SPECIES DIFFERENCES IN CYTOCHROMES P450 AND THEIR CORRESPONDING MESSENGER RNA'S

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1. INTRODUCTION

Cytochrome P-450 was first identified in 1958 by Klingenberg(1) and Garfinkel (2). These workers demonstrated the presence of a carbon monoxide binding pigment in liver microsomes which showed an unusual difference spectra, having a peak at 450 nm upon reduction and ligation with carbon monoxide. Since that time the study of cytochrome P-450 has broadened explosively and it is now widely accepted that cytochrome P-450 is the terminal oxidase in the hepatic microsomal mixed function oxidase system (3,4). This system plays a vital role in the oxidation of numerous foreign substances (drugs, carcinogens, insecticides, and environmental pollutants) as well as diverse endogenous compounds such as vitamin D, fatty acids and steroids (Table 1), and for this

TABLE 1 Cytochrome P450 Catalysed Reactions

	Reaction Type	Substrates
1.	Aliphatic hydroxylation	Cholesterol, fatty acids, pentobarbitone, prostaglandins, testosterone, vitamin D_3
2.	Aromatic hydroxylation	Aniline, amphetamine
3.	Dehalogenation	Carbon tetrachloride, chloroform, halothane
4.	Desulphuration	Carbon disulphide, disulphiram, parathion
5.	Epoxidation	Aldrin, benzo(a)pyrene, unsaturated fatty acids, styrene
6.	Oxidative deamination	Amphetamines
7.	N-dealkylation	Aminopyrine, chlorcyclizine, ethylmorphine, N-alkyl amphetamines
8.	O-dealkylation	Codeine, phenacetin
9.	S-dealkylation	6-methyl thiopurine
10.	N-oxidation	2-acetylaminofluorene, aniline, amphetamines
11.	S-oxidation	Chlorpromazine

reason a great deal of effort has gone into the purification and characterisation of cytochrome P-450. Early studies showed that different drug or xenobiotic inducers could manipulate the biochemical and biophysical properties of the microsomal hydroxylation system (5,6,7) and this in conjunction with the remarkably broad substrate specificity, regioselecti-

vity and stereoselectivity of cytochrome P-450 was attributed to the existence of a number of distinct species of the haemoprotein (8-11). Subsequent to these early studies, experimental evidence including sodium dodecyl suphate (SDS)-polyacrylamide gel electrophoresis, peptide mapping, amino acid composition and sequence determination, spectroscopy, chromatography, kinetic and immunological analyses, have conclusively shown that various purified cytochromes P-450 are distinct proteins (12-14).

This concept of haemoprotein multiplicity has been further extended in that it is now known that the metabolism of a wide variety of xenobiotics and endogenous substrates by these multiple forms of cytochrome P-450 varies with species, sex, age, tissue, hormonal status, and exposure of the animal to various foreign compounds (15). This would indicate that the different cytochromes and therefore their corresponding mixed function oxidase activities are under different genetic control mechanisms.

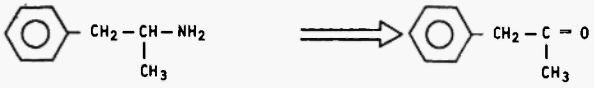
The biological significance of this multiplicity of cytochrome P-450 between different species is that since the mono-oxygenases catalyse both activation and detoxification pathways for a wide variety of substrates, their distinct enzymatic properties and variable occurences might result in marked dissimilarities in the pharmacology, toxicity or carcinogenicity of a given compound (Table 2). It is for this reason that species differences in drug oxidations and characterisation of the multiple forms of cytochromes P-450 is important in order that extrapolations from animal toxicity testing data may serve as an indicator of potential toxicity to man. With the development of procedures for the purification of homogenous forms of cytochromes P-450, reconstituted enzyme systems (16) have offered the possibility of greater detailed study of the more definitive roles of the multiple forms of cytochromes P-450 which exist in mammals. Similarly because of the absence of other interfering enzymes, the reconstituted enzyme system is ideal for kinetic and mechanistic studies and can therefore be used to establish and identify intermediates in the metabolism of a wide variety of substrates. Such parameters are important in view of the multitude of cytochrome P-450 isoenzymes that any particular species can possess, as it offers the possibility of a metabolic profile to be made for those different isoenzymes in a given species for particular substrates of the monooxygenase system. Subsequently as improved characterisation of the different isoenzymes are made available, such metabolic profiles could be used as an index of potential toxicity.

At the present time, although the existence of multiple forms of cytochrome P-450 (as judged by the present criteria for homogeneity) is well documented, the precise number of different forms in any given

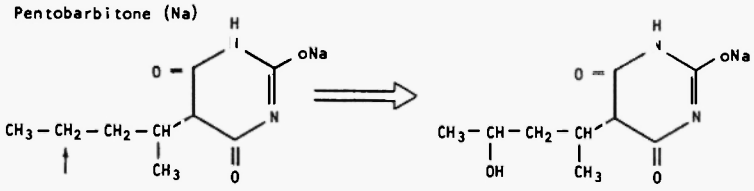
TABLE 2 Role of Cytochromes P450 in the Deactivation[a] or Activation[b] of Drugs and other Xenobiotics[c]

A. DEACTIVATION REACTIONS[a]

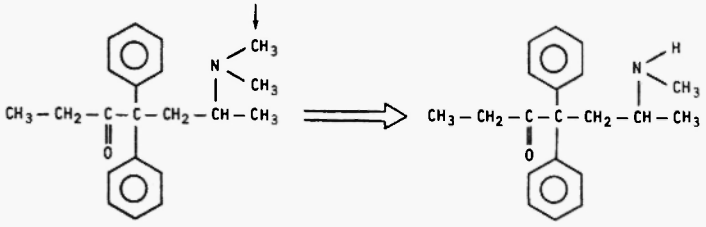
(i) Amphetamine :



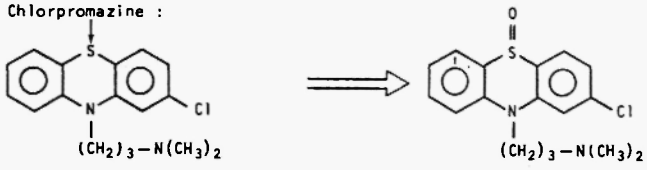
(ii) Pentobarbitone (Na)



(iii) Methadone :

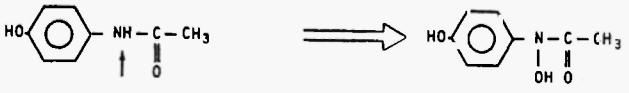


(iv) Chlorpromazine :

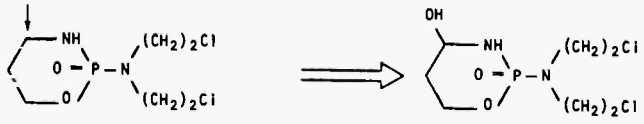


B. ACTIVATION REACTIONS[b]

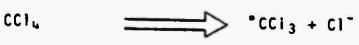
(i) Paracetamol :



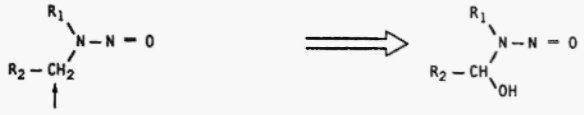
(ii) Cyclophosphamide :



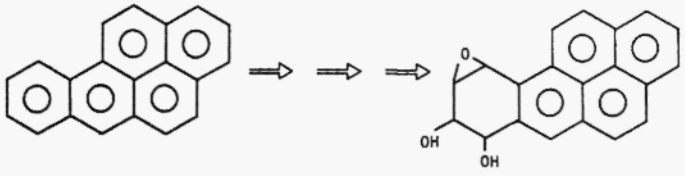
(iii) Carbon Tetrachloride :



(iv) Nitrosamines :



(v) Benzo(a)pyrene :



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a Deactivation reactions are defined as those resulting in the formation of a metabolite with decreased pharmacological activity when compared to the parent compound.

b Activation reactions are defined as the case where the metabolite is either pharmacologically more active or more toxic than the parent compound. In certain instances, the intermediate formed may not be the ultimate reactive species, but rather represents the first step in the bioactivation pathway.

c The arrow indicates the point of metabolic attack.

species is confusing, mainly because of a lack of uniformity in nomenclature amongst different research groups (12-14). However, it is generally accepted that a conservative estimate of the minimum number of forms in various microsomes would be:

a) Five to eight forms of cytochrome P-450 in rat liver microsomes (21,22,37,41,43).
b) Five to six forms of rabbit liver and lung microsomes taken collectively (13,47,50,52,58).
c) A minimum of four forms in mouse liver microsomes (60,62)
d) An undetermined number in human liver microsomes (65,67,69,74).

Further studies involving other species and tissues will no doubt show the existence of more forms. Nebert has postulated the existence of hundreds or thousands of discrete forms of cytochrome P-450 that would vary slightly in order to be able to efficiently metabolise the hundreds of substrates of widely differing chemical structures (17). Nebert argues that the cytochrome P-450 polypeptides may have both variable and constant regions (similar to the immunoglobins), but that at the present

time, immunochemical and amino acid sequencing techniques may not be sensitive enough to determine whether a purified cytochrome P-450 contains only one form or many forms of the hemoprotein. Verification will have to await a much greater understanding of genetic regulation and expression, although their is no doubt of the relationship associating genetic variables and individual differences in susceptibility to toxicity cancer seen in both experimental animals and humans (18,131).

