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COMMENTARY

THE CYTOCHROMES P-448—A UNIQUE FAMILY OF ENZYMES INVOLVED IN CHEMICAL TOXICITY AND CARCINOGENESIS

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The ubiquitous cytochromes P-450, the terminal oxygenases of the mixed-function oxidase systems of bacteria, yeasts, plants, insects, fish and other vertebrates, catalyse the oxidation, and reduction, of numerous substrates, both endogenous and exogenous, of widely diverse chemical structure. Most studies of this family of enzymes have been conducted in mammalia, in which the cytochromes are to be found in the endoplasmic reticulum (microsomes), mitochondria and the nuclear envelope, and are involved in the biosynthesis of cholesterol, the steroid hormones, bile acids and prostanoids, and the oxidative metabolism of fatty acids, lipophilic drugs and other chemicals. The mitochondrial cytochrome P-450 may function with adrenodoxin, ferredoxin or cytochrome P-450 reductase as the electron donor system, but the microsomal and nuclear cytochromes P-450 function only in the presence of the P-450 reductase [1]. The present review concentrates on the extensively studied cytochromes P-450 of the endoplasmic reticulum.

It was originally considered that the wide substrate specificity of the microsomal mixed-function oxidases was attributable to a single cytochrome P-450 haemoprotein, although early studies had indicated that at least two different forms could be induced by treatment of animals with the drug phenobarbitone (PB), (cytochrome P-450), or with the carcinogenic polycyclic aromatic hydrocarbon, 3-methylcholanthrene (MC), (cytochrome P-448). For several years it was considered that cytochrome P-448 was probably merely an adduct of cytochrome P-450 with the inducing polycyclic hydrocarbon, or its metabolite, but solubilisation of this membrane-bound enzyme system, and isolation of discrete haemoproteins associated specifically with cytochrome P-450 and cytochrome P-448 activities, have shown these to be distinctly different enzymes. A number of related haemoproteins, both constitutive and inducible, have now been isolated and purified, and their structures determined. The individual proteins exhibit overlapping specificity in respect to substrate and the type of oxygenation effected, but they also possess varying degrees of selectivity for certain substrates and reactions catalysed. The natural endogenous substrates of the cytochromes P-450 are considered to be steroids, sterols and fatty acids, but several constitutive forms of this family of enzymes, active in the metabolism of steroids, also exhibit oxygenation of a number of xenobiotic chemicals. Cytochrome P-450 proteins are therefore involved simultaneously in the metabolism of endogenous substrates and of structurally-unrelated xenobiotics.

The cytochrome P-450-mediated mixed-function oxidation of xenobiotics (drugs, pesticides, industrial chemicals, etc.) is concerned primarily with detoxication, namely, the formation of more polar, biologically-inactive, readily-excretable metabolites. However, the same microsomal enzyme system can also catalyse the oxidative formation of reactive intermediates and ultimate carcinogens, leading to covalent binding, DNA damage, mutations and malignancy, and other pathological processes. Gelboin [2] has described this catalysis of the opposing pathways of detoxication and activation of chemical carcinogens by the same family of enzymes as the paradox of chemical carcinogenesis and cancer. However, chemical carcinogenesis has long been associated with the specific induction of cytochrome P-448 [3] and, recently, it has been shown that the cytochrome P-448 family of enzymes is specifically concerned with the activation of chemicals to reactive intermediates, and the formation of mutagens and carcinogens [4]. The biogenesis of reactive intermediates and ultimate carcinogens is thus the result of these two competing processes of detoxication and activation, catalysed by different gene families of the haemoprotein, so that not only the cellular levels of total cytochrome P-450, but also the constituent isozyme population, will determine whether a chemical will be metabolised by activation to toxic reactive intermediates or be detoxicated and subsequently eliminated.

It has been suggested recently that there are at least four distinct gene families of cytochromes P-450 [5]. The two most extensively studied are those cytochromes whose prototype inducing agents are phenobarbitone and the carcinogenic polycyclic aromatic hydrocarbons. These two families have been solubilised, isolated and purified in a number of laboratories, and the only obstacle in comparing the various preparations is the confusing proliferation of nomenclature. In the present review the PB-inducible forms are referred to as "PB-cytochromes P-450" (P-450b and P-450e of rat), and the polycyclic aromatic hydrocarbon-inducible forms are referred to in their original terminology as "cytochromes P-448" (P-450c and P-450d of the rat). Although we

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appreciate that this terminology may be considered imprecise, it is nevertheless widely used and is necessary to distinguish between two distinctly different gene families of these cytochromes. It has now been established that rat P-450c and P-450d correspond to forms LM_6 and LM_4 of rabbit, and to P_1-450 and P_3-450 of mouse, respectively [6].

Similarities and differences between PB-cytochromes P-450 and cytochromes P-448

Similarities. These two gene families, although distinctly different from each other, do share some common characteristics. Both families of cytochromes occur at very low levels in livers of untreated animals and comprise less than 5% of the constitutive population of microsomal cytochromes in normal rat liver [7–9]. They are induced, by as much as 70-fold, by the appropriate inducing agents [10–12]; they may comprise as much as 80% of the total microsomal cytochrome P-450 content in the induced animal. Inducers of both of these families similarly induce a minor form of the haemoprotein, namely cytochrome P-450a [13], which exhibits high regioselectivity catalysing the 7α-hydroxylations of androstenedione, progesterone and testosterone [14, 15]; the induction is sex-dependent, and activity is increased by PB and MC only in female rats and in immature males [11].

