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MOLECULAR DIMENSIONS OF THE SUBSTRATE BINDING SITE OF CYTOCHROME P-448

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Abstract—The molecular geometries of specific substrates, inhibitors and inducers of cytochrome P-448 activity were determined using computer-graphic techniques for use in defining the molecular dimensions of the substrate binding site of this enzyme Specific substrates of cytochrome P-448 are essentially planar molecules characterised by a small depth and a large area/depth ratio In contrast, compounds that do not serve as substrates of cytochrome P-448 are bulky, non-planar molecules characterised by small area/depth ratios and greater flexibility in molecular conformation Specific inhibitors of cytochrome P-448 whose effect is mediated through interaction with the haem still meet the dimensional criteria for substrates indicating that they must also interact with the substrate binding-site, which is probably located in proximity to the haem Inducers of cytochrome P-448 activity exhibit similar molecular geometries to the substrates from which it may be inferred that the cytosolic receptor associated with the induction of cytochrome P-448 activity is structurally related to the active site of the cytochrome

The cytochrome P-450-dependent mixed-function oxidase system is unique, not only because of its biological ubiquity but also because of its capacity to effect the metabolism of numerous different chemicals, both endogenous and exogenous, having no apparent common structural or physico-chemical characteristics. The broad-specificity of the mixed-function oxidase system is due to the existence of families of cytochrome P-450 proteins exhibiting different substrate specificities [1]. There are at least four major gene families or classes of cytochromes P-450, two of which have been extensively studied [2, 3], namely the 3-methylcholanthrene (MC)-inducible forms and the phenobarbitone (PB)-inducible forms. Because of the diverse and confusing systems of nomenclature of the cytochromes currently in use, we prefer to distinguish these two classes by their original names of "cytochrome P-450" for the PB-inducible forms, and "cytochrome P-448" for the MC-inducible forms The cytochromes P-450 occur in relatively few tissues, mostly in the liver and metabolise non-planar, bulky, more hydrophilic compounds, whereas the cytochromes P-448 occur in almost all mammalian tissues and metabolise planar, aromatic, highly lipophilic chemicals [2]. The complete nucleotide sequences of rat liver cytochromes P-448 and cytochromes P-450 differ greatly, although some degree of sequence homology between the two families indicate that the two genes have evolved from a common ancestor [4] The two forms of cytochromes P-448 found in all MC-treated animals except the DBA/2J mouse [5] have similar gene organisation and homology in the deduced nucleotide and amino acid sequences, indicating that these two cytochromes constitute a different family from those cytochromes of the P-450 family [6, 7]. The distribution of the various isozymes of the cytochrome is genetically controlled [8], and can also be modified by environmental factors, such as diet and previous exposure to chemicals [1, 9] Many agents selectively induce the synthesis of primarily one form of the cytochrome which thus predominates and confers its own substrate specificity on the enzyme system Drugs such as phenobarbitone induce cytochromes P-450, and polycyclic aromatic hydrocarbons such as 3-methylcholanthrene induce cytochromes P-448 [1]

The cytochromes P-450 and P-448 have markedly different substrate specificities, and exhibit contrasting roles in the metabolic oxygenation of chemicals [10, 11]. The difference in substrate specificity reflects dissimilar substrate binding sites, as has previously been demonstrated by difference spectrophotometry [12]. Cytochrome P-450 possesses one substrate-binding site of broad specificity which can accommodate a diversity of substrates, while cytochrome P-448 possesses a different kind of binding site displaying narrow specificity. This difference in binding site between the two classes of cytochromes is in agreement with the work of Dus [13] who demonstrated homology between the haemopeptides of the various cytochrome P-450 isozymes, but very little homology between the haemopeptides of the cytochromes P-450 and that of cytochrome P-448, the haemopeptides comprise the haem moiety and the substrate-binding site

