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NOVEMBER 1984
INDUCTION AND CHARACTERISATION OF
CYTOCHROME P-450 MULTIPLE FORMS.

A thesis presented for the
Degree of Doctor of Philosophy

by

Ryszard Jan Zygmunt Makowski

August 1985
University of Surrey
Department of Biochemistry
Guildford, Surrey
England.
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SUMMARY

The isolation and purification of two major phenobarbitone-induced hepatic cytochromes P-450 from male Wistar rats is described. These forms (designated cytochromes P-450 B₁ and P-450 B₂) were extensively characterised and structurally and functionally compared to two other homogeneous isoenzymic forms, cytochromes P-452 (clofibrate-induced) and cytochrome P-447 (BNF-induced). These characterisation studies (including catalytic, immunological, physical and spectral analysis) indicated that all four isoenzymes were distinct, unique hemoproteins.

The above characterisation was extended towards the induction profiles of these hemoproteins in hepatic and renal microsomes, following single dose xenobiotic pretreatment. Of primary interest was the immunoquantitation data which revealed the presence of cytochrome P-452 as a major constitutive isoenzyme. This data in conjunction with the metabolic data also indicated that xenobiotic induction of cytochromes P-450 was both a specific and precise event.

In order to determine the induction profile of these hemoproteins following xenobiotic pretreatment, analysis of the mRNA species was also undertaken. In vitro rabbit reticulocyte translation systems in conjunction with immunoprecipitation analysis (using monospecific antibodies against the specific cytochrome P-450) revealed that following phenobarbitone and β-napthoflavone pretreatment the induction of the specific translatable mRNA species was due to a difference in the amount of specific mRNA and not to a change in its translatability. This induction profile corresponded to that found on analysis of the holoenzyme cytochromes P-450 present within hepatic microsomes. The temporal aspects of the induction of the mRNA species coding for cytochrome P-452 was not in agreement with the metabolic and immunonochemical data for this isoenzyme in hepatic microsomes. This discrepancy is possibly believed to relate to the activation/repression of genes coding for similar and related cytochrome P-452
A preliminary analysis of renal mRNA species coding for the cytochromes P-450 in conjunction with metabolic data was strongly suggestive for there being different modes of induction in the liver and kidney.
Chapter 1

INTRODUCTION

Cytochrome P-450 is the terminal hemoprotein component of an electron transport chain ubiquitously distributed throughout living organisms and functional in the aerobic metabolism of numerous structurally unrelated compounds. These include drugs, carcinogens, insecticides, environmental pollutants, as well as a wide number of diverse endogenous compounds such as vitamin D, fatty acids and steroids (table 1), and for this reason a great deal of effort has gone into the purification and characterisation of cytochromes P-450.

Early studies showed that different xenobiotic inducers could manipulate the biochemical and biophysical properties of the microsomal hydroxylation system (Alvares et al. 1971, Conney et al. 1960), and this in conjunction with the remarkably broad substrate specificity, regioselectivity and stereoselectivity of cytochrome P-450 was attributed to the existence of a number of distinct species of the hemoprotein (Thomas et al. 1976, Ullrich et al. 1977). Subsequent to these early studies, experimental evidence including sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS P.A.G.E.), peptide mapping, amino acid composition and sequence determination, spectroscopy, chromatography, kinetic and immunological analysis, have conclusively shown that various purified cytochromes P-450 are distinct proteins (Lu et al. 1980, Guengerich et al. 1979).

This concept of hemoprotein 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 (Parke 1975). This would indicate that the different cytochromes and therefore their corresponding mixed function oxidase activities (M.F.O.) are under different genetic control mechanisms.
<table>
<thead>
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<th>Reaction Type</th>
<th>Substrates</th>
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<tr>
<td>1. Aliphatic hydroxylation</td>
<td>Cholesterol, fatty acids, pentobarbitone, prostaglandins, testosterone, vitamin D₃.</td>
</tr>
<tr>
<td>2. Aromatic hydroxylation</td>
<td>Aniline, Amphetamine</td>
</tr>
<tr>
<td>3. Dehalogenation</td>
<td>Carbon tetrachloride, chloroform, halothane.</td>
</tr>
<tr>
<td>5. Epoxidation</td>
<td>Aldrin, benzo(a)pyrene, unsaturated fatty acids, styrene.</td>
</tr>
<tr>
<td>6. Oxidative deamination</td>
<td>Amphetamines.</td>
</tr>
<tr>
<td>7. N-dealkylation</td>
<td>Aminopyrene, chlorcyclizine, ethylmorphiline, N-alkyl amphetamines.</td>
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</table>
The biological significance of this multiplicity of cytochrome P-450 between species relates to the fact that the monooxygenases catalyse both activation and detoxification pathways for a wide variety of substrates, their distinct enzymatic properties and variable occurrences might result in marked dissimilarities in the pharmacology, toxicity or carcinogenicity of a given compound. 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 cytochrome P-450 and reconstituted enzyme systems (Lu et al. 1974) have offered the possibility of greater detailed study of the more definitive roles of the multiple forms of cytochrome P-450. Similarly, because of the absence of other interfering enzymes, the reconstituted enzyme system is ideal for kinetic and mechanistic studies involving cytochrome(s) P-450. This taken in conjunction with analysis of the messenger RNA (mRNA) species coding for the distinct cytochrome P-450 isoenzymes, offers an improved means of characterisation, with these profiles providing 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 species is confusing, mainly because of the lack of uniformity in nomenclature amongst different research groups. However, it is generally accepted that a conservative estimate of the minimum number of forms in various microsomes (for reviews see Lu et al. 1980, Makowski et al. 1982) would be:

a) eight to fifteen forms in rat liver microsomes.
b) five to six forms of rabbit liver and lung microsomes taken collectively.
c) a minimum of four forms in mouse liver microsomes.
d) an undetermined number in human liver microsomes.
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 vary slightly from each other in order to be able to efficiently metabolise the hundreds of substrates of widely differing chemical structures (Nebert 1979). Verification will have to await a much greater understanding of genetic regulation and expression, although there is no doubt of the relationship associating genetic variables and individual differences in susceptibility to toxicity and cancer, as seen in both experimental animals and humans (Vesell et al. 1977, Nebert 1980).

With increasing sophistication in immunological and amino acid sequencing techniques, evidence has mounted that many of the multiple forms of cytochrome P-450 within a given species have different primary and tertiary structures. Such data (Vlasuk et al. 1982, Levin et al. 1980) have indicated that these cytochromes were in fact separate gene products and not post-translational modifications of a single gene product. Recombinant DNA research has now shown this to be the case, with it becoming evident that the cytochromes P-450 are in fact coded for by a multigene complex within a given species (Atchison et al. 1983, Kumar et al. 1983). 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 characterised by different amino acid sequences and being immunologically distinct as a result of separate gene expression.

1.1 MULTIPLE FORMS OF RAT HEPATIC MICROSOMAL CYTOCHROME P-450

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 these isoenzymes are fundamentally different proteins which differ in their primary amino acid sequences, substrate specificity, immunological cross-reactivity and inducibility by different xenobiotics (Ryan et al. 1979, 1980). In general, no
specific structural similarities exist between the inducers of cytochrome P-450 which are responsible for the specific increase of a particular class of hemoprotein and hence its substrate specificity (Snyder et al. 1979). This latter diversity of substrate specificity between different species has been a major problem in adequate toxicity and carcinogenicity screening programmes.

According to current criteria for homogeneity (which includes SDS P.A.G.E., catalytic, spectral and immunological properties, sequencing of the NH$_2$- and COOH-termini and proteolytic peptide fragments of cytochrome P-450), eight to fifteen forms of cytochrome P-450 have been postulated to be present in rat hepatic microsomes. The amounts of these various isoenzymes has been shown to be a function of age, sex, strain and inducer (Sato et al. 1978, Pickett et al. 1981). Taking these variable factors into consideration, results indicate that the constitutive levels of the cytochromes P-450 and P-448 in rat liver microsomes is very low (less than 5% of the total cytochrome P-450, Pickett et al. 1981). Phenobarbitone and 3-methylcholanthrene induce cytochrome P-450 (15-30 fold) and cytochrome P-448 (40 fold) respectively. After phenobarbitone-induction, depending on the age and species of the animal, the phenobarbitone-induced isoenzymes represent 30-60% of the total microsomal cytochrome P-450, whereas after 3-methylcholanthrene pretreatment cytochrome P-448 levels can be as high as 80% of the total cytochrome P-450 population (Harada et al. 1980). This group also showed that phenobarbitone 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 phenobarbitone-inducible form of cytochrome P-450 in rat liver (designated P-450$_{b}$ by some authors, Ryan et al. 1979), can also be induced by polychlorinated biphenyls such as Aroclor 1254. The sub-unit molecular weight of cytochrome P-450$_{b}$ ranges from 50-53K, with an absorption maximum of its reduced CO-difference spectrum ranging 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 (Botelho et al. 1979). This form 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 residues of cytochrome P-450\textsubscript{b} and rabbit liver cytochrome P-450 LM\textsubscript{2} also induced by phenobarbitone (Botelho et al. 1979). Of the first twenty one amino acids, fourteen common residues are found with six of the differences being 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 2 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 phenobarbitone, 3-methylcholanthrene or polychlorinated biphenyls. Both enzymes exhibit a poor catalytic activity towards benzo(a)pyrene hydroxylation but they show almost absolute specificity for the 16\alpha hydroxylation of testosterone with little or no detectable activity for the 6\beta or 7\alpha hydroxylation of this substrate.

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 the C-terminal regions and do not co-electrophorease on SDS P.A.G.E. They are also immunologically non-identical (Botelho et al. 1979), and since antibodies are directed against several spatially distant sites (epitopes) on the protein surface, it indicates that they have dissimilar structures.

Cytochrome P-450\textsubscript{b} is predominantly a low spin hemoprotein although there is evidence that it may contain some high-spin component. Purified preparations from either phenobarbitone or Aroclor 1254-treated rats are usually low spin hemoproteins (Ryan et al. 1979).

The major 3-methylcholanthrene-induced form in rat liver has been termed cytochrome P-450\textsubscript{c}, also induced by 8-naphthoflavone, Aroclor 1254 and 3,4,5,3',5' pentachlorobiphenyl. Reports of sub-unit molecular weight of this cytochrome P-450 range from 53-56K, 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 are isoleucine and leucine respectively (Botello et al. 1979). Cytochrome P-450<sub>c</sub> 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 observed with liver microsomes suggesting that it is not the major isoenzyme responsible for this hydroxylation. Preliminary work also indicated the presence of a second form of cytochrome P-448 following induction by 3,4,5,3,4,4 hexachlorobiphenyl (Kohli et al. 1981). 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<sub>c</sub>. The other form has a sub-unit molecular weight of 52,000, but does not metabolise either benzphetamine or 7-ethoxyresorufin, substrates preferentially metabolised by cytochromes P-450<sub>b</sub> and P-450<sub>c</sub> respectively.

In the rat the major form (cytochrome P-450<sub>c</sub>) has been shown to represent at least 80% of the total polycyclic aromatic hydrocarbon inducible aryl hydrocarbon hydroxylase (AHH) activity (Thomas et al. 1981). However evidence has also been presented indicating the presence of further forms of cytochromes P-450 following induction by TCDD (Guenther et al. 1978, Chen et al. 1982). These have been termed cytochromes P<sub>1</sub>-450 and P<sub>3</sub>-450 and it has been suggested that these are related to the same species found within the mouse (Negishi et al. 1979). Cytochrome P<sub>1</sub>-450 is defined arbitrarily as the polycyclic aromatic hydrocarbon inducible form most closely associated with aryl hydrocarbon hydroxylase activity. Cytochrome P<sub>3</sub>-450 is defined 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 was some initial scepticism relating to the existence of these two forms of cytochrome P-450 in species other than mouse, data has emerged indicating that both the rat and mouse forms are immunologically indistinct (Chen et al. 1982).
A portion of the mouse cytochrome P\textsubscript{1}-450 gene has been cloned and there is sufficient nucleotide sequence homology between the mouse structural gene and its mRNA to that of the rat, to indicate that rat cytochrome P-450\textsubscript{c} is similar to mouse cytochrome P\textsubscript{1}-450 (Tukey et al. 1981, Negishi et al. 1981). It is interesting to note that cytochrome P\textsubscript{1}-450 (the form believed to be associated with the initiation of PAH carcinogenesis), has the highest molecular weight of all the cytochrome P-450 isoenzymes induced by PAH's in the rat, rabbit, mouse and fish (Nebert et al. 1982). No immunological identity is observed between the major 3-methylcholanthrene-induced cytochrome P-450\textsubscript{c} of rats and rabbits.

A third species, cytochrome P-450\textsubscript{a}, has also been purified from phenobarbitone, 3-methylcholanthrene and Aroclor 1254-treated rats (Ryan et al. 1979). It has a sub-unit molecular weight of 48K with an absorption maximum of its reduced CO complex at 452 nm. The hemoprotein preferentially hydroxylates testosterone at the 7α position but has low catalytic activity for the metabolism of benzphetamine, benzo(a)pyrene and 7-ethoxycoumarin. This isoenzyme contains methionine as both its N- and C-terminal amino acids and partial sequencing of the first 19 amino acids of the N-terminal region reveals little homology with the corresponding sequences of cytochromes P-450\textsubscript{b} and P-450\textsubscript{c}. The substrate specificities of cytochromes P-450\textsubscript{a,b,c} isolated from Aroclor 1254-treated rats are the same as the specificities of the corresponding hemoprotein isolated from 3-methylcholanthrene or phenobarbitone treated rats.

A further minor form, cytochrome P-450\textsubscript{e}, has also been isolated from rats treated with Aroclor 1254 and phenobarbitone but not 3-methylcholanthrene (Ryan et al. 1982). It has a minimum molecular weight of 52,500 with an absorption maximum of its reduced CO complex at 450.6 nm. It has been shown to be immunochemically related to the major phenobarbitone-induced cytochrome P-450\textsubscript{b} isoenzyme. Its substrate specificity is also similar to that of cytochrome P-450\textsubscript{b}, although certain substrates such as benzphetamine, benzo(a)pyrene and 16α testosterone hydroxylation were metabolised at only 15-25% of the rate seen with
cytochrome P-450\textsubscript{b}. However the metabolism of estradiol-17\beta at the 2-position was carried out more efficiently by cytochrome P-450\textsubscript{e} than P-450\textsubscript{b}. Analysis of the peptide fragments of the hemoproteins following chemical or proteolytic digestion (Ryan et al. 1982) as well as the gene structure (Mizukami et al. 1983) indicates that the two isoenzymes are very similar but not identical.

Another cytochrome P-450 variant, cytochrome P-450\textsuperscript{PCN\textsubscript{a}} has been isolated following induction by pregnenolone-16\alpha-carbonitrile (Elshourbagy et al. 1980). Animals pretreated with this catatotoxic steroid are resistant to the toxicity of many drugs and xenobiotics (including CCl\textsubscript{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 P-450 and NADPH-cytochrome P-450 reductase as a consequence of PCN administration (Baron at al. 1982). It has sub-unit molecular weight of 51K and an absorption maximum in its reduced CO difference spectrum of 450 nm. The purified isoenzyme has been shown to be distinct from cytochromes P-450\textsubscript{b}, and P-450\textsubscript{c} with respect to exhibiting reduced stability at room temperature and markedly different chromatographic, spectral, catalytic and immunological properties. In addition, it also contains as much as fifty per cent more lysine and proline residues as well as a far greater amount of methionine residues than observed with the other two isoenzymes. In reconstituted enzyme systems, the purified cytochrome catalysed the N-demethylation of ethymorphine and aminopyrene but at a much slower rate than is seen with the original pretreated liver microsomes.

The presence of microsomal cytochrome P-450\textsuperscript{PCN} following induction by other steroids such as dexamethasone, spironolactone and non-steroids (phenobarbitone) has also been demonstrated (Heuman et al. 1982). Dexamethasone is a potent glucocorticoid but its inductive effect towards cytochrome P-450\textsuperscript{PCN} is not believed to be mediated by glucocorticoid receptors, although the presence and involvement of second, lower-affinity dexamethasone receptors in liver has been suggested (Heuman et al. 1982, Ambellan et al. 1981).
A unique form of liver cytochrome P-450 induced in rats treated with the methylenedioxyphenyl compound isosafrole (Dickens et al. 1978) has been purified to apparent homogeneity (Ryan et al. 1980, Fisher et al. 1981). 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$_{a,b,c}$ have also been partially purified from isosafrole treated rats and these latter hemoproteins have the same properties as the enzymes from Aroclor 1254-treated rats. The highly purified high spin hemoprotein, designated cytochrome P-450$_d$, has a sub-unit molecular weight ranging from 52-53K and a reduced CO complex having an absorption maximum at 447 nm. It is purified as an isosafrole metabolite-cytochrome P-450 complex, but addition of certain displacers such as 7-ethoxycoumarin, or benzimidazoles results in removal of most of the bound metabolite. Cytochrome P-450$_d$, either before or after removal of the isosafrole 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. The most distinguishing feature of cytochrome P-450$_d$ is its high degree of specificity for isosafrole oxidation.

Cytochrome P-450$_c$ has also been shown to exhibit partial immunological identity with cytochrome P-450$_c$. No cross reaction is evident with antibodies prepared against cytochromes P-450$_a$ and P-450$_b$. Peptide maps resulting from enzymatic or chemical cleavage indicate that the primary structure of this isoenzyme differs from cytochromes P-450$_{a,b,c}$ (Botelho et al. 1982).

During the course of characterisation of the cytochrome P-450 species induced by PCN, it was suggested that other forms of cytochrome P-450 not then characterised could be involved in benzo(a)pyrene metabolism since inhibition by antibodies to cytochromes P-450$_{a,b,c}$ did not result in complete metabolic inhibition (Thomas et al. 1981). It was also demonstrated that high benzo(a)pyrene metabolising activity in PCN-treated rats was insensitive to an antibody directed against cytochrome P-450$_c$. Subsequent to the above observations, a low spin
hemoprotein designated cytochrome P-446 has been purified from β-napthoflavone pretreated rat liver (Saito et al. 1981). This form of cytochrome has the highest turnover for benzo(a)pyrene yet reported, and exhibits high activity with p-nitrophenotole, but has relatively low activity with 7-ethoxycoumarin. The isoenzyme was reported to have an apparent minimum molecular weight of 53.4K and an absorption maximum in the CO reduced difference spectrum at 446 nm. The amino acid composition indicates that the protein contain 35% hydrophobic residues and otherwise resembles that of most other cytochromes P-450. Subsequent work (Lau et al. 1982) 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 (Saito et al. 1981). This turnover number was comparable to that obtained with cytochrome P-450_c. This has led to speculation as to whether or not the cytochromes P-450_c and P-446 may be the same protein.

Comparative studies have indicated that the above two cytochromes P-450 were immunologically identical, having similar cleavage products following proteolysis, identical sub-unit molecular weights and similar absorption maxima (Lau et al. 1982). The only difference was their catalytic activity towards benzo(a)pyrene, where cytochrome P-446 was shown to be nearly twice as efficient in its hydroxylation than cytochrome P-450_c. It also appears that cytochrome P-446 produces higher amounts of the 9-hydroxy benzo(a)pyrene metabolite. Whether this catalytic difference is due to residual amounts of bound inducer or whether the two cytochromes are similar yet distinct proteins can not be fully ascertained at present.

Gibson et al. (1982) have also purified a unique form of cytochrome P-450 which is induced in the rat by the hypolipidaemic drug clofibrate. The total cytochrome P-450 was fractionated into four pools, with the first pool containing a cytochrome P-450 of sub-unit molecular weight 51,500 and having an absorbance maximum in the difference spectrum at 451.8 nm. This fraction, designated cytochrome P-452, preferentially hydroxylated lauric acid at the 12-position, and to
a far lesser extent at the 11-position. It has low catalytic activity towards benzphetamine in direct contrast to cytochrome P-450b. The cytochrome P-450 fraction 2 exists as a low spin form, and although less homogeneous than fraction 1, preferentially hydroxylated lauric acid at the 11-position. Cytochrome P-450 fraction 3, exhibited only trace laurate oxidase activities, but had a rapid turnover rate for benzphetamine at rates comparable to cytochrome P-450b.

Comparison of homogeneous cytochrome P-452 to cytochromes P-450b and P-447 indicated marked structural, immunological, catalytical and spectrophotometric differences between the 3 forms, indicating the uniqueness of the cytochrome P-452 isoenzyme (Tamburini et al. 1984).

Although the majority of work on cytochromes P-450 has been carried out on isoenzymes induced by various xenobiotics, the 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 et al. (1978) reported the partial purification of two forms of cytochrome P-450 in high yield from untreated rats. 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 52K. 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 within the same laboratory resulted in the purification of two further forms, designated RLM\textsubscript{3} and RLM\textsubscript{5}, (Cheng et al. 1982). These two forms have apparent minimum molecular weights of 50 and 51K, and absorbance maxima in the CO reduced difference spectra at 449 and 451 nm respectively. RLM\textsubscript{3} exists in a low spin form and preferentially hydroxylates testosterone at the 6α and 6β positions, as well as catalysing the formation of an unidentified highly
polar metabolite. RLM₅ contains some high spin component and preferentially hydroxylates testosterone at the 2β and 16α positions. It also exhibits a specific activity two or three times higher than RLM₃ towards the metabolism of benzphetamine, aminopyrene and ethylmorphine. N-terminal amino acid analysis indicates that the first residue of each form is methionine, with the first four residues being identical for both hemoproteins.

Although there is some homology between the above two forms based on the amino acids sequenced, they do not correspond to either cytochromes P-450b or P-450c. It is estimated that RLM₅ represents about 5%, and RLM₃ about 6% of the constitutive isoenzymes in untreated rat liver microsomes. Additional work demonstrated the existence of three further isoenzymes termed RLM₂ₐ, RLM₄ and RLM₆β, characterised by monomeric molecular weights of 49K, 51K and 53K respectively (Schenkman et al. 1982). The significance of these forms is unclear.

Although sex differences in cytochrome P-450 catalyzed drug and steroid metabolism have been known for some time (Kato et al. 1965, Pasleau et al. 1980), it is only recently that constitutive cytochrome P-450 isoenzymes have been purified which show sexual differentiation (Kamataki et al. 1983, MacGeoch et al. 1984, and Waxman 1984).

In female rats a low spin constitutive form designated cytochrome P-450 15β has been isolated which was shown to have an important physiological function in the metabolism of steroids (MacGeoch et al. 1984). Its unique ability to hydroxylate steroid sulphates in the 15β position accounts for the prevalence of 15β-hydroxylated sulphate metabolites of corticosterone in female excreta, and their absence in males.