Induction of one of these two families of cytochromes may suppress the already low levels of the other, and treatment of rats with MC decreases the levels of P-450b and other PB-specific forms in untreated animals, or the higher levels encountered in PB-treated animals [16–20], while administration of the mixed P-450/P-448 inducer, the polychlorinated biphenyl Aroclor 1260, simultaneously with phenobarbitone, decreases the amount of phenobarbital-inducible LM_2 in rabbit liver microsomes [21]. Both classes of inducing agent are known to induce the *de novo* synthesis of several distinct cytochrome P-450 proteins. PB induces P-450a, P-450b and P-450e, a fourth haemoprotein which is also induced by the synthetic steroid pregnenolone 16α-carbonitrile (PCN) [22] and by macrolide antibiotics [23], a fifth isoenzyme which is also present in untreated animals [24] and, probably even more, as preparations considered to be homogeneous on the basis of immunoprecipitation and limited NH_2-terminal sequence analysis were found to comprise three distinct proteins [25]. Similarly, treatment of rats with the cytochrome P-448 inducer, β-naphthoflavone, results in isolation from liver of five antigenically-distinct forms of the cytochrome exhibiting different catalytic activities towards a number of substrates [26] of which the two major forms are P-450c and P-450d [12]. More recently, Seidel and Shires [27] have isolated four isozymes of the cytochrome from liver of rats treated with MC; in addition to P-450c, P-450d and P-450a, a novel form (P-450 MC-B) with a unique N-terminal amino acid sequence was found. Generally it appears that there are more PB-P-450 proteins than cytochromes P-448 [28].

Differences in induction. Despite these similarities, there is unequivocal evidence, based on a number of criteria, that the PB-P-450 and P-448 cytochromes, although probably evolved from a common ancestor, are distinctly different gene families [29], which are selectively induced by different chemicals (see Table 1). PB-P-450 activity is enhanced by a variety of chemicals, in addition to PB, including DDT, γ-chlordane, SKF-525A, *trans*-stilbene oxide, and some non-planar polyhalogenated biphenyls [30]. In contrast, the cytochromes P-448 are induced by planar molecules such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), β-naphthoflavone, polycyclic aromatic hydrocarbons [4, 12, 31], planar polyhalogenated biphenyls [30], aminoazobenzenes [32] and, to a lesser extent, by aromatic amines [4, 33] and amides [34, 35]. Some inducing agents, such as the methylenedioxyphenyl compounds, safrole and isosafrole [12, 36], numerous polyhalogenated biphenyls (Aroclors) [30], and the drugs chlorpromazine and phenothiazine [12] act as mixed-type inducers, increasing the activities of enzyme associated with both families of cytochromes.

Differences in distribution. The hepatocellular distributions of PB-cytochromes P-450 and cytochromes P-448 also show differences. Both families are encountered in the centrilobular hepatocytes, but the cytochromes P-448 show greater concentration in the periportal zones while the PB-cytochromes P-450 are much higher in the centrilobular regions [37, 38]. It is significant that epoxide hydrolase and glutathione transferase activities are greatest in the centrilobular regions, where the PB-cytochromes P-450 have the highest concentration, and are lowest in the periportal regions, where cytochromes P-448 have their highest concentration [38], thus supporting the association of PB-cytochrome P-450-mediated oxygenations with subsequent conjugation and detoxication and the deficiency of these latter in the cytochrome P-448-mediated oxygenations.

Differences in immunological properties. Immunological studies have also defined the differences between the cytochromes P-448 and the PB-cytochromes P-450. Polyclonal antibodies against the purified proteins were the first tools to show the differences of the two gene families. The two major forms of the cytochromes P-448 isolated from the liver microsomes of rats treated with MC each cross-react with antibodies against the other [39] and, similarly, some cross-reactivity occurs between antibodies against the cytochrome P-450d induced by 3,4,5,3′,4′,5-hexachlorobiphenyl and the cytochrome P-450c induced by MC [9]. However, despite their antigenic similarity, these two forms of cytochromes P-448 differ in their substrate specificities, molecular weights, chromatographic properties and peptide maps [40]. In contrast to the two cytochrome P-448 forms, the two major PB-inducible cytochromes P-450b and P-450e show remarkable similarity, both immunologically and in substrate specificities, but differ in rates of hydroxylation and their electrophoretic profiles following digestion, emphasising their minor structural differences.

Isolation of a series of monoclonal antibodies to the PB-cytochromes P-450 and cytochromes P-448 allowed not only identification of the proteins within one gene family, but also further highlighted the differences between these two gene families. Of nine monoclonal antibodies prepared against cytochrome

P-450c, three cross-react strongly with cytochrome P-450d, showing the presence of common epitopes, but none cross-react with cytochromes P-450b and P-450e [41]. Similarly, of twelve monoclonal antibodies prepared against cytochrome P-450b, ten also recognise cytochrome P-450e, but none cross-react with cytochromes P-448 [42].