The substrate 7-ethoxyresorufin provides the most specific means of determining cytochrome P-448 activity, as demonstrated with purified enzyme preparations isolated from the rat [14] and rabbit [15] The specific inhibitor of cytochrome P-448 activity, 9-hydroxyellipticine [15, 16], like ethoxyresorufin, is a planar molecule of large dimensions. Similar observations have been made with other groups of inhibitors such as the benzimidazoles [17] and antimalarial drugs [18] where maximal inhibition of cytochrome

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P-448 activity was obtained with molecules having three of four fused aromatic rings. Furthermore, potent inducers of cytochrome P-448 activity such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [19], 3-methylcholanthrene, β-naphthoflavone, 2-aminoanthracene [10] and ellipticine [20] are also large planar molecules The similarity of the conformations of substrates, inhibitors and inducers of cytochrome P-448 must therefore reflect the dimensions of the active site of this enzyme, and it was considered that an appropriate approach to the characterization of the active site would be a study of the molecular dimensions characteristic of the specific substrates and inhibitors. Conventional methods that have been used for determining the dimensions and conformation of the active site of relatively simple enzymes, by X-ray crystallography of the enzyme in the presence and absence of its substrate, in the solid state, were considered to be highly inappropriate for studies of the active site of the cytochromes P-450/P-448. These membrane-bound enzymes require the presence of cytochrome P-450 reductase and phosphatidylcholine for enzymic activity, and are highly hydrated *in vivo*, with a water molecule possibly being concerned in the sixth ligand to the haem For such a complex enzyme system, it would seem most unlikely that data on the conformation and dimensions of the active site obtained in the solid state would have any validity *in vivo*.

The cytochromes P-450 direct the oxidative metabolism of xenobiotic chemicals primarily to deactivation and detoxication, whereas in contrast, cytochrome(s) P-448 paradoxically direct metabolism towards the formation of toxic reactive intermediates [10]. As cytochrome P-448 leads to increased toxicity it would be advisable to design drugs and other chemicals that act neither as substrates nor inducers of this form. The present study was therefore undertaken to determine the molecular dimensions of the active sites of cytochromes P-448 and P-450 from the molecular geometry of specific substrates, inhibitors and inducers of these enzymes, using computer-graphic techniques, in an attempt to define the molecular dimensions that would be associated with potential toxicity and carcinogenicity of chemicals

EXPERIMENTAL

Crystal data for benzo(*a*)pyrene [21], aflatoxin B_1 [22], cholesterol [23], ellipticine [24], ethylmorphine [25], hexobarbital [26], diphenylhydantoin [27], propranolol [28], dimethylaminoazobenzene [29], 7,8-benzoflavone [30], phenothiazine [31], *trans*-stilbene oxide [32], DDT [33], aldrin [34], theophylline [35], caffeine [36], 2-acetylaminofluorene [37], phenobarbital [38], quinacrine [39], chlorpromazine [40] and benzo(*a*)pyrene-7,8-diol [41] were used to obtain their molecular geometries For chemicals for which no crystal data was available the Modified Intermediate Neglect of Differential Overlap, 3rd version (MINDO/3) method of Bingham *et al* [42] was employed to determine the molecular orbital calculations, the program being obtained from The Quantum Chemistry Program Exchange at Indiana University, molecular orbital calculations were executed at the Control Data Corporation CDC 7600 at the University of Manchester Regional Computer Centre, and graphics on the Prime 750 computer systems at the University of Surrey Molecular graphic plots were obtained using the PLUTO crystallographic package. Molecular graphical plots are viewed both in, and perpendicular to, the molecular plane. It is not known to what extent, if any, substrate molecular conformations change during binding to cytochromes P-450/P-448 in the biophase, but the conformers of the substrates obtained from crystal data or from MINDO/3 optimization are as accurate as can possibly be known with certainty. Furthermore, many of the substrates are conformationally restricted by virtue of their molecular structure in that they contain fused aromatic rings Using molecular graphic display in the form of the space-filled structures by employing van der Waals radii, it has been possible to measure molecular dimensions of the substrates to within an accuracy of ±0 1 Å