The male equivalent, cytochrome P-450₂c (Waxman, 1984), is both catalytically and immunologically distinct from the female form. Its activity is primarily directed towards the endocrine regulated male specific 16α-hydroxylase, although a parallel induction at puberty of six different steroid hydroxylase
<table>
<thead>
<tr>
<th>Species(^a)</th>
<th>P-450 Nomenclature</th>
<th>Inducer(^b)</th>
<th>Monomeric Molecular weight</th>
<th>Absorbance maximum of Fe(^{2+})-CO complex</th>
<th>Preferred Substrate(^c)</th>
<th>Terminal Amino Acid N-(^{-}) C-(^{-})</th>
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<tr>
<td>Rat</td>
<td>P-450</td>
<td>ARO, 3MC, PB</td>
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<td>452</td>
<td>Testosterone</td>
<td>MET</td>
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<td>450</td>
<td>Benzphetamine</td>
<td>GLU</td>
</tr>
<tr>
<td>Rat</td>
<td>P-450</td>
<td>ARO, BNF, 3MC</td>
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<td>447</td>
<td>7-ethoxyresorufin</td>
<td>ISO</td>
</tr>
<tr>
<td>Rat</td>
<td>P-450</td>
<td>ISO</td>
<td>52,500</td>
<td>447</td>
<td>Isofurene</td>
<td>-</td>
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<tr>
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<td>ARO, PB</td>
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<td>Estradiol (17β position)</td>
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<tr>
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<td>447.5</td>
<td>Testosterone</td>
<td>-</td>
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<tr>
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<td>50,000</td>
<td>447.5</td>
<td>Testosterone</td>
<td>-</td>
</tr>
<tr>
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<td>None(^d), ethanol</td>
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<td>451</td>
<td>Testosterone</td>
<td>-</td>
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<td>Lauric acid, arachadonic acid</td>
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<td>450.5</td>
<td>Benzo(a)pyrene</td>
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<tr>
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<td>451</td>
<td>Benzphetamine</td>
<td>MET</td>
</tr>
<tr>
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<td>Form 3</td>
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<tr>
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<td>452</td>
<td>Ethanol</td>
<td>ALA</td>
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<tr>
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<td>451</td>
<td>Progestosterone</td>
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</tr>
<tr>
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<td>Form 3c</td>
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<td>449</td>
<td>Progestosterone</td>
<td>MET</td>
</tr>
<tr>
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<td>447-448</td>
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<td>-</td>
</tr>
<tr>
<td>Rabbit</td>
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<td>BNF</td>
<td>51-55,000</td>
<td>447</td>
<td>Benzo(a)pyrene</td>
<td>-</td>
</tr>
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<td>-</td>
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<td>3MC</td>
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<td>449.3</td>
<td>Benzo(a)pyrene</td>
<td>-</td>
</tr>
<tr>
<td>Mouse</td>
<td>P2(^{-})-450</td>
<td>3MC, ISO</td>
<td>55,000</td>
<td>448-449</td>
<td>Isofurene</td>
<td>-</td>
</tr>
<tr>
<td>Mouse</td>
<td>P3(^{-})-450</td>
<td>3MC</td>
<td>55,000</td>
<td>448</td>
<td>Acetanilide</td>
<td>-</td>
</tr>
</tbody>
</table>

\(a\) The information for each species may not be related to results from other laboratories as different strains of rats were used.

\(b\) Abbreviations: ARO, Aroclor 1254; BNF, β-naphthoflavone; Iso, Isofurene; 3MC, 3 Methylcholanthrene; PB, Phenobarbitone; TCDD, 2,3,7,8 - tetrachlorodibenzo-p-dioxin.

\(c\) The information on the preferred substrate of the cytochrome P-450 isoenzyme is only an indication of the substrate specificity of that particular form as each isoenzyme has not been tested with all the substrates indicated in this table.

\(d\) Indicates that this isoenzyme has been purified from uninduced rat liver (constitutive form).
activities have also been observed.

Three further isoenzymic forms have also been purified from the hepatic microsomes of untreated and ethanol pretreated Sprague-Dawley rats (Ryan et al. 1984). These forms designated cytochromes P-450\(_f\), P-450\(_g\), and P-450\(_h\) are all active in steroid metabolism, and on the basis of Ouchterlony double diffusion analysis do not react with any of the already characterised inducible cytochromes P-450. On the basis of minimum molecular weight, spectral characteristics and catalytic activity it is highly likely that cytochrome P-450\(_h\) corresponds to the male specific cytochrome P-450 purified by Kamataki et al. (1982), and the cytochrome P-450 RLM\(_5\) form purified by Cheng et al. (1982).

A summary of the major hepatic cytochrome P-450 isoenzymes isolated to date across species is given in table 1.1 which illustrates the major characteristics and dissimilarities of these isoenzymic forms with each other.

1.2 MOLECULAR EVENTS FOLLOWING INDUCTION OF HEPATIC CYTOCHROME P-450 BY XENOBOTICS

1.2.1 Biosynthesis of Cytochrome P-450 Directed by Rat Liver Messenger RNA

Although the complete mechanism of hemoprotein induction has still not been completely elucidated, many of the regulatory processes involved have been determined. Phenobarbitone pretreatment of experimental animals results in hyperplasia of the smooth endoplasmic reticulum which results in hypertrophy of the liver, characterised by an increase of cytochrome P-450 and other proteins (Orrenius et al. 1965, Ernster et al. (1964). These include NADPH-cytochrome P-450 reductase, epoxide hydrolase (Gonzalez et al. 1981), and several cytosolic enzymes such as glutathione-S-transferase and aldehyde dehydrogenases (Kuryama et al. 1969). This induction (as also in the case for 3MC pretreatment) is preceded by an increase in the activity of the "aggregate" RNA polymerase system within the liver, as a result of an increased efficiency of the chromatin as a template for RNA
synthesis (Piper et al. 1968). This results in an increase in the different RNA classes and utilisation of amino acids required throughout protein synthesis (Bresnick et al. 1969). This inductive response can be inhibited by drugs such as actinomycin D, ethionine and α-aminitin (protein synthesis inhibitors), thereby suggesting that the inductive mode of action is at the level of transcription and/or processing of mRNA for these inducers (Jacob et al. 1974). Furthermore using blockers of heme synthesis (aminotriazole), it was shown that the phenobarbitone-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 (Giger et al. 1981). Phenobarbitone also leads to increased stability and processing of ribosomal RNA precursors possibly due to decreased RNase 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. Furthermore the increase in protein synthesis as a result of xenobiotic pretreatment is also believed to be related to greater mRNA stability, possibly through decreased rates of degradation (Matsumura et al. 1983).

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 and the subsequent stability as well as translational fidelity of the mRNA within the cytoplasm. In fact current evidence strongly suggests that induction following xenobiotic pretreatment is modulated by transcriptional control of the specific gene coding for the particular cytochrome P-450 isoenzyme (Tukey et al. 1981, Hardwick et al. 1983). By such control, the regulation of specific mRNA levels coding for the isoenzymic variants can be achieved.
1.2.2 Expression of mRNA Activity following Phenobarbitone and 3-Methylcholanthrene Pretreatment

Following induction, analysis of total cytoplasmic RNA activity upon assaying with an in vitro rabbit reticulocyte system was shown to increase. Immunoprecipitation analysis with the corresponding monospecific antibody further substantiated this observation. These findings clearly indicated an increase in mRNA activity for the major phenobarbitone-induced cytochrome P-450 as a result of phenobarbitone administration.

Cloning analysis for the genes coding for cytochromes P-450 coupled with hybridisation studies has enabled the size of the corresponding RNA species to be determined. A number of groups have subsequently determined by such means the size of the mRNA coding for the major phenobarbitone-induced cytochrome P-450 isoenzyme. This has ranged from approximately 1.8 kb (Fujii-Kuriyama et al. 1982), 2.0 kb (Fagan et al. 1983), and 3.8 kb (Omiecinski et al. 1983). The reason for this size discrepancy is at present unclear, but is believed to relate to the fact that more than one size mRNA exists that would code for similar cytochromes P-450 (Walz et al. 1982). This hypothesis is strengthened by the fact that multiple size classes of mRNA are known to exist which code for identical proteins. These include dihydrofolate reductase mRNAs (Setzer et al. 1980), α-amylase mRNA, (Hagenbuckle et al. 1981) and α- and β-interferon mRNAs (Solymoss et al. 1971).

3-methylcholanthrene pretreatment also results in increased mRNA activity due to the activation of the Ah locus gene which codes for a cytosolic receptor. Such an induction response includes (Nebert et al. 1983); (i) high affinity binding of specific xenobiotics to the Ah receptor; (ii) temperature-dependent translocation of the "activated" inducer-receptor complex into the nucleus; (iii) binding of the complex presumably to chromatin components; (iv) transcriptional activation of specific genes; (v) maximal increases in intra-nuclear high molecular weight precursor mRNA that precedes by several hours the maximal increases in cytoplasmic mRNA; (vi) translation of the mRNA principally on membrane-bound
polysomes; and (vii) increases in the specific membrane-bound proteins that reflect enhanced specific drug-metabolising activities.

On sizing the mRNA coding for the major cytochrome P-450 isoenzyme, discrepancies in size (as in the case following phenobarbitone pretreatment) were observed. In contrast to phenobarbitone-induction however, it appears that the 3-methylcholanthrene-induced cytochromes P-450 were translated from two size classes of mRNA. These have been shown to consist of a 2.0 and 2.7 kb mRNA species (Fagan et al. 1982, 1983) and a 2.0 and 4.0 kb mRNA species (Lippman Morville et al. 1983). In mouse, two mRNA size classes of 2.7 and 3.5 kb have also been reported (Tukey et al. 1981). Why such large mRNA species coding for the cytochromes P-450 are present is unclear, especially since it has been estimated that a mRNA species of 1.5 kb coding information would quite adequately translate into a protein of molecular weight 50,000 (Omiecinski et al. 1983). Such large non-coding regions within the mRNA species are similarly not limited just to the cytochrome P-450 isoenzymic family. For example, the mRNA coding for the preparathyroid hormone has been shown to be 50% non-coding (Stolarsky et al. 1978). Whether or not this relates to the possibility that such large mRNAs could code for a number of subtly heterogeneous proteins, or alternatively simply code for longer peptides which undergo extensive proteolytic cleavage is not known and awaits further elucidation.

As to the size of the cytochrome P-450 gene family, it is currently estimated to consist of five gene clusters (Nebert et al. 1985). Although a slight degree of homology has been observed, the coding genes can be considered quite distinct from each other. The precise number of genes within the gene clusters coding for all the cytochrome P-450 variants is not known, although values in the range of 30-200 genes have been suggested (Nebert et al. 1985). Undoubtedly the number of genes isolated will increase, with it appearing to date that every cytochrome P-450 protein is derived from its own gene. Whether or not this will be the case for all the cytochrome P-450 isoenzymes or whether mechanisms of differential splicing and
processing, gene amplification, post-transcriptional or post-translational modification which would allow a single cytochrome P-450 gene to encode multiple protein products is not yet known. With time, further research will undoubtedly unravel the complex processes involved.

In conclusion one can state that species variation in drug metabolism can be rationalised by a variety of factors, one of the most important being the nature and amount of liver enzymes responsible for the biotransformation of the drug or xenobiotic. In this respect both the involvement and multiplicity of the cytochrome P-450 isoenzymes have been well documented. Their importance lies in their ability to both activate and detoxify drugs and xenobiotics. This differentiation causes major problems for both the pharmaceutical industry and the regulatory authorities, in 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 the activation/detoxification of drugs could be characterised on a genetic basis. In this respect, the multiplicity of cytochrome P-450 is well established, and knowledge relating to the regulation of the cytochrome P-450 system at the levels of mRNA transcription, processing, translation and degradation is rapidly advancing. Progress in the study of gene regulation and resolution of specific cytochrome P-450 gene structures has the potential to place the extrapolation of animal data to man on a more firm experimental basis. Indeed this is already being done with the successful isolation of a human cytochrome P_1-450 full-length complementary DNA clone (Jais et al. 1985). Clearly this is an important step forward as induction of cytochrome P_1-450 has been linked to susceptibility to certain chemically induced cancers in mouse and man.

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.

1.3 AIMS OF THE PRESENT INVESTIGATION

The present study was centered upon the purification, characterisation and
induction of the major phenobarbitone-induced cytochromes P-450. This
characterisation and comparison was then to be extended to two other previously
purified cytochromes P-450 (P-452 and P-447) available in this laboratory, both in a
homogeneous state and also within the endoplasmic reticulum environment of
hepatic and renal microsomes (chapter 4).

The induction of these isoenzymes following xenobiotic pretreatment was to
be investigated at both a biochemical (chapter 4) and molecular (chapter 6) level.
Towards this aim intensive development of the in vitro translation system coupled
with the technique for immunoprecipitation analysis had to be undertaken (chapter
5). This investigation was further extended in that the procedure for the synthesis
of complimentary DNA from purified poly (A)$^+$ mRNA derived from clofibrate-
induced rat liver was also undertaken.
METHODS RELATING TO THE PURIFICATION AND
CHARACTERISATION OF CYTOCHROME P-450 ISOENZYMES

2.1 MATERIALS

Sepharose 4B, DEAE sephacel, carboxymethyl sepharose CL 6B, carboxymethyl
sephadex-C50, and sephadex G-100 were obtained from Pharmacia (U.K.) Ltd.
(Hounslow, Middlesex). Acrylamide, N,N-methylene-bis-acrylamide, sodium dodecyl
sulphate, ammonium persulphate, coomassie brilliant blue R-250, G-250 and
bromophenol blue were obtained from Biorad Laboratories (Watford, Herts).
Emulgen 911 was purchased from K.A.O. Atlas Co. (Tokyo, Japan), and N\(^6\) - (6
aminohexyl) -adenosine - 2' 5' -triphosphate sepharose 4B and 2' AMP were obtained
from P-L Biochemicals Inc. (Milton Keynes, U.K.) Diaminoctane and cyanogen
bromide were purchased from the Aldrich Chemical Company (Poole, Dorset).

Sodium cholate, sodium deoxycholate, dithiothreitol, flavin mononucleotide,
Tris (Trizma base), cytochrome c, creatine phosphokinase, chymotrypsin, papain,
bovine serum albumin, chicken egg albumin, lactic dehydrogenase, catalase,
NADPH, L-\(\alpha\)-dilauroyl phosphatidyl choline (DLPC), \(\beta\)-napthoflavone, lauric acid,
aminopyrine, potassium thiocyanate and diamino-benzidine chloride were all
purchased from the Sigma Chemical Co. Ltd. (Poole, Dorset). Phosphorylase a and
glutamate dehydrogenase were obtained from the Boehringer Corporation (Lewes,
Sussex). Staphylococcus aureus V8 protease was purchased from Miles Laboratories
(Slough, Berks). Sodium phenobarbitone, sucrose, EDTA, glycine, agarose 25,
ammonium acetate, ammonium sulphate, sodium azide, sodium chloride, sodium
potassium tartrate, potassium phosphate, Folin Ciocalteau phenol reagent were all
purchased from British Drug Houses Ltd. (Poole, Dorset). Glycerol was obtained
from May and Baker Ltd. (Dagenham, Essex).
Benzphetamine was obtained from the Upjohn Co. (Kalamazoo, Michigan, U.S.A.), sodium clofibrate from I.C.I. Pharmaceuticals (Macclesfield, Cheshire) and $^{14}$C-lauric acid from the Radiochemical Centre (Amersham, Bucks).

Nitrocellulose filters (0.45 μm) were purchased from Anderman and Co. Ltd. (Kingston Upon Thames, Surrey). Affinity purified complexes of peroxidase, anti-peroxidase complexes of sheep and rabbit IgG were obtained from Nordic Immunology (Tilbury, The Netherlands). Control donkey sera was supplied from the Guildhays animals unit (University of Surrey).

All other chemicals were obtained as Analar grade wherever possible.

2.2 ANIMALS

Male Wistar Albino rats of approximately 160g were obtained from the University of Surrey breeders and were housed in cages with sawdust bedding, being allowed food (Spratts animal diet No. 1) and water ad libitum. A twelve hour light dark cycle was in operation (0700-1900 light) at 22°C and 50% humidity.

For antibody production, male half-lop New Zealand white rabbits or sheep were used. Rabbits were housed in the Universities animal unit and the sheep were maintained under field conditions within the Guildhays animal farm unit.

2.2.1 Pretreatment of Animals

Phenobarbitone

Phenobarbitone (sodium salt) was administered orally as a 0.1% (w/v) solution in the drinking water for 6 days. Normal drinking water was returned to the cages 24h before killing.

Clofibrate

Clofibrate (sodium salt) was administered as a solution in distilled water.
Rats received 400 mg kg\(^{-1}\) by i.p. injections once daily for 3 consecutive days and were killed 24h after the last injection.

\(\beta\)-Napthoflavone (BNF)

BNF induction involved i.p. injection of 80mg/kg in 0.25 ml corn oil once each day for 3 days prior to sacrifice. The animals were killed 24h after the last injection.

2.3 ISOLATION OF HEPATIC AND KIDNEY MICROSONES

Twenty rats were used for each purification of phenobarbitone-induced cytochromes P-450. The animals were killed by cervical dislocation with the livers being rapidly excised, perfused with 0.9% (w/v) aqueous NaCl, blotted dry and then weighed. All subsequent steps were carried out at 4°C. The livers (averaging between 13-15g in weight) were scissor-minced and homogenised in 3 volumes (w/v) of ice-cold 0.25M sucrose using a motor driven Potter-Elvehjem glass teflon homogeniser (3 return strokes). In the case of kidney microsome isolation, the outer cutaneous membrane was first removed, otherwise the procedures were essentially identical. The homogenate was adjusted to 25% (w/v) by the addition of 0.25M sucrose and centrifuged at 11,000 gav. for 30 mins using a 6 x 250 ml aluminium angle head rotor in an MSE HS 18 centrifuge. The supernatant was decanted and centrifuged at 105,000 gav. for 1 hr using a 6 x 94 ml titanium angle head rotor in a Beckman LK-65 refrigerated ultracentrifuge. The supernatant was discarded and the pellet resuspended in ice-cold 50mM potassium phosphate buffer pH 7.25 containing 25% (v/v) glycerol, using a motor driven Potter-Elvehjem glass teflon homogeniser, to an appropriate volume of 10 ml per gram rat liver (giving a protein concentration of 25-30 mg/ml). The microsomal suspension was stored at -80°C at which the cytochrome P-450 is stable for several months.
2.4 PURIFICATION OF MICROSOMAL ENZYMES

2.4.1 Preparation and Regeneration of Column Chromatography Media

8-AMINOCTYL SEPHAROSE 4B

Method of Synthesis

Diaminoctane was coupled to sepharose 4B utilising cyanogen bromide activation as described by Cuatrecasas (1970).

Finely divided cyanogen bromide (100g) was added with stirring to 400 ml (packed volume) of sepharose 4B present as a 1:1 suspension in distilled water. As the cyanogen bromide dissolved, the pH of the solution was maintained at pH 11 with 10N sodium hydroxide and the temperature maintained at 20°C by adding ice. When the pH of the solution no longer showed a tendency to drop, large amounts of ice were added, followed by washing under suction with 3 litres of 50mM sodium carbonate/bicarbonate buffer pH 10 on a sintered glass funnel as rapidly as possible. The activated sepharose 4B was then transferred to 400 ml of 25% (w/v) diaminoctane adjusted to pH 10, and gently stirred at 4°C for 20h. The fully derivatised sepharose 4B was then washed on a sintered glass funnel consecutively with 3L of 50mM Na₂CO₃/NaHCO₃ buffer pH 10, distilled water and 100mM potassium phosphate buffer pH 7.25 containing 20% (v/v) glycerol, 0.7% (w/v) sodium cholate, 1mM dithiothreitol, 1mM EDTA, 2μM FMN, and was stored in the last buffer containing 0.02% (w/v) sodium azide at 4°C.

Method of regeneration

For the most effective regeneration, it was important to elute cytochrome P-450 reductase and cytochrome b₅ from this material whilst packed in a column, by washing with the appropriate buffers (see purification methodology for these enzymes). Following column chromatography, the media was transferred to a sintered glass funnel and washed under suction consecutively with occasional stirring, with 2L of each of the following : 100mM potassium phosphate buffer pH 7.25 containing 0.4% (v/v) emulgen 911, 0.4% sodium deoxycholate; distilled water;
10% (v/v) aqueous dioxane; distilled water; 500mM potassium phosphate buffer pH 7.25 containing 500mM potassium chloride, 1mM EDTA; distilled water; 100mM potassium phosphate buffer pH 7.25 containing 0.7% (w/v) sodium cholate, 1mM dithiothreitol, 1mM EDTA, 2μM FMN.

HYDROXYLAPATITE
Preparation for Chromatography

Hydroxylapatite was routinely made in the laboratory by the method of Levin (1962) as it was shown to possess superior chromatographic properties to the commercially available hydroxylapatite. It is prepared by dripping 0.5M di-sodium hydrogen orthophosphate and 0.5M calcium chloride together at a flow rate of approximately 12-15 ml/min with continual stirring. The precipitated calcium phosphate was allowed to settle after which it was washed four times with 3L of water, the precipitate being allowed to completely settle between each wash. The precipitate was then taken up in 3L of water to which 100ml of 40% (w/v) sodium hydroxide had been added and was then boiled for 1 hour with continual stirring. The calcium phosphate was allowed to sediment for 5 mins following which the turbid supernatant was removed by aspiration. The precipitate was then resuspended for 5 mins, with the supernatant being removed by aspiration. This last procedure was repeated three times. The precipitate was then resuspended in 4L 10mM potassium phosphate buffer pH 6.8, stirred, heated until just boiling, allowed to sediment for 5 mins after which the turbid supernatant was removed. This procedure was repeated twice with 10mM KPO₄ buffer (pH 6.8), and then twice with 1mM KPO₄ buffer (pH 6.8). For each of the last four precipitations the boiling period was for fifteen mins. After the last boiling preparation was complete the hydroxylapatite was resuspended in 50mM KPO₄ buffer (pH 7.25) and stored at 4°C.

For column preparation the required amount of hydroxylapatite was transferred in a 5 fold excess volume of column equilibration buffer and gently swirled into suspension. The hydroxylapatite was then allowed to settle, leaving a
cloudy buffer volume above containing slowly sedimenting "fines" which were
decanted. Fresh buffer was then added and the procedure repeated four times.
The medium was discarded after use.

**DEAE SEPHACEL ANIONIC EXCHANGE MEDIUM**

**Preparation for Chromatography**

The required amount of medium was transferred to a sintered glass funnel and
washed under gravity with distilled water to remove the storage solution. The
chloride counter ion was exchanged with phosphate and the medium set to the
correct pH by transferring to an excess volume of 400mM potassium phosphate
buffer pH 7.25. After 12 hours, the pH was checked before transferring to the
column equilibration buffer.

**Regeneration after use**

DEAE sephacel was regenerated by standing in an excess volume of 400mM
potassium phosphate buffer pH 7.25 overnight, followed by extensive washing with
500mM NaCl and distilled water. The medium was stored in 400mM potassium
phosphate buffer pH 7.25, containing 20% (v/v) methanol, at 4°C.

**CARBOXY-METHYL SEPHADEX - C50 CATIONIC EXCHANGE MEDIUM**

Carboxymethyl sephadex - C50 was swollen by placing in excess distilled water
and heating to 90°C on a steam bath for 1hr. 1g of dry material swelled to between
30-40 ml. The swollen material was transferred to an excess of column equilibration
buffer prior to use. The medium was discarded after use.

**CARBOXY-METHYL SEPHAROSE CATIONIC EXCHANGE MEDIUM**

**Preparation for Chromatography**

Carboxymethyl sepharose was supplied pre-swollen. The required amount of
medium was washed with distilled water on a sintered glass funnel and transferred into column equilibration buffer before use.

Regeneration after use

Regeneration was achieved by standing the medium in 400mM potassium phosphate buffer pH 7.25, followed by washing with the same buffer and distilled water in a sintered glass funnel under gravity. The medium was stored in 50mM potassium phosphate buffer pH 7.25, containing 20% (v/v) glycerol, 0.02% sodium azide at 4°C.

SEPHADEX G-100

Sephadex G-100 was swollen in excess distilled water by heating at 90°C for 5h. The gel was discarded after use.

N^-{(6-AMINOHEXYL) - ADENOSINE 2', 5'-DIPHOSPHATE SEPHAROSE 4B
(ADP AGAROSE)}

ADP agarose was supplied pre-swollen and simply transferred to equilibration buffer prior to use.

After chromatography, the medium was washed with distilled water to remove excess AMP and stored in 20% glycerol, 0.02% azide at 4°C.

2.4.2 Column Pouring and Equilibration

A small volume (1-5 ml) of column equilibration buffer was poured into the bottom of an assembled chromatography column of the required dimensions (Pharmacia, Sweden). The buffer was partially allowed to run through the outlet tap to displace air trapped under the column support bed. With the outflow fully closed and the reservoir assembled in the pouring mode, the chromatography medium was carefully poured into the column as a 20% (v/v) suspension with the exception of
sephadex G-100 which was poured as a 50% (v/v) suspension.

After allowing the medium to settle for approximately 12 hours in the column, column packing was achieved under a constant pressure head at the flow rate required during chromatography.