Characterisation of the various cytochrome P-450 isozymes by immunological techniques may not always have the inferred specificity. Although the two cytochrome P-450c proteins isolated from the livers of rats treated with MC or β-naphthoflavone have identical immunological properties, electrophoresis patterns after proteolytic digestion, and the same subunit molecular weights, they display similar, but not identical, catalytic activities towards benzo[*a*]pyrene, thus indicating that although these two cytochromes P-450c are structurally very similar, they may be two distinct enzymic proteins [43].

Differences in amino acid sequences. The primary amino acid sequences of cytochromes P-448 and PB-cytochromes P-450 have been deduced and, although a high homology of 68% has been observed between cytochromes P-450c and P-450d [44–46] with identical sequences for the 18 NH_2-terminal amino acids [39], the homology between cytochrome P-450c and the PB-inducible cytochromes P-450b and P-450e is only 30% [46, 47]. Similarly, the MC-induced murine cytochromes, P_1-450 and P_3-450, show an amino acid sequence homology of 73% and exhibit a 93% homology with rat cytochromes P-450c and P-450d, respectively, but homology with rat PB cytochrome P-450e and rabbit LM_2 is only 15% [6]. The PB-inducible cytochromes P-450b and P-450e share an even higher homology, of more than 97%, differing only in 13 amino acids in a 491 amino acid sequence [47–49] and possessing identical NH_2-terminal sequences for the first 302 amino acids.

Differences in mRNA and cDNA. Differences are also seen in the mRNAs, which are compatible with the differences in amino acid structures of these two families of cytochromes. In rabbit, the mRNAs concerned in the expression of three genes associated with the PB-cytochromes P-450 showed 72–90% homology [50]. In mouse, the mRNAs associated with cytochromes P_1-450 and P_3-450 (cytochromes P-448) show homology of at least several hundred base pairs [51]. In rat, MC-induced mRNAs coding for three cytochrome P-450 peptides, and those that coded for cytochrome P-450c and P-450d were not induced by PB, were of different sizes, and shared homologous regions as well as having unique regions [52], whereas two PB-inducible mRNAs were of similar size and differed only in a few bases [48, 49, 53].

The DNA nucleotide sequences concerned with the genetic information for these two families of cytochromes also show marked differences. Genomic blot analysis has shown that the MC-inducible cytochrome P-448 proteins are smaller than those induced by PB [47, 54–56]. The cDNA sequences of the MC-inducible murine cytochromes P_1-450 and P_3-450 share 68% homology [6]; the two genes are located on the same chromosome [57], display remarkable similarity in the intron–exon patterns [58], and are regulated by the same cytosolic receptor protein [59], indicating that these two genes belong to the same family. The cDNA sequences of rat P-450c and P-450d show 75% homology, whereas rat P-450c shows 86% homology with mouse P_1-450 and that of rat P-450d shows 88% homology with murine P_3-450 [6, 46]. Human liver contains at least two cytochromes P-448, showing 80% homology with each other, which are related to the TCDD-inducible cytochromes P-448 of mice (P_1-450 and P_3-450) and rabbit (LM_6 and LM_4), respectively, with which they show 70–80% homology [60]. The nucleotide sequences of rat P-450c and P-450d show high homology particularly in the carboxyl-terminal regions and show 70% homology with murine P_3-450 [56]. In contrast, the nucleotide sequences of the murine cytochromes P_1-450 and P_3-450 show only 30% homology with the cDNAs coding for the PB-induced rat P-450e and rabbit LM_2 [6, 45, 61], and the gene structures for P-450c and P-450d differ greatly from those of P-450b and P-450e [29, 46].

However, although the gene structures for the PB-cytochromes P-450 and cytochromes P-448 families differ greatly, the degree of sequence homology that is common to the two types of cytochrome indicates that the two gene families have evolved from a common ancestor [29].

Differences in regulatory control. Cytochromes P-448 are induced simultaneously by a number of xenobiotics, although the levels of P-450c and P-450d are increased to different relative extents by the various inducing agents, e.g. 3,4,5,3′,4′,5′-hexachlorobiphenyl and safrole primarily induce P-450d whereas MC and β-naphthoflavone induce primarily P-450c [12], indicating that they are not under coordinate control. The mRNAs for P-450c and P-450d have different apparent induction kinetics and different stabilities, confirming that they are not under coordinate regulatory control [52, 62]. Moreover, when TCDD is administered to pregnant rabbits, LM_6 is induced in the neonate, whereas in the adult the predominant form is LM_4, although LM_6 is also induced [63]. In contrast, the PB-inducible cytochromes P-450b and P-450e are induced simultaneously, the levels of P-450b being greater, but the two enzymes are under coordinate regulatory control [18, 19].