Although Nebert's hypothesis regarding an infinite number of cytochrome P-450 multiple forms similar to the antibody system is undoubtedly attractive and at the present time cannot be proved or disproved, it is still insufficient to explain all the complexities encountered in the biochemical and biophysical regulation of cytochrome P-450: However with increasing proficiency in immunological and amino acid sequencing techniques, evidence is mounting that many of the multiple forms of cytochromes within a given species have different primary and tertiary structures. Such data(19,20) indicates that these cytochromes are in fact separate gene products and not post-translational modifications of a single gene product. Similar data from the use of DNA recombinant technology (which shall be referred to later) is also providing evidence that cytochromes P-450 are coded for by a multigene complex within a given species. Thus one should in fact consider the different cytochromes P-450 not as multiple enzyme forms but as distinct isoenzymes, because although these enzymes often catalyse the same enzymatic reaction, they do not necessarily have similar catalytic properties, being frequently characterised by different amino acid sequences and being immunologically distinct as a result of separate gene expression.

2. SPECIES DIFFERENCES IN DRUG OXIDATIONS AND CHARACTERISTICS OF MULTIPLE-FORMS OF CYTOCHROME P-450

A major problem associated with the analysis and characterisation of cytochrome P450 multiple forms is the confused status of the nomenclature used by various authors. At present, no consistent nomenclature for the isoenzymes exists, making a comparison across species (and even in the same species between different laboratories) extremely difficult, and each laboratory involved in separation, and purification and characterisation studies, adopts their own nomenclature system. This problem is further compounded by the usage of nomenclature systems that are a hybrid of other systems. Although space does not permit a full examination of the various systems used, the most widely used terminology are as follows:

(1) The IUPAC-IUB Commission on Biochemicals Nomenclature rec-

ommends that isoenzymes should be named according to the electrophoretic mobility, such that the lowest number is given to the form with the highest mobility towards the anode (lowest molecular weight). This then forms the basis for the naming of the rabbit liver cytochromes P450 forms 1-6.

(2) Some forms of cytochromes P450 are named after the absorbance maximum of the ferrous - CO adduct, eg cytochromes P448 or P452.

(3) A nomenclature system is based on the elution of supposedly distinct 'pools' of cytochromes P450 from chromatographic columns used in purification studies, e.g. cytochromes P450a, P450b, or P450I, P450II, or P450A, P450B.

(4) Some nomenclature systems identify the various cytochromes based on the chemical inducer used, e.g. isoenzymes isolated from phenobarbitone (PB) pretreated animals is defined as PB-P450. An extension of this terminology is to define further sub-fractions isolated by column chromatography as cytochrome PB-P450A or PB-P450B, etc.

2.1 *Multiple forms of cytochrome P-450 in rat liver microsomes*

Despite the close similarities among the cytochromes P-450 in terms of their localisation, function and molecular weight range, it is becoming increasingly clear that cytochrome P-450 species are different proteins which differ in their primary amino acid sequences, substrate specificity, immunological cross-reactivity and inducibility by different drugs or xenobiotics (21,22). In general, no specific structural similarities exist between the inducers of cytochromes P-450 which are responsible for the specific increase of a particular class of haemoprotein and hence its substrate specificity (23). This latter diversity of substrate specificity between different species has been a major problem in adequate toxicity and carcinogenicity screening programmes.

According to the current criteria for homogeneity (which includes SDS polyacrylamide gel electrophoresis, catalytic, spectral and immunological properties, as well as the sequencing of NH_2-terminus, COOH-terminus and proteolytic peptide fragments of cytochrome P-450), five to eight forms of cytochrome P-450 have been postulated to be present in rat liver microsomes. The amounts of the various forms of cytochrome P-450 have been shown to be a function of sex, age, strain and inducer (24,25). Taking these variable factors into consideration, it is generally accepted that the constitutive levels of cytochrome P-450 and P-448 in rat liver microsomes is very low (less than 5% of the total cytochrome P-450, 25). Phenobarbital (PB) and 3-methylcholanthrene (3MC) markedly induce cytochrome P-450 (15-30 fold) and cytochrome P-448 (40

fold) respectively. After PB induction, depending on the age and species of the animal,the PB-induced form represents approximately 30-60% of the total microsomal cytochrome P-450, whereas after 3MC treatment, cytochrome P-448 represents approximately 80% of the total cytochrome population (25,26). It was also shown by Harada and Omura (26) that PB pretreatment resulted in a corresponding increase in NADPH-cytochrome P-450 reductase almost in parallel with that of total microsomal cytochrome P-450.

The major PB inducible form of cytochrome P-450 in rat liver, (designated P-450$_b$ by some authors(27)), can also be induced by polychlorinated biphenyls such as Aroclor 1254 (21). The sub-unit molecular weight of cytochrome P-450$_b$ ranges from 50,000 to 52,000 and the absorption maximum of its reduced CO difference spectrum ranges from 450-451 nm. In Long-Evans rats, the N- and C-terminal amino acid residues of cytochrome P-450$_b$ are glutamic acid and serine respectively (28). This form of cytochrome P-450 efficiently catalyses the metabolism of substrates such as benzphetamine, N,N dimethylaniline, parathion, and N,N-dimethylphentermine. It is also interesting to note that there is a high degree of homology in the N-terminal regions of cytochrome P-450$_b$ and *rabbit* liver cytochrome LM$_2$ induced by PB (28). Of the first twenty-one amino acids, fourteen common residues are found; six of the differences can be accounted for by a single amino acid change and one by a double amino acid change in the nucleotide sequence. In addition to N-terminal homology, the overall amino acid composition of the above two enzymes are very similar, and are in fact even more closely related in their substrate specificities than are the three main forms isolated from rat liver microsomes (a,b,c) after treatment with phenobarbital, 3-methylcholanthrene or polychlorinated biphenyls (28). Both enzymes exhibit a poor catalytic activity towards benzo(a)pyrene hydroxylation but they show almost absolute specificity for the 16α hydroxylation of testosterone with little or no detectable activity for the 6β or 7α hydroxylation of this substrate (21). Despite their homology of N-terminal regions and similarity of amino acid composition and catalytic properties, the above two proteins have different amino acid sequences in their C-terminal regions and do not co-electrophorese on SDS-polyacrylamide gels. They are also immunologically non-identical (28), and since antibodies are directed against several spatially distant sites on the protein surface, the absence of immunochemical relatedness indicates that these proteins have dissimilar structures. Cytochrome P-450$_b$ is predominantly a low spin hemoprotein although there is evidence that it may contain some high spin component (21). Purified preparations from either PB- or Aroclor 1254- treated rats are usually low spin hemoproteins (21).

The major 3MC inducible form in rat liver has been termed cytoch-

rome P-450$_c$, and is also induced by BNF and polychlorinated biphenyls such as Aroclor 1254 and 3,4,5,3',5' pentachlorobiphenyl. Reports of sub-unit molecular weight of this cytochrome P-450 range from 53,000 to 56,000 and its reduced CO complex exhibits an absorption maximum of 447 to 448 nm. In Long-Evans rats, the N- and C-terminal amino acid residues of cytochrome P-450 are isoleucine and leucine respectively (28). Cytochrome P-450$_c$, preferentially catalyses the metabolism of such substrates as benzo(a)pyrene, 7-ethoxycoumarin, 7-ethoxyresorufin, and zoxazolamine. It also displays catalytic activity towards the hydroxylation of testosterone at the 6β position, but at a much lower rate than that seen with liver microsomes indicating that it may not be the major form of cytochrome P-450 responsible for this hydroxylation reaction (21). Preliminary work has also indicated the presence of a second form of cytochrome P-448 following induction by 3,4,5,3,4,4,-hexachlorobiphenyl (39). This compound is believed to induce two forms of cytochrome P-448, one of which has electrophoretic, catalytic and spectral properties similar to those of cytochrome P-450$_c$. The other form has a sub-unit molecular weight of 52,000, but does not metabolise either benzphetamine or ethoxyresorufin, substrates preferentially metabolised by cytochromes P-450$_b$ and P-450$_c$ respectively. In the rat, the major form of cytochrome induced by 3-methylcholanthrene (P-450$_c$) represents at least 88% of the total polycyclic aromatic hydrocarbon inducible aryl hydrocarbon hydroxylase activity (30). However evidence is mounting for the presence of a further form of cytochrome following induction by TCDD (29,132). Nebert *et al.* (29,132) have reported the possible induction of the above two forms of cytochrome, termed cytochrome P$_1$-450 and P-448, and indicate that they are related to the cytochromes P$_1$-450 and P-448 species in the mouse (62). Cytochrome P$_1$-450 is defined arbitrarily as the polycyclic aromatic hydrocarbon inducible form most closely associated with aryl hydrocarbon hydroxylase activity and cytochrome P-448 is defined arbitrarily as the polycyclic aromatic hydrocarbon inducible form (when reduced and combined with carbon monoxide) having a Soret peak shifted to approximately 448 nm. Although there had been scepticism relating to the existence of these two forms of cytochrome in species other than the mouse, data is now emerging that the rat and mouse cytochromes P$_1$-450 and P-448 are immunologically indistinct (132). A portion of the mouse cytochrome P$_1$-450 gene has been cloned and there is sufficient nucleotide sequence homology between the mouse structural gene and its mRNA and that of rat, to indicate that rat P-450$_c$ is similar to mouse P$_1$-450 (126,127). It is also interesting to note that the polycyclic aromatic hydrocarbon inducible cytochrome P$_1$-450 (the form believed to be associated with the initiation of polycyclic hydrocarbon carcinogenesis), has the highest

molecular weight of all the cytochrome P-450 isoenzymes induced by polycyclic aromatic hydrocarbon compounds in the rat, rabbit, mouse and fish (29).