All chromatography columns were equilibrated with at least 3 volumes of equilibration buffer, during which time the pH of the eluent was regularly monitored.

2.4.3 Purification of Cytochrome P-450 from the Hepatic Microsomes of Phenobarbitone - Induced Rats

All operations were carried out at 0-4°C.

Solubilisation of Microsomal Proteins

Microsomal proteins were solubilised with sodium cholate in the presence of 0.1M potassium phosphate buffer pH 7.25 containing 20% (v/v) glycerol to prevent conversion of cytochrome P-450 to cytochrome P-420 (Lu and Coon, 1968), 1mM dithiothreitol to keep sulphydryl groups in the reduced state, 1mM EDTA to inhibit lipid peroxidation and 2μM FMN to reverse any flavin dissociation from NADPH-cytochrome P-450 reductase (hereafter termed buffer A). The ratio of protein to detergent is critical in determining the recovery of holo-cytochrome P-450, with excess detergent leading to extensive destruction of cytochrome P-450. Our initial studies showed that solubilising microsomes at a protein concentration of 10 mg/ml in the presence of 1.4% (w/v) sodium cholate enabled us to consistently obtain recoveries of around 90%.

The microsomes were diluted in buffer A and gassed with nitrogen for 10 minutes with gentle stirring. Sodium cholate was then added slowly as a 21% (w/v) stock solution to the required concentration and solubilisation was allowed to proceed for 45 minutes under an atmosphere of nitrogen to prevent heme...
destruction arising from lipid peroxidation (Sato et al. 1973), before centrifugation at 105,000g for 45 minutes using a 6 x 94 ml titanium angle head rotor in a Beckman L5 65 ultracentrifuge.

The supernatant containing solubilised microsomal protein was diluted with buffer A to a final cholate concentration of 0.7% (w/v).

**Chromatography on 8 aminooctyl sepharose 4B**

The 10^5 g microsomal supernatant from 20 rats resulting from the cholate solubilisation was applied to 2 columns of 8-aminooctyl sepharose 4B (2.6 x 38 cm, Pharmacia) previously equilibrated with buffer A containing 0.7% (w/v) sodium cholate. Loading was carried out at a flow rate of approximately 30 ml/hr and was allowed to continue until just over the top third of the column was dark red. The columns were then washed with 3 column volumes of buffer A containing 0.42% (w/v) sodium cholate to remove unadsorbed protein including cytochrome P-420 and membrane lipids.

Elution of cytochrome P-450 was achieved by washing the columns with 1L of buffer A containing 0.33% (w/v) sodium cholate and 0.08% (v/v) emulgen 911, whereupon a dark red band was seen to sharpen as it migrated slowly down the column. The cytochrome P-450 content was monitored spectrophotometrically at 417 or 407 nm. Fractions displaying an absorbance greater than 1/3 of the peak value were pooled and dialysed against 2 successive 3.5L volumes of buffer, designated buffer B, containing 10mM potassium phosphate buffer (pH 7.7), 0.1mM EDTA, 20% (v/v) glycerol, 0.1% (v/v) emulgen 911 and 0.2% (w/v) sodium cholate, for 12h periods. The high absorbance at 280 nm of emulgen 911 present in all the subsequent buffers precluded the rapid estimation of the purity of peak cytochrome P-450 from the 417 to 280 nm absorbance ratio.

**DEAE Sepacel Anionic Exchange Chromatography**

The pooled dialysed cytochrome P-450 was then applied to a column of DEAE
sephacel (2.6 x 38 cm) previously equilibrated with buffer B. Upon loading at a flow rate of approximately 50 ml/h, the cytochrome P-450 was seen to bind as a tight red band at the top of the column. The column was then washed with a further 500 ml of buffer B to remove any unadsorbed cytochrome P-450; this eluate was designated fraction "A". Elution of cytochrome P-450 was then achieved by using a linear 0 to 0.25M NaCl gradient. The mixing flask contained IL of buffer B, and the second flask used to form the gradient contained IL of buffer B to which 0.25M NaCl had been added. On completion of the gradient the elution profile was determined spectrophotometrically and 2 peaks, designated fraction B₁ and B₂ respectively, were resolved. At this stage a third tight red band was still visible at the top of the column and this was eluted with buffer B containing 0.5M NaCl. This fraction was designated B₃. All the fractions from the column were analysed for cytochrome P-450 and protein, with fractions being finally pooled according to their purity on SDS polyacrylamide gel electrophoresis (SDS P.A.G.E.). Pooling of fractions was routinely carried out according to the purity of every second tube.

The two fractions B₁ and B₂ were shown to correspond to two different cytochrome P-450 isoenzymes requiring different protocols for their subsequent purification.

Subsequent Fractionation of Separate Cytochrome P-450 Isoenzymic Forms

The B₁ fraction (of lower molecular weight than the B₂ fraction) was dialysed against 2 successive 2.5L volumes of 10mM potassium phosphate buffer (pH 7.7), 20% glycerol, 0.2% emulgen 911, for 12h periods. It was then loaded onto a hydroxylapatite column (2.6 x 15cm) previously equilibrated with the same dialysis buffer. The column was then washed with one column volume of this buffer after which the cytochrome P-450 was eluted with 500 ml each of a 10 to 200mM potassium phosphate (pH 7.7), 20% glycerol, 0.2% emulgen 911, linear gradient buffer. As was the case for every column chromatographic stage, the primary criteria was purity and this was determined by SDS P.A.G.E. analysis of the elution
products. Following this stage the isolated cytochrome P-450 phenobarbitone-induced isoenzyme was homogeneous, although occasionally reapplication onto DEAE sephacel was required in order to remove trace higher molecular weight contaminants.

The cytochrome P-450 B₂ fraction was also dialysed against two successive 2.5L volumes of 10mM potassium phosphate buffer (pH 7.7), 20% glycerol, 0.2% emulgen 911, for 12h periods. This fraction was then reapplied to a column of DEAE sephacel (2.6 x 30 cm) previously equilibrated with the same dialysis buffer. On further washing of the tightly bound cytochrome P-450 with the dialysis buffer (normally 2 column volumes) the tight red band would be seen to split into two bands and migrate a few centimetres down the length of the column material. At this stage elution of cytochrome P-450 was achieved by washing with the same buffer in which the ionic strength was increased in 5mM increments up to a final concentration of 35mM. The cytochrome P-450 content of eluted fractions was then assayed and pooled as described above. The purest fractions were pooled, redialysed against the previous 10mM KPO₄ dialysis buffer and loaded onto a hydroxylapatite column (2.6 x 15 cm) which had been equilibrated with the same buffer. The column was then washed with one column volume of this buffer after which the cytochrome P-450 was eluted with 500 ml each of a 10 to 125mM potassium phosphate (pH 7.7), 20% glycerol, 0.2% emulgen 911, linear gradient buffer. Fractions were then pooled according to their purity on SDS P.A.G.E.

Removal of non-ionic detergent (Emulgen 911)

Non-ionic detergents have been shown to affect the spin equilibrium of purified cytochromes P-450 (Hashimoto-Yutsudo et al 1980) and their catalytic properties thereby necessitating their removal. This was achieved in a number of ways.

The pooled cytochrome P-450 was loaded directly onto a small column of CM sephadex - C50 (2.6 x 4 cm) previously equilibrated with the corresponding elution
buffer (Imai et al 1980). The cytochrome P-450 binds as an intense red band within the top centimetre of the column after which the column was rapidly washed with the same buffer containing zero emulgen 911 until the absorbance at 280nm in the eluate decreased to zero. Elution of the emulgen-free protein was achieved by slowly eluting the column (20 ml/hr) with 325mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol. All tubes containing cytochrome P-450 were pooled. This method enabled cytochrome P-450 to be obtained within a suitably concentrated stock solution of between 8-10 μM with column recoveries of over 90%. However problems were encountered when this method was employed to remove detergent from the isoenzyme pools eluted with low ionic strength (5-10mM), as aggregation occurred after removal of emulgen, giving rise to low recoveries.

This problem was overcome by utilising a hydroxylapatite column of the same dimensions as that of the CM sephadex -C50. The conditions employed were the same for both columns with the only disadvantage associated with hydroxylapatite usage being that of a slower flow rate.

2.4.4 Purification of Cytochromes P-450 from the Hepatic Microsomes of Clofibrate and β-Napthoflavone-Induced Rats

Clofibrate, a potent hypolipidaemic drug induces a unique form of cytochrome P-450, termed P-452 in hepatic liver microsomes and is of interest because of its high lauric acid turnover rate. The isoenzyme is purified according to the methodology of Gibson et al. (1982) and is summarised in figure I.

For isolation of the major cytochrome P-450 present in liver microsomes isolated from β-napthoflavone (5,6-benzoflavone) treated animals the procedures of Guengerich and Martin (1980) were employed. These are summarised in figure 2.
Figure 1

Flow Chart for the Purification of Four Fractions of Cytosochrome P-450 Isolated from the Endoplasmic Reticulum of Clofibrate-treated Rats

Microsomes
Solubilised Supernatant
8-Amino-octyl-Sepharose 4B

Hydroxylapatite
50 mM pool
50 mM pool
150 mM pool

DEAE Sephacel
5 mM pool
5 mM pool
25 mM pool
5 mM pool

CM-Sephadex
Cytochrome P-450 Fraction 1
Cytochrome P-450 Fraction 2
Cytochrome P-450 Fraction 3
Cytochrome P-450 Fraction 4

Figure 2

Flow Chart for the Purification of Two Fractions of Cytosochrome P-447 Isolated from the Endoplasmic Reticulum of 8-naphthoflavone Treated Rats

Microsomes
Solubilised Supernatant
8-Amino-octyl-Sepharose 4B

DEAE Sephacel
Fraction A
Fraction B1
Fraction B2

CM-Sephadex
Cytochrome P-447 Fraction 1
Cytochrome P-447 Fraction 2
2.4.5 Purification of Rat Hepatic Microsomal NADPH-Cytochrome P-450 Reductase (EC 1.6.2.4)

NADPH - cytochrome P-450 reductase was routinely purified to electrophoretic homogeneity utilising the method of Yasukochi and Masters (1976), or by a modification of this method when isolated as a by-product from the purification of phenobarbitone-induced cytochromes P-450.

Solubilisation of microsomal protein and DEAE sephacel column chromatography

All operations were performed at 0-4°C.

The hepatic microsomes from 60 male rats, (approx. 120g wt. each), pretreated with sodium phenobarbitone (0.1% (w/v) in drinking water for 6 days) were solubilised at a concentration of 14 mg ml\(^{-1}\) in 10mM Tris-HCL buffer pH 7.25 containing 25% (v/v) glycerol, 0.1mM EDTA, 0.1mM dithiothreitol, 0.3% (w/v) sodium cholate and 1% (v/v) emulgen 911 with gentle stirring for 30 minutes, followed by centrifugation at 105,000 g for 1h in a 6 x 94 ml titanium angle rotor head in a Beckman L5-65 ultracentrifuge. The supernatant fraction was applied to 2 columns of DEAE sephacel (2.6 x 38 cm), previously equilibrated with solubilisation buffer. After washing the columns with at least 3 column volumes of equilibration buffer, which was the same buffer as the solubilisation buffer except that the ionic strength had been increased to 25mM Tris-HCL, the cytochrome P-450 and NADH-cytochrome b\(_5\) reductase were eluted by washing with the same buffer in which the ionic strength had been further increased to 150 mM Tris-HCL. When all the cytochrome P-450 had been eluted, NADPH-cytochrome P-450 reductase and cytochrome b\(_5\) were co-eluted in the above buffer containing a 2 x 1L linear 0 to 350mM potassium chloride gradient. NADPH-cytochrome P-450 reductase was detected according to its ability to reduce cytochrome c.

Chromatography on 8-aminooctyl sepharose 4B

When purified as a by-product, NADPH-cytochrome P-450 reductase was
eluted from 8-aminoctyl sepharose 4B, subsequent to the removal of cytochrome P-450, by washing the column with 50mM potassium phosphate buffer (pH 7.25) containing 25% (v/v) glycerol, 0.35% (w/v) sodium cholate, 0.15% (w/v) sodium deoxycholate, 2 μM FMN. All fractions containing NADPH-cytochrome P-450 reductase activity were pooled.

Chromatography on N⁶-(6-aminohexyl)-adenosine 2',5'-diphosphate sepharose 4B (2',5' ADP agarose)

NADPH-cytochrome P-450 reductase pools, obtained either from DEAE or 8-aminoctyl sepharose 4B columns, were directly applied to a column of 2',5' ADP agarose (1.3 x 8 cm) previously equilibrated with the particular elution buffer used in the previous step. Upon loading, an intense yellow band formed at the top (0.5 cm) of the column. When loading was completed, the column was washed with a minimum of 25 column volumes of 200mM potassium phosphate buffer (pH 7.7), containing 25% (v/v) glycerol, 0.1% (v/v) emulgen 911, 0.4mM EDTA and 0.2mM dithiothreitol. Emulgen 911 was then removed from the column by washing with 10mM potassium phosphate buffer (pH 7.7), containing 25% (v/v) glycerol, 0.02mM EDTA and 0.2mM dithiothreitol until the A₂₈₀ of the eluate had decreased to baseline, followed by elution of the ADP bound reductase in the same buffer containing 0.7mM 2'-AMP. The NADPH-cytochrome P-450 reductase in the eluate was monitored spectrophotometrically at 451 nm. Under these conditions, the protein eluted in a broader peak than if emulgen was present, but could be readily concentrated by ultrafiltration to a usable stock concentration (10-20 units/ml).

Removal of bound AMP

AMP was routinely removed from final reductase preparations by dialysing against 2 successive volumes of 500mM potassium phosphate buffer (pH 7.25), containing 20% (v/v) glycerol. This procedure resulted in electrophoretically homogeneous reductase preparations with emulgen 911 removed to below the level
of detection using a colourometric assay.

Cytochrome b$_5$ was also routinely purified as a by-product from the purification of phenobarbitone - induced cytochrome P-450 according to the methodology of Chiang (1981).

2.4.6 Ultrafiltration of Purified Proteins

Purified preparations were routinely concentrated either as an integral part of ongoing purifications, or to obtain proteins in usable stock concentrations.

Method

A fresh membrane of the required molecular weight exclusion was placed in an ultrafiltration cell (American Instruments Co., U.S.A.) of capacity 10, 100 or 500 ml. The chamber was filled with distilled water and the instrument attached to a nitrogen cylinder via a high pressure tube. By applying pressures of up to 30 psi, the water was forced through the membrane, displacing glycerol contained in the pores. Upon emptying, the purified protein was introduced into the chamber and ultrafiltration was accomplished at 30 psi. The extent of filtration was assessed by measuring the outflow volume with time. Used membranes were washed in 1M NaOH and stored in 20% methanol at 4°C.

2.4.7 Storage of Purified Microsomal Proteins

Purified microsomal proteins were placed in 1ml aliquots in glass freeze drying vials. They were then sealed with a rubber septum held in position with a crimped aluminium cap. The vial contents were gently gassed with nitrogen using 2 fine syringe needles inserted into the septum to serve as a gas inlet and outlet. The gassed vials were then stored at -80°C. All purified proteins were found to be stable under these conditions even after a number of thawing and re-freezing cycles.
2.5 SPECTROPHOTOMETRY

Spectrophotometric determinations were performed using either an Aminco DW2 UV-visible split beam/dual wavelength recording spectrophotometer, a Varian /Cary 219 split beam spectrophotometer or a Pye Unicam SP 1800 split beam spectrophotometer. All three instruments were equipped with a thermostated cuvette housing. Spectral measurements were made in either the split beam mode ($\lambda_1 = \lambda_2$) or in the dual wavelength mode using a single cuvette ($\lambda_1 = \lambda_2$). Simple colourimetric assays were performed on either a Pye Unicam SP 500, or a Cecil CE 292 spectrophotometer.

2.6 SPECTROPHOTOMETRIC ENZYME ASSAYS

2.6.1 Cytochrome P-450

FERROUS CARBON MONOXIDE ADDUCTS

I. LIVER MICROSONES

Cytochrome P-450 was routinely measured as the reduced carbon monoxide adduct (Omura and Sato, 1964). Microsomes (1-2 mg/ml) or purified cytochrome P-450 in 50mM potassium phosphate buffer (pH 7.25), 20% (v/v) glycerol was divided between 2 cuvettes, and a base line was recorded between 400 and 500 nm. Reduction of the ferric to ferrous form of the hemoprotein was achieved by the addition of sodium dithionite to both sample and reference cuvettes and carbon monoxide (40 bubbles at approximately 1 bubble per second) was gassed through the sample cuvette only. The spectrum was repetitively scanned until there was no further absorbance development at 450 nm. The concentration of cytochrome P-450 was calculated using the difference extinction coefficient (450 minus 490 nm) of 91 mm$^{-1}$ cm$^{-1}$

II. KIDNEY MICROSONES

A different procedure was employed to that described for liver microsomes, because kidney microsomal preparations tend to have mitochondrial contamination.
Sodium succinate was added to the assay solution to reduce mitochondrial electron transport enzymes. NADH was also added to completely reduce any cytochrome b$_5$ present.

To 0.6 ml of kidney microsome suspension, 0.6 ml sodium succinate (100 mM) and 4.8 ml potassium phosphate buffer (50 mM, pH 7.25 containing 20% glycerol) were added. 3 ml were again pipetted into both sample and reference cuvettes and a baseline recorded. NADH (25 µl, 2% (w/v) solution) and a few grains of sodium dithionite were then added to each cuvette. Again, CO was bubbled for forty seconds into the sample cuvette, the spectrum was reassessed and concentration measurements calculated as for liver microsomes.

**Pyridine Hemochrome Assay**

This assay enables the total heme content of the preparation to be determined. 1M NaOH (1 ml) was added to 0.5 ml of diluted cytochrome P-450 followed by the addition of 60% (v/v) pyridine (2 ml). After adjusting the volume to 6 ml with distilled water and dividing between sample and reference cuvettes, a baseline of equal absorbance between 450 and 650 nm was established. A few grains of sodium dithionite were then added to the sample cuvette only, and the spectrum rescanned. The concentration of pyridine hemochrome was calculated using a difference extinction coefficient (557 minus 575 nm) of 32.4 mM$^{-1}$ cm$^{-1}$ (Omura and Sato, 1964a).

**Cytochrome P-450 Soret Absorbance**

Cytochrome P-450 was calculated from the absolute spectrum of the hemoprotein using an extinction coefficient of 90 mM$^{-1}$ cm$^{-1}$ at 407 nm. The extinction coefficient was calculated with respect to total heme content determined from the pyridine hemochrome measurements. 407 nm was selected, as this represents the isosbestic point between low and high spin soret bands, and is therefore independent of the hemoprotein spin states.
2.6.2 NADPH - Cytochrome P-450 Reductase (E.C. 1.6.2.4.)

NADPH - cytochrome c reductase activity

Cytochrome P-450 reductase was assayed enzymatically according to the ability of the protein to reduce the non-physiological acceptor cytochrome c as follows. The enzyme preparation was diluted to 2.5 ml in 100 mM Tris-HCl buffer (pH 7.25) containing cytochrome c (0.46 mg/ml). After pre-equilibration at 25°C the reaction was initiated with 2% (w/v) NADPH in 1% sodium bicarbonate (25 μl). The reduction of cytochrome c was measured with time as the increase in absorbance (550 minus 541 nm) measured in the dual wavelength mode. The concentration of reduced cytochrome c formed with time was calculated using a value of $A_{550} \text{ nm} = 19.6 \text{ mM}^{-1} \text{ cm}^{-1}$ for the reduced hemoprotein. One unit of activity was defined as 1 μmole cytochrome c reduced per minute.

NADPH - cytochrome P450 reductase absolute spectrum

The concentration of reductase in homogeneous preparations was determined from the absolute absorbance of the flavin moieties at 454 nm using an extinction coefficient of 21.4 mM$^{-1}$ cm$^{-1}$ (French and Coon 1979).

2.7 RECONSTITUTION METHODOLOGY FOR ASSAYING CYTOCHROME P-450 DEPENDENT MIXED FUNCTION OXIDASE ACTIVITY (IN VITRO) USING PURIFIED RECONSTITUTED PROTEIN COMPONENTS

The metabolism of model substrates by purified preparations of cytochrome P-450 was routinely determined using the standard reconstituted system of Haugen et al (1975).
Reagents

1. L-α-dilauroyl phosphatidyl choline (DLPC) 300 µg/ml, probe sonicated in water.
2. Purified cytochrome P-450 (emulgen free), or microsomal samples.
3. Purified NADPH - cytochrome P-450 reductase. (Fpt)
4. Sodium deoxycholate 500 µg/ml. (DOC)
5. 400 mM Potassium phosphate buffer (pH 7.25).
6. 150 mM Magnesium chloride.
7. Stock solution of substrate.
8. 40 mM NADPH in 1% sodium bicarbonate.

The components were added in the following order on ice to obtain final concentrations of:-

DLPC (30 µg/ml), Cytochrome P-450 (0.1-0.3 µM), Fpt (0.3-0.9 U/ml, DOC (50 µg/ml), 50mM potassium phosphate buffer (pH 7.25), MgCl₂ (15mM), saturating substrate, distilled water to the required volume (usually 1 ml).

The system was preincubated for the required length of time (usually 2-3 minutes) on a shaking water bath set to the required temperature (usually 37°C) prior to initiation of the reaction by the addition of a final concentration of 1mM NADPH. After allowing the reaction to proceed for the required length of time the reaction was terminated in various ways depending upon the substrate and subsequent assay for turnover employed. These details are given in the sections below.

Enzyme blank experiments where cytochrome P-450 was omitted from the complete reconstitution system were run in parallel with substrate turnover experiments to determine the non-enzymatic rates of substrate turnover.
2.7.1 Determination of Benzphetamine Demethylase Activity

The standard reconstituted system described above was employed. Formaldehyde produced during the demethylation of benzphetamine was determined by the method of Nash (1953).

The reconstituted system contained 0.3 \( \mu M \) cytochrome P-450, 0.9 U/ml of NADPH - cytochrome P-450 reductase and a final benzphetamine concentration of 1.5 mM. The pre-incubation and incubation times were 2 and 3 mins respectively. After the incubation period the reaction was terminated by the addition of 0.5 ml of ice-cold 12.5\% (w/v) trichloroacetic acid (TCA) to every 1 ml aliquot of the reconstituted system. Samples were mixed and allowed to stand on ice for 5 min following which they were centrifuged at 10,000 rpm for 10 minutes. The supernatant (1 ml) was then removed for determination of formaldehyde, using the Nash assay.

Nash Assay

Reagent

Ammonium acetate (150g) was dissolved in 900 ml of distilled water. Acetylacetone (2 ml) was added and completely dissolved. The pH was adjusted to pH 6.0 with glacial acetic acid, and the volume adjusted to 1l. The reagent was stored at 4\(^\circ\)C.

Method

The supernatant from the TCA precipitation (1 ml) was added to Nash reagent (1 ml), mixed and heated in a water bath at 58\(^\circ\)C for 10 mins in the dark. After 5 minutes cooling, the absorbance at 412 nm was recorded. A standard curve was constructed using 1 ml volumes containing 0,10,20,40, 60,100,140 nmol formaldehyde and treated in an identical manner to the reconstituted system. Under these
2.7.2 Determination of Lauric Acid Hydroxylation

The metabolism of $^{14}$C-lauric acid to 12- and 11-hydroxylated products was evaluated by t.l.c. analysis essentially as described by Parker and Orton (1980). The 1 ml incubation volume contained either cytochrome P-450 and NADPH-cytochrome P-450 reductase at concentrations of 0.3 μM and 0.9 units/ml respectively or microsomes at a concentration of 2 mg/assay. $^{12}$C-lauric acid (0.55 mM final concentration) was added as a methanolic solution (200 mM) and $^{14}$C-lauric acid (0.1 μCi) was added in 10 μl of methanol. The pre-incubation and incubation times for the purified cytochrome P-450 isoenzymes were both 6 mins. However, for liver and kidney microsomes, although the pre-incubation times remained the same, the incubation times were increased to 10 and 15 mins respectively. The reactions were terminated by the addition of 300 μl of 3M HCl to the incubation volume.