Differences in substrate specificity and enzyme activity sites. Perhaps the most notable differences in these two families of cytochromes are seen in the differences in their preferred substrate and in their distinctly different substrate binding sites. Spectral studies have revealed that pretreatment with PB increases the magnitude of the Type 1 spectral interaction between hepatic microsomes and substrates such as hexobarbital, benzphetamine or perhydrofluorene, while pretreatment with cytochrome P-448-inducing agents such as MC or benzo[*a*]pyrene either decreases or completely abolishes this spectral interaction but enhances the Type II spectral interactions seen with PB [64, 65]. Similar observations were made with purified rabbit proteins LM_2 and LM_4 instead of crude microsomes [65]. The PB-cytochromes appear to possess one substrate binding site of broad specificity which can accommodate a diversity of substrates, while, in contrast, cytochromes P-448 possess a substrate binding site which

exhibits narrow specificity [65]. This concept is supported by the work of Dus [66] who demonstrated homology between the excised haemopeptides (comprising the substrate binding site, plus haem moiety) of various PB-cytochromes P-450, but these shared only little homology with a cytochrome P-448 haemopeptide.

Chemicals which interact with cytochromes P-448, such as 7-ethoxyresorufin, 9-hydroxyellipticine and MC, are essentially rigid, planar molecules, whereas compounds that interact with the PB-cytochromes P-450, but elicit no interaction with cytochromes P-448, are non-planar, bulky molecules [67]. The importance of the planarity of chemicals in their interaction with cytochromes P-448 is further supported by extensive studies using polyhalogenated biphenyls as probes [68]. Those polychlorinated biphenyls exhibiting maximum coplanar conformation, i.e. substituted in the meta- and para-positions, preferentially induce cytochromes P-450c and P-450d, those with less planarity, i.e. substituted also in the ortho-position, exhibit induction of both PB-cytochromes P-450 and cytochromes P-448, whereas those with two or more ortho-substituents and unsubstituted in a vicinal para-/meta-position are non-coplanar and exhibit induction only of the PB-cytochromes P-450 [68].

A further instance of the significance of the planarity of chemical substrates is that the UDP-glucuronyltransferases that are selectively induced by MC also preferentially conjugate planar substrates, such as *p*-nitrophenol, 4-methylumbelliferone and 4-hydroxybenzo[*a*]pyrene [69, 70], indicating that all MC-inducible proteins, whether haemoproteins or not, interact predominantly with planar molecules. In contrast, the PB-inducible glucuronyltransferases preferentially conjugate globular molecules, such as morphine, 4-hydroxybiphenyl, hydroxy-diphenylhydantoin and chloramphenicol.

Initial studies aimed at identifying specific substrates for these two families of mixed-function oxidases were largely unsuccessful because of extensive overlap in substrate specificity among the cytochrome P-450 isozymes. As a result, researchers have become sceptical in using such an approach, or continue to use substrates such as benzo[*a*]pyrene or ethoxycoumarin which, although perhaps more favoured for hydroxylation by the cytochromes P-448, are non-specific for this family of enzymes. Furthermore, the continued use of non-specific substrates, such as benzo[*a*]pyrene, has contributed considerably to the failure to appreciate the fundamental differences between the PB-cytochromes P-450 and cytochromes P-448 [71]. However, there is now strong evidence testifying to the use of the O-deethylation of 7-ethoxyresorufin (EROD) to reflect only cytochrome P-448 activity; only cytochrome P-450c, and to a much lesser extent cytochrome P-450d, can catalyse this oxygenation while no other P-450 isozyme has any detectable activity [34, 40, 72].

Metabolic activation of chemicals and carcinogens

Undoubtedly, the most important distinction between the cytochromes P-448 and PB-cytochromes-450, as first observed over twenty years ago [3], is in their contrasting roles in the detoxication/activation of chemicals (Table 1). Cytochromes P-448 appear always to convert relatively innocuous chemicals to reactive electrophiles, which react covalently with cellular macromolecules giving rise to toxicity/carcinogenicity. In contrast, the PB-cytochromes P-450 generally direct the overall metabolism of a chemical towards the formation of inactive metabolites and detoxication (Fig. 1). Different types of chemical carcinogens may be activated by cytochromes P-448, including polycyclic aromatic hydrocarbons, aromatic amines and amides, heterocyclic amines and azo compounds.

Polycyclic aromatic hydrocarbons. These ubiquitous chemicals, the products of combustion, are preferentially metabolised by the cytochromes P-448, and those which are carcinogenic also lead to the induction of this family of cytochromes. Benzo[*a*]pyrene, the most extensively studied chemi-

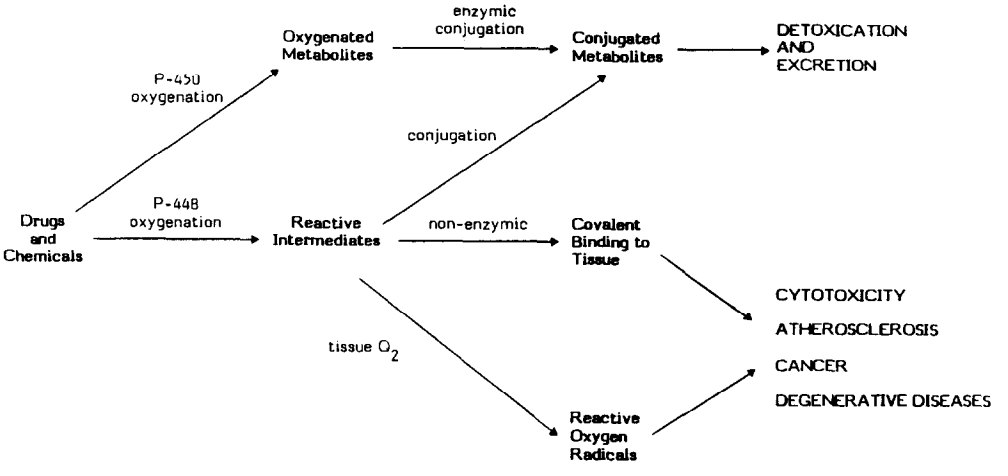

Fig. 1. Role of PB-cytochromes P-450 and cytochromes P-448 in the detoxication and activation of drugs, chemicals and carcinogens.

