed by mass spectrometry and electrochemical analysis (Grootveld and Halliwell, 1988). The hydroxylase enzyme producing gentisate may also produce the 2,3-dihydroxy isomer as a minor product. Alternatively, the latter compound might arise as a product after hydroxyl radical attack on salicylic acid (Halliwell and Gutteridge, 1985; Grootveld and Halliwell, 1986).

It has been reported that the concentrations of gentisic acid found in plasma and synovial fluid were in excess of those required to exert an antioxidant effect *in vitro* as determined by a chemiluminescence model (Cleland *et al.*, 1985). Experiments have been conducted in which human neutrophils were incubated in medium containing 10 mM salicylate, and thereafter stimulated with phorbol ester for 1 h (neutrophils (1×10^6 cells) produced 55 ng of gentisic acid and also smaller quantities of 2,3-dihydroxybenzoic acid). Inhibitor experiments indicated that SOD, haem protein inhibitors and glutathione blocked gentisic acid formation, whereas catalase, mannitol and desferrioxamine were inactive. Hydroxylation of

salicylate to give gentisic acid and DHBA occurs only in activated neutrophils (Davis *et al.*, 1989). Apparently, activated neutrophils are able to hydroxylate salicylate by a pathway which is still unknown. This pathway may contribute to the increased recovery of hydroxylated salicylates in patients with inflammatory disorders (Davis *et al.*, 1989). It is in this context interesting to note that also 5-ASA is metabolized to salicylate and gentisate by activated mononuclear cells and neutrophils (Dull *et al.*, 1987) and that 5-ASA is metabolized in a reaction in which 5-ASA decreases the luminol-dependent chemiluminescence of neutrophils (Williams and Hallet, 1989b).

The hydroxyl radical-scavenging activity of salicylic acid, dihydroxybenzoic acids and catechol have been compared in a test system based on the inhibition of the ESR signal of 5,5-dimethyl-2-hydroxypyrrolidine-*N*-oxide (DMPO-OH) obtained by addition of hydroxyl radical to 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Catechol was found to be 6600 times more potent than salicylic acid, 2,5-dihydoxybenzoic acid or 2,3-dihydroxybenzoic acid in this particular system (Capelle *et al.*, 1992).

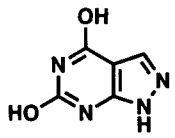
The anti-inflammatory action of salicylate derivatives is usually attributed to their effects on cyclooxygenase (Roth *et al.*, 1975; Higgs *et al.*, 1987; Vane and Botting, 1987). The observed therapeutic actions may also be derived, at least in part, from the metabolism of the salicylates at sites of inflammation by activated inflammatory cells (Sagone and Husney, 1987).

In principle, most anti-inflammatory drugs could protect cells from damage resulting from production of ROS at sites of inflammation. Hypochlorous acid reacts with most anti-inflammatory drugs, but not fast enough for scavenging to be feasible at normal concentrations of the drug at the site of inflammation *in vivo* (Wasil *et al.*, 1987). Furthermore, some NSAIDs have a weak capacity to bind iron ions in forms that are poorly active in the Fenton reaction *in vitro* (Wasil *et al.*, 1987). Concerning scavenging of the hydroxyl radical at the site of inflammation, it is likely that very high concentrations, in the millimolar range, should be required to trap this reactive radical. However, in some cases concentrations at that level can be achieved, for example in patients with rheumatoid arthritis given salicylate. The concentration of 2,3-dihydroxybenzoate was higher in rheumatoid patients treated with salicylate than in a control group (Grootveld and Halliwell, 1986). It also seems likely that anti-inflammatory drugs like aspirin at lower concentrations are capable to interact with less reactive oxygen-derived radicals and are consequently beneficial for attenuation of the inflammatory process. Besides the direct reaction of radicals with, or interference in their formation by, drugs, the resultant metabolites may be active as cyclooxygenase inhibitors or as antioxidants in the oxidative burst process. Thus, 2,5-dihydroxybenzoate was long ago reported to be a potent inhibitor of prostaglandin synthesis (Blackwell *et al.*, 1975; Holmes *et al.*, 1985). Graziano *et al.* (1974) observed that 2,3-dihydroxybenzoate scavenges hydrogen peroxide and also chelates

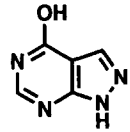
iron, which might be expected considering the structure of the molecule. It has also been reported that this metabolite interferes with iron-dependent events. For example, 2,3-dihydroxybenzoate chelates iron (Graziano *et al.*, 1974), interferes with iron-dependent radical reactions (Halliwell and Gutteridge, 1985; Halliwell *et al.*, 1985) and inhibits lipid peroxidation (Graziano *et al.*, 1976).

6.1.4 Miscellaneous

Oxypurinol (**633**) has a radical-scavenging effect and a novel mechanism of action for allopurinol (**634**) and oxypurinol in myocardial salvage has been postulated (Das *et al.*, 1987). Oxypurinol is an inhibitor of xanthine oxidase-catalysed production of superoxide radical (Spector, 1988) and can also scavenge HOCl (Grootveld *et al.*, 1987). Oxypurinol exerts a protective influence on the trinitrobenzene sulfonic acid (TNBS) model of inflammatory bowel disease in the rat. The accumulation of TBARS demonstrated a free radical-mediated component of the tissue damage (Siems *et al.*, 1992).



633
oxypurinol



634
allopurinol

Allopurinol is used in clinical medicine to inhibit uric acid accumulation in conditions such as gout, and oxypurinol is a major metabolite of allopurinol in the human body. Allopurinol has antioxidant capacity in biological systems (Faure *et al.*, 1990). Activated neutrophil granulocytes are an important source of oxygen free radicals in acute skin grafts. Allopurinol was superior to vitamin E in attenuating myeloperoxidase activity and lipid peroxidation in acute skin grafts (Goldstein *et al.*, 1992). One product of attack of hydroxyl radical upon allopurinol is oxypurinol, which is a superior hydroxyl radical scavenger. Hence, the protective actions of allopurinol against reperfusion damage after hypoxia need not be due entirely to xanthine oxidase inhibition (Moorhouse *et al.*, 1987). In fact, there is no strong evidence that xanthine oxidase makes any significant contribution to the production of reactive oxygen metabolites in human heart and brain (Wajner and Harkness, 1989; Werns and Lucchesi, 1990).

D-Penicillamine, used in the treatment of rheumatoid arthritis, can react with aldehydes and ketones to form thiazolidines, and forms disulfides with sulfhydryl group-containing molecules. The drug is also a powerful

chelator of transition metals. A combination of copper and penicillamine at physiological concentrations inhibits human fibroblast DNA synthesis. It is possible that the fibroblast proliferation, which is the process leading to destructive pannus formation, is inhibited by the copper–penicillamine combination as a result of hydrogen peroxide generation (Matsubara and Hirohata, 1988). According to this theory, hydrogen peroxide here provides an anti-inflammatory effect. With regard to copper itself, it was recently demonstrated that copper salts of indomethacin and acetylsalicylic acid exhibit a better anti-inflammatory effect than the parent drugs (Roch-Arveiller *et al.*, 1990). The role of the highly redox-active transition metal in the anti-inflammatory process is still obscure. Interestingly, the penicillamine–copper complex has also been suggested to act as a superoxide dismutase equivalent (Joyce, 1990). An anti-inflammatory effect is observed upon injection of superoxide dismutase into the inflamed human knee joint (Menander-Huber and Huber, 1977).

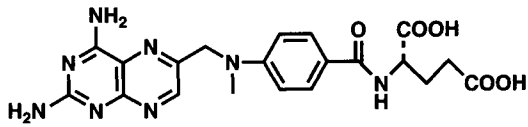
The role of penicillamine as a scavenger of hypochlorite (Cuperus *et al.*, 1987) and as an inhibitor of myeloperoxidase (Joyce, 1990) has been discussed. High concentrations of D-penicillamine have an ability to scavenge oxygen-derived free radicals (Betts *et al.*, 1984; Staite *et al.*, 1985) and this scavenging property may be a clinically relevant mechanism of action of the drug. The hydroxyl radical-scavenging activity of a series of slow-acting antirheumatic drugs (SARDs) including D-penicillamine, has been determined using the ESR–spin-trapping method in a cell-free system. D-penicillamine and most of the SARDs studied exhibited hydroxyl radical-scavenging activity. However, none of the drugs had a significant scavenging activity at concentrations observed in plasma from patients (Pronai *et al.*, 1990).

In one study the concentration of lipid peroxides in the plasma and synovial fluid of 65 arthritic patients was determined. Patients with rheumatoid arthritis receiving only NSAIDs had a significantly higher mean concentration of lipid peroxides in synovial fluid samples than osteoarthritic patients. No increase in plasma levels of lipid peroxides in the rheumatoid patients could be demonstrated. Treatment of rheumatoid arthritis with D-penicillamine was associated with a significant reduction of lipid peroxide levels, suggesting that this drug may function as an oxygen radical scavenger in the joint cavity. This result supports the concept that oxygen free radicals play an important role in the pathogenesis of chronic inflammatory disorders (Wade *et al.*, 1987).

Penicillamine therapy is often associated with many autoimmune reactions and it is possible that reactive thiyl radicals, derived from reactions with hydroxyl radicals or HOCl, contribute to the side-effects of the drug. One can also speculate that various disulfides, formed after radical-mediated coupling with a thiyl radical, may modulate the inflammatory response and consequently be beneficial in therapy.

Inhibitory effects on the generation of reactive oxygen species of the antimalarials chloroquine (**617**) and quinacrine (**616**) have been demonstrated both in polymorphonuclear leukocytes and in the xanthine/xanthine oxidase system. Although the plasma levels of antimalarials remain low (90–170 $\mu g\,l^{-1}$ after administration of therapeutic doses), the tissue concentrations are considered to be extremely high (McChesney *et al.*, 1957; Shaffer *et al.*, 1958). Thus, it has been suggested that the drugs can act as antioxidants at the site of inflammation, protecting against oxidative tissue damage with resulting anti-inflammatory effects (Miyachi *et al.*, 1986a). It has been reported that chloroquine and hydroxychloroquine interfere at multiple levels in the metabolic pathways leading to neutrophil superoxide release (Hurst *et al.*, 1988). As discussed above, chloroquine and quinacrine lack hydroxyl radical scavenging and Fe^{III} binding ability (Aruoma and Halliwell, 1988).

The folate antagonist methotrexate (**635**) has been used in a number of inflammatory diseases such as rheumatoid arthritis, severe asthma, systemic



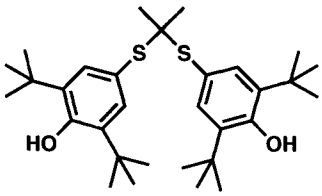
635
methotrexate

lupus erythematosus and sarcoidosis. Lipid peroxidation has been implicated in a number of inflammatory diseases and methotrexate was therefore studied *in vitro* in a lipid peroxidation assay, where malonaldehyde was measured. Methotrexate inhibited lipid peroxidation but only at high concentrations (Iqbal *et al.*, 1993).

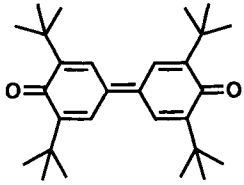
6.2 ANTIHYPERLIPOPROTEINAEMIC AND ANTIHYPERTENSIVE DRUGS

6.2.1 Probucol

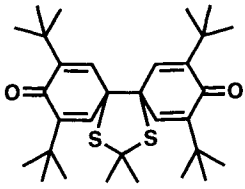
Probucol (**636**), used clinically to lower blood cholesterol levels, is a powerful antioxidant, as expected from the structural similarities to phenolic antioxidants. Probucol has been shown to inhibit oxidative modification of LDL (Parthasarathy *et al.*, 1986; McLean and Hagaman, 1989) and to prevent the secondary modification of the lysine groups on the surface of the LDL particle. *In vitro* evidence has been provided that the concentration of probucol in LDL determines the extent of oxidative modification of the



636
probucol



637



638

lipoprotein. Degradation of probucol to diphenoquinone (**637**) via a spiroquinone (**638**) precedes lipoprotein oxidation (Barnhart *et al.*, 1989). These metabolites have been detected in the serum of probucol-administered modified Watanabe heritable hyperlipidaemic (WHHL) rabbits (Mao *et al.*, 1991a).

The efficiencies of various antioxidants in linoleic acid micelles in the presence of sodium dodecyl sulfate have been studied. It was found that the peroxyl radical formed in this system was scavenged equally effectively by α-tocopherol and probucol (Pryor *et al.*, 1988). Oxidation of LDL that contained probucol produced probucol phenoxyl radical, and a synergistic interaction between probucol and ascorbate in inhibiting oxidation of LDL has been reported (Kalyanaraman *et al.*, 1992). Breugnot *et al.* (1990) produced model peroxyl radicals by irradiation of aerated ethanol and observed that probucol was inactive as scavenger. However, the same group reported that probucol was more efficient than vitamin E in protecting LDL against copper-induced peroxidation.

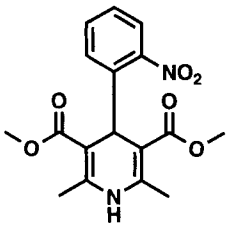
Ultraviolet irradiation of lipoproteins has been used as a model system for the study of the biological effects of lipid peroxides on cultured cells. The UV-induced lipid peroxidation of LDL was strongly inhibited by 25 μM probucol, but only poorly by 100 μM vitamin E. The UV-treated LDL protected by probucol was much less cytotoxic than unprotected UV-treated LDL. In contrast, LDL treated by UV in the presence of 100 μM vitamin E was cytotoxic, similar to unprotected LDL (Nègre-Salvayre *et al.*, 1991). Pretreatment with probucol prevented oxidative injury induced by organic hydroperoxides to endothelial cells to the same extent as pretreatment with α-tocopherol, but addition of probucol directly to medium containing hydroperoxide and oxidized LDL did not prevent endothelial cell toxicity. The authors suggested that it was necessary for probucol to be incorporated into the membrane to prevent cellular injury from hydroperoxide and oxidized LDL (Kuzuya *et al.*, 1991a). The same authors showed that the toxicity induced by copper-oxidized LDL was dependent on both the presence of transition metals and the lipid hydroperoxide content of LDL (Kuzuya *et al.*, 1991b).

Experimental and clinical studies suggest that probucol could be of interest in the prevention of atherosclerosis (Steinberg, 1988). The antiatherogenic effect of probucol seems unrelated to its hypercholesterolaemic effect. There is evidence that antioxidants can selectively inhibit *in vivo* LDL degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the WHHL rabbit (Carew *et al.*, 1987; Kita *et al.*, 1987; Finckh *et al.*, 1991; Mao *et al.*, 1991b). Probucol has been shown to inhibit atherogenesis in Watanabe rabbits. Atherosclerotic lesions contain macrophages capable of secreting interleukin 1 (IL-1) and other cytokines that could contribute to the pathogenesis. It was suggested that the observed inhibition of IL-1 secretion from macrophages by probucol contributes to its therapeutic effects in atherosclerosis and may also result in beneficial activity in some chronic inflammatory diseases (Ku *et al.*, 1990). Interestingly, O'Brien *et al.* (1991) reported that probucol did not suppress the levels of oxidized LDL in atherosclerotic lesions in the WHHL rabbit. Further, from a recent study entitled PQRST (probucol quantitative regression Swedish trial) involving 303 patients with hypercholesterolaemia, it was concluded that probucol had no effect on atherosclerosis. It was claimed that the lack of effect may be linked to the fact that, although probucol reduced atherogenic LDL-cholesterol level, it also lowered the concentration of protective HDL-cholesterol. This study was not designed to study probucol's antioxidant effects.

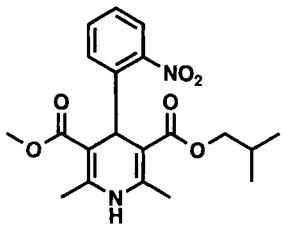
Oxygen free radicals have been implicated as mediators of pancreatic islet β-cell damage in autoimmune, insulin-dependent diabetes mellitus. A combination therapy with the antioxidant probucol and a corticosteroid prevented autoimmune diabetes in non-obesic diabetic (NOD) mice. (Rabinovitch *et al.*, 1992). The effects of probucol on the development of diabetes in BB rats have been studied, and it was suggested that the drug may alter the inflammatory process, resulting in β-cell destruction in these genetically diabetes-prone rats. The authors speculated that the beneficial effects observed after administration of probucol might be related to its antioxidant activity (Drash *et al.*, 1988).

6.2.2 Calcium Antagonists

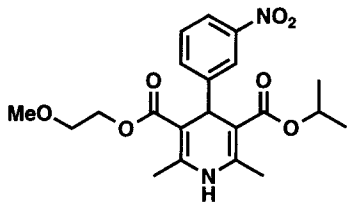
Janero *et al.* (1988) reported that cardiac membrane phospholipid was protected against oxidative injury by calcium antagonists. Relatively high concentrations of the calcium channel blocker nifedipine (**639**), unlike diltiazem and verapamil, protected cultured lymphoid cells against the cytotoxicity of oxidized LDL by two different mechanisms: (a) an antioxidant effect inhibiting LDL oxidation outside the cell and (b) a direct cytoprotective effect at lower concentrations, the mechanism of which is unknown. These data could explain in part the mechanism of action of nifedipine in the



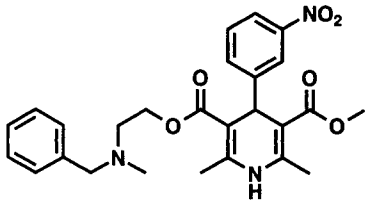
639
nifedipine



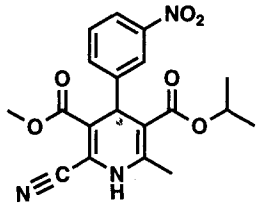
640
nisoldipine



641
nimodipine



642
nicardipine



643
nilvadipine

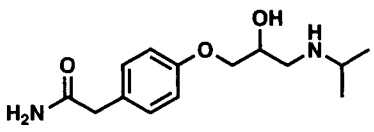
prevention of cellular damage potentially involved in atherogenesis (Nègre-Salvayre and Salvayre, 1992b). In a related study, lipid peroxidation of LDL, promoted by either UV irradiation or copper ions, was inhibited in a dose-dependent manner by nisoldipine (**640**) (IC_{50} around 10 μM) whereas nimodipine (**641**) was less potent (IC_{50} 50–100 μM) and nicardipine (**642**) were virtually inactive (Nègre-Salvayre *et al.*, 1992). Nilvadipine (**643**) inhibited ischaemic and carrageenan paw oedema as well as superoxide radical production from neutrophils and from xanthine oxidase. Oral dosing of nilvadipine suppressed carrageenan-induced paw oedema at a potency corresponding to that of the anti-inflammatory drug ibuprofen. Nifedipine, nicardipine and nimodipine were much less effective, and nitrendipine, diltiazem and verapamil were without effect. Nilvadipine inhibited superoxide radical production from xanthine oxidase both with the lactate dehydrogenase/NADH method and the cytochrome *c* method. It was suggested that the observed superior inhibitory activity of nilvadipine on active oxygen-related ischaemic damage and inflammation may prevent atheromatous development of blood vessels (Oyanagui and Sato, 1991). Provided that the antioxidant effect observed is a result of hydrogen abstraction from the bisallylic position and that the stability of the formed radical is essential, nilvadipine could be expected to be a superior antioxidant compared with the other calcium antagonists. An important stabilization of the radical by the nitrile function could be expected. Furthermore, the higher

potency of nisoldipine compared with that of nimodipine and nicardipine might result from radical stabilization by the nitro group in the former, although the structural dissimilarities make a relevant comparison difficult.

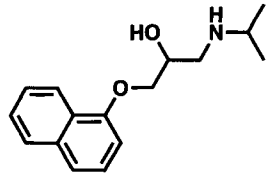
In an effort to distinguish the nature of cytoprotection, Mak and Weglicki (1994) examined the effects of pharmacologically active (+) and inactive (−) enantiomers of nicardipine. The compounds displayed identical dose-dependent inhibitory activities against lipid peroxidation of sarcolemmal membranes: (+)-nicardipine, EC_{50} 20.6 μM; (−)-nicardipine, EC_{50} 22.8 μM (to be compared to BHT, EC_{50} 5.2 μM).

6.2.3 *β-Adrenoceptor Blockers and Captopril*

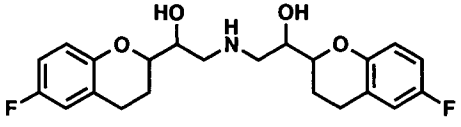
Iron-dependent sarcolemmal lipid peroxidation *in vitro* is reduced by β-receptor blockers, possibly via lipid membrane interactions rather than direct free radical scavenging (Tong Mak and Weglicki, 1988). A study was undertaken to evaluate the potential cardiac membrane lipid peroxidation inhibitory capacity and anti-ischaemic efficacy of a series of β-adrenoceptor blockers in relation to their β-adrenoceptor blockade. The hydrophilic atenolol (**644**) was less active than the more lipophilic agent propranolol (**645**) as an antiperoxidative agent (Mousa *et al.*, 1992). The antioxidant property of β-blockers might represent an additive factor against myocardial ischaemic injury. Atenolol is ineffective as an anti-ischaemic agent in an isolated perfused rabbit heart model of global ischaemia, while other β-blockers, such as propranolol and nebivolol (**646**), are effective, indicating that the



644
atenolol



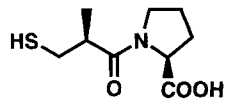
645
propranolol



646
nebivolol

myocardial protective effects cannot be explained solely by the β-blocking effect (Vandeplassche *et al.*, 1988).

The thiol captopril (**647**), an inhibitor of angiotensin-converting enzyme which reduces hypertension (Ondetti *et al.*, 1977), possesses anti-inflammatory properties (Martin *et al.*, 1984) and has been found to provide protection against reperfusion arrhythmias (vanGilst *et al.*, 1986). Captopril has been reported to be a potent free radical scavenger, with a scavenging power as effective as superoxide dismutase against superoxide, as effective as dimethylthiourea against hydroxyl radical and better than allopurinol against HOCl (Bagchi *et al.*, 1986). The free radicals were generated from various biochemical systems (Das *et al.*, 1987). More recently, it was reported that captopril does not scavenge superoxide, and that it rather prevents superoxide production by chelating copper (Jay *et al.*, 1991).



647
captopril

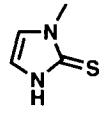
6.3 MISCELLANEOUS

Acetaminophen (**621**) has found increasing use as a substitute for acetylsalicylic acid as a mild pain-killer. The mechanism of action of acetaminophen is unknown (Clissold, 1986). Acetaminophen reduces the hydroperoxide levels in tissues. Since cyclooxygenase is activated by hydroperoxides, Lands (1981) suggested that acetaminophen could indirectly reduce prostaglandin synthesis. However, many other reducing agents do not share the pharmacological effects of acetaminophen. More recently, Vane (1994) suggested that a third isozyme of cyclooxygenase ("COX3"), located in the brain, might be inhibited by the agent. Acetaminophen does not inhibit lipoxygenases, but Su *et al.* (1995) found that the drug potently inhibited linoleic acid dioxygenase, a fungal enzyme. Whether the drug also inhibits oxygenation of polyunsaturated fatty acids in humans remains to be determined.

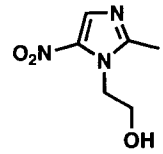
Fast flow systems have enabled the detection of the 4-acetamidophenoxyl radical, a species believed to be an important metabolic intermediate in the activity and toxicity of this drug (Fischer *et al.*, 1986). The effect of acetaminophen on the respiratory burst of isolated human polymorphonuclear leukocytes (PMNLs) has been studied. The drug inhibited the chemiluminescence peak response of cells stimulated with PMA or opsonized zymosan in a concentration-dependent manner. Generation of superoxide

was markedly inhibited. Furthermore, the phagocytic activity of PMNLs was significantly reduced in acetaminophen-treated cells. Thus, acetaminophen causes significant inhibition of the human PMNL functions *in vitro*. The therapeutic plasma level of acetaminophen is within the range of concentrations used in this study and it is possible, although there is no direct evidence, that the drug may have the same effect on PMNL function *in vivo* (Ali Shalabi, 1992).

When skin is exposed to UV radiation, free radicals are generated (Pathak and Stratton, 1969). Methimazole (**648**), 2-mercaptobenzimidazole and indomethacin abolished the erythema response after topical application. Methimazole is a potent scavenger of hydroxyl radicals (Taylor *et al.*, 1984) and it was suggested that the suppression of the UV-induced inflammatory lesions observed could be a consequence of oxygen free radical-scavenging by the thiocarbamide compounds. This action appeared more likely in influencing the inflammatory response *in vivo* than inhibition of the prostaglandin synthetase system. The topical use of radical scavenging compounds during radiotherapy may protect surface tissues (Ferguson *et al.*, 1985).



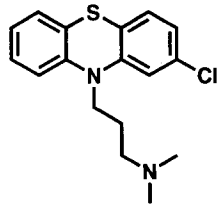
648
methimazole



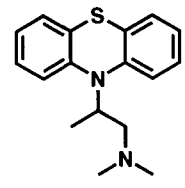
649
metronidazole

The effect of metronidazole (**649**) on the generation of ROS has been examined both in neutrophils and in a xanthine/xanthine oxidase system. Metronidazole had an antioxidant effect, which was not due to the scavenging of reactive oxygen metabolites, but to action on neutrophil function. It was proposed that the beneficial effects of metronidazole in the treatment of papulopustular rosacea might in part be attributable to this anti-inflammatory effect (Miyachi *et al.*, 1986b).

Chlorpromazine (**650**) and promethazine (**651**) are well established



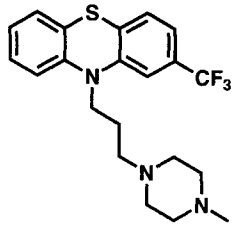
650
chlorpromazine



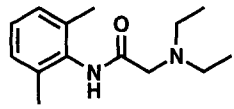
651
promethazine

to inhibit carbon tetrachloride-induced lipid peroxidation in rat liver microsomes (Slater, 1968; Poli *et al.*, 1989). Chlorpromazine inhibits lipid peroxidation in rat brain and liver (Hoshi *et al.*, 1990), as well as reducing oxidative injury to cardiac phospholipid by membrane stabilization (Janero and Burghardt, 1989). Polarographic studies demonstrated that the half-wave potential of substituted phenothiazines is dependent on the alkyl substituent on the nitrogen atom, and that unsubstituted derivatives have lower oxidation potentials (Kabassakalian and McGlotten, 1959). This has been confirmed in recent electrochemical studies of phenothiazines (Zimova *et al.*, 1990). Chlorpromazine and a series of related phenothiazines have been evaluated as cytoprotective agents using primary cultures of neuronal cells exposed to hydrogen peroxide. While the phenothiazines unsubstituted at the nitrogen atom exhibited cytoprotective effects, chloropromazine was totally ineffective. Although multiple mechanisms may be involved, the inhibition data reported are in the rank order of potency predicted by the compounds' antioxidant capacity as measured electrochemically and by their ability to inhibit iron-dependent lipid peroxidation *in vivo* (Yu *et al.*, 1992).

Chlorpromazine and trifluoperazine (**652**) were as effective as probucol in inhibiting copper-catalysed oxidation of LDL and its subsequent recognition and catabolism by mouse peritoneal macrophages (Breugnot *et al.*, 1990). The authors suggested that lipophilic agents such as probucol and phenothiazines, in addition to their antioxidant capacity, may alter the physical properties of the phospholipid membrane and thereby render the lipoprotein more resistant to oxidative attack. Trifluoperazine exhibits a protective effect on mitochondrial damage induced by copper II/pro-oxidants (Pereira *et al.*, 1992).



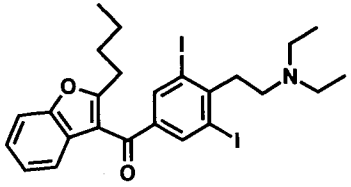
652
trifluoperazine



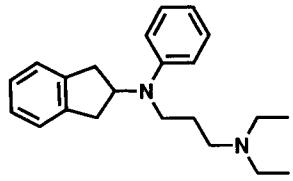
653
lidocaine

Lidocaine (**653**), an antiarrhythmic and local anaesthetic agent, reduced in coronary sinus the levels of conjugated dienes, a marker of lipid peroxidation, during reperfusion of postischaemic myocardium, possibly through membrane stabilization (Lesnefsky *et al.*, 1989). The effect of a series of antiarrhythmic agents on lipid peroxidation induced by iron/ascorbate, using rat hepatic microsomes, has been studied. A trend toward a parabolic

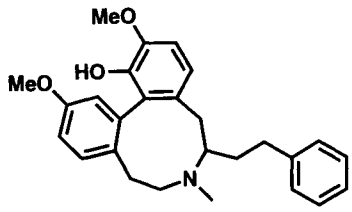
relationship between antioxidant potency and lipophilicity was observed. Three of the tested antiarrhythmics – the lipophilic amiodarone (**654**), aprindine (**655**) and asocainol (**656**) – were very potent antioxidants. Lidocaine had a negligible effect on lipid peroxidation up to a concentration of 1 mM. The authors suggested that, at least for some antiarrhythmic drugs, antioxidant activity may be part of the mode of action, and that this may form an additional beneficial feature for the treatment of cardiac failure (Rekka *et al.*, 1990).



654
amiodarone

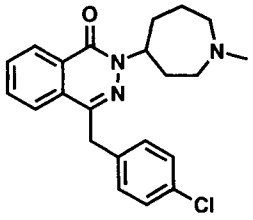


655
aprindine

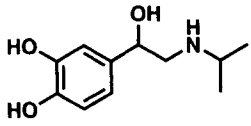


656
asocainol

Antiasthma drugs have been evaluated with regard to antioxidant actions. The inhibitory actions of antiallergy drugs, β-agonists, theophylline and corticosteroids on the *in vitro* generation of superoxide anion by human PMNLs were studied using a chemiluminescence method. Azelastine (**657**) and isoproterenol (**658**) inhibited fMLP-induced superoxide generation in a



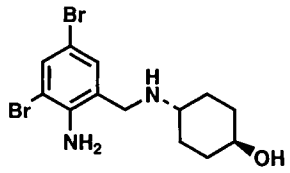
657
azelastine



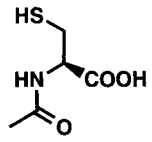
658
isoproterenol

dose-dependent fashion, whereas the other drugs lacked inhibitory actions except at very high concentrations. Isoproterenol inhibited generation from the hypoxanthine–xanthine oxidase system, unlike azelastine and the other drugs. The authors suggested that azelastine and isoproterenol inhibit the process of superoxide generation from PMNLs, while isoproterenol also scavenges superoxide. This may be beneficial in the treatment of airway inflammation due to superoxide generation in bronchial asthma (Kato *et al.*, 1991).

Ambroxol (**659**), a drug from the expectorant class, which stimulates the formation and release of surfactant by pneumocytes type II, exerts antioxidant activity *in vitro*. It was suggested that this antioxidant activity might have clinical significance in protecting lung tissue from oxidant-induced injury (Nowak *et al.*, 1994).



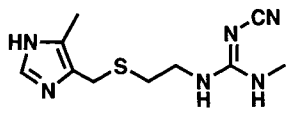
659
ambroxol



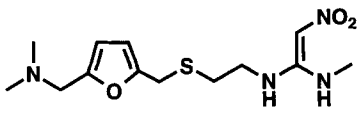
660
N-acetyl-L-cysteine

The antioxidant action of *N*-acetyl-L-cysteine (**660**) and its reaction with hydrogen peroxide, hydroxyl radical, superoxide and hypochlorous acid have been studied (Aruoma *et al.*, 1989). It has been demonstrated that both *N*-acetyl-L-cysteine and *N*-acetyl-D-cysteine protect rat lungs exposed to pure oxygen and attenuate the inflammatory response (Särnstrand *et al.*, 1995). Although the expectorant *N*-acetyl-L-cysteine used against chronic bronchitis is given in high doses, the oral bioavailability of the L-stereoisomer (but not the D-stereoisomer) is low (Sjödin *et al.*, 1989) and it is not likely that **660** exerts its therapeutic effect by direct interaction with the oxidative burst process.

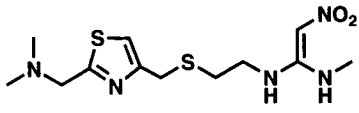
Histamine H_2-receptor antagonists in clinical use were found to be potent inhibitors of MPO-catalysed reactions under conditions resembling those in experiments with intact neutrophils. Since peak plasma concentrations of cimetidine (**661**), ranitidine (**662**) and nizatidine (**663**) are well within the



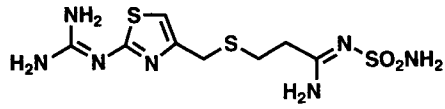
661
cimetidine



662
ranitidine



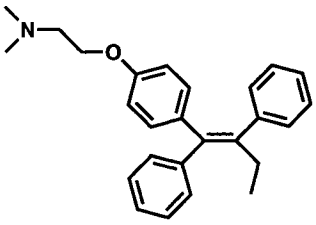
663
nizatidine



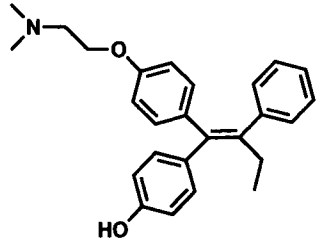
664
famotidine

micromolar range after oral therapeutic dosing, the results may be of clinical relevance. The inhibitory actions of cimetidine and nizatidine were largely due to scavenging of HOCl. In contrast to famotidine (**664**), ranitidine was also a potent scavenger of HOCl, while both drugs inhibited MPO reversibly. The HOCl-scavenging potencies of ranitidine and nizatidine were about three times higher than that of penicillamine, which had a potency similar to that of cimetidine. Using riboflavin as a probe, the H_2 antagonists were found to be inhibitors of hydroxyl radical generated in an iron–hydrogen peroxide reaction mixture. The drugs were also efficient chelators of iron. The gastrointestinal tract can contain potentially reactive iron and the authors proposed that the presence of H_2 antagonists might serve to suppress iron-driven steps in tissue damage (van Zyl *et al.*, 1993).

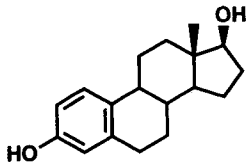
The antioestrogen tamoxifen (**665**) is now being assessed in clinical trials as a prophylactic agent against breast cancer (Powles, 1992; Bush and Helzlsouer, 1993; Jordan, 1993). Tamoxifen, 4-hydroxytamoxifen (**666**) and 17β-oestradiol (**667**) act as antioxidants *in vitro*, inhibiting metal ion-dependent lipid peroxidation (Wiseman *et al.*, 1990) and protecting human LDL against oxidative damage (Wiseman *et al.*, 1993a). Tamoxifen decreases lipid peroxidation in postmenopausal women with breast cancer (Thangaraju *et al.*, 1993). Wiseman and Halliwell (1994) reported that tamoxifen protects against the formation of genotoxic reactive intermediates and products of lipid peroxidation in the nuclear membrane. It was demonstrated that a membrane-stabilizing action against lipid peroxidation via decreased



665
tamoxifen



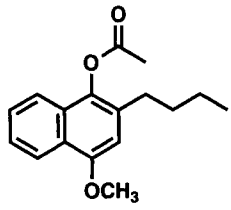
666
4-hydroxytamoxifen



667
17β-oestradiol

membrane fluidity by tamoxifen and related compounds is likely to be the mechanism of their antioxidant action (Wiseman *et al.*, 1993b). The authors suggested that this protection could be important in the prevention of nuclear DNA damage and that this possible anticarcinogenic effect could be of importance in long-term therapy with tamoxifen as well as in its proposed use in prevention of breast cancer.

As mentioned in section 1, a more subtle role of ROS as mediators involved in signal transduction, especially with respect to the transcription factor NF-κB, has attracted great interest over the last few years (Schreck and Baeuerle, 1991; Schreck *et al.*, 1992; Meyer *et al.*, 1993; Schenk *et al.*, 1994). Bunaprolast (**668**) has been described as the most potent antioxidant inhibitor of endothelial activation of NF-κB and gene expression of E-selectin and vascular cell adhesion molecule-1 (Manning and Anderson, 1994).



668
bunaprolast

7 Conclusions

The involvement of free radicals and oxidative stress in a number of major human diseases seems continuously to acquire experimental support. The pathological significance of reactive oxygen species has gradually gained acceptance in the biochemical community, even if the arguments sometimes reside upon circumstantial evidence. The recent topicality of the field is certainly reflected in the extensive use of dietary nutrients and antioxidant vitamin supplements as well as by the growing number of fundamental, clinical and epidemiological studies employing such vitamins and their analogues.

The participation of free radicals is sometimes offered as an ethiological possibility in situations that are not readily explained by other factors. This might have entailed an over-optimistic view of the therapeutic potential of pharmaceutical antioxidants. A word of caution has consequently been put forward by several authors regarding the risk of invoking radicals as a crucial factor in too many cases. Clinical and *in vivo* studies might have been expected to demonstrate a salvage role of endogenous antioxidants and

therapeutic agents in a larger number of instances if the view of the true believers was correct to the full extent. In our opinion, it has not been shown conclusively that any of the therapeutic agents in current use exerts its beneficial effect solely by an antioxidant mechanism. Clinically used therapeutics may still be acting by virtue of other pharmacological actions in conjunction with antioxidant properties.

The development of novel antioxidant compounds is perhaps no longer a challenge from a chemical point of view. Rather, the design of biologically relevant substances which are able to become properly assimilated into, and support, the endogenous defence network evolves as the primary objective for the development of pharmaceutical antioxidants. It appears that a multitude of sufficiently potent chain-breaking antioxidants with high intrinsic capacity are already at hand, not least the ones provided by evolution. The disappointing pharmacological experiences with synthetic agents encountered in many cases may result from inadequate incorporation of other essential physicochemical properties. The design of new therapeutics will benefit from the generation of indepth knowledge regarding the role and mechanistic features of oxidative stress in different pathophysiological conditions.

As discussed in Section 6, there are established examples where drugs are present at concentrations expected to result in significant interference with the oxidative burst process. However, it is not clear to what degree the pure antioxidant capacity of the drugs discussed contribute to the observed clinical effects. Only salicylates, aminosalicylates, sulphasalazine and possibly penicillamine and antihistamines seem to reach concentrations *in vivo* that would allow competition with biomolecules for reactions with reactive species such as hydroxyl radical and hypochlorous acid. Many of the drugs discussed in Section 6 are metabolized under oxidative pressure to new compounds expected, and sometimes known, to possess alternative properties that might be of clinical importance.

Prodrugs with suitable properties could be envisaged to be based on the array of compounds described in this review. A chain-terminating drug should ideally be regenerable by endogenous sacrificial reducing agents within the site of high oxidative burden, mimicking the catalytic performance of endogenous antioxidants. Some of the agents under development might prove the true potential of pharmaceutical antioxidants. Additionally, the recent progress in the art of mimicking enzymatic antioxidants promises to result in highly interesting substances in the future. Concerning anti-inflammatory drugs, it is a challenge for the future to develop antioxidants that are activated at the site of inflammation and that act as precursors for second-line compounds acting as potent inhibitors of the arachidonic acid cascade, iron chelators, scavengers, etc. Successful targeting would be beneficial, but the strategy is still associated with problems related to design of antioxidants with pre-determined sites of metabolism. The evolving insight

into the role of ROS in signal transduction pathways might provide novel approaches for intervention of, for example, inflammatory conditions via specifically designed antioxidants.

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Multidrug Resistance of Cancer Cells

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ALL	Acute lymphoid leukaemia
AML	Acute myeloid leukaemia
ATP	Adenosine triphosphate
CAT	Chloramphenicol acetyl transferase
CFTR	Cystic fibrosis transmembrane conductance regulator
CLL	Chronic lymphoid leukaemia
CML	Chronic myeloid leukaemia
CsA	Cyclosporin A
GPx	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione *S*-transferase
MDR, *mdr*	Multidrug-resistance genes
MRP	Multidrug-resistance-associated protein
NHL	Non-Hodgkin's lymphoma
Pgp	P-glycoprotein
PKA, PKC	Protein kinase A, protein kinase C
RT-PCR	Reverse transcriptase-polymerase chain reaction
TM	Transmembrane domain
TopoII	Topoisomerase II
VAD	Vincristine–doxorubicin–dexamethasone

1 Introduction and Overview

The development of multidrug-resistance remains a serious impediment to curative chemotherapy in patients with cancer. Operationally, multidrug-resistance is defined as the intrinsic or acquired resistance of cancer cells to multiple classes of structurally and mechanistically unrelated chemotherapy drugs. Perhaps the best-characterized mechanism of multidrug-resistance, at least in tissue culture model systems, is that mediated by *mdr* genes, which encode the multidrug transporter known as P-glycoprotein, P170 or Pgp. *Mdr*-related multidrug-resistance has been exhaustively reviewed and this chapter is not intended to be a comprehensive discussion of the *mdr* literature. Rather, it will focus primarily on recent reports relating to issues that remain unresolved in the field and on developments that have come to the forefront in the past few years.

After a brief overview of *mdr* genes and Pgp activity, we will discuss the clinical relevance of the human *MDR*1 gene and Pgp, the mechanism by which Pgp confers drug-resistance and the regulation of *MDR*1 gene activity in normal and cancer cells. We will briefly review what is known about other

cellular mechanisms for conferring resistance to *mdr*-related drugs. Finally, we will return to the clinical aspect of *mdr* by discussing prospects for overcoming multidrug-resistance in light of what is now known about its mechanisms of action. For additional information on clinical, biochemical and molecular aspects of multidrug-resistance, the reader is referred to recent reviews contributed by Bellamy and Dalton (1994), Germann (1993), Gottesman and Pastan (1993), Gottesman *et al.* (1994) and chapters in Goldstein and Ozols (1994).

Multidrug-resistance was originally characterized by the decreased intracellular accumulation of several classes of drugs (see Table 1). Reduced drug accumulation results in reduced cytotoxicity. Using hamster, mouse and human tissue culture cell lines selected for their resistance to one or more drugs, researchers identified a 170 000-dalton plasma membrane glycoprotein (Pgp) whose expression was associated with this type of resistance (see Section 3 and reviews by Kane *et al.*, 1990; Gottesman and Pastan, 1993). Complementary DNAs encoding Pgp were cloned from each of these species and designated *mdr* genes.

There is a small family of *mdr* genes in mammals, with two human genes, *MDR*1 and *MDR*2 (Chen *et al.*, 1986; van der Bliek *et al.*, 1988) and three rodent genes, *mdr*1a, *mdr*1b and *mdr*2, for example, in mice (Gros *et al.*, 1986, 1988; Hsu *et al.*, 1989; Devault and Gros, 1990). Expression of the *mdr*1 class of genes, but not *mdr*2 genes, is sufficient for conferring drug-resistance in drug-sensitive cell lines and in animals (Ueda *et al.*, 1987a; Gros *et al.*, 1988; van der Bliek *et al.*, 1988; Mickisch *et al.*, 1992). For the remainder of this review, *MDR*1 or *mdr* will be used generically to refer to mammalian genes functional in conferring multidrug-resistance, unless a different *mdr* gene is specifically being discussed.

Sequencing and functional analysis of *MDR*1 complementary DNAs (cDNAs) identified Pgp as a 1280-amino acid protein (in humans) that shows sequence and structural homology to the superfamily of proteins known as traffic ATPases or ABC transporters (ABC = ATP binding cassette) (reviewed by Ames *et al.*, 1992; Higgins, 1992). A large body of biochemical and genetic evidence supports a model of Pgp as an ATP-dependent drug transporter in the plasma membrane that prevents intracellular drug accumulation and thereby renders cells drug-resistant (see Section 3).

Substrate utilization by Pgp is broad, encompassing neoplastic drugs, peptides, steroids, immunosuppressive and antifungal agents, and a variety of other cytotoxic compounds (Table 1). A number of Pgp inhibitors have been identified which are able to reverse the effects of Pgp on multidrug resistance. These include verapamil, cyclosporin A, quinidine, reserpine, trifluoperazine and others. Photoaffinity drug analogues have been useful for probing drug binding to Pgp and for studying the mechanism of action of the drug transporter (Table 1).

*MDR*1 overexpression has been associated with intrinsic and acquired

TABLE 1

Partial list of agents that interact with Pgp

Antineoplastic drugs
- Vinblastine, vincristine
- Doxorubicin, daunomycin, mitoxantrone
- Bisantrene
- Etoposide, teniposide
- Topotecan
- Mitomycin C
- Paclitaxel, taxotere
- Mithramycin
- Actinomycin D

Other agents
- Colchicine
- Rhodamine 123
- Ethidium bromide
- Erythromycin
- Ketoconazole
- Puromycin
- Lipophilic cations (TPP^+, TPA^+)

Steroid hormones[a]
- Progesterone (not transported)
- Aldosterone (transported)
- Cortisol (transported)
- Tamoxifen
- Megestrol
- Dexamethasone (transported)

Modulators/chemosensitizers[a]
- Verapamil (transported), diltiazem (transported), bepridil
- Azidopine (transported), nitrendipine (not transported), nifedipine, nicardipine
- Quinidine, amiodarone
- Cyclosporin A (weakly transported), FK506 (transported), SDZ PSC-833, rapamycin
- Terfenadine
- Trifluoperazine, flupenthixol
- Monensin
- Quinine, quinicrine
- Reserpine
- Forskolin

Peptides
- Gramicidin D
- Valinomycin
- *N*-acetyl-leucyl-leucyl-norleucine (ALLN)

Photoaffinity drug analogues
- Azidopine
- 6-*o*-[[2-[3-(4-azido-3-iodophenyl)proprionamido]ethyl]-carbamyl]forskolin
- *N*-(*p*-azido-3-iodosalicyl)-*N'*-(β-aminoethyl)vindesine (NASV)
- Iodoarylazidoprazosin (IAAP)
- *N*-(*p*-azido-3-iodophenethyl)spiperone
- Tamoxifen aziridine
- Analogues of colchicine, daunomycin, verapamil and others

[a]Modulators and steroid hormones are classified as being substrates for transport by Pgp or inhibitors of drug transport without themselves being transported (see text).
TPP^+ tetraphenylphosphonium; TPA^+ tetraphenylarsonium.

resistance of many types of human cancers, but its contribution to clinical resistance to chemotherapy is still being determined. Early clinical trials using chemotherapy in combination with Pgp inhibitors have had mixed results, the most promising coming in the treatment of some types of blood-borne cancers (see next section).

2 *MDR*1 and Clinical Multidrug Resistance

Despite an overwhelming literature on *MDR*1 expression and Pgp activity in tissue culture model systems of multidrug resistance (see Nielsen and Skovsgaard (1992) for a review of multidrug-resistant cell lines), the association of *MDR*1 with the clinical phenomenon of multidrug resistance remains controversial. This is pointedly illustrated by a recent pair of articles supporting *MDR*1 as a good target for reversing drug resistance in certain haematopoietic malignancies on the one hand (Dalton and Sikic, 1994), but emphasizing its limited contribution to resistance of non-haematological cancers on the other (Houghton and Kaye, 1994). While the case for *MDR*1 involvement in clinical resistance is strongest for blood-borne disease, however, it is probably premature to dismiss its association with either the intrinsic or acquired resistance of solid tumours.

In general, inherently resistant tumours tend to arise from tissues that normally express high levels of *MDR*1 (see below), and continue to express *MDR*1 in the tumour. Furthermore, many untreated cancers show expression of *MDR*1 even though they derive from tissues that do not normally express the gene or express it at low levels. These tend to show higher levels or higher incidence of *MDR*1 in relapse samples than in untreated samples, but the association between *MDR*1 expression and response to chemotherapy is not clear for these cancers. There has been some suggestion that Pgp expression is associated with more aggressive disease (Weinstein *et al.*, 1991), but this does not seem to be a general observation with most cancers. Below is a more detailed discussion of the methods used to measure *MDR*1 in cancer and the general results of such studies to date.

2.1 METHODOLOGY

2.1.1 Measurement of MDR*1 Expression*

The limitation in resolving the question of the clinical relevance of *MDR*1 continues to be the tools and methods with which the appropriate studies can be approached. There has been a general lack of uniformity in the type and quality of assays used to measure *MDR*1 messenger RNA (mRNA) or Pgp protein in clinical samples, making it difficult to compare results from studies using different methods or even from different laboratories using the same methods. RNA-based assays include Northern and slot blots, reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridization. *In situ* hybridization has the advantage of being able to detect cell-to-cell heterogeneities in expression, but it is tedious to perform. RT-PCR is useful for screening large numbers of specimens, but it does not distinguish between normal and tumour cells within a sample and can be difficult to perform quantitatively.