Extraction and analysis of hydroxylauric acid

The incubation mixtures were transferred to 15ml screw cap sovrl tubes using ether (10ml), and then rotated end over end for 10 mins. After centrifugation at 2,000 rpm for 10 mins to clarify the phases, the upper (ether) layer (approx. 9 ml) was transferred to a test tube and evaporated to dryness under a steam of nitrogen.

Thin layer chromatography (TLC) analysis of total 11- and 12- hydroxylaurate

The dried ether extracts were reconstituted in 60 μl of methanol, of which 20 μl was spotted onto silica gel GF plates 250μ (Merck, 20 x 20 cm). The plates were developed in hexane: diethyl ether: acetic acid, (70 : 20: 1.5 by vol) with the solvent front being allowed to migrate up 3/4 of the length of the plate. The radioactive areas corresponding to unmetabolised lauric acid (Rf = 0.29) and 11-and 12-hydroxylauric acid (Rf = 0.06) were located by plating against Kodak CX-Industrex X-ray film for 3 days, after which the spots corresponding to the metabolites
formed were scraped off, suspended in 5 ml CAB-O-SIL scintillant and counted in an
LKB 1216 Rackbeta liquid scintillation counter. The rates of hydroxylation were
calculated from the fractional conversion of substrate to total hydroxy products
making the estimation of recoveries unnecessary.

High Performance liquid chromatography (HPLC) procedure for the separate
quantification of 11- and 12-hydroxylauric acid

The dried ether extracts were reconstituted in 150 μl of eluting solvent, water:
methanol : glacial acetic acid (45 : 55 : 0.5 by vol.). 50-100 μl aliquots were then
injected into the HPLC system of the following specifications:

- Column: Micropak MCH -10 (30 x 0.4 cm, Varian Associates Ltd.).
- Guard Column: Whatman column packed with CO : PELL ODS.

Elution of the products was achieved by using a linear gradient of water:
methanol : glacial acetic acid (45 : 55 : 0.5 by vol.) to 100% methanol over a 30 min
period at a flow rate of 1.5 ml/min. A pressure of 350-500 psi was maintained with
the 11- and 12-hydroxylauric acid being successively eluted followed by the
unmetabolised lauric acid. The elution profile of the radioactive products was
monitored using a Berthold LB 503 HPLC radioactivity monitor (Laboratory Impex
Ltd., Middx., U.K.) and radioactive metabolites were quantitated using a liquid
scintillation technique. Alternatively the D.C. output from the radioactivity
monitor was interfaced (hexagan interface, ICI) with a PET 4032 microcomputer,
linked to a chart recorder for data analysis.

2.7.3 Determination of Ethoxyresorufin O-deethylation

The O-deethylation of ethoxyresorufin was routinely determined by the
method of Burke et al (1977), using the difference in fluorescent properties of
ethoxyresorufin (excitation wavelength = 456 nm, emission wavelength = 560 nm)
and the product resorufin (excitation wavelength = 510 nm, emission wavelength =
586 nm).
The standard reconstituted system contained cytochrome P-450 and NADPH-cytochrome P-450 reductase at concentrations of 0.3 μM and 0.5 units/ml respectively. Ethoxyresorufin was added to a final concentration of 50 μM in 10 μl of ethanol. The complete system was then transferred to a fluorimeter cuvette and placed in a thermostated cuvette housing of a Perkin-Elmer MPF-3 fluorimeter, set at an excitation wavelength of 510 nm and emission wavelength of 586 nm. After preincubation at 37°C for 2 min a steady base line with time was established. The reaction was initiated with a final concentration of 1mM NADPH and the production of resorufin with time monitored as the increase in fluorescence at 586 nm. The fluorimeter was calibrated with multiples of 10 μl of resorufin standard (10 μM in ethanol).

2.7.4 Antibody Inhibition Studies of Catalytic Activity

For the determination of catalytic activity of specific cytochrome P-450 isoenzymes with preferred substrates the standard reconstitution systems as described were utilised. The order of additions was essentially the same, except that after the addition of cytochrome P-450 and its corresponding reductase the mixture was allowed to equilibrate on ice for 5 min. After this the specific antibodies were added - either as sera or IgG in potassium phosphate buffer (pH 7.4) - and preincubation was allowed to proceed at 25°C for 10 mins. The remaining substrates and co-factors for the specific reactions were then added and the reactions allowed to continue as per normal. For all analyses the corresponding inhibition studies were carried out with either pre-immune sera or IgG as well as utilising the appropriate blanks.

2.8 DISCONTINUOUS SODIUM DODECYL SULPHATE POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS

The method of Laemmli (1970) was routinely used for the assessment of the purity and molecular weight of liver and kidney microsomal proteins. The proteins
were first rendered monomeric by solubilising them with SDS in the presence of 2-mercaptoethanol and then separated on polyacrylamide using a discontinuous buffer system.

Reagents

Gel monomer stock : 30% (w/v) acrylamide (Electran) containing 0.8% (w/v) N,N-methylene-bis-acrylamide.

Separating gel buffer: - 1.5M Tris-HCl (pH 8.8) containing 0.4% (w/v) SDS.
Stacking gel buffer : 0.5M Tris-HCl (pH 6.8) containing 0.4% (w/v) SDS.
Electrode buffer : 25mM Tris-HCl (pH 8.3) containing 192mM glycine and 0.1% (w/v) SDS.

Sample solubilisation buffer:- 62.5mM Tris-HCl (pH 6.8) containing 2.3% (w/v) SDS, 15% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue.
Ammonium persulphate 10% (w/v).
N-N-Tetramethylethylenediamine (TEMED).

Method:

Electrophoresis was carried out at room temperature in either one of two vertical slab gel assemblies. One system was laboratory manufactured in which the gel was contained in a glass and perspex cassette of internal dimensions 120 x 130 x 15 mm. The other was a commercially available LKB 2000 vertical gel electrophoresis unit in which the internal dimensions of the poured gel were 160 x 140 x 1.5 mm. The respective plates were stored in nitric acid and washed in detergent, tap water, distilled water and acetone prior to use. The cassettes were assembled from the dry, dust-free plates and perspex spacers, sealed and then clamped in a vertical position. The lower (separating) gel was prepared by mixing separating gel buffer with an amount of gel stock to give the correct running gel acrylamide concentration. The volume was then adjusted with distilled water and the polymerisation process initiated with the addition of TEMED and freshly
prepared ammonium persulphate solution. This solution was then poured into the 2 different electrophoresis cassettes to a height of 80 and 115 mm respectively. A layer of distilled water was introduced above the gel mixture to ensure a flat interface between the separating and stacking gels after polymerisation. When polymerisation was complete (20-30 min), the water layer was removed and the upper (stacking) gel consisting of a mixture of stacking gel buffer, gel stock and water - polymerisation being initiated as before - was poured on top of the separating gel. A perspex comb was introduced into the stacking gel before polymerisation to form the sample wells. After polymerisation the bottom edge spacer and sample comb were removed and the gel slab immediately placed into the electrophoresis tank. Electrode buffer was poured into the anode and cathode reservoirs with any air bubbles trapped beneath the gel combs or underneath the gel being removed using a syringe and fine tubing.

The samples to be analysed were diluted in sample buffer and placed in a boiling water bath within a fume cupboard for 3 minutes. After cooling, 5-20 µl aliquots were applied to the stacking gel sample wells using a Hamilton syringe. The amount of protein applied for the separation of microsomal bands was generally 15 µg/well for 20-well gels, and 80 µg/well for 6-well gels. The amount of purified protein applied depended upon the purity of the preparation. Generally 2.5 to 10 pmol of cytochrome P-450 per well were applied to 20-well gels. Samples were run through the stacking gel under a 20 mA constant current until the bromophenol blue indicator entered the running gel, whereupon the current was increased to 40 mA. The current was switched off when the indicator dye had moved to within 0.5 cm of the bottom of the gel. The gel slab holder was then removed from the tank, the plates separated, and the lower running gel cut out and removed for staining.

2.8.1 Detection of Proteins on Polyacrylamide gels

Electrophoretic bands were detected by using coomassie brilliant blue R-250 as the protein stain.
Reagent

Staining solution: propan-2-ol/acetic acid/water (25 : 10 : 65 by vol.) containing 0.05% (w/v) coomassie brilliant blue R-250.

De-staining solution: propan-2-ol/acetic acid/water (1 : 1 : 8 by volume).

Method

Staining of protein bands was achieved by placing the gel overnight in staining solution. The gel background was destained by extraction with successive volumes of de-staining solution on a gently rocking water bath over a 12h period.

2.8.2 Standard protein Molecular Weight Markers used in Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis

The following proteins of known molecular weight were routinely used to calibrate polyacrylamide gels. Phosphorylase a (rabbit muscle Mr = 92,500), bovine serum albumin (Mr = 68,000), catalase (beef liver, Mr = 60,000), glutamate dehydrogenase (beef liver, Mr = 53,000), chicken egg albumin (Mr = 43,000), creatine phosphokinase (rabbit muscle, Mr = 36,000), cytochrome c (horse heart, Mr = 12,400).

The stock solution of standard proteins (5ml) contained 1mg of each protein made up to volume with solubilisation buffer, was stored in aliquots (0.5ml) at -20°C. Before use, an aliquot was diluted 1:3 and heated in a boiling water bath for 3 minutes. The volume applied was 5μl per well for 20 track gels and 20μl per well for 6 track gels.

Molecular weight studies of the purified proteins were also performed using calibrated SDS P.A.G.E. and the degree of non-ideality of migration of the cytochrome P-450 isoenzymes was determined according to the method of Fergusson (1964).
2.8.3 Limited Proteolysis of Homogeneous Cytochrome P-450 Isoenzymes

Proteolytic maps were prepared according to the method of Cleveland et al (1977) and Koop et al (1981).

Samples of cytochrome P-450 were diluted in 0.25M Tris-HCl buffer (pH 6.8) containing 0.1% (w/v) SDS to a final concentration of 0.33 mg/ml in 50 µl. Incubations were carried out at 37°C for the required length of time after the addition of proteolytic enzyme and terminated by the addition of 30µl of 50% (v/v) 2-mercaptoethanol, 10% (w/v) SDS followed by heating in a boiling water bath for 3 minutes. Glycerol and bromophenol blue were then added to final concentrations of 10% (v/v) and 0.004% (w/v) respectively and 20µl samples (3.5 µg cytochrome P-450) were submitted for electrophoresis on 12.5% (w/v) polyacrylamide gels as previously described.

2.9 ANTISERUM PRODUCTION

The specificity and resolution of immunochemical techniques are responsible for their increasing application to problems in biochemistry and molecular biology enabling specific and sensitive methods for both qualitative and quantitative analyses of protein antigens to be provided. In this study antibodies have been raised against separate, unique, homogeneous cytochrome P-450 isoenzymes in order to demonstrate: (a) immunological differences between purified rat cytochromes P-450, (b) identification and analysis of these different cytochrome P-450 isoenzymes following xenobiotic induction, (c) the presence of these multiple forms of cytochrome P-450 in different tissues, (d) as a convenient and very precise means of identifying and isolating these different multiple cytochrome P-450 isoenzymes, during a number of in vitro experimental procedures.

2.9.1 Preparation of Antigens for Immunisation

Antisera was raised against a single hepatic protein antigen designated
cytochrome P-450 B\textsubscript{2}, following phenobarbitone-induction. In order to obtain as specific antisera as possible, the purity of the injected antigen is critical such that only entirely homogeneous cytochrome P-450 fractions were employed. For the first immunisation 700 \mu g of the antigen was used, which was added to an equal volume of non-ulcerative Freund's complete adjuvant containing heat-killed BCG mycobacteria. The use of Freund's complete adjuvant results in a far greater antibody response than is stimulated by antigen in a simple water-in-oil emulsion because of the greater persistence of the antigenic complex within the tissues of the test animal. Similarly the presence of the heat-killed mycobacteria serve to stimulate the entire reticuloendothelial system of the animal. The antigen-adjuvant emulsion is obtained by the Double-hubbed needle method first suggested by Berlin et al. (1953), which has the advantage that materials are kept sterile at all times, with there being no loss of material and that the finished emulsion can be delivered into one of the syringes involved ready for immediate use. The technique employs two syringes which are clamped together by a double hubbed needle. The antigen solution is rapidly passed into the adjuvant with the mixture then being passed backwards and forwards between the syringes until the right viscosity is attained. The stability of the emulsion was always ascertained by placing a single drop in a beaker of cold water. If it was stable then the first droplet spreads evenly across the liquid surface with subsequent drops remaining on or just below the surface but not breaking up. If this was the case then immunisation was immediately initiated.

2.9.2 Immunisation Sites, Schedules and Antiserum Processing

Female New Zealand white half lop rabbits were immunised intramuscularly and subcutaneously with 0.7 mg cytochrome P-450 B\textsubscript{2}. Prior to immunisation animals were bled to obtain non-immune (control) serum. Immunisation occurred at six sites intramuscularly in the legs and four sites subcutaneously in the back. After 6 weeks, the first post-immunisation bleed was taken and the sera collected. Subsequent bleeds were taken every 2 weeks with the immune response being
conveniently estimated by immunoprecipitation analysis. An additional boost of 700 µg cytochrome P-450 B2 in Freunds complete adjuvant was administered 12 weeks later with subsequent bleeds again being analysed for highest titres by immunoprecipitation analysis.

Blood was obtained by sectioning of the rabbit marginal ear veins, from which 80ml of blood could easily be obtained. In order to obtain a good flow rate the ears were first swabbed with xylene (an irritant) at a position distal to the point of incision, with this being removed afterwards by washing with ethanol.

Immunisation schedule for the production of antibodies against cytochrome P-452

Antibodies to clofibrate-induced cytochrome P-452 were raised in female sheep following immunisation with 600 µg of a highly purified electrophoretically homogeneous preparation of cytochrome P-452 in Freunds complete adjuvant. Four weeks later the sheep were boosted with 300 µg of purified antigen with blood being collected 7 days later from the jugular vein. The IgG enriched fractions were prepared from the sera by a combination of ammonium sulphate precipitations and DEAE cellulose chromatography after which they were concentrated to a volume of 70-80 mg/ml using an Amicon PM 30 membrane. Control sera and its respective IgG were prepared from the blood of pre-immunised sheep in the same manner.

2.9.3 Purification of Immunoglobulins

Blood from rabbits immunised with cytochrome P-450 B2 was obtained as previously described after which it was allowed to clot for a few hours at room temperature. The sera was then carefully decanted from the clot and the immunoglobulin fraction isolated by a modification of the method of Walker and Mayer (1977). The sera was brought to 50% saturation with solid ammonium sulphate and the precipitate collected by centrifugation on a MSE HS 18 centrifuge at 10,000 rpm for 10 min. It was then washed several times with ice cold 1.75 M
ammonium sulphate until it was white, in order to remove any albumin, transferrin and α-proteins including haptoglobin and hemoglobin that may be present. The final white pellet was then dissolved in 10 mM potassium phosphate buffer (pH 7.0) and dialysed overnight against 5L of water. The subsequently precipitated lipoproteins were removed by centrifugation at 2,000 rpm for 20 min with the remaining supernatant being redialysed against 2 x 3L of 10 mM potassium phosphate buffer (pH 8.0) over a period of 16 hours. This preparation was then loaded onto a column of DEAE cellulose (Whatman DE 52) previously equilibrated with the same buffer with the column then being washed with the same buffer until all the IgG was eluted. The height of the column corresponded to 1ml of DEAE cellulose per 4 ml of sera initially fractionated. Elution was measured spectrophotometrically at an absorbance of 280 nm with the protein peak being concentrated by ultrafiltration using an Amicon PM-30 membrane. Finally the IgG was dialysed against 10 mM potassium phosphate buffer (pH 7.25) after which it was aliquoted and frozen at -40°C.

2.10 ASSESSMENT OF ANTIBODY SPECIFICITY

In order to determine the specificity of each antiserum several immunodiffusion and immunoelectrophoretic techniques were employed. This was done not only to achieve quantitative data for specific antibodies raised against the corresponding cytochrome P-450 antigens, but also to ensure that there were no artifacts existing peculiar to any specific system which might or might not give misleading information about the multispecificity of any particular sera.

2.10.1 Ouchterlony Immunodiffusion Analyses

Double-diffusion analyses as demonstrated by Ouchterlony (1967) was used to identify the number of antigen-antibody systems between a tissue extract and a specific antiserum.

Precoated glass plates were used as a base for the immunodiffusion media.
The (8 x 8 cm) plates were repeatedly washed with detergent, distilled water and ethanol. Precoating was achieved by spreading a few ml of 0.2% (w/v) agar, 0.1% (w/v) thiomersal along the plate by gently moving the edge of another glass plate at a 45° angle along its surface. The agar was then dried to a fine film in hot air from a hair dryer. The immunodiffusion media which consisted of 0.9% agarose 25, 80 mM NaCl, 15 mM sodium azide, 1M glycine, being adjusted to pH 7.4 with sodium hydroxide was then gently poured onto the glass plates on a horizontal levelling table. Fourteen ml of gel media was required per plate giving a gel thickness of 2 mm. The plates were allowed to set for 10 min in moisture boxes maintained damp with a solution of 0.1% (w/v) thiomersal in water. Four millimetre diameter wells were punched with a template resulting in a pattern of wells with center to center distances of 10 mm. After filling the wells with the appropriate proteins, the plates were incubated at room temperature in a humid atmosphere for at least 3 days. After this period of time any non-precipitated proteins are removed by washing the plate a number of times with deproteinising solution containing 3% NaCl, 0.01% sodium azide and 0.01% Tween 20 with intermittent pressings. The plates were pressed by covering the gel with a layer of filter paper (avoiding air bubbles) and a 2-3 cm layer of soft tissue paper. A slight pressure of about 10 g/cm² is sustained by means of a number of glass plates for a period of 30 minutes. At this stage the gel would be reduced to a thin film and would either be rewashed and pressed or else dried in a hot air cupboard as a prelude to staining. The number of washings and pressings would vary from 3-6 depending upon the purity and amount of protein loaded onto the gel. The plates were then stained with 1.25% (w/v) coomassie brilliant blue R-250, 45% (v/v) 96% ethanol, 10% (v/v) acetic acid, in water for a period of 10 minutes, after which they were rapidly destained in 33% (v/v) ethanol, 13.3% (v/v) acetic acid, 53.7% (v/v) water.

2.10.2 Radial Immunodiffusion

The technique of single radial diffusion developed by Mancini et al., (1965) was
utilised in order to quantitate antigen within specific tissues.

The immunodiffusion media contained 1.0M glycine, 0.2mM EDTA, 86mM sodium chloride, 15mM sodium azide, 5% glycerol, 0.9% agarose 25, 0.5% cholate, 0.2% emulgen 911. After melting the agarose media it is allowed to cool to 50°C on a waterbath at which point specific anti-sera is added. After mixing, 10ml aliquots of the media are poured onto pre-heated, pre-coated (8 x 8 cm) glass plates on a precisely level surface. After standing at room temperature for 10 minutes the antisera-agar solution solidifies into a gel layer of approximately 1.5mm thickness. The plates are then stored overnight at 4°C in a moisture chamber. Holes of uniform size (2mm diameter) are punched into the layer and exact amounts of antigen samples of between 1-5 μl are added using a Hamilton microliter syringe. In the case of microsomes, solubilisation has to be carried out by the addition of 1mg cholate, 0.2 mg emulgen 911 per mg of protein solubilised with stirring for 20 min on ice. All plates had wells containing purified protein as a means of internal standard. The plates were left for 3 days following which they were washed, pressed, dried, stained and destained using the same protocol and solutions as for ouchterlony diffusion plates.

The immunoprecipitin rings were then measured with a measuring microscope utilising a 0.1mm scale with the resulting squares of the diameters being plotted against antigen concentrations. Standard curves enabled quantitation of specific fractions to be subsequently made.

2.10.3 Western Blotting Analysis

This technique entails the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets which are then subject to immunological analysis. The procedures employed were modifications of the basic procedures of Towbin et al. (1979) and Burnette, W. (1981).

SDS - polyacrylamide gel electrophoresis was carried out on samples to be
analysed as previously described. The resulting gels were soaked for 45 min in transfer buffer (20mM Tris base, 150mM glycine). This pre-transfer stage is included to allow any changes in the size of the gel due to swelling or shrinking to occur at this stage rather than during transfer. Actual transfer is achieved by using a physical assembly sandwich apparatus. In essence, a sheet of nitrocellulose (0.45 µm in pore size) was briefly wetted with transfer buffer and laid on 2 sheets of Whatmann 3MM paper lying on top of a Scotch Brite scouring pad. The whole assembly is supported by a stiff plastic grid. The gel to be blotted was then put on the nitrocellulose sheet with a further 2 sheets of filter paper and a scouring pad being placed on top. It is important to ensure that no air bubbles are caught between the filter and the gel because of the possibility of low efficiency transfer and band distortion occurring in these areas. The immobilised assembly was then inserted into the electrophoretic transfer chamber with the nitrocellulose paper facing the anode. The chamber was filled with transfer buffer with electrophoretic transfer being achieved overnight at 5 V/cm.

For direct visualisation of all proteins transferred, the nitrocellulose sheet could be stained for 5 mins in a solution containing 0.2% coomassie brilliant blue R-250, 40% methanol and 10% acetic acid. Rapid destaining was accomplished in 90% methanol, 2% acetic acid.

After electrophoretic transfer the nitrocellulose sheet was washed in phosphate buffered saline (PBS) containing 20% pre-immune donkey sera and 0.2% Triton X-100. Washing was carried out for 15 min with gentle agitation. Two further washes were then carried out as before except that the control donkey sera concentration was reduced by 5% each time. After the last PBS wash the nitrocellulose sheet was washed for 1 hr in the final wash buffer (PBS containing 10% pre-immune donkey sera, 0.2% Triton X-100) containing the primary antibody of interest. The amounts of primary antibody utilised varied depending on the experiment and specific details are given in the appropriate figure legends. After this period, 3 further washes of 15 min with the PBS wash buffer were again carried
out. The next wash of 60 min consisted of the PBS wash buffer and either the secondary antibody or else a horseradish peroxidase labelled antibody. If the first antibody was isolated from sheep then a second sandwich antibody was required before the horseradish peroxidase labelled double antibody. This approach gave rise to far greater sensitivity. Again 3 further washes in PBS wash buffer of 15 min duration were required followed by a final wash in PBS alone. The nitrocellulose sheet was then developed in 0.1M Tris-HCl buffer (pH 7.5) containing 0.5 mg/ml diaminobenzidine chloride and 1:5,000 H$_2$O$_2$ (30 vols). Once the desired image intensity was observed the sheet was then rinsed in distilled water which effectively stopped the reaction.

2.11 PRACTICAL PROCEDURES

2.11.1 Protein Assays

Protein concentrations were routinely determined by a modified method of Lowry et al. (1951) using bovine serum albumin as a standard.

Reagents

- 2% (w/v) Sodium carbonate in 0.1M NaOH
- 1% (w/v) Hydrated copper sulphate
- 2% (w/v) Sodium potassium tartrate.

Protein samples were diluted with distilled water to give appropriate concentrations. A range of 4 dilutions were used in duplicate assays. The protein standard (20 mg/ml) was diluted in distilled water to give 0, 8, 16, 24, 32, 40 µg/assay/tube in duplicate. Copper sulphate, sodium potassium tartrate and sodium carbonate/hydroxide solution were mixed sequentially to give the ratio (1 : 1 : 100 by vol) immediately before use. The protein standards, samples and blanks (0.5ml) were mixed with the above reagent (2.5ml) and allowed to stand at room temperature for 10 minutes. Folin Ciocalteau phenol reagent, diluted 1:1 with distilled water, was added (0.25ml) and the solutions were immediately vortexed.
After allowing to stand for a minimum of 30 minutes, the absorbance at 750nm was recorded. Most of the purified preparations assayed by this procedure contained concentrations of glycerol and detergents (emulgen 911), which are known to interfere with the colour forming reaction. On such occasions the presence of these compounds was compensated for by appropriate additions to the standard curve. The extent of colour development is a partial function of the amino acid composition since tyrosine residues react with the Lowry reagent.