Table 1. Differences between PB-cytochromes P-450 and cytochromes P-448

Property	PB-cytochromes P-450	Cytochromes P-448
Number of isozymes	At least five.	At least three.
CO-absorption maximum	450 nm	448 nm
Tissue distribution	Present at low levels in most tissues but not detectable in gut and placenta; readily induced in liver but not in other tissues and not inducible in placenta.	Present at low levels in most tissues; inducible in lung, kidney and gut; highly inducible in liver and placenta.
Perinatal development	Low in neonate, increase with age.	Predominate in neonate, decrease postnatally.
Antigenic properties		
Polyclonal and monoclonal antibodies	Cross-reactivity within the family but none with cytochromes P-448.	Cross-reactivity within the family but none with PB-cytochromes P-450.
Structure	High amino acid and nucleotide homology within the family but not with cytochromes P-448.	High amino acid and nucleotide homology within the family but not with PB-cytochromes P-450.
Enzyme induction	Phenobarbitone is inducer, reductase is simultaneously induced. No receptor identified. Inducible in both rats and hamsters. More inducible in neonate. Under coordinate control.	3-Methylcholanthrene is typical inducer, reductase is not simultaneously induced. Cytosolic receptor has been identified. Poorly inducible in hamsters, inducible in rats. More inducible in adult. Not under coordinate control.
Substrate binding site		
Substrate specificity, molecular dimensions of substrates, and regioselectivity	Broad substrate specificity; accept globular and planar molecules with small area/depth ratio and large depth. Cannot oxygenate sterically-hindered positions.	Narrow substrate specificity; accept only planar molecules with large area/depth ratio and small depth. Oxygenate sterically-hindered positions.
Specific substrate	7-Pentoxyresorufin	7-Ethoxyresorufin
Specific inhibitor	Metyrapone	9-Hydroxyellipticine
Bioactivation of carcinogens	Do not activate polycyclic hydrocarbons, aromatic amines, amides, heterocyclic amides or aminoazo compounds to mutagens by C- or N-oxygenations. Activate nitrosamines to mutagens.	Activate polycyclic hydrocarbons, aromatic amines and amides, heterocyclic amines and aminoazo compounds by hindered C- and N-oxygenations, to form mutagens and carcinogens. Do not activate nitrosamines.
Endogenous substrates	Hydroxylate testosterone at 16α-, 16β- and 17-positions. Hydroxylate prostaglandins at the ω- and (ω-1)-positions. Require cytochrome b_5.	Hydroxylate prostaglandins at the (ω-1)- and (ω-2)-positions. No requirement for cytochrome b_5.

cal carcinogen, is metabolically activated to mutagens by liver microsomal S9 preparations from MC-treated rats, whereas similar preparations from phenobarbital-induced animals are much less active [73]. This mutagenesis is inhibited by 9-hydroxyellipticine, a specific inhibitor of cytochrome P-448 activity [74], and also is inhibited markedly by antibodies to cytochrome P-450c but is unaffected by antibodies to cytochrome P-450b [75]. Furthermore, purified cytochromes P-448 are more efficient than PB-cytochromes P-450 in converting benzo[*a*]pyrene and *trans*-benzo[*a*]pyrene-7,8-dihydrodiol to mutagens [76]. This preferential activation of polycyclic aromatic hydrocarbons by cytochromes P-448 applies also to MC, benzo[*a*]anthracene, dibenz[*a*,*h*]anthracene, and dibenz[*a*,*c*]anthracene [75, 77–81].

Aromatic amines. In the metabolism of aromatic amines, the pathway of activation, N-hydroxylation, is catalysed by two hepatic enzyme systems, namely, the FAD-monooxygenase system and the cytochromes P-448. 4-Aminobiphenyl [82, 83] and 2-aminofluorene are readily N-hydroxylated and converted into mutagens by cytochromes P-450c and P-450d but much less readily by cytochromes P-450b and P-450e [78, 83, 84]. 2-Naphthylamine is activated to mutagens more readily by liver microsomal preparations from Aroclor 1254- and MC-induced rats than by PB-induced microsomes [85], and the isoenzyme responsible appears to be cytochrome P-450d [84]. Similarly, the activation of 2-aminoanthracene to mutagenic intermediates is catalysed by rabbit LM_4 but not LM_2 [86]. Despite the structural similarities of cytochromes P-450c and P-450d, they do have different substrate specificities, in that cytochrome P-450d is especially active in the N-hydroxylation of aromatic amines, amides and aminoazobenzenes, whereas P-450c is particularly efficient in forming arene oxides of the polycyclic aromatic hydrocarbons.