Protein-based assays include Western blotting, immunohistochemistry and flow cytometry. Like *in situ* hybridization, the latter two have the advantage of being able to detect heterogeneity of Pgp expression in tumour samples. A number of antibodies are available for these assays, but they are variable in their specificity and sensitivity. The most useful are those that detect extracellular epitopes of Pgp and thus only measure presumably functional protein expressed at the cell surface. These antibodies include MRK-16, UIC2 and 4E3 (Hamada and Tsuruo, 1986; Mechetner and Roninson, 1992; Arceci *et al.*, 1993; Schinkel *et al.*, 1993a).

Brophy *et al.* (1994) recently did side-by-side comparisons of four methods (*in situ* hybridization, mRNA slot blot, RT-PCR and immunohistochemistry with MRK-16) and measured *MDR*1 expression in cell lines established from clinical samples. In 39% of the cases, all techniques agreed as to whether the cell line was positive or negative for *MDR*1 expression. In a further 39%, there was agreement among all but one of the techniques. Taking the consensus result of these assays as the correct one, the conclusions of the study were that *in situ* hybridization and RT-PCR are the most accurate techniques (fewest false positives and negatives and most true positives and negatives), but that immunohistochemistry is predictive for a clinical complete response of the patient on the basis of Pgp levels.

Herzog *et al.* (1992) also found that RT-PCR is the most sensitive and quantitative method for detecting low levels of *MDR*1 expression in cell lines, similar to levels seen in most clinical samples. Quantitation with antibody-based assays was difficult at the lowest levels of expression in this study. It should be noted that RT-PCR assays vary greatly from laboratory to laboratory and the accuracy of this method cannot necessarily be extrapolated to other studies.

One functional assay for Pgp activity is to measure intracellular accumulation of a fluorescent drug (e.g. daunorubicin) in the absence or presence of a Pgp modulator. For clinical studies, this method is best applied to haematopoietic diseases on which analysis can be performed without the need for cell culturing. However, care must be taken in choosing the correct drugs and inhibitors for these studies, and they should not be performed in the absence of any independent measure of *MDR*1. One recent report demonstrated no correlation between *in vitro* daunorubicin accumulation and *MDR*1 mRNA levels in neoplastic cells (protein was not measured) (Marie *et al.*, 1993) and some non-Pgp mechanisms of drug-resistance can apparently affect drug accumulation (see Section 5). Furthermore, reversal of drug uptake by the Pgp modulators verapamil or cyclosporin A can sometimes also be associated with non-*MDR*1 forms of resistance (see Cole *et al.*, 1994; Gruber *et al.*, 1994) and not all inhibitors are equally effective in reversing specific drug accumulation in some cell types (Herweijer *et al.*, 1990; Cardarelli *et al.*, 1995).

Finally, it is still not clear how to define a positive result once a reliable

measurement of *MDR*1 expression has been attained. It is not known what level of positivity or what proportion of *MDR*1-positive cells in a population might be significant for interfering with clinical response to chemotherapy. While there have been attempts to correlate *MDR*1 expression level with drug-resistance, these generally rely on *in vitro* determinations of resistance which may or may not correspond to clinical drug-resistance.

2.1.2 Experimental Design

In addition to problems encountered when different methods of measurement are used, studies of *MDR*1 in cancer have been limited by the types of clinical specimens that are available. Early studies were mostly retrospective measurements of *MDR*1 at a single time-point in disease. Given the problems associated with heterogeneity of tumours, different standards of positivity, different drug combinations used to treat patients and patient-to-patient variability in *MDR*1 expression, it is not surprising that there is significant variability in the results of these studies.

Recently, attempts have been made to measure *MDR*1 in patients prospectively, before treatment, then to correlate clinical outcome with *MDR*1 levels. An extension of this approach is to follow *MDR*1 expression within the same patient as a function of drug-resistant disease progression. A higher incidence of *MDR*1 expression associated with drug-resistant disease and an enrichment for *MDR*1-positive cells during disease progression would be clear indications that *MDR*1 is contributing to the drug-resistant phenotype. However, prospective, longitudinal studies are difficult, particularly with non-haematological cancers. Furthermore, if chemotherapy protocols to treat patients include both *MDR*1- and non-*MDR*1-related drugs, then the correlation (or lack of it) between *MDR*1 and disease progression is difficult to interpret. If non-*MDR*1 drugs are included in a treatment protocol, then some *MDR*1-positive cells could be killed by those drugs while other cells survive, presumably by a combined mechanism of resistance to *MDR*1 and non-*MDR*1 drugs. Thus a low level of *MDR*1-positive cells in relapsed patients could nevertheless be significant in terms of drug-resistant disease if those cells were able to expand in the face of further drug treatments. These issues must be taken into account when assessing *MDR*1 and clinical multidrug-resistance.

2.2 *MDR*1 IN CANCER

2.2.1 Haematological Malignancies

It is not uncommon to detect *MDR*1 expression in untreated haematological malignancies (see reviews by Licht *et al.*, 1994; Nooter and Sonneveld, 1994).

Incidence of *MDR*1 positivity tends to be higher in samples from treated patients (Nooter and Herweijer, 1991). In prospective studies of acute leukaemia, there is generally good association between *MDR*1 expression and poor response to chemotherapy in acute myeloid leukaemia (AML), but less of an association for acute lymphoid leukaemia (ALL) (Table 2). In a statistical overview of 12 AML studies using a technique called meta-analysis, Holmes and West (1994) found a significant difference in remission rates between *MDR*1-positive and *MDR*1-negative patients.

On the other side of the coin, Pieters *et al.* (1992) found no evidence of *MDR*1 expression in childhood ALL, either in 28 cases before treatment or in 14 cases at relapse, and a similar result has been obtained for adult AML and ALL (Ito *et al.*, 1989). In longitudinal studies, the pattern of *MDR*1 expression before and after relapse is less clear, but it is worth noting the results of Musto *et al.* (1991) who measured the percentage of neoplastic cells expressing cell surface Pgp as a function of disease progression. There is a dramatic enrichment for Pgp-expressing cells at relapse in this study, in patients with AML and ALL.

Less information is available about chronic leukaemia. Chronic myeloid leukaemia (CML) tends to be positive for *MDR*1 when patients are in blast crisis but negative in the chronic phase. Kuwazuru *et al.* (1990) found that three patients with CML who were Pgp negative, by Western analysis, at the time of complete remission, were Pgp positive when they relapsed and then they did not respond to chemotherapy. Keith *et al.* (1990) observed a single patient with CML who was *MDR*1 negative before treatment but positive after anthracycline-based chemotherapy.

In chronic lymphoid leukaemia (CLL), there appears to be variable *MDR*1 expression levels in samples from untreated and treated patients and no overall difference in expression between the two populations of patients (Shustik *et al.*, 1991). This conclusion was borne out in a recent study by Sparrow *et al.* (1993) who found widely varying percentages of Pgp positive cells in samples from both untreated and treated patients at various stages of disease. Wulf *et al.* (1994) also observed a wide range of Pgp levels in treated and untreated samples, but they found a greater incidence and higher average expression of *MDR*1 in patients treated with *MDR*1-related drugs than in untreated patients or those treated with non-*MDR*1 drugs. *MDR*1-negative patients had a median survival time nearly 2.5 times longer than *MDR*1-positive patients.

Results in patients with multiple myeloma are once again conflicting. In a small study, of eight patients who had received no prior therapy or therapy with non-*MDR*1 drugs, two were positive for *MDR*1 mRNA and did not respond to subsequent treatment that included *MDR*1 drugs. Of the six *MDR*1-negative patients, five responded to subsequent *MDR*1-related therapy (Linsenmeyer *et al.*, 1992). Grogan *et al.* (1993) found striking evidence for selection of *MDR*1-positive neoplastic cells in patients treated

TABLE 2

Correlation of Pgp/*MDR*1 with clinical outcome in acute leukaemias

Reference	No. positive[a]	CR[b]	No. negative[a]	CR[b]	Method[c]	Criteria[d]
AML						
Campos *et al.* (1992)	71	23	79	64	Flow cytometry	>20% positive
Gruber *et al.* (1992)	14	9	20	15	RNAse protection	Any detectable
Kuwazuru *et al.* (1990)	9	2	8	7	Western blot	Any detectable
Marie *et al.* (1991)	16	5	7	5	Slot blot	Twofold over K562
Pirker *et al.* (1991)	44	24	17	16	Slot blot	Any detectable
Sato *et al.* (1990)	20	8	13	10	Northern blot	Any detectable
te Boekhorst *et al.* (1993)	30	8	22	16	Immunocytochemistry	>30% positive
Zhou *et al.* (1992)	19	6	42	32	Immunocytochemistry	>5% positive[e]
Total	223	85 (38%)	208	165 (79%)		
ALL						
Brophy *et al.* (1994)	5	3	6	6	Multiple[f]	Consensus result[f]
Goasguen *et al.* (1993)	21	16	38	35	Immunocytochemistry	>1% positive[g]
Gruber *et al.* (1992)	4	3	8	8	RNAse protection	Any detectable
Kuwazuru *et al.* (1990)	4	2	7	6	Western blot	Any detectable
Total	34	24 (71%)	59	55 (93%)		

[a]Refers to the Pgp/*MDR*1 status of the sample.
[b]Number of patients showing complete response (also includes partial responses for Kuwazuru *et al.*).
[c]Method used to measure Pgp/*MDR*1.
[d]Criteria for Pgp/*MDR*1 positivity.
[e]This study also used slot blots to quantify *MDR*1 mRNA and found similar results. No difference was seen if two- to threefold higher thresholds were used for Pgp/*MDR*1 positivity.
[f]See text for explanation of methods.
[g]81% of the Pgp-positive patients subsequently relapsed and only 15% of those went on to a second remission; 37% of the Pgp-negative patients subsequently relapsed and 38% of those went into second remission.

with vincristine and doxorubicin. Whereas *MDR*1-positive cells were detected in only 3 of 47 (6%) untreated patient samples, the incidence increased steadily in patients exposed to one or more *MDR*1-related drugs. Thus, there were *MDR*1-positive cells in 2 of 16 (12%) patients receiving a low cumulative dose of both vincristine and doxorubicin, in 7 of 13 (54%) patients if either of the drugs was received at high dose and in 11 of 11 (100%) patients receiving high doses of both drugs. Cornelissen *et al.* (1994) reported on 63 patients who failed primary therapy with alkylating agents (six of whom received doxorubicin as part of front-line therapy). Thirty-three of the patients had more than 60% of cells positive for Pgp expression on the cell surface, but there was no correlation between *MDR*1 expression and subsequent response to therapy with vincristine, doxorubicin and dexamethasone. Despite this latter report, studies with *MDR*1-reversing agents suggest that the Pgp component in multiple myeloma can contribute significantly to its multidrug resistance phenotype (see below).

There appears to be some association between *MDR*1 and clinical outcome in non-Hodgkin's lymphoma (NHL), although the data are again inconsistent (reviewed by Yuen and Sikic, 1994). Generally, though not uniformly in all studies, there is lower incidence of *MDR*1 expression in untreated samples than in those taken at recurrence after chemotherapy. Schlaifer *et al.* (1990) reported significant Pgp levels in macrophages and epithelial cells of patients with NHL and Hodgkin's lymphomas, but no evidence of expression in neoplastic cells. Table 3 provides a compilation of results showing the relationship between *MDR*1/Pgp and response to chemotherapy, combining both previously untreated and treated patients with NHL. Except in two studies, *MDR*1/Pgp seems to be a prognostic indicator for poor response. As always, however, these results are confounded by the fact that different chemotherapy regimens were used to treat patients, some had been previously treated and others not, and different assays and criteria were used to assess *MDR*1/Pgp levels.

2.2.2 Solid Tumours

It is much more difficult to assess the contribution of *MDR*1 to multidrug-resistance of solid tumours than with haematological diseases. In addition to differences in architecture between three-dimensional tumour masses and free-flowing neoplastic cells of the blood, there are important limitations on the availability of appropriate clinical samples from patients with solid tumours, as well as a greater likelihood that those samples will be contaminated with non-malignant cells. This is especially a problem when using bulk techniques to measure *MDR*1 expression, such as RT-PCR, slot blot or Western analysis.

Nevertheless, some general conclusions can be drawn regarding *MDR*1

TABLE 3

Correlation of Pgp/*MDR*1 with clinical outcome in lymphomas

Reference	No. positive[a]	CR/PR[b]	No. negative[a]	CR/PR[b]	Method[c]	Criteria[d]
Cheng *et al.* (1993)	11	4	10	9	Immunohistochemistry	≥10% positive
Dan *et al.* (1991)	3	1	9	7	Northern blot	Any detectable
Moscow *et al.* (1989)	6	4	9	6	Northern blot	≥Fourfold over MCF-7
Niehans *et al.* (1992)	20	17	19	12	Immunohistochemistry	>10% positive
Pileri *et al.* (1991)	25	6	35	23	Immunohistochemistry	≥2% positive
Rodriguez *et al.* (1993)	17	5	24	10	Dot blot	≥Twofold over K562
Total	82	37 (45%)	106	67 (63%)		

[a]Refers to the Pgp/*MDR*1 status of the sample.
[b]Number of patients showing complete or partial response.
[c]Method used to measure Pgp/*MDR*1.
[d]Criteria for Pgp/*MDR*1 positivity.

in solid tumours. In general, tumours that are inherently resistant to chemotherapy arise in organs that normally express *MDR*1 at significant levels and continue to express the gene in the tumour. These include colonic, kidney, liver, adrenocortical and pancreatic cancers, all of which have significant levels of Pgp in at least 70% of untreated tumours (Fojo *et al.*, 1987a, b; Thiebaut *et al.*, 1987; Kakehi *et al.*, 1988; Goldstein *et al.*, 1989; Kanamaru *et al.*, 1989; Cordon-Cardo *et al.*, 1990; Mizoguchi *et al.*, 1990; Weinstein *et al.*, 1991; Flynn *et al.*, 1992; Pirker *et al.*, 1993; Itsubo *et al.*, 1994). It now appears that endometrial cancer belongs in this class as well, as Schneider *et al.* (1993) observed Pgp in ten of ten normal endometrial glands and 23 of 23 tumours, most with 50–100% of the cells expressing Pgp. Carcinoid tumours and phaeochromocytomas also usually overexpress *MDR*1 (Goldstein *et al.*, 1989).

In a second class of tumours, *MDR*1 is expressed in many samples taken from untreated patients, even though the tissue of origin expresses the gene at low, heterogeneous, or undetectable levels. This class includes cancer of the breast (Goldstein *et al.*, 1989; Weinstein *et al.*, 1993; Charpin *et al.*, 1994), ovary (Holzmayer *et al.*, 1992; Arao *et al.*, 1994), cervix (Riou *et al.*, 1990; Schneider *et al.*, 1992), stomach (Mizoguchi *et al.*, 1990; Robey-Cafferty *et al.*, 1990; Wallner *et al.*, 1993), oesophagus (Robey-Cafferty *et al.*, 1990), lung (Lai *et al.*, 1989; Scagliotti *et al.*, 1991; Holzmayer *et al.*, 1992; Abe *et al.*, 1994), head and neck (Keith *et al.*, 1990) and bladder (Benson *et al.*, 1991; Naito *et al.*, 1992; Clifford *et al.*, 1994), sarcomas (Gerlach *et al.*, 1987; Chan *et al.*, 1990; Noonan *et al.*, 1990; Roessner *et al.*, 1993; Stein *et al.*, 1993) and neuroblastomas (Bourhis *et al.*, 1989a; Goldstein *et al.*, 1990; Chan *et al.*, 1991).

In many of these cancers, there is a tendency towards poorer clinical outcome associated with *MDR*1 expression, and higher expression after chemotherapy, but the contribution of *MDR*1 to drug resistance is still not clear. There are almost assuredly other resistance mechanisms working in these cancers as well. In cases where it has been studied, there are often lower levels of *MDR*1 expression in tumour tissue than in corresponding normal tissue from the same patients, suggesting either contamination of the tumour with other cell types or clonal outgrowth of a malignant cell expressing lower than average levels of *MDR*1. Nevertheless, it is possible that the low-expressing cells are able to contribute to drug-resistant disease.

For a few solid tumours, we have information about whether *MDR*1 is a prognostic indicator of clinical outcome. Some of these are summarized here. *MDR*1 expression is low and heterogeneous in all types of lung cancer, probably reflecting the gene's activity in normal lung tissue. In non-small cell lung carcinoma, there does not appear to be a correlation between *MDR*1 levels in pretreatment specimens and clinical outcome in patients (Lai *et al.*, 1989; Scagliotti *et al.*, 1991; Shin *et al.*, 1992; Abe *et al.*, 1994). In a small study of small cell lung carcinoma, Holzmayer *et al.* (1992) found that three

of three *MDR*1-negative tumours responded to chemotherapy that included *MDR*1-related drugs while four of four *MDR*1-positive tumours did not respond, even though some of these had only low levels of expression.

The same group studied ovarian carcinoma and found that five of seven *MDR*1-negative tumours responded to chemotherapy while nine of ten *MDR*1-positive ones did not. They suggest that subpopulations of *MDR*1-positive cells contribute to clinical resistance in these cases (Holzmayer *et al.*, 1992). Arao *et al.* (1994) analysed 52 untreated ovarian carcinomas and eight normal ovaries. They saw *MDR*1 expression in all tumours, but never higher than the low expression seen in normal tissue. There was little correlation between expression and clinical outcome, although all four of the patients who had recurrent disease after resection and chemotherapy had shown increased *MDR*1 expression before treatment. Earlier studies showed elevated Pgp/*MDR*1 in ovarian carcinoma after chemotherapy but not before treatment (Bell *et al.*, 1985; Bourhis *et al.*, 1989b; Rubin *et al.*, 1990).

Neuroblastomas and astrocytomas frequently express *MDR*1 at presentation and at relapse, with generally higher levels after chemotherapy (Bourhis *et al.*, 1989a; Goldstein *et al.*, 1990) and a higher response rate to chemotherapy in Pgp-negative patients (Chan *et al.*, 1991). In a single follow-up case, Tishler *et al.* (1992) reported on a neuroectodermal tumour that was negative at initial resection, before chemotherapy, but positive at recurrence.

Untreated adult and childhood sarcomas (soft tissue and bone) frequently express *MDR*1 (Gerlach *et al.*, 1987; Roessner *et al.*, 1993; Stein *et al.*, 1993; Vergier *et al.*, 1993). There was good correlation between expression and clinical outcome in a recent study of Ewing's sarcoma: 2 of 12 Pgp-positive patients responded to chemotherapy while six of nine Pgp-negative patients responded (Roessner *et al.*, 1993). *MDR*1 is expressed in about 30% of untreated gastric carcinomas (Robey-Cafferty *et al.*, 1990; Wallner *et al.*, 1993) and in six of ten untreated oesophageal cancers (Robey-Cafferty *et al.*, 1990). Of 18 previously treated oesophageal cancers, six of six from patients with a partial or complete response were Pgp negative while 6 of 12 with no response were Pgp positive.

In breast cancer the data are still conflicting. In general, *MDR*1 mRNA or Pgp is detected in about 50% of breast carcinomas (Weinstein *et al.*, 1993; Charpin *et al.*, 1994), although two studies failed to detect any *MDR*1/Pgp in 121 cases (Merkel *et al.*, 1988; Dixon *et al.*, 1992). On closer inspection, there is a trend toward low levels and heterogeneous expression in untreated breast cancer, with slightly higher expression and a greater proportion of Pgp-positive cells in samples taken after one or more rounds of chemotherapy with at least one *MDR*1-related drug (Goldstein *et al.*, 1989; Schneider *et al.*, 1989; Sanfilippo *et al.*, 1991; Weinstein *et al.*, 1993; Charpin *et al.*, 1994).

There have been a few attempts to correlate Pgp expression with clinical outcome in breast cancer, but no real pattern has emerged. Verrelle *et al.*

(1991) observed that six of seven patients with intense immunopositivity of at least 75% of cancer cells showed no change or progressive disease upon subsequent chemotherapy. Ro *et al.* (1990) also suggested a good correlation between Pgp positivity, measured by immunohistochemistry, and less than partial response to therapy; of eight patients in their study who showed complete response to induction chemotherapy, all had been Pgp negative before therapy. In a separate set of patients, however, this same group found no differences in expression in successive samples taken before and after chemotherapy and upon recurrence. Again, given our lack of understanding about what levels of Pgp are clinically significant, any Pgp positivity before or after chemotherapy could be important with respect to response or recurrence.

2.3 TRIALS WITH REVERSING AGENTS

Perhaps the litmus tests for *MDR*1 involvement in clinical multidrug resistance are the clinical trials that attempt to modulate multidrug resistance with agents that inhibit Pgp transport activity (Tsuruo *et al.*, 1982). If *MDR*1/Pgp is involved in at least some component of clinical multidrug resistance, then chemotherapy in the presence of inhibitors should have some therapeutic benefit over the same chemotherapy alone. A number of phase I, II and III trials have now been initiated with modulators as diverse as verapamil, quinidine, cyclosporin A and trifluoperazine (reviewed by Raderer and Scheithauer, 1993; Dalton and Sikic, 1994; Houghton and Kaye, 1994). Early trials with verapamil as the Pgp modulator had promising results for overcoming drug resistance in haematological diseases, but unacceptably high toxicities were associated with the doses of modulator required for Pgp inhibition.

Subsequent results have generally been less encouraging for treating solid tumours, even those that frequently express raised *MDR*1 such as colon and breast (Raderer and Scheithauer, 1993; Houghton and Kaye, 1994). However, these studies have suffered from the same difficulties and inconsistencies associated with measuring *MDR*1/Pgp in clinical samples. Mostly, they point out the need for carefully designed trials to gain useful information about *MDR*1 and multidrug resistance. To be most informative, trials should be performed on patients who are truly refractory to a particular drug regimen; *MDR*1 levels should be assessed to know whether a Pgp-related mechanism is expected to be involved in resistance (with the obvious caveats concerning our lack of knowledge about clinically relevant levels of *MDR*1); and the same drug regimen used before treatment failure should subsequently be used in combination with modulator, to assess the specific effect of the inhibitor on the resistance mechanism at hand. Adequate pharmacological analysis should also be performed with each modulator to know its effect on cytotoxic drug pharmacokinetics.

Somewhat informative phase I/II trials have recently been performed with cyclosporin A (CsA) as a modulator in the treatment of haematological diseases (Raderer and Scheithauer, 1993; Dalton and Sikic, 1994). Results of these studies also speak more directly to the issue of whether *MDR*1/Pgp is involved in clinical multidrug resistance. Trials with CsA in combination with cytotoxic drugs (anthracyclines, etoposide, vincristine) have demonstrated serum concentrations of the modulator in the range known to reverse drug-resistance completely *in vitro* and in animal models (Lum *et al.*, 1992; Yahanda *et al.*, 1992; List *et al.*, 1993a; Bartlett *et al.*, 1994). That this level of CsA has some clinical significance is suggested by the CsA dose-dependent, transient hyperbilirubinaemia seen in these trials and the concomitant increase in serum steady-state levels of cytotoxic drug. The most straightforward explanation for these results is that CsA is inhibiting liver- and kidney-associated Pgp or other transporters in a way that prevents renal clearance of drug and enhances serum bilirubin levels (see Section 4.1 on the expression and function of Pgp in normal tissue). Hepatic and other non-haematological toxicities of CsA were deemed to be manageable and not dose limiting in these studies.

Haematological toxicities are substantial with CsA in combination with cytotoxic drug. This could be due to the aforementioned higher steady-state serum concentrations of drug in the presence of CsA and the resulting prolonged exposure of haematopoietic cells to that drug. Alternatively, there could be inhibitory effects of CsA on Pgp-expressing haematopoietic cells, making them more susceptible to drug uptake and toxicity (Lum *et al.*, 1992; List *et al.*, 1993a). To control for the former possibility, investigators have recommended that modified doses of cytotoxic agent be used in combination with CsA so as to compensate for the effects of the modulator on drug pharmacokinetics (Yahanda *et al.*, 1992; Bartlett *et al.*, 1994). This type of approach will be necessary to assess the effectiveness and mechanism of Pgp chemosensitizers in reversing drug-resistance.

In a phase I/II trial of daunorubicin plus CsA, treating poor-risk patients with AML and CML in blast crisis, the remission rate was high (List *et al.*, 1993a). Sixty-nine per cent of patients achieved complete or partial response or non-progression of the disease. However, this study did not include direct comparison of drug with and without CsA, so it is difficult to assess whether CsA-mediated inhibition of Pgp is contributing to the response rate. Interestingly, however, seven patients in this study were tested for *MDR*1 status before and after treatment with modulator. Five patients were *MDR*1 positive before treatment. Four of these had a complete response with daunorubicin plus CsA and then were *MDR*1 negative at relapse; one had a partial response and lower *MDR*1 levels at relapse than before treatment. Two patients were *MDR*1 negative before treatment, had a complete response and remained *MDR*1 negative at relapse.

These results are consistent with the findings of Sonneveld *et al.*

(1994), who treated eight patients with VAD (vincristine–doxorubicin–dexamethasone)-refractory multiple myeloma with three rounds of VAD plus CsA. Four patients were followed sequentially throughout the trial. All four were Pgp negative before VAD alone but they were refractory to therapy; 50–99% of neoplastic plasma cells were Pgp positive after VAD failure and before VAD plus CsA; all had lower (5–40%) percentages of Pgp-positive neoplastic cells one month after VAD plus CsA; the two with the lowest Pgp positivity had continued low Pgp representation at subsequent relapse. Of the four other patients in the study, three had lower Pgp positivity after VAD–CsA than before; the one patient with higher Pgp positivity after VAD–CsA had progressive disease during the third course of treatment. Of all eight VAD-refractory patients, five showed partial response to VAD–CsA. Finally, cells from one patient tested *in vitro* showed CsA-enhanced accumulation of vincristine and doxorubicin in cells isolated before VAD–CsA administration. In cells taken after treatment there was higher accumulation of drug *in vitro* and no enhancement by CsA.

Taken together, the results of these last two studies suggest that: (1) *MDR*1 does play a role in drug-resistance of AML and multiple myeloma; (2) Pgp inhibition by CsA selects against cells expressing functional Pgp on their cell surface; (3) remitting activity of CsA appears to be due at least partially to Pgp modulation and not just altered drug pharmacokinetics in the presence of modulator; and (4) non-Pgp mechanism(s) can emerge to allow relapse of disease after complete or partial response to combination therapy (List *et al.*, 1993a; Sonneveld *et al.*, 1994).

While these studies are encouraging and indicate that further clinical trials with modulators are warranted, they also point to the need for better chemosensitizing agents, appropriate animal models to test novel Pgp modulators and their pharmacological effects, and more information about alternative mechanisms of resistance to Pgp-related drugs. We will return to these issues in the final sections of this review. One other point is that the focus has been on correlating *levels* of *MDR*1/Pgp with clinical outcomes. It is also possible that mutations or polymorphisms in *MDR*1, if they occurred in functionally important domains of Pgp, could contribute to enhanced drug resistance at relapse, without the need for raised *MDR*1 expression. Such sequence changes in Pgp could also potentially alter its interaction with reversing agents, thus accounting for the emergence of drug-resistant disease that also no longer responds to Pgp modulators. Very little has been done to look for *MDR*1 mutations in clinical samples (see Holmes *et al.*, 1992).

Some of the uncertainty about *MDR*1 in cancer also comes from our lack of basic information about the mechanism of action of Pgp and the regulation of *MDR*1 gene expression. As we learn more about how specific drugs and inhibitors interact with Pgp, we will be able to design more rational drug–modifier combinations that should circumvent *MDR*1 and have therapeutic benefit if *MDR*1 is a major contributor to resistance. As we learn

more about the normal tissue, temporal and developmental regulation of *MDR*1 gene expression, we might understand whether *MDR*1 should contribute to resistance depending on the location, differentiation state or growth state of cancer cells. As we learn more about the function of Pgp in normal cells, we might understand the possible contribution of its natural substrates to the development of resistance. These areas of *MDR*1 research will be addressed in the next sections of this review.

3 Mechanism of Action of the *MDR*1 Gene Product

Pgp was originally identified in hamster, mouse and human cell lines as a 170-kDa membrane glycoprotein whose overexpression was associated with the phenomenon of multidrug-resistance. Cell lines that overexpressed Pgp were able to take up but not accumulate drugs such as vinblastine, colchicine and Adriamycin (Dano, 1973; Juliano and Ling, 1976; Fojo *et al.*, 1985; Willingham *et al.*, 1986). Experiments using plasma membrane vesicles and polarized epithelial cell systems demonstrated that Pgp is able to bind and apparently efflux drugs in an ATP-dependent fashion (Cornwell *et al.*, 1986; Safa *et al.*, 1986; Horio *et al.*, 1988, 1989; Kamimoto *et al.*, 1989; Lelong *et al.*, 1992). Drug binding and transport of drug substrates are inhibited by other drugs that are part of the multidrug resistance phenotype and by agents that reverse multidrug resistance in cultured cells. Cloning of *mdr* cDNAs encoding Pgp identified it as a 1280-amino-acid (in humans) protein bearing striking structural similarities to a number of ATP-dependent transport systems known at the time (see Section 3.3.3). This homology supported the notion that Pgp might be a drug transporter.

Current issues in drug transport centre on defining the exact mechanism of Pgp-mediated drug transport. What biochemical activities – ATP hydrolysis, drug binding, drug transport, ion channel – can be directly attributed to Pgp and what are the characteristics of those activities? From which compartment(s) in the cell is drug extracted and what are the definable steps involved in drug transport? In what steps is ATP hydrolysis involved? What structural and functional domains on Pgp are responsible for drug binding and the substrate specificity of the transporter? A combination of biochemical and genetic techniques is being used to approach these questions, and recent advances in these areas are discussed here. Current models for Pgp's mechanism of action are presented at the end of this section.

3.1 FUNCTION OF PGP

3.1.1 Biochemical Activities Associated with Pgp

Purification of functional Pgp has been problematic, perhaps because of the need for a proper membrane environment to maintain biochemical activity.

Recent successes in measuring biochemical activities associated with Pgp have come from using heterologous expression systems, membrane vesicles prepared from multidrug resistant cells and *mdr*-transfected cells, and partially purified Pgp reconstituted into proteoliposomes.

3.1.1.1 ATP hydrolysis. Several lines of evidence suggest that ATPase activity should be associated with Pgp: (1) Pgp is able to bind 8-azido-[^{32}P]ATP, a photoactivatable analogue of ATP (Cornwell *et al.*, 1987); (2) Pgp-associated drug transport by membrane vesicles and epithelial cell monolayers requires hydrolysable ATP (Cornwell *et al.*, 1987; Horio *et al.*, 1988, 1989; Kamimoto *et al.*, 1989; Tamai and Safa, 1990; Doige and Sharom, 1992; Lelong *et al.*, 1992); (3) Pgp, like other transport proteins, contains two consensus ATP binding domains encoded in its primary amino acid sequence (Chen *et al.*, 1986); and (4) altered forms of Pgp carrying mutations in either of these domains are unable to confer multidrug resistance in transfected cells (Azzaria *et al.*, 1989).

Initial attempts to demonstrate ATPase activity with immunoprecipitated Pgp produced measurable but relatively low specific activities (Hamada and Tsuruo, 1988; Shimabuku *et al.*, 1992). More convincing demonstration of Pgp's ATPase activity has come from Pgp overexpression systems and from reconstituting partially purified Pgp into proteoliposomes. Sarkadi *et al.* (1992) expressed Pgp in Sf9 insect cells and measured high ATPase activity in plasma membranes. Similarly, Al-Shawi and Senior (1993) prepared membranes from Chinese hamster ovary cells expressing Pgp at levels constituting up to 32% of the total plasma membrane protein. In both these studies, ATPase activity correlated with the amount of protein produced by Pgp-expressing cells and it was stimulated up to fivefold by a variety of compounds involved in the multidrug-resistance phenomenon. Finally, using partially purified Pgp solubilized from mammalian plasma membranes, a number of groups have reconstituted Pgp into proteoliposomes to show definitively that it is able to hydrolyse ATP (Ambudkar *et al.*, 1992; Sharom *et al.*, 1993; Shapiro and Ling, 1994).

Some of the observations from these various studies could be significant to the mechanism of Pgp-mediated drug transport (see Table 4 for a summary). All groups see basal levels of ATPase activity in the absence of drug, possibly because there are endogenous substrates present in the Pgp preparations. It is also possible that a cellular regulatory factor(s) is lost during the purification–reconstitution procedure, so that ATPase activity and drug transport are coupled in the cell but not in proteoliposomes.

All groups observe stimulation of ATP hydrolysis in the presence of *MDR*1-related drugs and chemosensitizers but not unrelated drugs. Stimulation is uniformly higher with chemosensitizers (verapamil and trifluoperazine in particular) than with drugs. The reason for this is not clear, but one possibility is that chemosensitizers work by uncoupling ATP hydrolysis from

TABLE 4

ATPase activity of reconstituted Pgp

	Reference[a]				
	(1)	(2)	(3)	(4)	(5)
Species[b]	Hamster	Human (wt)	Human (V185)	Hamster	Hamster
V_{max} w/o drug[c]	1.9	5–12	1	0.3	1.5–2
V_{max} + verapamil (fold)[c]	9 (5)	15–38 (3, vbl)	3–5 (5)	6.9 (22)	—
K_m ATP	1.4 mM	285 μM	500 μM	510 μM	—
Stimulation[d]					
Verapamil	4.9 (0.9)	3.8 (35)	5.2 (0.5)	10.3 (1)	2 (10)*
Trifluoperazine	—	—	4.8 (5)	5.2 (5)	2 (10)*
Nifedipine	—	—	5.0 (10)	2.2 (5)	—
Vinblastine	1.8 (0.9)*	3.5 (4)	3.1 (0.5)	4.8 (0.5)	None
Colchicine	1.6 (50)*	1.7 (45)	2.2 (20)	1.4 (2)	1.4 (90)
Daunorubicin	1.9 (0.8)*	2.1 (6)	2.1 (2)	1.5 (2.5)	None
Actinomycin D	—	3.1 (10)	—	1.1 (0.5)	—

[a]References are: (1) Al-Shawi and Senior (1993); (2) Ambudkar *et al.* (1992); (3) Sarkadi *et al.* (1992); (4) Shapiro and Ling (1994); (5) Sharom *et al.* (1993).

[b]Species from which Pgp was derived. Study (2) used wild-type human Pgp, and study (3) used a mutant Pgp (see text).

[c]Units are μmol ATP hydrolysed per min per mg Pgp (corrected for Pgp content in the proteoliposomes). V_{max} in the absence and presence of verapamil are shown, with the fold-stimulation by verapamil given in parentheses. Study (2) used vinblastine instead of verapamil.

[d]Maximum fold-stimulation of ATPase activity for each of the agents shown. The concentration required for half-maximal stimulation is given in parentheses. An asterisk indicates that values are estimates derived from graphs. A dash indicates that the agent was not included in the study. "None" indicates that the agent was included but there is no stimulation of ATPase activity.

drug transport. Some chemosensitizers could also be better substrates for transport than chemotherapy drugs. In the Sf9 expression system, the immunosuppressant cyclosporin A does not stimulate ATPase activity but is a potent competitive inhibitor of verapamil-stimulated ATP hydrolysis (Rao and Scarborough, 1994), suggesting that there are at least two mechanisms for inhibiting Pgp: one mechanism that inhibits only drug binding and another that inhibits binding and ATP hydrolysis.

Stimulation of ATPase activity by *MDR*1-related drugs is variable from study to study, with different drugs showing different relative stimulatory activities and different concentrations of drugs required for maximal stimulation (Table 4). Again, the reasons for these differences are not clear, but they could be due to: (1) different methodologies used to purify and reconstitute Pgp; (2) different membrane compositions of different species and cell types; or (3) different species of Pgp used in the studies (human versus hamster). It will be important to understand the underlying basis for the different drug activities in these systems – differential sensitivities of drugs

to the various proteoliposome–membrane compositions, differential sensitivities of Pgp species to lipid composition, purity of Pgp, assay conditions – before these systems can be used for meaningful structure–function studies with variants of Pgp (see Section 3.3).

3.1.1.2 Drug binding and transport. Two reconstitution studies have tried to demonstrate Pgp's drug transport activity. Sharom *et al.* (1993) report that proteoliposomes reconstituted with partially purified human Pgp can take up [^{3}H]colchicine in an ATP-dependent, osmotically sensitive fashion against a concentration gradient. Vinblastine, verapamil and daunomycin all inhibit uptake (in that order of efficiency), while methotrexate and cytosine arabinoside (Pgp-unrelated drugs) do not. Curiously, ATP hydrolysis in this system is stimulated only slightly by colchicine and not at all by vinblastine and daunomycin, while verapamil and trifluoperazine stimulate about twofold over the basal ATPase activity. Using proteoliposomes reconstituted with rat liver Pgp, Eytan *et al.* (1994) showed indirect evidence for ATP-dependent transport of valinomycin and gramicidin D, two peptide ionophores that are known to be part of the *MDR*1 phenomenon. An unrelated ionophore, nigericin, does not have activity in this system and doxorubicin, vinblastine and daunorubicin all inhibit transport of valinomycin and gramicidin D.

The ATPase stimulation studies described above suggest that there are two steps in drug transport: drug binding and a second step that involves ATP hydrolysis. Additional transport data support this notion. For example, a recent study using membrane vesicles derived from multidrug-resistant murine erythroleukaemia cells distinguishes two phases of [^{3}H]vinblastine transport (Schlemmer and Sirotnak, 1994). The first phase, perhaps drug binding, is rapid, ATP independent and osmotically insensitive. It is inhibited by chemosensitizers and cold vinblastine (other drugs were not studied), but not by C219 monoclonal antibody, vanadate or a nonhydrolysable ATP analogue. The second phase, suggested to be the actual translocation of drug into the vesicle, is sevenfold slower than the first phase and is rate limiting. It is ATP dependent, osmotically sensitive and inhibited by all the agents listed above. Therefore, ATP hydrolysis appears to be required for drug transport but not for binding and both processes are saturable.

Ueda and co-workers looked directly at drug and inhibitor transport using a transepithelial transport system in porcine kidney proximal tubule cells transfected with human *MDR*1 and grown as epithelial monolayers. Pgp is expressed preferentially on the apical surface of these monolayers and net basal-to-apical transport of Pgp substrates can be measured. In this system, there is transepithelial transport of steroid hormones cortisol, aldosterone and dexamethasone, a cardiac glycoside digoxin, the *MDR*1 drug vinblastine, and of Pgp modulators azidopine, diltiazem and immunosuppressive agents cyclosporin A and FK506 (Tanigawara *et al.*, 1992; Ueda *et al.*, 1992; Saeki *et al.*, 1993a, b). The steroid progesterone is not transported across the

monolayers even though it exhibits high affinity for binding by Pgp and interferes efficiently with transport of other Pgp substrates (Yang *et al.*, 1989; Ueda *et al.*, 1992). Another Pgp modulator, nitrendipine, also is not transported in this system (Saeki *et al.*, 1993a).

Again, this is evidence for there being two kinds of Pgp modulators: (1) inhibitors that bind to Pgp and interfere with drug binding but are not themselves translocated out of the membrane; and (2) inhibitors that bind and are translocated and thereby modulate both drug binding and the ATPase-dependent translocation of drug. The relative effectiveness of the two types of chemosensitizers with respect to reversing multidrug resistance has not been carefully studied.

Finally, yeast is another useful heterologous expression system for studying *MDR*1 function. *MDR*1-transformed yeast are resistant to the Pgp substrates valinomycin and FK520 (Kuchler and Thorner, 1992; Raymond *et al.*, 1994). *MDR*1 present in plasma membranes of transformed yeast binds photoactivatable drug and ATP analogues (Kuchler and Thorner, 1992; Raymond *et al.*, 1994). Inside-out membrane vesicles prepared from yeast transformed with the mouse *mdr*1a gene are capable of accumulating colchicine and vinblastine in an ATP-dependent, osmotically sensitive manner that is inhibited by verapamil and C219 monoclonal antibody (Ruetz *et al.*, 1993). However, no ATPase activity could be measured over control vesicles in this study, probably because of high endogenous H^+-ATPase activity present in the plasma membrane.

A slightly different approach using yeast involves the temperature-sensitive *sec*6-4 strain that is defective in the final step of the vesicular secretory pathway (fusion of vesicles to the plasma membrane). Temperature shift causes accumulation of large amounts of secretory vesicles carrying newly synthesized, properly oriented, plasma membrane proteins. Highly pure, homogeneous vesicles can be readily isolated and used for *in vitro* biochemical assays. Using this system, Ruetz and Gros (1994a) have shown specific vinblastine accumulation in secretory vesicles that is mediated by Pgp.

3.1.1.3 Other activities of Pgp. In addition to transporting drugs, it has been suggested that Pgp can act as a chloride channel and an ATP channel. Valverde *et al.* (1992) first reported ATP-dependent, volume-regulated, outward-rectifying chloride channel activity associated with *MDR*1-transfected NIH3T3 cells. Their results and a subsequent report by Altenberg *et al.* (1994a) are consistent with there not being co- or counter-transport of drug and chloride. More extensive analysis by Gill *et al.* (1992) suggests that the putative chloride channel of Pgp requires ATP binding but not hydrolysis for its activation. Pgp-related drugs inhibit channel activation but only when they can actually be transported (i.e. under conditions of ATP hydrolysis). Thus, substituting nonhydrolysable forms of ATP, or using Pgp mutants that are defective in ATP hydrolysis, does not prevent channel activity but does

eliminate the ability of drugs to inhibit channel activation. Once the channel is activated, drugs are ineffective in inhibiting chloride current (Gill *et al.*, 1992).

According to the authors of this study, these results suggest that drugs themselves do not block channel function but that the process of drug transport prevents channel activation. They propose that channel activity and transport function are mutually exclusive events: the channel is activated under conditions of hypotonicity and this locks out a transporter configuration; the transporter is functional under isotonic conditions. However, the recent report by Altenberg *et al.* (1994a), using MCF-7 cells transfected with *MDR*1, indicates that hypotonic conditions do not prevent drug transport and some drugs do not prevent activation of chloride current. This group also does not see volume regulation of chloride channel activity associated with multidrug-resistant Chinese hamster ovary cells.

A number of other studies argue against drug transport and chloride channel activities residing within the same molecule (see Ehring *et al.*, 1994, and references within). Most recently, Hardy *et al.* (1995) suggested that phosphorylation of Pgp (see Section 3.3.4) downregulates the activity of a cellular chloride channel that is distinct from Pgp. Clearly, these studies on Pgp chloride channel activity remain controversial. It appears that channel activity can be associated with Pgp but does not require the presence of Pgp in all cell lines. However, data are now most consistent with there being an indirect effect of ATP binding by Pgp, possibly regulated by phosphorylation, on activation of a non-Pgp chloride channel.

The same type of controversy surrounds reports of ATP channel activity (Abraham *et al.*, 1993). These studies show an association between Pgp expression and rate of release of ATP by multidrug-resistant cells. The effect is not seen with mutant Pgp that is unable to hydrolyse ATP, and results of patch-clamp experiments suggest that the mutations affect channel opening but not ATP movement through the channel (Abraham *et al.*, 1993). For both of these channel activities (ATP and chloride), demonstration of a more direct role for Pgp will require the development of Pgp mutants that disable the channels without affecting ATP binding or hydrolysis, with or without altering drug transport function.

3.1.2 Drug Interaction with Pgp

Central to the mechanism of drug-resistance is where in the cell and when in the course of drug influx–efflux Pgp interacts with its substrate. It is well established that drugs can bind directly to Pgp present in plasma membranes (Cornwell *et al.*, 1986; Safa *et al.*, 1986; Horio *et al.*, 1988) and progress is being made in mapping the exact sites on Pgp that bind substrate (see Section 3.3.1.2). But a more detailed understanding of Pgp–drug interaction has been

difficult to attain. Raviv *et al.* (1990) used energy transfer from photo-excitable doxorubicin to a radioactively tagged membrane probe, with subsequent labelling of nearby membrane proteins. Their results indicate that doxorubicin is almost exclusively associated with Pgp in drug-resistant cells. It is non-specifically associated with membrane proteins in sensitive cells and also in resistant cells incubated with doxorubicin plus the reversing agent verapamil. These results suggest that doxorubicin and Pgp can interact directly in the membrane.

Kinetic studies by Stein *et al.* (1994b) suggest that Pgp plays a role in both reducing drug influx and increasing drug efflux, and that these are functionally distinct processes. These authors propose that Pgp extracts drug from both the inner and outer leaflets of the lipid bilayer. However, Altenberg *et al.* (1994b) did not observe reduced drug influx associated with Pgp expression and they argue that Pgp extracts drugs from the cytosol. Differences between these two studies could be due to different cell lines, drugs or methodologies used in the respective experiments.

Aside from kinetic data, the localization of Pgp substrates is consistent with their being extracted directly from the membrane. In drug-sensitive cells, substrates such as rhodamine 123 and hydrophobic peptides are thought to accumulate preferentially in membrane environments, suggesting that they interact with Pgp from that compartment in drug-resistant cells (Germann, 1993). Another useful set of probes are hydrophobic acetoxymethylester derivatives of fluorescent dyes. In their neutral forms, these reagents are Pgp substrates. In their free acid form, to which they are rapidly converted upon entry into the cytoplasm, they are no longer substrates. Sarkadi and co-workers have found that acetoxymethylester dyes do not accumulate in Pgp-expressing cells, suggesting that the compounds are never exposed to the cytoplasm and are actively extruded from the plasma membrane (Homolya *et al.*, 1993; Hollo *et al.*, 1994).

3.2 STRUCTURE OF Pgp

The exact structure of Pgp in plasma membranes is still somewhat controversial. Hydropathy plots of Pgp amino acid sequences predict proteins composed of two homologous halves, each with six transmembrane domains (TMs) and an ATP binding domain (see Fig. 1A). This prediction is consistent with models for other members of the ABC transporter family (see Section 3.3.3.1), but little is known about membrane topologies for any of these proteins.

Limited data with sequence-specific antibodies are consistent with the predicted topology of Pgp indicating that the first and fourth loops are in fact extracellular, the nucleotide binding domains are both intracellular, and the carboxyl terminus is intracellular (Kartner *et al.*, 1985; Yoshimura *et al.*,

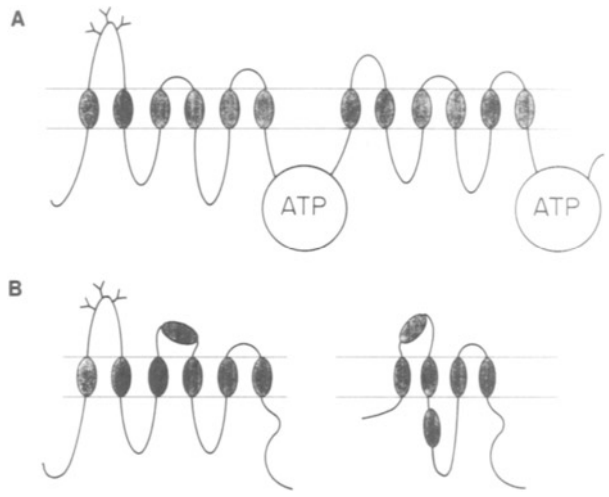

FIG. 1. Alternative models for the topological configuration of Pgp. (A) Topology of Pgp predicted by hydropathy plots and supported by data from Loo and Clarke (1995). Grey ovals are the transmembrane domains. Glycosylations are shown as Y sticks on the first extracellular domain. ATP binding domains are drawn as circles. (B) Possible alternative topologies for the amino (left) and carboxyl (right) halves of Pgp, according to models proposed by Bibi and Beja (1994) and Zhang and Ling (1991), respectively (see text for other variations on these models).

1989; Georges *et al.*, 1993). Conflicting data have arisen from attempts to study Pgp structure directly.

Using a mouse protein, fusions with alkaline phosphatase as a reporter of membrane orientation and a bacterial expression system, Bibi and Beja (1994) concluded that the predicted TM4 domain (aa214–232 in the mouse) is actually located externally and residues 243–260 traverse the membrane instead. Their model shifts the placement of TM4 but preserves the overall six-TM structure predicted by hydropathy plots (Fig. 1B). Zhang *et al.* (1993b), using an *in vitro* translation system with hamster pgp1, found variable results with the TM3–5 membrane assignments. Their model proposes four or six TMs in the amino half of Pgp depending on whether TMs three and five span the membrane or not. Skach and Lingappa (1994) have studied human Pgp in both *in vitro* translation and *Xenopus* oocyte expression systems, using an epitope tag as a reporter for membrane orientation. This work suggested that TM3 and 4 do span the membrane in orientations predicted by computer analysis. In all of these studies, TMs 1, 2 and 6 are as originally predicted (Zhang *et al.*, 1993b; Bibi and Beja, 1994; Skach and Lingappa, 1994).