2.11.2 **Determination of Emulgen 911 Detergent Content**

The residual emulgen content of purified cytochrome P-450 preparations was routinely determined using a general procedure for the assay of polyethoxy non-ionic detergents (Goldstein and Belcher 1975).

**Reagents**

Ammonium cobaltothiocyanate solution: made up as 17.8% ammonium thiocyanate, 2.8% (w/v) cobalt nitrate hexahydrate stored in a polythene bottle.

Emulgen 911 standard: 2 mg/ml emulgen 911 in 50% (v/v) ethanol.

Dichloroethane.

Samples were diluted with ethanol and 50% (v/v) ethanol to give an appropriate concentration of emulgen 911 in 50% (v/v) ethanol. The emulgen 911 standard was diluted to give 25, 50, 100, 200, 300 and 400 µg/assay.

Ammonium cobaltothiocyanate (0.4ml) was added to the blanks, standards and samples (0.3ml), mixed, and allowed to stand at room temperature for 5 minutes. Dichloroethane (1.5ml) was added and the solutions were vortexed for 2 minutes followed by centrifugation at 6000 g for 10 minutes to clarify the phases. The upper aqueous phases were discarded and the organic phases were scanned from 500 to 700 nm using the reagent blank as reference. The absorbance difference 625 nm minus 700 nm was used to construct a standard curve.
Deoxyribonucleic acid which is the genetic material in eukaryotes, performs two functions that are basic to life. It replicates itself and it ultimately directs the synthesis of all proteins. But, DNA itself is not used as a direct template for induced proteins, instead, it acts as the template for a corresponding RNA molecule called messenger RNA (mRNA). The mRNA being a product complimentary to the base sequence of DNA receives the information for the sequence of the respective protein in the order of its own nucleotides and subsequently directs its synthesis by a process known as translation. Thus in order to study the molecular biology of the induction of the different cytochrome P-450 isoenzymes the mRNA sequences coding for these isoenzymes have been isolated and characterised.

3.1 MATERIALS

Oligo (dT) cellulose type 7, oligo (dT)_{12-18}, 2'-deoxy-guanosine 5' triphosphate, sodium (dGTP), 2'-deoxythymidine 5' triphosphate, sodium (dTTP), 2-deoxyctydine 5'-triphosphate, sodium (dCTP), 2' deoxyadenosine 5'-triphosphate, disodium (dATP), were obtained from PL-Biochemicals (Milton Keynes, U.K.). Creatine phosphokinase, S_1 nuclease (Aspergillus oryzae) were obtained from Boehringer Mannheim (Lewes, East Sussex). Rabbit reticulocyte lysate (amino acid depleted, nuclease treated), vanadyl ribonucleoside complex, rabbit globin mRNA, E. coli DNA polymerase I "Klenow" fragment, reverse transcriptase (AMV), cesium chloride, formamide (ultra-pure) were obtained from Bethesda Research Laboratories (Glasgow, U.K.). ^{35}S-Methionine in aqueous solution containing 0.1% 2-mercaptoethanol, ^{14}C methylated protein mixture containing labelled molecular weight protein markers were obtained from Amersham International. These
included myosin (Mr = 20,000), phosphorylase a, b (Mr = 92,500), bovine serum albumin (Mr = 69,000), ovalbumin (Mr = 46,000), carbonic anhydrase (Mr = 30,000), lysozyme (Mr = 14,300). Unlabelled amino acid mixture containing 19 of the 20 amino acids at a concentration of 1mM and 2mM dithiothreitol as an antioxidant, deoxyadenosine 5'- α³²P triphosphate, triethylammonium salt in stabilised aqueous solution and Amplify were also obtained from Amersham (Bucks, U.K.). Guanidinium hydrochloride, oligo (dT) cellulose, diethyl pyrocarbonate, heparin (porcine intestinal mucosa), 2-mercaptoethanol, lithium chloride, phenylmethanesulphonyl fluoride, Nonidet P-40, Triton X-100 were obtained from Sigma Chemical Co. Ltd (Poole, Dorset). Whatmann GF/C filters were obtained from Whatmann Chemical Separation Ltd. (Maidstone, Kent). Hind III DNA markers, Protosol, Enhance were all obtained from New England Nuclear (Southampton, Hants). Sucrose (RNase free) was obtained from Cambrian Chemicals (Croydon, Surrey) and Pansorbin cells were obtained from Calbiochem-Behring Corporation (San Diego, U.S.A.). Protein A agarose, sephadex G-50 and G-75 were obtained from Pharmacia (U.K.) Ltd. (Hounslow, Middx.). Boric acid, RNA molecular weight markers, tri-sodium citrate, ethidium bromide, sodium acetate, trichloroacetic acid were all obtained from the British Drug House (Poole, Dorset).

All other chemicals used were either as mentioned in chapter 2, or else of Analar grade to ensure maximum purity.

3.2 ANIMAL PRETREATMENT

Male Wistar Albino rats were obtained and maintained as previously described. However the dosing schedule was slightly altered in order to maximise the induction conditions for the mRNA levels coding for the cytochrome P-450 isoenzymes.

Phenobarbitone and clofibrate were both administered as solutions in drinking water. In the case of phenobarbitone, animals received one i.p. injection of 125 mg/kg, and for clofibrate one i.p. injection of 400 mg/kg. BNF induction involved
one i.p. injection of 80 mg/kg in 0.25 ml of corn oil. All animals were killed 16-18 hr after injection by cervical dislocation.

3.3 EXTRACTION OF TOTAL CELLULAR RNA

The major technical problem in experiments involving the isolation of RNA is one of preventing RNA hydrolysis by ribonucleases. While all the precautions taken throughout the nucleic acid experiments might not have always been absolutely necessary, it was learned by experience that extra caution minimised the unexpected failure of some RNA preparations, especially when these were involved in in vitro translation experiments.

Inhibition of Ribonuclease activity

The primary objective therefore, was to denature any ribonucleases contained within the biological material being excised, such that if there was any subsequent contamination it was due to exogenous ribonuclease sources. Diethyl pyrocarbonate is an effective inhibitor of ribonuclease reacting with the imidazole nitrogens of histidine residues and the free amino groups causing subsequent loss of enzyme activity. Deionised water was treated with 0.1% (v/v) diethyl pyrocarbonate with stirring for 12 hr, after which it was autoclaved twice at 20 p.s.i. for 30 minutes. Following such treatment the water was considered sterile, with such water being used to make up all buffers, media etc. An additional measure was the addition of heparin, (a sulphated polysaccharide, which is a particularly effective competitive inhibitor of ribonuclease) to all media in which the tissues were initially homogenised.

Latex gloves were worn at all times in order to avoid contact between the experimentors fingers (a source of exogenous RNase contamination) and glassware or solutions. All glassware was thoroughly washed, rinsed with deionised water and then treated at 250°C for 5 hours. Where applicable solutions were also filtered
through sterile prerinsed 0.45 µm nitrocellulose filters (Millipore). During the synthesis of complimentary DNA (cDNA) sequences vanadyl-ribonucleoside complexes were employed as the most effective ribonuclease inhibitors. All experiments were carried out under the most stringent and RNase free conditions possible.

**Isolation of Total Cellular RNA**

The method used was a modification of the procedures of Chirgwin et al. (1979) and Deeley et al. (1977).

After killing, the livers or kidneys of the test animal were removed, weighed and then homogenised in a 7.5M guanidinium hydrochloride stock solution (pH 7.0) containing; 25mM Tri-sodium citrate, 10mM 2-mercaptoethanol, 5mM dithiothreitol and 0.1% (v/v) Triton X-100. Preliminary experiments showed that optimum amounts of RNA were isolated when a ratio of 21 ml of the above guanidinium hydrochloride stock buffer /g liver was employed. The tissues were then solubilised in this medium by ultraturraxing for a few minutes at maximum speed. The homogenate was then centrifged at 10,000 g, av. for 20 minutes using a 6 x 250 ml aluminium angle head rotor in a MSE HS 18 centrifuge at 4°C in order to sediment any particulate matter. The supernatant was then decanted into a flask and the RNA precipitated by the addition of 0.025 volumes (relative to the original volume of homogenisation buffer) of 1M acetic acid to lower the pH from 7 to 5 and 0.75 volumes ethanol. The sample was thoroughly mixed and precipitation was allowed to proceed at -25°C overnight. Next day the RNA precipitate was collected by centrifugation at 10,000 g at -10°C for 20 minutes with the resultant RNA pellet being again dissolved in guanidinium hydrochloride stock buffer (0.5 volume of original volume). RNA was reprecipitated by adding 0.025 volumes of 1M acetic acid, 0.5 volumes of ethanol. The solution was kept at -25°C for 12 hours and then centrifuged as before. This reprecipitation cycle was repeated twice more with a
further halving of the total volume for each precipitation. After the final precipitation the RNA pellet was dissolved in sterile water and 3M sodium acetate (pH 5.5) was added to give a final concentration of 0.3M. Precipitation was initiated by the addition of 2.5 volumes ethanol and storing overnight at -25°C. The RNA was pelleted as before and the precipitation repeated. Finally the RNA pellet was washed once with 80% (v/v) ethanol and once with 96% (v/v) ethanol with the RNA being collected each time by low spin centrifugation. The ethanol was then removed by drying under vacuum and the RNA pellet was dissolved in sterile water. The RNA was stored in aliquots of 20μl at -80°C.

Occasionally an additional purification step was used according to the procedure of Glisin, V. et al (1974) in order to ensure that the RNA preparation had absolutely no DNA contamination. This was achieved by dissolving the RNA pellet after the final guanidinium hydrochloride precipitation in 10mM Tris-HCl buffer (pH 7.5) and 0.1mM EDTA. This was then gently layered onto 1.2ml cushions of 5.7M cesium chloride in 0.1M EDTA (pH 7.5). Centrifugation was carried out in a 6 x 5 ml AH 650 swinging bucket rotor at 35,000 g for 12 hours at 20°C on a Sorval ultracentrifuge. Any DNA in the preparation remains at the top of the cesium chloride cushion whereas the RNA is pelleted. The RNA pellet is then precipitated with sodium acetate and finally washed with ethanol as before.

3.4 ISOLATION OF POLY (A) CONTAINING RNA

The criteria which distinguishes the majority of eukaryotic mRNA's from other cellular RNA species is the occurrence of a poly (A) segment at the 3' end of the molecule. This property provides a useful means of isolating poly (A) + mRNA with the method of choice being chromatography on oligo (dT) cellulose as described by Aviv and Leder (1972).
Method

Loading Buffer: 20mM Tris-HCl (pH 7.6), 0.5M LiCl, 1mM EDTA, 0.1% (w/v) SDS.

Wash Buffer: 20mM Tris-HCl (pH 7.6), 0.1M LiCl, 1mM EDTA, 0.1% (w/v) SDS.

Elution Buffer: 10mM Tris-HCl (pH 7.5), 1mM EDTA, 0.05% (w/v) SDS.

A 6 ml oligo (dT) cellulose (Sigma Chemical Co.) column equilibrated in loading buffer was poured into a 10 ml plastic syringe plugged with glass wool. The column was then washed with 5 column volumes each of: 1. Sterile water, 2. 0.1M NaOH, 3. 5mM EDTA, 4. Sterile water.

The pH of the column eluent is checked to ensure that it is less than pH 8 following which the column is equilibrated with 10 volumes of loading buffer. The total RNA fraction to be purified is then heated at 65°C for 5 minutes, rapidly cooled in ice and diluted 1:1 (v/v) with 2 x loading buffer. The solution was then applied to the column with the eluate being again reheated at 65°C for 5 minutes, rapidly cooled in ice and then reapplied onto the column. The subsequent eluate consists of all the unbound RNA species lacking a polyadenylic tail. The column is then washed with loading buffer until the $A_{260}$ of the eluate is zero. It is next washed with wash buffer so that any non-specifically bound RNA is also removed. Again, washing continues until the eluate $A_{260}$ reading is zero. The degree of non-specific binding is related to the purity of the oligo (dT) cellulose preparation. The enriched poly (A)$^+$ mRNA is then eluted by washing with the elution buffer until the $A_{260}$ readings again reach zero.

In order to further enrich the poly (A)$^+$ mRNA, it can be selected again for oligo (dT) cellulose chromatography by adjusting the LiCl concentration of the eluted mRNA to 0.5M. The mRNA was then heated and cooled as described previously and applied onto a 0.5ml high affinity oligo (dT) cellulose (type 7) column which had been washed and pre-equilibrated as before. The subsequent procedures for the isolation of the enriched poly (A)$^+$ mRNA were identical to that used in the
first column. A solution of 3M sodium acetate (pH 5.5) was added to the resulting poly (A)$^+$ mRNA eluate to a final concentration of 0.3M and the RNA precipitated by the addition of 2.5 volumes of ethanol at -25°C for 12 hours. The poly (A)$^+$ RNA was pelleted by centrifugation at 10,000 g, at -10°C for 20 minutes after which it was washed once each with 80% (v/v) and 96% (v/v) ethanol. The poly (A)$^+$ mRNA was then dried under vacuum, resuspended in a small volume of sterile water and stored in 10-20μl aliquots at -80°C.

After completion of the oligo (dT) fractionation the columns were washed with 5 column volumes each of:

1. Water,
2. 0.1M NaOH,
3. 5mM EDTA,

The columns were finally re-equilibrated with loading buffer after which they were stored at 4°C until again required.

At this stage the RNA solution would routinely give an absorbance $A_{260} : A_{280}$ nm ratio of between 1.8 and 2. The concentration of RNA can be determined spectrophotometrically with an optical density of 1 corresponding to 40 μg/ml RNA. (Maniatis et al 1982).

3.5 SUCROSE GRADIENT FRACTIONATION OF POLY (A)$^+$mRNA

Sucrose gradient centrifugation is a convenient preparative fractionation procedure which was used to further purify the enriched poly (A)$^+$ mRNA by fractionating the RNA into different size classes which were then analysed to determine which contained the mRNA sequences coding for specific induced cytochrome P-450 isoenzymes. It was these fractions which were subsequently used for synthesis of corresponding cDNA strands. The procedure utilised was a
The gradient mixer, cell tubing, eppindorf and centrifuge tubes were pretreated in a 0.1% (v/v) solution of diethyl pyrocarbonate in water for 12 hours prior to autoclaving. A linear 15 to 30% RNase free sucrose gradient made up in 10 mM Tris-HCl (pH 7.5), 10 mM EDTA and 0.2% (w/v) SDS was prepared in 14 ml Beckman SW40 centrifuge tubes. The flow rates for the gradients were such that each tube took approximately 30 minutes to fill, after which it was left standing at room temperature for a number of hours in order to remove any possible inhomogeneities within the gradient.

The RNA to be fractionated was vacuum dried and then resuspended in 99% dimethylsulphoxide, 10 mM Tris-HCl (pH 7.5). Gentle mixing was required with it being possible to solubilise up to 100 µg of RNA in 200 µl of this DMSO solution. The solubilised RNA solution was then heated for 5 minutes at 37°C after which it was diluted 4-fold with 5 mM Tris-HCl buffer (pH 7.5), 1 mM EDTA, 0.5% (w/v) SDS. The fraction was mixed and then further heated for 1 minute at 65°C after which it was rapidly cooled in ice. The fractions were then gently layered onto the sucrose gradient with centrifugation being carried out at 30,000 g for 16 hours at 20°C in a Beckman SW40 6 x 14 ml swing out rotor in the corresponding ultracentrifuge. Following centrifugation the tubes were removed with great care in order not to disturb the gradient with 0.4 ml fractions being collected dropwise into eppindorf tubes by piercing the base of the centrifuge tube. The RNA profile was then determined spectrophotometrically. The RNA was recovered by adding 3M sodium acetate (pH 5.5) to a final concentration of 0.3M and precipitating with ethanol as already described. The RNA pellet was then washed once each with 80% (v/v) and 96% (v/v) ethanol, vacuum dried, resuspended in a small quantity of water, after which it was subsequently characterised.
3.6 **GEL ELECTROPHORESIS OF RNA**

Slab gels of RNA fractions denatured by either formamide, (Maniatis et al 1980) or formaldehyde, (Boedtker, 1971) were routinely run to determine the structural integrity of the isolated RNA species.

**Electrophoresis of RNA through gels containing formamide**

The gel consisting of 5% (w/v) acrylamide, 1.2% (w/v) bisacrylamide, 98% (v/v) formamide, 20μM Na₂HPO₄, 6μM NaH₂PO₄ was poured into the glass gel apparatus (120 x 130 x 1.5mm) as already described (section 2.8). Polymerisation was initiated by the addition of TEMED. The formamide (ultra pure) was first deionised by passing it through a mixed bed resin (BIO-RAD AG 501-X8) until the pH was neutral. The RNA samples (5-10μg) were dissolved in 25μl formamide, 25% (v/v) glycerol, 0.01% (w/v) bromophenol blue and then incubated at 65°C for 15 minutes. The gels were run at a constant voltage of 100V utilising gel buffer consisting of 0.16M Na₂HPO₄ and 0.004M NaH₂PO₄ until the dye front was within a few centimetres of the bottom of the gel. The gel pattern was visualised by staining with ethidium bromide (0.5 μg/ml) for 30 minutes in the dark and then viewing over a shortwave ultra violet light box.

**Electrophoresis of RNA through gels containing formaldehyde**

1.2% (w/v) agarose gels containing 2.2M formaldehyde made up in Tris-borate (TBE) buffer were also routinely used. TBE buffer consists of 89mM Tris-borate, 2mM EDTA (pH 8.0). The samples were prepared by mixing RNA (4.5μl) with 10 x TBE buffer (2.0μl), formaldehyde (3.5μl), and formamide (10μl) after which they were incubated at 65°C for 15 minutes. The running gel buffer was TBE buffer and the gel was first pre-run for 30 minutes at 35 mA prior to loading the samples. Four μl of gel loading buffer (50% (v/v) glycerol, 1mM EDTA, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol) was added to the RNA sample prior to loading. Gel
electrophoresis apparatus and staining conditions were as for the previous gel system.

3.7 MESSENGER RNA TRANSLATION

In *vitro* protein synthesising systems from eukaryotic cells have been successfully used in studies to elucidate the basic mechanism of protein synthesis and the identity and structure of the reacting components. In addition these systems identify specific mRNAs enabling their properties to be studied as well as the properties of the proteins for which they code by analysing their translation products. Furthermore, *in vitro* systems which accurately translate mammalian mRNAs are extremely useful to investigate the regulation of mRNA translation and to quantitate biologically active mRNAs from whole cells or from cellular subfractions by their translational capacity.

3.7.1 Principles of the translation assay

A number of *in vitro* systems have now been isolated capable of translating heterologous mRNA. These systems are either broken cell preparations containing ribosomes and factors necessary for protein biosynthesis or intact cells which can be injected with the RNA to be tested. However it should be noted that all these systems have different advantages and limitations (Shafritz 1977), therefore it is important to choose the correct system most suited for ones requirements. In all these systems addition of mRNA fractions to responsive translation systems, incubated in the presence of radioactive amino acids, will synthesise a large array of labelled proteins coded for by the individual mRNAs. Subsequently, in order to monitor the translation of a specific mRNA a method yielding high and consistent recoveries of the corresponding protein from other radioactive products is required.

The most commonly utilised systems at present are as follows:-

Biologically active extracts of wheat germ have been shown to faithfully translate mRNAs of viral, plant, fungal and animal origin. Among the advantages of this system are that it is easy to prepare and use, it is economical, and it has a relatively low endogenous background of mRNA activity. Its disadvantages however are that its translational activity is low, with mRNA molecules being translated only 1-5 times, and that sub-optimal ion concentrations cause the production of incomplete peptide fragments as a result of premature termination of protein synthesis with the release of peptidyl-tRNA. Longer RNA species possibly due to a lack of normal post-translational modification of the polypeptides also appears to be particularly sensitive to this problem (Taylor 1979).

B. The Rabbit Reticulocyte Lysate (Pelham and Jackson, 1976)

Its translational efficiency is the highest of all eukaryotic systems developed so far and possesses the capability of translating mRNA molecules more than fifty times. The initial disadvantage of the system was the high level of endogenous messenger activity (approximately 90% of the product represents α- and β-globin chains) which acted in competition for the elements of the translation machinery when exogenous mRNA was added. This problem was overcome by preincubating the lysate with micrococcal nuclease which is itself inactivated after it has completed its task of destroying any endogenous mRNA present. The system has been shown to translate messages coding for proteins with molecular weights of up to 200,000 daltons and has a low incidence of premature chain termination. A wide variety of viral, prokaryotic and eukaryotic mRNAs have been translated using this system (Shafritz, 1977). Its primary disadvantage is its more complex preparation and determination of optimum conditions necessary to ensure consistent high performance.
C. Intact Xenopus Oocytes (Lane et al., 1971)

The injection of mRNA into Xenopus oocytes to stimulate protein synthesis is not strictly in vitro protein synthesis but it is frequently used, to achieve the same aims and objectives as in vitro translation systems. The advantage of the system however, is that synthesis of heterologous protein may continue for up to 2 weeks and that as little as 1-10 ng of purified mRNA can achieve saturation. This is close to the sensitivity of cDNA : RNA hybridisation. There is little requirement for mRNA specific factors and only slight competition between exogenous RNA and endogenous RNA unless very high levels of mRNA are added. The systems disadvantages include; difficulty in the microinjection technique, maintaining a supply of oocytes and that as only minute quantities of mRNA can be translated in a single egg only limited quantities of product are available for analysis.

3.7.2 The Nuclease Treated Rabbit Reticulocyte Lysate

Due to the very high translational activity and capacity for generating high molecular weight polypeptides as well as having a low incidence of protein termination, this was the in vitro translation system of choice used throughout this study relating to cytochrome P-450 induction.

Initially the lysate was prepared according to the method of Pelham and Jackson (1976) but problems of stability were encountered after nuclease treatment even though storage was at -80°C. The lysate still showed superior protein translational activity to the commercially available reticulocyte lysate from Amersham International but even this was exceeded when the BRL reticulocyte lysate was employed. Eventually it was this system which was consistently employed because of the important saving in preparation time and because of the reproducibility obtained in protein translation experiments.

The radioactive amino acid employed was $^{35}$S-methionine because of its
availability at a very high specific radioactivity which enables a much higher incorporation of radioactivity into protein to be obtained, even though methionine occurs relatively infrequently in many proteins. Another advantage is that the endogenous pool size of methionine in the reticulocyte lysate is relatively low and does not greatly dilute the added $^{35}$S-methionine.