Heterocyclic aromatic amine premutagens, formed during the pyrrolysis of proteins and amino acids, are also activated to mutagens by N-hydroxylation catalysed by cytochromes P-448 and not by PB-cytochromes P-450 [87, 88]. Trp-P-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole), Glu-P-1 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole), Trp-P-2 (2-amino-6-methyldipyrido[1,2-α:3′,2′-*d*]imidazole) and IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline) are activated to mutagens by cytochromes P-448, particularly the high spin form cytochrome P-450d [87–90], and the mutagenicity of Trp-P-2 is inhibited 85% by antibodies to cytochrome P-448 but only 8% by antibodies to PB-cytochromes P-450 [44].

Aromatic amides. The aromatic amide, 2-acetylaminofluorene (AAF), like the corresponding amine, is activated to a mutagen/carcinogen by N-hydroxylation catalysed exclusively by cytochromes P-448 [34], which is inhibited 60% by antibodies to cytochrome P-448 but only 35% by antibodies to cytochrome P-450 [75]. As with the aromatic amines, cytochrome P-450d is the most active P-448 isozyme, and cytochrome P-450c is devoid of AAF N-hydroxylating activity [91].

Azo compounds. Similarly, the activation of the azo carcinogens *N*-methyl-4-aminoazobenzene, *N*-dimethyl-4-aminoazobenzene, and 3′-methyl-*N*,*N*-dimethyl-4-aminoazobenzene involves N-hydroxylation catalysed by cytochromes P-448 [92–94]. The N-hydroxylation of *N*-methylaminoazobenzene is catalysed exclusively by the flavoprotein monooxygenase in the untreated rat but by the flavin monooxygenase and cytochromes P-448 in the MC-treated rat [95]; in the MC-treated rat the N-hydroxylation is catalysed by cytochrome P-450d at twice the rate of cytochrome P-450c, although the latter is more active in ring-hydroxylation [93]. In the activation of *o*-aminoazotoluene to mutagens, only cytochrome P-450c is effective [78].

Polyhalogenated hydrocarbons. Hexachlorobenzene and polyhalogenated biphenyls result in porphyria, which is attributed to inhibition of uroporphyrinogen decarboxylase by a metabolite of the halogenated biphenyls formed exclusively by the cytochromes P-448 [96].

Drugs and other chemicals. Other miscellaneous carcinogens, such as aflatoxin [97] and 2-nitrofluorene [78], are also activated preferentially by cytochromes P-448. However, activation of chemicals to toxic intermediates by cytochromes P-448 is not confined to carcinogens, and drugs such as the analgesic paracetamol are similarly activated by rat and rabbit cytochromes P-448 but not by PB-cytochromes P-450 [98, 99]. The naturally-occurring pulmonary toxin, 4-ipomeanol, is also activated by MC-inducible cytochromes P-448 [100].

However, in conflict with the general hypothesis that it is the cytochromes P-448 that specifically activate chemical carcinogens, the nitrosamines are selectively activated and metabolised by the ethanol- and phenobarbital-induced isozymes of rabbit liver [101], and cyclophosphamide is selectively activated to cytotoxic, mutagenic and teratogenic products by PB-cytochromes P-450 [102].

Endogenous substrates. PB-Cytochromes P-450 and cytochromes P-448 also show differences in their hydroxylation of endogenous substrates, such as the steroids, polyunsaturated fatty acids, and prostanoids. Regioselectivity in the site of hydroxylation is again exhibited, e.g. cytochromes P-450c and P-450d hydroxylate testosterone at the conformationally hindered 6β-position, while cytochromes P-450b and P-450e hydroxylate the same substrate in the non-hindered 16α-, 16β- and 17-positions [103]; cytochrome P-450a, induced by both groups of inducing agents, selectively catalyses the 7α-hydroxylation [103]. Regioselectivity by cytochromes P-448 and PB-cytochromes P-450 is also evident in the metabolism of prostaglandins, e.g. LM_2 hydroxylates prostaglandins primarily at the ω- and (ω-1)-positions [104]; in contrast, LM_4 and LM_6 hydroxylate prostaglandins at the (ω-1)-position and form no ω-hydroxylated metabolites, while LM_6 also hydroxylates the (ω-2)-position [105–107].

Mechanisms of activation and chemical carcinogenesis

Although toxic chemicals and carcinogens may be activated by enzymic reduction (e.g. mitomycin C, or reduction of azo compounds to aromatic amines) or by conjugation (e.g. sulphate conjugation of 7-

hydroxymethyl-12-methybenz[*a*]anthracene [108]), the most important of all activation mechanisms in chemical toxicity are those involving oxidations, including C-oxidation (hydrocarbons), N-oxidation (amines), S-oxidation (thioethers), and S-replacement (activation of malathion to malaoxon). This activation of toxic chemicals and carcinogens by biological oxidations may be catalysed by (i) the cytochromes P-448, (ii) the flavoprotein monooxygenases, and (iii) non-enzymic free radical hydroxylations from hydroxyl radicals produced in "redox cycling" of quinones [109] and from transoxygenations resulting from the conversion of HPETEs to HETEs in prostaglandin biogenesis [110].