RESULTS

Table 1 shows the MINDO/3 optimized dimensions of a variety of substrates, the metabolism of most of which is preferentially or exclusively catalysed by cytochrome P-448. Generally these substrates have large dimensions of width and length but a very small depth, i.e. exhibit a high area/depth (A/D) ratio. The specific substrate ethoxyresorufin has an A/D ratio of 34.1, while that of aldrin, hexobarbitone and ethylmorphine, which are metabolised by cytochrome P-450 but not by cytochrome P-448, have A/D ratios of 10 0, 10.9 and 14.4 respectively. The carcinogens, benzo(*a*)pyrene, 4-aminobiphenyl, dimethylaminoazobenzene as well as the ultimate carcinogen, benzo(*a*)pyrene-7,8-diol, which are preferentially activated to mutagens by cytochrome P-448 [43–45] have a small depth and an A/D ratio of over 20 The pyrolysis products of amino acids, Trp-P-1, Glu-P-1 and 2-amino-3-methyl-α-carboline, whose activation to mutagens is also catalysed by cytochrome P-448 [46–48] also exhibit A/D ratios of over 20. Phenacetin, the activation and *N*-hydroxylation of which is catalysed exclusively by cytochrome P-448, has an A/D ratio of 23 3 [49] Ethoxycoumarin, whose deethylation is catalysed by both cytochromes P-450 and P-448 [50], has an A/D ratio of 17.7, above those ratios observed for specific substrates of cytochrome P-450, such as aldrin and hexobarbitone [51, 52] and well below those specific for cytochrome P-448 such as ethoxyresorufin Surprisingly, benzphetamine, a substrate whose *N*-demethylation is not catalysed by cytochrome P-448, displays a relatively large A/D ratio of 18 9 However, the aromatic hydroxylation of benzphetamine is selectively induced by 3-methylcholanthrene indicating that it may also serve as a substrate for cytochromes P-448 [53].

A similar picture emerges when inhibitors of cytochrome P-448 activity are considered (Table 2). 9-Hydroxyellipticine [16] has a large A/D ratio of 29 2, in contrast to metyrapone and phenylimidazole which act as inhibitors of other forms of cytochrome P-450 [54, 55] but not of cytochrome P-448, and exhibit A/D ratios of 13 6 and 10 7 respectively

Table 1. Molecular dimensions of aromatic substrates for cytochromes P-450 and P-448

Molecule	Length (Å)	Width (Å)	Depth (Å)	Area/Depth (Å)	Substrate for cytochrome P-448	References for enzyme activity
Dibenzo(*a*,*h*)anthracene	15 9	9 3	3 2	46 2	+*	74
Benzo(*a*)pyrene	13 6	9.0	3 2	38 3	+	43
Ethoxyresorufin	14 6	9 1	3 9	34 1	+	14,15
Dimethylaminoazobenzene	15.1	6 6	3 2	31 1	+	45
2-Aminoanthracene	12 9	7 3	3 2	29 5	+	75
4-Aminobiphenyl	13 8	7.4	3 6	28.4	+	44
Trp-P-1	12 6	9 1	4 2	27 3	+	46
Propranolol	16 1	9 4	6 0	25 2	+	76
Glu-P-1	12.2	8 0	4.0	24 5	+	47
Phenacetin	14 1	8 9	5 4	23 3	+	49
2-Acetylaminofluorene	14.4	7 3	4 6	22 9	+	77
Benzo(*a*)pyrene-7,8-diol	13 6	9 0	5 5	22 3	+	43
Cholesterol	19 0	7.5	6 4	22.3		
2-Amino-3-methyl-α-carboline	12 2	7 6	4.2	22.0	+	48
Zoxazolamine	11 1	7 1	3 6	21 9	+	78
Methoxyresorufin	12 0	7 3	4 1	21 4	+	79
Aflatoxin	12.3	10 6	6 4	20 4	+	80
Caffeine	10 0	8 5	4.2	20 2	+	81
Theophylline	10.0	8 5	4 2	20 2	+	82
Paracetamol	11 6	7.3	4 2	20 2	+	83
Benzphetamine	15 5	7 1	5.8	18 9	+	67
7-Ethoxycoumarin	11 9	7.0	4 7	17 7	+	50
Azaprocarbazine	12 4	8 1	5.9	17 0	+	84
Ethylmorphine	11 5	9 3	7.4	14 4	−	67
Hexobarbitone	9 0	7 5	6 2	10 9	−	52
Aldrin	10 0	9 0	9 0	10 0	−	51