Preparation of Rabbit Reticulocyte Lysate

Six month old New Zealand white rabbits were injected in the scruff of the neck with 4,4 and 3 ml of 1.25% (w/v) acetylphenyl hydrazine in water for 3 days respectively. On day 8 they were anaethetised with lignobarb and injected with 2,000 units of heparin into the marginal ear vein after which the animals were maximally bled by heart puncture. This is achieved by entering the chest cavity just level with the elbow from the rabbits left side and feeling for the heart with the point of the needle. Once the heart has been punctured and the maximum amount of blood obtained, the chest cavity is opened and any remaining blood is removed with a pasteur pipette. An average of 80 ml blood per animal is obtained in this manner. The blood is filtered through cheese cloth to remove hair and debris and is then spun at 2,000 rpm for 10 minutes in a 6 x 250 ml rotor in a MSE HS 18 centrifuge at 4°C. The supernatant is then removed by aspiration and the cells suspended in buffered saline composed of 0.134M NaCl, 5mM KCl, 7.5mM MgCl$_2$, 5mM D-glucose, 10mM HEPES (pH 7.2). The cells are spun down as before with these washes being carried out a total of 4 times. During the final wash the volume of cells is determined. After this spin 1.5 volumes (with respect to the packed cell volume) of ice-cold distilled water containing 1mM dithiothreitol is added to the red cells and the suspension thoroughly mixed. The lysate is then spun at 10,000 rpm for 10 minutes at 2°C in the MSE HS18 centrifuge after which it is filtered through Nybolt 123 nylon mesh to prevent any glutinous stroma entering the lysate which inhibits protein synthesis.
Nuclease treatment of Lysate

For every 10 ml of lysate, 0.2 ml of 1 mM hemin, 0.1 ml of 5 mg/ml creatine kinase in 50% (v/v) glycerol, 0.1 ml of 0.1M CaCl₂, and 0.1 ml of 15,000 units/ml micrococcal nuclease (staphylococcal) are added. The solution is thoroughly mixed and incubated at 20°C for 20 minutes. The digestion is terminated by the addition of 0.1 ml of 0.2M EGTA and 60 μl of 10 mg/ml tRNA (calf liver) in water. Again the solution is mixed after which the micrococcal nuclease treated rabbit reticulocyte lysate is aliquoted into small volumes and stored at -70°C.

3.8 PROCEDURE FOR IN VITRO PROTEIN TRANSLATION CODED FOR BY EXOGENOUS mRNA

To ensure that a given mRNA will be efficiently translated using the rabbit reticulocyte cell free system it is important to determine optimum values for a) mRNA concentration, b) length of incubation time, c) potassium ion concentration and, d) magnesium ion concentration. Also it is vital to ensure that no calcium is introduced into the lysate as this will reactivate the micrococcal nuclease which was used to destroy the endogenous mRNA and that the RNA preparation to be translated is free of any trace amounts of ethanol, detergent or sodium ions. Even residual amounts can cause inhibition of the translation machinery.

The in vitro translations are carried out in sterile plastic eppindorf tubes which are prechilled at -70°C for several hours prior to the start of the experiment. The lysate is extremely sensitive to environmental conditions such that only the required amounts are thawed for each experiment with all the additions being performed rapidly at 0°C on ice. Typical final volumes utilised for translations varied from 5-10 μl. Precise measurements of volume were achieved with the aid of graduated sterile micro-pipettes. The order of additions for the translation is as follows:-

The lysate is added to the eppindorf tube to a final concentration of 30% (v/v).
A mixture of 19 unlabelled L-amino acids which are approximately 1 mM with respect to each amino acid is then added at a ratio of 1 part to 15 parts of total lysate volume. The labelled amino acid is added, the concentration used being dependant upon the nature of the experiment. If it was a standard translation experiment a concentration of 0.5 - 0.7 μCi/μl of $^{35}$S-methionine was found to be sufficient, but if it was an immunoprecipitation analysis of the translation products then this concentration had to be increased to 1 μCi/μl. The next stage is the addition of potassium acetate and magnesium acetate (if required) ions followed by the addition of 100 mM creatine phosphate to a final concentration of 30 mM. At this stage the lysate master mix is gently vortexed with intermittent microfuging between mixes to ensure that all the components are thoroughly distributed. It is important to avoid frothing the mix, as this not only prevents good mixing but also results in some inhibition of protein synthesis through oxidation or denaturation of proteins. The master mix is then fractionated into the appropriate number of tubes following which RNA is added to the desired concentration and then sterile water to the required total lysate volume. Thorough mixing is again achieved as before. The tubes are then removed from the ice bath and transferred to a water bath where they are allowed to incubate at 30°C for 90 minutes. At the end of this period of time, an aliquot (normally 1-2 μl) is removed for analysis of incorporation of labelled amino acid into protein with the remaining lysate fraction being prepared directly for analysis onto SDS P.A.G.E. or for subsequent immunoprecipitation analysis. For every translation experiment a minimum of two controls was utilised. One was a zero mRNA "blank" control, the other was the translation of a characterised mRNA fraction of known translated activity in order to serve as an internal standard.

**Preparation of RNA for in vitro Protein Translation**

The primary means involved was that of heat denaturation of the RNA sample...
just prior to translation by incubating at \(65^\circ\text{C}\) in a water bath for 5 minutes after which it was rapidly cooled in ice. The RNA fractions in any translation experiment were never reused in order to avoid any loss in translational activity as a result of thawing and denaturation.

A second procedure utilised - although to a lesser degree because of the inherent dangers involved - was denaturation by methyl mercury hydroxide according to the method of Payvar and Schimke (1979). A stock solution of 1M methyl mercury hydroxide (a gift from Sittingbourne Research Centre) was diluted to the appropriate concentration with water and a equal volume added to the RNA sample prepared for translation. The RNA/methyl mercury hydroxide mix was mixed as described and incubated at room temperature for 5 minutes. The treated RNA was then added to the lysate master mix and the translation was allowed to proceed as before. All procedures involving methyl mercury hydroxide were carried out in a fume cupboard with utmost caution being exercised at all times.

Assay of Incorporation of Radioactivity into Proteins

After the incubation, the aliquots removed for measurement of incorporation of labelled amino acid into protein are delivered into 1ml of water in 12 x 75 mm glass tubes with the delivery micropippette being rinsed several times. To this is added 0.5 ml of decolourising solution containing 0.1% (w/v) DL-methionine, 5% (v/v) of 30% (w/v) hydrogen peroxide ("100 volume") in 1N sodium hydroxide with the tubes then being incubated in a \(60^\circ\text{C}\) water bath for 15 minutes. This deacetylates charged tRNA and bleaches the red colour due to the large amounts of heme present. 2ml of 25% (w/v) TCA is added to each tube with the proteins then being allowed to precipitate at \(0^\circ\text{C}\) for one hour.

The solutions are then filtered through Whatmann GF/C filters with all tubes being subsequently mixed with 2 x 10 ml of 25% (w/v) TCA and 2 x 10 ml of 8% (w/v) TCA, with the filters also being washed with the rinsings. Finally the filters
are washed with 2 x 10 ml of 96% ethanol. This last wash is important as it not only aids in drying the filters, but also removes a lot of the brown colour resulting from the action of TCA on the heme of the lysate, which if present in large amounts acts as a quencher during liquid scintillation counting. The filters are then dried, individually cut up into small squares and then counted in 5ml of liquid scintillation fluid.

Optimisation Conditions of mRNA Translation

In order to achieve maximum translatable activity it is important to maintain optimum concentrations of monovalent and divalent cations for the different mRNAs tested. The type of ion is also important with higher rates of protein synthesis being obtained with potassium rather than sodium, and magnesium rather than calcium (Weber, et al. 1977). The other ion of the salt is also important with acetate being preferred to the chloride ion. This is because the high chloride concentrations required inhibits the binding of mRNA to ribosomes in the formation of initiation complexes, and because the use of acetates rather than chlorides gives a much broader potassium optimum range. Accordingly it is important to test the ion concentration optima for translation of various mRNA's from different sources in order to ensure efficient and correct translation of the RNA.

Similarly, a concentration response curve for the optimum mRNA concentration has also to be determined. These concentration optima vary for different RNA fractions i.e. total RNA, poly A+ mRNA, polyribosomal RNA, and as such have to be determined for each preparation.

The final optimisation criteria required is that of translational dependance upon time in order to determine how active the system is under ideal conditions.
Although the precise mechanism of cytochrome P-450 induction is not understood, studies on the subject indicate that the induction of these specific isoenzymes after administration of certain xenobiotics to rats or mice results from an increase of the corresponding mRNAs in the cytoplasm of hepatocytes. Accordingly, this investigation has utilised in vitro translation studies followed by specific immunoprecipitation of the translation products in order to quantitate the level of functional cytochrome P-450 mRNAs in response to xenobiotic administration.

3.9.1 Treatment of mRNA Coded Lysate Translational Mix

mRNA at specific time points after chemical induction was isolated and translated as described in sections 3.3, 3.4 and 3.8. After this period of translation an aliquot was removed to determine total T.C.A. precipitable activity with the remaining fraction being utilised for immunoprecipitation analysis. Experiments showed that the optimum lysate volume required for each fraction analysed to be 40 μl, such that as a general indication a minimum of 500,000 d.p.m. were present. Three different methodologies were utilised for the isolation of the subsequent antigen - antibody complex, either by means of a double antibody sandwich, the use of a protein A - sepharose slurry or fixed Staphylococcus aureus cells. Each technique required different modifications with each having its own corresponding advantages and disadvantages. Similarly even using the same technique slight variations had to be employed for the quantitation of specific mRNAs because of the significant effects different detergent conditions had on the stability of the isolated antigen - antibody complexes.

3.9.2 Pretreatment Stages, Detergent Conditions and Treatment of the Isolated Antigen - Antibody Complexes

One of the major problems concerned with qualitative immunoprecipitation is
that of a high background of non-specific precipitation. This is believed to occur either as a result of chance recognition of some cell proteins with antibodies present within the antisera or alternatively some cellular proteins having weak but significant affinity for similar hydrophobic regions within the immunoglobulin or Staphylococcus aureus molecule. Consequently different techniques were shown to be more useful for different analysis with these being utilised accordingly. For all the immunoprecipitation stages eppindorf tubes were used throughout to limit protein adsorption onto their walls.

Double Antibody Immunoprecipitation

The procedure followed was similar to those developed by Treadwell et al (1980), and Fujii-Kurinyama et al (1981).

After translation the lysate mixes were diluted ten fold with phosphate buffered saline containing 0.01M phenylmethanesulphonyl fluoride (PMSF) and 1% (v/v) Triton X-100 (pH 7.6), then being microfuged for fifteen minutes in a Beckman Eppindorf Microfuge to remove any insoluble material. The supernatants were removed to separate tubes and specific antibody directed against the antigen of interest was added. Because the amount of antibody addition varied with different experiments, the precise volumes utilised will be documented with each experiment. The translation mix was then incubated at 37°C for one hour and then overnight at 4°C with end over end rotation. Next morning (usually 12-16 hours later) 3 μg of the second antibody (donkey anti-rabbit IgG) was added and the mix was further incubated at 37°C for 30 minutes and then at 4°C with end over end rotation for four hours. The antibody specificity of the secondary antibody was also checked by Ouchterlony double immunodiffusion analysis. The immunoprecipitates were recovered by centrifugation in the Beckman Microfuge at 12,000 rpm for 15 minutes and were then resuspended in 200 μl of 10mM Tris buffer (pH 7.4), 2mM EDTA, 0.1% (w/v) SDS. This was then gently layered on top of a 0.3ml cushion of 1M sucrose, 0.5%
(w/v) sodium deoxycholate, 0.5% (v/v) Triton X-100, 10mM unlabelled methionine. The initial tube was then rinsed with a further 100 μl of the wash buffer and this was also gently layered on top of the sucrose layer. The mix was then centrifuged at 12,000 rpm for 15 minutes after which the supernatant was discarded with the immunoprecipitate being resuspended and washed through the sucrose layer as before. The pellet was then resuspended in 1ml of 10mM Tris buffer (pH 7.4), 6mM EDTA following which it was spun down in the microfuge for 10 minutes. This final washing procedure was repeated a total of three times. The resulting immunoprecipitate was then resuspended in 60μl of fluorography sample buffer, boiled for 5 minutes and then analysed on SDS P.A.G.E.

Protein A aided immunoprecipitation

Protein A is a single polypeptide chain of apparent molecular weight 41,000 isolated from the cell wall of Staphylococcus aureus. Its characteristic biological property is its ability to interact with, and form precipitates with, a wide variety of IgG molecules (via their Fc regions) from several species. For this type of immunoprecipitation the method of Pickett et al. (1980) was followed with modifications.

After translation the mixes were made 2% (w/v) with SDS, boiled for three minutes and then diluted with 4 volumes of buffer 1 which consisted of 2.5% (v/v) Triton X-100, 190mM NaCl, 5mM EDTA, 50mM Tris-HCl (pH 7.4) and 0.01M PMSF. The mixture was then preincubated for 2 hours with 10μl of the appropriate preimmune IgG and 100μl of 50% (v/v) protein A slurry at room temperature with end over end rotation in order to decrease non specific binding. The protein A sepharose slurry was made up in buffer 2 which consisted of 150mM NaCl, 50mM Tris-HCl (pH 7.4), 5mM EDTA, 0.05% (v/v) Nonidet NP40 and 0.2% (w/v) sodium azide. The preimmune IgG/protein A - sepharose complex was removed by centrifugation at 12,000 rpm for 15 minutes after which the required volume of
specific antibody was added to the decanted supernatant. Immediately following the
addition of the appropriate immune IgG 150μl of 50% (v/v) protein A slurry was
added with the mix then being allowed to incubate for 12-16 hours at 4°C with end
over end rotation. The immune complexes bound to protein A -sepharose were
recovered by centrifugation and resuspended in 200μl of buffer 2. This was then
layered onto a 0.3ml sucrose cushion as described previously for the double antibody
immunoprecipitation with the initial tube being rinsed with a further 100μl of buffer
2 which was also layered on top of the sucrose cushion. The washing procedure was
then repeated as before with two sucrose washes and subsequently 3 washes in 10mM
Tris-HCl buffer (pH 7.4), 6mM EDTA. The antigen was then eluted from the IgG by
boiling in 60μl of fluorography sample buffer after which the mix was centrifuged at
12,000 rpm for 1 minute to pellet the protein A slurry. An aliquot was removed to
determine the amount of radioactivity present with the supernatant containing the
antigen of interest then being subjected to SDS P.A.G.E.

Staphylococcus aureus aided immunoprecipitation

The immunoprecipitation procedures utilising protein A-bearing strains of
Staphylococcus aureus bacteria were modifications of the methods of Phillips et al.
(1981), and Pickett et al. (1980).

Staphylococcus aureus cells were stored in 0.5ml aliquots as a 10% (w/v)
suspension in phosphate buffered saline at -80°C. Cells were not reused after
thawing as freeze-thaw cycles were shown to considerably increase the degree of
non specific binding. Immediately prior to use the required amount of cells was
taken and centrifuged down at 12,000 rpm for 1 minute after which they were
resuspended and equilibrated in 1 ml of the extraction/washing buffer (150mM NaCl,
50mM Tris-HCl (pH7.4), 5mM EDTA, 2mM unlabelled methionine, 0.5% (v/v) Triton
X-100 and 0.02% (w/v) sodium azide). The cells were thoroughly resuspended in this
buffer after which they were spun down as before. The supernatant was decanted
and the cells were again resuspended in 1 ml of the above buffer. This procedure was repeated 3 times after which the cells were resuspended in the same buffer with the exception that the detergent concentration had been reduced to 0.5% (v/v) and unlabelled methionine was omitted. The cells were washed a further 2 times as before after which they were resuspended in this buffer to the original volume. This procedure serves to eliminate fragmented and poorly fixed staphylococci.

When using Staphylococcus aureus cells a preincubation stage was shown to be essential otherwise gross non specific binding was evident. Following the translation the mix was adjusted to 5% (w/v) SDS and then boiled for 3 minutes after which it was diluted 1:10 with the buffer 1 utilised during the protein A aided immunoprecipitation. The mixture was then preincubated for 5 minutes at 30°C after which the cells were removed by centrifugation (5 minutes). The supernatant was decanted, to which was added the appropriate antibodies. This mix was then incubated at 30°C for 1 hour and then at 4°C with end over end rotation for 12-16 hours. The antigen-antibody complexes were precipitated by the addition of 75μl of prewashed Staphylococcus aureus cells followed by a further incubation period of 1 hour at 4°C with end over end rotation. The antigen-antibody-staphylococci complex was then isolated by centrifugation in a microfuge (15 minutes), after which it was thoroughly resuspended in 10mM Tris-Cl buffer (pH 7.4), 6mM EDTA. If the immunoprecipitation had been directed towards the isolation of either the clofibrate-induced cytochrome P-452 or BNF-induced cytochrome P-447 antigens, then the isolated immune complexes would be washed a total of 3 times in the above buffer by means of resuspension and subsequent centrifugation (10 minutes) of the resulting pellet. However, if the immunoprecipitation was directed towards the isolation of phenobarbitone-induced cytochrome P-450 antigens then the resulting immunoprecipitate complex would be layered onto a 0.3ml sucrose cushion containing 0.3M sucrose, 0.5% (w/v) sodium deoxycholate, 0.5% (v/v) Triton X-100 and 10mM unlabelled methionine. The immunoprecipitates were then collected by
centrifugation (10 minutes), resuspended in the 10mM Tris-HCl (pH 7.4), 6mM EDTA buffer and then washed 3 times as before. After the final wash the immunoprecipitated complexes were resuspended in 60μl of fluorography sample buffer, boiled for 5 minutes and then centrifuged for 1 min to remove the residual Staphylococcus cells. The supernatant containing the immunoprecipitated translation products was then assayed for total radiolabelled amino acid incorporation and then analysed on SDS P.A.G.E.

3.9.3 Quantitation of Cytochrome P-450 apoprotein Synthesis

Quantitation of the immunoprecipitates resulting from the translation products of in vitro protein synthesis was achieved by fluorographic analysis of the SDS polyacrylamide gels which allowed visualisation and subsequent excision of the radioactive bands of interest. Two different methods were used of which the second was judged to be the most efficient.

The first method utilising the digestion procedure of Mahin and Lofberg (1966) involves the use of perchloric acid and hydrogen peroxide. The gel slice was placed in a 10 ml glass vial to which was added 0.2 ml of 60% (v/v) perchloric acid followed by 0.4 ml of 30% (v/v) hydrogen peroxide. The vial was then capped and incubated at 60°C in a shaking water bath for a period of five hours by which time the gel slice had dissolved. The vial was allowed to cool after which 10 ml of Econofluor scintillation fluid (New England Nuclear) was added with counting being carried out after 1 hour.

In the second method the gel slice was rehydrated in a glass vial by the addition of 150 μl water over a period of five hours. To this was added 10 ml of Econofluor containing 3% (v/v) Protosol (New England Nuclear) with incubation at 37°C being allowed to proceed for 12-16 hrs. The vials were then cooled and subsequently counted. Radioactivity was monitored using an LKB Rackbeta liquid scintillation counter giving a counting efficiency for $^{35}$S of 66%.
3.10 FLUOROGRAPHIC ENHANCING

The primary reason for the utilisation of fluorographic techniques in the analysis of radiolabelled products within polyacrylamide gels is that fluorographic scintillators can increase sensitivity in detection by as much as 10-15 fold for $^{35}$S-methionine. Three fluorographic scintillators were utilised during the course of this study; sodium salicylate and the commercially available scintillators Amplify (Amersham) and Enhance (New England Nuclear). For isotope detection Kodak X-Omat AR or Kodak Industrex CX X-ray film was used. Fluorographic sample buffer utilised throughout the immunoprecipitation analysis consisted of 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10mM EDTA, 30% (v/v) glycerol, 0.01% (w/v) bromophenol blue.

Fluorographic detection of radioactivity with the water soluble fluor, sodium salicylate, was carried out according to the procedure of Chamberlain (1979). Following electrophoresis the gels were fixed for 1 hour on a shaking water bath in 30% (v/v) methanol, 10% (v/v) glacial acetic acid and 10% (w/v) TCA. They were then washed for 30 minutes in water and then for 30 minutes in 1M sodium salicylate after which they were dried on a Bio-Rad gel drier on Whatman 3MM paper and then plated against X-ray film at -70°C. The only disadvantage of this method was that a slightly mottled image of the radioactive bands was obtained which is believed to result from the reflection of light from the surface of large crystals of salicylate.

Amplify was probably the safest fluorographic reagent used, being also both fast and convenient with it being possible to treat a gel within 2 hours on following the manufacturers instructions. However, of the 3 fluorographic reagents Enhance was deemed to be the most efficient and was therefore the most utilised despite the longer preparative time.

The polyacrylamide gels were first fixed in 30% (v/v) methanol, 10% (v/v) glacial acetic acid and 10% (w/v) TCA for one hour applying gentle agitation. They
were then impregnated for a further hour with Enhance after which the fluor is precipitated by immersing the gel in water. The precipitation stage was carried out for fifty minutes with gentle agitation after which the gel was dried at 60-70°C on Whatman 3MM paper using a Bio-Rad gel drier. The dried gel was placed against X-ray film and exposed at -70°C. Generally an exposure period of 2 weeks would be required for the analysis of immunoprecipitations and 1 week for the analysis of total translation products.

3.11 SYNTHESIS OF COMPLIMENTARY DNA FROM CLOFIBRATE-INDUCED CYTOCHROME P-452 mRNA

The final experimental stage in this project was the synthesis of a double stranded complimentary DNA (cDNA) sequence to the corresponding clofibrate induced poly(A)^+mRNA. This was carried out with a primary viewpoint of determining and subsequently optimising the conditions required for attaining full length copies of cDNA, which would eventually be cloned and then utilised as highly specific hybridisation probes for the analysis of the gene structure of the highly related cytochrome P-450 isoenzyme family.

Although the enzymatic conversion of poly(A)^+mRNA to double stranded cDNA is now a well established procedure the task for optimisation for both size and yield is influenced by so many factors (e.g. ribonuclease degradation, pH, monovalent and divalent cation concentration etc) that the successful isolation of full length double stranded cDNA (ds-cDNA) becomes a complex assignment. For this reason no specific methodology was found to be entirely successful and as such modifications of a variety of procedures had to be employed.

3.11.1 Synthesis of Single Stranded cDNA

The first stage is the further enrichment of the mRNA of interest and this is achieved by size fractionation of the mRNA through a sucrose gradient as described
in section 3.5. Each fraction of the mRNA is then translated in vitro and the protein product of interest is identified by a combination of immunoprecipitation and SDS polyacrylamide gel electrophoresis.

Solutions utilised

1. Reverse transcriptase buffer: 500mM Tris-HCl (pH 8.3), 50mM MgCl₂, and 50mM dithiothreitol.
2. Unlabelled nucleotide (dNTP) solution; containing GTP, CTP, and TTP at a concentration of 20mM and ATP at a concentration of 10mM.
3. Oligo dt₁₂₋₁₈ primer at a concentration of 660 µg/ml in water.
4. T/E/N buffer: 20mM Tris-HCl (pH 8.0), 1mM EDTA and 100mM NaCl.
5. T/E buffer: 10mM Tris-HCl (pH 8.0), 1mM EDTA

The pH of the reverse transcriptase buffer is measured at 42°C because of the pH variability of Tris with temperature. The amount of poly(A)⁺mRNA utilised varied from 0.5 to 2.5 µg per assay with an optimal amount of 50µCi of (α-³²P) ATP utilised as the labelled precursor. All stock solutions, buffers and apparatus utilised were sterilised.