In the carboxyl half of the protein, TMs 7, 11 and 12 appear to be as predicted by hydropathy plots (Zhang and Ling, 1991, 1993; Skach *et al.*,

1993), but there are discrepancies with TMs 8–10. Zhang and Ling (1991), using the *in vitro* translation system, suggested that TM8 is actually extracellular, TM9 is in the membrane but oriented in, towards the cytoplasm, and TM10 is intracellular. Skach *et al.* (1993), using the *Xenopus* oocyte expression system, obtained results that were mostly consistent with those of Zhang and Ling (1991), but they proposed a model with both TM 8 and 9 being extracellular and TM10 traversing the membrane in its predicted inward orientation. Both of these models predict only four TMs in the carboxyl half of the protein (Fig. 1B).

The three systems used in these studies (*in vitro* translation, bacterial expression and *Xenopus* oocyte expression) are all artificial in some sense. While their results are consistent with alternate membrane topologies for Pgp, they are also not entirely inconsistent with the standard model for Pgp, and the alternate topologies are generally detected for only a fraction of the expressed Pgp. However, the most recent report on this topic looked at human Pgp expressed in mammalian cells and is, therefore, perhaps the closest reflection of the *in vivo* structure of the transporter (Loo and Clarke, 1995). Using site-specific cysteines as reporters for accessible and inaccessible regions of Pgp, this study concluded that the computer model of Pgp is entirely correct, with the predicted TMs, extracellular loops and intracellular domains in each half of the protein. Though unlikely according to these data, models proposed by Bibi and Beja (1994) and Zhang and Ling (1991) cannot be ruled out completely.

The higher-order structure of Pgp in plasma membranes has not been determined unequivocally. Most data are consistent with dimers of Pgp, and possibly monomers, being the functionally active form of the transporter (Naito and Tsuruo, 1992; Germann, 1993; Poruchynsky and Ling, 1994).

3.3 STRUCTURE–FUNCTION ANALYSIS OF Pgp

The mechanism of action of Pgp and the basis for its broad substrate utilization will be understood only by deciphering exactly how substrates interact with the protein. To this end, biochemical and genetic approaches have been used to map functional domains on Pgp and to determine which residues contribute to the substrate utilization and specificity of the transporter.

3.3.1 Functional Domains of Pgp

3.3.1.1 ATP binding and hydrolysis. Pgp was originally classified as an ATP-dependent transporter in part because of its consensus nucleotide binding domains in each half of the protein (Ames *et al.*, 1992; Higgins,

1992). Amino acids that make up these domains in Pgp contain Walker A and B sequence motifs (Walker *et al.*, 1982) separated by about 110 residues, plus a "centre" region about midway between Walker A and B and a "linker" peptide just upstream of Walker B. These centre and linker sequences are highly conserved among ABC transporters, with the latter now considered as the ABC motif (L-S-G-G-$(X)_3$-R-hydrophobic-X-hydrophobic-A) (Shyamala *et al.*, 1991; Germann, 1993). Point mutations in either of the Walker A motifs abolish the ability of Pgp to confer multidrug resistance, but not to bind ATP, indicating the importance of ATPase activity for transport function and suggesting that both halves of Pgp are required for drug transport (Azzaria *et al.*, 1989).

Nevertheless, each half retains the ability to hydrolyse ATP when synthesized individually in heterologous expression systems, albeit at reduced levels relative to the ATPase activity of full-length Pgp (Shimabuku *et al.*, 1992; Loo and Clarke, 1994c). However, both halves are needed to achieve drug stimulation of ATPase activity (Loo and Clarke, 1994c), suggesting that coupling of ATP hydrolysis to drug binding requires interaction between the two halves (also see next section and Section 3.3.2). More direct evidence that consensus nucleotide binding domains are required for ATPase activity comes from the recent report that ATP can bind to a purified soluble form of the *C*-terminal nucleotide binding domain (Baubichon-Cortay *et al.*, 1994).

3.3.1.2 Drug binding. Attempts to map the drug binding site(s) on Pgp have used radioactively tagged photoaffinity drug analogues to label Pgp covalently, followed by enzymatic digestion and immunoprecipitation with region-specific antibodies to identify the labelled polypeptides (reviewed by Germann, 1993; Greenberger *et al.*, 1994). Mapping with [^{3}H]azidopine identified labelling domains in both halves of Pgp. The two sites are equally labelled, suggesting either a single drug binding domain or two identical domains in each half of the protein (Bruggemann *et al.*, 1992). Finer mapping with [^{125}I]arylazidoprazosin has identified the symmetrical regions in or immediately *C*-terminal to TM6 and TM12 as the labelling sites in mouse Pgp (Greenberger, 1993). The most likely domain in the *C*-terminal half is within residues 998–1044 of the mouse protein but the exact labelling sites have not been determined. [^{125}I]arylazidoforskolin also labels a fragment spanning TM5-6 of human Pgp and an unidentified region in the *C*-terminal half of the protein (Morris *et al.*, 1994). There are probably minor contributions to drug binding by other transmembrane sequences as well (Greenberger, 1993).

Similar labelling results have been obtained with multiple affinity reagents (Morris *et al.*, 1994). However, it is important to remember that actual drug binding domains could be offset from the sites detected by photolabelling, since covalent attachment of photoaffinity drugs could occur at some distance

from their sites of binding. Furthermore, these results do not preclude the possibility that there are multiple drug binding sites within an overall drug interaction domain. In fact, kinetic studies of drug binding and transport in the presence of inhibitors (Tamai and Safa, 1991; Ferry *et al.*, 1992), as well as genetic studies with Pgp mutants (Section 3.3.2), suggest that different classes of drugs have different binding sites. Comparing photolabelling characteristics of [^{125}I]arylazidoprazosin and an iodoazidophenethyl analogue of spiperone, Safa *et al.* (1994) observed slightly different labelling sites with the two drug analogues. They also found that labelling with the two agents was inhibited differently by various Pgp substrates and modulators. Clearly, more precise methods will be needed to understand the exact nature of Pgp–drug interaction at the molecular level.

3.3.2 Substrate Utilization

As stated earlier, substrate utilization by Pgp is extremely broad (Table 1), but the mechanism by which a single protein is able to recognize such a wide variety of substrates is not known. No common structural element present in all transported compounds has yet been identified (Ford and Hait, 1990; Beck and Qian, 1992; Ramu and Ramu, 1992; Tang-Wai *et al.*, 1993). The best substrates and Pgp modulators are hydrophobic with amphipathic characteristics. Most chemical substrates have a planar structure with a basic nitrogen atom and are charged at neutral pH. Those substrates that partition best in the lipid bilayer tend to be the best substrates for Pgp-mediated resistance as well (Zamora *et al.*, 1988; Nogae *et al.*, 1989; Gros *et al.*, 1992), suggesting that one component of recognition is simply access to the transporter.

To learn more about substrate recognition, the approach has been to use natural variants and site-directed mutants of Pgp as well as chimeric Pgp proteins to determine the exact amino acids involved in drug binding and to probe the mechanism and specificity of Pgp–drug interaction. In general, mutations all along the Pgp polypeptide chain can inactivate transporter activity, and neither half of the protein can act alone in conferring drug-resistance (Currier *et al.*, 1989; Loo and Clarke, 1994c). This is consistent with biochemical mapping of the drug binding domain in both halves of Pgp. More detailed studies of substrate utilization are summarized here.

3.3.2.1 MDR*1/2 chimeras.* The *mdr* gene family consists of two human genes, *MDR*1 and *MDR*2, and three rodent genes, such as *mdr*1a, *mdr*1b, and *mdr*2 in mice (see Section 1.1). There is a high degree of sequence homology among all the proteins encoded by *mdr* genes, but only the *MDR*1-like genes are functional in conferring multidrug resistance (Gros *et*

al., 1988; van der Bliek *et al.*, 1988; Schinkel *et al.*, 1991; Buschman and Gros, 1994). Nevertheless, it is likely that *mdr*2 genes have transporter activity as well (Smit *et al.*, 1993; Ruetz and Gros, 1994b; see Section 4.1). The inability of mouse *mdr*2 to confer drug resistance appears to be due to a defect in drug binding but not in ATP binding (Buschman and Gros, 1991, 1994). Chimeric proteins containing sequences from mouse *mdr*1b and *mdr*2 identify TM5–6 and TM7–8 as regions required for maintaining drug transport activity (Buschman and Gros, 1991). Currier *et al.* (1992) demonstrated that changing as few as four amino acids within the first intracytoplasmic loop of Pgp from *MDR*1-encoded residues to corresponding *MDR*2-encoded residues destroys the ability of Pgp to confer multidrug resistance.

Despite the remarkably broad substrate utilization by Pgp, there is also a degree of specificity in the preferential use of some substrates over others, and the pattern of substrate recognition can vary considerably with the amino acid sequence of Pgp. There is a high level of sequence homology among all of the proteins encoded by *mdr* genes. Proteins encoded by mouse *mdr*1a and *mdr*1b share 85% amino acid sequence identity, but they confer different patterns of cross-resistance (Devault and Gros, 1990; Yang *et al.*, 1990). Attempts to map the determinants of those patterns of cross-resistance failed to localize any one region on Pgp that is responsible for substrate utilization (Dhir and Gros, 1992).

Following up on their biochemical mapping of the drug binding domain to the TM12 vicinity, Zhang *et al.* (1995) have used *MDR*1/*MDR*2 chimeras to demonstrate that substrate specificity can also be partially mapped to this region. Substitution of TM12 from *MDR*2 into *MDR*1 (six amino acid differences) yields a protein which is defective in conferring resistance to actinomycin D, doxorubicin and vincristine, but is still able to confer colchicine resistance. A TM11 substitution (also six amino acid differences) has no effect or slightly enhances resistance to all those drugs. Substitution of the loop between TM11 and 12 (seven amino acid differences) results in enhanced resistance (up to threefold) for all four drugs.

3.3.2.2 MDR*1 mutants*. Early on it was discovered that a single amino acid change can alter the pattern of cross-resistance conferred by *MDR*1 genes (Choi *et al.*, 1988; Gros *et al.*, 1991; Devine *et al.*, 1992). Since then, systematic mutational analyses have been performed to map the exact determinants of substrate specificity. Results of these studies are summarized in Table 5 and Fig. 2. Intracytoplasmic loop 1, TM5–6 and TM11–12 appear to be the most critical regions for substrate interaction. In TM6, five of the predicted 21 amino acids in the membrane are important for either substrate specificity or overall function of the protein (Devine *et al.*, 1992; Loo and Clarke, 1993b, 1994b). Two other residues have smaller effects on substrate utilization (Loo and Clarke, 1994b). Mutations in TM3, TM7 and TM8 tend

TABLE 5

Pgp mutations that alter substrate utilization

Mutation[a]	COL[b]	VBL	DOX	ACTD	[^{3}H]AZD[c]	Site[d]	Reference[g]
G141V	2.9	0.8	2.6	0.9	1.0	IC1	(1)
G185V	2.1	0.4	0.5	0.1	3.8	IC1	(2)
N183S/G185V	2.7	1.0	1.1	1.2	—	IC1	(3)
G187V	3.1	0.8	2.7	0.3	1.0	IC1	(1)
P223A	0.3	1.6	0.4	0.2	1.0	TM4	(4)
G288V	2.1	1.1	1.4	0.4	1.0	IC2	(1)
F335A	1.3	0.1	1.6	0.2	5.4	TM6	(5)
G338A/A339P[e]	0.2	—	—	1.6	—	TM6	(6)
V338A	2.2	0.3	1.9	0.9	1.0	TM6	(7)
G341V	0.1	0.8	0.1	0.8	1.0	TM6	(7)
G812V	2.2	0.8	2.0	0.9	1.0	IC4	(1)
G830V	2.9	0.8	3.0	0.5	1.0	IC4	(1)
P866A	0.3	1.0	0.4	0.3	1.0	TM10	(4)
S939F[f]	0.1	0.7	0.1	0.1	0.1–0.5	TM11	(8)
F978A	<0.1	0.2	0.6	0.2	1.0	TM12	(5)

[a]Mutations are designated according to the position of their substitution preceded by the one-letter code for the wild-type amino acid and followed by the mutant amino acid.

[b]Drug resistance profiles of NIH3T3 cells expressing the mutants are given relative to cells expressing wild-type Pgp (COL, colchicine; VBL, vinblastine; DOX, doxorubicin; ACTD, actinomycin D). A dash indicates the drug was not tested.

[c]Results of photoaffinity labelling with [^{3}H]azidopine (AZD) are shown relative to wild-type protein.

[d]Indicates the location of mutations in the linear model of Pgp (IC, intracytoplasmic loop; TM, transmembrane domain).

[e]A natural mutation in hamster *pgp*1 which arose in cells selected for high resistance to actinomycin D. The corresponding amino acid positions in human Pgp are 341 and 342. Analysis was done on transfected hamster DC-3F cells. Each independent hamster mutation affects substrate interaction with Pgp (P. Melera, personal communication).

[f]A site-directed mutation in mouse *mdr*1a and *mdr*1b. Relative resistances are shown for *mdr*1a. The corresponding position in human Pgp is 943. Analysis was done on transfected Chinese hamster LR73 cells. Substitutions of other amino acids at this site have variable effects on substrate utilization, with bulky side-chains being the most deleterious and VBL transport affected less than that of other drugs (Dhir *et al.*, 1993).

[g]References are: (1) Loo and Clarke (1994a); (2) Choi *et al.* (1988); (3) Currier *et al.* (1992); 4) Loo and Clarke (1993b); (5) Loo and Clarke (1993a); (6) Devine *et al.* (1992); (7) Loo and Clarke (1994b); (8) Gros *et al.* (1991).

not to have effects on Pgp transport activity (Loo and Clarke, 1993a, b, 1994a).

Genetic data are generally consistent with the physical mapping of the drug binding domain to both halves of Pgp. Mutations in each half of the protein can alter substrate specificity. The two halves are sometimes but not

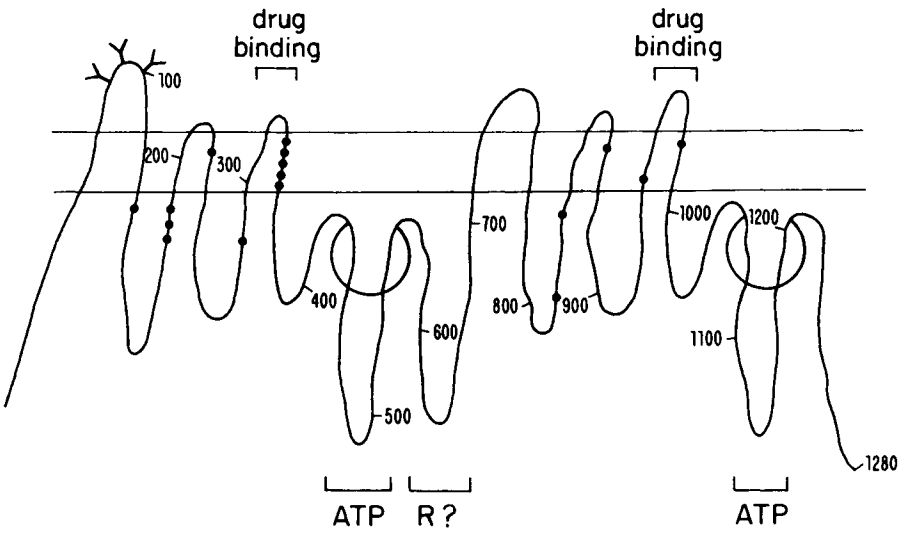

FIG. 2. Linear representation of Pgp. A two-dimensional drawing of Pgp is shown, with numbers referring to amino acid residues in the linear sequence. Braces indicate residues comprising the ATP binding domains (within the circles), the putative drug binding domain(s) contributed by each half of the molecule, and linker region (R?) that might be equivalent to the CFTR R domain (see text). Filled circles represent sites at which amino acid substitutions alter the substrate specificity of the multidrug transporter (see also Table 5).

always equivalent in their contributions to drug transport. For example, mutations to pro223 and pro866, in analogous positions in TM4 and TM10, affect drug-resistance profiles similarly, but mutations to phe335 (TM6) and phe978 (TM12) have different effects on drug-resistance and on drug binding (Table 5).

Just as biochemical data suggest that different drugs interact with different sites on Pgp (see Section 3.3.1.2), trends in the genetic data are also consistent with this notion. Mutations generally affect colchicine and doxorubicin resistance in the same direction (enhanced or decreased) and vinblastine and actinomycin D in the same direction, implying that drugs within each of these pairs interact similarly with the transporter but that the two pairs of drugs are different from each other in their recognition by Pgp. Other substrates and modulators might fall into one or the other of these putative interaction domains.

Mutants have begun to shed light on the molecular events in drug transport. Different mutations can affect different steps in the drug transport process. For example, mouse Pgp with a mutation at residue 939 confers lower resistance to vinblastine and binds less azidopine than wild type Pgp

at steady state, indicating a defect in drug binding in this mutant (Kajiji *et al.*, 1993). Conversely, human Pgp variants with a mutation at amino acids 185 or 335 confer lower resistance to vinblastine but bind more azidopine and vinblastine at steady state, relative to wild-type protein (Safa *et al.*, 1990; Loo and Clarke, 1993b). Likewise, the 185 mutant confers high colchicine resistance but binds less colchicine than wild-type protein (Safa *et al.*, 1990). These results suggest that the 185 and 335 mutations do not alter initial drug binding but might affect release of drug from the transporter. Conflicting results have been obtained in another study of the 185 mutant, in which the initial binding of vinblastine was thought to be altered (Bruggemann *et al.*, 1992). Finally, it has been suggested that TM6 mutations affect the coupling between drug transport and ATP hydrolysis (Loo and Clarke, 1994b).

Pgp modulators appear to be sensitive to the same sites on Pgp that affect drug transport (Kajiji *et al.*, 1993). Furthermore, different modulators are differentially affected by mutations, which might reflect their different mechanisms of action in inhibiting Pgp or their different binding sites on the transporter (Kajiji *et al.*, 1994; Stein *et al.*, 1994b; Cardarelli *et al.*, 1995; P. Melera, personal communication). Kajiji *et al.* (1994) observed that structurally distinct inhibitors have varying modulating abilities depending on the exact amino acid at position 939 of the mouse *mdr*1a gene product. For example, the modulating ability of CP117227, a tricyclic synthetic reagent, is reduced nearly fiftyfold in the phe939 mutant of *mdr*1a, relative to wild-type, while two other inhibitors, cyclosporin A and CP100356, are unaffected by this mutation. With a set of six other substitutions at residue 939, each of these modulators has a distinct profile of potency – enhanced, reduced or unaltered – against individual mutants. This further implicates TM11 in the drug binding site and suggests that the region is very sensitive to the exact structure of substrates and modulators (Kajiji *et al.*, 1994).

In a recent study with the mutant encoding valine at position 185 (V185) versus the wild-type glycine 185 (G185) (Cardarelli *et al.*, 1995), several observations are noteworthy. (1) The combination of drug and inhibitor is important. Of cyclosporin A, verapamil and quinidine, cyclosporin A is the most effective inhibitor of colchicine resistance, whether G185 or V185 is used. Verapamil is the best modulator of taxol, vinblastine and daunorubicin resistance with the wild-type protein. (2) Consistent with the *mdr*1a studies above, the combination of inhibitor and Pgp type is important. Cyclosporin A works best as an inhibitor of V185-mediated resistance, with all four drugs tested. Verapamil works better with G185, for all drugs except colchicine. (3) The combination of drug and Pgp type is important. Colchicine resistance is reversed more effectively, by all three modulators, with V185 than with G185. This is interesting because the V185 mutant also confers enhanced resistance to colchicine, relative to G185, even though colchicine binding at steady state is lower to V185 than to G185 (Choi *et al.*, 1988; Safa *et al.*, 1990). One interpretation of these results is that the modulators lower

colchicine binding to both G185 and V185 but that this has a lesser effect on drug efflux mediated by G185. That is, colchicine release from G185 is slower and rate limiting, so the effect of modulators that alter binding is less pronounced than with V185 where the release of colchicine is rapid.

A TM6 double mutation in hamster Pgp (A338P339 substituted for G338A339) destroys the modulating capacity of cyclosporin A. This effect has been further localized to the P339 mutation. Furthermore, the double mutant confers nearly tenfold lower colchicine resistance than wild-type and verapamil modulation is also reduced in the mutant (P. Melera, personal communication). This is consistent with the common effect of the V185 mutation on enhancing both colchicine resistance and the reversal of colchicine resistance by Pgp modulators (Cardarelli *et al.*, 1995).

Studies on the pharmacological behaviour of mutants are therefore informative about how specific substrates and modulators interact with the transporter. Many of the systems developed for screening novel substrates and inhibitors of Pgp are also potentially applicable to this work. The transepithelial transport system of Ueda and colleagues should be particularly useful for studying the specificity of drug transport by Pgp mutants. The yeast secretory vesicle system might prove useful for this purpose, although these analyses could be complicated by differences between yeast and mammalian cells in membrane composition and by the presence of numerous endogenous transport proteins in yeast.

3.3.3 Structural Homologues

As mentioned earlier, *MDR*1 belongs to a superfamily of genes that encode ABC transporters. While a comprehensive review of ABC transporters is beyond the scope of this article, some main points are worth mentioning in relationship to the function and mechanism of Pgp.

3.3.3.1 Structural and functional similarities among ABC transporters. Literally scores of ABC transporters have been identified and their genes cloned from bacteria, yeast and higher eukaryotes (Kane *et al.*, 1990; Ames *et al.*, 1992; Higgins, 1992; Balzi and Goffeau, 1994; Lewis, 1994). At the last count, there were 168 sequences with ABC transporter homology recorded in DNA databases (K. Kuchler, personal communication). Identification of these homologues is based on their structural similarities with other transporters and their functional behaviour consistent with some type of transport activity. Substrates for this class of proteins range from ions and heavy metals to amino acids, sugars, polysaccharides, toxins, peptides and proteins.

In bacteria, prototypical ABC transporters are *hly*B, one of the proteins responsible for export of a 107-kDa toxin from the cytoplasm of *Escherichia*

coli, and the histidine permease system of *Salmonella typhimurium* that imports histidine via a multisubunit complex (Higgins *et al.*, 1982; Felmlee *et al.*, 1985). In yeast, *STE6* encodes the protein that transports a-factor mating pheromone, a 12-residue farnesylated peptide (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). A number of other yeast transporters appear to be involved in mediating resistance to a wide range of toxic substances (reviewed by Balzi and Goffeau, 1994; Egner *et al.*, 1995). In *Drosophila*, three ABC transporters encoded by *white*, *brown* and *scarlet* genes are involved in the transport of guanine and tryptophan (precursors in the synthesis of eye pigments) (see Ewart *et al.*, 1994).

Mammalian transporters include Pgp; CFTR, the voltage-regulated chloride channel associated with cystic fibrosis (Riordan *et al.*, 1989); TAP1 and TAP2, transporters involved in peptide translocation into the endoplasmic reticulum as part of major histocompatibility complex class I antigen presentation (reviewed by Hill and Ploegh, 1995); two peroxisomal proteins, pMP70 and ALD (Kamijo *et al.*, 1990; Mosser *et al.*, 1993); and MRP, a multidrug resistance associated protein that appears to transport drugs as well as glutathione conjugates (Cole *et al.*, 1992; see Section 5.1). In addition, a number of prokaryotic and eukaryotic genes have been cloned that share homology and structural features with ABC transporter structure, but their functions are unknown (e.g. Wu *et al.*, 1991; Allikmets *et al.*, 1993; Gerrard *et al.*, 1993; Luciani *et al.*, 1994).

The hallmarks of ABC transporters are their transmembrane domains (TMs), generally consisting of six membrane spanning α-helices, and their ATP binding cassettes (ABC), containing Walker A and B motifs, a linker peptide and a centre region that are highly conserved among ABC transporters. The configuration of TM and ABC domains is variable, however. Bacterial permeases tend to encode different functions (TM, ABC and sometimes substrate binding proteins) on separate subunits that come together to form a transport complex. Pgp, Ste6, CFTR and MRP have all of these functions fused on a single polypeptide, with six TMs followed by an ABC domain in each of two halves of the protein (Fig. 1A). The Pdr5/Sts1 and Snq2 drug transporters of yeast are also single polypeptides, but they have the ABC domain preceding the TM in each half of the molecule (Balzi and Goffeau, 1994). Mammalian *TAP1* and *TAP2* each encode "half-transporters" with 6TM–ABC configurations that apparently come together to form the peptide translocator. Finally, *Drosophila brown*, *scarlet* and *white* encode ABC–6TM half molecules that also appear to act as heterodimers with each other. The yeast single-chain transporters Pdr5/Sts1 and Snq2 appear to be the topological homologues of the *Drosophila* half-molecules.

Despite the flexibility in organization of TM and ABC domains, in most, perhaps all, systems it appears to be critical to have at least 12 membrane-spanning helices and two ATP binding cassettes within the transport

structure. At least in the case of eukaryotic transporters, the contribution of each 6TM–ABC unit appears to be unequal. Thus, co-expression of *N*-terminal and *C*-terminal half-molecules of Ste6 or Pgp reconstitutes a functional transport complex, but neither half alone is functional (Berkower and Michaelis, 1991; Loo and Clarke, 1994c). This is consistent with the putative drug binding domain of Pgp being composed of regions from both halves of the molecule (see above). Cystic fibrosis transmembrane conductance regulator (CFTR) might be an exception to this, since an amino half-molecule is functional as a chloride channel, although regulation of channel activity is slightly different than for wild-type CFTR (Sheppard *et al.*, 1994). CFTR may also have some unknown transport function that does require both halves of the protein.

There might be some functional overlaps between transporters as well. Mouse and human *MDR*1 genes are able to rescue **a**-factor transport in yeast strains deleted for *STE*6 (Kuchler *et al.*, 1992; Raymond *et al.*, 1992). Mutations that alter the substrate specificity of Pgp also destroy its **a**-factor transport activity (Kuchler and Thorner, 1992; Raymond *et al.*, 1992). A number of transporters appear to be involved in pleiotropic drug resistance in yeast, and at least some of their substrates can also be transported by Pgp (Balzi and Goffeau, 1994; Egner *et al.*, 1995). Pgp and MRP also appear to share some substrates, although the mechanism of MRP-mediated drug-resistance has not been entirely worked out (see Section 5.1). As stated earlier, Pgp might have channel activities that are distinct from its transport function, linking it functionally to CFTR as well as sodium, calcium and potassium channels that share the 6TM motif (see Greenberger and Ishikawa, 1994).

3.3.3.2 Structure–function analysis of ABC transporters. The structural and functional similarities among ABC transporters suggest that we can learn about Pgp's drug transport mechanism by studying the mechanisms of other transport proteins (Kuchler, 1993; Greenberger and Ishikawa, 1994). For the TAP1/2 heterodimer, ATP is not needed for binding of peptide but ATP hydrolysis is required for translocation of peptide into the endoplasmic reticulum (Hill and Ploegh, 1995).

In CFTR (reviewed by Welsh *et al.*, 1992; Riordan, 1993), ATP binding appears to regulate the opening and closing of the chloride channel. Mutational analysis suggests that the two ABC domains are functionally distinct in this process, with one site possibly initiating a burst of channel activity and the other site terminating the burst (Carson *et al.*, 1995). Unlike most other ABC transporters, CFTR has a large (over 200 residues) charge-rich R (regulation) domain following the first ABC domain. Channel activation is linked to protein kinase A-mediated phosphorylation within the R domain, which is thought to interact with the ABC domains to mediate channel regulation (Welsh *et al.*, 1992).

Mutations in highly conserved ATP binding domains that alter CFTR activity also reduce or alter transporter activity when the mutations are introduced into analogous positions of Pgp or Ste6 (Berkower and Michaelis, 1991; Hoof *et al.*, 1994). For Pgp, this includes a single amino acid deletion (ΔF508) that alters CFTR processing and reduces its appearance at the cell surface. The analogous deletion in Pgp appears to confer a similar phenotype on the multidrug transporter (Hoof *et al.*, 1994). This is not the case with Ste6, however, where a ΔF508-like deletion has no apparent effect on **a**-factor transport (Berkower and Michaelis, 1991).

The ΔF508 phenotype is at least partially conferred by the CFTR ABC domain. CFTR/Ste6 chimeras carrying the *N*-terminal ABC sequence from CFTR in place of the analogous Ste6 sequence are partially functional in transporting **a**-factor in yeast but transport is reduced if the ΔF508 mutation is present. Transport of **a**-factor is partially restored by a second mutation at position 553 within the CFTR sequences, which lies in the highly conserved ABC motif dodecapeptide just upstream of the Walker B motif. This same mutation, within a full-length ΔF508 CFTR molecule, also partially restores chloride channel activity (Teem *et al.*, 1993). These results suggest that ABC domains of different transporters are interchangeable, as previously observed for Pgp (Buschman and Gros, 1991); highly conserved sequences within the ABC domains are critical for transporter function; and regions containing amino acids 508 and 553 might interact with each other, at least in CFTR.

In a fashion analogous to Pgp, mutations in TM6 of CFTR are particularly associated with altered anion selectivity of the channel, while TM1 is linked to this as well (Anderson *et al.*, 1991; Tabcharani *et al.*, 1993). Mutations in *Drosophila* transporters *brown* and *white* implicate TMs 5 and 6 in substrate interaction and further suggest an intersubunit interaction in this part of the *brown–white* heterodimer (Ewart *et al.*, 1994). Mutations in hlyB that rescue transport of mutated hlyA substrates map to sequences in or near TMs, but primarily in the third and fifth sectors of hlyB (Zhang *et al.*, 1993a). Membrane domains of other transporters, including the Plasmodium *mdr* homologue (*pfmdr*) (Foote *et al.*, 1990; van Es *et al.*, 1994) and TAP2 (Powis *et al.*, 1992), are also implicated in substrate specificity. Finally, there appear to be similarities between Pgp and sodium and calcium channels in the location of their substrate binding domains near TM6 of each channel motif, suggesting a conserved tertiary structure in these proteins (Greenberger and Ishikawa, 1994).

Therefore, despite the diversity in substrates for a wide variety of transporters, the site of substrate interaction appears almost invariably to be within the membrane. While there might be primary contact with substrates in the TM5–6/11–12 region, other residues throughout the transporter seem to contribute to substrate interaction as well. These contributions could be either direct or indirect, through some effect on overall transporter structure.

3.3.4 *Post-translational Modification of Pgp*

The role of post-translational modifications in Pgp activity is not well understood. Human Pgp has three carbohydrates on the first extracellular loop. There is ample evidence that glycosylation is not required for drug transport activity (Germann *et al.*, 1990b; Kuchler and Thorner, 1992; Bibi *et al.*, 1993; Gottesman and Pastan, 1993; Schinkel *et al.*, 1993b; Raymond *et al.*, 1994), but it could contribute to the stability of the protein (Schinkel *et al.*, 1993b).

Pgp is phosphorylated as well (Carlsen *et al.*, 1977; Hamada *et al.*, 1987; reviewed by Germann *et al.*, 1995). There is considerable indirect evidence suggesting that phosphorylation is involved in some aspect of drug-resistance. In a number of tissue culture model systems, there is an association between protein kinase C (PKC) activation and enhanced Pgp phosphorylation, stimulation of drug transport and appearance of the multidrug resistance phenotype (Germann *et al.*, 1995). Treatment of cells with PKC inhibitors, such as staurosporine and calphostin C, also results in decreased Pgp phosphorylation, increased drug accumulation, and decreased multidrug resistance (Ma *et al.*, 1991; Bates *et al.*, 1992, 1993; Chambers *et al.*, 1992; Utz *et al.*, 1994).

However, some of the effects of PKC agonists and inhibitors could be due to non-specific effects of the reagents on cell metabolism or effects of signal transduction signals on *MDR*1 gene activity (Germann *et al.*, 1995; see Section 4). Some PKC inhibitors can also have a more direct effect on drug transport by competing with drug substrates for binding to Pgp (Sato *et al.*, 1990). Pgp might also be a substrate for protein kinase A (PKA) or other uncharacterized kinases, but the effects of these phosphorylations on multidrug resistance is not known (Mellado and Horwitz, 1987; Staats *et al.*, 1990; Sampson *et al.*, 1993; Chambers *et al.*, 1994).

Both PKC and PKA have been shown to phosphorylate Pgp *in vitro*, with similar sites of phosphorylation observed on Pgp isolated from cells (Chambers *et al.*, 1992, 1993). The major sites of PKC and PKA phosphorylation have now been mapped to the highly charged linker region of Pgp (Chambers *et al.*, 1993, 1994; Orr *et al.*, 1993). It has been suggested, therefore, that the linker might be analogous to the larger R domain of CFTR that is thought to regulate channel activity via a phosphorylation mechanism (Chambers *et al.*, 1993). However, mutating the major phosphorylation sites in mouse *mdr*1b protein and in human Pgp has no obvious effect on the drug transport activity of Pgp (Gottesman and Pastan, 1993; Germann *et al.*, 1996). Recent results with CFTR indicate that even a low level of phosphorylation at a minor site can influence activity of the channel (Seibert *et al.*, 1995), so the same might also be true for Pgp. In addition, there could be subtle effects of phosphorylation, such as mediating the activity of Pgp as a chloride

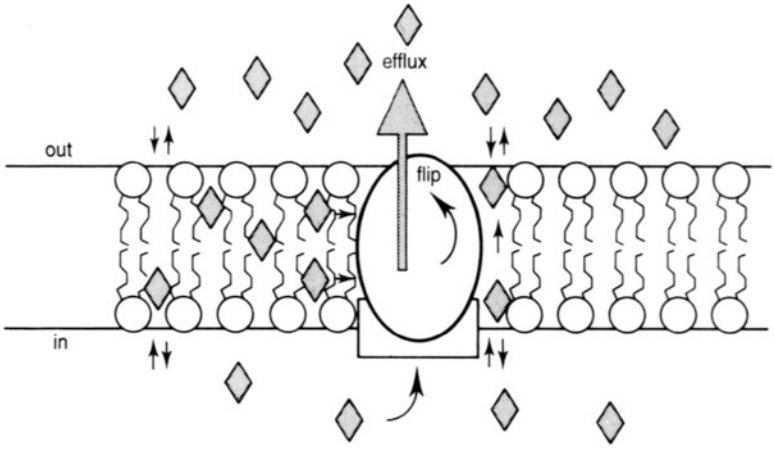

FIG. 3. Schematic representation of Pgp mechanism of action. Two alternative models for the mechanism of action of Pgp (oval–box structure) are shown: (1) Pgp as an efflux pump that removes drug (◈) from the plasma membrane, and possibly from the cytoplasm (indicated by arrows), and extrudes the drug to the outside of the cell; (2) Pgp as a flippase that moves drug from the inner leaflet to the outer leaflet of the membrane, thus driving release of the drug to the outside by diffusion (Higgins and Gottesman, 1992).

channel regulator (Hardy *et al.*, 1995) or affecting the drug substrate specificity of the transporter.

Clearly, our lack of understanding about the possible function of Pgp phosphorylation comes in part from our lack of information about the normal biological function of Pgp itself. It is difficult to attribute a regulatory role to phosphorylation when we do not know what activity is being regulated. It is perhaps inappropriate to study Pgp phosphorylation in multidrug-resistant cells where kinase activities might be disregulated themselves or where Pgp activity might be constitutively on. Certainly a direct demonstration that phosphorylation somehow regulates Pgp activity will come from the *in vitro* reconstitution systems described earlier.

3.4 MODELS FOR Pgp MECHANISM OF ACTION

Using the information outlined above for Pgp and other transporters, various models have been proposed for how Pgp mediates multidrug resistance. Pgp has been labelled variously as an efflux pump, a hydrophobic vacuum cleaner and a flippase (Higgins and Gottesman, 1992; Gottesman and Pastan, 1993), descriptions that are difficult to distinguish based on currently available data (Fig. 3). The important feature of any of these models, consistent with most

data, is that Pgp primarily removes drugs directly from the plasma membrane. In so doing, it both reduces the influx of drug into the cell and increases its efflux. There appears to be a single "channel" with which drugs interact during transport, and this channel is made up of residues from both halves of the protein. Mutational analysis suggests that different drugs interact with different, possibly overlapping sites within the transport channel. But it is also possible that there is only a single site of interaction and that drugs are differentially accessible to that site. If that is the case, then mutations might alter the secondary and tertiary structure of Pgp in a way that perturbs the transporter–membrane interface; this could differentially affect drug entry into or release from the transport channel rather than altering actual interaction with the drug binding site.

ATP hydrolysis apparently is not required for the initial binding of drug to Pgp but it is required for the translocation of drug to the outside of cells. By analogy with CFTR, and consistent with mutational studies, the two ABC domains might function differently in the transport process, but it is not known what alternative activities might be contributed by the two domains. One possibility is that one site triggers a conformational change to mediate drug translocation while the other site triggers the return to a conformation able to accept more drug. The short linker region between the two halves of the protein (but of unknown topology within the folded structure of Pgp) might serve some regulatory function in mediating the two putative activities of the ABC domains.

Alternative models of Pgp mechanism propose that it does not act as a drug transporter *per se* but has an indirect effect on drug accumulation through effects on the plasma membrane electrochemical potential or membrane fluidity (Roepe *et al.*, 1993; Luz *et al.*, 1994; Simon and Schindler, 1994; Simon *et al.*, 1994). In these models, the recently reported Cl^- and/or ATP channel activity associated with Pgp could contribute to the altered membrane potential and thus indirectly mediate drug efflux in Pgp-expressing cells. However, two recent studies conclude that Pgp-mediated drug efflux is not influenced by changes in pH and is not coupled directly or indirectly to transport of Cl^- or Na^+ (Altenberg *et al.*, 1993, 1994a). Ruetz and Gros (1994a), in their yeast secretory vesicle system, suggest that drug transport can occur in the absence of a membrane electrical potential and in the presence of a transmembrane proton gradient. However, there is some question as to the extent to which the electrical potential was perturbed in these studies, and additional concerns about other components of the vesicle system that might contribute to membrane electrochemical potential but were not taken into account (see Ruetz and Gros, 1994b, and the response by Roepe, 1995).

The difficulty with most, if not all, models of Pgp is that they use multidrug-resistant cell lines as their model systems and these probably have multiple abnormalities contributing to their phenotype. Indeed, an active drug transport model and effects of electrochemical potential on drug

distribution are not necessarily mutually exclusive concepts and could both contribute to multidrug resistance of cells. A resolution of the controversies must come from cleaner systems, but these have been difficult to establish. It seems clear from work with reconstituted proteoliposomes and membrane vesicle systems that Pgp is capable of translocating drug against a concentration gradient in an osmotically sensitive, ATP-dependent fashion. Drugs interact with Pgp in a specific and inhibitable manner.

At the same time, there appear to be difficulties in reconciling unusual kinetic and thermodynamic properties of Pgp with a biologically relevant transport activity (Bornmann and Roepe, 1994; Simon and Schindler, 1994). While these concerns must be addressed in considering how Pgp contributes to multidrug-resistance, it is important to remember that drug transport is in itself an artificial measure of Pgp activity. Chemotherapy drugs are surely not the physiological substrate for Pgp and therefore probably interact only fortuitously with the transporter. The true kinetic and thermodynamic parameters of Pgp will be understood only when we know what its true substrate(s) is. Clearly, if Pgp-mediated drug transport (be it efficient or inefficient, direct or indirect) does contribute to clinical multidrug resistance, it remains important to understand how drugs, modulators, ions or other substrates interact with the transporter to affect drug accumulation in cancer cells. The judicious analysis of Pgp mutants in an appropriate reconstitution system will provide this information.

4 *MDR*1 Gene Regulation

As discussed earlier, *MDR*1 is expressed most commonly in cancers derived from tissues that normally express the gene. It is also detected, either before or after chemotherapy, in some cancers of tissues that do not normally express *MDR*1. These observations suggest that: (1) *MDR*1 expression is tightly regulated in a tissue-specific fashion that probably relates to the normal function of Pgp; (2) abnormal expression of *MDR*1 might be due to the disregulated growth or differentiation state of cancer cells; and (3) chemotherapy drugs might induce *MDR*1 expression. A better understanding of *MDR*1 gene regulation will help determine how multidrug resistance arises in the first place and how best to avoid the effects of *MDR*1 expression on that phenomenon.

4.1 *MDR*1 EXPRESSION IN NORMAL TISSUE

4.1.1 Tissue Specificity of Expression

Highest *MDR*1 expression in humans has been detected in the liver on the biliary canalicular surface of hepatocytes and on the apical surface of small

biliary ductules; in small and large intestine on the apical surface of columnar epithelial cells; in the kidney on the brush border of proximal tubules; in the pancreas on the apical surface of small ductules; in the adrenal gland on medullary and cortical cells; and in the placenta and uterus during pregnancy. Expression is also seen in endothelial cells of capillaries in the brain and testis (reviewed in Borst *et al.*, 1993; Gottesman and Pastan, 1993). Functional Pgp has been detected on the cell surface of $CD34^+$ bone marrow stem cells and to a lesser but measurable extent on several subpopulations of bone marrow and circulating blood cells, particularly $CD56^+$ natural killer (NK) cells and $CD8^+$ T suppressor cells (Chaudhary *et al.*, 1992; Drach *et al.*, 1992; Klimecki *et al.*, 1994).

In mice, there are two *mdr* genes that can confer drug resistance: *mdr*1a (also known as *mdr*3) and *mdr*1b (*mdr*1). They have distinct tissue distributions, perhaps reflecting different transport functions for their respective gene products. *Mdr*1a, which is the more homologous to human *MDR*1, is expressed primarily in liver, intestine, testis and brain. *Mdr*1b predominates in placenta, the pregnant uterus and adrenal gland. Both *mdr*1a and *mdr*1b are expressed in kidney, heart, lung, thymus and spleen (Borst *et al.*, 1993).

The human *MDR*2 gene product, which is non-functional in conferring drug resistance but probably has some other transport activity, is predominantly detected in the liver at the canalicular membrane of hepatocytes, throughout the lobule (Smit *et al.*, 1994). Low levels of *MDR*2 mRNA, but not protein, are detectable in adrenal, heart and striated muscle, spleen and tonsil. Mouse *mdr*2 is detected primarily in these same locations (Croop *et al.*, 1989).

4.1.2 Normal Function of Pgp

The tissue specificity of *MDR*1 expression has led to a number of proposals about the normal function of Pgp, including transport of endogenous or exogenous toxic compounds at the apical borders of epithelial cell layers, transport of steroid hormones in the adrenal gland, maintenance of the blood–brain barrier, and regulation of epithelial cell volume through chloride channel activity. The expression of *MDR*1 in subpopulations of bone marrow and circulating blood cells suggests a possible role for Pgp in NK cell- or T cell-mediated cytotoxicity (Kobayashi *et al.*, 1994b).

The best clues as to the normal functions of Pgp come from mouse knock-out experiments (Borst *et al.*, 1993; Smit *et al.*, 1993; Schinkel *et al.*, 1994). Each of the three *mdr* genes has been knocked out independently in mice and no developmental or fertility effects have been observed. However, *mdr*1a knock-out mice have increased sensitivity to toxic effects of vinblastine and ivermectin, a neurotoxic pesticide. Levels of both of these drugs are

significantly raised in tissues throughout the body of homozygous *mdr*1a$^-$ mice and they are dramatically concentrated in the brains of these mice (Schinkel *et al.*, 1994).

These results suggest that one normal function of *mdr*1a is in maintaining the blood–brain barrier to some hydrophobic agents that might normally be able to traverse the tight endothelial lining of capillaries. Consistent with this, brain capillary endothelial cell monolayers exhibit basal-to-apical flux of Pgp substrates in a verapamil-inhibitable fashion (Tatsuta *et al.*, 1992; Shirai *et al.*, 1994). If *MDR*1 serves the same function in humans, this could have significant implications for efforts to use Pgp modulators to reverse the effects of multidrug resistance. The *mdr*1a gene product could normally function elsewhere in the body as well, but its knock-out might be compensated for by overexpression of *mdr*1b (Schinkel *et al.*, 1994). It will be interesting to know the effect of simultaneously knocking out both *mdr*1a and *mdr*1b on mouse development and sensitivity to drugs.

Mouse *mdr*2 knock-outs suffer from severe liver disease. The primary defect leading to this disease appears to be an inability to secrete phospholipid into the bile. This suggests that *mdr*2 might be a phospholipid transporter in the liver, possibly a "flippase" that translocates phospholipid from the inner to the outer leaflet of the membrane (Smit *et al.*, 1993). This function does not appear to be compensated for by *mdr*1a and *mdr*1b gene products which are overexpressed in the livers of the knock-out mice.

4.2 REGULATION OF *mdr* GENE EXPRESSION

While gene amplification mediates the huge overexpression of *MDR*1 in most multidrug-resistant cell lines, this mechanism does not appear to play a role in normal cells or in the vast majority of cancer. It is therefore more clinically relevant to understand the mechanisms of *MDR*1 gene regulation at the RNA level. Initial studies proposed that *MDR*1 gene regulation occurred mainly at the level of mRNA stabilization (Chin *et al.*, 1990; Marino *et al.*, 1990), but more recent work has focused on transcriptional control of the *MDR*1 gene. This work is summarized here (also see reviews by Chin *et al.*, 1993; Greenberger *et al.*, 1994; Gottesman *et al.*, 1996).

The normal tissue-specific expression of *mdr* genes suggests that they are under the control of developmental or differentiation signals. Consistent with this, steroid hormones, pregnancy and differentiating agents have all been observed to induce *mdr* gene expression in various cell types. Progesterone induces *mdr*1b, but not *mdr*1a, in the mouse endometrium and in tissue culture cells expressing progesterone receptors (Arceci *et al.*, 1990; Piekarz *et al.*, 1993). Knocking out one allele of *mdr*1b in the Y1 adrenal cell line causes an upregulation of the remaining allele, suggesting that there is a feedback control mechanism of the progesterone response (Altuvia *et al.*, 1993).

There is strong evidence to indicate that *MDR*1 gene expression is part of a signal transduction pathway(s) involved in normal cellular growth control. Oncogenes v-*ras* and v-*raf* and PKC activators all induce endogenous *mdr* gene expression in rat liver cells (Burt *et al.*, 1988). PKC agonists TPA and diacylglycerol induce *MDR*1 in haematopoietic cells (Chaudhary and Roninson, 1992). In addition, c-Ha-*ras* and mutant but not wild-type p53 reportedly cause activation of the *MDR*1 promoter in transient CAT reporter systems in murine fibroblasts and Chinese hamster ovary cells (Chin *et al.*, 1992; Dittmer *et al.*, 1993; Zastawny *et al.*, 1993). A conflicting report, also using a transient CAT assay system, suggests that wild-type but not mutant p53 stimulates *MDR*1 promoter activity in some human cell lines (Goldsmith *et al.*, 1995). Care must be taken in interpreting the results of these studies, given the difficulty of using transient transfection systems to measure regulated gene transcription by often limiting amounts of regulatory factors.

Cornwell and co-workers have studied the growth response of endogenous and transfected *MDR*1 sequences. In a transient assay system that measures activity of a transfected *MDR*1 promoter linked to a luciferase reporter gene, *MDR*1 promoter activity peaks just before the onset of S phase in NIH3T3 cells. Promoter activation in this system appears to be mediated in part by c-Raf kinase, a central component of the mitogen-activated signal transduction pathway (Cornwell and Smith, 1993a). The TPA-mediated induction of *MDR*1 in K562 cells (Chaudhary and Roninson, 1992) involves promoter activation by EGR-1 (early growth response factor; M. Cornwell, personal communication).

Several classes of exogenous stimuli can also enhance *mdr* mRNA levels, including heat shock, chemotherapy drugs and Pgp modulators (Chin *et al.*, 1993; Gottesman *et al.*, 1996). Given the probable position of *MDR*1 within a signal transduction pathway(s), it seems likely that these inducing agents exert their influence on *MDR*1 via direct or indirect effects on growth- or stress-related transcription factors. Consistent with this, induction of *MDR*1 gene expression by a variety of agents is abrogated by protein kinase inhibitors (Uchiumi *et al.*, 1993).