* These chemicals are metabolised by cytochrome P-448 but may also serve as substrates of other forms of the cytochrome

Quinacrine, a specific inhibitor of cytochrome P-448 [18] has a small A/D ratio of 16 0, largely the result of a very large depth.

Table 3 shows the molecular dimensions of mixed-function oxidase-inducing agents that induce selectively cytochrome P-448 and other forms of cytochrome P-450. It is once again evident that model inducers of cytochrome P-448 such as 3-methylcholanthrene, β-naphthoflavone and TCDD [19, 56, 57] have a very large A/D ratio of over 20, while typical inducers of the phenobarbital form of the haemoprotein such as phenobarbitone, diphenyl hydantoin and DDT [57–59] exhibit very small A/D ratios of less than 10. Inducers of the cytochrome P-450 form that preferentially catalyses the oxidation of ethanol and benzene such as benzene and imidazole [60, 61] and the model inducer of cytochrome P-452, clofibrate [62] exhibit A/D ratios of about 15, i.e intermediate to those of cytochrome P-448 and cytochrome P-450

Figure 1 shows side-views of 9-hydroxyellipticine, ethoxyresorufin and dibenz(*a*)anthracene, specific inhibitor, substrate and inducer respectively of cytochrome P-448 while Fig. 2 shows side-views of metyrapone, hexobarbital and phenobarbitone which act as specific inhibitor, substrate and inducer respectively of cytochrome P-450. In Fig. 3 the structures of (*a*) benzo(*a*)pyrene and ethoxyresorufin, (*b*) benzo(*a*)pyrene and paracetamol, and (*c*) benzo(*a*)pyrene and 9-hydroxyellipticine are overlayed and viewed perpendicular to the molecular plane

DISCUSSION

Four immunologically distinct forms of microsomal cytochrome have been purified from livers of rats treated with phenobarbitone (the low-spin major PB_1 and minor PB_2), and 3-methylcholanthrene (the high-spin major MC-1 and minor MC-2); PB-1 and

Table 2 Molecular dimensions of inhibitors of cytochromes P-450 and P-448

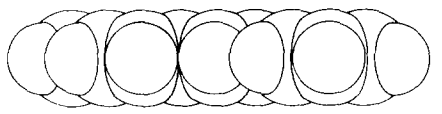
Molecule	Length (Å)	Width (Å)	Depth (Å)	Area/depth (Å)	Cytochrome specificity	References for enzyme inhibition
9-Hydroxyellipticine	13 5	9 1	4 2	29.2	P-448	16
Quinacrine	14 8	12 1	11 2	16.0	P-448	18
SKF-525 A	14 9	7 3	6 7	16 2	P-450	85
Metyrapone	13 4	6 8	6 7	13 6	P-450	54
Phenylimidazole	10 5	6 7	6 6	10 7	P-450	55