The first optimisations required to be carried out, are the determination of the ratio of reverse transcriptase to mRNA template in order to get maximal yield of cDNA per µg mRNA as measured by the incorporation of radioactive label (as described in section 3.12.2). The sequence of additions for the synthesis of single stranded cDNA (as cDNA) is as follows:-

1.25 µl of stock unlabelled dNTP solution is added to 2.5 µg of enriched clofibrate poly(A)⁺mRNA followed by 50 µCi of (α-32p) -ATP in a polypropylene eppindorf tube. The mRNA prior to addition was heated at 65°C for 3 minutes following which it was cooled in ice. The mix was vortexed and then pelleted by centrifuging for a few seconds in a microfuge. The components were then
lyophilised in order to remove trace amounts of ethanol and possible contaminating triethyl ammonium bicarbonate utilised as stabilising agents for the radiolabel which would otherwise inhibit the activity of the reverse transcriptase enzyme. Five µl of 250mM KCl, 5µl of reverse transcriptase buffer, 4µg of oligo (dt) primer and 0.5µl of 40mM sodium pyrophosphate were then added in succession with the contents of the tube again being mixed by gentle vortexing. Avian myeloblastosis reverse transcriptase was then added at concentrations ranging from 0 to 100 units of enzyme per µg mRNA template. Sterile water was finally added to a final volume of 25 µl after which the contents of the tube were mixed by gentle vortexing and then returned to the bottom of the tube by centrifugation for 15 seconds in a microfuge. The mixture was then incubated at 42°C for 2 hours, with aliquots being regularly removed throughout this period so that the amount of cDNA being synthesised could be monitored. The use of sodium pyrophosphate as a ribonuclease inhibitor was shown to increase the amount of cDNA synthesised in excess of the amount when vanadyl-ribonucleoside complexes were utilised as inhibitors. After the incubation period the reaction was extracted with an equal volume of phenol/chloroform in order to separate the proteins from nucleic acids. The fractions were throughly mixed by vortexing after which they were centrifuged for 50 seconds in a microfuge. The aqueous phase was then transferred to a fresh eppindorf tube, with the organic phase being re-extracted with an equal volume of T/E/N buffer. The aqueous phase was then isolated as before with the two phases then being combined. The synthesised cDNA was separated from the unincorporated dNTPs by chromatography on sephadex G-75 equilibrated in T/E/N buffer by the procedure outlined in section 3.12.4. Fractions of 0.2 ml were collected with the amount of radioactivity present being monitored by Cerenkov counting. Those fractions corresponding to ss cDNA are pooled and to which is added one tenth volume of 3M NaOH and 10mM EDTA in order to hydrolyse the mRNA template. The fractions were then incubated at 68°C for 1 hour after which the ss cDNA was
precipitated by the addition of 3M sodium acetate (pH 4.7) to give a final concentration of 0.3M followed by the addition of 2 volumes of ethanol. It is important to ensure that the alkali is neutralised and this is checked by spotting universal pH litmus paper. The ss cDNA is allowed to precipitate overnight at -70°C following which it is centrifuged down in a microfuge for 15 minutes at 4°C. The pellet is washed twice in ice-cold 70% ethanol, being pelleted each time as described. Finally the ss cDNA is lyophilised to remove all traces of the ethanol and is resuspended in water. The size of the resulting first strand cDNA is determined by electrophoresis through a 1.2% alkaline agarose gel as described in section 3.12.3. Aliquots are removed at all the relevant steps because as the specific activity of the radioactive precursor is known this gives a means of estimating the yield of the reaction product.

3.11.2 Synthesis of Double Stranded cDNA

To 20μl of ss cDNA, 5μl of dNTP stock is added followed by 2.5μl of 20mM ATP in order that all the dNTPs are at a final concentration of 1mM. A further 25μCi of (α-32P)-ATP is added and the reaction mixture gently vortexed. The Klenow fragment of E. coli DNA polymerase I is added to a final concentration of 30 units per μg of initial mRNA template with the reaction mixture then being brought to a total volume of 50μl with sterile water. A further 50μl of 2 x second strand buffer consisting of 0.2M HEPES (pH 6.9), 20mM MgCl₂, 5mM dithiothreitol and 0.14M KCl was then added. The contents of the tube was again mixed and brought to the base of the tube by centrifugation after which it was incubated at 15°C for 20 hours. The long incubation period is required to allow the enzyme to find the first strand cDNA molecules with hairpin loops at their 3' ends. The reaction is terminated by the addition of 2.0μl of 0.5M EDTA and the mixture is chilled on ice. The solution was then extracted by the addition of 100 μl phenol/chloroform as described previously. The two aqueous phases obtained were pooled and the ds
cDNA was separated from the unincorporated nucleotides by chromatography on Sephadex G-50. The final extraction was carried out utilising T/E buffer which was also the buffer used for the equilibration of the Sephadex G-50. The fractions containing ds cDNA were pooled, precipitated and washed as before. After lyophilisation the ds cDNA was resuspended in 20µl water.

In an attempt to increase the yield of full-length ds cDNA, the reaction mixture was then treated with reverse transcriptase. This is to re-initiate synthesis at strong-stop sequences in the initial ss cDNA. To the ds cDNA, 5µl of 1M Tris-HCl buffer (pH 8.3), 7µl of 1M KCl, 2µl of 250mM MgCl₂, a solution of all four dNTPs at a final concentration of 1mM, 2µl of 700mM 2-mercaptoethanol, 50 units of reverse transcriptase and water is added to give a final volume of 50µl. The solution was then mixed by gentle vortexing, briefly microfuged and then incubated at 42°C for 1 hour. The reaction is stopped by the addition of 2.0 µl of 0.5M EDTA and by chilling the mixture on ice. The sample was then extracted with an equal volume of phenol/chloroform with a second extraction then carried out with T/E buffer. The aqueous phases were combined with the ds cDNA then being separated from the unincorporated nucleotides by chromatography on Sephadex G-50 as described. The ds cDNA was subsequently precipitated with ethanol, and washed twice with ice cold 70% ethanol, lyophilised and then resuspended in water. In order to ensure that the synthesis of the second strand was successful, the length of the ds cDNA is visualised on an alkaline 1.4% agarose gel. As this was shown to be the case the experiment was terminated at this stage.

3.12 GENERAL ANALYTICAL PROCEDURES

3.12.1 Preparation of and Extraction with Phenol/Chloroform

One of the key steps in ds cDNA preparations is the removal of utilised enzymes from aqueous solutions of nucleic acids with a 1:1 mixture of phenol/chloroform. Commercial phenol has to be further purified in order to
remove contaminants that cause breakdown or cross linking of RNA and DNA. This is achieved by redistilling phenol at 160°C to which is then added 8-hydroxyquinoline at a final concentration of 0.1% (w/v). The melted phenol is then extracted twice with an equal volume of 1M Tris buffer (pH 8.0) followed by a final extraction in 0.1M Tris buffer (pH 8.0), 0.2% (v/v) 2-mercaptoethanol and 0.1% (w/v) 8-hydroxyquinoline. The phenol is then stored in small aliquots at -40°C. Chloroform is defined as a mixture of chloroform : isoamyl alcohol at a ratio of 24: 1 (v/v). This is stored at room temperature.

Extraction is achieved by the addition of an equal volume of 1:1 (v/v) phenol/chloroform. The solution is thoroughly mixed until an emulsion forms at which stage the phases are separated by centrifugation in a microfuge for 50 seconds. The aqueous phase is carefully removed and transferred to a fresh polypropylene tube. The organic phase is then re-extracted as before with an equal volume of T/E/N or T/E buffer. The aqueous phases are pooled and the lower organic phases discarded.

3.12.2 Measurement of Radioactivity in Nucleic Acids by Trichloroacetic Acid Precipitation

An aliquot of labelled nucleic acid solution is added to a tube containing 200 µl of 200 µg/ml salmon sperm DNA in 20 mM EDTA. Precipitation is achieved by the addition of 1.75 ml of ice-cold 10% (w/v) TCA, mixing, and chilling on ice for 1 hour. The precipitate was collected by filtering through a GF/C filter. The filter was then rinsed twice with 5 ml of ice-cold 10% (w/v) TCA, twice with 5 ml 8% (w/v) TCA, twice with 96% (v/v) ethanol, dried and subsequently counted.

3.12.3 Sizing of cDNA in Alkaline Agarose Gels

Alkaline electrophoresis buffer: 30mM NaOH and 1mM EDTA.

Alkaline loading buffer: 50mM NaOH, 1mM EDTA, 2.5% (w/v) Ficoll (type 400) and
A 1.2% (w/v) slurry of nucleic acid electrophoretic grade agarose was made up in 50mM NaCl and 1mM EDTA. The mixture was melted on a water bath, cooled to 55°C and then poured into the gel apparatus described in section 2.8. The gel was allowed to set for one hour after which it is covered with electrophoresis buffer and allowed to soak for a further hour. Initial migration of the DNA samples into the gel was carried out at 25V with the voltage subsequently being increased to 50V. Electrophoresis was carried out until the dye had migrated approximately 8cm down the length of the gel. At the end of the run the gel was removed from its glass cassette, soaked in 7% (w/v) TCA for 30 minutes and then dried by blotting with Whatmann 3MM filter paper. The gel was then covered with cling film and plated against Kodak X-ray film at room temperature.

3.12.4 Sephadex Column Chromatography

The required amount of Sephadex G-50 or G-100 was swollen overnight in T/E or T/E/N buffer. The slurry was then autoclaved for 15 minutes at 15-lb/square inch on liquid cycle, allowed to cool and stored at room temperature after a further buffer change. Columns utilised were 10 ml sterile, disposable, sterilin pipettes plugged at one end with sterile glass wool. The sephadex was poured to a column height of 9 inches and was then further equilibrated with the appropriate buffer. In order to fractionate cDNA from unincorporated nucleotides 5μl of 1 mg/ml (w/v) Orange G transfer dye was added to the cDNA solution. The tracker dye migrates with the unincorporated nucleotides. Fractions of approximately 200 μl were collected into sterile appindorf tubes. It was also possible to follow the progress of the incorporated and unincorporated (32P) dNTPs down the column using a hand-held minimonitor.
3.13 SCINTILLATION FLUIDS

For the quantification of radioactivity in areas of silica gel originating from TLC plates CAB-O-SIL was utilised. This consisted of 4% (w/v) CAB-O-SIL, 0.5% (w/v) PPO and 0.05% (w/v) dimethyl POPOP in scintillation grade toluene (sulphur free).

Routinely used standard scintillation fluid consisted of 0.5% (w/v) PPO, 0.01% (w/v) dimethyl POPOP, 33% (v/v) symperonic-NX detergent in scintillation grade toluene.
Chapter 4

PURIFICATION AND CHARACTERISATION OF MICROSONAL ENZYMES

4.1 INTRODUCTION

An important feature of cytochrome P-450 is the ability of several xenobiotics to cause the proliferation of smooth endoplasmic reticulum with a concomitant increase in CO binding microsomal proteins having an absorbance maximum at or near 450 nm. This increase was associated with augmented drug metabolising activity and is now known to be due to induced mixed function oxidase isoenzymes. It is now recognised that different xenobiotics induce different cytochromes P-450 and that at least 15 submembers of the hepatic cytochrome P-450 family exist. However the precise mechanisms that control the regulation and expression of these multiple forms is far from clear. This is partially due to the fact that until recently the existence of techniques specific enough to identify and quantitate the exact complement of cytochrome P-450 isoenzymes were just not available, let alone mentioning the occurrence of species, age, tissue differences and the repressive or destructive effects of various exogenous chemicals.

Accordingly, this study was initiated with a view of isolating the major phenobarbitone-induced cytochrome P-450 isoenzyme and characterising it at both a biochemical and molecular biology level.

Utilising protein purification procedures two major forms of phenobarbitone-induced cytochrome P-450 were isolated to electrophoretic homogeneity. The study was expanded to include two further forms of electrophoretically homogeneous cytochrome P-450 also purified within our laboratories, in order to study, the inductive heterogeneity of these cytochrome P-450's.

One of these forms, cytochrome P-452, is induced following pretreatment of animals with the hypolipidaemic agent clofibrate (ethyl 2-4-chlorophenoxy)-2-methyl propanoate, (Gibson et al., 1982). Induction resulted in significant and selected increases in cytochrome P-450 levels with the cytochrome P-452 having a
high activity towards the \( \omega \) and \((\omega-1)\) hydroxylation of fatty acids such as lauric (dodecanoic) acid.

The final form of cytochrome P-450 utilised was also purified within our laboratory and was the major isoenzyme induced following induction by \( \beta \)-napthoflavone. This form (cytochrome P-447) has a very high ethoxyresorufin O-deethylase activity and also corresponds to the major cytochrome P-450 induced in rat liver following 3-methylcholanthrene administration, sometimes termed cytochrome P-450c.

This chapter describes the purification of the two phenobarbitone-induced cytochrome P-450 isoenzymes and the associated flavoprotein NADPH cytochrome-c \((P-450)\) reductase. These purified cytochrome P-450 isoenzymes were subsequently characterised by a variety of criteria being compared and contrasted to the two other cytochromes described above. In addition a number of parameters relating to the induction of these specific isoenzymes following single dose induction have been investigated in both hepatic and renal microsomes.

RESULTS

4.2 INDUCTION OF MICROSOMAL CYTOCHROMES P-450 BY PHENOBARBITONE, CLOFIBRATE AND \( \beta \)-NAPTHOFLAVONE

The induction of microsomal cytochrome P-450 isoenzymes was monitored by a variety of criteria, one of which was their electrophoretic separation on SDS P.A.G.E. An example of this is illustrated in Fig. 4.1 which shows the electrophoretic separation of microsomal proteins derived from the hepatic microsomes of uninduced (control), phenobarbitone, clofibrate and \( \beta \)-napthoflavone - pretreated rats. The primary region of interest is in the 40,000 to 60,000 molecular weight range where many of the bands observed are believed to represent cytochrome P-450 isoenzymes. The most important points to note however is that within this region not only are there significant differences observed between the different microsomal fractions but also in the relative staining intensities of the electrophoretic bands. These observations suggest that pretreatment of rats with
Microsomal proteins were applied in the amounts indicated within the
brackets. In this gel 10 pmol of cytochromes P-450 B₁, P-450 B₂, P-447 and 20
pmol of cytochrome P-452 was added to the relevant tracks. Tracks 1, 10 contained
0.2 μg of each of the standard proteins listed in section 2.8.2. Migration was from
top to bottom. The nature of the inducing agents were:

<table>
<thead>
<tr>
<th>Track</th>
<th>Protein Type</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Control Microsomes</td>
<td>(20 μg)</td>
</tr>
<tr>
<td>3</td>
<td>Cytochrome P-450 B₁</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Phenobarbitone-induced microsomes</td>
<td>(12 μg)</td>
</tr>
<tr>
<td>5</td>
<td>Cytochrome P-450 B₂</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Clofibrate-induced microsomes</td>
<td>(10 μg)</td>
</tr>
<tr>
<td>7</td>
<td>Cytochrome P-452</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>β-naphthoflavone-induced microsomes</td>
<td>(12 μg)</td>
</tr>
<tr>
<td>9</td>
<td>Cytochrome P-447 (partially purified)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.2

**SDS PAGE of Phenobarbitone-Induced Microsomes Following Different Dosing Regimens**

![SDS PAGE image]

**Tracks**

- **Track 1**: 0.2 µg of each of the standard proteins.
- **Tracks 2, 7**: 12.5 and 25 µg of microsomal protein. Animals were dosed orally with 0.1% (w/v) phenobarbitone in their drinking water for 4 days, on the 5th day given an i.p. dose of 40 mg/kg and killed on day 6.
- **Tracks 3, 8**: 12.5 and 25 µg of microsomal protein following oral dosing with 0.1% (w/v) phenobarbitone in their drinking water for two weeks.
- **Tracks 4, 9**: 12.5 and 25 µg of microsomal protein following oral dosing with 0.1% (w/v) phenobarbitone in their drinking water for 4 days.
- **Tracks 5, 10**: 12.5 and 25 µg of microsomal protein following an i.p. dosage of 75 mg/kg for 4 days. Killed on day 5.
- **Tracks 6**: 5 pmol each of cytochrome P-450 B1 and P-450 B2.
these compounds results in alterations in the balance of cytochrome P-450 isoenzymes in the endoplasmic reticulum of rat liver. These changes in the hepatic microsomal protein content are also influenced by the mode of induction as shown in Fig. 4.2. This illustrates the electrophoretic separation of phenobarbitone-induced microsomes following four different dosing regimens. The differences observed are shown to relate not to the fact that different proteins may be induced following different routes of dosing by the same xenobiotic, but to the relative degree of induction of specific isoenzymes. For this reason it is important to maintain a standardised dosing regime as well as using purified cytochrome P-450 hemoproteins in order to characterise microsomal induction trends by different xenobiotics.

4.3 PURIFICATION OF MICROsomAL Enzymes

4.3.1 Purification of Hepatic Microsomal Cytochrome P-450 from Phenobarbitone-induced Rats

Cytochromes P-450 were purified from male Wistar rats by a modification of the method of Guengerich and Martin (1980). The purification protocol given in materials and methods is represented schematically in figure 4.3, and a typical purification result is given in table 4.1.

The recoveries at each step were similar to those reported by other authors up to the DEAE sephacel chromatographic stage, after which smaller yields were seen due to subsequent subfractionations. In the purification reported (table 4.1), the specific contents expressed as nmoles cytochrome P-450 per milligram of protein did not always appear to increase as would be expected, for a number of reasons, including the fact that during the course of purification, cytochrome P-450 undergoes heme loss to some extent, generating spectrally detectable apoprotein and decreasing the apparent purity. Also the presence of buffer components such as Emulgen 911 and glycerol in samples assayed for protein content, interfered with the Lowry protein determinations, often making them unreliable.
<table>
<thead>
<tr>
<th>Purification Stage</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total Protein (mg)</th>
<th>P-450 (nmol/ml)</th>
<th>Total P-450 (nmoles)</th>
<th>Specific Content (nmol/mg)</th>
<th>Yield (%)</th>
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<td></td>
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</tr>
<tr>
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<td>246</td>
<td>0.59</td>
<td>494</td>
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<tr>
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<td>0.59</td>
<td>41.6</td>
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<tr>
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<td>0.75</td>
<td>113</td>
<td>6.82</td>
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</tr>
<tr>
<td>B₁</td>
<td>688</td>
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<td>32.3</td>
<td>1417</td>
<td>286.9</td>
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<tr>
<td>CM-sephadex C₅₀</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₁</td>
<td>33</td>
<td>0.72</td>
<td>23.7</td>
<td>6.85</td>
<td>225</td>
<td>9.55</td>
<td>2.32</td>
</tr>
<tr>
<td>DEAE sephacel</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>B₂</td>
<td>78</td>
<td>0.34</td>
<td>26.8</td>
<td>3.41</td>
<td>265</td>
<td>9.91</td>
<td>2.75</td>
</tr>
<tr>
<td>CM-sephadex C₅₀</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₂</td>
<td>21</td>
<td>0.75</td>
<td>15.7</td>
<td>9.01</td>
<td>189</td>
<td>12.1</td>
<td>1.96</td>
</tr>
</tbody>
</table>
In more recent purifications the purity of eluting fractions was subsequently assessed with the use of SDS P.A.G.E. Here all increases in purity are clearly observed at each stage of the purification.

Solubilisation of microsomal protein was achieved with a final concentration of 1.4% (w/v) sodium cholate in the presence of 10 mg/ml protein, followed by dilution to 0.7% (w/v) cholate. This cholate concentration gave maximal recovery of solubilised microsomal protein (routinely between 80-90%) with minimal conversion of cytochrome P-450 to P-420 as judged by pilot solubilisation studies utilising final cholate concentrations of between 0.5 to 2.0% (w/v). The inclusion of the protease inhibitor PMSF (0.4mM) (Guengerich and Martin, 1980) at this stage had no apparent effect upon the recoveries at any purification stage.

Figure 4.3
Flow Chart Summarising the Purification Protocol Adopted for the Fractionation of Chromatographically Distinct Phenobarbitone-induced Cytochrome P-450 Pools
The elution profile of cytochrome P-450 from 8-aminooctyl sepharose 4B is shown in figure 4.4. Recoveries from this column were dependent upon the column batch and age. New columns would give recoveries of approximately 45% which would decrease to between 20-30% with subsequent usage. The cytochrome P-450 pool was free of NADPH-cytochrome P-450 reductase and cytochrome b$_5$ (which were subsequently eluted) but contained NADH-cytochrome b$_5$ reductase activity. The major fractions containing cytochrome P-450 were pooled and chromatographed on DEAE-sephacel according to the procedures outlined in section 2.4.3. No significant degradation of cytochrome P-450 was observed on this column or any others throughout the purification. NaCl was used to form the linear gradient. The elution of cytochrome P-450 from DEAE-sephacel is shown in Figure 4.5A, together with the corresponding SDS P.A.G.E. analysis of the eluted fraction (Figure 4.5B). The total yield of cytochrome P-450 recovered was routinely 50-60% for this step. Of this between 15-20% would represent the cytochrome P-450 B$_1$ form and 40-45% the cytochrome P-450 B$_2$ form. However of these total pools only a small proportion could be further purified because of contamination.

The cytochrome P-450 B$_1$ pool contained predominant protein contamination of apparent subunits molecular weights of 69,000, 54,000, 50,000 and 34,000. The proportion of contaminants was dependent upon the point of elution, with the most homogeneous fractions being found in the tail region of the elution profile. Here the cytochrome P-450 B$_1$ form was the major protein present with none of the contamination being present in amounts greater than 10%. Those fractions considered most homogeneous as judged by electrophoretic analysis were further purified on hydroxylapatite in order to remove these contaminants. The elution of the electrophoretically homogeneous cytochrome P-450 B$_1$ as judged by SDS P.A.G.E. analysis is shown in figure 4.6. A double peaked elution profile was obtained with the homogeneous protein being seen within the first peak. The initial fractions contain contaminating protein of apparent molecular weight 69,000 which gradually disappears. Towards the end of the peak a further contaminant appears of
Figure 4.4 Elution of Phenobarbitone-induced Cytochrome P-450 and NADPH-Cytochrome P-450

Reductase from 8-aminooctyl Separose 4B
apparent molecular weight 45,000 which runs throughout the second peak fractions. The cytochrome P-450 B\textsubscript{1} protein of the second peak was otherwise homogeneous. Those fractions considered homogeneous from the first peak were pooled and then chromatographed on CM-sephadex-C50 in order to remove the detergent. The specific content of the cytochrome P-450 B\textsubscript{1} isoenzyme ranged from 9 to 12 nmol/mg.

On analysis of the fractions obtained following elution of the cytochrome P-450 B\textsubscript{2} peak from DEAE sephacel (Figure 4.5A) three distinct pools became evident. The initial fractions were usually the least pure containing 3 major bands of apparent molecular weight 52,000, 53,000 and 54,000. Further minor contamination was seen with protein bands corresponding to apparent molecular weights of 69,000, 40,000 and 34,000. The contamination seen with these minor forms was fairly uniform throughout the whole of the elution profile (see figure 4.5B). At the peak of the elution profile the major cytochrome P-450 species was double-banded with these forms corresponding to the major cytochrome P-450 B\textsubscript{1} and P-450 B\textsubscript{2} isoenzymic forms. As for the cytochrome P-450 B\textsubscript{1} proteins, the most homogeneous fractions were seen in the tail region of the elution curve with the degree of minor contamination here also being at its lowest. The very last fractions contained the cytochrome P-450 B\textsubscript{2} isoenzyme in a relatively pure state, but with an underlying protein of apparent molecular weight of 52,000 which could not be separated chromatographically. At this stage the most homogeneous fractions subsequently had to be further purified on DEAE-sephacel which normally ensured that homogeneous cytochrome P-450 B\textsubscript{2} was obtained. This was achieved by utilising a potassium phosphate buffer stepwise gradient in which the ionic strength was increased in 5mM increments. Homogeneous cytochrome P-450 B\textsubscript{2} was obtained following elution with 20mM potassium phosphate buffer. Subsequent elution with increased ionic strength buffers gave rise to a double banded cytochrome P-450 isoenzyme fraction. This double banded fraction could be further purified on a hydroxylapatite column in order to remove trace higher molecular weight
Chromatography of the Phenobarbital - Treated Rat Liver 8-aminooctyl

Sepharose 4B Fractions on DEAE - Sephadex.
The following volumes of protein were loaded per track

Fractions:
- 50 - 56 = 12 µl
- 59 - 66 = 10 µl
- 92 - 101 = 8 µl
The following volumes of protein were loaded per track:

<table>
<thead>
<tr>
<th>Fractions</th>
<th>105 - 110</th>
<th>120 - 129</th>
<th>132 - 141</th>
<th>144</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 µl</td>
<td>30 µl</td>
<td>35 µl</td>
<td>45 µl</td>
</tr>
</tbody>
</table>

The fractions were pooled as follows for the further purification of the cytochrome P-450 B\textsubscript{1} and P-450 B\textsubscript{2} isoenzymes.