A third species of cytochrome P-450 has been purified from PB-, 3MC-, and Aroclor 1254-treated rats and is termed cytochrome P-450_a (21). This isoenzyme has a sub-unit molecular weight of 48,000 and the absorbtion maximum of its reduced CO complex is at 452nm. The haemoprotein preferentially hydroxylates testosterone at the 7α position but has low catalytic activity for the metabolism of benzphetamine, benzo(a)pyrene, . 7-ethoxycoumarin and zoxazolamine. This isoenzyme contains methionine as both its N- and C-terminal amino acid residues, and partial sequencing of the first 19 amino acids of the N-terminal region indicates little homology with the corresponding sequences of cytochromes P-450_b and P-450_c. However the substrate specificities of cytochromes P-450a, P-450_b and P-450_c isolated from Aroclor 1254 treated rats are the same as the specificities of the corresponding haemoproteins isolated from 3MC- and PB- treated rats. This amino acid sequence data, as well as immunological reactivity and differences in primary and tertiary structure, indicate that the above cytochromes are separate gene products and not post translational modifications of a single gene product. It should also be noted that cytochrome P-450_c is immunologically distinct from the major 3MC-inducible form in *rabbit* liver microsomes.

A further, minor form of cytochrome P-450, has also been isolated from rats treated with Aroclor 1254 and phenobarbital, but not by 3-methylcholanthrene (133). This minor isoenzymic form termed cytochrome P-450_e has been purified to apparent homogeneity having a minimum molecular -weight of 52,500 and an absorption maximum of its reduced CO complex at 450.66 nm (134). It is immunochemically related to cytochrome P-450_b, but not to cytochromes P-$450_{a,\ c,\ d}$. Its substrate specificity is similar to that of cytochrome P-450_b, although certain substrates such as benzphetamine, benzo(a)pyrene, 7-ethoxycoumarin, hexobarbital and testosterone at the 16α position were metabolised at only 15-25% of the rate seen with cytochrome P-450_b. However the metabolism of estradiol -17β at the 2-position was carried out more efficiently by cytochrome P-450_e than P-450_b. Analysis of the peptide fragments of the two haemoproteins following chemical or proteolytic digestion indicates that cytochromes P-450 and P-450_b are very similar but nevertheless have minor structural differences indicating that they are not identical (134). To date no form of cytochrome P-450 comparable to rat cytochrome P-450 has been identified in other species.

Another species of cytochrome termed cytochrome P-450_{PCN} has been

shown to be induced by pregnenolone 16α carbonitrile (PCN, 32) which increases the specific content of cytochrome P-450, but has no effect on levels of either cytochromes P-450$_a$, P-450$_b$ or P-450$_c$. Animals treated with this catatoxic steroid are resistant to the toxicity of many drugs and xenobiotics (including CCl_4), and are less susceptible to the formation of liver cancer by dimethylnitrosamine. It has been suggested that this might be due to the different intra-lobular distribution of specific cytochromes and NADPH-cytochrome c (P-450) reductase as a consequence of the administration of PCN (33). This purified form of cytochrome P-450 was shown to be distinct from the major forms of cytochrome P-450 induced by either PB or 3MC with respect to exhibiting reduced stability at room temperature and markedly different chromatographic, spectral, catalytic and immunological properties (32). This isoenzyme has a sub-unit molecular weight of 51,000 and an absorption maximum in its reduced CO difference spectrum of 450 nm, and, also differs from cytochromes P-450$_b$ and P-450$_c$ by containing as much as fifty percent more lysine and proline residues, and a greater number of methionine residues. In reconstituted enzyme systems, the purified cytochrome catalyses the N-demethylation of ethylmorphine and aminopyrine but at a much slower rate than is seen with the original liver microsomes. This loss of functional activity is believed to be due to an artifact of solubilisation and purification. The presence of microsomal cytochrome P-450$_{PCN}$ following induction by other steroids such as dexamethasone, spironolactone and non steroids (e.g. phenobarbital) has also been demonstrated (135). Dexamethasone is a potent glucocorticoid but its inductive effect towards cytochrome P-450$_{PCN}$ is not believed to be mediated by glucocorticoid receptors, although the presence and involvement of second, lower-affinity dexamethasone receptors in liver microsomes has been suggested (135, 136).

A unique form of liver cytochrome P-450 induced in rats treated with the methylenedioxyphenyl compound isosafrole (34) has been purified to apparent homogeneity (22,35). Isosafrole (a plant constituent) is a known *in vivo* hepatocarcinogen, and, as such, may be transformed to an ultimate carcinogen by cytochrome P-450 mediated oxygenation. Cytochromes P-450$_a$, P-450$_b$ and P-450$_c$ have also been partially purified from isosafrole treated rats and these latter haemoproteins have the same properties as the enzymes from Aroclor 1254-treated rats. The highly purified high spin haemoprotein, designated cytochrome P-450$_d$, has a sub-unit molecular weight ranging from 52,000 to 53,000 (comparable to cytochrome P-450$_h$), and its reduced CO complex has an absorption maximum of 447 nm, indistinguishable from cytochrome P-450$_c$ (22,35). It is purified as an isosafrole metabolite -cytochrome P-450 complex, but addition of certain displacers such as 7-ethoxycoumarin, or

benzimidazoles results in the removal of most of the bound metabolite (22). Cytochrome P-450$_d$, either before or after removal of the methylenedioxyphenyl metabolite has poor catalytic activity with respect to the metabolism of benzo(a)pyrene, benzphetamine, 7-ethoxycoumarin and testosterone in the 7α, 16α or 6β positions. Thus the observed catalytic activity present in the microsomal fraction of isosafrole-treated rats may well arise from the participation of additional cytochrome P-450 isoenzymes. The substrate specificities of the haemoproteins from isosafrole-treated rats are the same as the cytochromes from Aroclor 1254-treated rats (21): The most distinguishing feature of cytochrome P-45$_d$ is the high degree of specificity for isosafrole oxidation. The metabolite(s) which binds to the cytochrome P-450 has been proposed to be a carbene which attacks the haem moiety of the enzyme (36). Cytochrome P-450$_d$ also shows partial identity with cytochrome P-450$_c$, but does not cross react with antibodies prepared against cytochromes P-450$_a$ or P-450$_b$. Peptide maps resulting from enzymatic or chemical cleavage indicate that the primary structure of this isoenzyme differs from cytochromes P-450$_a$, P-450$_b$, and P-450$_c$ (137).

During the course of characterisation of the cytochrome P-450 species induced by PCN, it has been suggested that other forms of cytochrome P-450 not yet characterised may be involved in benzo(a)pyrene metabolism since inhibition by antibodies to cytochromes P-450a, P-450$_b$ and P-450$_c$ did not result in complete metabolic inhibition (30). It has also been shown that high benzo(a)pyrene metabolising activity in PCN-treated rats was insensitive to an antibody directed primarily against P-450$_c$. Subsequent to the above observations, a low spin hemoprotein designated cytochrome P-446 has been purified from β-napthoflavone (BNF) pretreated rat liver (37). This form of cytochrome has the highest turnover for benzo(a)pyrene yet reported (52 nmol/min/nmol of cytochrome P-446), and exhibits high activity with p-nitrophenotole, but has relatively low activity with 7-ethoxycoumarin. The isoenzyme has an apparent minimum molecular weight of 53,400 and has an absorbance maximum in the CO reduced difference spectrum at 446 nm. The amino acid composition indicates that the protein contains 35% hydrophobic residues and otherwise resembles that of most other cytochromes P-450. Subsequent work (138) has shown that the molecular weight of this cytochrome P-446 was in fact 56,500 and that it had a larger turnover number for ethoxycoumarin than was previously reported (37), the turnover number being comparable to that obtained with cytochrome P-450$_c$. This has led to speculation as to whether or not the cytochromes P-446 and P-450$_c$ may be the same protein. Comparative studies have indicated that the two cytochromes were immunologically identical, had similar cleavage products following proteolysis, appeared to

have identical sub-unit molecular weights and similar absorption maxima (446 ± 0.5 nm, 139). The only difference was their catalytic activity towards benzo(a)pyrene and cytochrome P-446 was shown to be nearly twice as efficient in hydroxylating benzo(a)pyrene than cytochrome $P\text{-}450_c$. It appears that cytochrome P-446 produces higher amounts of the 9-hydroxy benzo(a)pyrene metabolite than does cytochrome $P\text{-}450_c$ (139). Whether this catalytic difference is due to residual amounts of bound inducer or whether the two cytochromes are similar yet distinct proteins is not known at present time.

Two further isoenzymes have been purified from the livers of phenobarbital-induced Sprague-Dawley rats (140). Termed cytochromes P-450 PB-4 and PB-5, these low spin hemoproteins have molecular weights of 49,000 and 51,000 respectively. Both cytochromes have similar spectral properties, but cytochrome P-450 PB-5 exhibits approximately 20% of the catalytic activity of cytochrome P-450 PB-4. The presence of cytochrome b_5 markedly stimulates the catalytic acitivity of both cytochromes in a reconstituted system. NH_2-terminal analysis of the two hemoproteins indicates that they have identical primary structures for the first 31 (and probably 34) amino acids. The sequences are highly hydrophobic, and homologous with 22 NH_2-terminal residues derived from phenobarbital-induced Long-Evans rat liver cytochrome $P\text{-}450_b$ (21), and rabbit liver cytochrome form 2 (51). When cytochrome $P\text{-}450_b$ is compared to cytochromes P-450 PB-4 and PB-5, the only differences in the NH_2-terminal sequences is the absence of the terminal methionine residue and the presence of threonine instead of serine as found in position 4 of cytochromes P-450 PB-4 and PB-5. It has been suggested that this absence of the NH_2-terminal methionine is an experimental discrepency similar to that seen with the sequencing of cytochrome P-450 LM_{3b}, where it is present in some preparations and absent in others (141). The presence of the highly hydrophobic sequences followed by a series of basic residues at the NH_2-terminal segment is believed to represent signal sequences relating to membrane binding. Immunochemical analysis indicates that cytochromes P-450 PB-4 and PB-5 are similar proteins and immunochemically indistinguishable from cytochrome $P\text{-}450_b$ derived from Long-Evans rats. On the basis of these findings it has been suggested that cytochromes P-450 PB-4 and PB-5 are analogous to cytochromes $P\text{-}450_b$ and $P\text{-}450_e$ in Long-Evans rats (134), despite the fact that they do not co-electrophorese when subjected to SDS polyacrylamide gel electrophoresis. Cytochrome P-450 PB-4 was also shown to be more susceptible than cytochrome P-450 PB-5 to suicide inactivation by allylisoproplyacetamide and secobarbital (140). It is unknown whether these two closely related

isoenzymes are separate gene products or are related by post-translational modification.