Table 3 Molecular dimensions inducers of cytochromes P-450 and P-448

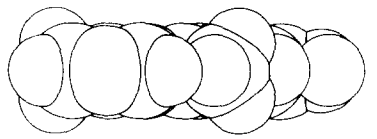
Molecule	Length (Å)	Width (Å)	Depth (Å)	Area/depth (Å)	Cytochrome P-448 inducer	References
Ellipticine	13 5	9 1	3 8	32 3	+*	20
3-Methylcholanthrene	14 6	8 6	4 0	31 4	+	56
TCDD	13 8	7 4	3 6	28 4	+	19
β-Naphthoflavone	13 5	8 3	5 3	21 1	+	57
Rifampicin	18 6	13 8	14 0	18 3	–	10
Phenothiazine	11 5	7 2	4 5	18 1	+	86
Chlorpromazine	11 4	6 2	3 9	18 1	+	86
Benzene	7 4	7 4	3 2	17 1	–	60
p-Xylene	9 0	6 8	4 2	14 6	–	87
Trans-stilbene oxide	13 5	6 9	6 6	14 1	–	88
Clofibrate	14 8	6 7	7 1	14 0	–	62
Chlordane	11 1	9 8	8 1	13 4	–	85
Imidazole	6 6	6 0	3 3	12 0	–	61
DDT	13 6	8 1	11 5	9 6	–	59
Phenobarbitone	10 1	7 3	8 1	9 1	–	57
Diphenylhydantoin	11 4	7 1	9 0	9 0	–	58

* Agents induce primarily cytochrome P-448 but may also induce other forms to a lesser extent

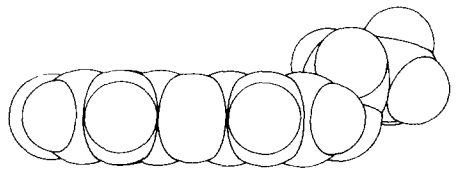
PB-2 were not induced by methylcholanthrene, and MC-1 and MC-2 were not induced by phenobarbitone [63] Recent studies employing recombinant DNA techniques have provided indisputable evidence that cytochrome P-448 (MC-1), although related to, is a different haemoprotein from the cytochromes P-450 (PB-1 and other forms) [4, 64, 67]. Apoenzyme and haemoprotein homologies between cytochrome P-448 and cytochrome P-450 are low, but are very high among the forms of cytochromes P-450 [6, 13] The two classes of enzymes possess distinctly different substrate-binding sites [12], are under different mechanisms of regulation, are encoded by distinctly different m-RNAs [64], and their development and inducibility with age follow contrasting patterns [66]



Dibenz (a, h) anthracene

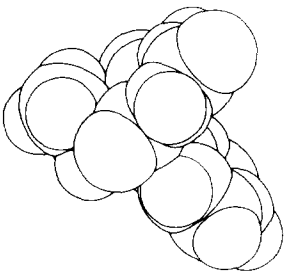


9-Hydroxyellipticine

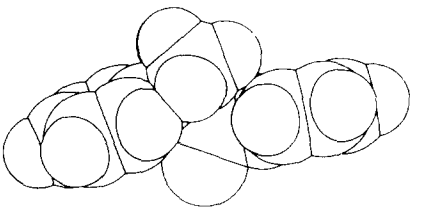


7-Ethoxyresorufin

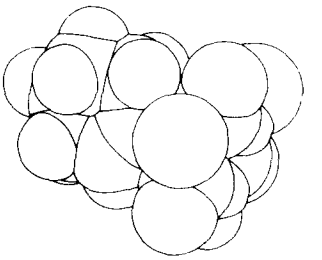
Fig 1 Space-filled models of compounds exhibiting high specificity for cytochrome P-448 Models were drawn using the PLUTO computer program and utilising the following Van der Waals radii to generate computer graphical plots of molecular geometries, carbon 1 6 Å, nitrogen 1 5 Å, oxygen 1 4 Å and hydrogen 1 2 Å



Phenobarbital



Metyrapone



Hexobarbital

Fig 2 Space-filled models of compounds exhibiting high specificity for cytochrome P-450 Legend as in Fig 1