4.3 STRUCTURE OF *mdr* PROMOTERS

To study *MDR*1 gene regulation more closely, the 5′-flanking sequences have been isolated and sequenced from human and rodent cell lines. While there is now some information about the general factors involved in basal expression of the family of *mdr* genes, it has been difficult to determine the basis for the differential regulation of these genes in specific tissues, differentiation states or induction conditions.

In general, the human *MDR*1 promoter does not contain consensus TATA

and CCAAT sequences normally present in very active cellular promoters but it does contain numerous other consensus binding sites for transcription factors (Ueda *et al.*, 1987b). Initial studies identified a basal promoter (within −132 to +82) that is sufficient for the accurate initiation of *MDR*1 gene transcription *in vitro* (Cornwell, 1990) and *in vivo* (Madden *et al.*, 1993; van Groenigen *et al.*, 1993). This region contains a consensus initiator sequence (Inr) straddling the transcription start site (−6 to +7). The Inr is required for proper initiation while additional upstream and downstream sequences probably contribute to efficient transcription (Madden *et al.*, 1993; van Groenigen *et al.*, 1993). The Inr site specifically binds the YY1 transcription initiator protein (M. Cornwell, personal communication), but the functional significance of this is not known.

More detailed analysis of the basal promoter region has identified several additional sequence elements that apparently contribute to *MDR*1 expression. A GC-rich domain centred around −50 (−41 to −69) contains overlapping SP-1 and EGR-1 transcription factor binding sites. Both SP-1 and EGR-1 are known to bind this region of the promoter (Cornwell and Smith, 1993b). The EGR-1 site appears to be involved in phorbol ester-stimulated promoter activity in haematopoietic cells, while c-Raf kinase acts through the SP-1 site in this promoter (Cornwell and Smith, 1993a, b; Miltenberger *et al.*, 1995; M. Cornwell, personal communication). A consensus binding site for the NF-Y family of proteins is found at −73 to −82 and is required for efficient basal expression of *MDR*1 in some cell types but not in drug resistant KB-8-5 epidermoid cells (Cornwell and Smith, 1993b; Goldsmith *et al.*, 1993). The exact factor mediating this effect is not known.

A region from −120 to −100 contains possibly two elements that negatively influence *MDR*1 transcription. Mutations from −121 to −115 or −110 to −103 result in enhanced expression of *MDR*1–reporter constructions in transfected cell lines (Ogura *et al.*, 1992; Cornwell and Smith, 1993b). There appear to be multiple factors mediating repression in this region. NF-R1 and NF-R2, purified from drug-resistant K562/ADR cells, both bind sequences from −121 to −115 (Ogura *et al.*, 1992; Takatori *et al.*, 1993). An unknown factor present in KB-8-5 cells does not recognize these same sequences but does bind to unrelated sequences from −112 to −96 (Cornwell and Smith, 1993b). The functional relationship between these putative repressor factors and their cognate binding sites is not known. Finally, an element from −157 to −131 is important for *MDR*1 activation, apparently through the action of NF-IL6, a member of the CCAAT box/enhancer binding protein family of transcription factors (Combates *et al.*, 1994).

Additional information about *mdr* gene regulation has also come from studies on rodent *mdr* promoters, but these have not completely clarified the issue of tissue specificity and inducibility of transcription. As stated earlier, mouse *mdr*1a and *mdr*1b genes are differentially expressed in normal mouse

tissues. There is also differential activation of the promoters in J774.2 mouse cell lines independently selected for resistance to vinblastine, colchicine or paclitaxel (Hsu *et al.*, 1989). The exact basis for these differential expression patterns is not known, but sequence analyses of *mdr*1a and *mdr*1b promoters have found some transcription factor binding sites unique to one or the other promoter as well as some shared recognition sequences also found in the human *MDR*1 promoter (Hsu *et al.*, 1990; Raymond and Gros, 1990; Cohen *et al.*, 1991). Again, the functional significances of these differences and similarities are not entirely known.

Dissection of these promoters indicates that *mdr*1a basal activity is mediated by sequences between −155 to +89, probably through the action of SP-1 and a second factor binding at −160 to −133 (Cohen *et al.*, 1994). These sites appear to be functionally equivalent to the SP-1 and NF-IL6 sites in human *MDR*1 (Cornwell and Smith, 1993b; Combates *et al.*, 1995).

*Mdr*1b promoter activity is influenced by at least three nuclear factors (Yu *et al.*, 1993), including an AP-1-like site and the NF-IL6 site that are also found in other rodent and human *mdr* promoters (Ueda *et al.*, 1987b; Hsu *et al.*, 1990; Silverman *et al.*, 1991; Teeter *et al.*, 1991; Zastawny and Ling, 1993; Cohen *et al.*, 1994). Some cell-type specificity appears to be conferred by a −93 to +84 fragment of the *mdr*1b promoter (Raymond and Gros, 1990). Progesterone responsiveness is conferred by the first exon of *mdr*1b (+1 to 98) and by a glucocorticoid/progesterone response element at −234 to −206 in *mdr*1b that is not present in *mdr*1a (Piekarz *et al.*, 1993).

4.4 *MDR*1 GENE REGULATION IN CANCER

The regulation of *MDR*1 gene expression in tumour cells is not well characterized. Clearly, some *MDR*1 expression in tumours is accounted for by the tissue-specific expression of *MDR*1 in normal cells. Furthermore, the growth regulation of *MDR*1 in normal cells suggests that its aberrant expression in cancer cells might occur through changes in relative levels or activities of growth-related transcription factors. The appearance of novel *mdr* transcripts due to activation of alternative *mdr* promoters or transcription start sites is associated with the emergence of drug resistance in some cell lines and might occur through the recruitment of novel factors to the promoters (Ueda *et al.*, 1987b; Hsu *et al.*, 1989; Lepage *et al.*, 1993). The appearance of novel transcripts could in turn have effects on mRNA stability or on the production of alternative Pgp translation products. Thus, relative levels of SP-1, AP-1, EGR-1, NF-IL6, NF-R1, NF-R2 and other factors that are not yet characterized could affect the expression of *MDR*1 and the emergence of the multidrug resistance phenotype in different tumour types.

*MDR*1 expression might be related to the differentiation state of cancer

cells. The detection of *MDR*1 mRNA and Pgp in haematopoietic stem cells but only a subpopulation of mature cells suggests that *MDR*1 may be downregulated during differentiation in the haematopoietic system. An association between CD34 and *MDR*1 expression has been suggested by some, but not all, clinical studies (List *et al.*, 1991; Campos *et al.*, 1992; Zhou *et al.*, 1992; Miwa *et al.*, 1993; te Boekhorst *et al.*, 1993). In solid tumours, there is no clear-cut association between *MDR*1 expression and differentiation or de-differentiation (Kanamaru *et al.*, 1989; Mizoguchi *et al.*, 1990; Weinstein *et al.*, 1991; Bradley *et al.*, 1992; Pirker *et al.*, 1993; Arao *et al.*, 1994), but more studies along this line are warranted.

There is mounting evidence to suggest that the *in vivo* sensitivity of a tumour depends on the organ environment in which it resides, with cancers in visceral organs generally being more resistant than those in lymph nodes and skin (reviewed by Fidler *et al.*, 1994). At least some of the environmental influence appears to be due to effects on *MDR*1 gene expression. For example, in a mouse model with CT-26 colonic carcinoma cells, subcutaneous tumour formation is sensitive to intravenous injection of doxorubicin, while the development of lung metastasis is substantially resistant to the drug. This is not just a difference in drug accessibility to the tumours since accumulation of doxorubicin is the same at the two sites (Wilmanns *et al.*, 1992). The lung metastases have raised *MDR*1 expression and are resistant to doxorubicin but not 5-fluorouracil in culture. The *in vitro* resistance is transient: once removed from the lung environment, drug resistance reverts to that of the parent cell line in a matter of days (Dong *et al.*, 1994). In cultured CT-26 cells, *MDR*1 expression and drug resistance are inversely related to cell density, suggesting that organ-specific effects could be mediated by some aspect of cell–cell interaction (I. Fidler, personal communication). Similar effects of organ environment on drug sensitivity and *MDR*1 expression are seen with a human colonic carcinoma cell line in the mouse model (Wilmanns *et al.*, 1993).

These results suggest that something about specific organ environments (e.g. cell–cell or cell–matrix interactions or organ-specific growth factors) both promote the formation of metastases and induce the expression of *MDR*1 (and probably other drug resistance-related mechanisms), making certain sites prone to the development of drug-resistant disease. The mechanism responsible for inducing *MDR*1 in specific organs has not been entirely elucidated and it is not known whether the effect on *MDR*1 is transcriptional or post-transcriptional. But these observations once again suggest that *MDR*1 is under the control of growth or differentiation regulatory signals.

Cis-acting mutations within *MDR*1 promoter sequences could contribute to its altered regulation in cancer cells. Chen *et al.* (1994) recently reported that *MDR*1 overexpression emerges during single-step selection for doxorubicin resistance of a human sarcoma cell line. The overexpression is

consistent with the occurrence of spontaneous mutational events leading to a selective advantage of a small number of cells. The nature of such mutations has not been characterized but they could be within the *MDR*1 promoter, thus altering the transcriptional regulation of the gene. In a study of osteosarcomas with high *MDR*1 expression, seven of nine samples had point mutations in the promoter region downstream of the transcription start site (two cases at +103, five cases at +137). There were no differences from wild-type sequence in eight malignant fibrous histiocytomas, which had low levels of *MDR*1 mRNA. The +103 mutation conferred slightly raised *MDR*1 promoter activity on a CAT reporter construct transfected into COS cells (Stein *et al.*, 1994a). It is not known whether promoter mutations occur in other human cancers nor whether such mutations might contribute to overexpression of *MDR*1 in tumours.

Whether mediated by altered *trans*-acting signals or by mutated *cis*-acting promoter elements, aberrant *MDR*1 expression in tumours is most likely related to aberrant growth stimulation of those cells. In that case, we can think of Pgp as a tumour marker that happens to confer a selective advantage on neoplastic cells because of its ability to transport drugs. This is consistent with findings of *MDR*1 expression in many untreated cancers derived from cell types that do not normally express the gene. The enhanced expression of *MDR*1 in some tumours after chemotherapy suggests that there might be some direct or indirect induction of *MDR*1 by chemotherapy (Kohno *et al.*, 1989), again resulting in a selective advantage for cells expressing *MDR*1.

5 Other Mechanisms of Multidrug Resistance

There are several other defence mechanisms that cancer cells use to elude the cytotoxic effects of chemotherapy. In addition to Pgp overexpression, other cellular changes can be associated with multidrug-resistance, but it has been difficult to determine which changes are significant. Part of the problem is not knowing what to look for. As discussed recently by Simon and Schindler (1994), the basic challenge to understanding drug resistance can be defined in terms of understanding why cancer cells are more susceptible to chemotherapy drugs than normal cells in the first place, and then determining how some cells lose that susceptibility to become resistant.

One feature of cancer cells is their tendency to accumulate drugs to a higher concentration than normal cells, although the mechanism for this phenomenon is not entirely clear. Higher accumulation should lead to greater access of drugs to their cellular targets. Another characteristic of cancer cells is their altered regulation of cell cycle and DNA repair events. Chemotherapy drugs that directly or indirectly induce DNA damage, therefore, would be expected to have enhanced toxicity in cells lacking their normal "checkpoint"

mechanisms for repair and cell growth. Resistance mechanisms that lead to altered drug compartmentalization (either to the outside of cells or to vesicles within cells), detoxification of drugs, changes in their intracellular targets, or changes in the cell's response to DNA damage could thereby counteract the accumulation of drug in cancer cells and may abrogate their effects even when they do reach their cellular targets (Simon and Schindler, 1994).

In this section, we will briefly discuss some of these mechanisms of resistance, particularly those associated with resistance to Pgp-related drugs.

5.1 ALTERED DRUG ACCUMULATION AND COMPARTMENTALIZATION

A number of cell lines have been described that do not overexpress *MDR*1 but nevertheless exhibit resistance to many Pgp substrates (compiled by Nielsen and Skovsgaard, 1992). Some of these display altered accumulation of drug and some appear to sequester drug into vesicles as a mechanism of reducing the amount of biologically available drug. One such cell line, a human small cell lung carcinoma cell line selected for high resistance to doxorubicin (H69AR), was used to clone the gene for a novel multidrug resistance-associated protein (MRP or P190) (Cole *et al.*, 1992). Sequence analysis of MRP cDNA indicates that the protein is another member of the ABC transporter family, but its actual homology with other human transporters is low (14% for Pgp) and is limited to the ABC domain.

Since its initial discovery, considerable progress has been made on characterizing MRP's biochemical and biological properties. Expression of MRP has been detected at the RNA level in every normal tissue so far tested (Cole *et al.*, 1992; Zaman *et al.*, 1993). Overexpression of MRP and amplification of the MRP gene have been detected in many but not all multidrug-resistant cell lines that do not overexpress Pgp (Krishnamachary and Center, 1993; Slovak *et al.*, 1993; Zaman *et al.*, 1993; Barrand *et al.*, 1994; Futscher *et al.*, 1994).

MRP expression in clinical samples has not been extensively characterized. It is expressed at detectable levels in all normal haematopoietic cell types (Cole *et al.*, 1992; Abbaszadegan *et al.*, 1994; Burger *et al.*, 1994). Although raised MRP levels have been reported in a small number of patients with AML, particularly those previously treated with chemotherapy (Burger *et al.*, 1994; Schneider *et al.*, 1995), its levels are generally low in *de novo* AML, ALL, CML and multiple myeloma samples compared with its expression in normal haematopoietic cells (Abbaszadegan *et al.*, 1994; Burger *et al.*, 1994; Hart *et al.*, 1994; Schneider *et al.*, 1995). Expression is higher in CLL than in the other haematopoietic diseases, but there is no correlation between prior chemotherapy and MRP levels (Burger *et al.*, 1994). There also does not appear to be a correlation between MRP and lack of *MDR*1 expression,

with co-expression of the two genes not uncommon (Burger *et al.*, 1994; Hart *et al.*, 1994).

Transfection of the MRP gene can confer modest multidrug resistance on otherwise drug-sensitive cell lines (Grant *et al.*, 1994; Kruh *et al.*, 1994; Zaman *et al.*, 1994). The cross-resistance profile of MRP-transfected cells is qualitatively similar to that of Pgp-expressing cells and suggests that MRP can confer resistance to doxorubicin, daunorubicin, vincristine and etoposide, and to a lesser extent to vinblastine, colchicine and taxol. MRP-transfected cells accumulate about 50–60% as much drug as their parent cells and this effect appears to depend on ATP (Cole *et al.*, 1994; Zaman *et al.*, 1994). Verapamil and cyclosporin A markedly reverse the vincristine and doxorubicin resistance of MRP-transfected cells, but they have less of an effect on drug accumulation in these cells and probably do not directly interact with MRP to mediate chemosensitization (Cole *et al.*, 1994).

It has been suggested that one mode of action for MRP might be to mediate the altered compartmentalization of drug in cells, via a drug transport mechanism. Although some cell lines selected for multidrug resistance have significant levels of MRP in the membrane of intracellular organelles or endosomes, which would be consistent with a role in drug compartmentalization, the primary localization of MRP is on the plasma membrane (Marquardt *et al.*, 1990; Krishnamachary and Center, 1993; Hipfner *et al.*, 1994; Almquist *et al.*, 1995). The protein is glycosylated, phosphorylated, and is able to bind ATP (Almquist *et al.*, 1995), but direct binding of drug to MRP has not yet been demonstrated (Cole *et al.*, 1994; Almquist *et al.*, 1995). It remains to be determined, therefore, whether MRP is a drug transporter at the cell surface or in vesicles.

Two recent reports have suggested that MRP is the same protein as the glutathione S-conjugate (GS-X) pump (also known as the multiorganic anion transporter and the leukotriene transporter) (Jedlitschky *et al.*, 1994; Müller *et al.*, 1994). Substrates for this pump generally contain a hydrophobic alkyl chain and at least two negative charges. Removal of such compounds from the cell first requires that they be conjugated to glutathione (GSH) and then the conjugate is exported from the cell via the action of the GS-X pump. If MRP is the GS-X pump, its apparent ability to confer resistance to Pgp-related drugs, many of which are not thought to be substrates for direct conjugation to GSH, must be explained. Either these drugs must undergo some sort of intracellular metabolism and conjugation to GSH, or MRP has dual transport capability for anionic conjugates and for basic or neutral amphophilic compounds.

The mechanism of GS-X pump-mediated export of drugs requires that GSH levels remain high in cells despite its constant loss in the form of GS-X conjugates. This is consistent with observations that MRP-mediated resistance to doxorubicin, daunorubicin and vincristine can be reversed by GSH depletion with buthione sulfoximine (Müller *et al.*, 1994; Meijer *et al.*, 1987).

A requirement for GSH could also explain why it is difficult to achieve very high-level drug resistance by overexpressing MRP. Such resistance might also require the concurrent upregulation of the GSH biosynthesis system and of glutathione *S*-transferase (GST) to mediate the conjugation of drugs to GSH (also see next section).

Another protein whose overexpression is associated with non-Pgp multidrug resistance is a 110-kDa protein termed LRP56. Monoclonal antibody against LRP56 detects primarily a cytoplasmic distribution of the protein that is indicative of a vesicular localization (Scheper *et al.*, 1993). LRP56 expression has been analysed in patients with AML and found to be prevalent in *de novo* and relapse AML, and more so in secondary AML (along with Pgp). Both LRP56 and Pgp are associated with poor response to induction chemotherapy in these patients, with co-expression of LRP56 and Pgp being least favourable for achieving a complete or partial response (List *et al.*, 1993b; A. List, personal communication). LRP56, in particular, appears to be associated with mitoxantrone-resistant disease (List *et al.*, 1993b).

Interestingly, LRP56 is expressed in patients who relapse from previous response to daunorubicin plus cyclosporin A, while Pgp is low or undetectable in these same patients (List *et al.*, 1993a, b; A. List, personal communication). These results suggest that the combination of daunorubicin and a Pgp modulator selects against Pgp-expressing cells and that LRP56 can emerge as a secondary mechanism of resistance in patients with AML. The mechanism of action of LRP56 is not known. Cloning of the LRP56 gene and further characterization of the protein should shed light on another potentially important mediator of multidrug resistance. Other mechanisms that alter the uptake or intracellular compartmentalization of antineoplastic drugs are also possible (Simon and Schindler, 1994).

5.2 DETOXIFYING MECHANISMS

Glutathione *S*-transferases (GST) and glutathione peroxidases are parts of a complex intracellular oxidation–reduction system which protects cells against oxidative damage. Members of the GST isozyme family catalyse the addition of glutathione (GSH) to electrophilic xenobiotics, including *cis*-platin and a variety of alkylating agents, thus acting to detoxify those drugs (Colvin *et al.*, 1993). Because of the frequently increased levels of GST isozymes and GSH detected in classically multidrug-resistant tumour cell lines, much effort has focused on studying GST-mediated GSH conjugation as a mechanism of detoxifying Pgp-related drugs as well (discussed recently by Tew, 1994). A role for GST in resistance to most Pgp substrates has generally been ruled out because the drugs are not good substrates for GST isozymes. The case of doxorubicin is still not entirely settled though. While the drug is probably not itself a substrate for conjugation to GSH, it has been

proposed that the semiquinone species of doxorubicin or lipid peroxidation byproducts of quinone-induced free radicals might be subject to detoxification by the GST–GSH system (Tew, 1994).

GSH depletion experiments indicate that doxorubicin resistance can be abolished in the absence of GSH (Hamilton *et al.*, 1985; Meijer *et al.*, 1987; Dusre *et al.*, 1989). However, transfection studies to overexpress GST isozymes have had variable results, with only low levels of doxorubicin resistance achieved in some cell lines and no change in resistance in most others (e.g. Moscow *et al.*, 1989; Nakagawa *et al.*, 1990). Expression of human GST isozymes in yeast confers up to 15-fold resistance to doxorubicin (Black *et al.*, 1990).

Glutathione peroxidase (GPx) has been proposed as another enzyme involved in detoxifying reactive oxygen byproducts (superoxide, hydroxyl radicals, hydrogen peroxide, lipid peroxides) of anticancer quinones (doxorubicin and daunorubicin in particular), using GSH as a reductant (Sinha *et al.*, 1989; Samuels *et al.*, 1991; Doroshow *et al.*, 1990; Benchekroun *et al.*, 1993). Recent experiments demonstrated that depletion of GPx with antisense cDNA results in enhanced sensitivity to doxorubicin in a Chinese hamster ovary cell line (Taylor *et al.*, 1993). A 25-fold overexpression of the cDNA in the sense orientation or scrape-loading of GPx protein into MCF-7 cells confers about twofold enhanced survival in the presence of doxorubicin but not to redox-inactive analogues mitoxantrone and 5-iminodaunorubicin (Doroshow *et al.*, 1991, 1993). More modest increase (five- to tenfold) of GPx expression by transfection of T47D breast cells gives no enhanced resistance to daunomycin (Lavoie *et al.*, 1992).

The inability to get high resistance by transfection of GST or GPx into mammalian cells might not be conclusive. Given the cellular requirement for tightly controlled GSH homoeostasis, it might be deleterious to perturb one component of the system (GST or GPx) without concomitantly upregulating other components of the GSH system (either GSH biosynthesis or recycling of oxidized glutathione to the reduced state). The level of GST or GPx overexpression that can be achieved may therefore be constrained, particularly in cell lines that have limited capacity for GSH synthesis (Tew, 1994). With the recent discovery that MRP may be the GS-X pump that excretes GSH conjugates from the cell (see above) and as other GSH biosynthetic enzymes are further characterized, the role of the GSH redox system in conferring resistance to Pgp-related drugs may soon be clarified.

5.3 ALTERED CELLULAR TARGETS

Another way to elude the cytoxicity of chemotherapy drugs is to alter the cellular targets at which the drugs are directed. One example of this mechanism is topoisomerase II (topoII). Several Pgp substrates

(epipodophyllotoxins and anthracyclines) are inhibitors of topoII, which uses strand breakage–unwinding–ligation activities to resolve topological constraints associated with DNA replication (reviewed recently by Pommier *et al.*, 1994). TopoII inhibitors somehow stabilize DNA-topoII "cleavable complexes" by blocking the re-ligation step of the unwinding mechanism.

Two general modes of topoII-mediated drug-resistance have been observed in tissue culture cell lines: (1) downregulation of topoII levels in the cell, thus reducing the amount of drug target; and (2) mutation of topoII within the ATP binding domain of the enzyme, thus reducing the activity of the target (reviewed by Beck *et al.*, 1994; Pommier *et al.*, 1994). Both of these mechanisms ultimately prevent the accumulation of stable DNA–topoII cleavable complexes. The prevalence of altered topoII activities in human tumours is not entirely known. Danks *et al.* (1993) detected no topoII mutations in blast cells of relapsed patients with ALL previously treated with topoII inhibitors.

Although it is not completely understood how DNA–topoII complex stabilization is cytotoxic, one possibility is that cells perceive this as DNA damage and thus initiate an apoptotic response to that damage (Walker *et al.*, 1991; Ling *et al.*, 1993). Indeed, one early effect of topoII inhibitors is the induction of c-*jun* and of AP-1 DNA binding activity in a manner that parallels the formation of DNA–topoII complexes (Rubin *et al.*, 1991; Kim and Beck, 1994). These events appear to be a prelude to apoptosis in response to DNA fragmentation (Gewirtz, 1993). Induction of c-*jun*, AP-1 binding activity and apoptosis are abrogated in multidrug-resistant cells exhibiting altered topoII activity, possibly because the initial signal for induction (DNA damage) is absent (Kim and Beck, 1994).

The relationship between apoptosis and drug resistance has been explored recently in a number of tissue culture model systems. A variety of DNA damaging agents, including chemotherapy drugs, have been shown to lead ultimately to cell death, either programmed or non-programmed (see Gewirtz, 1993; Reed, 1994). The exact chain of events connecting those two endpoints (DNA damage and cell death) is not entirely known, but probably involves a complex cascade of signals initiated by the immediate early induction of AP-1 transcription factor complexes (thus explaining the results with topoII inhibitors). The cascade of signals ultimately can lead to cell cycle arrest followed by cell death or can proceed directly to apoptosis in the absence of growth arrest (Gewirtz, 1993; Yonish-Rouach *et al.*, 1993).

A late event in the apoptotic response to many DNA-damaging agents appears to be blocked by the action of the *bcl*-2 gene product, although the mechanism of this block is not known (Reed, 1994). Bcl-2 overexpression, possibly through its effect on blocking apoptosis, appears to protect cells from cytotoxic effects of many chemotherapeutic drugs and thus may be another mechanism of drug resistance (see, for example, Miyashita and Reed, 1993; Reed, 1994). Failures in other steps of the DNA damage–apoptosis/cell death

signal transduction pathway could also conceivably contribute to drug resistance, but such mechanisms need to be explored further.

6 Prospects for Pgp-related Chemotherapy

With some understanding of the mechanisms of resistance to Pgp-related drugs, recent efforts have focused on developing ways to overcome or circumvent the multidrug transporter. The final section of this review will discuss two general areas of such research: (1) inhibiting Pgp activity or synthesis; and (2) using gene therapy to protect normal cells from the normally dose-limiting toxicities of chemotherapy.

6.1 INHIBITING Pgp

Initial clinical studies using Pgp modulators to disable the multidrug transporter have had promising but mixed results (see Section 2.3). Currently available, proven chemosensitizers can be delivered to patients at levels that should be therapeutically beneficial, but the toxicities associated with these doses are often unacceptably high. As a result, much attention has focused on developing more potent Pgp modulators that can be used at lower doses and that have reduced biological activity other than their modulation of drug transport. Of interest are two non-immunosuppressive analogues of cyclosporin D, SDZ PSC-833 and SDZ 280-446 (Boesch *et al.*, 1991; Loor *et al.*, 1992), the *trans*-stereoisomer of flupenthixol (Ford and Hait, 1990; Ford *et al.*, 1990), cinchonine, a natural product antimalarial analogue of quinine (Genne *et al.*, 1994) and pure anti-oestrogenic derivatives of tamoxifen (Kirk *et al.*, 1994).

Several novel compounds have been synthesized or identified as candidate chemosensitizers because they have structural features thought to be important for Pgp modulating activity (Pearce *et al.*, 1989; Beck and Qian, 1992). This approach has been taken to screen novel phenothiazines, phenoxazines, dipyridamoles, dihydropyridines and propafenones (Asoh *et al.*, 1989; Nogae *et al.*, 1989; Ramu and Ramu, 1989, 1992; Shinoda *et al.*, 1989; Thimmaiah *et al.*, 1990; Hait and Aftab, 1992; Chen *et al.*, 1993; Kraus-Berthier *et al.*, 1994; P. Chiba, personal communication), but no clear criteria for *a priori* identification of the best modulators have yet been determined.

One hindrance to the discovery of effective Pgp modulators has been the lack of appropriate animal models with which to test candidate compounds, but several human xenograft models have recently been described which may alleviate this problem. These include xenografts of: (1) a human ovarian multidrug-resistant cell line 2780AD, used to identify the tiapamil analogue

Ro11-2933 as a potent reversing agent (Plumb *et al.*, 1994); (2) a small cell lung carcinoma SCLC-6, which expresses *MDR*1 and exhibits acquired resistance to Pgp and non-Pgp drugs (Arvelo *et al.*, 1993); (3) a panel of soft tissue sarcoma xenografts that show a range of inherent and secondary resistances to doxorubicin, ifosfamide, dacarbazine and cisplatin (Budach *et al.*, 1994); and (4) a multidrug-resistant multiple myeloma model in SCID mice that has been evaluated with respect to doxorubicin effects (Bellamy *et al.*, 1993). Each of these systems should prove useful for further testing of chemosensitizers in combination with Pgp-related drugs.

In addition to "classical" inhibitors of drug transport, monoclonal antibodies to Pgp and antibody-toxin conjugates are known to modulate multidrug resistance *in vitro* and *in vivo* (FitzGerald *et al.*, 1987; Tsuruo *et al.*, 1989; Efferth *et al.*, 1991; Mechetner and Roninson, 1992; Rittmann-Grauer *et al.*, 1992; Efferth and Volm, 1993). More recent work shows that antibodies can have a potentiating effect on some (cyclosporin A) but not all (verapamil and FK506) Pgp modulators, possibly by enhancing the accumulation of the modulator in target cells (Naito *et al.*, 1993).

Alternative methods of delivering cytotoxic drugs appear to be able to circumvent resistance. Encapsidation of doxorubicin in liposomes (Fan *et al.*, 1990; Sadavisan *et al.*, 1991; Rahman *et al.*, 1992; Warren *et al.*, 1992; Thierry *et al.*, 1993b) or in polyisohexylcyanoacrylate nanospheres (Bennis *et al.*, 1994; Colin de Verdiere *et al.*, 1994) leads to enhanced intracellular distribution of drug and enhanced cytotoxicity against multidrug-resistant cell lines. Furthermore, liposome-encapsidated vincristine, which is more toxic than free vincristine against multidrug-resistant HT-29 colonic cancer cells, can be further potentiated by monoclonal antibody MRK-16 or verapamil (Sela *et al.*, 1995). Finally, conjugation of doxorubicin to bovine serum albumin can also enhance toxicity against multidrug-resistant cells, again by apparently altering the uptake and/or efflux of conjugated drug relative to free drug (Ohkawa *et al.*, 1993).

A different approach to Pgp inhibition is to use antisense or ribozyme technology to block synthesis of the drug transporter. A number of groups have reported the successful downregulation of *MDR*1 mRNA and cell surface Pgp using this approach, with concomitant complete or partial reversal of multidrug resistance *in vitro* (Thierry *et al.*, 1993a; Holm *et al.*, 1994; Kiehntopf *et al.*, 1994; Kobayashi *et al.*, 1994a; Scanlon *et al.*, 1994). Up to 300-fold modulation has been seen in pancreatic and leukaemic cell lines previously selected for resistance to daunorubicin or vincristine, respectively (Holm *et al.*, 1994; Kobayashi *et al.*, 1994a).

Chemosensitization can be long term if *MDR*1-directed ribozymes are stably expressed from transfected expression vectors, or it can be shorter term if synthetic oligonucleotides are delivered to cells by liposome-mediated transfection. In both cases, however, only a percentage of treated cells take up or express ribozyme/antisense sequences at levels sufficient to inhibit Pgp

synthesis significantly. Clearly, the drawback of this approach to cancer chemotherapy is the need to deliver the therapeutic sequences to every resistant or potentially resistant cell in a tumour in order to overcome or avoid Pgp-mediated multidrug resistance completely. Indeed, the efficacy of any of these alternative approaches to chemotherapy will need to be worked out in more detail in animal model systems before proceeding to clinical trials in humans.

6.2 GENE THERAPY WITH *MDR*1

Gene therapy with drug-resistance genes has been proposed as a way to protect normal cells from the normally dose-limiting toxicities associated with chemotherapy (Bertino, 1990; Kane *et al.*, 1990; Gottesman *et al.*, 1996). The beauty of this concept is that it no longer matters whether a particular gene is truly responsible for the clinical phenomenon of drug resistance of cancer cells. Rather, it is only important that transfer and overexpression of that gene (either a wild-type or a mutant version of it) be *sufficient* for conferring resistance to a drug(s) in normal cells. In effect, the drug resistance gene must act as an *in vivo* dominant selectable marker so as to confer survival on cells that take up and express it.

6.2.1 Chemoprotection of Normal Cells

In terms of the *MDR*1 gene, the rationale for this idea is based on *in vitro* tissue culture and *in vivo* mouse experiments. *In vitro*, the human *MDR*1 gene can be transfected or retrovirally transduced into tissue culture cells and its expression is sufficient for conferring resistance to Pgp substrates (Ueda *et al.*, 1987a; Pastan *et al.*, 1988; McLachlin *et al.*, 1990). *In vivo*, transgenic mice expressing *MDR*1 in their bone marrow show reduced myelosuppression when injected with Pgp-related drugs (Galski *et al.*, 1989; Mickisch *et al.*, 1991, 1992). Furthermore, lethally irradiated mice can be reconstituted with bone marrow transduced with *MDR*1 retrovirus. Transplanted mice exhibit *MDR*1 DNA in their circulating blood cells; an enrichment for those sequences is seen if transplanted mice are treated with taxol, a Pgp substrate; and there is reduced myelosuppression when the mice are further treated with taxol (Podda *et al.*, 1992; Sorrentino *et al.*, 1992).

Recent results suggest that *MDR*1-reconstituted mice in fact express *MDR*1 mRNA in their bone marrow and spleen colonies at levels exceeding endogenous mouse *mdr*1a and *mdr*1b expression. In a bone marrow population enriched for stem cells, *MDR*1 expression is detectable up to eight months after transplant after a single dose of taxol administered ten days earlier (Sorrentino *et al.*, 1995). *MDR*1 mRNA also appears to be expressed

and functional in recipient mice obtained through serial transplantation of the original reconstituted marrow (Hanania and Deisseroth, 1994; Hanania *et al.*, 1995a).Taken together, these mouse studies are consistent with *MDR*1 acting as an *in vivo* drug-selectable and chemoprotective gene.

Recent advances in this field have demonstrated the use of *MDR*1 retroviruses to transduce human $CD34^+$ haematopoietic progenitor cells derived from either the bone marrow or peripheral blood. The transduced gene is selectable with Pgp drugs and is functional in substrate transport at the cell surface (Ward *et al.*, 1994; Hanania *et al.*, 1995b; P. Yam *et al.*, unpublished observations). Despite these promising results, retroviruses are probably not the most effective vehicle for delivering therapeutic genes to stem cells, because of their inherent inability to infect non-dividing cells (Miller *et al.*, 1990). Alternative delivery systems are needed. Adeno-associated virus (AAV) is one such alternative system that appears to transduce resting cells as well as dividing cells (Podsakoff *et al.*, 1994). We have recently obtained high-titre AAV-*MDR*1 virus which is capable of efficiently (50–70% on average) transducing human $CD34^+$ progenitor cells and producing functional cell surface Pgp (E. Shaughnessy *et al.*, unpublished results).

Gene therapy protocols are approved or underway to determine whether *MDR*1-transduced $CD34^+$ cells can be sufficiently enriched *in vivo* to provide a chemoprotective effect in humans and to test whether constitutive expression of *MDR*1 is deleterious to the differentiation programme of haematopoietic cells (Deisseroth *et al.*, 1994; Hesdorffer *et al.*, 1994; Mickisch and Schroeder, 1994; O'Shaughnessy *et al.*, 1994). As better gene delivery systems are developed by the gene therapy field, we may also be able to protect other tissues in the body against the side-effects of chemotherapy. Furthermore, as we learn more about the substrate specificity determinants of Pgp, we may be able to design "custom-made" versions of *MDR*1 to provide very specific chemoprotection against defined therapeutic agents without compromising modulator trials.

The chemoprotection concept can be extended to other drug resistance genes as well, as shown, for example, for dihydrofolate reductase (resistance to folates) (Corey *et al.*, 1990; Zhao *et al.*, 1994) and glutathione *S*-transferase (resistance to alkylating agents) (Greenbaum *et al.*, 1994). Our own group is exploring a combined approach, using *MDR*1 plus a second drug resistance gene to confer chemoprotection against a broad range of potential chemotherapeutic agents. For this purpose, we have developed a series of retroviral vectors (Fig. 4) capable of encoding *MDR*1 plus a heterologous gene under the control of either an internal promoter or an internal ribosome entry site (IRES) element (Ghattas *et al.*, 1991). In practice, we have inserted genes encoding human glutathione *S*-transferase π, human glutathione peroxidase or bacterial neomycin resistance (as a reporter). These vectors support the production of high-titre ecotropic and amphotropic retroviruses,

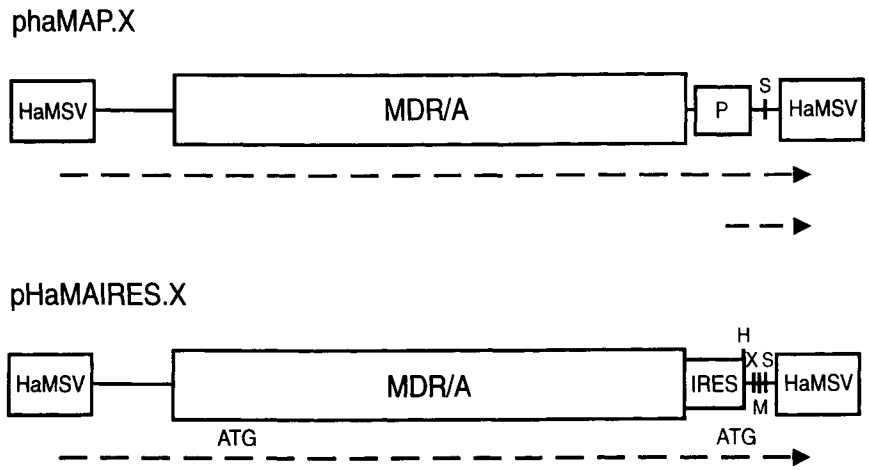

FIG. 4. Schematic drawings of *MDR*1 retroviral vectors. Two types of retroviral vector are shown, which use *MDR*1 as a selectable marker for gene transfer. The vectors have Harvey murine sarcoma virus long terminal repeats (HaMSV) flanking *MDR*1 and unique cloning sites for inserting a second gene of interest (X). The heterologous gene inserted into pHaMAP.X is expressed from an internal promoter (P) on a separate messenger RNA from *MDR*1, as shown (dotted lines) (see Gazit *et al.*, 1995; Metz *et al.*, 1995, 1996). With pHaMAIRES.X, *MDR*1 and the second gene are both transcribed on a single mRNA that contains two functional initiation codons (ATG) for translating two independent gene products (Metz *et al.*, 1996). Unique cloning sites in the vectors are: H, *Hpa*I; M, *Mlu*I; S, *Sal*I; X, *Xho*I.

transduced cells selected for expression of the *MDR*1 gene also express the second drug resistance gene at high levels and cells are resistant to both Pgp substrates and substrates specific to the heterologous gene (Gazit *et al.*, 1995; Metz *et al.*, 1995, 1996; J. Doroshow and S. Kane, unpublished observations).

6.2.2 MDR*1 as an* In Vivo *Selectable Marker*

In the two-gene approach, *MDR*1 serves a dual role as both a therapeutic gene and a selectable marker for achieving overexpression of the second resistance gene of interest. It is also conceivable that *MDR*1 could serve just the latter function in a gene therapy setting. In this case, the heterologous gene would be therapeutic for a metabolic disease, for example. *MDR*1 would provide a selective *in vivo* growth advantage to transduced target cells so as to achieve and maintain long-term stable expression of the therapeutic gene.

The activity of *MDR*1 as a selectable marker has been described *in vitro*.

In this technique, vectors that carry *MDR*1 plus a heterologous non-selectable gene are transfected into sensitive host cells; cells that take up and express *MDR*1 are isolated by selection with a Pgp substrate; drug-resistant cells are shown to express high levels of the non-selectable gene (Kane *et al.*, 1989; Kane and Gottesman, 1993). Furthermore, *MDR*1, like a few other mammalian markers, is amplifiable: increased copy number and expression of *MDR*1 and of the linked heterologous gene are obtained by growing transfected cells in increasing concentrations of drug (Kane *et al.*, 1988).

Within retroviral vectors, there are three general modes of expressing two genes and all three have been successfully modelled *in vitro* using *MDR*1 as the selectable marker. (1) The heterologous sequences can be part of a fusion gene with *MDR*1, thus encoding a chimeric protein with two functional domains. This has been demonstrated with a bifunctional Pgp–adenosine deaminase chimera, potentially therapeutic for severe combined immune deficiency (Germann *et al.*, 1990a). (2) The heterologous sequences can be expressed as an independent mRNA under the control of an internal promoter cloned downstream of *MDR*1. We have demonstrated this approach using the vectors shown in Fig. 4 and *neo* as the heterologous gene (Gazit *et al.*, 1995; Metz *et al.*, 1995, 1996). (3) The heterologous sequences can be encoded on the same mRNA as *MDR*1 but downstream or upstream of an internal ribosome entry site so that the genes are expressed as independent translation products. Vectors for this purpose have been described (Fig. 4; Sugimoto *et al.*, 1994; Metz *et al.*, 1996). We and others have used this method to express heterologous sequences that are either other drug resistance genes or are potentially therapeutic for treating a lysosomal storage defect leading to Gaucher disease (Aran *et al.*, 1994; Metz *et al.*, 1996).

The use of *MDR*1 as an *in vivo* selectable and amplifiable marker for expressing a heterologous gene has not been directly demonstrated in an animal model, although results with transgenic and marrow-reconstituted mice are consistent with *MDR*1 being selectable *in vivo*. Pre-clinical studies will need to be performed to determine whether long-term expression of heterologous genes can be maintained *in vivo* by selecting for *MDR*1 expression. If it turns out to be feasible to use *MDR*1 in this manner, this would bring the *MDR*1 gene full circle – from a deleterious gene in the clinical phenomenon of multidrug resistance, to a therapeutic gene for the chemoprotection of normal cells during chemotherapy, and finally to a selectable but benign gene for the transfer and maintained expression of therapeutic genes in the treatment of metabolic diseases.

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Bioactivation of Organic Nitrates and Other Nitrovasodilators

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1 Introduction

Organic nitrates such as glyceryl trinitrate are among the oldest and most widely used drugs for the treatment of myocardial ischaemia and its principal symptom angina pectoris. Bearing in mind that the antianginal effect of glyceryl trinitrate was described more than 100 years ago (Murrell, 1879), it seems astonishing that the cellular mechanism underlying this action has remained completely unknown until the 1970s. Since then it has become increasingly clear that the nitric oxide free radical and its second messenger cyclic guanosine monophosphate (GMP) mediate the vasodilatory and antianginal effects of organic nitrates (for reviews see Parratt, 1979; Rapoport and Murad, 1983; Ignarro and Kadowitz, 1985; Noack, 1990; Harrison and Bates, 1993). Thus, organic nitrates have to be classified as prodrugs releasing the pharmacologically active metabolite nitric oxide. Although several different pathways have been suggested, recent evidence points to a cytochrome P450 system playing an important role in the process of enzymatic nitric oxide generation from organic nitrates.

[1]Dedicated to Professor Ernst Mutschler on the occasion of his 65th birthday.

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2 Nitric Oxide and Cyclic GMP as Mediators of Organic Nitrate Action

The antianginal action of organic nitrates is due to their capacity to relax vascular smooth muscle in veins and to a lesser degree in arteries. The resulting reduction in preload and afterload decreases the myocardial requirement for oxygen, thus counteracting the inadequate oxygenation during ischaemia and anginal attacks. Moreover, dilatation of collateral vessels and large coronary arteries directly improves blood flow to ischaemic myocardium (for review see Bassenge *et al.*, 1981).

That the second messenger cyclic GMP might be involved in the smooth muscle-relaxing effect of certain nitrogen-containing compounds was first suggested in the mid-1970s when the smooth muscle relaxants sodium azide, hydroxylamine and sodium nitrite were shown to increase tissue cyclic GMP levels and to activate soluble guanylate cyclase (Kimura *et al.*, 1975; Mittal *et al.*, 1975; Miki *et al.*, 1976). Subsequently, Diamond and co-workers demonstrated glyceryl trinitrate to increase cyclic GMP levels in rat myometrium and canine femoral artery (Diamond and Holmes, 1975; Diamond and Blisard, 1976). Further studies in several laboratories revealed that sodium nitroprusside and other vasodilators containing nitrogen oxide constituents (nitrovasodilators; Fig. 1) produced relaxation of vascular and non-vascular smooth muscle via stimulation of cyclic GMP formation (Arnold *et al.*, 1977; Katsuki and Murad, 1977; Schultz *et al.*, 1977; Böhme *et al.*, 1978; Axelsson *et al.*, 1979; Janis and Diamond, 1979; Kukovetz *et al.*, 1979; Gruetter *et al.*, 1981). The mechanism by which nitrovasodilators increase intracellular cyclic GMP levels became clear when the unstable free radical nitric oxide was found to be a potent and reversibly relaxing agent which activated soluble guanylate cyclase (Arnold *et al.*, 1977; Miki *et al.*, 1977; Gruetter *et al.*, 1979). From these findings it was concluded that prior to cyclic GMP stimulation and vasodilatation, nitrovasodilator drugs have to be acted upon either enzymatically or non-enzymatically to release free nitric oxide.

Cyclic GMP-dependent vasodilatation has been shown to involve activation of a cyclic GMP-dependent protein kinase followed by a decrease in cytosolic calcium levels (for reviews see Lincoln, 1989; Walter, 1990). However, it was not until recently that the physiological relevance of nitric oxide as endothelium-derived relaxing factor and endogenous activator of soluble guanylate cyclase has emerged (Palmer *et al.*, 1987; Ishii *et al.*, 1989; Knowles *et al.*, 1989; Schröder and Schrör, 1989; Moncada *et al.*, 1991).

3 Nitric Oxide Formation from Organic Nitrates in Cell-Free Systems

Compounds that spontaneously release nitric oxide in aqueous solution, such as sodium nitroprusside and sydnonimines (Fig. 1), markedly raise cyclic GMP levels in various organs and activate soluble guanylate cyclase in

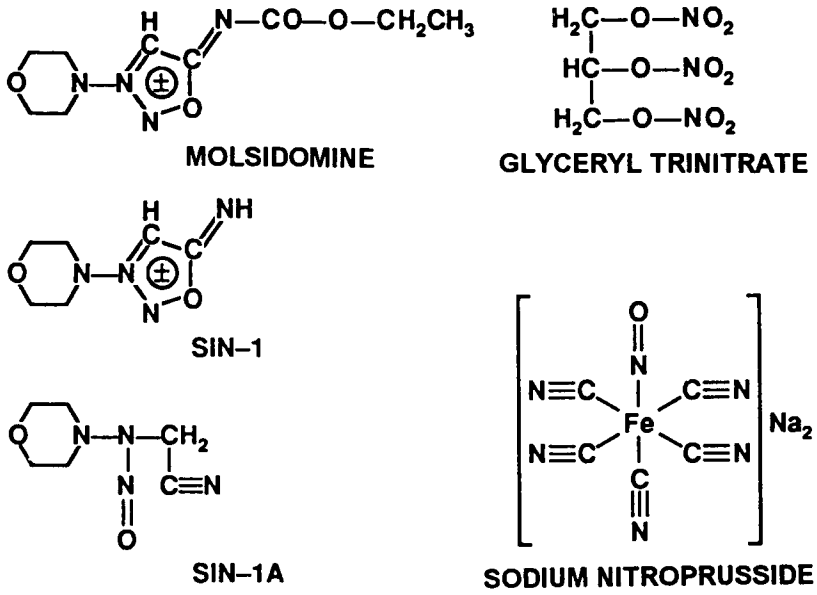

FIG. 1. Chemical structure of different nitrovasodilators. The antianginal agent molsidomine is a meso-ionic compound, and is metabolized enzymatically to 3-morpholinosydnonimine (SIN-1). SIN-1 is unstable in aqueous solution and is converted to *N*-nitroso-*N*-morpholinoaminoacetonitrile (SIN-1A) by pH-dependent, non-enzymatic opening of the oxdiazole ring. SIN-1A and sodium nitroprusside, but not glyceryl trinitrate, spontaneously release nitric oxide in aqueous solution.

cell-free systems (DeRubertis and Craven, 1976; Arnold *et al.*, 1977; Katsuki and Murad, 1977; Schultz *et al.*, 1977; Böhme *et al.*, 1978; Böhme *et al.*, 1984; Feelisch and Noack, 1987). Azide and hydroxylamine, which are known to form and release nitric oxide in the presence of catalase (Craven *et al.*, 1979), have been shown to act similarly (Kimura *et al.*, 1975). Glyceryl trinitrate also increases cyclic GMP levels in vascular smooth muscle between 10- and 130-fold (Axelsson *et al.*, 1979; Kukovetz *et al.*, 1979) but it fails to activate soluble guanylate cyclase in cell-free preparations from vascular smooth muscle (Ignarro and Gruetter, 1980; Ignarro *et al.*, 1981). Obviously, organic nitrates require conversion to nitric oxide by a metabolic process that is lost on homogenization of the tissue.