Gibson, Orton and Tamburini(43) have also purified a unique form of cytochrome P-450 which is induced in the rat by the hypolipidemic drug clofibrate (44). The total cytochrome P-450 was fractionated into four pools, with the first pool containing a cytochrome P-450 of apparent minimum molecular weight of 51,500 and having an absorbance maximum in the CO difference spectrum at 451.8 nm. This fraction was judged to be the most pure and preferentially hydroxylated lauric acid at the 12 position and to a lesser extent at the 11 position, but has low catalytic activity towards benzphetamine in direct contrast to cytochrome $P\text{-}450_b$. The cytochrome P-450 fraction 2 exists as a low spin form and although less homogenous than fraction 1, preferentially hydroxylates lauric acid at the 11 position. Cytochrome P-450 fraction 3 exhibits only trace laurate oxidase activity but rapidly turns over benzphetamine at a rate comparable to cytochrome $P\text{-}450_b$.

Although the majority of work on cytochromes P-450 has been carried out on isoenzymes induced by various xenobiotics, purification and characterisation of multiple forms of cytochrome P-450 in uninduced animals has also been studied. At the present time however, the relationship between these various forms is still unclear.

Gibson and Schenkman have reported the partial purification of two forms of cytochrome P-450 in high yield from untreated rats (40). Cytochrome P-450 I, had a reduced CO absorption spectrum at 449.5 nm, whereas cytochrome P-450 II had a maximum at 448.5 nm, the latter preparation appearing to have a sub-unit molecular weight of 52,000. Differences were observed in the catalytic activities of the two cytochromes towards several substrates, (particularly ethylmorphine) when activity was assayed in the presence of cumene hydroperoxide as an oxidant. Subsequent studies by the same laboratory has resulted in the purification of two further cytochrome isoenzymes from untreated rats (41). These two forms (designated RLM_3 and RLM_5) have apparent minimum molecular weights of 50,000 and 51,000, and absorbance maxima in the CO reduced difference spectra at 449 and 451 nm respectively. RLM_3 exists in a low spin form and preferentially hydroxylates testosterone at the 6α and 6β positions and catalyses the formation of an unidentified, highly polar metabolite. RLM_5 contains some high spin component and preferentially hydroxylates testosterone at the 2β and 16α positions, and exhibits a specific activity two or three times higher than RLM_3 towards benzphetamine, aminopyrine and ethylmorphine metabolism. N-terminal amino acid analysis indicates that the first residue of each form is methionine, with the first four residues being identical for both haemoproteins. Although their is some homology

between the two forms based on the amino acids sequenced, they do not correspond with those of either rat cytochrome P-450$_b$ or P-450$_c$ (42). It is estimated that RLM_5 represents about 5% of the constitutive cytochrome P-450 isozymes in untreated rat liver microsomes, and RLM_3 about 8% (41). Further work has demonstrated the existence of three additional isoenzymes termed RLM_{2a}, RLM_4 and RLM_{6B}, characterised by monomeric molecular weights of 48.5 - 49,000, 50.8 - 51,000 and 52.7 - 53,000 respectively (142).

Agosin, *et al.* have also purified two forms of cytochrome P-450 (fractions A and B) from untreated rats (45). The sub-unit molecular weight of both forms are 52,200 and 52,400 respectively and the absorbance maximum of the reduced CO complex is 450 nm for fraction A and 450.8 for fraction B. The levels of glycine, serine and alanine for these isoenzymes are approximately double that found in the RLM_3 and RLM_5 isoenzymes (41). Neither fraction A nor B forms is active in catalysing the metabolism of substrates such as benzphetamine, 7-ethoxyresorufin or aniline, but both enzymes actively catalyse the metabolism of the juvenile hormone analog (1-(4-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-trans-2-octene) to a polar metabolite with the highest activity observed with cytochrome P-450 fraction B.

2.2 Multiple forms of cytochrome P-450 in rabbit liver and lung microsomes

On the basis of several criteria, at least five different forms of cytochrome P-450 have been purified from rabbit liver and lung microsomes (13,46,47), although evidence has recently been presented for the presence of eleven forms in liver microsomes from untreated rabbits (143). Similarly as with the other isoenzymic forms of cytochrome P-450 purified to date, much confusion has arisen from the indiscriminate use of nomenclature, and this has led to the recommendation by the IUPAC-IUB commission on Biochemical Nomenclature that the multiple forms of an enzyme should be differentiated on the basis of electrophoretic mobility, with the number 1 being assigned to that form having the highest mobility towards the anode.

Johnson and Müller-Eberhard (49) have purified a cytochrome P-450 species termed form 1 from the liver microsomes of TCDD-treated rabbits, and subsequently showed that it was also present in the livers of untreated animals. This hemoprotein was reported to have a monomeric molecular weight of 48,000 and its reduced CO complex to have an absorption maximum of 450.5 nm. Antibodies to cytochrome P-450 forms 2,4 and 6 do not react with this preparation, and it can be distinguished from these species by peptide mapping. This form cata-

lyses the metabolism of benzphetamine, benzo(a)pyrene, 7-ethoxyresorufin, acetanilide and the 16α- and 6β-hydroxylation of progesterone. These activities are generally low especially if compared to cytochrome forms 4 and 6 which are also induced by TCDD. However form 1 differs in that it is the only isoenzyme capable of catalysing the 21-hydroxylation of progesterone to a significant degree, resulting in the conversion of progesterone to deoxycorticosterone (31).

The form 2 cytochrome P-450 is the major PB-inducible form in the livers of adult rabbits and has been purified to electrophoretic homogeneity (50,54). Termed LM_2, it has a sub-unit molecular weight range of 48,700 - 52,000 (47,51), with the absorption maximum of the reduced CO complex at 451 nm. The N- and C-terminal acid residues are methionine and arginine respectively, with a high proportion of hydrophobic amino acids being found in the first twelve residues of the N-terminal sequence. The cytochrome contains one residue of glucosamine and two of mannose per polypeptide subunit (51). LM_2 is most active in catalysing the metabolism of substrates such as benzphetamine, cyclohexane, biphenyl, aniline, warfarin and parathion, but is relatively inactive in metabolising benzo(a)pyrene.

In the lung, two cytochrome P-450 isoenzymes, $P\text{-}450_I$ and $P\text{-}450_{II}$, have been purified and characterised extensively (47, 50, 144). These isozymes have molecular weights of 52,000 and 58,000, with absorption maxima for their CO difference spectrums at 450 and 452 nm respectively. On the basis of their amino-acid compositions, amino terminal sequences and peptide maps of their protease digests the two cytochromes have been shown to be distinct protein moities. Using purified systems cytochrome $P\text{-}450_{II}$, but not cytochrome $P\text{-}450_I$, has been shown to be involved in the activation of the promutagens 2-aminoanthracene, 2-aminofluorene, 2-acetylaminofluorene and aflatoxin B_1 (145). However on the basis of structural, catalytic, spectral and immunochemical properties, cytochromes $P\text{-}450_I$ and liver cytochrome form 2 are indistinguishable (47,50). The isozymes have identical sequences for the first five residues at their amino terminals and very similar amino acid compositions, although discrepancies in their carboxyl terminal amino acid sequences exist. Immunofluorescence studies have shown the presence of cytochrome form II in the proximal tubules and lungs of both untreated and phenobarbital treated animals (146). A further lung cytochrome isozyme termed cytochrome $P\text{-}450_{III}$ has also been reported following induction by 3-methylcholanthrene (147). On the basis of electrophoretic and spectral data it is distinct from cytochromes $P\text{-}450_I$ and $P\text{-}450_{II}$ and may be related to form 6 of rabbit liver cytochrome. Work is currently being carried out to further characterise this hemoprotein (147).

Form 3 has been purified from untreated adult rabbits, and although it is present in microsomes following pretreatment of the animal with either PB or TCDD, it does not appear to be induced by these compounds (52). It is a low spin haemoprotein with a sub-unit molecular weight of 51,000-52,600 and has an absorption maximum in its reduced CO difference spectrum of 450 nm (25). It preferentially metabolises aminopyrine and also displays activity towards benzphetamine and biphenyl, but shows little or no activity with benzo(a)pyrene and 7-ethoxyresorufin as substrates. Form 3 can also be distinguished from forms 2,4 and 6 by its sub-unit molecular weight, peptide mapping and immunological properties (53) suggesting that these forms are not derived from a common protein precursor.

Further purification of isozymes which possess electrophoretic mobility in the "3 region" has led to increased characterisation of a number of distinct hemoproteins. Chronic ethanol administration has been shown to cause the induction of a cytochrome, termed form 3a, which is very active in the oxidation of ethanol to acetaldehyde, and in the p-hydroxylation of aniline (148). This high spin haemoprotein has a molecular weight of 51,000 and has an absorption maximum of its CO complex at 452 nm. Its NH_2-terminal and COOH-terminal amlno acid residues are alanine and leucine respectively. On the basis of amino acid composition, peptide mapping, spectral, electrophoretic and catalytic properties, this isoenzyme is believed to represent a unique gene product (148).