In the detoxication of drugs, toxic chemicals and carcinogens, the oxidative reactions of Phase 1 metabolism result in the formation of metabolites which subsequently undergo conjugation (Phase 2 metabolism) involving epoxide hydrolase, glutathione transferase, UDP-glucuronyltransferase, etc., to form conjugates, which are readily eliminated, resulting in overall detoxication. In contrast, in the activation of carcinogens and toxic chemicals, oxidations result in the formation of proximate carcinogens or reactive intermediates which are generally poor substrates for the conjugating enzymes and interact non-enzymically with proteins, RNA, DNA and other intracellular constituents, leading to covalent binding, formation of neo-antigens, mutations, malignancy and cell death (see Fig. 1).

Activation of carcinogenic polycyclic aromatic hydrocarbons to their ultimate carcinogens requires oxygenation in bay region positions [111] which, in the case of benzo[*a*]pyrene, involves oxygenation of benzo[*a*]pyrene-7,8-dihydrodiol in the 9,10 bay region position to give the ultimate carcinogen benzo[*a*]pyrene-7,8-diol-9,10-epoxide [76]. Using purified PB-cytochrome P-450 and cytochrome P-448, Levin *et al.* [76] showed unequivocally that the 9,10-epoxidation is catalysed only by cytochrome P-448 and that this bay region epoxide is a poor substrate for epoxide hydrolase.

Activation—A function of molecular shape. The realisation that the detoxication and activation of chemicals involve two different modes of oxygenations, catalysed by two different families of microsomal cytochromes, with markedly different active sites, led us to investigate further the molecular dimensions of the active site of these cytochromes. We used computer graphic techniques and established that the optimal molecular geometries of specific substrates, inhibitors and inducers of the PB-cytochromes P-450 are non-planar, globular molecules characterised by small area/depth ratios, large depth, and flexibility in molecular conformation. In contrast, the specific substrates, inhibitors and inducing agents of the cytochromes P-448 are essentially rigid, planar molecules characterised by a small depth and large area/depth ratios [67] (Table 2). The marked differences in the molecular dimensions of the typical substrates of these two families of enzymes, and hence in the active sites of the two enzymes, offer an explanation for the different modes of oxygenation catalysed by the two different families of cytochromes and hence for the alternative pathways of detoxication and activation. Chemical toxicity may therefore be considered to be a function of the molecular dimensions and spatial conformations of the xenobiotic molecule.

Conformationally-hindered oxygenations discourage subsequent conjugations. The bay region concept of Jerina and Daly [111], although applicable to the oxidative activation of carcinogenic polycyclic aromatic hydrocarbons, needs extension to the hypothesis of conformationally-hindered oxygenations when other classes of carcinogens and toxic chemicals are considered. As the cytochromes P-448 show a preference for rigid, planar molecules, the spatial conformations of the substrates, and the positions and stereochemistry of the oxygenations catalysed, assume considerable importance. The

Table 2. Molecular dimensions of carcinogens, toxic chemicals and drugs and their activation by cytochrome P-448

Chemical	Molecular dimension (area/depth^2)	Preferred cytochrome	Toxicity
Dibenz[*a*,*h*]anthracene	14.4	448	Carcinogen
Benzo[*a*]pyrene	12.0	448	Carcinogen
Dimethylaminoazobenzene	9.7	448	Carcinogen
4-Aminobiphenyl	7.9	448	Carcinogen
9-Hydroxyellipticine	7.0	448	Carcinogen
Trp-P-1	6.5	448	Carcinogen
2-Acetamidofluorene	5.0	448	Carcinogen
Paracetamol	4.8	448	Hepatotoxin at high dosage
Aflatoxin	3.2	448, 450	Hepatotoxin Hepatocarcinogen
Metyrapone	2.0	450	Adrenal suppression
Ethylmorphine	2.0	450	Analgesic, non-carcinogen
DDT	1.9	450	Cholinesterase inhibitor Non-carcinogen
Hexobarbitone	1.8	450	Hypnotic, non-carcinogen
Phenobarbitone	1.1	450	Hypnotic, non-carcinogen

cytochromes P-448, by virtue of the planarity of the enzyme active sites, are able to oxygenate chemicals and carcinogens in conformationally-hindered positions, whereas the PB-cytochromes P-450, with their globular active sites, oxygenate substrates only in unhindered positions.