3.1 THIOL-MEDIATED FORMATION OF NITRIC OXIDE

As early as 1950 it was demonstrated by Heppel and Hilmoe that reduced glutathione undergoes a spontaneous reaction with glyceryl trinitrate and other organic esters of nitric acid to form inorganic nitrite and oxidized

glutathione. A similar reductive hydrolysis of organic nitrates was found to take place in the presence of cysteine or cysteinylglycine (Heppel and Hilmoe, 1950). These and other findings suggesting a thiol-induced biotransformation of organic nitrates (Needleman *et al.*, 1973) prompted Ignarro and colleagues to include cysteine in their guanylate cyclase assay. Under these conditions, glyceryl trinitrate produced a marked activation of guanylate cyclase in cell-free preparations from vascular tissues (Ignarro and Gruetter, 1980; Ignarro *et al.*, 1981). It was proposed that free thiols such as cysteine were necessary co-factors for nitric oxide generation from organic nitrates and that this process involved the formation of *S*-nitrosothiols as intermediates. Cysteine was later shown to release nitric oxide from organic nitrates in a non-enzymatic chemical reaction and in correlation to the observed degree of guanylate cyclase activation (Schröder *et al.*, 1985; Feelisch and Noack, 1987; Schröder and Noack, 1987). The thiol dependence of guanylate cyclase activation by organic nitrates was found to be highly specific for cysteine and structurally related sulfhydryl compounds such as *N*-acetylcysteine and thiosalicylic acid (Schmidt *et al.*, 1985; Schröder *et al.*, 1985). Whereas other thiols release only nitrite during the non-enzymatic reaction with organic nitrates, the three thiols mentioned above were shown to yield both nitrite and nitric oxide (Feelisch *et al.*, 1988).

3.2 CYTOCHROME P450-MEDIATED FORMATION OF NITRIC OXIDE

The generation of nitric oxide upon incubation of organic nitrates with rat liver microsomal fractions was first reported by Wolf and Werringloer (1987, 1988). Servent and co-workers (1989) demonstrated the formation of a cytochrome P450-Fe^{II}-NO complex during microsomal hepatic denitration of glyceryl trinitrate. In the same paper, it was shown that inhibitors of microsomal cytochrome P450 such as carbon monoxide, metyrapone and miconazole lead to a marked attenuation of glyceryl trinitrate denitration. Moreover, this reaction was reported to be dependent on reduced nicotinamide adenine dinucleotide phosphate (NADPH) and sensitive to inhibition by dioxygen (Servent *et al.*, 1989; McDonald and Bennett, 1990) suggesting microsomal denitration of glyceryl trinitrate to be a reductive process according to the following equation (Servent *et al.*, 1989):

$$RO\text{–}NO_2 + P450\text{–}Fe_{(II)} \xrightarrow{NADPH} ROH + P450\text{–}Fe_{(III)}\text{–}NO$$

These findings are consistent with the view that organic nitrates serve as an electron acceptor for cytochrome P450 and may compete directly with dioxygen or carbon monoxide for binding to the haem moiety of cytochrome

P450. A similar reductase activity of cytochrome P450 has been reported for the conversion of nitro compounds to amines (Gillette *et al.*, 1968), the dehalogenation of haloalkanes (Ahr *et al.*, 1982; Nastainczyk *et al.*, 1982) and for other cytochrome P450-catalysed reactions (for review see Archakov and Zhukov, 1989). Interestingly, nitric oxide synthase, the enzyme being responsible for endogenous nitric oxide formation from L-arginine has also been demonstrated to be a haem–thiolate protein and to behave in many respects like a cytochrome P450-dependent enzyme (Bredt *et al.*, 1991; White and Marletta, 1992).

3.3 GLUTATHIONE *S*-TRANSFERASE-MEDIATED FORMATION OF NITRIC OXIDE

The question whether enzymes other than cytochrome P450 may also catalyse nitric oxide formation from organic nitrates was addressed by Wolf and Werringloer (1987, 1988). Using rat liver cytosolic fractions they found that degradation of glyceryl trinitrate by the cytosolic glutathione *S*-transferase resulted in the formation of both nitrite and nitric oxide as metabolites. However, the ratio of nitrite to nitric oxide formed during this reaction (150:1) and the respective K_m value for glyceryl trinitrate (4 mM) were both rather high compared with the cytochrome P450-catalysed reaction (15:1; $K_m < 200\ \mu M$). Thus, metabolism by cytochrome P450, but not that by glutathione *S*-transferase, exhibits a high efficiency for nitric oxide formation at low concentrations of glyceryl trinitrate. Moreover, it has been shown that the glutathione-dependent biotransformation of glyceryl trinitrate is not accompanied by activation of soluble guanylate cyclase in rat liver cytosolic fractions (Schröder, 1992). This finding demonstrates that, although glutathione *S*-transferase may generate nitric oxide from organic nitrates, the amount of nitric oxide formed is not high enough to produce a significant increase in guanylate cyclase activity. Thus, biotransformation by glutathione *S*-transferase, which mainly yields the pharmacologically inactive metabolite nitrite (Oberst and Snyder, 1948; Needleman, 1975; Keen *et al.*, 1976), can be characterized as a pathway for elimination but not bioactivation of organic nitrates.

4 Cytochrome P450 as a Mediator of Nitric Oxide Formation from Organic Nitrates in Intact Cells

According to Ignarro and colleagues, cysteine or related sulfhydryl compounds were the only co-factors required for the non-enzymatic formation of nitric oxide from organic nitrates (Ignarro and Gruetter, 1980; Ignarro *et al.*, 1981). This so-called nitrosothiol hypothesis was considered to hold true not only in cell-free systems but also in intact blood vessels, an assumption

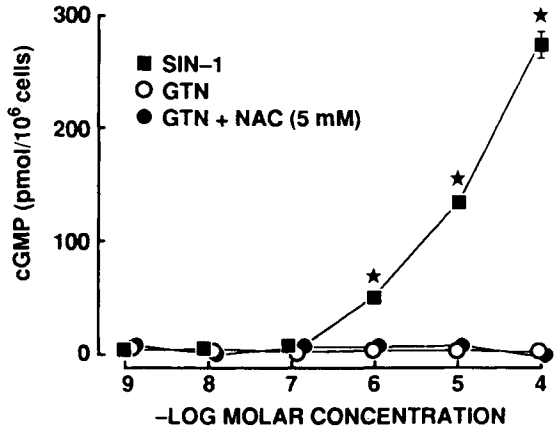

FIG. 2. Cyclic GMP accumulation in porcine aortic endothelial cells in the presence of 3-morpholinosydnonimine (SIN-1), glyceryl trinitrate (GTN), and GTN in combination with 5 mM *N*-acetyl-cysteine (NAC). After adding GTN or SIN-1, incubation was continued for a further 10 min. $^*P < 0.05$ (treatment versus basal cyclic GMP levels (4.9 ± 0.4 pmol per 10^6 cells), two-tailed *t* test). The data are mean ± SEM of six observations. (Methods as in Schröder (1992).)

based on earlier observations by Needleman and colleagues (1973). These authors had found ethacrynic acid, a known thiol alkylating agent, to antagonize glyceryl trinitrate-induced relaxation of rabbit thoracic aortic strips (Needleman *et al.*, 1973). Subsequent studies by Kenkare and Benet (1993) led to similar results. However, the inhibitory effect of ethacrynic acid on vascular relaxation was not specific for organic nitrates but was also observed with isoproterenol, sodium nitroprusside, cyclic adenosine monophosphate, cyclic GMP and other relaxing agents (Needleman *et al.*, 1973; Rapoport and Murad, 1988). Thus, the effect of ethacrynic acid and other thiol-blocking reagents on vascular relaxation (Rapoport and Murad, 1988) may be due to interaction with the contractile apparatus rather than to inhibition of organic nitrate metabolism. Moreover, the nitrosothiol hypothesis was challenged by Gruetter and Lemke (1985) who reported the dissociation of cysteine and glutathione levels from glyceryl trinitrate-induced relaxation in bovine coronary arteries. Using cultured porcine aortic endothelial cells, we found that sodium nitroprusside but not glyceryl trinitrate markedly increases cyclic GMP levels (Schrör *et al.*, 1989; Schröder, 1992). Even in the presence of *N*-acetylcysteine, the cells remained unresponsive to organic nitrates (Schrör *et al.*, 1989; Schröder, 1992), demonstrating that in intact cells nitric oxide formation from organic nitrates is not mediated via free thiols. A similar experiment with the spontaneous nitric oxide donor and molsidomine metabolite SIN-1 is shown in Fig. 2. These and other findings (Schröder *et al.*, 1987, 1988; Brien *et al.*, 1988; Bennett *et al.*, 1989;

TABLE 1

Effect of SKF 525A on cyclic GMP stimulation by glyceryl trinitrate, isoidide dinitrate and sodium nitroprusside in RFL-6 cells

	Cyclic GMP (pmol per 10^6 cells)		
Addition	10 μM GTN	10 μM IIDN	1 μM SNP
None (control)	33.6 ± 1.5	26.9 ± 2.0	37.2 ± 2.3
100 μM SKF 525A	10.4 ± 0.5*	7.3 ± 0.3*	39.0 ± 1.9

Cell culture conditions and incubation procedures as in Schröder *et al.* (1987).
Basal cyclic GMP levels were 1.5 ± 0.1 pmol per 10^6 cells.
*$P < 0.05$ (treatment versus control, two-tailed t test).
Data are mean ± SEM of six observations.

Chung and Fung, 1990; Seth and Fung, 1993) argue for an enzymatic rather than a thiol-dependent mechanism for the cellular bioconversion of organic nitrates to nitric oxide.

That cytochrome P450 is involved in cellular nitric oxide formation from organic nitrates was first suggested by findings demonstrating inhibition of organic nitrate-induced cyclic GMP increase in cultured cells by cytochrome P450 inhibitors such as cimetidine or miconazole (Schröder and Schrör, 1990; Schröder, 1992). The RFL-6 and LLC-PK_1 cells used in these investigations are established model systems to study organic nitrate action. Particularly with respect to the regulation of organic nitrate metabolism and cyclic GMP synthesis, these cell lines display strong similarities to vascular smooth muscle cells (Schröder *et al.*, 1987, 1988; Bennett *et al.*, 1988, 1989). An experiment with the cytochrome P450 inhibitor SKF 525A, proadifen (Mannering, 1971) is shown in Table 1. Cyclic GMP stimulation by glyceryl trinitrate and isoidide dinitrate is reduced to a similar extent by SKF 525A in cultured RFL-6 cells. In contrast, sodium nitroprusside-induced cyclic GMP stimulation remains unaltered under these conditions, precluding a direct inhibitory effect of SKF 525A on activated guanylate cyclase (Table 1). In the same cell type, a 24-h preincubation with 3-methylcholanthrene augmented cyclic GMP stimulation by glyceryl trinitrate by up to 100% (Schröder, 1992), indicating the involvement of an inducible cytochrome P450 system. Other compounds that have been found to induce bioactivation of organic nitrates include phenobarbital, β-naphthoflavone and *Escherichia coli* lipopolysaccharide (Bennett *et al.*, 1992a; Salvemini *et al.*, 1992).

Whether cytochrome P450 actually mediates nitric oxide release from organic nitrates in intact blood vessels has remained unclear since classical P450 inhibitors such as the above-mentioned SKF 525A or cimetidine do not alter vasodilatation by organic nitrates (Bornfeldt and Axelsson, 1987;

Bennett *et al.*, 1992a). However, recent studies have shown that 7-ethoxyresorufin, a cytochrome P450 substrate and inhibitor, as well as diphenyleneiodonium sulfate, an inhibitor of NADPH cytochrome P450 reductase, inhibit the biotransformation and vasodilatory action of organic nitrates in isolated blood vessels (Bennett *et al.*, 1992b; McGuire *et al.*,1994). Also in line with a role for the cytochrome P450 system in mediating vasodilatation by organic nitrates are findings by Pistelli *et al.* (1994), who demonstrated that nitroblue tetrazolium, an inhibitor of NADPH-dependent redox processes, reduces the hypotensive response to glyceryl trinitrate in rats.

Although in cell-free systems, glutathione *S*-transferase has been shown to generate small amounts of nitric oxide from glyceryl trinitrate (Wolf and Werringloer 1987, 1988), the affinity of this pathway for organic nitrates is very low in intact cells and therefore its relevance for the bioactivation of nitrates is questionable (Bennett *et al.*, 1989; Schröder, 1992). Yeates *et al.* (1989) reported that sulfobromophthalein, a known inhibitor of this enzyme (Yalcin *et al.*, 1983), antagonizes the pharmacological action of glyceryl trinitrate. However, this observation was not confirmed in later studies (Lau and Benet, 1992; Schröder, 1992). According to Nigam and colleagues (1993), inhibitors of glutathione *S*-transferase may also decrease the vasodilatory action of organic nitrates by directly inhibiting guanylate cyclase rather than by reducing nitric oxide formation. Moreover, it has been shown that the potency of organic nitrates for relaxation and cyclic GMP stimulation correlates well with their respective degree of biotransformation by cytochrome P450 but not by cysteine or glutathione *S*-transferase (Schröder *et al.*, 1987; Bennett *et al.*, 1989; McDonald and Bennett, 1990). However, at present, it cannot be excluded that, to some extent and under certain physiological conditions, the glutathione *S*-transferase pathway contributes to the metabolic activation of organic nitrates.

5 Unanswered Questions

5.1 IS CYTOCHROME P450 RELEVANT FOR THE ACTION OF ORGANIC NITRATES *IN VIVO*?

Although there is now considerable evidence that cytochrome P450 mediates nitric oxide formation from organic nitrates *in vitro*, the relevance of this pathway *in vivo* has still to be determined. This is particularly important considering the high sensitivity to inhibition by dioxygen. The reductive dinitration of organic nitrates by cytochrome P450 under aerobic conditions *in vitro* is only 20%, and less under anaerobic conditions (Wolf and Werringloer, 1987, 1988; Servent *et al.*, 1989; McDonald and Bennett, 1990). Thus, optimal expression of this pathway *in vivo* should be expected to occur in tissues with low oxygen tension, for instance in the venous system (Kratz and Staudinger,

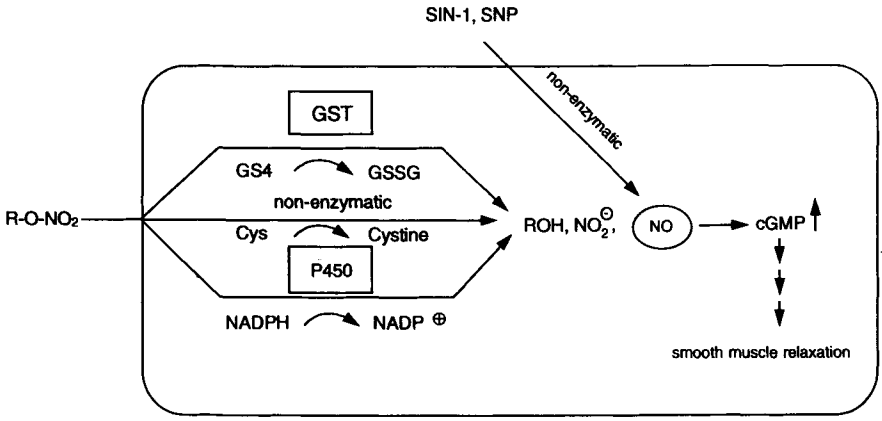

FIG. 3. Schematic diagram of possible mechanisms for nitrovasodilator-induced relaxation of vascular smooth muscle. In cell free systems, nitric oxide (NO) formation from organic nitrates ($RO\text{-}NO_2$) has also been observed in the presence of cysteine (Cys) or glutathione *S*-transferase (GST). Recent evidence, however, suggests that cytochrome P450 plays a key role in cellular bioactivation of organic nitrates. SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside.

1965; Nastainczyk *et al.*, 1977). In fact, venous capacitance vessels are more sensitive to the relaxant effect of organic nitrates than arterial resistance vessels, a phenomenon that is well documented *in vivo* (Imhof *et al.*, 1980; Kober *et al.*, 1981) and *in vitro* (MacKenzie and Parratt, 1977; Edwards *et al.*, 1984). Moreover, it has been suggested that the sensitivity of blood vessels to organic nitrates is related to their nitrate-metabolizing capacity (Fung *et al.*, 1984; Kawamoto *et al.*, 1987). In this context, it is interesting to note that cytochrome P450 has been identified in vascular wall tissues (Juchau *et al.*, 1976; Abraham *et al.*, 1985; Serabjit-Singh *et al.*, 1985), although its isoenzyme characteristics (for review see Guengerich, 1989) and relevance for biotransformation of organic nitrates have yet to be examined in more detail. On the other hand, blood cells such as neutrophils, which in terms of cyclic GMP increase are unresponsive to organic nitrates (Schröder *et al.*, 1990), may lack the required metabolizing system, i.e. cytochrome P450. Clearly, more research is needed, but to date most observations made in this field are well in line with the assumption that one or more than one cytochrome P450 enzyme also plays a role in the action of organic nitrates *in vivo* (Fig. 3).

5.2 IS CYTOCHROME P450 INVOLVED IN NITRATE TOLERANCE?

Prolonged exposure to organic nitrates is known to induce tolerance to the cardiovascular effects of these drugs in humans and experimental animals

(Crandall *et al.*, 1931; Schelling and Lasagna, 1967; Bogaert and De Schaepdryver, 1968). This desensitization phenomenon still poses an unsolved clinical problem. Presently, two mechanisms underlying tolerance development to organic nitrates are being discussed: (1) neurohormonal activation, e.g. activation of endogenous vasoconstrictor mechanisms limiting the drug's circulatory effects; and (2) reduced bioactivation, i.e. transformation into the active metabolite, nitric oxide. Neurohormonal activation as a physiological negative feedback response to nitrate therapy has mainly been characterized as an increase in the activity of the renin–angiotensin system resulting in increased sodium levels and water retention. Although there is a close time correlation between neurohormonal activation and the loss of nitroglycerin's haemodynamic efficiency (Packer, 1990), a causal relationship between these two events has not yet been established. Thus, the angiotensin converting enzyme inhibitor captopril failed to prevent nitrate tolerance in a study of patients suffering from congestive heart failure secondary to coronary artery disease (Dakak *et al.*, 1990). Moreover, an increase in plasma renin activity and water retention is an unspecific counter-regulatory mechanism observed during therapy with most vasodilatory and antihypertensive drugs, e.g. calcium channel blockers, diuretics or reserpine. However, despite neurohormonal activation, these drugs are not known to lose their pharmacological efficacy in a fashion comparable to nitrate tolerance. Obviously, nitrate tolerance is a substance-specific phenomenon, for even within the group of nitric oxide donors desensitization is confined to nitric acid esters such as glyceryl trinitrate or isosorbide dinitrate. Thus, it has been demonstrated that the vascular effects of the nitric oxide donor and molsidomine metabolite SIN-1 are not diminished by tolerance and that nitrate-tolerant patients are still responsive to molsidomine (Mülsch *et al.*, 1989; Störk *et al.*, 1993). Interestingly, neurohormonal activation has also been observed during the therapy with molsidomine/SIN-1, underlining the notion that neurohormonal activation is not necessarily followed by tolerance (Unger *et al.*, 1991).

There is now substantial evidence that nitrate tolerance is caused by a decrease in biotransformation of organic nitrates (Brien *et al.*, 1986; Bennett *et al.*, 1989) and subsequent nitric oxide formation (Förster *et al.*, 1991) resulting in the reduced cyclic GMP response in vascular tissue described previously (Wikberg *et al.*, 1978; Keith *et al.*, 1982; Rapoport *et al.*, 1987). That tolerance is specific for glyceryl trinitrate and derivatives can be explained by their bioactivation via cytochrome P450, whereas spontaneous nitric oxide donors such as SIN-1 or sodium nitroprusside do not require enzymatic transformation into nitric oxide (Feelisch *et al.*, 1988, 1989; Schröder, 1994). Presumably, due to downregulation or irreversible inhibition of cytochrome P450, the biotransformation and bioactivation of organic nitrates is inhibited in tolerant vascular tissue. By contrast, nitric oxide release from tolerance-free spontaneous nitric oxide donors remains unaltered under these conditions (Brien *et al.*, 1986; Bennett *et al.*, 1989; Förster *et al.*, 1991).

In a previous study we found that the reversal of nitrate tolerance in intact cells requires *de novo* synthesis of proteins (Schröder *et al.*, 1988). These results may now be reinterpreted as evidence for a tolerance-induced downregulation or irreversible inactivation of an enzyme responsible for organic nitrate bioactivation such as cytochrome P450. In addition to downregulation, other mechanisms leading to a decrease in enzymatic activity are also conceivable, e.g. reversible inhibition of cytochrome P450 by product or substrate (Kühn-Velten *et al.*, 1990; for reviews see Whitlock, 1986; Guengerich, 1989).

According to the nitrosothiol concept, it was previously suggested that nitrate tolerance is due to intracellular depletion or oxidation of cysteine and other thiols (Needleman and Johnson, 1973; for review see Noack, 1990). However, the results of early attempts to reverse tolerance with different thiol-containing compounds were contradictory (Axelsson *et al.*, 1982; Gruetter and Lemke, 1985; Torresi *et al.*, 1985). Recently, it has become clear that nitrate tolerance cannot be reversed by cysteine or related compounds and that the apparent restoration of nitrate responsiveness observed in earlier studies can be attributed to tolerance-independent interactions between cysteine and organic nitrates (Schröder *et al.*, 1988; Münzel *et al.*, 1990; Salvemini *et al.*, 1993; Boesgaard *et al.*, 1994).

6 Outlook

Although, today, cytochrome P450 has emerged as a possible site for the induction of nitrate tolerance it is at this point questionable whether this pathway will open up new opportunities to prevent or reverse tolerance in patients. Inducers of cytochrome P450 that have been shown to enhance the pharmacological action of nitrates *in vitro* are not suitable for combined therapy with glyceryl trinitrate, e.g. the carcinogen 3-methylcholanthrene or phenobarbital (Bennett *et al.*, 1992a; Schröder, 1992). At present the most popular and successful approach to the prevention of tolerance is an intermittent or on–off dosing strategy with oral as well as transdermal nitrate preparations. This is based on the observation that a nitrate-free period of a few hours in each 24-h period is beneficial in avoiding tolerance and maintaining the effectiveness of treatment (Abrams, 1992). However, during the nitrate-free interval the patient is without angina protection and β-adrenergic blockers or calcium channel blocking agents may not always be the appropriate substitute. Therefore, newly developed nitric oxide donors such as iron–sulfur cluster nitrosyls (Flitney *et al.*, 1992) are designed to prevent tolerance by spontaneous, yet controlled, nitric oxide release. Also, with the discovery of endogenous nitric oxide formation new concepts of nitric oxide delivery to vascular smooth muscle cells have become conceivable. Thus, it has recently been shown that it is possible to restore nitric oxide production

and inhibit intimal hyperplasia in endothelium-denuded rat carotid arteries by *in vivo* transfer of the endothelial cell nitric oxide synthase gene (Von der Leyen *et al.*, 1995). This finding suggests nitric oxide synthase to be a potential target for gene therapy in disease states such as atherosclerosis or restenosis following balloon angioplasty. However, for patients with angina or ischaemic heart disease, vasodilatation by exogenous nitric oxide, i.e. nitric oxide donors, has been and will remain a very useful and efficient therapeutic approach.

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The Vitamin D Endocrine System and its Therapeutic Potential

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LIST OF ABBREVIATIONS

$1,25\text{-}(OH)_2D_3$	1,25-Dihydroxyvitamin D_3
$25\text{-}OH\text{-}D_3$	25-Hydroxyvitamin D_3
cDNA	Complementary DNA
mRNA	Messenger RNA
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAF	Nuclear accessory factor
RXR	Retinoid X receptor
VDR	Vitamin D receptor
VDRE	Vitamin D response element

1 Historical Introduction

The disease rickets has been known since antiquity and was first thoroughly described by Whistler in 1645 (Hess, 1929). Undoubtedly, the disease appeared periodically in the human population but did not reach epidemic proportions until the turn of the century at the time of the industrial revolution (Hess, 1929). The urbanization of the population together with industrial smoke prevented exposure of skin to ultraviolet light, resulting in an absence of vitamin D. As a result, in northern Europe, especially the UK, northern Asia and in North America, rickets appeared in epidemic proportions. At this time, the concept of accessory food factors began to make its appearance (Funk, 1911). This led McCollum, at the Wisconsin Experiment Station, to demonstrate very clearly the presence of a lipid-soluble material in the diet essential for growth and reproduction which he termed "fat-soluble A" (later named vitamin A) (McCollum and Davis, 1913; McCollum *et al.*, 1916). Similarly, in the milk salts and whey, another factor termed water-soluble B (later named vitamin B), which prevented the appearance of polyneuritis in birds, was identified by McCollum and his colleagues (1916) and by Osborne and Mendel (1917). This led Sir Edward Mellanby (1919) of Great Britain to consider that rickets might also be a deficiency disease of some dietary component. He, therefore, fed dogs a diet which approximated that being consumed in England and Scotland where the incidence of rickets was highest. Unknowingly, he maintained the dogs indoors which prevented their exposure to ultraviolet light. These dogs developed severe rickets, and Mellanby found that the disease could be cured by cod liver oil, which contains the fat-soluble vitamin A described by McCollum. Mellanby concluded that the healing of rickets might be another property of vitamin A. McCollum, on the other hand, demonstrated that he could destroy the vitamin A activity in cod liver oil but still retain the ability to heal rickets. He, therefore, concluded that he had discovered a new fat-soluble vitamin called "vitamin D" (McCollum *et al.*, 1922). Meanwhile, Huldshinsky (1919) in Vienna found that he could cure rickets in children by simply exposing

them to sunlight or ultraviolet light. Steenbock and Hart (1913) at Wisconsin, who had been working with goats, found that exposure of goats to sunlight led to positive calcium balance. Steenbock conceived that sunlight or ultraviolet light might activate a factor in ordinary food and skin that might become vitamin D, and then demonstrated that ultraviolet irradiation of foods and animals could eliminate the disease rickets (Steenbock and Black, 1924). This led to the elimination of rickets as a major medical problem and set the stage for identification of a substance in the non-saponifiable fraction of diet which upon irradiation becomes vitamin D. Vitamin D_2 was isolated from irradiated plant sterols and identified by Askew and colleagues (1931) and vitamin D_3 was identified by Windaus *et al.* (1936). This vitamin therefore became known as the vitamin responsible for the prevention of bony diseases such as rickets and osteomalacia (Hess, 1929). The discovery and identification of the structure of vitamin D led to its rapid application not only in the healing of rickets but in its use in the treatment of many calcium-related diseases.

Soon after the discovery of vitamin D, the physiological actions of vitamin D in preventing bony diseases were being investigated. As early as 1923, Orr and colleagues suggested that vitamin D stimulates intestinal absorption of calcium by observing that rachitic infants fail to retain calcium and phosphorus. However, it was Nicolaysen (1937) who demonstrated that *in vivo* vitamin D accelerates intestinal absorption of calcium. Schachter and Rosen (1959) provided direct evidence that intestinal calcium transport is markedly stimulated by vitamin D by an *in vitro* demonstration using everted intestine from experimental animals. In 1955, Carlsson and colleagues provided evidence that vitamin D not only functions to facilitate the absorption of calcium but is also responsible for mobilizing calcium from bone when required (Bauer *et al.*, 1955).

Kodicek (1963), after a decade of investigating the metabolism of vitamin D, concluded that it is active directly, without metabolic alteration. However, DeLuca and colleagues clearly demonstrated by using radiolabelled vitamin D_3 of high specific activity that vitamin D_3 is rapidly converted *in vivo* to two or more biologically active metabolites (Lund and DeLuca, 1966). In 1968, the major circulating form of vitamin D_3 was isolated and identified as 25-hydroxyvitamin D_3 (25-OH-D_3) (Blunt *et al.*, 1968) and in 1971 the metabolically active form of vitamin D_3 was identified as 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (Holick *et al.*, 1971). These compounds were then chemically synthesized and made available to the medical world for use in the treatment of disease (Blunt and DeLuca, 1969; Semmler *et al.*, 1972).

This chapter is devoted to describing the vitamin D endocrine system, how the active hormonal form of vitamin D works, and the potential for treatment of a variety of diseases, ranging from metabolic bone diseases to such diverse disorders as psoriasis and cancer.

2 Biosynthesis of Vitamin D

There are two forms of vitamin D considered to be nutritional forms. One of these is normally produced in skin as a result of ultraviolet irradiation. These two compounds, vitamin D_2 and vitamin D_3, are illustrated in Fig. 1. Vitamin D_1 does not exist and was misidentified by Windaus and Linsert (1928). It turned out to be a mixture of vitamin D_2 and an irradiation product, tachysterol$_2$. Vitamin D_2 is the product of ultraviolet irradiation of the plant sterol, ergosterol (DeLuca *et al.*, 1967), while vitamin D_3 is the product of ultraviolet irradiation of 7-dehydrocholesterol in the skin of animals (Windaus *et al.*, 1936; Windaus and Bock, 1937; Esvelt *et al.*, 1978). For many years, irradiated ergosterol or vitamin D_2 was the predominant form of vitamin D used for the treatment of disease and for supplementation of diet for the prevention of rickets. However, with the development of improved methods for synthesizing 7-dehydrocholesterol, vitamin D_3 ultimately replaced vitamin D_2 in most circumstances.

The food supply is largely devoid of vitamin D except for unusual dietary items such as fish liver oils (Scott, 1971; DeLuca, 1988a). It is believed that the accumulation of vitamin D in fish livers results from a food chain beginning with plankton and ending in large fish such as tuna. Plankton and organisms found on the surface of the sea when irradiated with ultraviolet light are expected to have small amounts of vitamins D_2 and D_3. It has been reported that sea animals, especially fish, do not metabolize vitamin D and hence it accumulates in the liver of fish (Oizumi and Monder, 1972). Enzymatic production of vitamin D_3 in fish livers has also been suggested (Blondin *et al.*, 1967). Thus, the origin of the high concentration of vitamin D in some fish livers remains uncertain. However, it is of great historical significance that cod liver oil and other fish liver oils are often used as therapeutic agents because of their high content of vitamin D (Kramer and Knof, 1954). Certainly, until 1925 cod liver oil was the only known treatment for rickets.

In this chapter, some reference will be made to vitamin D_2 but the discussion will largely centre around vitamin D_3. For all practical purposes, as least in humans, vitamins D_2 and D_3 are handled similarly and have almost identical biological activity (DeLuca, 1988a).

Vitamin D_3 under natural circumstances is not a dietary substance but is normally manufactured in skin and hence must be considered a prohormone rather than a vitamin (DeLuca, 1974). The reaction that takes place in skin is illustrated in Fig. 2. 7-Dehydrocholesterol, which is found in surprisingly abundant quantities in skin (Kandutsch, 1964), absorbs ultraviolet light of between 282 and 315 nm wavelength. This results in a photolysis followed by a 1,7-sigmatropic shift, producing a compound called "previtamin D" (Velluz and Amiard, 1949). Previtamin D itself remains in skin because the vitamin D binding protein does not recognize this form of vitamin D (Holick

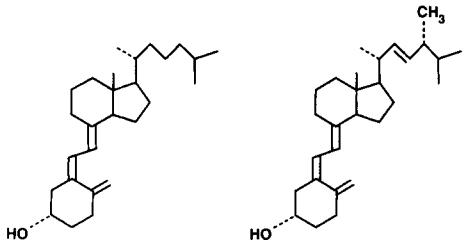
and Clark, 1978). Subsequently, previtamin D equilibrates to vitamin D_3, which is bound by the vitamin D binding protein and carried into the circulation. At body temperatures, achievement of equilibrium requires approximately 24–36 h and is largely in favour of vitamin D_3 (Velluz *et al.*, 1949). Over-irradiation results in biologically inert products such as tachysterol$_3$ and lumisterol$_3$ (Holick *et al.*, 1981). This reaction in skin is believed to be largely chemical since the reaction products found in skin are similar to those produced when the irradiation is carried out in organic solvents (Holick *et al.*, 1980).

The reaction of ultraviolet light on skin is very efficient in the production of vitamin D_3 (Holick, 1985). Ten minutes of summer sun on hands and face are reputed to be sufficient to produce the daily vitamin D requirement of 10 μg. Pigmented skin is fully able to carry out this conversion; however, the type of pigmentation affects the time required for maximum previtamin D_3 formation (Holick *et al.*, 1981). Sun screen prevents the cutaneous production of vitamin D_3, and thus could result in a deficiency of vitamin D_3 (Holick, 1987). It has been theorized that the races evolved to prevent vitamin D_3 toxicity or to permit adequate synthesis of vitamin D_3 (Loomis, 1967). That is, humans found at the equator required an increase in skin pigmentation, whereas those found north and south of the equator required hypopigmentation. Contrary to this hypothesis, vitamin D_3 intoxication has never been reported to result from prolonged sun exposure. A great deal of information is known concerning the skin system for producing vitamin D and interested readers are referred to other reviews (Holick, 1985, 1987, 1991).

3 Metabolism of Vitamin D

3.1 HISTORICAL CONSIDERATIONS

In attempting to learn how vitamin D functions, Kodicek and his colleagues were the first to use bioassays and low-specific activity radioactive vitamin D compounds for studying metabolism (Kodicek, 1963). Following more than a decade of investigation, Kodicek and co-workers concluded that vitamin D_3 was active as such without further metabolism, a conclusion also reached by Haussler and Norman (1967). However, when a physiological concentration of radioactive vitamin D_3 of high specific activity was administered to vitamin D-deficient animals, the vitamin D_3 largely disappeared and instead the radiolabel appeared in products much more polar than vitamin D_3 (Lund and DeLuca, 1966). The most abundant radiolabelled product when assayed biologically proved to be more active than vitamin D_3 itself and appeared to act more rapidly (Morii *et al.*, 1967). This led to the isolation and identification of this product from the plasma of pigs given large amounts of vitamin D_3. It was identified as 25-OH-D_3 by both chemical identification



vitamin D_3
cholecalciferol
calciol

vitamin D_2
ergocalciferol
ercalciol

FIG. 1. The nutritionally significant forms of vitamin D.

(Blunt *et al.*, 1968) and chemical synthesis (Blunt and DeLuca, 1969). However, this compound is also very rapidly metabolized to several more polar metabolites (Cousins *et al.*, 1970). One of these metabolites appeared to lose tritium from carbon-1 of vitamin D_3 (Lawson *et al.*, 1969) and in 1971 this metabolite was isolated from intestine and chemically identified as 1,25-$(OH)_2D_3$ (Holick *et al.*, 1971). Chemical synthesis of 1,25-$(OH)_2D_3$ provided the ultimate proof that the 1-hydroxyl is in the α-position and that the active form of vitamin D_3 is 1α,25-$(OH)_2D_3$ (Semmler *et al.*, 1972; Paaren *et al.*, 1977). The 1β,25-$(OH)_2D_3$ proved to be largely biologically inactive (Paaren *et al.*, 1977). As a result of this work, the first important vitamin D_3 analogue, 1α-OH-D_3, was prepared as an exercise in learning how to insert the 1-hydroxyl into the A-ring chemically (Holick *et al.*, 1973). As described below, this compound was to assume major importance as a therapeutic agent in Europe and Japan.

3.2 METABOLISM REQUIRED FOR FUNCTION

The pathway of metabolism of vitamin D_3 required for function is illustrated in Fig. 3. 25-Hydroxylation of vitamin D_3 was shown to be largely but not exclusively carried out in the liver (Horsting and DeLuca, 1969; Ponchon and DeLuca, 1969; Ponchon *et al.*, 1969; Olson *et al.*, 1976). In contrast, 1-hydroxylation of 25-OH-D_3 takes place in the proximal convoluted tubule cells of the kidney (Fraser and Kodicek, 1970; Gray *et al.*, 1971; Brunette *et al.*, 1978; Kawashima *et al.*, 1981; Reeve *et al.*, 1983; Shultz *et al.*, 1983).

25-Hydroxylation is carried out by two enzymes in the rat, one found in the microsomes and the other in the mitochondria of hepatic cells (Björkhem and Holmberg, 1979; Madhok and DeLuca, 1979). Microsomal vitamin D_3

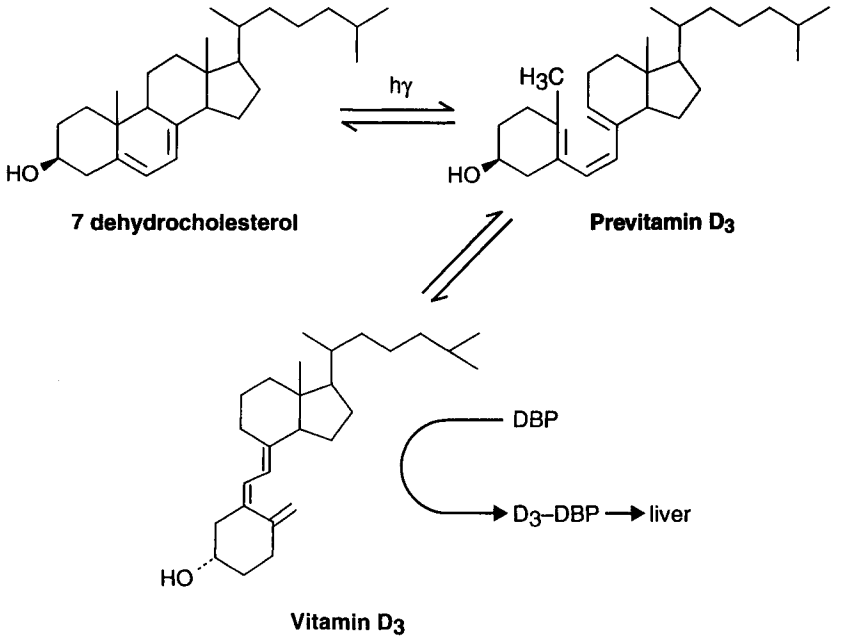


FIG. 2. Photochemical conversion of 7-dehydrocholesterol to previtamin D_3. Previtamin D_3 in turn equilibrates to vitamin D_3. Vitamin D binding protein (DBP) binds vitamin D_3 but not previtamin D_3.

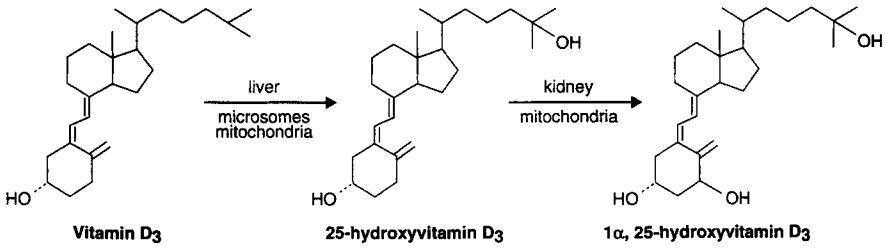


FIG. 3. The pathway of metabolism of vitamin D_3 required for function.

25-hydroxylase activity is lower in females than in males and distinct from the male enzyme (Dahlbäck and Wikvall, 1987; Hayashi *et al.*, 1988), whereas mitochondrial vitamin D_3 25-hydroxylase is indistinguishable between male and female. However, in human liver, vitamin D_3 25-hydroxylase activity exists only in the mitochondrial fraction (Saarem *et al.*, 1984; Saarem and Pedersen, 1985). These results suggest that the mitochondrial 25-hydroxylase must play an important role in the metabolism of vitamin D_3, at least in some species.

The vitamin D_3 25-hydroxylases have been further isolated and characterized. In 1990, a rat liver mitochondrial vitamin D_3 25-hydroxylase complementary DNA (cDNA) was isolated from a male rat liver λgt11 expression library (Usui *et al.*, 1990a) and a female rat liver λZAP expression library (Su *et al.*, 1990). The cDNA sequence contained a cytochrome P450 haem consensus sequence and was 73% similar to a cytochrome P450 isolated from rabbit liver mitochondria catalysing 5β-cholestane-3α,7α,12α-triol 27-hydroxylation (Su *et al.*, 1990; Usui *et al.*, 1990a). The rabbit liver sterol 27-hydroxylase cDNA was used as a probe to clone a human homologue (Cali and Russell, 1991). Subsequently, the possibility that one enzyme might have two different catalytic activities was tested by expressing both rat and human cDNAs and demonstrating that the expressed enzymes have both 5β-cholestane-3α,7α,12α-triol 27-hydroxylase and vitamin D_3 25-hydroxylase activities (Su *et al.*, 1990; Usui *et al.*, 1990b; Akiyoshi-Shibata *et al.*, 1991; Guo *et al.*, 1993). The microsomal vitamin D_3 25-hydroxylase, however, has not been cloned. This enzyme has been purified to homogeneity from male rat liver, and is a cytochrome P450 enzyme requiring reduced nicotinamide adenine dinucleotide phosphate (NADPH) and NADPH–cytochrome P450 reductase for catalytic activity (Hayashi *et al.*, 1986).

The 25-OH-D_3 is transported on the plasma transport protein that is responsible for transport of virtually all vitamin D metabolites. This is the same protein as the group-specific protein and has been studied by Van Baelen and Bouillon (Bouillon *et al.*, 1976). The vitamin D_3 26,23-lactone is the most tightly bound vitamin D_3 metabolite, followed by 25-OH-D_3 and 1,25-$(OH)_2D_3$ (Wichmann *et al.*, 1979). This 56-kDa protein is present in large excess. As a result, more than 99% of the vitamin D_3 metabolites are bound to the vitamin D binding protein (Bouillon and Van Baelen, 1981). Interested readers are directed elsewhere for details concerning this protein (Van Baelen *et al.*, 1988).

In normal animals and humans, 25-OH-D_3 is 1-hydroxylated exclusively in the proximal convoluted tubule cells of the kidney (Fraser and Kodicek, 1970; Gray *et al.*, 1971; Brunette *et al.*, 1978; Shephard *et al.*, 1979; Kawashima *et al.*, 1981; Reeve *et al.*, 1983; Shultz *et al.*, 1983). The sole site of 1-hydroxylation in normal animals is the kidney. This was conclusively demonstrated by administering 25-OH-D_3 of the highest available specific activity to vitamin D-deficient anephric rats and examining blood, intestine and bone for the presence of 1,25-$(OH)_2D_3$ (Reeve *et al.*, 1983; Shultz *et al.*, 1983). Thus, *in vivo* in normal animals, production of 1,25-$(OH)_2D_3$ occurs solely in kidney, and the reports of production of this important form of vitamin D_3 by non-renal tissue were most likely not physiologically relevant. The only exception to this is the placenta in which production of 1,25-$(OH)_2D_3$ *in vivo* (Gray *et al.*, 1979) and *in vitro* (Tanaka *et al.*, 1979) has been demonstrated. Also, in the disease sarcoidosis and certain types of lymphomas, ectopic production of 1,25-$(OH)_2D_3$ occurs and is probably

responsible for the hypercalcaemia found in these disease states (Barbour *et al.*, 1981; Mudde *et al.*, 1987). At least in sarcoidosis, 1,25-$(OH)_2D_3$ production probably takes place in activated macrophages (Adams *et al.*, 1983, 1994).

The enzyme involved in 1α-hydroxylation of 25-OH-D_3 is a cytochrome P450 that requires molecular oxygen, NADPH, ferredoxin and ferredoxin reductase for activity (Ghazarian *et al.*, 1973, 1974; Pedersen *et al.*, 1976). The known properties of the 25-OH-D_3 1α-hydroxylase rely on experiments performed with intact and cholate solubilized mitochondria, as the 25-OH-D_3 1α-hydroxylase has not been purified in sufficient quantity. Since 1974, when the 25-OH-D_3 1α-hydroxylase from chicken was initially solubilized (Ghazarian *et al.*, 1974), numerous investigators have attempted to purify the 25-OH-D_3 1α-hydroxylase (Hiwatashi *et al.*, 1982; Gray and Ghazarian, 1989; Mandel *et al.*, 1990). Limited success is most likely the result of the low abundance of this enzyme and most certainly due to the lability of the 25-OH-D_3 1α-hydroxylase to removal from its natural environment.

1-Hydroxylation is a tightly regulated step that responds to physiological needs for calcium and phosphorus. 25-OH-D_3 1α-hydroxylase activity is induced by hypocalcaemia which acts through parathyroid glands and parathyroid hormone (Boyle *et al.*, 1971; Garabedian *et al.*, 1972; Hughes *et al.*, 1975; Tanaka and DeLuca, 1984). Both cyclic adenosine monophosphate (cAMP) (Horiuchi *et al.*, 1977) and protein kinase C (Janulis *et al.*, 1993), which are second messengers for parathyroid hormone in the kidney, have been implicated as regulators of 25-OH-D_3 1α-hydroxylase activity. 25-OH-D_3 1α-hydroxylase activity is also induced by hypophosphataemia (Tanaka and DeLuca, 1973, 1984). This induction occurs by a mechanism that is independent of parathyroid hormone (Tanaka and DeLuca, 1973). The product of the 1-hydroxylation reaction, 1,25-$(OH)_2D_3$, suppresses 25-OH-D_3 1α-hydroxylase activity (Tanaka and DeLuca, 1974, 1984). Thus, the intricate regulation of the 25-OH-D_3 1α-hydroxylase provides a mechanism for producing 1,25-$(OH)_2D_3$ when calcium and phosphorus are needed.

In anephric animals, 1,25-$(OH)_2D_3$ is capable of carrying out its function effectively, whereas 25-OH-D_3 has no effect on target organs (Boyle *et al.*, 1972). Since 1,25-$(OH)_2D_3$ and not 25-OH-D_3 is required to produce a physiological response in anephric animals, it is clear that 1,25-$(OH)_2D_3$ or a metabolite of 1,25-$(OH)_2D_3$ is the active form of vitamin D. Since no metabolites have been identified that either exceed or even approach 1,25-$(OH)_2D_3$ in its biological activity, the available evidence suggests that 1,25-$(OH)_2D_3$ is the metabolically active form of vitamin D. For a review of this subject, interested readers are referred to Brommage and DeLuca (1985). Furthermore, the existence of vitamin D-dependent rickets type 1, as discovered by Prader and others (1961), illustrates that physiological amounts of vitamin D_3 or 25-OH-D_3 are unable to cure rickets in affected

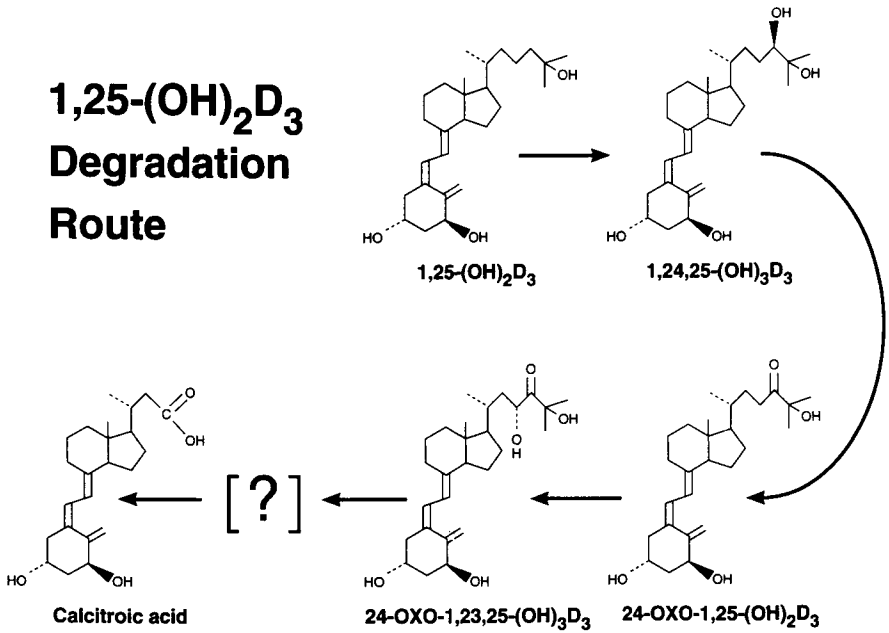


FIG. 4. Metabolism of 1,25-$(OH)_2D_3$ to its excretion product, calcitroic acid.

children, whereas 1,25-$(OH)_2D_3$ given in physiological amounts can heal that disease (Fraser *et al.*, 1973). These results demonstrate clearly that the activity of vitamin D_3 in humans is dependent upon the two-step hydroxylation process to produce the final active hormone, 1,25-$(OH)_2D_3$.

3.3 METABOLISM NOT REQUIRED FOR FUNCTION

Many metabolites of vitamin D_3 have been isolated from plasma and identified, i.e. 25,26-dihydroxyvitamin D_3 (Suda *et al.*, 1970), 24,25-dihydroxyvitamin D_3 (Holick *et al.*, 1972), 1,24,25-trihydroxyvitamin D_3 (Holick *et al.*, 1973), 23,25-dihydroxyvitamin D_3 (Tanaka *et al.*, 1981), and 25-hydroxy-26*R*,23*S*-lactone (Wichmann *et al.*, 1979). The only known excretory form of 1,25-$(OH)_2D_3$ is calcitroic acid (Esvelt *et al.*, 1979; Onisko *et al.*, 1980). The pathway of metabolism of 1,25-$(OH)_2D_3$ to its excretion product, calcitroic acid, is illustrated in Fig. 4. A number of investigators have contributed to the evolution of this pathway and interested readers are referred to the papers elucidating this path of metabolism (Mayer *et al.*, 1983; Reddy *et al.*, 1987; Makin *et al.*, 1989; Reddy and Tserng, 1989).