Two constitutive cytochromes, forms 3b and 3c, have also been purified to apparent homogeneity (149). Subsequent characterisation (149) showed that form 3b corresponded to form 3 (52), and it became evident that form 3b was subject to both functional and structural polymorphism (150). It appears that in outbred New Zealand white rabbits, two or more structurally similar forms exist. One form exhibits a high affinity for the hydroxylation of progesterone at the 6β- and 16α-positions, whereas the other catalyses the 16 α- but very little 6β-hydroxylation. Both forms show differential inhibition by 16α-methyl progesterone. Conversely, form 3b purified from an inbred rabbit strain appears to lack the ability to catalyse the 6β-hydroxylation of progesterone and is also insensitive to inhibition by 16α-methyl progesterone. When the proteins from both strains are subjected to tryptic peptide mapping considerable differences in peptide maps are observed, but apart from this difference the proteins appear identical (150). Whether or not these differences are due to post-translational modification of the protein, genetic variation between the species or post-transcriptional processing of the mRNA is now under study. The other constitutive cytochrome purified, form 3c, has a molecular weight of 53,000 and an absorption maxima in its reduced CO difference spectra of 449 nm. Its NH_2-terminal amino acid

residue is methionine followed by an acidic residue and then hydrophobic amino acids (149). The physiological role of these constitutive forms is not known at the present time.

Form 4 (LM_4) is the liver microsomal form of cytochrome P-450 induced in adult rabbits by 3MC, TCDD or BNF (11,50,51,55). It has also been isolated from PB-treated and untreated animals (51). The reported molecular weight of the high spin haemoprotein ranges from 51,000 to 55,000 and has an absorption maximum in its reduced CO difference spectrum at 447-448nm. The C-terminal amino acid is lysine whereas the N-terminal residue is believed to be blocked (51). No cross reactions observable by precipitate band formation were detected between anti-LM_2 serum and cytochrome P-450 LM_4, or between anti LM_4 serum and cytochrome P-450 LM_2, indicating that the two cytochromes have structural differences (56). Unlike cytochrome P-450_c isolated from the liver microsomes of 3MC-treated rats, LM_{4a} is relatively inactive in catalysing the metabolism of polycyclic aromatic hydrocarbons but catalyses the metabolism of acetanilide, biphenyl, ethoxyresorufin and benzo(a)pyrene. These reactions are sensitive to inhibition by α-napthoflavone (151). In addition to the major form4 cytochrome P-450, another form of cytochrome P-450 (termed LM_{4a}) has been partially purified from the liver microsomes of BNF-treated rats (12). This latter protein has the same sub-unit molcular weight as LM_4, but preferentially metabolises benzo(a)pyrene at a much faster rate (57).

Form 6 of cytochrome P-450 (58) is induced in both adult and neonate liver by TCDD. This particular form of the hemoprotein has a sub-unit moelcular weight of 57,000 and the absorption maximum of its CO complex is at 448 nm. This cytochrome catalyses the metabolism of ethoxyresorufin, and is approximately 100-fold more active than forms 2,3 and 4 in metabolising polycyclic aromatic hydrocarbons such as benzo(a)pyrene. Both activities are sensitive to inhibition by α-napthoflavone (151). Isosafrole also induces a cytochrome, termed LM_6, which on the basis of electrophoretic, catalytic, spectral, immunological and one dimensional peptide mapping is believed to be the same as form 6 (152).

Immunofluorescence studies indicate that cytochrome forms 4 and 6 are predominantly located in the centrilobular and mid-zonal regions of the liver. Following pretreatment by TCDD, the presence of these forms in the renal and proximal tubular epithelium was observed. In the lungs, forms 2, 6 (and to a lesser degree form 4) can also be identified following TCDD pre-treatment (146).

It should be noted that the extent of the structural differences exhibited by the four main multiple forms (forms 2,3,4 and 6) suggests that these haemoproteins are not derived from a common protein precursor.

Other forms of rabbit liver microsomal cytochrome P-450 are also known to exist, but as these have not been fully characterised, the extent of multiplicity remains uncertain (12,13,14).

2.3 Multiple forms of cytochrome P-450 in mouse liver microsomes

In general, more research effort has been directed towards an understanding of the genetic aspects of cytochrome P-450 induction in mouse liver rather than the purification and characterisation of the isoenzymes present. Four forms of cytochrome P-450 (A_1, A_2, C_1, C_2) have been purified (60) from PB-treated male mice of which only two forms were judged homogenous (A_2, C_2). The reported sub-unit molecular weight of form A_2 is 50,000, and for C_2 is 56,000. The reduced CO complexes of fractions A_1, A_2, C_1 and C_2 absorbed respectively at 450, 451, 449 and 450 nm. Benzphetamine was metabolised most rapidly by fraction C_1, benzo(a)pyrene by A_1 and A_2, 7-ethoxycourmarin by A_1 and C_1, ethoxycoumarin by A_1 and ethylmorphine by C_2 (14). Ouchterlony double diffusion analysis indicated cross reaction between the mouse cytochrome P-450 C_1 fraction and cytochrome P-450_b of rat liver microsomes, whereas the other forms (A_1, A_2 and C_2) cross reacted very poorly, indicating fundamental differences between those particular isoenzymes of PB-treated rats and mice. Pregnenolone 16α-carbonitrile also induces a form of cytochrome P-450 from mouse liver microsomes which has a molecular weight of 53,000, but has not been further purified or characterised (59).

The induction of cytochromes P-450 by polycyclic aromatic hydrocarbons such as 3MC, TCDD or BNF has been shown to be under the genetic control of the Ah locus (61), which also regulates numerous other drug metabolising enzyme activities in the liver (61,62). Inducers are capable of binding with different affinities to a cytosolic receptor, with the inducer-receptor complex then undergoing a temperature dependent translocation to the nucleus (17). Induction specific m-RNA is then transcribed in the nucleus which results in the initiation of translation of the specifically induced cytochrome P-450. The presence of such a receptor appears necessary for induction to occur and mouse strains possessing such receptors are termed responsive.

Negishi and Nebert have purified two forms of cytochrome P-450 (termed P_1-450 and P-448) from inbred mice treated with 3MC (62). Both forms of cytochrome P-450 have an apparent molecular weight of about 55,000 and the soret peaks of the reduced hemoprotein CO complex are at approximately 449.3 and 448.0 nm for P_1-450 and P-448 respectively. Cytochrome P_1-450 is more closely associated with polycyclic aromatic hydrocarbon compound inducible aryl hydrocarbon hy-

droxylase activity and NADPH oxidation in the presence of benzo(a)-pyrene, whereas cytochrome P-448 is more closely associated with polycyclic aromatic hydrocarbon-inducible acetanilide 4-hydroxylase activity and NADPH oxidation in the presence of acetanilide (62). These two forms of 3-methylcholanthrene induced cytochrome P-450 have now been sufficiently characterised to indicate that they are structurally distinct gene products (62,153). The Ah locus is also known to regulate the induction of a further cytochrome, termed cytochrome P_2-450, following induction by isosafrole and 3MC. This isozyme has a molecular weight of approximately 55,000 and exhibits soret peaks of the reduced hemoprotein CO complex in microsomes at 449 nm, and in purified preparations at 448 nm (154). Immunological evidence indicates that it is also present in untreated and phenobarbital-treated mice. Cytochrome P_2-450 metabolises isosafrole and does not display polycyclic aromatic hydrocarbon-induced aryl hydrocarbon hydroxylase activity.

2.4 Multiple forms of cytochrome P-450 in human liver microsomes

Although the existence of multiple forms of cytochrome P-450 have now been shown in a variety of animal species (21,22,13,60), of greater pharmaco-toxicological interest is the determination and characterisation of these forms in humans and the relationship between these forms and those isoenzymic forms already characterised in experimental animals. However such evidence for multiple forms of cytochrome P-450 in man have only recently began to appear, primarily because of purification difficulties but also because of ethical problems encountered in obtaining sufficient amounts of starting material, low concentrations of cytochrome P-450, variability of human samples and the inability to ethically modulate the levels of these haemoproteins with inducers (63,64).

A number of laboratories have recently reported on the purification of cytochromes P-450 from human liver. Beaune, *et al.* (66) has partially resolved cytochrome P-450 into four fractions. The fraction purified to the greatest homogeneity (as judged by SDS polyacrylamide gel electrophoresis) was fraction A_2, having a sub-unit molecular weight of 45,000 and a soret peak of the reduced CO complex at 450 nm. This haemoprotein fraction catalyses the demethylation of p-nitroanisole, hydroxylation of aniline and the 16-α hydroxylation of pregnenelone. The other main forms (B_1 and B_2) are more active in metabolising p-nitroanisole, 7-ethoxycoumarin, benzo(a)pyrene and testosterone and thus clear differences are seen amongst the substrate specificities of the different forms (65).

Bösterling and Trudell have also purified human cytochrome P-450 and NADPH-cytochrome P-450 reductase (67). These workers obtained three haemoprotein fractions, of which the most highly purified (fraction HA-2) was shown to have a sub-unit molecular weight of 52,500. Magnetic circular dichroism spectra of human cytochrome P-450 HA-2 and rat cytochrome P-450$_b$ showed differences which indicated that although the proteins appeared to have similar spin states, the actual haem environment of the two proteins were different (67). Differences were also observed between human and rat NADPH-cytochrome P-450 reductase by circular dichroism spectra (67,68).