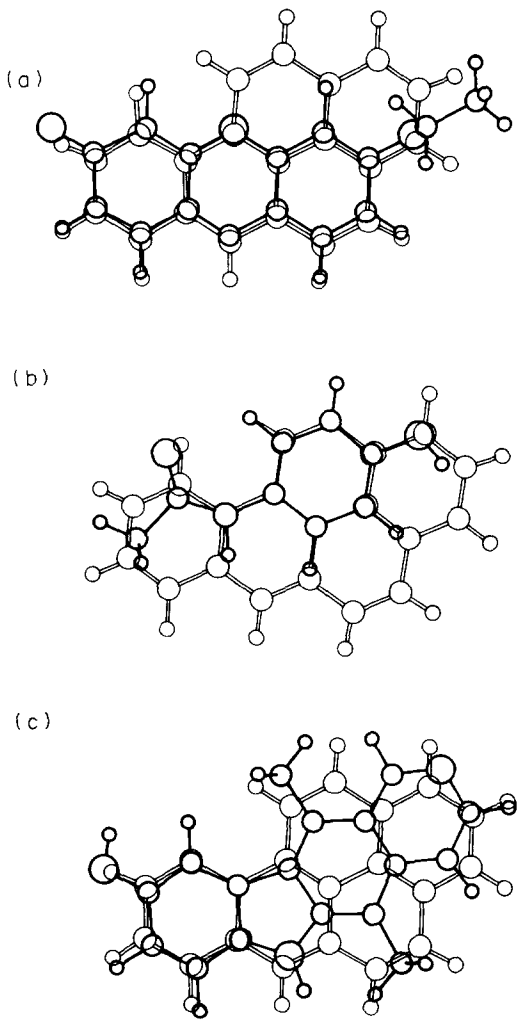


Fig 3 Ball and spoke models of compounds displaying high specificity for cytochrome P-448 (a) ——, Ethoxyresorufin, ═══, benzo(*a*)pyrene (b) ——, paracetamol, ═══, benzo(*a*)pyrene (c) ——, 9-hydroxyellipticine, ═══, benzo(*a*)pyrene All models were drawn using the PLUTO computer program and the following atomic radii, carbon 0 3 Å, nitrogen 0 35 Å, oxygen 0 4 Å and hydrogen 0 2 Å

Substrates of cytochrome P-448 such as 7-ethoxyresorufin exhibit an A/D ratio two to three times that of substrates of cytochromes P-450 such as aldrin, ethylmorphine and hexobarbitone These observations lend support to our previous spectral studies where hexobarbitone failed to generate a type I spectral interaction with purified cytochrome P-448, indicative of no enzyme–substrate interaction, while such a spectrum was readily obtained with purified cytochrome P-450 [12]. It is evident that cytochrome P-448 substrates are essentially planar, rigid molecules with large molecular dimensions and large A/D ratios, but with small depths. This is seen when the side-views of ethoxyresorufin, 9-hydroxyellipticine and dibenz(*a*)anthracene are considered and compared to those of phenobarbital, metyrapone and hexobarbital (Figs 1 and 2) Ethoxyresorufin can be seen to have a smaller depth than hexobarbital, a drug substrate not metabolised by cytochrome P-448 [67] One of the most striking features is the ability to overlay one molecule on the plane of another substrate, e.g benzo(*a*)pyrene and ethoxyresorufin, and benzo(*a*)pyrene and paracetamol (Fig 3). The features similar to all cytochrome P-450 substrates, but not found in those of P-448, are molecular non-planarity, small A/D ratios and greater flexibility in molecular conformation.