Of the enzymes involved in the metabolism of vitamin D, the most is known about the 24-hydroxylase that catalyses 24-hydroxylation of both 25-OH-D_3 and 1,25-$(OH)_2D_3$ (Burgos-Trinidad and DeLuca, 1991; Ohyama and

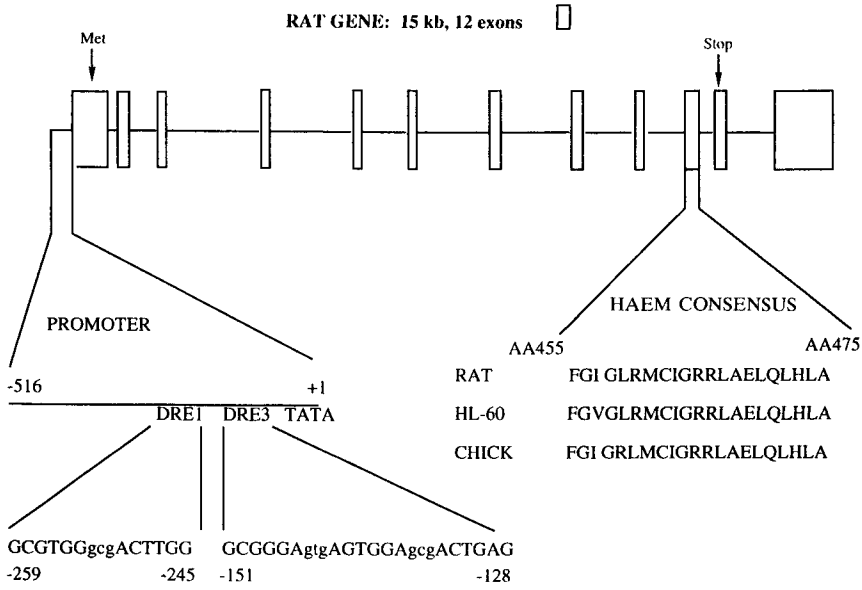


FIG. 5. The gene structure of 25-OH-D_3-24-hydroxylase from rats. Note the vitamin D response elements in the promoter and the haem consensus sequence in exon 10.

Okuda, 1991). The 24-hydroxylase, like the 1-hydroxylase, is a mitochondrial cytochrome P450 enzyme which requires molecular oxygen, NADPH, ferredoxin and ferredoxin reductase for it to catalyse 24-hydroxylation of its substrates (Knutson and DeLuca, 1974; Madhok *et al.*, 1977; Ohyama *et al.*, 1989). 24-Hydroxylation has been described in many target tissues of the vitamin D hormone, including intestine (Kumar *et al.*, 1978), kidney (Omdahl *et al.*, 1972), bone cells (Howard *et al.*, 1981), human promyelocytic leukaemia HL-60 cells (Inaba *et al.*, 1991), cartilage (Garabedian *et al.*, 1978), fibroblast (Gamblin *et al.*, 1985), placenta cells (Weisman *et al.*, 1979), chorioallantoic membrane (Puzas *et al.*, 1980) and yolk sac (Danan *et al.*, 1982).

The intense efforts of Okuda and colleagues resulted in the isolation to homogeneity of the rat kidney 24-hydroxylase (Ohyama *et al.*, 1989). This effort allowed the generation of polyclonal antibodies that were used to clone this enzyme from a λgt11 and λZAP rat kidney cDNA libraries (Ohyama *et al.*, 1991). The rat kidney 24-hydroxylase cDNA was used to isolate a genomic DNA clone and the entire primary structure of the kidney 24-hydroxylase is now known and illustrated in Fig. 5 (Ohyama *et al.*, 1993). As will be discussed in the following section, the promoter region has yielded a very powerful vitamin D responsive element (VDRE) (Zierold *et al.*, 1995).

The 24-hydroxylase VDRE permits 1,25-$(OH)_2D_3$ to induce transcription of the 24-hydroxylase, thus stimulating degradation of the vitamin D hormone.

4 Mechanism of Action of 1,25-$(OH)_2D_3$

4.1 DISCOVERY OF THE 1,25-$(OH)_2D_3$ NUCLEAR RECEPTOR

Following radiochemical synthesis of 1,25-$(OH)_2D_3$ of high specific activity, it became possible to determine cellular and subcellular localization of radiolabel before the initiation of target organ responses. Within 2 h following intravenous injection of a physiological dose of high specific activity 1,25-$(OH)_2D_3$, the radiolabel was found to be localized in the nuclei of proven target organs such as the enterocyte of the small intestine, the crypt cells of the small intestine, osteoblasts and distal renal tubule cells of the kidney (Stumpf *et al.*, 1979, 1981). This localization was diminished or abolished by unlabelled 1,25-$(OH)_2D_3$, indicating specificity of localization. Since 1,25-$(OH)_2D_3$ is a steroid and localizes specifically in the nucleus, the presence of a nuclear receptor was anticipated. Brumbaugh and Haussler (1973) first demonstrated the existence of a protein that specifically binds 1,25-$(OH)_2D_3$. Similarly, Kream and colleagues (Kream *et al.*, 1976; Kream and DeLuca, 1977) provided further evidence for the existence of the receptor protein. Since that time, numerous reports of the existence of the vitamin D receptor (VDR) have appeared in both target and not previously appreciated target organs of vitamin D action (Walters, 1981; Dokoh *et al.*, 1983; Merke *et al.*, 1983; Sandgren *et al.*, 1991). The VDR, unlike the oestrogen and glucocorticoid receptors, is found to be localized in the nuclei with little or no VDR in the cytoplasm (Nakada *et al.*, 1984; Clemens *et al.*, 1988). The molecular weight of the VDR varies between species and is in the 52–60 kDa range (Dame *et al.*, 1985; Mangelsdorf *et al.*, 1987; Pike *et al.*, 1987). The K_d for 1,25-$(OH)_2D_3$ is 10^{-10} to 10^{-11} M in crude preparations (Link and DeLuca, 1985).

4.2 CLONING OF THE VITAMIN D RECEPTOR

Two groups attempted purification of this protein of extremely low abundance, with only limited success (Pike and Haussler, 1979; Simpson *et al.*, 1983). Often impure preparations resulted following a multiple-step procedure or only small amounts of apparently homogeneous VDR were isolated. The quantity or purity of the receptor isolated by these procedures was insufficient for biochemical characterization and thus a different strategy was

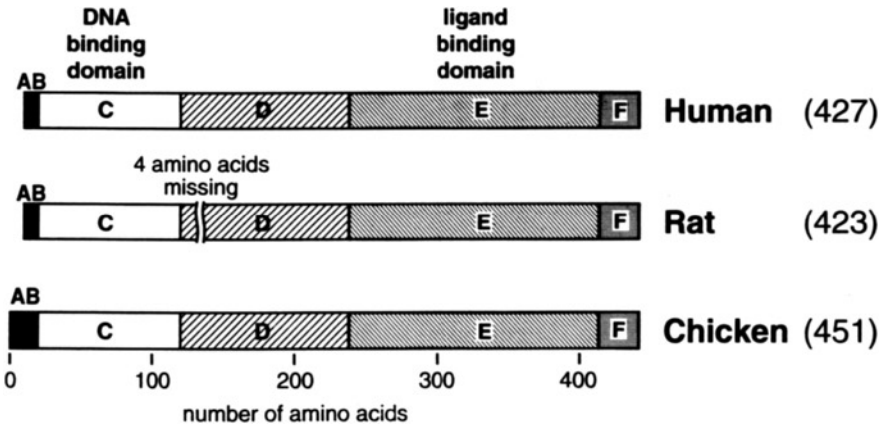


FIG. 6. Structure of the human, rat and chicken vitamin D receptors.

required to determine the primary structure of the VDR. With the partially purified preparations, two groups prepared monoclonal antibodies directed against the VDR (Pike *et al.*, 1983; Dame *et al.*, 1986). These monoclonal antibodies were utilized to clone the VDR from λgt11 expression libraries (McDonnell *et al.*, 1987; Burmester *et al.*, 1988a). Thus, the structure of the VDR was deduced for rat (Burmester *et al.*, 1988b) and human (Baker *et al.*, 1988). The human VDR has a sequence of 427 amino acids, while the rat VDR has a sequence of 423 amino acids. The avian VDR has just recently been cloned and its structure determined (Elaroussi *et al.*, 1994). Continued work has established that there are three more amino acids in the avian receptors than originally reported (Z. Lu *et al.*, unpublished results). Both the Japanese quail and chicken VDRs have 451 amino acids, being larger in size than the mammalian receptors.

Figure 6 illustrates the general structure of the VDR as it is currently understood. The VDR is the smallest member of the steroid–thyroid hormone superfamily of receptors (Baker *et al.*, 1988; Wahli and Martinez, 1991). The small size of the VDR can be attributed to an extremely small amino-terminal domain. The DNA binding domain is 100% conserved between the amino acid sequence of human and rat, and contains nine conserved cysteines believed to form two "zinc fingers" (Weinberger *et al.*, 1985; Burmester *et al.*, 1988b). The 3′ zinc finger is different in the avian species than in the mammalian species (Elaroussi *et al.*, 1994). It is likely, therefore, that the avian receptor may not bind to all of the response elements to which the mammalian receptor will bind and vice versa. However, there are no examples currently available. The steroid binding domain is 93% identical between the amino acid sequence of human and rat and 87.5%

identical between mammalian and avian receptors (Burmester *et al.*, 1988b; Elaroussi *et al.*, 1994). The carboxy-terminal domain may be responsible for binding to a nuclear accessory factor (NAF) during the course of activation of transcription of target genes (Nakajima *et al.*, 1994).

The VDR has been overproduced using an expression plasmid in yeast (Sone *et al.*, 1990b), in bacteria (Kumar *et al.*, 1992), and also with a baculovirus system in insect cells (MacDonald *et al.*, 1991; Ross *et al.*, 1991). The most prolific VDR production is in the bacterial expression systems, giving rise to large amounts of VDR protein. However, these are found in inclusion bodies and, when solubilized, are largely denatured. The most effective of the expression systems used so far to obtain native VDR is the baculovirus system in insect cells. From this source, milligram amounts of the receptor are available and will probably be used for deduction of the three-dimensional structure with crystallography.

4.3 MUTATIONS OF THE VITAMIN D RECEPTOR

The importance of the VDR for the action of 1,25-$(OH)_2D_3$ is attested by the existence of the genetic disorder, vitamin D-dependent rickets type II (Brooks *et al.*, 1978; Marx *et al.*, 1978). In this disorder, patients present with severe rickets, alopecia and high plasma levels of 1,25-$(OH)_2D_3$ (Liberman and Marx, 1990). The high plasma levels of 1,25-$(OH)_2D_3$ indicate that the ricketic phenotype results from a defect in the VDR. This was clearly demonstrated by analysis of VDR genomic DNA from fibroblasts or lymphocytes of type II kindreds by polymerase chain reaction technology (Hughes *et al.*, 1988). A number of defects in the VDR have been identified including mutations in the VDR DNA binding domain (Hughes *et al.*, 1988; Sone *et al.*, 1990a; Saijo *et al.*, 1991) and truncated versions of the VDR which result in dysfunctional ligand binding domains (Ritchie *et al.*, 1989; Wiese *et al.*, 1993). A defective VDR in type II kindreds underscores that the function of the vitamin D hormone is through a mechanism involving the VDR.

4.4 MECHANISM OF VITAMIN D RECEPTOR FUNCTION

The VDR is believed to function by binding to response elements located in the promoter region of target genes (Darwish and DeLuca, 1993). So far, at least seven such genes have been described, and nine response elements are illustrated in Table 1. These response elements are generally characterized by a six-base repeat sequence, separated by three non-specified bases. The three non-specified bases are required spacing for VDREs (Umesono *et al.*, 1991). If the spacing is four, the response element is specific for the

TABLE 1

The vitamin D response elements found in target genes

Gene	Sequence	Position
Calbindin D-9k	GGGTGT . . . AAGCCC	−488 to −474
Rat osteocalcin	GGGTGA . . . AGGACA	−456 to −442
Human osteocalcin	GGGTGA . . . GGGGCA	−511 to −486
Mouse osteopontin	GGTTCA . . . GGTTCA	−757 to −743
Rat 24-hydroxylase distal	GGTTCA . . . GGTGCG	−262 to −238
Human 24-hydroxylase distal	AGTTCA . . . GGTGTG	−293 to −273
Rat 24-hydroxylase proximal	GAGTCA . . . AGGTGA . . . AGGGCG	−151 to −125
Human 24-hydroxylase proximal	GAGTCA . . . AGGTGA . . . AGGGCG	−171 to −143
Suppression		
Human parathyroid hormone	NNNNNN TGAACCT	−106 to −100

thyroid hormone receptors, and five bases separating the repeat sequences are characteristic of the retinoic acid receptors (Umesono *et al.*, 1991). The first gene in which a VDRE was identified and characterized was the osteocalcin gene (Demay *et al.*, 1990; Ozono *et al.*, 1990). This was followed by the osteopontin gene (Noda *et al.*, 1990) and the calbindin D-9k gene (Darwish and DeLuca, 1992). The most recent of the genes shown to be responsive is the 25-OH-D_3 24-hydroxylase (Ohyama *et al.*, 1994; Zierold *et al.*, 1994; Chen and DeLuca, 1995). This enzyme is induced by 1,25-$(OH)_2D_3$ which programs its own destruction, as the 24-hydroxylase is an enzyme that begins the path of degradation of the vitamin D_3 hormone to its excretion product, calcitroic acid. The unique feature of the response element in this gene is that there are two VDREs which differ from each other and which are separated by 93 base pairs (Chen and DeLuca, 1995; Zierold *et al.*, 1995). Furthermore, the second response element has three repeats, each separated by three nucleotide bases. This is the most powerful of the VDREs found. The entire 24-hydroxylase VDRE system results in a tenfold stimulation of the chloramphenicol acetyltransferase (CAT) reporter gene (Zierold *et al.*, 1995) or a 100-fold stimulation of the luciferase reporter gene by 1,25-$(OH)_2D_3$ in the presence of expressed VDR (T. Ross *et al.*, unpublished results). A VDRE in the calbindin D-28k gene has also

been identified; however, it differs significantly from the other reported VDREs (Gill and Christakos, 1993).

Of great importance is the suppression of the parathyroid hormone gene by 1,25-$(OH)_2D_3$ (Silver *et al.*, 1986). A single six-base sequence has been located as the site of binding of the VDR (Demay *et al.*, 1992). This results in transcriptional suppression of the parathyroid hormone gene in response to 1,25-$(OH)_2D_3$. This six-base sequence is the reverse orientation of one of the two osteopontin six-base sequences, which is a positive response element. Many genes will likely be found that have response elements to the vitamin D hormone. Thus, at least a portion of the mechanism of action of the vitamin D hormone is via its receptor binding to these response elements, subsequently causing an altered rate of transcription of the target gene.

In order for the VDR to bind to the response elements thus far found, a NAF is required (Sone *et al.*, 1990b; Ross *et al.*, 1992). This NAF can be replaced by retinoid X receptors (RXRs) that have been reported to bind 9-*cis*-retinoic acid and that serve as co-regulators of thyroid hormone receptor and retinoic acid receptor function (Yu *et al.*, 1991; Kliewer *et al.*, 1992). Recently, the NAF found in porcine intestine has been substantially purified (Munder *et al.*, 1995). This protein has a molecular weight of 55–60 kDa and will serve as an accessory factor for binding of the retinoic acid receptor to its response element. Furthermore, this protein reacts with an antibody prepared to a peptide sequence of the RXRs. The highly purified NAF also binds 9-*cis*-retinoic acid indistinguishably from RXRs. Finally, an anti-RXR antibody binds the VDR–VDRE–NAF complexes. Thus, the NAF found in porcine intestine is an RXR (Munder *et al.*, 1995). The binding of this RXR and the VDR to VDREs occurs in the absence of ligand, although the presence of 1,25-$(OH)_2D_3$ may increase the affinity of the VDR to VDREs (Ross *et al.*, 1993). It is important to emphasize that 1,25-$(OH)_2D_3$, however, is not required for the binding of the VDR to its response element. The ligand of the glucocorticoid, oestrogen and progesterone receptors is also not required for binding of these receptors to their response elements (Darwish and DeLuca, 1993).

Another important feature of the VDR is that it becomes phosphorylated very quickly following the addition of 1,25-$(OH)_2D_3$ (Brown and DeLuca, 1990). This has been clearly demonstrated in chicken intestinal organ cultures in which the phosphorylation of the VDR occurs within 1 h after ligand administration. This phosphorylation event occurs before the uptake of calcium and the induction of calcium binding protein. Furthermore, stimulation of VDR phosphorylation by activators and inhibitors of protein kinase A can be shown to increase transcriptional activity of the VDR (Darwish *et al.*, 1993). This effect occurs in the absence of ligand; however, the addition of ligand results in a further increase in the transcriptional activity of the VDR (Darwish *et al.*, 1993). Thus, phosphorylation of the receptor appears to play an important role in the subsequent transcriptional activity of the

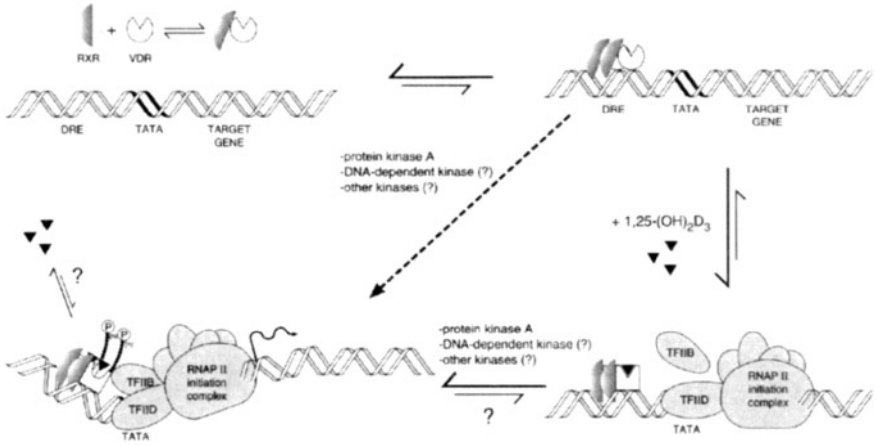


FIG. 7. Diagrammatic representation of the current hypothesis of how 1,25-$(OH)_2D_3$ initiates transcription of a target gene. DRE, vitamin D response element; RNAP, ribonucleic acid polymerase; TF, transcription factor; VDR, vitamin D receptor.

VDR. The site of the 1,25-$(OH)_2D_3$-induced phosphorylation has been localized to a 23-kDa peptide which spans the designated hinge region and one-half of the ligand binding domain, and involves a serine residue (Brown and DeLuca, 1991).

Figure 7 provides a model of the molecular mechanism of action of the vitamin D hormone in eliciting a target gene response. In this model, the VDR together with the RXR, also known as NAF, binds to the response element in the absence of ligand. The next step is the addition of ligand which then allows the complex to be a substrate for phosphorylation. This then converts the VDR into a transcriptionally active protein which likely interacts with several other factors to elicit transcription of the subsequent gene. It is unknown whether following phosphorylation there is a further need for the ligand. Possibly, inactivation of the VDR as a transcriptive agent involves dephosphorylation and loss of ligand. Much remains to be learned concerning the molecular mechanism of action of 1,25-$(OH)_2D_3$ in eliciting gene transcription.

4.5 NON-GENOMIC ACTIONS OF 1,25-$(OH)_2D_3$

Some reference should be given to the reports of non-genomic actions of 1,25-$(OH)_2D_3$ (Nemere *et al.*, 1994). These observations are of an *in vitro* nature and are not supported by *in vivo* results. For example, Nemere and

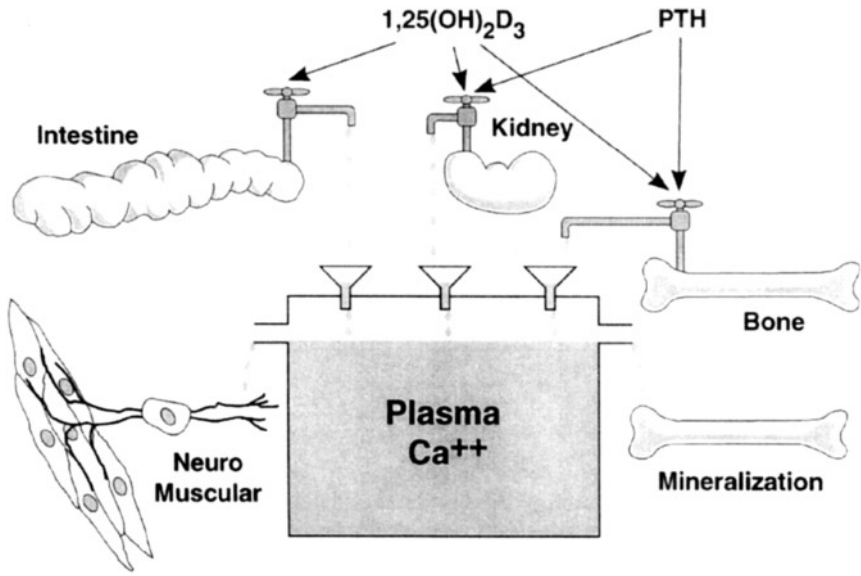


FIG. 8. Sites of physiological action of 1,25-$(OH)_2D_3$ in raising blood calcium and phosphorus levels. This effect is, in turn, responsible for mineralization of the skeleton and prevention of hypocalcaemic tetany.

colleagues (1984) reported, using a duodenal loop system from vitamin D-replete chickens, that 1,25-$(OH)_2D_3$ induces calcium transport within 14 min after administration. In contrast, the administration of 1,25-$(OH)_2D_3$ to rats does not cause an immediate increase in serum calcium concentration, even when calcium is present in the small intestine (Halloran and DeLuca, 1981; Krisinger *et al.*, 1991). Instead, approximately 4 h are required before serum calcium rises in response to 1,25-$(OH)_2D_3$ when calcium is obtained from intestinal sources. Thus, the claimed non-genomic stimulation of calcium transport by 1,25-$(OH)_2D_3$, termed transcaltachia (Nemere and Norman, 1986) is not supported by *in vivo* evidence.

5 Functions of Vitamin D

The classical actions of 1,25-$(OH)_2D_3$ result in the mineralization of the skeleton, the prevention of hypocalcaemic tetany and the suppression of the parathyroid gland (Fig. 8). As was presented above, the VDR plays a crucial role in the molecular mechanism of action of 1,25-$(OH)_2D_3$. Following the discovery of the VDR in the enterocyte and crypt cells of the small intestine, osteoblasts and distal renal tubule cells, came the finding of the VDR in a

TABLE 2

Cells or tissues that are known to be targets of 1,25-$(OH)_2D_3$ action or are expected to be from the presence of either receptor or nuclear localization of 1,25-$(OH)_2D_3$

Proven	Putative
Intestinal enterocyte	Islet cell (pancreas)
Osteoblast	Endocrine cell (stomach)
Distal renal cell	Pituitary cell
Parathyroid cell	Ovarian cell
Skin keratinocyte	Placenta
Promyelocyte, monocyte	Epididymis
Lymphocyte	Brain (hypothalamus)
Colonic enterocyte	Myoblast (developing)
Shell gland	Mammary epithelium
Chick chorioallantoic membrane	Aortic endothelial cell
	Skin fibroblast

number of tissues not previously appreciated as targets of 1,25-$(OH)_2D_3$ action (Stumpf *et al.*, 1981; Sandgren *et al.*, 1991). These sites, listed in Table 2, include bone marrow cells such as promyelocytes (Tanaka *et al.*, 1982), skin cells (Simpson and DeLuca, 1980), activated lymphocytes (Provvedini *et al.*, 1983), ovarian cells (Dokoh *et al.*, 1983), islet cells of the pancreas (Stumpf *et al.*, 1979; Pike, 1982), basal cells of the hair follicle (Stumpf *et al.*, 1984) and others. As a result, roles for 1,25-$(OH)_2D_3$ in cellular differentiation, the skin, the immune system, female reproduction, insulin secretion and hair growth have been suggested. In addition to the classical functions of 1,25-$(OH)_2D_3$, these roles will be discussed.

5.1 CLASSICAL FUNCTIONS OF VITAMIN D

Vitamin D was first discovered because of its activity in healing hypocalcaemic tetany, rickets and osteomalacia (DeLuca, 1988b). In the hypocalcaemic tetany disorder, when plasma calcium concentration falls, nerves continuously excite muscle giving rise to a convulsive state which will quickly result in death unless ameliorated. This disorder is corrected by infusion of calcium or by raising plasma calcium concentration. The disease rickets is one in which the organic matrix of the skeleton is synthesized by the osteoblasts but it fails to acquire the hydroxyapatite mineral that provides the rigidity for skeletal function (Kramer and Gribetz, 1971). This is corrected by increasing plasma calcium and phosphorus levels. Similarly, osteomalacia

is the adult disorder in which the newly synthesized organic matrix produced during remodelling fails to mineralize because of a lack of calcium and phosphorus (Kramer and Gribetz, 1971). This is also healed by an increase in plasma calcium and phosphorus concentration. In a vitamin D-deficient model, it has become clear that only calcium and phosphorus are needed to bring about this mineralization and that vitamin D is not required (Underwood and DeLuca, 1984; Weinstein *et al.*, 1984; Holtrop *et al.*, 1986). Thus, the essence of vitamin D action in these classical functions of vitamin D is to increase plasma calcium and phosphorus to levels that will then be used to mineralize skeleton and prevent hypocalcaemic tetany. The vitamin D hormone does this by stimulating intestinal transport of calcium and phosphorus, by mobilizing calcium from bone and by reabsorption of calcium in the renal distal tubule (DeLuca *et al.*, 1990).

The vitamin D hormone stimulates transport of calcium and phosphorus, independently, in the enterocytes of the small intestine by an active process (Corradino, 1973; Wasserman and Taylor, 1973; Halloran and DeLuca, 1981). Little is known concerning the molecular events involved in the intestinal calcium or phosphorus transport process. Two proteins have been identified that are believed to play a role in the calcium transport process. One is a cytosolic calcium binding protein called calbindin D-9k in mammals and calbindin D-28k in avian species (Wasserman and Feher, 1977). Calbindin D-9k binds 2 moles of calcium and calbindin D-28k binds 4 moles. How calbindin D functions in the transport process is unknown; however, it is transcriptionally upregulated by 1,25-$(OH)_2D_3$ in the intestine and kidney (Christakos *et al.*, 1989). The second protein is the calcium pump found in the basal lateral membrane of intestine which has been shown to be increased by 1,25-$(OH)_2D_3$ at the messenger RNA (mRNA) level (Cai *et al.*, 1993).

Because intestinal calcium is not a reliable source of calcium, the body must have available to it an immediate source of calcium when needed to prevent fatal hypocalcaemic tetany. For this purpose, the skeleton serves not only in a structural capacity but as a source of calcium. Calcium is brought into the circulation under the influence of the vitamin D hormone working together with the parathyroid hormone (Tanaka and DeLuca, 1971; Garabedian *et al.*, 1974). The osteoblasts, which are the cells that actually synthesize and catalyse the mineralization of the skeleton and not osteoclasts, the skeletal resorption cells, are targets for 1,25-$(OH)_2D_3$ and parathyroid hormone (Stumpf *et al.*, 1981; Rouleau *et al.*, 1986; Clemens *et al.*, 1988). There is controversy as to the role of the osteoblast and the osteoclast in the mechanism of bone calcium mobilization. Talmage (1970) proposed that calcium transport occurs across the osteoblast. Many scientists believe that mobilization of calcium from bone is a result of stimulation of osteoclastic-mediated bone resorption (Suda *et al.*, 1992). McSheehy and Chambers

(1986, 1987) carried out an important experiment in which conditioned medium taken from osteoblasts treated with 1,25-$(OH)_2D_3$ stimulated osteoclastic-mediated bone resorption. This result suggests that osteoblasts activated by 1,25-$(OH)_2D_3$ in some fashion provide a signal to the osteoclast to begin resorption. Whether this is part of the mechanism by which 1,25-$(OH)_2D_3$ adjusts serum calcium concentration by mobilizing bone requires further investigation.

Another mechanism that can be considered a classical action of vitamin D_3 is the renal reabsorption of calcium. The vitamin D_3 hormone stimulates renal tubular reabsorption of calcium and also facilitates the effect of parathyroid hormone on renal tubular reabsorption of calcium (Yamamoto *et al.*, 1984). Both hormones are known to have receptors in the distal renal tubule of the nephron (Nissenson and Arnaud, 1979; Simpson *et al.*, 1980; Stumpf *et al.*, 1980; Rouleau *et al.*, 1986). Conservation of filtered calcium contributes to the increase in plasma calcium concentration, which then results in mineralization of the skeleton and correction of hypocalcaemia.

Although it may not be considered a classical action, the suppression of the parathyroid gland is an important function of the vitamin D hormone and constitutes some of the feedback mechanism involved in the regulation of plasma calcium concentration (DeLuca, 1992). Following the discovery of the VDR, at least two groups provided evidence for the existence of the VDR in the parathyroid gland (Hughes and Haussler, 1978; Stumpf *et al.*, 1979). Of particular interest is that the vitamin D hormone when given *in vivo* or provided to cells will suppress secretion of parathyroid hormone (Silver *et al.*, 1985, 1986). Furthermore, this has now been traced to a VDRE found in the promoter region of the parathyroid hormone gene (Demay *et al.*, 1992). There is also suppression of parathyroid gland proliferation by a mechanism which is not totally understood (Dietel *et al.*, 1979). In any case, the control of parathyroid gland size and secretion of the parathyroid hormone are important regulatory mechanisms. In the disease renal osteodystrophy, inadequate control of the parathyroid gland results in secondary hyperparathyroidism (DeLuca and Avioli, 1979), as is discussed below.

5.2 ROLE OF 1,25-$(OH)_2D_3$ IN CELLULAR DIFFERENTIATION

An important discovery was made by Abe and Suda in which they demonstrated that the addition of the vitamin D hormone to cultures of promyelocytes brings about a differentiation of these cells to macrophages and a suppression of their growth (Abe *et al.*, 1981). In addition to the effect of 1,25-$(OH)_2D_3$ on the promyelocyte, the hormone functions in the evolution of the giant osteoclast from the macrophage (Abe *et al.*, 1983).

Therefore, in this system at least, the development of the giant osteoclast comes under control of the vitamin D hormone as a regulator of differentiation and development. Similarly, 1,25-$(OH)_2D_3$ plays a role in the differentiation of the keratinocyte and the suppression of its growth (Hosomi *et al.*, 1983; Smith *et al.*, 1986). In a number of cancer cell lines, as well as *in vivo*, the vitamin D hormone will cause a suppression of cell growth and a change in morphological structure (Colston *et al.*, 1981; Frampton *et al.*, 1983; Eisman *et al.*, 1987). These studies are suggestive for a role for the vitamin D hormone in differentiation and development.

5.3 OTHER FUNCTIONS OF 1,25-$(OH)_2D_3$

5.3.1 Role of 1,25-$(OH)_2D_3$ in Skin

Although the exact biological function of 1,25-$(OH)_2D_3$ in skin is not known, the presence of the VDR in keratinocytes and fibroblasts suggests that it is a target organ for 1,25-$(OH)_2D_3$ (Stumpf *et al.*, 1979; Simpson and DeLuca, 1980; Clemens *et al.*, 1983). In culture, proliferation of dermal fibroblasts and epidermal keratinocytes is inhibited by 1,25-$(OH)_2D_3$ (Clemens *et al.*, 1983; Smith *et al.*, 1986). In addition, 1,25-$(OH)_2D_3$ stimulates a morphological differentiation of cultured human keratinocytes (Smith *et al.*, 1986). In conjunction with its morphological effect, 1,25-$(OH)_2D_3$ stimulates transglutaminase activity, an enzyme responsible for cross-linking the proteins of the cornified envelope. The effect of 1,25-$(OH)_2D_3$ on skin cells has been utilized to develop treatments for the disease psoriasis (Holick, 1989).

5.3.2 Role of Vitamin D in the Immune System

The finding that T lymphocytes, upon activation with lectin, possess the VDR prompted the notion that vitamin D_3 plays a role in the immune system (Provvedini *et al.*, 1983). It is clear from *in vivo* studies that vitamin D_3 plays a role in cell-mediated immunity. In the absence of vitamin D_3 there is a marked decrease in delayed-type hypersensitivity and thymic proliferation in truly vitamin D-deficient mice (Yang *et al.*, 1993b). The impaired immune response can be restored with vitamin D_3; however, this requires more than 3 weeks. Interestingly, not only does vitamin D_3 deficiency impair the immune system but 1,25-$(OH)_2D_3$, at pharmacological doses, acts as an immunosuppressant (Lemire and Archer, 1991; Yang *et al.*, 1993a). 1,25-$(OH)_2D_3$ and 19-nor-1,25-$(OH)_2D_2$, a non-calcaemic analogue, suppress immunoglobulin production and thymic lymphocyte proliferation when administered to mice (Yang *et al.*, 1993a). The mechanism by which 1,25-$(OH)_2D_3$ exerts its immunosuppressive effect is not known. However,

in vitro studies have demonstrated a suppressive effect of 1,25-$(OH)_2D_3$ on interleukin (IL) 2 secretion from T lymphocytes (Rigby *et al.*, 1984), a down-regulation of λ interferon mRNA levels in T lymphocytes (Rigby *et al.*, 1987) and an inhibition of IL-1 secretion by monocytes (Manolagas *et al.*, 1989). More detailed studies of the role of 1,25-$(OH)_2D_3$ in the immune system are clearly needed; however, these results suggest that an important development may be the use of vitamin D analogues as immunosuppressants.

5.3.3 Vitamin D and Reproduction

Female reproduction is compromised in vitamin D deficiency. Vitamin D-deficient rats are less successful in mating, and the percentage of pregnant rats giving birth to normal litters is dramatically decreased (Kwiecinski *et al.*, 1989). This is not the result of hypocalcaemia but is a specific function of 1,25-$(OH)_2D_3$ (Kwiecinski *et al.*, 1989). Ovary (Dokoh *et al.*, 1983) and uterus (Walters, 1981) contain receptors for 1,25-$(OH)_2D_3$; therefore, it seems likely that 1,25-$(OH)_2D_3$ is involved in mechanisms of female reproduction. However, considerably more work must be done before the role of 1,25-$(OH)_2D_3$ in female reproduction is fully understood.

Male reproduction is also impaired by vitamin D deficiency, but this appears to be the result of hypocalcaemia (Uhland *et al.*, 1992). Vitamin D-deficient rats made normocalcaemic by dietary means are fully able to produce adequate amounts of functional sperm.

5.3.4 Miscellaneous Functions of Vitamin D

The islet cells of the pancreas are target organs of 1,25-$(OH)_2D_3$ as determined by autoradiography (Stumpf *et al.*, 1981) and receptor measurements (Pike, 1982). Furthermore, there is an impaired insulin secretion in vitamin D-deficient rats challenged with glucose (Chertow *et al.*, 1983). Some controversy exists as to whether this is a specific action of 1,25-$(OH)_2D_3$ or is secondary to the hypocalcaemia that results from vitamin D deficiency. Additional work is required on this system as well.

The basal cells of the hair follicle are a target for 1,25-$(OH)_2D_3$ (Stumpf *et al.*, 1984). There has been one recent report that cytotoxic agents used in cancer chemotherapy cause hair loss but can be prevented by topical administration of 1,25-$(OH)_2D_3$ (Jimenez and Yunis, 1992). Of considerable interest is that patients with vitamin D-dependent rickets type II, who have a defect in the VDR, present with a lack of hair (Feldman *et al.*, 1982; Liberman and Marx, 1990). This suggests that vitamin D does play a role in hair growth and maintenance. However, experiments that directly address this possible role for 1,25-$(OH)_2D_3$ are required to examine this hypothesis.

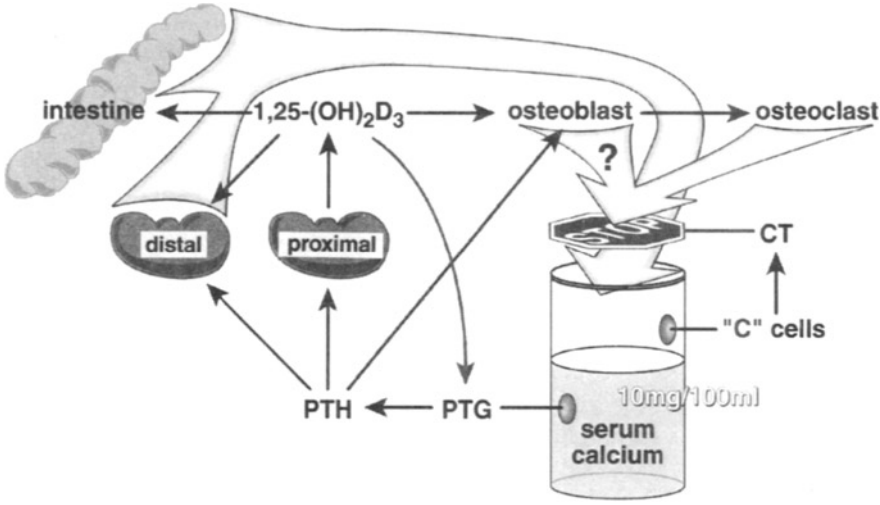


FIG. 9. Diagram illustrating how the vitamin D endocrine system maintains plasma calcium concentration at a constant level. Note the low calcium sensing organ is the parathyroid gland (PTG) and the high calcium sensing organ is the C cells of the thyroid gland. CT, calcitonin; PTH, parathyroid hormone.

6 **Regulation of the Vitamin D Endocrine System**

One of the most tightly regulated substances in plasma is ionized calcium concentration. Except under conditions of disease, plasma calcium levels remain at a constant level, at 10 mg per 100 ml total calcium or 1 mM ionized calcium (Bronner, 1982). The vitamin D endocrine system is intimately involved in maintaining this plasma calcium concentration. As illustrated in Fig. 9, the parathyroid glands are triggered by hypocalcaemia and the C cells of the thyroid respond to hypercalcaemia (Bronner, 1982). Parathyroid glands will rapidly secrete parathyroid hormone in response to a hypocalcaemic challenge (Sherwood *et al.*, 1968). The parathyroid hormone is an 84-amino-acid peptide whose lifetime can be measured in minutes in plasma (Kemper, 1984). Its major binding site is the entire length of the nephron of the kidney and the osteoblast of the skeleton (Rouleau *et al.*, 1986). In the proximal convoluted tubule cells, parathyroid hormone binds to a membrane receptor where it facilitates phosphaturia (Muff *et al.*, 1992) and of great importance stimulates the 25-OH-D_3 1α-hydroxylase, bringing about increased production of 1,25-$(OH)_2D_3$ (Garabedian *et al.*, 1972; Horiuchi *et al.*, 1977; Kawashima *et al.*, 1981; Tanaka and DeLuca, 1984). 1,25-$(OH)_2D_3$ is then transported from the proximal convoluted tubule cells to the enterocytes of the small intestine, the osteoblasts of bone and the distal renal tubule cells (Stumpf *et al.*, 1980, 1981). 1,25-$(OH)_2D_3$ stimulates the

enterocyte of the small intestine to transport calcium and phosphorus against an electrochemical potential gradient, thereby increasing the levels of these two components of plasma required for bone formation (Wasserman and Taylor, 1973; Halloran and DeLuca, 1981). In the osteoblast, 1,25-$(OH)_2D_3$ acts to signal transport of calcium from bone into the plasma, a process that requires the presence of the parathyroid hormone (Garabedian *et al.*, 1974). In addition, there is stimulation of osteoclasts, both in formation and in activation of existing osteoclasts to stimulate bone resorption, giving rise to an increased bone remodelling activity (Suda *et al.*, 1992). In the distal renal tubule, 1,25-$(OH)_2D_3$, together with parathyroid hormone, functions to facilitate renal reabsorption of calcium (Yamamoto *et al.*, 1984). These functions cause plasma calcium to rise which in turn shuts off parathyroid hormone secretion, and subsequently production of 1,25-$(OH)_2D_3$ is shut down, thereby providing an exquisitely regulated feedback loop system. If calcium in the plasma rises to higher-than-normal levels, the C cells of the thyroid gland react by secreting calcitonin. Calcitonin functions by binding a membrane receptor on the osteoclasts to diminish bone calcium resorption, bringing plasma calcium concentration into the normal range (Zaidi *et al.*, 1990).

Of considerable importance is the function of 1,25-$(OH)_2D_3$ to inhibit parathyroid gland proliferation and to suppress preproparathyroid hormone gene expression (Dietel *et al.*, 1979; Silver *et al.*, 1986). The importance of this regulatory function is exemplified by the increases in parathyroid gland size and parathyroid hormone secretion found in patients with renal osteodystrophy (Silverberg *et al.*, 1975; Slatopolsky *et al.*, 1984). It is very clear that disruption of the vitamin D endochrine system results in disease, as is described below.

The vitamin D hormone plays an important role in protecting bone mass. Under hypocalcaemic conditions, parathyroid hormone is synthesized and secreted, followed by production of the vitamin D hormone which in turn stimulates the intestine to absorb calcium. This brings calcium in from the environment rather than mobilizing it from the skeleton. If, however, there is a lack of calcium in the intestine or intestinal calcium transport system does not function properly or the vitamin D hormone is not synthesized in response to the hypocalcaemic challenge, the parathyroids will continue to secrete parathyroid hormone and become hyperactive. The presence of high concentrations of parathyroid hormone and whatever 1,25-$(OH)_2D_3$ is present will result in mobilization of bone calcium. Thus, the vitamin D hormone protects bone mass by facilitating intestinal calcium transport, which diminishes the need for bone calcium mobilization, and also by suppressing parathyroid hormone secretion, which essentially shifts bodily needs for calcium to the intestine rather than from the skeleton. The vitamin D hormone is an important contributor to protecting bone mass and preventing osteoporosis, as is described below.

The molecular mechanism by which parathyroid hormone stimulates 25-OH-D_3 1α-hydroxylase is at present unknown. This is due largely to the inability of numerous investigators to successfully purify 25-OH-D_3 1α-hydroxylase in sufficient quantity. As a result, antibodies that recognize 25-OH-D_3 1α-hydroxylase and a 25-OH-D_3 1α-hydroxylase cDNA are not available to use in elucidating this mechanism.

Of considerable importance is the degradative pathway of 1,25-$(OH)_2D_3$. 24-Hydroxylation of 1,25-$(OH)_2D_3$ is the initial step in the subsequent oxidative removal of 1,25-$(OH)_2D_3$ to calcitroic acid, the only known excretory product of 1,25-$(OH)_2D_3$ (Onisko *et al.*, 1980; Makin *et al.*, 1989; Reddy and Tserng, 1989; Burgos-Trinidad and DeLuca, 1991). 1,25-$(OH)_2D_3$ induces the 24-hydroxylase by a transcriptive mechanism, resulting in the rapid oxidation of not only 1,25-$(OH)_2D_3$ but also its precursor (Zierold *et al.*, 1995). Parathyroid hormone inhibits the induction of the 24-hydroxylase by 1,25-$(OH)_2D_3$ (Shinki *et al.*, 1992), and thus inhibits the degradative pathway for 1,25-$(OH)_2D_3$, thereby providing a much longer lifetime for this important hormone. It is not clear at this stage how parathyroid hormone functions in reducing the mRNA encoding for the 24-hydroxylase.

Much remains to be learned concerning the molecular mechanisms of regulation of the vitamin D endocrine system. However, it is clear that the vitamin D endocrine system plays a key role in calcium homoeostasis. Therefore, understanding this system is important in providing treatment of calcium regulatory diseases.

7 Therapeutic Uses of Vitamin D Metabolites and Analogues

7.1 RENAL OSTEODYSTROPHY

Perhaps the first disease to come under treatment by 1,25-$(OH)_2D_3$ and its analogues is renal osteodystrophy (Brickman *et al.*, 1974; Silverberg *et al.*, 1975; Slatopolsky *et al.*, 1984). This disorder occurs as kidneys cease to function. As a result, there is an increase in serum parathyroid hormone levels and reduced levels of 1,25-$(OH)_2D_3$ (Brickman *et al.*, 1974; Silverberg *et al.*, 1975; Slatopolsky *et al.*, 1984). With increased levels of parathyroid hormone, osteitis fibrosa cistica occurs that will eventually lead to fragile bones which fracture easily. Renal osteodystrophy, therefore, results from two basic causes: (1) the organ that eliminates phosphate found in abundant quantities in the environment is now absent; and (2) the organ that produces 1,25-$(OH)_2D_3$ is absent (DeLuca, 1981). The physician treats the high plasma level of inorganic phosphate by reducing phosphate intake and by using phosphate binders. Aluminium hydroxide was the method of choice until aluminium toxicity was discovered to be a serious problem. Aluminium appears to inhibit the mineralization process of the skeleton, resulting in

osteomalacia (Ott *et al.*, 1982). Now calcium carbonate is the primary method of reducing phosphate absorption (Baker, 1991).

As was pointed out above, 1,25-$(OH)_2D_3$ has an important function in the parathyroid glands. It suppresses growth of the parathyroid gland and suppresses production of the hormone itself (Silver *et al.*, 1985, 1986). The response element for the VDR has been located in the parathyroid hormone gene promoter and is one of the sites where 1,25-$(OH)_2D_3$ and the VDR function in an important physiological action to control the parathyroid glands (Demay *et al.*, 1992). It is this function that appears to be the manner in which 1-hydroxylated vitamin D compounds are used to treat renal osteodystrophy. Many reports have appeared on the use of orally administered 1,25-$(OH)_2D_3$ and 1α-OH-D_3 to treat renal osteodystrophy (Nielsen *et al.*, 1980; Nordal and Dahl, 1988; Baker *et al.*, 1989). 1α-OH-D_3 must enter the bloodstream to allow its conversion to the active hormone before function (Holick *et al.*, 1976). On the other hand, 1,25-$(OH)_2D_3$ can act directly on the intestine as it is absorbed, which causes a predisposition to hypercalcaemia, thus limiting the usefulness of oral 1,25-$(OH)_2D_3$ in this disorder. However, intravenously administered 1,25-$(OH)_2D_3$ at the time of dialysis has been found to be extremely effective in controlling hyperparathyroidism (Slatopolsky *et al.*, 1984). Nevertheless, hypercalcaemia is a risk with 1α-hydroxylated vitamin D_3 compounds. Recently, non-calcaemic analogues of 1,25-$(OH)_2D_3$ have been developed. The most important are: (1) Leo Pharmaceuticals MC-903 (Calverley, 1987); (2) Chugai Pharmaceutical Company 22-oxa-1,25-$(OH)_2D_3$ (Slatopolsky, 1993); (3) Hoffmann-La Roche 16-ene-1,25-$(OH)_2D_3$ (Uskokovic *et al.*, 1991); and (4) University of Wisconsin 24-homologated 1,25-$(OH)_2D_3$ (Perlman *et al.*, 1990a), 19-nor-1,25-$(OH)_2D_3$ (Perlman *et al.*, 1990b) and 1α-OH-pregnacalciferol series (Lam *et al.*, 1975; Ostrem *et al.*, 1987). Of these compounds, the 22-oxa-1,25-$(OH)_2D_3$ is being developed in Japan for renal osteodystrophy. Another non-calcaemic compound, 19-nor-1,25-$(OH)_2D_2$, is being developed at Abbott Laboratories for the treatment of this disorder (Slatopolsky *et al.*, 1994). These compounds suppress the parathyroid gland without causing hypercalcaemia. The mechanism whereby this occurs is not fully understood; however, it is likely that metabolism of the analogues plays an important role in limiting its hypercalcaemic activity.