The most successful attempt at purifying cytochrome P-450 to date is the studies of Wang, *et al.* (69). These workers have purified cytochrome P-450 from autopsy material using cholate extraction and chromatography on n-octylamino-Sepharose 4B, hydroxyapatite and DEAE-cellulose columns. The human cytochromes P-450 obtained were shown to exist in a low spin form with sub-unit molecular weights of 53,000 and 55,000, with the reduced CO spectra being in the range 449-451 nm. (N.B. for comparison; PB-treated rat cytochrome P-450$_b$, 53,000; 3MC treated rat cytochrome P-450$_c$, 55,000; PB treated rabbit LM_2, 51,000; and BNF-treated rabbit LM_4, 53,500). The above haemoprotein preparations catalysed the metabolism of benzphetamine and 7-ethoxycoumarin when coupled with either rat or human liver NADPH-cytochrome P-450 reductase in a reconstituted, drug-metabolising enzyme system. On analysis of the metabolic profiles of various substrates, a marked degree of inter-individual variation was observed (70), which was particularly pronounced in the case of benzo(a)pyrene metabolism. The amino acid composition of the human cytochrome P-450 preparations showed some similarities to the major cytochrome P-450 isolated from PB- and 3MC- treated rats (71). The most significant difference was in the composition of basic amino acid residues in that the human preparation contained very little histidine and much more lysine residues than either of the two rat cytochromes P-450. Immunological analysis indicates that distinguishable forms of cytochrome P-450 exist within individual human liver samples and that there exists a marked degree of variability in the forms of human cytochrome P-450 present amongst different individuals (71). Rabbit antibodies raised to PB-treated rat cytochrome P-450 were more effective in inhibiting benzphetamine N-demethylase activity in human liver microsomes than were antibodies raised to 3MC-treated rat cytochrome P-450. These antibodies also inhibited benzo(a)pyrene hydroxylation in human liver microsomes, although the inhibition profiles did not follow a general pattern as in the case of benzphetamine as substrate (72). Other experiments (69) have also indicated that benzo(a)pyrene hydroxylase

TABLE 3 SUMMARY OF SPECIES DIFFERENCES IN THE MAJOR FORMS OF HEPATIC CYTOCHROME P450 ISOENZYMES[a]

Species[b]	P450 Nomenclature	Inducer[c]	Monomeric molecular weight	Absorbance maximum of Fe^{2+}—CO complex	Preferred Substrate(s)[d]	Terminal Amino Acid N-	Terminal Amino Acid C-	Reference
Rat	$P450_a$	ARO, 3MC, PB	48,000	452	Testosterone	MET	MET	21
Rat	$P450_b$	ARO, PB	52,000	450	Benzphetamine, Dimethylaniline	GLU	SER	21, 27, 28
Rat	$P450_c$	ARO, BNF, 3MC	56,000	447	Benzphetamine, 7-ethoxycoumarin 7-ethoxyresorufin	ISO	LEU	21, 28
Rat	$P450_d$	ISO	52-53,000	447	Isosafrole	-	-	22, 35
Rat	$P450_e$	ARO, PB	52,500	450.6	Estradiol (17B position)	-	-	133, 134
Rat	RLM_3	None[e]	50,000	449	Testosterone	MET	-	41
Rat	RLM_5	None[e]	51,000	451	Testosterone	MET	-	41
Rabbit	Form 1	TCDD	48,000	450.5	Benzo(a)pyrene, Benzphetamine progesterone	-	-	49
Rabbit	Form 2 (LM_2)	PB	48-52,000	451	Benzphetamine, cyclohexane	MET	ARG	47, 50, 51
Rabbit	Form 3	PB, TCDD	51-52,600	450	Aminopyrine	-	-	52, 53
Rabbit	Form 3a	Ethanol	51,000	452	Ethanol	ALA	LEU	148
Rabbit	Form 3b	None[e]	52,000	451	Progesterone	-	-	149, 150
Rabbit	Form 3c	None[e]	53,000	449	Progesterone	MET	-	149
Rabbit	Form 4 (LM_4)	BNF, 3MC, PB, TCDD	51-55,000	447-448	Acetanilide, Benzo(a)pyrene, Biphenyl	-	LYS	50, 51, 55
Rabbit	Form 4a (LM_{4a})	BNF	51-55,000	447	Benzo(a)pyrene	-	-	12
Rabbit	Form 6	TCDD, (ISO)	51,000	448	Ethoxyresorufin	-	-	58, 152
Mouse	A_2	PB	50,000	451	Benzphetamine, Testosterone	-	-	60
Mouse	C_2	PB	56,000	450	Ethylmorphine	-	-	60
Mouse	P_1450	3MC	55,000	449.3	Benzo(a)pyrene	-	-	62, 153
Mouse	P448	3MC	55,000	448	Acetanilide	-	-	62, 153
Mouse	P_2450	3MC, ISO	55,000	448-449	Isosafrole	-	-	154

[a] This table serves as a summary of the major, well-characterised forms of cytochrome P450 and the reader is referred to the text for a description of other characterised isoenzymes.

[b] The information for each species may not be related to results from other laboratories as different strains of rats were used.

[c] Abbreviations : ARO, Aroclor 1254; BNF, B-naphthoflavone; ISO, Isosafrole; 3MC, 3-methylcholanthrene; PB, phenobarbitone; TCDD, 2, 3, 7, 8 - tetrachlorodibenzo-p-dioxin.

[d] The information on the preferred substrates of the cytochrome P450 isoenzymes are only an indication of the substrate specificity of that particular form because each isoenzyme has not been tested with all the substrates indicated in this table.

[e] Indicates that the enzyme has been purified from un-induced rat liver (constitutive form).

activity in human lymphocytes and monocytes is more readily inhibited by 3MC rat IgG than by PB rat IgG.

Thus although the number of unique cytochromes P-450 that potentially constitute the mixed function monooxygenase system in a single mammalian species is presently unknown, different isoenzymic forms within a given species have been characterised by a variety of experimental criteria (12,13,14). No direct homology has yet been shown for any two cytochromes P-450 in different species, although similarities have been shown to exist (159). That some of these proteins represent unique gene products is now accepted, but the picture is complicated by the fact that structural and functional polymorphism of different cytochromes P-450 is exhibited within different strains of the same species. In Long-Evans and Holtzman rats for example, four immunochemically identical but molecularly distinct forms of phenobarbital-induced hepatic cytochrome P-450 have been shown to exist in unique combinations for that particular strain (19). Subsequent *in vitro* translation experiments indicated that those proteins were in fact coded for by different mRNA's (157). The elucidation of such functional polymorphism will aid in the understanding of the molecular multiplicity of cytochrome P-450. Towards this aim, recombinant DNA technology is being directed in order to determine the size of the cytochrome P-450 multi-gene family.

3. MOLECULAR EVENTS FOLLOWING INDUCTION OF HEPATIC CYTOCHROME P-450 BY XENOBIOTICS

3.1 Biosynthesis of cytochrome P-450 directed by rat liver messenger RNA

The discovery that the activity of hepatic microsomal enzyme systems could be increased by different drug or xenobiotic inducers was made in the mid 1960's, when it was observed that phenobarbitone and polycyclic aromatic hydrocarbons such as 3-methylcholanthrene were potent inducers of cytochromes P-450. Subsequent to these early observations, many drugs and chemicals have been shown to induce liver microsomal cytochrome P450 in various species, and the compounds shown in Table 4 represent only a partial list of known inducing agents. Although the complete mechanism of haemoprotein induction has still not been completely elucidated, many facts are known (15,106). Phenobarbitone pretreatment of experimental animals results in hyperplasia of the smooth endoplasmic reticulum with resulting hypertrophy of the liver, characterised by an increase of cytochrome P-450 and other proteins. These include NADPH-cytochrome P-450 reductase (77), epoxide hydrolase (79), and several cytosolic enzymes such as glutathione-S-transferases

(80) and aldehyde dehydrogenases (81). This induction is preceded by an increase in the level of messenger-type RNA and an increase in the utilisation of amino acids for protein synthesis. This inductive response can be inhibited by drugs such as actinomycin D, ethionine and α-aminitin (protein synthesis inhibitors, 77, 78), thereby suggesting an inductive mode of action at the level of transcription and/or processing of mRNA for these inducers (93). Furthermore, using blockers of heme synthesis (aminotriazole), it was shown that the PB-induced increase in liver microsomal cytochrome P-450 was due to *de novo* protein synthesis which was not dependent upon heme synthesis, but which did require a supply of heme (107). PB also leads to increased stability and processing of ribosomal RNA precursors possibly due to decreased RNAase activity. Nucleocytoplasmic transport of newly synthesised ribosomal sub-units is stimulated and is believed to be dependent on the synthesis of cytoplasmic factors, possibly ribonucleoprotein particles (RNP's). This increase in protein synthesis as a result of PB induction may be related to greater mRNA stability, possibly through decreased rates of degradation (108). Thus in considering the molecular events following induction of cytochrome P-450, the crucial question which needs to be answered is whether the molecular action of the inducer is to enhance the expression of a specific gene coding for a particular cytochrome P-450, or whether a single gene could code for a number of different but closely related proteins by means of post-transcriptional control. This could include the regulation of RNA processing control within the nucleus, transport of the mRNA from the nucleus and the subsequent stability as well as translational fidelity of the mRNA within the cytoplasm. Ultimately this question will be answered by the sequencing and characterisation of the various cytochrome P-450 structural genes by means of DNA recombinant technology.