Thus, the reason for oxygenation resulting in activation of toxic chemicals and carcinogens, in preference to detoxication, is that insertion of oxygen into "conformationally-hindered" positions by cytochromes P-448 results in the formation of oxygenated "reactive intermediates", which are poor substrates for subsequent conjugation and detoxication. In contrast, oxygenation at "conformationally-unhindered" positions of chemicals by cytochromes P-450 is followed by rapid conjugation which results in detoxication. Studies by Oesch and colleagues [112, 113] provide evidence that metabolites oxygenated in conformationally-hindered positions undergo conjugation with epoxide hydrolase or glutathione transferase much less readily than do metabolites oxygenated in conformationally-unhindered positions. Epoxide hydrolase is highly inhibitory to the binding of the non-carcinogenic 9-hydroxybenzo[*a*]pyrene-4,5-oxide to nuclear DNA, but it is not inhibitory to the binding of the ultimate carcinogen benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide [112]. Similarly, the glutathione transferases are much less active (200- to 2000-fold) in decreasing the mutagenicity of the carcinogen/mutagen benz[*a*]anthracene-8,9-diol-10,11-oxide than they are in decreasing the mutagenicity of the less-toxic, non-carcinogenic benz[*a*]anthracene-5,6-oxide [113]. Likewise, the carcinogenicities of 2-naphthylamine, 4-acetylaminobiphenyl and 2-acetylaminofluorene are inversely related to the ease of glucuronidation of their hydroxamic acid derivatives [114]. In contrast, sulphate conjugation of reactive intermediates does occur but results in the further activation of the ultimate carcinogen, e.g. in the activation of estragole [115], 4-aminoazobenzene [116] and 7,12-dimethylbenz[*a*]anthracene [108].

Induction of cytochromes P-448 and chemical carcinogenesis. The cytochromes P-448 are induced by a mechanism involving one or more cytosolic receptors, similar to the mechanism of action of steroid hormones. The inducing agent, or its metabolite, binds to the cytosolic receptor and is translocated into the nucleus where it effects genomal derepression, mRNA and protein synthesis by a mechanism, the details of which are still unknown. From our structural studies it is seen that the overall conformation of the active site of the cytochromes P-448 is essentially the same as that of the cytosolic receptor associated with the regulation of the cytochrome P-448 genome [67]. Hence, chemicals that fit the cytochrome P-448 active site, and consequently are metabolised to reactive intermediates or proximate carcinogens, have molecular structures which may lead to the increased *de novo* synthesis of this activating family of enzymes. Thus, exposure to a chemical carcinogen, or toxic chemical, leads not only to metabolic activation to a proximate carcinogen, but also to an increase of the enzymes catalysing this activating process. However, despite the similarity of the spatial dimensions of substrates and inducers of the cytochromes P-448, not all substrates are effective inducing agents (e.g. paracetamol) and not all potent inducing agents are readily metabolised by the enzyme (e.g. TCDD).

It has been shown recently that inducers of cytochromes P-448, such as TCDD, decrease the binding of epidermal growth factor (EGF) to its receptor, mediated by the increased formation of reactive intermediates of co-administered polycyclic aromatic hydrocarbons [117]. Other carcinogens such as 2-acetaminofluorene and diethylnitrosamine also decrease the binding [118], while glucocorticoids, which inhibit polycyclic aromatic hydrocarbon carcinogenesis, result in the opposite effect, namely, an increase of EGF binding [119]. EGF is one of a number of polypeptide growth factors which act by binding to transmembrane receptors, thereby activating a protein-tyrosine kinase and initiating a pleiotropic growth response which results in DNA transcription, protein synthesis and cell division. EGF and other growth factor receptors function to stimulate cell growth, and they play a key role in oncogenesis [120]. Recently, several oncogenes have been shown to be similar to growth factors or growth factor receptors, regulating the same mRNA [121, 122]. It has also been suggested that steroid receptors may be homologous to oncogenes [123]. Substrates/inducers of the cytochromes P-448 are therefore complete carcinogens in that they can effect damage to the DNA (genotoxic effects) and also effect changes in DNA regulation and cell division (epigenetic effects).

Genetic differences in the inducibility of the cytochromes P-448, and hence in susceptibility to tumorigenesis, have been studied extensively in mice [59]. The B6 inbred mouse exhibits high inducibility of P-448 and high susceptibility to cancer, whereas the D2 mouse has poor inducibility of P-448 and low susceptibility to cancer. MC at high doses induces both P_1-450 and P_3-450 in B6 mice but not in D2 mice, whereas high levels of the more potent inducer, TCDD, induce both cytochromes in both strains of mice, by increased rates of gene transcription [124]. This indicates that the genetic differences in P-448 induction seen in mice are attributable to differences in the affinities of the cytosolic *Ah* receptors for the inducing agents. Genetic and regulatory differences are seen in other species. For example, guinea pigs, a species known to be resistant to chemical carcinogens, are relatively resistant to induction of the cytochromes P-448 by MC, in marked contrast to rats and mice [125].

The metabolism of carcinogens and toxic chemicals to reactive intermediates which then react with vital cellular macromolecules or result in the generation of reactive oxygen species, culminating in neoantigen formation, cell death, mutations and malignancy, has long been associated with the causation of cancer and, more recently, with the aetiology of atherosclerosis [126], rheumatoid arthritis, and other degenerative disease states (see Fig. 1). The identification of the role of the cytochromes P-448 in this activation process, an understanding of the enzymic mechanisms involved, and of the mechanism of regulation of this enzyme, are but the first steps to a deeper understanding and prevention of malig-

nancy and degenerative disease. The screening of human populations for high activities of cytochrome P-448, the identification of chemicals such as combustion products and polyhalogenated biphenyls which increase this, and of nutrients which decrease cytochrome P-448 activity or increase the activity of the detoxicating PB-cytochromes P-450, and possibly the design of selective inhibitors of P-448 or of its induction, are distinct possibilities of the future.

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