7.2 HYPOPARATHYROIDISM

The basis for hypoparathyroidism is the autoimmune destruction of the parathyroid glands, surgical removal of the gland because of malignancy or other such problems, and finally, some subjects are born without functional parathyroids. These patients present with hypocalcaemia and tetany (Sherwood, 1990). Since calcium cannot be mobilized from the skeleton with-

out the presence of the parathyroid hormone, even with 1,25-$(OH)_2D_3$ (Garabedian *et al.*, 1974), it is clear that the source of calcium for maintenance of plasma calcium levels must be the intestine. Thus, a dietary supplement of calcium is required to provide approximately 1–2 g calcium per day orally, and a small physiological dose of 1,25-$(OH)_2D_3$ of 0.5–1.0 μg per day is provided (Kooh *et al.*, 1975; Sherwood, 1990). This treatment is successful in correcting the hypocalcaemia of hypoparathyroidism. Similarly, 1α-OH-D_3 was also developed in Japan and in Europe for this condition (Russell *et al.*, 1974; Kind *et al.*, 1976). The 1-hydroxylated forms of vitamin D together with oral calcium are the method of choice for the treatment of this disorder.

7.3 VITAMIN D-RESISTANT RICKETS

The most common form of vitamin D-resistant rickets, namely X-linked hypophosphataemic rickets, is a disorder of phosphate loss in the kidney. This results in very low blood phosphorus concentrations with approximately normal serum calcium concentrations (Chesney *et al.*, 1983). Although there is some disagreement, it appears that these patients also have low blood levels of 1,25-$(OH)_2D_3$, especially in the face of low plasma phosphorus concentrations (Chesney *et al.*, 1980). Since these patients suffer from a defect in phosphorus homoeostasis and not in vitamin D metabolism, they are treated with frequent oral doses of phosphate compounds to raise blood phosphorus concentration (Glorieux *et al.*, 1972). However, this treatment causes secondary hyperparathyroidism because plasma calcium falls to low levels. Treatment of these patients is best achieved by frequent oral doses of phosphorus together with approximately 0.5–1.0 μg per day of 1,25-$(OH)_2D_3$ or 1α-OH-D_3 (Chesney *et al.*, 1983; Glorieux, 1990). The 1-hydroxylated vitamin D compound increases plasma calcium levels and directly suppresses the parathyroid gland, thus preventing secondary hyperparathyroidism.

Vitamin D-dependency rickets type I is a disorder discovered in 1961 by Prader and colleagues. These children have an autosomal recessive defect resulting in low plasma levels of 1,25-$(OH)_2D_3$, hypocalcaemia, hypophosphataemia and severe rickets in the face of normal intake of vitamin D or normal irradiation of skin from sunlight (Scriver *et al.*, 1978). They can be treated with large amounts of 25-OH-D_3 or vitamin D_3 or with physiological doses of 1,25-$(OH)_2D_3$ (Fraser *et al.*, 1973). It is believed, but has not been proven, that this disease is a defect in the 25-OH-D_3 1α-hydroxylase gene. However, since this hydroxylase has not been cloned, it is unclear whether this hypothesis is correct. It is curious that, if the defect is in the 25-OH-D_3 1α-hydroxylase, 25-OH-D_3 in large amounts should heal this disorder. Since 25-OH-D_3 binds to the VDR when present in large amounts (Eisman *et al.*, 1976), it is indeed possible that it can act as an analogue of 1,25-$(OH)_2D_3$

in inducing transcription of target genes. Thus, high concentrations of 25-OH-D_3 can substitute for 1,25-$(OH)_2D_3$. In any case, this relatively uncommon disease can best be treated with 1-hydroxylated forms of vitamin D, either 1α-OH-D_3 or 1,25-$(OH)_2D_3$.

As discussed in a previous section, vitamin D-dependent rickets type II is the result of an autosomal recessive defect in the VDR (Hughes *et al.*, 1988). These children present with high plasma levels of 1,25-$(OH)_2D_3$, severe rickets, alopecia, hypocalcaemia, hypophosphataemia and danger of tetany (Brooks *et al.*, 1978). In the more severe form of the disease, patients do not respond to 1,25-$(OH)_2D_3$; however, their skeleton appears to mineralize if calcium is infused directly into the bloodstream to facilitate mineralization (Balsan *et al.*, 1986). The prognosis of these children is, indeed, very poor.

7.4 OSTEOPOROSIS

There are at least three basic types of osteoporosis which can be defined as a disorder in which total bone mass is markedly reduced to a point where the patient is at risk to fracture under normal skeletal use (Slemenda and Johnston, 1990). Perhaps the best known form of osteoporosis is that suffered by women during menopause, termed postmenopausal osteoporosis (Johnston, 1989). It is generally accepted that total bone mass of human beings reaches a maximum somewhere between 25 and 30 years of age, after which a decline begins to occur. When menopause occurs, there is a rapid loss of bone due to the lack of oestrogen. If untreated with oestrogen, the bone loss will continue until the patient is put at severe risk of fracture. Clearly, the administration of oestrogen will prevent the bone loss and thus prevent postmenopausal fractures (Lindsay *et al.*, 1976). This is considered the method of choice for the treatment of postmenopausal osteoporosis, although there is some risk of endometrial cancer which may be reduced by oestrogen replacement therapy which includes progesterone (Mack and Ross, 1990). However, the reinitiation of menstrual periods is not a desirable side-effect, plus the risk of cancer results in a lack of compliance or acceptance of this mode of therapy. Treatment of postmenopausal women with osteoporosis at 0.5 μg per day of 1,25-$(OH)_2D_3$ has been shown to increase bone mass and reduce the rate of fracture (Gallagher *et al.*, 1982; Aloia *et al.*, 1988; Tilyard *et al.*, 1992). Doses below that level are considered ineffective (Christiansen *et al.*, 1981; Ott and Chesnut, 1989) and this is the reason for the reports that state that the vitamin D hormone is inactive in the treatment of this disorder. Clearly, 1α-OH-D_3 and 1,25-$(OH)_2D_3$ have been in use in Japan for well over a decade, with excellent success (Shiraki *et al.*, 1985; Orimo *et al.*, 1987; Fujita, 1990).

The largest category of osteoporosis is age related, and afflicts both males

and females, especially those of Caucasian and Oriental races. Certainly there is a decrease in the ability to produce 1,25-$(OH)_2D_3$ as a function of age (Gallagher *et al.*, 1979). Furthermore, Slovik and colleagues (1981) demonstrated that injection of parathyroid hormone fails to increase plasma 1,25-$(OH)_2D_3$ levels in elderly subjects while increasing plasma 1,25-$(OH)_2D_3$ levels in younger persons. Thus, the capacity to produce 1,25-$(OH)_2D_3$ is diminished with age. Treatment with 1,25-$(OH)_2D_3$ or one of its analogues is clearly indicated for this disorder. In Japan, its effectiveness has been demonstrated (Orimo and Shiraki, 1990). Similarly, Caniggia in Italy has demonstrated the usefulness of 1-hydroxylated vitamin D in the treatment of a mixed population of postmenopausal women and patients with age-related osteoporosis (Caniggia *et al.*, 1986, 1990).

A final form of osteoporosis to be considered is glucocorticoid-induced osteoporosis (Baylink, 1983). When glucocorticoids are used as anti-inflammatory agents, there is clearly a loss of skeletal mass which results in the risk of fracture. It might be expected that 1-hydroxylated forms of vitamin D might be effective in the treatment of this disorder but so far trials have been virtually absent.

In considering the various forms of osteoporosis and the fact that the actual predisposition to bone loss is often related to oestrogen lack, the logical question is why would one expect the vitamin D compounds to be effective in the treatment of this disorder? In the case of postmenopausal women with a lack of oestrogen, calcium is mobilized from bone, which suppresses parathyroid hormone, which, in turn, suppresses plasma levels of 1,25-$(OH)_2D_3$. This diminishes calcium absorption, and soft tissue needs of calcium are satisfied by the mobilization of skeletal calcium. Thus, these patients have reduced calcium absorption and loss of bone mass (Gallagher, 1990). When 1,25-$(OH)_2D_3$ is administered to these subjects, calcium absorption increases, bone formation also increases, bone resorption is decreased by suppression of the parathyroids, and these women go into positive calcium balance (Gallagher *et al.*, 1982). In addition, 1,25-$(OH)_2D_3$ causes development of and activation of osteoclasts, thus increasing bone turnover or remodelling (Suda *et al.*, 1992). Furthermore, 1,25-$(OH)_2D_3$ has anabolic actions on osteoblasts, increasing the synthesis of important non-matrix proteins such as osteocalcin (Lian and Gundberg, 1988) and osteopontin (Butler, 1989). How they help in bone formation is uncertain, but are indicative of an anabolic action on bone. Figure 10 illustrates in a diagrammatic way how 1,25-$(OH)_2D_3$ or an analogue might be useful in increasing bone mass and thus be used in the therapy of osteoporosis.

Treatment of osteoporosis with 1,25-$(OH)_2D_3$ is limited because of immediate stimulation of intestinal calcium transport when it is absorbed and the resultant tendency to produce hypercalcaemia and hypercalciuria (Aloia, 1990). The therapeutic window is very narrow as 0.5 μg per day is effective and 0.75 μg per day often results in hypercalcaemia and, thus, this compound

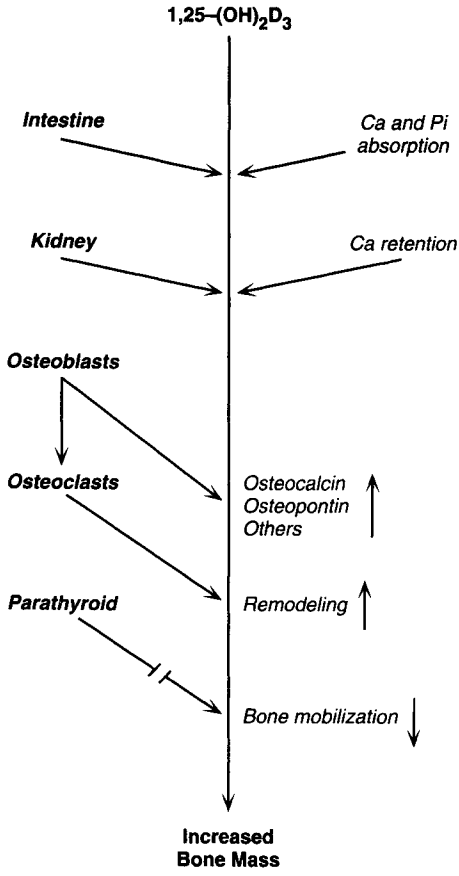


FIG. 10. Use of 1-hydroxylated vitamin D compounds in osteoporosis: sequence of events in response to 1,25-$(OH)_2D_3$.

may not be considered safe in the hands of a practising physician. For this reason, in countries such as the USA where intake of calcium is high, this compound has not been approved for the treatment of osteoporosis. On the other hand, 1,25-$(OH)_2D_3$ and 1α-OH-D_3 are effective in countries where the calcium intake is below 500 mg per day (Gallagher, 1990). Because of this problem, interest in 1,25-$(OH)_2D_3$ compounds that do not raise blood and urinary calcium levels has been generated. Substitution on the 24-carbon with a 24-methyl group in the epi-position in the case of 1,25-$(OH)_2D_2$ provides a compound that has minimal bone resorption activity while being effective in stimulating intestinal calcium transport and bone formation (DeLuca *et al.*, 1988). Furthermore, another compound called ED-71 is under development at Chugai Pharmaceutical Company, and appears to have

major action in increasing bone formation (Okano *et al.*, 1991). Its turnover is very slow, which may account for its high potency.

Of considerable importance is 1α-OH-D_2, which has gone through phase I and II clinical trials with proven effectiveness in increasing bone mass after 2 years of treatment of postmenopausal women with osteoporosis (Gallagher *et al.*, 1993a, b, 1994). Thus, in the future, derivatives of 1,25-$(OH)_2D_3$ may well be developed that favour bone formation versus bone resorption and which may prove to be safe in terms of hypercalcaemia and hypercalciuria.

7.5 PSORIASIS

Because of the actions of 1,25-$(OH)_2D_3$ on keratinocytes and fibroblasts, interest has been developing in the use of vitamin D compounds for treatment of skin disorders (Clemens *et al.*, 1983; Smith *et al.*, 1986). One of the first to receive a great deal of attention is the disease, psoriasis, which is a hyperproliferative disorder of the epidermis (Baden and Pugliese, 1973; Holick, 1989). 1,25-$(OH)_2D_3$ and its analogues actually suppress proliferation of psoriatic keratinocytes and fibroblasts (MacLaughlin *et al.*, 1985; Smith *et al.*, 1988). Topical application of 1,25-$(OH)_2D_3$ has been greatly successful in the treatment of psoriasis, although some cases of hypercalcaemia and hypercalciuria have occurred (Holick, 1989). By administering 1,25-$(OH)_2D_3$ orally at bedtime, Holick (1989) found that 1–2 μg per day could be used to treat psoriasis without significant hypercalcaemia or hypercalciuria. Leo Pharmaceuticals has developed a compound called calcipotriol (MC903) which has minimal calcaemic activity while having reasonable effectiveness against psoriasis (Kragballe *et al.*, 1988). However, even with this compound, some hypercalcaemia has been reported (Holick, 1993). The concentrations of this analogue, however, are much higher than that of 1,25-$(OH)_2D_3$, which may account for some of the side-effects observed. Many new compounds are under development for the treatment of psoriasis and an optimal form will probably be found eventually. In the meantime, calcipotriol is marketed for this disorder in Europe and the USA.

7.6 OTHER POSSIBLE THERAPIES

One of the most important possible applications is the use of 1,25-$(OH)_2D_3$ analogues that do not have calcaemic activity for the treatment of malignancy. Certainly 1,25-$(OH)_2D_3$ treatment of cancer cell lines results in the arrest of their growth and in their differentiation into non-aggressive cells (Colston *et al.*, 1981; Frampton *et al.*, 1983). The concentrations of 1,25-$(OH)_2D_3$ needed to achieve this are, however, quite high. By feeding a low-calcium

diet to mice, it has been possible to administer sufficient amounts of $1,25\text{-}(OH)_2D_3$ to suppress growth of malignant tissue having a receptor for $1,25\text{-}(OH)_2D_3$ (Eisman *et al.*, 1987).

Of considerable interest is the development of the non-calcaemic analogues described above that may well be used to suppress malignancy without causing hypercalcaemia. So far, at least two reports have appeared that use non-calcaemic analogues in the treatment of malignant growth in experimental animals (Abe *et al.*, 1991; Abe-Hashimoto *et al.*, 1993). So far no human trials have been reported. It appears very possible that, in the future, forms of vitamin D will be developed for the treatment of malignancy and other disorders. Thus, vitamin D, its metabolites and analogues are likely to provide a wide array of uses far beyond those initially imagined.

8 Conclusions and Future Directions

There has been enormous progress in our understanding of the molecular mechanism of action of vitamin D since 1960. With this has come not only the discovery of the metabolically active forms of vitamin D, its detailed metabolism, and the vitamin D endocrine system, but also the intracellular molecular mechanisms of action of the final vitamin D hormone. There has also been a great deal of progress in understanding how this hormone is degraded and what regulates its degradation, especially the vitamin D 24-hydroxylase enzyme that is induced by the vitamin D hormone.

With the discovery of the activation of the vitamin D molecule has come the application of the metabolites of vitamin D and its analogues to human disease. Furthermore, the discovery of the vitamin D receptor in tissues not previously appreciated as target tissues has resulted in the discovery of new functions of vitamin D, as, for example, its involvement in the differentiation and development of the monocyte from the promyelocyte. The regulation of the keratinocytes in skin is another important area of vitamin D function that has resulted in treatment of the disease psoriasis. Thus, on the basis of these developments have come methods of treating renal osteodystrophy, hypoparathyroidism, osteoporosis, vitamin D-resistant rickets and psoriasis. There is promise that there will be adjunct therapies for cancer and other important disorders.

To guide the synthesis of new analogues that will be selective in action, the three-dimensional structure of the VDR is required and will likely represent an important new development in the future. A further detailed analysis of the role of $1,25\text{-}(OH)_2D_3$ in initiating transcription of target genes will yield much new information that will be of value not only in understanding these complex systems but in providing guidance to maximize treatment of disease. It can be expected that new and important develop-

ments will continue to occur in our understanding of the vitamin D endocrine system, and that these will be rapidly applied to the treatment of human disease.

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Dopamine Receptors: Studies on Structure and Function

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The recognition of dopamine as an independent neurotransmitter in the brain has led over the past 30–40 years to much interest in its function and mechanism of action. This interest has been strengthened from the realization that dopamine systems are important in certain diseases, e.g. Parkinson's disease, and that the alteration of dopamine activity may be important for its treatment and that of, for example, schizophrenia (for review see Strange, 1992). Particularly active targets for research have been the receptors for dopamine, and in this chapter I shall first consider the new information that has emerged recently on dopamine receptors from the application of

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TABLE 1

Dopamine receptor subtypes defined from physiological, pharmacological and biochemical studies

	D_1	D_2
Pharmacological characteristics		
Selective antagonists	SCH23390	(−)-Sulpiride YM09151-2
Selective agonists	SKF38393	Quinpirole N-0437
Specific radioligands	[^{3}H]SCH23390[a]	[^{3}H]YM09151-2 [^{3}H]spiperone[b]
Physiological functions	Aspects of motor function (brain), cardiovascular function	Aspects of motor function and behaviour (brain), control of prolactin and α-MSH secretion from pituitary, cardiovascular function
Biochemical responses	Adenylyl cyclase ↑ Phospholipase C ↑	Adenylyl cyclase ↓ K^+channel ↑ Ca^{2+} channel ↓
Localization	Caudate nucleus, putamen, nucleus accumbens, olfactory tubercle, cerebral cortex (brain), cardiovascular system	Caudate nucleus, putamen, nucleus accumbens, olfactory tubercle, cerebral cortex (brain), anterior and neurointermediate lobes of pituitary gland, cardiovascular system
Size of receptor protein (Da)		
Affinity labelling	72 000	85–150 000 (major species of 94 000, 118 000, 140 000)
Purification	—	92–95 000 (brain) 120 000 (pituitary)

With the advent of molecular biological studies (Table 2), these subtypes should be termed D_1-like and D_2-like receptors. The localization data are from functional and ligand binding studies on dispersed tissues and tissue slices. The data on the size of the receptor proteins are from affinity-labelling studies of tissues and from the purification of receptors by affinity chromatography.

[a][^{3}H]SCH23390 can also bind to 5-HT_2 receptors if present.

[b][^{3}H]spiperone can also bind to 5-HT_{1A}, 5-HT_2 receptors and α_1-adrenoceptors if present. For more details, see Strange (1993).

MSH melanocyte stimulating hormone.

the techniques of molecular biology to the dopamine receptors. Then I shall consider the mechanism of ligand binding to dopamine receptors from a theoretical and experimental standpoint. It should be clear throughout that the knowledge gained with molecular biological techniques has revolutionized our ability to study and conceptualize dopamine receptors.

1 Multiple Dopamine Receptors

The application of biochemical, pharmacological and physiological techniques to the study of dopamine receptors showed clearly that there were multiple receptors for dopamine, and in the late 1970s it was proposed that there were two subtypes of dopamine receptor (D_1 and D_2) (Kebabian and Calne, 1978). These had distinct biochemical and pharmacological properties; in Table 1 a summary of the properties of the D_1 and D_2 subtypes is given.

This relatively simple picture of two dopamine receptor subtypes was swept aside by the application of molecular biology techniques to the field in the late 1980s. This showed that there were at least five dopamine receptor subtypes (D_1, D_2, D_3, D_4, D_5) (reviewed in Strange, 1991, 1994; Sibley and Monsma, 1992; Civelli *et al.*, 1993; O'Dowd, 1993) so that the two subtypes defined pharmacologically and biochemically correspond to at least five gene products. From a consideration of the structural and functional properties of the subtypes defined using molecular biology, however, they can be divided into subgroups (D_1, D_5 and D_2, D_3, D_4) (Table 2) which resemble the pharmacologically defined receptor subtypes and so may be termed D_1-like and D_2-like subfamilies, respectively. The application of molecular biology techniques has provided much new information about dopamine receptors, as described below. When I refer subsequently to one of the dopamine receptor isoforms defined by gene cloning, I shall use the D_1–D_5 nomenclature, whereas if the receptor is defined only by its pharmacological profile I shall use the D_1-like/D_2-like nomenclature.

2 Common Properties of Dopamine Receptor Subtypes Defined by Molecular Biology

From the application of molecular biological techniques the DNA sequence and hence the amino acid sequence of the different subtypes was obtained. This showed that the different subtypes each consisted of about 400 amino acids with a molecular weight of about 50 000 Da and that there were significant homologies between the dopamine receptors and other G protein-linked receptors (see, for example, Attwood *et al.*, 1991; Trumpp-

TABLE 2

Dopamine receptor subtypes from molecular biological studies

	D_1-like		D_2-like		
	D_1	D_5/D_{1b}	$D_{2(short)/(long)}$	D_3	D_4
Amino acids[a]	446 (h,r)	477 (h) 475 (r)	414/443 (h) 415/444 (r)	400 (h) 446 (r)	387 (h) 368 (r)
Pharmacological characteristics (K_d, nM)[b]	SCH23390 (0.35) Dopamine (2340)	SCH23390 (0.30) Dopamine (228)	Spiperone (0.05) Raclopride (1.8) Clozapine (56) Dopamine (1705)	Spiperone (0.61) Raclopride (3.5) Clozapine (180) Dopamine (27)	Spiperone (0.05) Raclopride (237) Clozapine (9) Dopamine (450)
Homology[c]					
With D_1 receptor	100	82	44	44	42
With $D_{2(short)}$ receptor	44	49	100	76	54
Localization[d]	Caudate/putamen, nucleus accumbens, olfactory tubercle, frontal cortex	Hippocampus, hypothalamus	Caudate/putamen, nucleus accumbens, olfactory tubercle, cerebral cortex (low)	Nucleus accumbens, olfactory tubercle, islands of Calleja, cerebral cortex (low)	Frontal cortex, midbrain, amygdala, medulla (all low), cardiovascular system, retina
Response	Adenylyl cyclase ↑	Adenylyl cyclase ↑	Adenylyl cyclase ↓	?	?
Introns in gene	None	None	Yes	Yes	Yes

Organization of amino acid sequence					
Putative third intracellular loop	Short	Short	Long	Long	Long
Carboxy-terminal tail	Long	Long	Short	Short	Short
Reference (examples)	Monsma *et al.* (1990)	Sunahara *et al.* (1991) Tiberi *et al.* (1991)	Bunzow *et al.* (1988)	Sokoloff *et al.* (1990)	Van Tol *et al.* (1991)

The properties of the principal dopamine receptor subtypes identified by gene cloning are shown. They are divided into D_1-like and D_2-like groups to reflect amino acid homology, functional similarity, structural similarity and pharmacological properties. This grouping conforms with a previous classification based on pharmacological and biochemical properties (Table 1).

[a]h and r refer to human and rat sequences, respectively.

$D_{2(short)}$ and $D_{2(long)}$ refer to different alternatively spliced forms of the D_2 receptor gene, as outlined in the text.

[b]Values are the dissociation constants (nM) of selected ligands taken from the published data; some are for rat, some human. The values for dopamine are in the presence of Gpp(NH)p. $D_{2(short)}$ and $D_{2(long)}$ do not differ greatly pharmacologically where antagonist affinities are concerned, although small differences have been reported for the substituted benzamide drugs (Castro and Strange, 1993a; Malmberg *et al.*, 1993).

[c]The homology values are for the transmembrane-spanning regions and are taken from Jarvie and Caron (1993).

[d]The localizations shown are the principal ones known at present from *in situ* hybridization and use of the polymerase chain reaction, and in some cases have not been examined exhaustively.

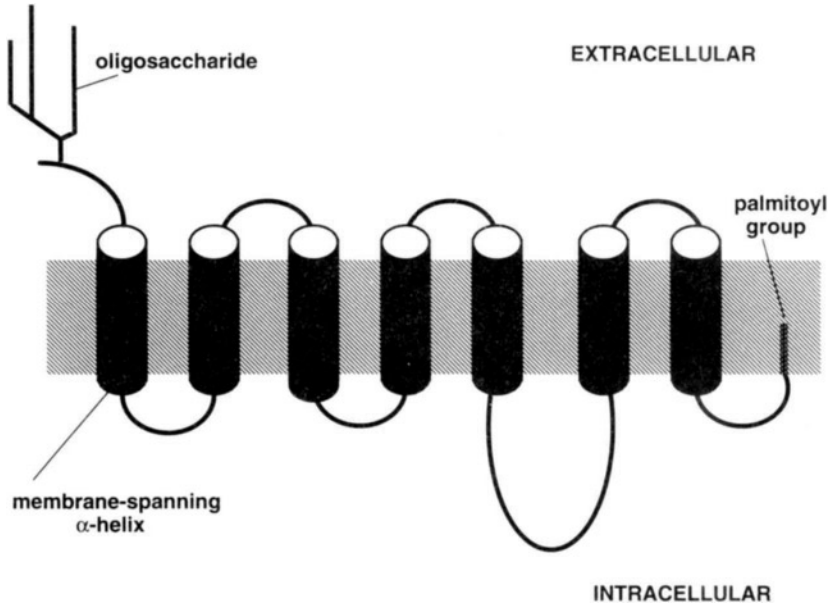


FIG. 1. Structure of a G protein-coupled receptor. The diagram shows the seven putative membrane spanning α-helices linked together by loops of protein that protrude outside the membrane. The amino-terminal is extracellular and is thought to contain the oligosaccharide moieties and the carboxy-terminal is thought to be intracellular and may be linked to the membrane via a palmitoyl moiety. The large third intracellular loop, which is an important site for the interaction with the G protein, is also shown.

Kallmeyer *et al.*, 1992). Homologies were greater within the putative transmembrane-spanning regions. For example, within the transmembrane-spanning regions the D_2 dopamine receptor shows 48% homology with the α_2-adrenergic receptor, 41% homology with the $5HT_{1A}$ serotonin receptor, 27% homology with the M_1 muscarinic receptor, 26% homology with the NK3 tachykinin receptor and 32% homology with the thyrotrophin releasing hormone (TRH) receptor (Bunzow *et al.*, 1988; Straub *et al.*, 1990). Within the dopamine receptor family, homologies were greater between D_1 and D_5 (82% within the transmembrane regions) and between D_2, D_3, D_4 (54–76% within the transmembrane regions) than between, for example, D_1 and D_2 (44% within the transmembrane regions) (Jarvie and Caron, 1993), supporting the division of the receptors into D_1-like and D_2-like subfamilies (see Table 2). For each subtype, however, when the amino acid sequence was subjected to hydropathy analysis seven stretches of hydrophobic amino acids were recognized. These stretches were long enough to form membrane-spanning α-helices and so the dopamine receptors form part of the

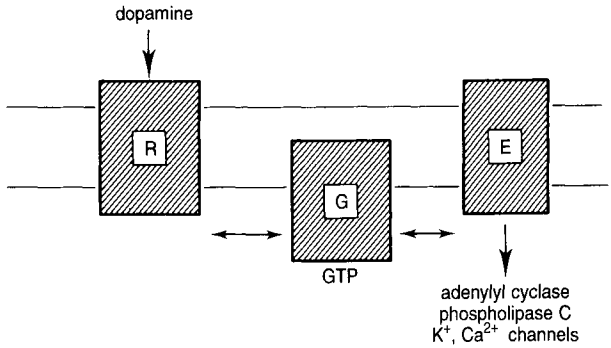


FIG. 2. The signalling machinery associated with G protein-coupled receptors. The diagram shows the receptor (R), a G protein (G) (with bound GTP) and an effector (E) (adenylyl cyclase, phospholipase C, K^+ or Ca^{2+} channels for the dopamine receptors) that interact to effect transmembrane signalling in response to the interaction of an agonist, e.g. dopamine with the receptor.

superfamily of G protein-linked receptors which are thought to possess this structure with the seven helices linked by loops of protein outside the membrane (see Fig. 1 for a representation of this). The D_1-like receptors have short third intracellular loops and long carboxy-terminal tails, whereas the D_2-like receptors have long third intracellular loops and short carboxy-terminal tails. Not only does this support the division of the dopamine receptors into D_1-like and D_2-like subfamilies but the division may have functional significance. The third intracellular loop of G protein-coupled receptors is thought to be important for the interaction of the receptor with the G proteins so that the two subfamilies may interact differently with G proteins. In general, this seems to be associated with the interaction of the D_1-like receptors with G_s and of the D_2-like receptors with members of the G_i/G_o family. The mechanisms of action of the dopamine receptors are considered in more detail below. A general scheme for the actions of G protein-coupled receptors is given in Fig. 2.

For each dopamine receptor the amino terminus (extracellular) has one or more consensus sites for glycosylation and there may be an additional potential glycosylation site in the putative second extracellular loop, e.g. the D_1-like receptors. The actual glycosylation status of these receptors has, however, not been defined. Each receptor subtype also contains potential sites for phosphorylation by protein kinases A and C on the internal surface, particularly on the third intracellular loop and C-terminal tail. There is also in each subtype a cysteine residue in the C-terminal section that, by analogy with studies on the β_2-adrenergic receptor (Kobilka, 1992), may be post-translationally modified with a palmitoyl residue; association of this palmitoyl residue with the membrane may form a fourth intracellular loop. Recent studies have shown that both the D_1 and D_2 receptors are palmitoylated (Ng

et al., 1994a, b). Whereas the role of the oligosaccharide moieties has not been defined for these kinds of receptor, the other post-translational modifications probably play a role in regulating the activity of the receptors, as has been demonstrated for the β_2-adrenergic receptor (Hausdorff *et al.*, 1990; Kobilka, 1992; Ostrowski *et al.*, 1992; Strader *et al.*, 1994).

The ligand binding site of these receptors is thought to be formed from the membrane-spanning α-helices, which are considered to bundle in the membrane creating a cavity into which the ligands bind (Tota *et al.*, 1991; Baldwin, 1993; Donnelly *et al.*, 1994). Consistent with this idea is the observation that within each dopamine receptor subtype certain amino acids are found at similar positions in the helices. These are an aspartic acid in the third helix, aromatic residues in the third, fifth, sixth and seventh helices, and three serine residues in the fifth helix. These are found about one-third of the way in from the outer surface of the membrane and are likely to be important in providing electrostatic, hydrophobic and hydrogen bond interactions with certain ligands. This will be discussed in more detail below when the mechanism of ligand binding at these receptors is considered.

3 Distinct Properties of the Dopamine Receptor Subtypes Defined by Molecular Biological Techniques

3.1 D_1-LIKE RECEPTORS

The first member of this subfamily to be studied was the human D_1 dopamine receptor. This was cloned in 1990 and corresponded to a protein of 446 amino acids (Dearry *et al.*, 1990; Monsma *et al.*, 1990; Sunahara *et al.*, 1990; Zhou *et al.*, 1990). When the complementary DNA (cDNA) was expressed in mammalian cells and the properties evaluated, it was clear that this corresponded to the D_1 receptor defined previously on the basis of pharmacological and biochemical studies. Thus the receptor showed a high affinity for the D_1 selective radioligand [^{3}H]SCH 23390 and in competition studies high affinities were seen for *cis*-flupenthixol, *cis*-piflutixol and (+)-butaclamol. The receptor was also found to be linked via G proteins to the stimulation of adenylyl cyclase. The localization of the D_1 receptor has been assessed from the distribution of the messenger RNA (mRNA), and this showed high levels of the receptor mRNA in the neostriatum (caudate nucleus and putamen), nucleus accumbens and olfactory tubercle. More recently antibodies specific for the D_1 receptor have been raised against fusion proteins containing the carboxy terminal of the receptor (Levey *et al.*, 1993). These antibodies have been used in immunocytochemical localization studies and have confirmed the distribution of the receptor in the neostriatum, olfactory tubercle and nucleus accumbens. D_1 receptors were

also seen in the subtantia nigra (pars reticulata), which presumably reflects D_1 receptors on the terminals of striatonigral neurons. In the neostriatum, D_1 receptors could be seen on the dendrites and spines of medium spiny neurons, where they are presumably postsynaptic to mesostriatal afferents (see Strange, 1992, for more discussion).

Subsequently a second member of this subfamily was cloned from human and rat sources and termed the D_5 and D_{1b} receptor, respectively (Sunahara *et al.*, 1991: Tiberi *et al.*, 1991). The two receptors are species variants and are 82% homologous (within the putative transmembrane-spanning regions) (Jarvie and Caron, 1993). The properties of the D_5/D_{1b} receptor are very similar to those of the D_1 receptor except that the human (D_5) receptor shows a higher affinity for the natural agonist dopamine (tenfold) and the human (D_5) and rat (D_{1b}) receptors show lower affinities for certain antagonists. The D_5/D_{1b} receptor is found at much lower levels than the D_1 receptor based on comparisons of mRNA levels. In addition the distribution is quite different, the D_5/D_{1b} receptor being found in the hippocampus and hypothalamus with very little in the neostriatum. The D_5/D_{1b} receptor is also linked via G proteins to stimulation of adenylyl cyclase.

There has been some speculation that other D_1-like receptors are yet to be identified (Andersen *et al.*, 1990). This is based on the observation that in brain, in addition to the archetypal D_1-like stimulation of adenylyl cyclase, there is evidence that a D_1-like receptor can stimulate phospholipase C activity. This stimulation of phospholipase C has not been seen yet in experiments where cloned receptors are expressed in mammalian cells. It is not clear whether this represents a deficiency in the mammalian cell expression systems or the existence of an additional phospholipase C-linked D_1-like receptor.

3.2 D_2-LIKE RECEPTORS

3.2.1 Properties of the D_2-like Receptors

The first member of this subfamily to be studied was the rat D_2 dopamine receptor. This was cloned in 1988 and corresponded to a protein of 415 amino acids (Bunzow *et al.*, 1988). The properties of this receptor expressed in mammalian cells were consistent with it being related to the D_2 receptor defined in biochemical and pharmacological tests: the receptor showed a high affinity for typical radioligands such as [^{3}H]spiperone and [^{125}I]iodosulpride, and in competition experiments the receptor showed a high affinity for D_2-selective ligands such as (+)-butaclamol, haloperidol and sulpiride (see also Table 2). Subsequently two other members of this subfamily have been identified (Sokoloff *et al.*, 1990; Van Tol *et al.*, 1991) and termed D_3 and

D_4 receptors; some of their properties are outlined in Table 2. Both the D_3 and D_4 receptors have pharmacological properties that show their relationship with the original pharmacologically defined D_2 receptor; thus the original D_2 receptor corresponded to three or more gene products. Nevertheless the three receptor subtypes exhibit significantly different pharmacological properties, underlining that they are different receptor subtypes. This point is underlined by comparisons of the amino acid sequences, which show that within the transmembrane-spanning regions the D_2 receptor is 76% homologous with the D_3 receptor and 54% homologous with the D_4 receptor (Jarvie and Caron, 1993). Notable pharmacological differences between the D_2-like receptors are the high affinities for some agonists shown by the D_3 receptor, and for the D_4 receptor its low affinity for raclopride and its apparently higher affinity for clozapine (see Strange, 1994, for more discussion of some of these points).

The D_2-like subfamily of receptors is notable for the complexity of the genes (including the 5′ untranslated regions), which contain multiple introns (O'Malley *et al.*, 1990; Gandelman *et al.*, 1991; Minowa *et al.*, 1992), and also for the existence of variants generated by alternative splicing of the gene. The variants are based on differences in the protein sequence in the putative third intracellular loop of these receptors and, given that this part of the receptor is thought to be important for coupling to G proteins, it has been speculated that these variants may differ in their interaction with G proteins. This point is considered in more detail below. For the D_2 receptor, two variants have been described termed $D_{2(short)}$ and $D_{2(long)}$, which are identical except for an insertion of 29 amino acids in the third intracellular loop of $D_{2(long)}$ (Giros *et al.*, 1989; Monsma *et al.*, 1989). For the D_3 receptor, the picture is somewhat less clear. In the mouse, $D_{3(short)}$ and $D_{3(long)}$ variants have been described differing by a 21-amino-acid insertion in the third intracellular loop (Fishburne *et al.*, 1993). The $D_{3(long)}$ in the mouse corresponds to the D_3 receptor originally described in the rat. $D_{3(short)}$ and $D_{3(long)}$ variants have, however, not been described in rat or human, but the rat and human receptors are 89% homologous overall (Jarvie and Caron, 1993) and differ in length by 46 amino acids, this difference being in the third intracellular loop (Giros *et al.*, 1990; Sokoloff *et al.*, 1990). Whether this difference represents the existence of other short/long variants remains to be seen. For the D_4 receptor, an even more complex picture exists, with multiple variant species being seen in the human based on insertions of multiples of 16 amino acids (32, 48, 64, 80, 96, 112, 128, 160) in the third intracellular loop of the receptor (Van Tol *et al.*, 1992; Seeman and Van Tol 1994). These variants are found as polymorphic variants in the human population; the 64-amino-acid insertion is the most common, being found in about 60% of the human population. Other variants are, however, found to significant extents, e.g. the 112- and 32-amino-acid isoforms being found in 14% and 10% of the population, respectively.

3.2.2 *Localization of the D_2-like Receptors*

The localization of the D_2-like receptors has been analysed at the level of mRNA and at the protein level. mRNA for the D_2 receptor is found at high levels in typical dopamine-containing regions of the brain such as the neostriatum (caudate and putamen), nucleus accumbens and olfactory tubercle, as well as regions containing dopamine neuron cell bodies, e.g. substantia nigra, ventral tegmental area (Meador-Woodruff *et al.*, 1989), indicating that D_2 receptors are found both pre- and post-synaptically. The $D_{2(short)}$ and $D_{2(long)}$ variants are found to different extents in different regions of brain with, in the rat, a greater proportion of $D_{2(long)}$ in most regions but with a differential distribution in certain regions: the anterior pituitary contains a high proportion of $D_{2(long)}$ whereas in other regions there is proportionately more $D_{2(short)}$, e.g. in the pituitary the long/short ratio is approximately 50 whereas in the neostriatum it is about 4 (Giros *et al.*, 1989). In other species the ratio of $D_{2(short)}/D_{2(long)}$ is somewhat different (Gandelman *et al.*, 1991). The use of antibodies has confirmed the overall distribution pattern of the D_2 receptor, but the strength of the antibody approach lies in the ability to describe the detailed distribution pattern at the subcellular level. This is just beginning to emerge but the data show that the D_2 receptor is found on dendrites and spines of medium spiny neurons in the neostriatum where modulation of cortical and thalamic inputs to these medium spiny neurons may take place (Levey *et al.*, 1993). For the D_3 and D_4 receptors, studies at the mRNA level showed that, in the rat, there were much lower levels of the mRNA compared with those for the D_2 receptor. The distributions of the mRNA showed that these receptors were distributed differently compared with the D_2 receptor. The D_3 and D_4 receptor mRNA showed a more limbic localization, indicating a role in cognitive/emotional aspects of dopamine function (Bouthenet *et al.*, 1991; Van Tol *et al.*, 1991). The $D_{3(short)}$ and $D_{3(long)}$ variants in the mouse are found in different ratios in different brain regions (Fishburne *et al.*, 1993). For the D_4 receptor, it seems that the distribution in the human may be somewhat different compared with its distribution in the rat, with higher levels of mRNA and possibly higher levels of the receptor protein (reviewed in Strange, 1994). Antibodies specific for D_3 receptors have recently been employed to show that D_3 receptors are expressed in 65% of medium spiny neurons in the striatum (Ariano and Sibley, 1994). This must reflect the low-level expression of the D_3 receptor by these neurons combined with the sensitivity of the antibody technique, as when levels of D_3 receptors in the striatum are determined using ligand binding only very low levels are detected.

3.2.3 *Mechanisms of Action of the D_2-like Receptors*

The mechanisms of action of the D_2-like receptors have been a focus of much interest. From biochemical and physiological studies on D_2-like receptors it

was shown that these receptors could be coupled, via G proteins, to the inhibition of adenylyl cyclase, the stimulation of potassium channels and the inhibition of calcium channels (Vallar and Meldolesi, 1989). With the discovery of the different cloned D_2-like receptor isoforms, it was of some interest to discover whether these different responses were associated with different isoforms. This problem has been approached by the expression of the cloned receptors in mammalian cells. For the D_2 receptors, inhibition of adenylyl cyclase was reported in recombinant mammalian cells (e.g. CHO (Hayes *et al.*, 1992), C6 glioma (Cox *et al.*, 1992), CCL 1.3 fibroblasts (MacKenzie *et al.*, 1994)) expressing D_2 receptors. In a comparative study the D_2 receptor expressed in GH_4C_1 cells and in Ltk^- cells was evaluated for effects on second messenger systems (Vallar *et al.*, 1990). Whereas in both recombinant systems the receptor could inhibit adenylyl cyclase, in the GH_4C_1 cell line stimulation of K^+ channels was observed and in the Ltk^- cells stimulation of phospholipase C was seen. This suggests that the inhibition of adenylyl cyclase is a primary response for this receptor but that in recombinant systems effects on other effector systems can be seen, presumably depending on the cellular complement of G proteins and effector molecules.

Initial studies on the variants of the D_2 receptor showed that these coupled differentially to G proteins (Castro and Strange, 1993a) and exhibited different abilities to inhibit adenylyl cyclase (Montmayeur and Borelli, 1991; Hayes *et al.*, 1992). A subsequent, more detailed, study showed that the 29-amino-acid insertion conferred on $D_{2(long)}$ the ability to couple to the G protein $G_{i\alpha 2}$ (Montmayeur *et al.*, 1993). In contrast, Senogles (1994) has shown that $D_{2(short)}$ and $D_{2(long)}$ couple selectively to $G_{i\alpha 2}$ and $G_{i\alpha 3}$, respectively. Despite these contradictions it is clear that, as predicted from the structural difference between the two isoforms, namely the insertion of the 29-amino-acid segment in the third intracellular loop, there are differences in the coupling of the two isoforms to signalling mechanisms. It has also been shown that the two isoforms exhibit slightly different pharmacological properties. $D_{2(short)}$ shows slightly higher affinities for certain drugs, notably the substituted benzamides (Castro and Strange, 1993b; Malmberg *et al.*, 1993). This shows that, as well as altering the ability of the isoforms to interact with G proteins, the 29-amino-acid insertion alters the overall conformation of the receptor including altering the folding of the transmembrane helices to form the binding site so that the two isoforms display slightly different drug binding profiles. It has also been shown that the two isoforms expressed in recombinant cell lines are regulated differently in response to treatment with dopamine (Zhang *et al.*, 1994). Whereas dopamine treatment results in the desensitization of both isoforms, there is a downregulation of $D_{2(short)}$ and a contrasting upregulation of $D_{2(long)}$.

For the D_3 receptor, initial studies were notable in failing to demonstrate any coupling of the receptor to G proteins or signalling systems when the

rat receptor was expressed in African green monkey kidney cells (COS cells) or Chinese hamster ovary (CHO) cells (Sokoloff *et al.*, 1990). The human receptor expressed in CHO cells did show some evidence of coupling to G proteins, but much less than for the corresponding cell line containing the D_2 receptor (Sokoloff *et al.*, 1992). Similarly the D_3 receptor expressed in CHO (Sokoloff *et al.*, 1992) or GH_4C_1 (Seabrook *et al.*, 1992) cells failed to demonstrate any effects on adenylyl cyclase, phospholipase C, arachidonic acid release or ion channels. In contrast to these rather negative results, there are a few positive ones. In CHO cells three reports exist of coupling to G proteins (Castro and Strange, 1993b; Chio *et al.*, 1994; MacKenzie *et al.*, 1994) and in one of these cell lines we have been able to demonstrate that the receptor occupied by dopamine will inhibit adenylyl cyclase by up to 50% (Hall and Strange, 1994). The corresponding figure for the D_2 receptor is 80% (Hall and Strange, 1994). Chio and colleagues (1994) have also reported inhibition of adenylyl cyclase in recombinant CHO cells expressing the D_3 receptor. In differentiated NG108 × 15 cells expressing D_3 receptors, it has been shown that the receptor will inhibit calcium currents (Seabrook *et al.*, 1994), and in HEK 293 cells expressing D_3 receptors inhibition of adenylyl cyclase by dopamine has been described (S. Freedman, personal communication). This forms a very confusing picture and it has to be concluded that the D_3 receptor does couple to G proteins and second messenger systems but that this coupling is dependent on the cell type used for expression and perhaps the particular recombinant cell clone chosen for study. Whether under these circumstances the mechanisms described bear any relation to those for the receptor *in vivo* is yet to be determined.

For the D_4 receptor it has been possible to demonstrate linkage to inhibition of adenylyl cyclase via a G protein when the receptor is expressed in MN9D cells (Tang *et al.*, 1994a) and HEK 293 cells (McHale *et al.*, 1994) but no other responses have yet been described. The variants of the D_4 receptor have not been studied in respect of differential coupling to G proteins as has been done for variants of the D_2 receptor. Some evidence has, however, been provided that the variants have slightly different pharmacological profiles when the affinities of certain drugs such as clozapine are compared (Van Tol *et al.*, 1992; Asghari *et al.*, 1994). This is presumably a reflection of the different length third intracellular loops altering the overall conformation of the receptor and hence altering its ability to bind drugs.

This forms a rather unsatisfying picture at present and much further work is needed to clarify the coupling mechanisms of the different D_2-like receptor subtypes and the relation of the results to *in vivo* systems. A recent development in systems for assaying the effects of these receptors comes from the work of Swarzenski and co-workers (1994), who have shown that in recombinant neuronal MN9D cells containing D_2, D_3 or D_4 receptors treatment with dopamine results in morphogenic changes. This not only provides a new assay system for the effects of these receptors but may relate

to the roles these receptors play in the development of the nervous system. The same workers (Tang *et al.*, 1994b) have also shown that the D_2 and D_3 receptors but not the D_4 receptors expressed in these cells will inhibit dopamine release, providing a further interesting correlate of dopamine receptor activation. Chio *et al.* (1994) have shown that the D_2 and D_3 receptors expressed in CHO cells will both increase the rate of extracellular acidification and stimulate cell division, but the molecular bases of these changes are uncertain.

It should be clear from the preceding discussion that the application of molecular biological techniques to the dopamine receptors has revolutionized the way these receptors can be studied and has already provided much new information and understanding.

4 The Mechanism of Binding of Ligands to Dopamine Receptors

In this next section I shall consider in some detail how ligands bind to the dopamine receptors. This is clearly of interest in terms of a basic understanding of receptor function but it has immense practical relevance for the design of drugs. It seems likely that if we could understand in detail what makes a substance bind with high affinity to a receptor, and in the case of agonists activate the receptor, then it would be possible to design more selective therapeutic agents. I shall approach this firstly from a theoretical standpoint and then I shall consider how these theoretical considerations have been tested experimentally.

4.1 THEORETICAL CONSIDERATIONS

4.1.1 Energetics of Ligand Binding

It is clear that the dopamine receptors have to perform at least two vital functions. They have to bind ligands (both agonists and antagonists) and, in the case of agonists, the receptor–ligand complex must activate effector mechanisms via the intermediacy of G proteins. The binding of a ligand to a receptor involves both favourable and unfavourable processes but the net result must be the favourable free energy change associated with high-affinity binding. This apparently favourable free energy change results from the compensation for large unfavourable free energy changes by larger favourable free energy changes; some of these are summarized in Table 3.

The unfavourable changes are the result of the intrinsically improbable event of ligand binding. Ligand binding restricts the freedom of the ligand and this results in a loss of rotational and translational entropy in the ligand

TABLE 3

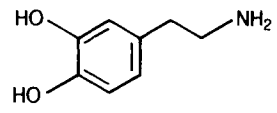
Energetics of ligand–receptor interaction

	Contribution to dissociation constant (M)
Unfavourable factors	
Loss of rotational and translational entropy	10^{10}
Restrictions of internal rotations upon binding	10 (per rotation restricted)
Conformational changes in receptor	?
Favourable factors	
Electrostatic interactions	$>10^{-4}$
Hydrogen bonds	10^{-1} to 10^{-3}
Hydrophobic interactions	10^{-4} (for one aromatic ring)
Van der Waals' interactions	?

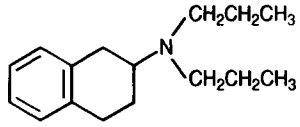
Estimates of the contributions of the various factors that constitute the ligand binding process are given, based on some of the arguments in the text. Values are for the process occurring in aqueous solution as discussed in the text; they are necessarily only estimates and would need to be determined empirically for a particular case. No values are given for the energy of conformational changes or van der Waals' interactions because these will be specific for the particular ligand–receptor pair.

Unfavourable factors will contribute at least a factor of 10^{11} M to the dissociation constant if the process includes the restriction of a single rotation on binding. Given that the dissociation constant for a typical ligand at a receptor is $< 10^{-6}$ M it can be seen that favourable factors must provide contributions equivalent to at least 10^{-17} M. This can easily be achieved for a ligand that interacts using a mixture of the interactions discussed here.