Inhibitors of cytochrome P-448 activity show a similar pattern. 9-Hydroxyellipticine has a large A/D ratio, in contrast to metyrapone and phenylimidazole, which are inhibitors of other forms of cytochrome P-450 but not cytochrome P-448 Indeed, 9-hydroxyellipticine can be overlayed on the plane of the cytochrome P-448 substrate benzo(*a*) pyrene (Fig. 3) Quinacrine, although a specific inhibitor of cytochrome P-448 exhibits a relatively small A/D ratio (Table 2), presumably because of the bulky amino substituent which results in it having a depth of 11 2 compared to that of 4 2 for 9-hydroxyellipticine. However, in the absence of the bulky side chain the molecule has a width, length and depth of 7.1, 14.9 and 4.3 Å giving it an A/D ratio of 24.6 It is likely that the side chain of quinacrine is not involved in its interaction with the cytochrome, which it presumably approaches by the pyridinic nitrogen Inhibitors of mixed-function oxidase activity may act by binding at the active site or by liganding to the haem moiety of the cytochrome preventing the binding and activation of oxygen and thus oxygenation of the substrate. With crude microsomal preparations or purified forms of the cytochromes these liganding inhibitors yield type II spectral changes, indicative of an interaction with the haem [12]. It might therefore have been expected that these inhibitors would not exhibit the molecular dimensions that are necessary for binding to the substrate active site. The observation that these inhibitors whose effect is mediated through interaction with the haem, still meet the dimensional criteria for substrates, indicates that they may also have a second site of interaction with the cytochromes, namely the active site Indeed the inhibitor 9-hydroxyellipticine interacts also with the substrate binding site of the cytochromes and the generated spectrum has a type I component indicative of such an interaction [68] Moreover, following its inhibitory phase, 9-hydroxyellipticine acts as an inducer of cytochrome P-448 activity providing further evidence for an interaction with the substrate binding site Studies with methylenedioxyphenyl compounds, such as safrole and isosafrole, have demonstrated that following metabolism, by either cytochromes P-448 or P-450, these are converted to intermediates which ligand to the haem to form complexes; these complexes can be dissociated only by type I substrates from which it may be inferred that an interaction with the active site must occur for the haem ligand complex to dissociate [69, 70] These findings indicate that the active site of cytochrome P-448 is probably located in proximity to the haem moiety, so that inhibitors liganding to the haem must also

meet the dimensional requirements for type I substrates

With inducers of cytochromes P-450 and P-448 similar differences are again evident. Inducers of cytochrome P-448 such as 3-methylcholanthrene, β-naphthoflavone and TCDD exhibit markedly higher A/D ratios than phenobarbitone and DDT Our findings agree with previous work [71] where coplanar, in contrast to non-coplanar isomers of hexachlorobiphenyl, induce cytochrome P-448 as measured by increased ethoxyresorufin *O*-deethylase activity. Rifampicin, a specific inducer of cytochrome P-450 has a relatively large A/D ratio, however, its large depth of 14 0 prevents it from fitting into the cytochrome P-448 active site. It is interesting that inducers of the ethanol form of cytochrome P-450 such as benzene and imidazole, and clofibrate, an inducer of cytochrome P-452, have A/D ratio values between those of cytochromes P-450 and P-448 It may therefore be possible to categorise the various cytochrome P-450 isoenzymes by computergraphic studies of their specific substrates, inducers and inhibitors

Induction of cytochrome P-448 activity appears to be dependent on an interaction between the inducing agent and a cytosolic binding protein receptor which has been isolated from liver and other tissues [72]. An excellent correlation exists between the avidity with which a chemical interacts with this receptor and its capacity to induce cytochrome P-448 activity It can therefore be inferred that the cytosolic receptor also accepts only planar molecules having large A/D ratios and small depths, and it is reasonable and attractive to speculate that the cytosolic receptor may be structurally related to the haemopeptide (active site moiety) of cytochrome P-448

In conclusion, the present study confirms that the substrate binding of cytochrome P-448 is distinct and markedly different from that of cytochromes P-450 It accepts only planar molecules having large dimensions and large A/D ratios, but with small depths. Since metabolism by cytochrome P-448 invariably leads to increased toxicity, and to the activation of carcinogens, an appreciation of the dimensions of its active site will enable the design of chemicals that cannot act as substrates and do not induce cytochrome P-448 activity, and therefore are unlikely to be associated with carcinogenicity or frank toxicity A similar approach has been employed successfully in the production of triazole fungicides that inhibit the cytochrome P-450-dependent 14α-demethylation of 24-methylene-24,25-dihydrolanosterol [73].

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