PB and 3MC pretreatment of experimental animals results in a 2-3 fold increase in the liver microsomal content of cytochrome P-450 (26,90), and immunological measurements indicate that pretreatment with either xenobiotic inducer leads to a 24-50 fold increase in the levels of different molecular forms of cytochrome P-450, each of which is present at low levels in the liver of uninduced animals (25,26,90). PB treatment for four days was shown to result in a large increase in liver weight (55%) and although the level of polyribosomal RNA per gram of liver increased only slightly (12%), there was a marked enhancement of poly $(A)^+$ mRNA in the liver microsomal fraction (82). Although the majority of eukaryotic mRNA's contain a poly (A) region at their 3'-OH end, the precise role of this sequence is still far from clear(83). Similarly it should be noted that some mRNA species do not normally possess a poly (A) sequence and this absence apparently does not influence their

TABLE 4 Xenobiotic Inducers of Hepatic Cytochrome P450

A.	Drugs	Example
	1. Hypnotics/sedatives	most barbiturates, including phenobarbitone; chlordiazepoxide; nitrazepam
	2. Anti-inflammatories	phenylbutazone
	3. Anti-histamines	chlorcyclizine, diphenyhydramine
	4. Anti-convulsants	phenytoin, carbamazepine
	5. Hypolipidaemics	most oxyisobutyrates including clofibrate and bezafibrate
	6. Narcotic analgesics	pethidine
	7. Psychotropics	chlorpromazine, imipramine
	8. Anti-fungals	griseofulvin
	9. Antibiotics	oleandomycin, triacetyloleandomycin
	10. Respiratory stimulants	nikethamide, bemegride
	11. Steroids	pregnenolone-16α-carbonitrile
B.	Chemicals	
	1. Polycyclic aromatic hydrocarbons	many combustion products including 3-methylcholanthrene, benzo(a)pyrene, 1,2:5,6-dibenzanthracene, 1,2-benzanthracene, naphthalene, phenanthrene, chrysene
	2. Insecticides	DDT (dichlorodiphenyl-trichloroethane), aldrin, dieldrin
	3. Alcohols	ethanol
	4. Food anti-oxidants	ethoxyquin and 3,5-di-tert-butyl-4-hydroxytoluene
	5. Dibenzo-p-dioxins	TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin)
	6. Flavones	5,6-benzoflavone
	7. Methylenedioxyphenyls	isosafrole, piperonyl butoxide
	8. Halogenated biphenyls	many including 3,3′,4,4′-tetrachlorobiphenyl
	9. Indoles	indole-3-acetonitrile
	10. Aromatic amides	2-acetylaminofluorene

translatable activity (84). However it is believed that polyadenylation is involved in the conversion, and hence stability, of primary mRNA transcripts to functional mRNA molecules (83). In addition, the transport of mRNA from the nucleus to the cytoplasm of liver cells is also believed to be dependant upon polyadenylation (85). These two factors are probably interrelated as the occurrence of the poly (A) sequence at the 3 end of the RNA chain would serve to protect it from nucleolytic enzymes which would otherwise destroy the mRNA sequence before it reached the cytoplasm (86). It has subsequently been shown that translation of total liver poly $(A)^+$ mRNA in a heterologous rabbit reticulocyte cell-free system resulted in the synthesis of a polypeptide corresponding to cytochrome P-450, indicating that the corresponding mRNA existed in rat liver as a polyadenylated form(87). By means of immunoprecipitation with specific cytochrome P-450 antibodies of the translation products, the major protein synthesised was shown to have a monomeric molecular weight of 52,000, strongly suggesting that the precipitated species was indeed 52 K cytochrome P-450. Partial sequencing of the *in vitro* translation product, in which five amino acids were radiolabelled in the mature protein, (representing 14 of the first 20 residues) have been carried out and the positions of the chosen amino acids in the *in vitro* synthesised cytochrome P-450 corresponded exactly to their position in the mature protein (88). This would indicate that the above phenobarbital induced form of cytochrome P-450 is probably synthesised as the mature polypeptide which does not require to undergo extensive post translational modification (88,89).

Various groups have shown that PB administration results in an increase in the mRNA coding for cytochrome $P\text{-}450_b$ (90,91), and Phillips, *et al.* (90) have estimated that there is approximately an 18 fold increase of 52 K cytochrome P-450 mRNA in the livers of Sprague Dawley rats. However following PB administration, it has been observed that the increase of spectrophotometrically detectable cytochrome P-450 reaches a maximum at about 45 hours after a single injection of phenobarbital whilst the increase in cell free translation of apo-cytochrome P-450 directed by the corresponding mRNA, reaches a maximum at 16 hours following PB administration (89). This time delay could perhaps reflect the stability and translational efficiency of the message at this particular time, possibly representing a subtle method of cytoplasmic gene control. Alternatively the rate limiting step in the biosynthesis of cytochrome P-450 may involve a post translational process such as heme insertion into the newly synthesised apoprotein. This is indeed conceivable as it is known that the apoprotein of cytochrome P-450 and the heme group turn over at different rates *in vivo* (107). With respect to the time delay between inducer administration, apoprotein biosynthesis,

and the presence of spectrophotometrically detectable cytochrome P-450 as described for PB induction, it is interesting to note that the induction of cytochrome P-450 by BNF as measured in the cell free system follows a similar temporal pattern (92).

Lechner and Sinogas (94) have suggested that the time course of the nuclear pre-mRNA processing into functional polyadenylated mRNA and its subsequent expression following induction by PB may be modulated by the presence of messenger ribonucleoprotein particles (RNP's). This is because potentially functional mRNA is frequently stored in the cytoplasm in a repressed state as RNP complexes (95), and the equilibrium between polysomes and free mRNP plus ribosomal sub-units is known to constitute an important device for translational control. They showed that such RNP's and their corresponding poly $(A)^+$ mRNA displayed a much higher and durable template activity following xenobiotic induction, indicating that these particles could conceivably be acting as potentially active message vehicles for regulation mechanisms. It is also postulated that the RNP's may play a role in the transport of mRNA, possibly by interacting with the nuclear pore complex. It has been shown that in newly synthesised mRNA, an increase in the amount of poly (A) present can be correlated with an increase in the amount of a 74,000-78,000 molecular weight protein associated with the complex which is believed to be a component of the mRNP complex(96). It was further suggested (94) that the RNP's could be involved, possibly through some form of specific attachment mechanism, in the direct association between the mRNA sequence and the endoplasmic reticulum membrane because of an increased binding affinity between endoplasmic reticulum membranes and RNP complexes. Such binding could modulate the equilibrium between latent and active forms of mRNA's so that translational efficiency is increased (97). This concept is given credence by the observation that endoplasmic reticulum membrane bound polysomes from the livers of PB treated rats are capable of synthesising, in an *in vitro* translation system, approximately ten times higher levels of cytochrome P-450 than do free polysomes (88). In fact, on considering the ratio of bound to free polysomes in the rat liver(98), this indicates that the membrane bound polysomes are responsible for approximately 95% of the apocytochrome P-450 synthesised. Such a function for RNP complexes has also been suggested in adenovirus infected KB cells where it is thought that the mRNP's are specific for the attachment of viral mRNA to host cell ribosomes (99). Thus although the precise role of mRNP's in induction is still unclear, the pharmacological and toxicological implications are important when considering the question of drug action and toxicity, as it appears that modification of these mRNA

binding proteins may have the potential to modulate protein translational efficiency.

It is interesting to note that no time delay is observed between the binding of the specific mRNA containing polysomes to the endoplasmic reticulum and the presence of immunoprecipitable translated product in the endoplasmic reticulum following induction of NADPH-cytochrome P-450 reductase and epoxide hydrolase by phenobarbital (75). Evidence suggests that PB acts primarily at the level of the nucleus increasing the rate of transcription or post transcriptional processing and nucleocytoplasmic transport of the specific mRNA's (101). This inductive effect is inhibited by actinomycin D (77) and partially blocked by cordycepin which causes the termination of RNA chain synthesis (101). These findings suggest similarities between the modes of action of PB and steroid hormones in which hormone-receptor complexes interact with chromatin causing an increase in the rate of transcription of specific mRNA's (101). The existence of cytoplasmic receptors which would modulate the induction of PB has also been postulated (100), although none have been identified to date. However in the mouse and rat, such receptors (termed Ah receptors) have been identified (100). These bind polycyclic aromatic compounds such as TCDD and 3-MC and their binding affinity correlates well with their potency as inducers.

A further means of cytoplasmic gene control may be achieved by post-translational modification, although as yet, such modification has only been reported in one case in which a 3-MC form of cytochrome P-448 appeared to be synthesised as a polypeptide precursor of monomeric molecular weight 59,000 (103). Significant conversion of the 59,000 species to a 53,000 species could be demonstrated when the translation was carried out in the presence of microsomal membranes isolated from rat liver. Whether these differences could be explained by post-translational.modification involving cleavage of the polypeptide precursor giving rise to a different species, or whether the synthesis and translation of individual mRNA's gives the option that each species can have its own precursor, is unknown at the present time. Thus although the induction by PB and 3MC appears to be directed at the transcriptional or translational levels, the possibility of minor post-translational modifications which might possibly occur as a result of phosphorylation (104) or glycosylation (105) should not be discounted.

3.2 In Vitro Translation and Purification of Cytochrome P-450 mRNA

In vitro protein synthesising systems from eukaryotic cells have been successfully used in studies to elucidate the basic mechanisms of protein synthesis and to identify specific mRNA's by their translation products.

Moreover, *in vitro* experiments which accurately translate mRNA's are extremely useful in understanding both the regulation of mRNA translation and as an experimental tool in the quantitation of specific mRNA's derived from whole cells or sub-cellular fractions. Specific cytochrome P-450 mRNA's can now be identified by using a combination of *in vitro* translation analysis of mRNA followed by immunoprecipitation using a suitable antibody (88,90). However, these studies have only succeeded in partially purifying cytochrome P-450 mRNA and subsequent work has focused on cloning of the cDNA sequence of cytochrome P-450 mRNA in order to investigate, in detail, the molecular multiplicity of cytochrome P-450.

Initial experiments have shown that following PB induction, the nascent peptides of cytochrome P-450 are mainly located on the membrane bound polysomes in rat liver (110). When free and membrane bound polysomes are first separated on a discontinuous sucrose density gradient and the mRNA is removed and subsequently translated in an *in vitro* protein synthesising system, it has been shown that the apoprotein of cytochrome P-450 is predominantly synthesised by the membrane bound polysomes, with less than 10% of the total cytochrome P-450 being synthesised by the reaction mixture containing mRNA from free polysomes (88,111). The levels of specific translatable cytochrome P-450 mRNA ranges from 0.2 to 4.0% (112) in PB induced rat liver, and 0.63% in rabbit liver (113).