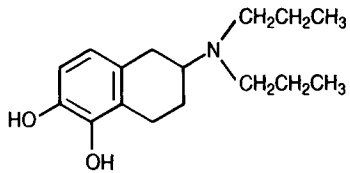
upon binding to the receptor (Page and Jencks, 1971). The restriction of internal rotations of the ligand when it binds to the receptor also has entropically unfavourable results (Page and Jencks, 1971). If the ligand also induces a conformational change in the receptor, as in the activation of effectors by agonists, then this will also consume free energy. Some estimates of the magnitudes of these effects are given in Table 3. The loss of rotational and translational entropy upon ligand binding has been estimated for the binding of substrates to enzymes and contributes a free energy change (ΔG_{t+r}) of approximately 55 kJ mol^{-1} to the process equivalent to a factor of $>10^9$ M in the dissociation constant (Page and Jencks, 1971). (The free energy change (ΔG) and the effect on the dissociation constant (K) are related by the formula $\Delta G = -RT \ln K$. Dividing the free energy change (in kJ mol^{-1}) by 5.7 gives the effect on $\log_{10}$ K at room temperature.) Williams *et al.* (1991) have provided a relationship between the unfavourable free energy of association (ΔG_{t+r}, kJ mol^{-1}) and the molecular weight of a ligand associating with a larger receptor molecule. For example, the



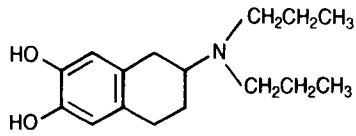
dopamine



2-dipropylaminotetrahydronaphthalene



5,6 dihydroxy-2-dipropylaminotetrahydronaphthalene (5,6-ADTN)



6,7 dihydroxy-2-dipropylaminotetrahydronaphthalene (6,7-ADTN)

FIG. 3. Structures of some aminotetralins and their relation to dopamine.

association of a molecule of 150 Da with a receptor will contribute an unfavourable factor of 10^{10} M to the dissociation constant.

For the restriction of internal rotations, Page and Jencks (1971) showed that binding is adversely affected by approximately 5 kJ mol^{-1} for each rotation restricted. This corresponds to an unfavourable factor of about 10 M in the dissociation constant for each restricted rotation. For the dopamine receptors this can be seen in the binding of dopamine compared with the 2-amino, dihydroxy tetrahydronaphthalenes (ADTNs). The ADTNs (Fig. 3) contain the structure of dopamine but in a conformationally restricted form where the molecule has had some of the internal rotations frozen out. Mutagenesis studies described below indicate that for the D_2 receptor the rotameric form in which dopamine binds corresponds to 6,7-ADTN rather than 5,6-ADTN. 6,7-ADTN binds to the D_2 receptor with a tenfold higher affinity than dopamine (Woodward *et al.*, 1996). This difference in affinity corresponds to a free energy change of approximately 5 kJ mol^{-1}, equivalent to the energy of freezing out of a single rotation. This can only be an approximation as there are in fact two internal rotations that are frozen out in the conversion of dopamine to 6,7-ADTN, but possibly there are already

restrictions to free internal rotation within the dopamine molecule. Nevertheless, the difference must reflect the loss of internal rotations in dopamine when it binds to the receptor, whereas for 6,7-ADTN this unfavourable process does not have to occur. Many drugs which have a high affinity for dopamine receptors, e.g. phenothiazines and butyrophenones, have large rigid moieties which would alleviate this unfavourable process, thus contributing to the tightness of binding. This rigidity may indeed be one important attribute of ligands with high affinity for receptors. Another example of this effect concerns conformationally constrained analogues of gonadotrophin-releasing hormone. These analogues have a higher affinity for the corresponding receptor than the natural hormone (Flanagan *et al.*, 1994), presumably due to the need to constrain fewer internal rotations in the analogue upon binding.

Flexibility in the ligand may, however, become favourable where the interacting groups of the ligand are not optimally positioned in the ground state. In this case some adjustment of the conformation of the ligand may help to achieve better interaction with the receptor (see Portoghese, 1970, for more discussion of this point).

4.1.2 Favourable Interactions Between Ligands and Receptors

The unfavourable aspects of the binding of ligands to receptors together may contribute factors of 10^{11} M or more to the dissociation constant. The binding of ligands is in fact favourable with dissociation constants lower than 10^{-6} M so that there must be significant favourable free energy changes (equivalent to factors of 10^{-17} M or less in the dissociation constant; this would be even lower if conformational changes in the receptor also occurred) to compensate for these unfavourable processes. These must derive from the interaction of the ligand with the receptor. If we take as an example the agonist dopamine, then from the structure of dopamine it can be predicted that these interactions between receptor and ligand may depend on electrostatic interactions with the cationic amino group, hydrogen bonds to the catechol hydroxyl groups and hydrophobic interactions with the aromatic ring of the catechol. Van der Waals' interactions may also contribute either favourable or unfavourable interactions to the overall free energy change. It is important to realize that, in considering these interactions, the binding of a ligand to a receptor in aqueous solution is an exchange process whereby ligand (L) and receptor (R) solvated by water bind together releasing the bound water, and the free energy change reflects the difference in energy between the ligand solvated by water and the ligand bound to the receptor (equation 1).

$$R\cdots H_2O + L\cdots H_2O \rightleftharpoons R\cdots L + H_2O\cdots H_2O \qquad (1)$$

I shall now consider the different forces that may be involved in the binding of ligands to receptors. The free energy change resulting from an *electrostatic interaction* between a ligand and a receptor is due to the actual electrostatic force (enthalpic) between the two charges and in addition the release of water solvating the charges providing an entropic drive; the water molecules in bulk water are less restricted than when involved in solvating the charged groups on the receptor or ligand (equation 2).

$$[\text{R-COO}^- \cdots \text{H}_2\text{O}] + \text{L}^+ \cdots \text{H}_2\text{O} \rightleftharpoons [\text{R-COO}^- \cdots \text{L}^+] + \text{H}_2\text{O} \cdots \text{H}_2\text{O} \quad (2)$$

Estimation of the magnitude of the contribution of an electrostatic interaction to the overall binding energy is difficult owing to uncertainties in defining the energies of desolvation and in defining the local dielectric constant which will affect the energy of the charge–charge interaction. For the β_2-adrenergic receptor, deletion of an active site aspartic acid residue, which is thought to participate in an electrostatic interaction with the cationic head group of noradrenaline, reduces the apparent affinity of noradrenergic agonists to stimulate adenylyl cyclase by a factor of about 10 000 (Strader *et al.*, 1988). The effect of mutation of such a charged residue to an uncharged one is outlined in equation 3, and may alter both the actual electrostatic interaction and the desolvation process.

$$[\text{R}\ ] + \text{L}^+ \cdots \text{H}_2\text{O} \rightleftharpoons [\text{R}\ \text{L}^+] + \text{H}_2\text{O} \cdots \text{H}_2\text{O} \quad (3)$$

It is difficult to define the relative contributions of these effects and, for the muscarinic acetylcholine receptor, it has been suggested (Hulme *et al.*, 1993) that these components may play different roles in the binding of agonists to the free receptor and the form of the receptor coupled to G proteins (see below). In addition if the mutation also affects the transduction process, this will complicate matters further. Fersht (1985) cites two estimates of the energy of electrostatic interactions between charged groups from measurements on enzymes. The energy for the electrostatic interaction between the substrate and tyrosyl-transfer RNA synthetase is $-18\,\text{kJ mol}^{-1}$, and the energy of the electrostatic interaction that stabilizes the active conformation of chymotrypsin is $-12.1\,\text{kJ mol}^{-1}$. Novotny *et al.* (1989) calculated the energy for the interaction between the negatively charged headgroup of phosphocholine and an arginine residue in the McPC 603-specific antibody as $-48\,\text{kJ mol}^{-1}$. These values correspond to stabilizations that contribute factors of approximately 10^{-3}, 10^{-2} and 10^{-8} M, respectively, to the dissociation constant, and suggest that there may be some variability in the contribution of these interactions to binding energies, presumably dependent on the particular environment.

As is discussed below, an important contributor to the stabilization of

positively charged ligands in the binding sites of receptors is interaction with the π electrons of aromatic residues.

For *hydrogen bond* interactions, the free energy of the overall interaction consists of the actual energy of the hydrogen bond plus the free energy of the desolvation of the participating groups. Consideration of this process for formation of a hydrogen bond between two species in aqueous solution (equation 4) (Fersht *et al.*, 1985) shows that the change actually involves the making of two hydrogen bonds and the breaking of two hydrogen bonds. The enthalpy change of the overall process is therefore small and the driving force for the interaction is likely to derive from the release of water which has been ordered in solvating the two participating groups on the left-hand side of equation 4 and is less ordered in bulk water.

$$[\text{R-X}\cdots\text{H}_2\text{O}] + \text{L}\cdots\text{H}_2\text{O} \rightleftharpoons [\text{R-X}\cdots\text{L}] + \text{H}_2\text{O}\cdots\text{H}_2\text{O} \qquad (4)$$

One way to probe the possible participation of amino acid side-chains in hydrogen bonds is to mutate the side-chain by site-directed mutagenesis to remove the hydrogen bond ability, and this is described experimentally below. The consequences of the deletion of a hydrogen bonding group in a receptor are illustrated in equation 5.

$$[\text{R}\ ] + \text{L}\cdots\text{H}_2\text{O} \rightleftharpoons [\text{R L}] + \text{H}_2\text{O}\cdots\text{H}_2\text{O} \qquad (5)$$

Although there may be a change in the affinity of the receptor resulting from the mutation, this will not directly reflect the loss of the free energy of the hydrogen bond between the ligand and receptor. The binding of a ligand to the mutant receptor now involves the formation of one hydrogen bond and the breaking of another, and so will not differ greatly in free energy from the process for the native receptor. Effects of the mutation must therefore reflect differential solvation of the native and mutant receptors and changes in the entropic contributions of water release. There may also be effects of the creation of a cavity in the receptor–ligand complex where the hydrogen bonding group is normally found. Williams (1991) has estimated the energies associated with the formation of different hydrogen bonds in aqueous solution; some of these are given in Table 4. The free energy of a hydroxyl–hydroxyl hydrogen bond interaction is important for discussion below on the interaction of ligands with dopamine receptors. The value in Table 4 suggests that this may be of little importance for the energetics of receptor–ligand interaction. In fact, small but significant free energies for this interaction in aqueous solution (approximately $-2\,\text{kJ mol}^{-1}$, corresponding to factors of $10^{-1}\,\text{M}$ in the dissociation constant) have been determined experimentally for this kind of hydrogen bond (Fersht *et al.*, 1985), indicating that the considerations of equations 4 and 5 are an oversimplification. Also, the experiments described below on the mutation of serine hydroxyl groups

TABLE 4

Free energy of some hydrogen bond interactions in aqueous solution

Interaction	ΔG (kJ mol^{-1})
Amide–amide	−20
Amide–oxygen	−12
Amide–carboxylate	−28
Amide–imidazole	−24
Hydroxyl–hydroxyl[a]	~0
Carboxylate–arginine	−65

[a]The value for hydroxyl–hydroxyl interaction is a theoretical figure taken from Cox *et al.* (1991), and experimental measurements discussed in the text indicate that it is an underestimate.

From Williams (1991).

in dopamine receptors and the effects on the binding of ligands indicate the practical importance of this interaction.

Hydrophobic interactions derive their favourable free energy from the entropically unfavourable ordering of water molecules by the separate hydrophobic moieties and the relief of this ordering when the two hydrophobic species interact (equation 6).

$$[R\cdots H_2O] + L\cdots H_2O \rightleftharpoons [R\cdots L] + H_2O\cdots H_2O \qquad (6)$$

The hydrophobic nature of the ligand and a part of the receptor leads to the ordering of water molecules, which are unable to interact with the hydrophobic species. Interaction of the ligand with the hydrophobic part of the receptor leads to the release of the water molecules from this ordering effect, an entropically favourable process that provides a favourable free energy change.

The energy of hydrophobic interactions can be estimated from the area of the hydrophobic moiety that is buried in the complex. For every square ångström of hydrophobic residue removed from exposure to water by the binding process, an energy of 0.19 kJ mol^{-1} has been calculated (Williams, 1991). For example, the surface area of the side-chain of a phenylalanine residue has been estimated as 120 square ångströms (Chothia, 1974), giving a hydrophobic interaction energy with a receptor of −22.8 kJ mol^{-1} contributing a factor of approximately 10^{-4} M to the dissociation constant.

Van der Waals' interactions may be important too, and Jencks (1975) has suggested that the binding of a substrate to an enzyme, and presumably also of a ligand to a receptor, may provide a tightly packed configuration that optimizes these interactions. Calculation of the van der Waals' energy of interaction is difficult owing to the difficulty in defining the interactions.

Nevertheless these weak interactions may make a significant contribution to the overall energetics of ligand binding. Fersht (1985) has calculated that each methylene group in a crystalline hydrocarbon has 8.4 kJ mol^{-1} of van der Waals' energy. He also cites the calculations for the van der Waals' energy of the binding of substrate to the enzyme lysozyme where these interactions contribute -58.5 kJ mol^{-1} (equivalent to a factor of about 10^{-10} M in the dissociation constant) for the interaction of one of the sugar rings with its subsite on the enzyme (Warshel and Levitt, 1976).

It is clear, therefore, that when a ligand binds to a receptor the unfavourable aspects of the ligand binding process can be overcome by a combination of the favourable interactions considered here. These interactions occur at the ligand binding sites of the receptors so that, in terms of understanding them further, it will be necessary first to consider the structure of the ligand binding site in more detail.

4.2 STRUCTURE OF THE LIGAND BINDING SITE

The ligand binding site for these kinds of receptors for small molecules is thought to be formed from the membrane-spanning α-helical regions of the receptor which bundle in the membrane to form the cavity into which ligands bind. This is based on homologies that have been detected between the G protein-linked receptors and the G protein-linked light-harvesting pigment rhodopsin (see, for example, Baldwin, 1993; Donnelly *et al.*, 1994). Rhodopsin is in turn thought to be related to the bacterial light-harvesting protein bacteriorhodopsin (Findlay and Pappin, 1986). Although bacteriorhodopsin is not itself G protein linked, hydropathy analysis of its amino acid sequence reveals seven hydrophobic stretches that could form α-helices, and this has been confirmed from electron diffraction structural studies which clearly show the seven α-helical regions (Henderson *et al.*, 1990). A high-resolution structure of rhodopsin is not yet available but low-resolution studies confirm the seven-helix structure (Schertler *et al.*, 1993). It therefore seems reasonable to assume that this basic structure will hold for the G protein-linked receptors (see also Baldwin, 1993), and the amino acids lining the cavity will then interact with ligands to generate the free energy of ligand–receptor interaction. As will be developed below for receptors that bind small molecule ligands, including the dopamine receptors, there is a cluster of amino acids within the helical regions about one-third of the distance from the membrane surface, and these are thought to form the interaction points for ligands. There is some experimental support for the contention that ligands bind in this region of the receptors (see, for example, Tota *et al.*, 1991).

In the absence of a high-resolution structure of a G protein-linked receptor, attempts have been made to produce models of G protein-linked receptors

which can be used to make structural predictions. For the most part these have been based on the structure of bacteriorhodopsin (see, for example, Hibert *et al.*, 1991, 1993; Livingstone *et al.*, 1992; Trumpp-Kallmeyer *et al.*, 1992; Nordvall and Hacksell, 1993; Teeter *et al.*, 1994). The amino acid sequences of the receptors are overlaid on the sequence and coordinates of bacteriorhodopsin (Henderson *et al.*, 1990) and a crude model obtained. The model can be refined by using data from biological studies, e.g. the requirement for certain amino acids to be present in the receptor binding site cavity based on the results of mutagenesis studies. The structure is also subjected to energy minimization and ligands can be docked into the putative binding site. In these structures the interaction of the ligands with various side-chains around the binding site can be seen. For the most part these are located about one-third of the way in from the membrane surface, which is presumably the site of binding of the ligands. In particular an aspartic acid residue in the third helix can be seen which forms an electrostatic interaction with the cationic ligands. For the dopamine receptors, serine residues in the fifth helix (which may interact with catechol-containing ligands through hydrogen bonds; see below) and aromatic residues (which may interact directly with ligands or may stabilize the positive charge of the cationic ligand, and which may be important for signal transduction; see Hibert *et al.*, 1991, 1993) were also seen.

Given the very weak homology between the receptors and bacteriorhodopsin at the amino acid level this approach has some hazards; for example, it is difficult to define the precise points of overlap of the receptor and bacteriorhodopsin helices. The low-resolution structure of rhodopsin (Schertler *et al.*, 1993), however, confirmed the seven-helix structure and the overall packing of the helices, although it also showed that the helix packing was slightly different. For example, helix VII lies closer to helices III and V, and the binding pocket for small ligands seems to be formed by the apposition of helices III, V, VI and VII. Experimental confirmation that helices II and VII are spatially close has been obtained for the gonadotrophin-releasing hormone receptor (Zhou *et al.*, 1994). The information derived from the rhodopsin structure has been used by Donnelly and co-workers (1994) to generate new models that may be more realistic. The crude structural predictions of earlier models were, however, mostly confirmed in this model.

4.3 MECHANISM OF BINDING OF LIGANDS TO DOPAMINE RECEPTORS: EXPERIMENTAL OBSERVATIONS

In the next section I shall consider the experimental support for the existence of interactions that may stabilize ligand–receptor interaction as outlined earlier theoretically.

4.3.1 *Electrostatic Interactions with the Cationic Moiety of Ligands*

For the dopamine receptors, all the ligands that interact with the receptors possess some kind of amino function with a $pK_a > 7$, so that the ligand will be mostly positively charged at neutral pH. It has been assumed that it is the positively charged form of the ligand that interacts with the receptor so that electrostatic interactions with negatively charged groups on the receptor will be important for generating some of the free energy of ligand–receptor interaction. It had been speculated that carboxyl groups on the receptor could fulfil this function, and this speculation was supported by observations on the mechanism of action of rhodopsin where carboxyl residues were implicated as counter-ions for cationic moieties during the activation of this protein (Khorana, 1992). As soon as amino acid sequences of receptors for cationic amines (adrenergic, muscarinic cholinergic, dopamine) became available, it was clear that there were two candidate aspartic acid residues within the putative membrane-spanning regions of these receptors that were conserved. For the D_2 dopamine receptor these are Asp 80, about two-thirds of the distance down the second putative α-helix, and Asp 114, about one-third of the distance down the third putative α-helix. Aspartic acid residues equivalent to Asp 80 are found in almost every G protein-linked protein, so it seems unlikely that this residue is involved in specific interactions with ligands. It is more likely to be involved in some aspect of the function of the protein, and there is now evidence to suggest that this residue is important in the allosteric regulation of the receptor by Na^+ and H^+ ions and for regulating the conformation of the receptor so that it can participate in transmembrane signalling (Neve *et al.*, 1991). Aspartic acid residues equivalent to Asp 114 are found only in receptors that bind cationic amines such as the adrenergic, muscarinic and dopaminergic receptors, so this residue seems a good candidate for interacting directly with the ligands via an electrostatic interaction.

For the D_2 receptor, evidence that carboxyl groups are important for the binding of ligands has been obtained from the use of carboxyl specific reagents such as *N,N'*-dicyclohexylcarbodiimide. This reagent eliminates the binding of [^{3}H]spiperone and [^{3}H]YM 09151-2 to D_2 receptors and the effects are blocked by preincubation with another antagonist, showing that the carboxyl group concerned (Asp 114?) is at the ligand binding site (Williamson and Strange, 1990; D'Souza and Strange, 1995). Analysis of the pH dependence of the binding of antagonists such as haloperidol and spiperone to the D_2 receptor has shown that a single ionizable group (pK_a approximately 6) on the receptor is responsible for the pH dependence of the binding of these ligands (Williamson and Strange, 1990; D'Souza and Strange, 1995). This pH dependence is likely to correspond to the protonation of Asp 114. Firmer evidence for the participation of Asp 114 in the binding of ligands to the D_2 receptor comes from site-directed mutagenesis studies. Mutation

of Asp 114 to Asn abolishes the ability of the receptor to bind ligands such as [^{3}H]raclopride (Mansour *et al.*, 1992).

The magnitude of the contribution of the electrostatic interaction to ligand binding must be estimated, but at present there is no experimental information on this for the dopamine receptors. The only quantitative information available relates to the β_2-adrenergic receptor, as summarized above and in Table 3. The corresponding aspartic acid residue (Asp 105) has been investigated for the muscarinic acetylcholine receptor where an Asn 105 mutation substantially reduces antagonist binding to the receptor (Fraser *et al.*, 1989), in agreement with an important role for this residue in ligand binding. The role of this aspartic acid residue in the muscarinic acetylcholine receptor has also been analysed by a Glu 105 mutation which preserves the negative charge but perturbs the interactions with cationic drugs by altering the bond lengths. This mutation has a relatively small effect on the binding of some antagonists and a slightly larger effect on the binding of agonists to the free receptor but substantially disrupts the interaction of agonists with the form of the receptor coupled to G proteins (Hulme *et al.*, 1993). It was suggested that binding of agonists to the free receptor could be via a relatively loose association with Asp 105 where the cationic moiety is stabilized by interactions with aromatic residues, whereas in the form of the receptor coupled to a G protein there is a closer interaction more dependent on electrostatic forces. It seems therefore that the determinants of agonist binding to this receptor may be different for the receptor and for the receptor–G-protein complex, and this point will be returned to later for the D_2 dopamine receptor.

The nature of this electrostatic interaction has also been probed for the D_2 dopamine receptor by testing the binding and activities of a series of analogues of dopamine agonists and antagonists that are either permanently uncharged or permanently positively charged (Miller *et al.*, 1988; Harrold *et al.*, 1993). For the antagonists, whereas the permanently charged analogues were active at the receptor, the permanently uncharged analogues were inactive, supporting the view that it is the charged form of the drugs (protonated on the basic amino group) that bind to the receptor. These authors also suggested, based on the different affinities of the native and permanently charged drugs, that the electrostatic interaction actually consists of a reinforced ionic bond (Jeffrey and Mitra, 1984) consisting of a combination of ionic and hydrogen bonding. Scott (1989) has, however, argued that a simple electrostatic interaction may be present. Miller *et al.* (1988) also reported interesting findings with some permanently uncharged analogues of dopamine. These were unable to act as agonists but were able to bind to the D_2 receptor and appeared to bind to the form of the receptor coupled to G proteins. Further experimental work is required to understand these findings but they imply a dissociation between forms of the receptor

involved in signalling and the form coupled to G proteins detected in ligand binding studies (see below for further discussion).

It is generally assumed that this electrostatic interaction with the conserved aspartic acid residue in the third helix will be an important contributor to the free energy of binding for all cationic ligands to these kinds of receptors. There are, however, indications that this may be an oversimplification. For example, in the $5HT_{1A}$ serotonin receptor, mutation of this residue eliminates the binding of serotonin to the receptor but has little effect on the binding of pindolol (also a cationic amine) (Ho *et al.*, 1992). Similarly, for the $5HT_{2A}$ receptor, mutation of this residue has rather modest effects on the binding of certain cationic ligands (Wang *et al.*, 1993). For the muscarinic acetylcholine receptor, Asselin and colleagues (1983) concluded from pH dependence studies that the binding of the antagonist *N*-methyl scopolamine to the receptor depended more on hydrophobic interactions than on electrostatic interactions. The findings of Hulme *et al.* (1993) outlined above also indicate that, in some cases, the binding of ligands to muscarinic receptors depends only to a limited extent on electrostatic interactions. Of some interest here are the observations on ligand binding to the nicotinic receptor: the binding of the cationic ligands does not depend on electrostatic interactions at all but is stabilized by interactions with aromatic residues (Fu and Sine, 1992). As is discussed below, there are other examples of the binding of cationic ligands to enzymes and antibodies by interactions with aromatic residues rather than via electrostatic interactions.

Thus it may be that for certain cationic ligands and certain receptors electrostatic interaction plays a rather modest role in binding the ligand, with the positive charge being stabilized by interactions with aromatic residues and the desolvation of the positive charge providing further driving force. In the case of the D_2 dopamine receptor the evidence available points to an important role for the electrostatic interaction.

4.3.2 Hydrogen Bond Interactions Between Ligands and Serine Residues

For agonists to act at dopamine receptors it has been pointed out that one or two hydroxyl groups on the agonist are desirable (see, for example, Seeman, 1980). These effects can be seen from the addition of hydroxyl groups to the aminotetralin nucleus where affinities (K_i (free receptor); Seeman *et al.*, 1985) for binding to the D_2 receptor are greater for 5,6-dihydroxy 2-dipropylaminotetralin (40 nM) and 6,7-dihydroxy 2-dipropylaminotetralin (2100 nM) compared with dipropylaminotetralin (9160 nM) (see Fig. 3 for structures).

It is then presumed that these hydroxyl groups form hydrogen bonds with suitable amino acid side-chains. For the β_2-adrenergic receptor, serine residues in the fifth α-helix were examined as potential hydrogen bond

acceptors. There are three Ser residues (203, 204, 207) in the fifth α-helix of the β_2-adrenergic receptor and these are conserved in the D_1 receptor (198, 199, 202) and D_2 receptor (193, 194, 197), and partially conserved in the α_2-adrenergic receptors where the three serines are replaced by the motif Ser, Cys, Ser (200, 201, 204). These potential hydrogen bond acceptors are found about one-third of the distance down the fifth helix at a similar level to the conserved Asp residue in the third helix, suggesting that they may form a cluster of residues interacting with the ligands. This also supports the idea that helices III and V are important contributors to the binding site structure (see above). The conservation of these hydrogen-bonding residues between receptors that bind catecholamines also suggests that they are important for receptor function. It has generally been assumed that these Ser residues are mainly important for agonist binding and do not play a major role in antagonist binding but, as will be discussed below, this is an oversimplification.

The role that these residues play in receptor function has been investigated for the β_2-adrenergic receptor by mutagenesis of each Ser individually to Ala (reviewed in Strader *et al.*, 1994). From the data it was concluded that the meta- and para-hydroxyl groups of noradrenaline interacted with Ser 204 and 207, respectively. The Ala 203 mutant, however, did not express an active protein, so these conclusions may be incomplete, and Ser 203 may play an as yet undefined role in the binding of ligands. Wang *et al.* (1991) analysed mutant α_2-adrenergic receptors where Ser 200 and 204 had been replaced by alanine, showing that mutation of these residues did affect the binding of catechol agonists to the mutant receptors. Firm conclusions could not, however, be drawn about the role of the two residues. It will be necessary to ascertain the role that is played by Cys 201 to understand the relative importance of the different residues in interactions with agonists.

The corresponding serine residues have been mutated to alanine in both the D_1 and D_2 receptors. For the D_1 receptor Ala 198, Ala 199 and Ala 202 mutant receptors were prepared (Pollock *et al.*, 1992) and characterized with a narrow range of ligands, so the conclusions that can be drawn are limited. Nevertheless, it is clear from the data that each of the mutations affects the binding of dopamine to the receptor, with the greatest effects being seen with Ala 198 ($>$ Ala 202 $>$ Ala 199). Hence the conclusions of Strader *et al.* (1994) on the β_2-adrenergic receptor, that catecholamines interact primarily with only two serine residues, cannot be extrapolated to the D_1 receptor. Effects are also seen on the binding of antagonists such as SCH 23390, with Ala 198 ($>$ Ala 199 $\gg$ Ala 202) having the greatest effect. These mutations do not affect the ability of the receptor to activate adenylyl cyclase apart from the apparent reduction in potency of dopamine related to the reduction in binding affinity. Tomic *et al.* (1993) prepared the Val 199–Ala 202 double mutant receptor and reported in contrast no effect on antagonist binding but a 15-fold reduction in the binding of dopamine. Both of these studies would

have benefited from the use of a wider range of compounds, but nevertheless the conclusion is clear that dopamine interacts with the D_1 receptor via the three serine residues rather than via two serine residues as suggested by Strader *et al.* (1994) from studies on the β_2-adrenergic receptor.

For the D_2 receptor, three studies have been performed on the mutation of the three Ser residues, and the different conditions used in each study render exact comparisons difficult. All three Ala mutants will express as active proteins and the consequences of the mutations were then evaluated using ligand binding (Cox *et al.*, 1992; Monsour *et al.*, 1992; Woodward *et al.*, 1996). For antagonists, effects of these Ala mutations were mostly either small or absent indicating that the mutations do not affect the gross conformation of the receptor. For the rat $D_{2(long)}$ receptor, the Ala 193 mutation led to increases in the affinity of a number of substituted benzamide drugs, although not all the members of this class of drug were affected and no classical antagonists were affected (Woodward *et al.*, 1996). This may reflect a subtle change in the conformation of the receptor, the substituted benzamide drugs being particularly sensitive to the conformation of the receptor. The Ala 194 mutation led to reductions in affinity for a set of substituted benzamide drugs and there was some tendency for this to be compounds with a 5-sulfonamide moiety, e.g. sulpiride. This may reflect the disruption of a hydrogen bond between Ser 194 and these drugs. The Ala 197 mutation reduced the affinity for binding only three drugs, domperidone, NCR 181 and raclopride. These drugs are so different in structure that it is not possible to draw any conclusions about the origins of the effects. The effects of these mutations on the human $D_{2(long)}$ and rat $D_{2(short)}$ receptors have been evaluated with a limited number of antagonists (Cox *et al.*, 1992; Mansour *et al.*, 1992) and, although there are some differences, the results generally concur with those for the rat $D_{2(long)}$. It is clear that for a small group of antagonists these Ser residues play a role in generating part of the free energy of binding, presumably by interacting with hydrogen bonding moieties on the ligands, e.g. carbonyl, amino and hydroxyl groups.

For agonists the different states of the receptor (free receptor and receptor–G protein complex) have different agonist affinities. It is important, therefore, to consider effects of the mutations on the binding of the agonist to these different states of the receptor. The binding of agonists to the free receptor can be assayed in the presence of guanine nucleotides and the effects of the Ala mutations may be evaluated with a range of carefully chosen agonists. For dopamine all three mutations alter its affinity for the free receptor, with Ala 193 having the greatest effect (Cox *et al.*, 1992; Woodward *et al.*, 1996). The conformationally constrained dopamine analogues 5,6-ADTN and 6,7-ADTN were affected differently by the three mutations: for 5,6-ADTN the effects were Ala 193 > Ala 197 > Ala 194, and for 6,7-ADTN the effects were Ala 193 ≫ Ala 1994 >Ala 197 (no effect). These observations show that all three serine residues can participate in interactions with agonists

(as concluded for the D_1 receptor above) and that there is no unitary mode of interaction of catecholamines with the D_2 receptor. It is also possible to deduce that for the ADTNs there are preferential interactions between Ser 193, Ser 194 and Ser 197 and the 6-, 7- and 5-hydroxyl groups, respectively, when these are present. It is not possible to specify the precise mode of binding of dopamine from the data but it appears to be similar to that for 6,7-ADTN, suggesting that dopamine may bind in this conformation. These data are quite different from those obtained for the β_2-adrenergic receptor using mutagenesis (Strader *et al.*, 1994) and those suggested for the interactions of dopamine agonists with receptors from pharmacophore analysis (Seeman, 1980; Seeman *et al.*, 1985). To examine the effects of Ala mutations on the binding of agonists to the receptor–G protein complex, it is necessary to assay agonist binding in the absence of added guanine nucleotides. This can be done in competition experiments versus a radiolabelled antagonist in which case higher- and lower-affinity agonist binding sites can be detected. From these data the ratio of affinities of the two sites (K_l/K_h) can be used as a measure of the ability of the agonist to stabilize receptor–G protein interaction (Wreggett and DeLean, 1984) and then the effects of the mutations determined on this parameter. Alternatively a radiolabelled agonist may be used to label the higher-affinity sites and effects of the mutations on the binding of the radiolabelled agonist will reflect effects on the binding of the agonist to the receptor–G protein complex as well as effects on its stability. Mostly the Ala 193 and Ala 194 mutations did not greatly alter the agonist stabilization of receptor–G protein interaction as judged by effects on the K_l/K_h ratio (Woodward *et al.*, 1996). For some compounds, however, these mutations did alter the agonist effect on receptor–G protein interaction and this was particularly true for the Ala 197 mutation where, for several compounds, the formation of the coupled state was abolished by this mutation based on effects on the K_l/K_h ratio. Based on studies with a radiolabelled agonist, the Ala 197 mutation was also found to have large effects on the binding of some agonists (Mansour *et al.*, 1992). This must mean that the interactions agonists make with these Ser residues are different for the free receptor and for the receptor coupled to the G protein. This can be seen particularly clearly for the effects of the Ala 197 mutation on the binding of 6,7-ADTN: the mutation has no effect on the binding of this compound to the free receptor whereas it abolishes formation of the receptor–G protein complex for this compound (Woodward *et al.*, 1996). The conclusion that the determinants of agonist binding to the free and coupled states of the receptor are different has important implications for the design of selective agonists. A similar conclusion has been reached by Hulme *et al.* (1993) from analysis of the muscarinic acetylcholine receptor (see above). Based on their findings it may be that interaction of agonist with the free receptor is relatively loose and that interaction of agonist with the receptor such that coupling to G proteins is stabilized involves the formation

of tighter interactions both with the aspartic acid residue in the third helix and with other residues, such as the serines in the fifth helix.

Cox *et al.* (1992) also examined the effects of these Ala mutations on the ability of the mutant receptors to inhibit adenylyl cyclase, i.e. activate effectors. The Ala 194 mutant was notable in that dopamine was unable to inhibit adenylyl cyclase. This is of some interest in that the Ala 194 mutation does not greatly affect receptor–G protein coupling for dopamine as determined in ligand binding assays (Woodward *et al.*, 1996). Based on these results and those discussed above, it may be that there are different determinants of agonist binding for the receptor in its free state, its state coupled to G protein in the absence of added guanine nucleotides and in its state signalling to effectors.

Cox *et al.* (1992) have mutated another serine residue in the D_2 dopamine receptor (Ser 391) and shown that this reduces the affinity of the receptor for dopamine. This serine residue is, however, unlikely to be involved in generating the specificity of ligand binding as it is highly conserved in many receptors for cationic amines.

4.3.3 Other Hydrogen Bond Interactions

4.3.3.1 Histidine residues. A notable feature of the D_2-like family of receptors (D_2, D_3, D_4) is the existence of a His residue in the sixth putative α-helix. This provides a unique ionizable residue within the ligand binding site of these receptors which could potentially form hydrogen bonds or electrostatic interactions with ligands. His residues are not found within the ligand binding site of the other biogenic amine receptors, although they are found within receptors for some neuropeptides, e.g. opiate receptors (see, for example, Surratt *et al.*, 1994) and adenosine receptors (see Trumpp-Kallmeyer *et al.*, 1992; Dudley *et al.*, 1993; IJzerman *et al.*, 1994), where a His residue is found in the sixth helix. The presence of such an ionizable residue within the ligand binding site of the D_2-like dopamine receptors could potentially influence the pH dependence of ligand binding. Indeed initial interest in this residue was kindled from the observation that the substituted benzamide drugs are selective D_2-like antagonists and that their binding to the D_2 receptor was pH dependent and influenced by an ionizable group of pK_a approximately 7, which could be this histidine (Williamson and Strange, 1990; Presland and Strange, 1991) (see below for a fuller discussion of this pH dependence).

To probe the possible role of this residue (His 394 in the D_2 receptor) in the binding of ligands and their pH dependence, this residue was mutated to a leucine residue to eliminate its potential for forming hydrogen bonds and electrostatic interactions whilst retaining some of its bulk and hydrophobicity (Woodward *et al.*, 1994). The Leu 394 mutant receptor was

evaluated by testing the binding of a series of antagonists; for many of the antagonists tested the mutant showed very similar affinities to the native receptor, indicating that the overall conformation of the receptor had not been changed. The Leu 394 mutant receptor did, however, show decreased affinities for binding a group of drugs, e.g. sulpiride, and increased affinities for binding another group of drugs, e.g. clebopride, and all the drugs affected by the mutation were members of the substituted benzamide family. Therefore, for the compounds tested, the Leu 394 mutation had an orthopramide-specific effect. By testing a series of structurally related compounds it was possible to dissect these effects further. Compounds whose affinities were reduced by the Leu 394 mutation all possessed either a sulfonamide or sulfone moiety at the 5-position on the benzamide ring, so it is a reasonable deduction that the mutation is disrupting an interaction between His 394 and these groups. Given the size of the effect, which is consistent with the breaking of a hydrogen bond (see above), it seems likely that the interaction affected is a hydrogen bond between His 394(H^+) and the S-O^- moiety. The compounds whose affinity is increased by the mutation all possess the 4-amino, 5-chloro substitution pattern on the benzamide ring. One possibility is that there is some unfavourable interaction between His 394 and this part of these molecules when they bind to the receptor and this is relieved in the Leu 394 mutant, leading to the increase in affinity. Alternatively the mutation allows a hydrogen bond to form which was impossible in the native receptor. Compounds that contain the 4-amino, 5-sulfone/sulfonamide substituent pattern are not greatly affected by the mutation so that the two effects to some extent can internally compensate.

The effects of the Leu 394 mutation illustrate an interesting principle. From the effects of the mutation it was possible to divide the substituted benzamide drugs into different subgroups according to the effects of the mutation on their behaviour. Without any further characterization this shows that the different subgroups of drug interact with the receptor differently. This could be termed a "reverse structure–activity determination" as it is the reverse of the typical structure–activity determination where the structure of the ligand is varied and the effect on the activity of the receptor determined.

4.3.4 The Role of Aromatic Residues in Receptor Function

Within the putative ligand binding pocket of the dopamine receptors, a number of aromatic amino acid residues can be seen. Some of these are conserved or substituted conservatively in all of the receptors that bind cationic amine ligands (e.g. for the $D_{2(long)}$ receptor Phe 110 (helix III), Trp 386, Phe 389 (helix VI)), whereas others are conserved in the receptors that bind natural ligands containing aromatic rings such as the adrenergic,

dopamine and serotonin receptors (e.g. Phe 390 (helix VI)). The aromatic residues that are conserved in all of the cationic amine receptors are probably not involved in specific interactions, at least with the natural ligands, and it has been proposed that these residues may be important for stabilizing the positive charge of the cationic ligands via interactions with the π electrons (Hibert *et al.*, 1991, 1993; Trumpp-Kallmeyer *et al.*, 1992). This stabilization would be in addition to that afforded by the electrostatic interaction with the aspartic acid residue in the third helix, and this point was considered earlier. Some support for the idea that interactions with aromatic residues can stabilize cationic ligands comes from the structure of the enzyme acetylcholinesterase where the cationic head group of the acetylcholine is bound in this manner (Sussman *et al.*, 1991) and from studies on model acceptors for cations (Dougherty and Stauffer, 1990). No systematic study has been made of the effects of these interactions in the cationic amine receptors.

One of these aromatic residues that is found in many cationic amine receptors (e.g. adrenergic, muscarinic acetylcholine, dopamine D_2, D_3 and D_4 receptors) is a tyrosine residue in the seventh α-helix. Hulme *et al.* (1990) have suggested for the muscarinic acetylcholine receptor that possibly this residue (Tyr 417 in the $D_{2(long)}$ receptor) interacts with the aspartic acid residue (Asp 114 in the D_2 receptor) in the third α-helix to play some role in the structure or function of the receptors. Hibert *et al.* (1993) have proposed in addition that this residue may interact with the conserved aspartic acid residue in the second helix (Asp 80 in the D_2 receptor) but that upon receptor activation interaction with Asp 114 occurs. In the D_2 dopamine receptor this residue has been mutated to a phenylalanine in order to eliminate the hydrogen bonding potential (Daniell, S., Strange, P. G. and Naylor, L. H., unpublished work). The Phe 417 mutant was evaluated in terms of the binding of a series of antagonist drugs and it was found that for every drug tested the Phe 417 receptor showed a reduced binding affinity (approximately 2–6-fold). The diversity of the structures of the drugs tested suggests that this cannot reflect the disruption of an interaction with specific moieties of drugs; rather it must reflect either a conformational change in the receptor or the disruption of some interaction that is common to all the drugs, and in either case the binding of all the drugs tested is affected. If the effect reflects a common specific interaction then one possibility is that the tyrosine residue is normally involved in stabilizing the positive charge of the amino group of the bound ligand via a hydrogen bond.

Modelling studies of the D_2 receptor have implicated certain aromatic residues in providing hydrophobic stacking interactions with ligands, e.g. Phe 199 (helix V) and Phe 390 (helix VI) in the $D_{2(long)}$ receptor (Hibert *et al.*, 1991; Livingstone *et al.*, 1992; Trumpp-Kallmeyer *et al.*, 1992; Teeter *et al.*, 1994), but these have not been studied in mutagenesis experiments on the D_2 receptor. It has been suggested that these aromatic residues may

provide stacking interactions with the aromatic rings of the natural ligands to help bind the ligands and interactions with these aromatic residues may also be important in triggering the conformational changes associated with receptor activation (Hibert *et al.*, 1991, 1993; Teeter *et al.*, 1994). The residue corresponding to Phe 390 in the D_2 dopamine receptor has been studied for the β_2-adrenergic (Phe 290; Dixon *et al.*, 1988) and $5HT_2$ serotonin (Phe 340; Choudhary *et al.*, 1993) receptors and shown to reduce agonist binding and, in the case of the $5HT_2$ receptor, antagonist binding. For the β_2-adrenergic receptor another aromatic residue (Phe 289, helix VI) has been mutated and shown to affect agonist but not antagonist binding (Dixon *et al.*, 1988). The effect of this mutation is larger than for mutation of Phe 290 above, suggesting that some of the predictions of the models may not be accurate and that other specific interactions between ligands and aromatic residues are occurring.

In summary, aromatic residues are likely to be very important in both the binding of ligands and in the mechanism of receptor activation. The binding of ligands may be stabilized by interactions of the aromatic residues with the positive charge of the ligands as well as through hydrophobic interactions with aromatic moieties of ligands. The optimal formation of both these sets of interactions may cause conformational changes in the receptor, leading to alterations in the packing of the helices in relation to one another, changes in the structure of the intracellular loop regions of the receptors and hence to receptor activation.

4.4 THE pH DEPENDENCE OF THE BINDING OF DRUGS TO RECEPTORS AS A PROBE OF THE LIGAND BINDING PROCESS

The binding of drugs to receptors such as that for dopamine and other monoamines involves at least one electrostatic interaction (between the aspartic acid residue in the third α-helix and the cationic amino group of ligands). There are also within the helices other ionizable residues; for example, there is the aspartic acid residue in the second α-helix which is conserved in the majority of G protein-coupled receptors, and in the D_2-like receptors there is a histidine residue in the fifth α-helix. Not only will the protonation state of these residues vary as the pH is altered but the charges on the residues will interact and influence one another's protonation states. For the D_2-like receptors this is potentially very interesting as there are at least three ionizing residues within the binding site (Asp 80, Asp 114, His 394) which may interact.

The pH dependence of the binding of a range of drugs to the D_2 dopamine receptor has been examined (Williamson and Strange, 1990; Presland and Strange, 1991; D'Souza and Strange, 1995). The results of these studies show that the binding of classical antagonists ((+)-butaclamol, haloperidol, spiperone) is pH dependent, and a careful analysis of the data indicates that

their binding is affected by a single ionizing residue on the receptor whose pK_a is about 6. From these data it is not possible to identify the residue responsible for this pH dependence but it seems reasonable to speculate that it is Asp 114, whose protonation interferes with receptor–ligand interaction by disrupting the electrostatic interaction between drug and receptor. This value for the pK_a is rather high for a carboxyl group but may reflect the hydrophobic environment created by the bundle of α-helices or interactions with the other ionizing groups. In studies on the pH dependence of ligand binding to the muscarinic acetylcholine receptor (Birdsall *et al.*, 1989), pK_a values of approximately 5.4 and 6.8 have been inferred for the receptor in binding different ligands and speculated to correspond to aspartic acid residues in the receptor.

When the pH dependence of the binding of the substituted benzamide drugs to the D_2 dopamine receptor was determined, it was found in contrast that their binding depended on the protonation of two ionizable groups on the receptor whose pK_a was between 6 and 7. These data show that the classical and substituted benzamide drugs bind differently to the D_2 receptor and there is much other evidence in support of this contention based on the differential sensitivity of the two classes of drug to the concentration of Na^+ (Theodorou *et al.*, 1980), and to mutations of the receptor (Leu 394 (Woodward *et al.*, 1994), Asn 80 (Neve *et al.*, 1991)), and based on the differential affinities of the short and long isoforms for the substituted benzamides but not classical antagonists (Castro and Strange, 1993a). One of the ionizable groups is presumably the same residue that affected the binding of the classical antagonist drugs and is therefore likely to be Asp 114. The second must reflect some other ionizable residue on the receptor; good candidates would be Asp 80 or His 394. His 394 can be ruled out, as in the Leu 394 mutant receptor the binding of the substituted benzamides is more pH sensitive than for the native receptor, rather than less so (Woodward *et al.*, 1994). Mutation of Asp 80 to Asn does reduce the pH sensitivity of the substituted benzamides (Neve *et al.*, 1991; D'Souza and Strange, 1995). Given that the pH sensitivity of these drugs will be a function of the ionization state of two residues, these observations provide reasonable evidence that the second ionizing group affecting the binding of the substituted benzamide drugs may be Asp 80. This would have an unusually high pK_a for a carboxyl group but this may be a result of the microenvironment of the receptor binding site. It is unlikely that the effects of protonation of Asp 80 reflect a direct interaction between Asp 80 and the drugs. As outlined above Asp 80 is probably important for regulating the conformation of the receptor and protonation of this residue switches the receptor to a state with lower affinity for the substituted benzamides, hence reducing their binding.

Some evidence can be obtained that these three ionizable residues (Asp 80, Asp 114, His 394) do interact from studies of the pH dependence of ligand binding to the Leu 394 mutant receptor (D'Souza, 1995). The

binding of both spiperone and sulpiride to the Leu 394 mutant receptor is more sensitive to pH than their binding to the native receptor. For example, the effect of a change of pH from 7.5 to 6 is to alter the affinity of spiperone by about twofold for the native receptor and sevenfold for the Leu 394 mutant. This presumably reflects the removal from the binding site of the positively charged His 394 residue so that protonation of Asp 114 becomes easier.

The data on the pH dependence of the binding of dopamine antagonists to the D_2 receptor allow the classical and substituted benzamide antagonists to be separated in terms of their behaviour; the binding of the classical antagonists depends on the ionization of a single ionizing group (Asp 114?), whereas that of the substituted benzamides depends on the ionization of two groups (Asp 80, Asp 114?). Within one of these subgroups of drug, however, different drugs show slightly different pH dependencies. For example, when the pH dependence of the binding of the classical antagonists was examined, the drugs were found to depend on the ionization of a single residue but the pK_a of this residue varied from 5.5 to 6.1. This variability may reflect the existence of different conformations of the receptor that bind the different drugs, and in these conformations the single ionizable group (Asp 114) has a slightly different pK_a.

Limited data are available on the effects of pH on agonist binding to the form of the D_2 receptor uncoupled from G proteins (Neve, 1991), and the results show that different agonists have different sensitivities to alterations of the assay pH. For example, the binding of dopamine is quite sensitive to a change of the assay pH from 8 to 6.8, whereas that of bromocriptine is rather insensitive. The data are only preliminary but may indicate different modes of binding for different agonists.

These data show that, from the use of a simple technique such as the pH dependence of ligand binding, useful information may be obtained about receptor function.

5 Conclusions

The mechanism of the binding of ligands to dopamine receptors is beginning to be understood but much information is still to be obtained. There is much direct and circumstantial evidence that an important factor in the interaction of ligands with the D_2 receptor is an electrostatic interaction between Asp 114 and the cationic amino group found in all ligands. For agonists containing catechol moieties, hydrogen bond interactions with two or three serine residues (Ser 193, 194, 197) contribute to the free energy of binding. These serine residues, as well as His 394, play a role in the binding of some antagonists as outlined above. As also indicated above, hydrophobic interactions with aromatic residues are likely to provide an important

contribution to the free energy of binding but these have not yet been studied for dopamine receptors. The contributions of these interactions to the overall free energy of the ligand binding process have been estimated earlier and the experimental observations reported here are consistent with the need for several favourable interactions between ligand and receptor to compensate for the unfavourable process of ligand binding and to lead to receptor activation.

This dissection of the interactions that contribute to the binding of ligands to receptors will be very important in understanding the precise mechanism of receptor action. Similar studies are in progress for antigen–antibody interactions and here calculations have been made that in some cases agree with experimental observations (Novotny *et al.*, 1989). These kinds of studies will also, in time, help in the understanding of the basic molecular forces involved in the recognition between molecules.

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