Analysis of PB induced Sprague-Dawley *rat* livers by Fujii-Kuriyama *et al.* (111), showed that approximately half of the cytochrome P-450 mRNA possessed a poly (A) tail, as is often the case with eurkaryotic mRNA's (114). Sucrose density fractionation in a linear density gradient indicated that this poly(A)$^+$mRNA was approximately 18S in size which corresponds to 2 - 2.3 Kb. It was also interesting to note that the poly (A-)mRNA was also able to code for cytochrome P-450$_b$ when translated in an *in vitro* system. However the actual content of this fraction was not sufficient to be exploitable. The stability of poly (A-) mRNA as compared to poly(A)$^+$mRNA has not been determined.

The characterisation of mRNA associated with *mouse* liver cytochrome P_1-450 has revealed the presence of two distinct forms of mRNA present following induction by 3MC (120). This phenomenon was shown to be strain dependent with genetically responsive 3MC pretreated B6 mice producing at least ten times more mRNA associated with inducible cytochrome P_1-450, as the non-responsive 3MC-pretreated D2 or control B6 or D2 mice (117). The fact that two distinct forms of mRNA existed became clear only under denaturing conditions and is believed to reflect changes in the secondary or tertiary structures of the mRNA. Sucrose density gradient centrifugation yielded a 22S to 23S

mRNA associated with cytochrome P_1 -450, whereas under denaturing conditions (5 mM methyl mercury hydroxide, 1% agarose gel) two mRNA sub-units became apparent, one slightly less than 21S the other.23S, corresponding to 2,700 and 3,500 nucleotides respectively (116). The observed size of the smaller mRNA is almost twice as large as the 1,500 nucleotides required for translation into a Mr = 55,000 sub-unit, and the size of the larger mRNA is big enough to be bicistronic. Subsequent studies have shown that it was the 23S mRNA which was most closely associated with cytochrome P_1-450 activity (117). The precise reason for the existence of the two mRNA species is not known but one possibility is that the antibody to cytochrome P_1-450 immunoprecipitated at least two different forms of cytochrome P-450 associated with the Ah^b allele. This is given support by the findings that one or more 20S mRNA's associated with Ah^b Allele are also induced following administration of either isosafrole or low doses of 3-methylcholanthrene (156). These mRNA species code for one or more forms of cytochrome P-450 other than cytochrome P_1-450. It is believed that both the 20S and 23SmRNA's possess homologous nucletotide sequences.

Partial purification of *rabbit* liver cytochrome P-450 LM_2 mRNA has also been carried out(1l3). As in the case of rat, PB induction results in an increased amount of cytochrome P-450 LM_2 mRNA which sedimented in the 18Sregion of a sucrose density gradient (5-25%) (115). Acrylamide agarose gel electrophoresis of the partially purified mRNA suggests that it has a sub-unit molecular weight of 700,000. The major protein coded for by this mRNA, like that coded for by its rat liver counterpart, migrated on SDS polyacrylamide gels with cytochrome P-450 LM_2. As in the case for the purification of other mRNA's(113,117), the mRNA sequence coding for cytochrome P-450 LM_2 contains large 5 and 3 untranslated nucleotide sequences (113) as only 1272 of its nucleotides would be required to code for the 424 amino acids in cytochrome P-450 LM_2 (51). The significance or reason for the presence of these silent nucleotides in the specific mRNA sequences is at present unknown (118,119).

The significance of large amounts of untranslated nucleotides in the 5 and 3 regions of cytochrome P-450 (as well as for other mRNA's) is unknown (119), and has made identification and characterisation of the structural gene more difficult. It is for this reason that DNA recombinant techniques have been utilised for mRNA quantitation and elucidation of the structure and multiplicity of cytochrome P-450 genes.

3.3 Recombinant DNA studies involving molecular cloning of complimentary DNA to inducible cytochrome P-450 mRNA

Nucleic acid hybridisation can be used to measure the degree of complimentarity between the base sequences of two polynucleotide molecules (121). Recently with the development of techniques for synthesising and cloning double stranded DNA copies of poly (A) containing mRNA, it has been possible to obtain pure mRNA sequences in relatively large amounts (121,122) and complementary DNA's to a number of different induced cytochrome P-450's have now been successfully cloned. For example, Fujii-Kuriyama *et al.* have isolated a cDNA to PB induced cytochrome P-450$_b$ mRNA which on translation, resulted in the synthesis of a single protein corresponding to that of cytochrome P-450$_b$ (111,123). Subsequent studies with several cDNA clones of phenobarbital induced cytochrome mRNA enabled the coding nucleotide sequences of these cloned cDNA's and the primary amino acid sequence of the cytochrome P-450 peptide for which they code to be elucidated (118). On sequencing the cDNA's it was shown that although the nucleotide sequences were highly homologous, substitutions occurred in a limited portion of their sequence, indicating that the cloned cDNA's were probably derived from two very similar but nevertheless distinct mRNA's. It was estimated that the cytochrome P-450 apoprotein consisted of 491 amino acids equivalent to a molecular weight of 55,900 (118), which is similar to the estimated apparent molecular weight of the pure protein (28). Structural analysis of the total gene coding for cytochrome P-450 indicated that it was approximately 13 Kb in length being separated into 9 exons and 8 intervening sequences of various sizes (116). On the basis of this gene structure, it was estimated that the mRNA was 1920 nucleotides long plus a poly A sequence, of which 30 bases are for the leader sequence, 1473 bases for the coding sequences and 416 bases for the trailer sequence followed by the poly A stretch (116).

Adesnik *et al.* (38) has also reported the isolation of cloned c DNA's encoding the major PB-induced cytochrome P-450 in rat liver. Using this cDNA probe in hybridization experiments, these authors demonstrated that PB treatment results in a 30 fold increase in polysomal cytochrome P-450 mRNA. Bresnick, *et al.* (124) has also successfully constructed a cDNA clone containing the information for rat cytochrome P-450 induced by 3MC. They showed that maximum induction of cytochrome P-450 mRNA occurred after 15 hours although a detectable increase could already be seen 7 hours following a single injection of 3MC. The levels of cytochrome P-450$_c$ mRNA began to return to control levels after 24 hours (125). Using their cDNA cytochrome P-450 clone, the translated product migrated with authentic cytochrome P-450

when subjected to SDS page analysis (124). These latter authors were unable to find any evidence for the presence of a precursor segment of cytochrome P-450$_c$ as had been previously reported (103).

A further DNA complementary to a mRNA of 2000 nucleotides coding for 3-methylcholanthrene-induced cytochrome P-450 has also been constructed (129). Comparison of this clone to the one constructed by Bresnick *et al.* (124) indicated that both possess cloned sequences from different mRNA's. On the basis of translational and immunoprecipitation evidence, a further mRNA (2.8 Kb) coding for 3-MC cytochrome P-450 peptides has also been identified and is believed to be different in sequence to the 2.0 Kb mRNA species described above (129). In addition, induction with 3-MC was shown to result in a 4 fold increase in the levels of 3-MC cytochrome P-450 mRNA.

Cloned DNA sequences have also been prepared which have allowed characterisation of mouse cytochrome P$_1$-450 mRNA (126,127). By use of the clone (designated clone 46), it was shown that induction of cytochrome P$_1$-450 23SmRNA is under transcriptional control (126), and that polysomes bound to the endoplasmic reticulum were the principle site of synthesis for cytochrome P$_1$-450 (128). In addition, clone 46 hybridises with rat mRNA but not with 3-methylcholanthrene-induced 23S mRNA in the rabbit (132). The P$_1$-450 structural gene has been localised to mouse chromosome number 2 and is estimated to span 5kb possessing at least 5 exons and 4 introns. There is also some evidence suggesting that other genes coding for polycyclic aromatic hydrocarbon-inducible forms of P-450 are located in tandem on the same chromosome (158).

4. CONCLUSIONS

Species variation in drug metabolism can be rationalised by a variety of factors, one of the most important being the nature and amount of the liver enzymes responsible for biotransformation of the drug substrate. Many studies have emphasised the crucial role played by the hepatic cytochromes P-450 in the process of drug metabolism(5-7) and it is now well documented that cytochrome P-450 exists as multiple enzyme forms (8-11). Furthermore, some forms of the cytochrome biologically function to detoxify drugs and enhance their excretion from the body, whereas other forms of cytochrome P-450 may metabolize drugs to produce potentially toxic metabolites (130). Ultimately these reactive intermediates have the ability of binding to cellular genetic material (including RNA and DNA), resulting in mutagenic and possibly carcinogenic responses in experimental animals and man. A major problem which faces both the pharmaceutical industry and the regulatory au-

thorities is that when a new drug is introduced for use in man, it must first be tested in laboratory animals and existing methods of toxicity testing can only *extrapolate* from animal data and hence only *indicate* a potential toxicity in man.

This problem may be overcome on an *in vitro* basis if the catalytic properties of the different cytochromes P-450, which are known to be responsible for activation/detoxification of drugs, could be characterised on a genetic basis. In this respect, the multiplicity of cytochrome P-450 is well established, but knowledge relating to the regulation of the cytochrome P-450 system at the levels of mRNA transcription, processing and degradation is at present lacking. Progress in the study of gene regulation and resolution of the specific cytochrome P-450 gene structures has the potential to place the extrapolation of animal data to man on a more firm experimental basis. Accordingly, identification and characterisation of the cytochrome P-450 structural genes will not only provide valuable insight into an understanding of the genetic regulation of drug metabolising enzyme induction, but may also aid in the development of new potentially useful assays for determining genetic differences in individual risks of cancer and drug toxicity in both human and animal populations.

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