\textbf{B}_{1} : fractions 62 - 70  
\textbf{B}_{2} : fractions 101 - 120
Figure 4.6 Elution and SDS-PAGE analysis of Cytochrome P-450 B$_1$ from Hydroxylapatite

Aliquots of 34 µl were applied to the respective tracks. Fractions 130-160 were pooled for chromatography on CM-sephadex-C50.
contamination, but it was not found possible to separate the two isoenzymic forms. The final stage was that of removal of detergent on CM-sephadex-C50. A SDS P.A.G.E. analysis and elution profile of the 20mM eluted cytochrome P-450 B₂ fraction is shown in figure 4.7.

The specific content of the cytochrome P-450 B₂ pool ranged from 12-15 nmol/mg. These values were found to be slightly higher for both cytochrome P-450 B₁ and B₂ isoenzymes when the heme content was determined using the pyridine hemochrome assay. This difference was believed to be due to the instability of this enzyme in the presence of sodium dithionite, which was used as the chemical reductant in the former assay system. The final preparations described above were both shown to be free of Emulgen 911. The ferric, ferrous and ferrous - carbon monoxide absolute spectra of these purified cytochrome P-450 isoenzymes are shown in figure 4.12A,B (section 4.4.4.).

Fraction A isolated from the initial DEAE sephacel column, containing all the unadsorbed proteins from this column was subjected to electrophoretic analysis and subsequently discarded. This was primarily because the fraction did not contain the major cytochrome P-450 forms of interest and because of its low specific content.

The final fraction isolated from the DEAE column-designated fraction B₃ -was shown to consist of a single seemingly homogeneous protein of apparent molecular weight 15,500. This information coupled with its ferrous-carbon monoxide spectra indicates that it is probably cytochrome b₅ which was carried over from the previous 8-aminooctyl sepharose 4B column.
Figure 4.7

Elution Profile and SDS P.A.G.E. Analysis of Cytochrome P-450 \( \beta_2 \) from DEAE-Sephacel following Step-wise Gradient Elution

35\( \mu \)l aliquots were applied to the respective tracks.
4.3.2 Purification of Rat Hepatic NADPH-Cytochrome P-450 Reductase

NADPH-cytochrome P-450 reductase (Fpt) was purified according to the methodology of Yasukochi and Masters (1976) as described in section 2.4.5. Fpt was also purified by a modification of this method in which it was eluted as a by-product from the 8-aminoethyl sepharose 4B column in the course of a cytochrome P-450 purification. Subsequent steps were as described. Both forms of cytochrome P-450, $B_1$ and $B_2$, could be isolated in a partially purified state following elution of the
solubilised microsomes from DEAE-sephacel. An elution profile and the corresponding analysis of the fractions obtained throughout the Fpt purification is illustrated in figures 4.9A and B. The cytochrome P-450 pools were not utilised further however, due to the presence of large amounts of cytochrome P-420. The partially purified Fpt was then further purified on 2', 5', -ADP-sepharose 4B, utilising the property of 2'-AMP being a potent competitive inhibitor of NADPH-cytochrome c reductase activity. At this stage the Fpt would normally be present in a homogeneous state although occasionally trace of a lower molecular weight fragment was discernable. This was subsequently removed on the final column of hydroxylapatite which was used to remove the detergent present and to concentrate the protein.

Figure 4.9A

Elution of Cytochromes P-450 and Fpt from DEAE-Sephacel
The volumes of each fraction loaded per track are indicated within brackets.

<table>
<thead>
<tr>
<th>Tracks</th>
<th>1, 8</th>
<th>Standard molecular weight proteins.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Fraction 50</td>
<td>(20 μl)</td>
</tr>
<tr>
<td>3</td>
<td>Fraction 75</td>
<td>(20 μl)</td>
</tr>
<tr>
<td>4</td>
<td>Fraction 115</td>
<td>(15 μl)</td>
</tr>
<tr>
<td>5</td>
<td>Fraction 130</td>
<td>(20 μl)</td>
</tr>
<tr>
<td>6</td>
<td>Pooled Fpt from DEAE-sephacel</td>
<td>(20 μl)</td>
</tr>
<tr>
<td>7</td>
<td>Fpt following chromatography on 2',5'-ADP agarose</td>
<td></td>
</tr>
</tbody>
</table>

A purification table for a large scale Fpt preparation involving 60 rats orally dosed with 0.1% (w/v) phenobarbitone in drinking water for 6 days is shown in table 4.2. The final preparations yielded an electrophoretically homogeneous monomeric protein of molecular weight 77,000 as judged by SDS P.A.G.E. analysis on 9% acrylamide gels. All preparations were free of Emulgen 911, as determined by a colourimetric method.
Table 4.2

Purification of NADPH-Cytochrome P-450 Reductase from Hepatic Microsomes of Phenobarbitone-Induced Rats.

<table>
<thead>
<tr>
<th>Purification Stage</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total (mg)</th>
<th>Pkt Activity (units/ml)</th>
<th>Total Units</th>
<th>Specific Content Units (mg/ml)</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>355</td>
<td>36.47</td>
<td>12,947</td>
<td>8.2</td>
<td>2,911</td>
<td>0.22</td>
<td>100</td>
</tr>
<tr>
<td>Solubilised Microsomes</td>
<td>900</td>
<td>14.35</td>
<td>12,915</td>
<td>3.11</td>
<td>2,806</td>
<td>0.22</td>
<td>96</td>
</tr>
<tr>
<td>DEAE-SEPHACEL</td>
<td>265</td>
<td>3.24</td>
<td>861</td>
<td>5.56</td>
<td>1,473</td>
<td>1.71</td>
<td>51</td>
</tr>
<tr>
<td>2',5'-ADP Agarose</td>
<td>40</td>
<td>0.56</td>
<td>22.5</td>
<td>14.5</td>
<td>580</td>
<td>25.7</td>
<td>20</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>16</td>
<td>1.27</td>
<td>20.3</td>
<td>34</td>
<td>550</td>
<td>27.1</td>
<td>19</td>
</tr>
</tbody>
</table>
4.4  CHARACTERISATION OF PURIFIED CYTOCHROME P-450 ISOENZYMES

4.4.1 Substrate Turnover Studies

With the development of procedures for the purification of homogeneous forms of cytochrome P-450, reconstituted enzyme systems have offered the possibility of greater detailed studies of the differential substrate specificities of cytochrome P-450 multiple forms. Benzphetamine, ethoxyresorufin, and lauric acid were utilised as substrates, being widely accepted as suitable marker substrates for cytochrome P-450, P-447 and P-452 respectively. The corresponding turnovers for these cytochromes P-450 are shown in table 4.3. The results revealed clear differences in substrate specificities between the isoenzymes. For example, the cytochrome P-450 B^1 and P-450 B^2 forms exhibited exceptionally high rates of benzphetamine N-demethylase activity, whereas only limited activity was seen for the metabolism of lauric acid and ethoxyresorufin.

Cytochrome P-452 displayed a marked substrate specificity for lauric acid. HPLC analysis of the 11- and 12- hydroxylauric acid metabolites indicates preferential formation of 12- hydroxylaurate metabolites by cytochrome P-452 (Tamburini et al., 1984). In contrast cytochrome P-452 exhibited only trace turnovers with both benzphetamine and ethoxyresorufin as substrates.

In contrast, cytochrome P-447, exhibited a distinct substrate specificity for ethoxyresorufin deethylation, an activity that was not shown by any of the other isoenzymes to any significant extent.

In addition to the substrates listed in table 4.3, cytochrome P-450 B^2 was shown to be responsible for the metabolism of 1-napthol in both NADPH and cumene hydroperoxide dependent reconstitution reactions. (d'Arcy Doherty et al. 1984) yielding napthoquinones and covalently binding species. In the presence of NADPH the formation of 1,4-napthoquinone (the major metabolite) was observed within 30 seconds and was shown to have an almost total dependence on cytochrome P-450 and NADPH cytochrome P-450 reductase. In the absence of NADPH and NADPH-
<table>
<thead>
<tr>
<th>Form of Cytochrome P-450</th>
<th>Lauric acid Hydroxylase (11+12)</th>
<th>Benzphetamine N-Demethylase</th>
<th>Ethoxyresorufin Deethylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-450 B₁</td>
<td>5.0</td>
<td>265</td>
<td>0.08</td>
</tr>
<tr>
<td>P-450 B₂</td>
<td>5.0</td>
<td>215</td>
<td>0.35</td>
</tr>
<tr>
<td>P-452</td>
<td>35.5</td>
<td>27.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NDA&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>P-447</td>
<td>1.7</td>
<td>22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> all activities expressed as nmol product/nmol cytochrome/min. With the exception of cytochrome P-450 all other components of the reconstituted system were present in excess (Tamburini et al. 1984).

<sup>b</sup> These levels were at the minimum level of detection of the colourimetric method

<sup>c</sup> NDA: No detectable activity.
cytochrome P-450 reductase, 1-napthol was metabolised in a cumene hydroperoxide and cytochrome P-450 dependent reaction to 1,2- and 1,4-naphthoquinone and covalently bound products. Glutathione and EDTA inhibited both the NADPH and cumene hydroperoxide formation of covalently bound products.

4.4.2 Molecular Weight Studies

Subunit Mr values of the homogeneous cytochrome P-450 isoenzymes, P-450 B₁, P-450 B₂, P-452 and P-447 were estimated from calibrated SDS P.A.G.E., by plotting $\log_{10} \text{Mr}$ for the standard proteins versus the relative mobilities ($R_f$), as shown in figure 4.9A. Electrophoresis was carried out in gels of varying acrylamide concentrations from 7-12% in order to determine the degree of non-idealilty of SDS P.A.G.E. in these determinations.

According to the Fergusson equation (Fergusson 1964)

$$\log_{10} R_f = -K_r t + \log_{10} Y_0$$

where $K_r$ = retardation coefficient

t = acrylamide coefficient

$Y_0$ = relative mobility of the protein - SDS complex in solution

The relative mobilities of the protein standards (figure 4.9B) and cytochrome P-450 isoenzymes (figure 4.10) were plotted against acrylamide concentration. The close intersection of all such points on the y-axis clearly showed that the values of $Y_0$ for protein standards and cytochrome P-450 isoenzyme - SDS complexes were, within error the same, and therefore independent of protein charge and size. This in turn, implies a uniform high degree of SDS binding and a constant ratio of effective charge to friccional coefficient. An analysis of this type was essential since certain hydrophobic proteins such as cytochrome oxidase (Rubin and Tzagoloff 1973) and glycoproteins, bind abnormally high concentrations of detergent or are inherently highly charged, giving rise to atypical Fergusson plots.
The standards (0.3μg) used in order of increasing mobility were phosphorylase a, bovine serum albumin, catalase, glutamate dehydrogenase, chicken egg albumin, creatine phosphokinase, lactic dehydrogenase and cytochrome C (section 2.8.2.). Migrations represent the average of 4 determinations.
Figure 4.10

Ferguson Plot for Electrophoretically Homogeneous Cytochrome P-450 Isoenzymes

Electrophoretic Analysis (SDS P.A.G.E.)
of Purified Cytochrome P-450 Isoenzymes

Migrations represent the average of 4 determinations.

1 = cytochrome P-452
2 = cytochrome P-450 B1
3 = cytochrome P-450 B2
4 = cytochrome P-447 (partially purified)
The Mr values of the electrophoretically homogeneous isoenzymes analysed show distinct differences with the values being given in figure 4.10. These differences are further emphasised when the hemoproteins are viewed on SDS P.A.G.E. (figure 4.10).

4.4.3 Peptide Mapping Studies

Limited proteolysis of cytochromes P-450 followed by SDS P.A.G.E. of the resulting fragments has been used in other laboratories as a probe for differences in protein primary or possibly secondary structure (Koop et al. 1981). This technique was used in the present study for the comparative analysis of cytochromes P-450 B₂, P-452 and P-447. Limited digestion was carried out in the presence of either papain (Papaya latex), chymotrypsin (bovine pancrease) or S. aureus V8.

In preliminary studies, the dependence of the fragmentation patterns upon protease concentration and incubation time were evaluated. Protease was varied as follows: (µg in 50 µl volume); papain (0.005-0.5), chymotrypsin (0.05-5), S. aureus V8 (0.125-12.5), with the incubation time being varied from 5 to 90 minutes for each protease.

The conditions selected for presentation (figure 4.11) were such that some undigested apoprotein remained to serve as a point of reference on polyacrylamide gels. The resulting fragmentation patterns (figure 4.11) clearly showed that in the presence of each protease, under similar conditions, no common fragments arising from cytochromes P-450 B₂, P-452 and P-447 were observed. This strongly suggests that the amino acid sequences of these three isoenzymes are (fundamentally) different from each other.
Cytochrome P-450 isoenzymes were incubated with Staph. aureus V8 (1.25 μg for 5 minutes), chymotrypsin (0.5 μg for 30 minutes) and papain (0.05 μg for 5 minutes). The amounts of each digested isoenzyme applied per track were; cytochrome P-450 B₂ (2 μg), cytochrome P-447 (2 μg) and cytochrome P-452 (3.5 μg).
4.4.4 Spectral Characterisation

As a further means of characterisation of the two isolated phenobarbitone-induced cytochrome P-450 isoenzymes their absolute absorption spectra were compared (figures 4.12A,B). Prior to spectrophotometric determinations both preparations were checked to ensure freedom from Emulgen 911 whose presence is capable of causing spectral shift changes. Both oxidised isoenzymic forms showed typical b-type cytochrome spectra displaying \( \alpha, \beta \) and soret bands at around 570, 534 and 418 nm respectively. The general shape of the spectrum obtained for the oxidised hemoproteins is characteristic of low-spin hemoproteins. Although the differences between the two ferric isoenzymes remained subtle, there was a small difference in the 390/418 nm ratio between the two hemoproteins which could also reflect a dissimilarity between the spin states of their heme irons.

In the reduced forms both isoenzymes exhibit a soret peak at the same wavelength position. The major difference however was that cytochrome P-450 B\( _1 \) exhibited a far more intense absorbance within the \( \alpha \)-band region and also exhibited a peak at 468 nm which was not evident within the cytochrome P-450 B\( _2 \) spectrum.

This peak was also apparent for cytochrome P-450 B\( _1 \) when the reduced carbon monoxide difference spectra was monitored. Both exhibited intense soret peaks within the 450 nm region. For cytochrome P-450 B\( _1 \) this was located at 451.5 nm and for cytochrome P-450 B\( _2 \) at 450.5 nm.

The absolute absorption spectrum of fully oxidised NADPH-cytochrome P-450 reductase is given in figure 4.13. Peaks are displayed at 380 nm and 454 nm due to the absorbance of the FAD and FMN prosthetic groups. The final preparations were free of Emulgen 911 with preparations routinely giving \( A_{280}/451 \) ratios of between 9-10.0, a value which is an agreement with other authors observations (French and Coon, 1979).
The spectra portrayed are of the oxidized (**--**), sodium dithionite reduced (-----), sodium dithionite reduced in the presence of carbon monoxide (-x-x-x-x-). The cytochromes P-450 concentration was 0.75 μM.
Figure 4.13

UV/Visible Absorbance Spectra of Electrophoretically Homogeneous NADPH-Cytochrome P-450 Reductase
Metyrapone (2-methyl-1,2,di(3 pyridyl)-1- propanonone) is commonly used as an inhibitor of some cytochrome P-450 mediated oxidations (Jonen, H. et al. 1974), and its clinical use in the diagnosis of impaired pituitary function (ACTH release) is based on its ability to inhibit cytochrome P-450 mediated steroid hydroxylation in the adrenal gland. In this study however its use is related to its ability to provide a specific assay for the major phenobarbitone-inducible forms of cytochrome P-450.

Metyrapone reacts with the heme iron of ferrous cytochrome P-450, forming a spectral complex with a characteristic peak at 446 nm. Although this ligand is not absolutely specific for a single form of cytochrome P-450, it is capable of distinguishing between certain cytochrome P-450 isoenzymes. Of the major forms isolated to date, only cytochrome P-450\textsubscript{b} and P-450\textsubscript{e} have been shown to effectively bind to the ligand, whereas cytochromes P-450\textsubscript{a}, P-450\textsubscript{c} and P-450\textsubscript{d} exhibit no significant metyrapone spectral complex formation (Luu-The, V. et al. 1980). In fact the specificity for the phenobarbitone-induced forms of cytochrome P-450 is so great that it has been used as a means of being able to quantitate the levels of this cytochrome within rat liver microsomes (Mitani, F. et al. 1982).

Utilising the homogeneous isoenzymes isolated from phenobarbitone-induced rats -cytochromes P-450 B\textsubscript{1} and P-450 B\textsubscript{2} - the apparent spectral dissociation constants (K\textsubscript{s}) of the metyrapone-reduced cytochrome P-450 ligand complexes were determined (table 4.4). The data was analysed utilising a linear regression statistics program (University of Surrey Computing Unit) by both double reciprocal and Eadie-Hofstee plot analysis. Clear differences were seen to emerge with cytochrome P-450 B\textsubscript{1} having a far greater affinity for metyrapone than the cytochrome P-450 B\textsubscript{2} form. This K\textsubscript{s} value obtained was slightly lower than that obtained by other authors (Luu-The et al. 1980) but their determination was for the major phenobarbitone cytochrome P-450\textsubscript{b} form isolated from Sprague Dawley rats. A K\textsubscript{s} value was also obtained for phenobarbitone-induced microsomes (0.1% (w/v) Pb in drinking water, oral dosing for 6 days). Here, the derived K\textsubscript{s} constant was very similar to that derived by other authors (Mitani, F. et al. 1982, Leibman, K. et al. 1969).
Table 4.4

Apparent Dissociation Constants ($K_s$) for Metyrapone Binding to Purified Cytochrome P-450 and Phenobarbitone-induced Microsomes

<table>
<thead>
<tr>
<th>Cytochrome P-450 B$_1$ (a)</th>
<th>Cytochrome P-450 B$_2$ (b)</th>
<th>Phenobarbitone-induced microsomes (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_s$ ($\mu$M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.01 + .19</td>
<td>5.56 + .49</td>
<td>0.69 + .28</td>
</tr>
</tbody>
</table>

a - values represent the mean ± S.D of 9 determinations
b - values represent the mean ± S.D of 6 determinations
c - values represent the mean ± S.D of 12 determinations.

Because of the specificity of metyrapone for the phenobarbitone-induced cytochromes, its inhibitory influence upon their drug metabolising activity in a reconstituted system for both purified isoenzymic preparations and microsomal fractions was determined. The turnover of benzphetamine was monitored in the presence of different concentrations of metyrapone. The results (figure 4.14) clearly indicate the specificity of metyrapone inhibition, with a corresponding difference being seen in the extent of inhibition as one would have expected due to the differences in their $K_s$ constants.

The inhibition profile relating to benzphetamine N-demethylase activity with phenobarbitone-induced microsomes was also examined (table 4.5). A temporal change became evident with the relative degree of inhibition rapidly increasing after 12 hours induction. This effect is seen to be both dose and time dependent and as such undoubtedly reflects the inhibition of the concomitantly induced cytochrome P-450 species being induced with time.
Inhibition of Purified Cytochrome P-450 B\textsubscript{1} and P-450 B\textsubscript{2} Mediated Benzphetamine N-demethylation Activity by Metyrapone

Values represent the mean ± S.D of 4 determinations. For cytochrome P-450 B\textsubscript{1} and P-450 B\textsubscript{2} the corresponding 100% activity values equal 275 and 223 nmol HCHO/nmol P-450/min respectively.

<table>
<thead>
<tr>
<th>Metyrapone Concentration (μM)</th>
<th>Control</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.7</td>
<td>13.7</td>
<td>17.3</td>
<td>36.6</td>
<td>30.8</td>
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</tr>
<tr>
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<td>80</td>
<td>77.4</td>
<td>2000</td>
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</tbody>
</table>

Values represent the mean ± S.D of 4 determinations.
4.5 PURIFICATION AND CHARACTERISATION OF ANTIBODIES

The gross multiplicity of the M.F.O. system has resulted in there being a noticeable absence of specific assays for each form of cytochrome P-450, which consequently has hindered the elucidation of the regulation of these different forms of cytochrome P-450. To overcome this researchers have turned towards antibodies because being highly specific they are uniquely suited as reagents capable of identifying and discriminating between functionally related proteins such as the multiple forms of cytochrome P-450.

The procedures utilised in the isolation and purification of the specific antibody fractions is described in sections 2.9 to 2.10. If the antibody was required in a purified immunoglobulin state then salt fractionation of the sera followed by ion exchange chromatography was shown to suffice. Using different batches or types of ion exchange resins did not influence the purity of the IgG fraction. Separation of the isolated IgG into its corresponding sub-fractions was not carried out. On subjecting the IgG to SDS P.A.G.E. analysis two bands became evident (data not shown). In calibrated gels these bands were shown to possess molecular weights of 50,000 and 25,000 which correspond to the heavy and light chains of γ globulins respectively.

4.5.1 Antibody Specificity

The most important parameter relating to any antibody is its relative degree of specificity towards its corresponding antigen. This was routinely determined utilising the technique of double diffusion within an agarose medium as described by Ouchterlony (1967). The antibodies isolated (against purified cytochromes P-450 B₂, P-452 and P-447) all gave visible precipitin bands against their corresponding antigens by such analysis. No differences in immunoprecipitation were observed when either the corresponding sera or IgG fraction was used.
Figure 4.15 shows an analysis in which pre-immune IgG and anti-P-450 B<sub>2</sub> IgG were directed against purified cytochrome P-450 B<sub>2</sub>.

![Ouchterlony Double Diffusion Analysis of Purified Cytochrome P-450 B<sub>2</sub> Against its Corresponding Antibody and Pre-immune IgG](image)

The centre well in A contained 30 μg of pre-immune IgG and in B contained 25 μg anti-P-450 B<sub>2</sub> IgG. The outside wells in a clockwise order from position 1 contained 6.7, 6.7, 13.4, 13.4, 20, 20 pmoles of purified cytochrome P-450 B<sub>2</sub> protein.

As can be seen only a single band is obtained on reaction with its homologous antigen. In the control experiment no precipitation reaction was observed and this was the case with all cytochrome P-450 isoenzymes analysed. To ensure the possibility that any possible lack of reactivity in the Ouchterlony double diffusion test being due to poor antigen diffusibility, the tests were repeated both in the presence and absence of Emulgen 911 within the diffusion media. No relative
immunoprecipitation differences were observed in either of the two systems, although the media with no detergent had a tendency to exhibit slightly less intense immunoprecipitation bands.

An interesting observation (figure 4.16) was that both phenobarbitone-induced cytochromes P-450 B₁ and P-450 B₂ appear to share common antigenic determinants in that both hemoproteins were precipitated by anti-cytochrome P-450 B₂ IgG.

**Figure 4.16**

*Ouchterlony Double Diffusion Analysis of Highly Purified Cytochromes P-450 B₁ and P-450 B₂*

Top centre wells contain cytochrome P-450 B₁ and bottom centre wells cytochrome P-450 B₂ at a concentration of 33 pmoles. Increasing amounts of anti-P-450 B₂ antibody were added in anti-clockwise directions from well position 1. The same antibody additions were carried out in both top and bottom wells.
Single immunoprecipitation bands were obtained in both cases and as such would indicate that the two forms are at least immunologically similar if not structurally similar. Such cross-reactivity was not seen with any of the other antibodies (figure 4.17). Here all the antibodies showed immunochemical identity only with their homologous antigens. No cross-reactivity was obtained between these purified antibodies and their heterologous antigens. This would indicate that for these major isoenzymic forms major structural dissimilarities exist at least within the framework of antigen-antibody recognition binding sites. Further conclusions relating to the immunochemical relationships between these forms is not really possible because of the relative inflexibility of the test system itself.

**Figure 4.17** Ouchterlony Double Diffusion Analysis of Highly Purified Cytochromes P-447, P-450 and P-452

Centre wells A, B, C, D contain IgG fractions corresponding to anti-P-450 B₂ (9 µg), anti-P-447 (9 µg) and anti-P-452 (20 µg), and a mixture of the last 3 IgG fractions (equivalent amounts of protein) respectively. All the outer wells numbered 1-6 contain highly purified cyt. P-450 isoenzymes as follows; Well 1, cyt. P-450 B₂ (60 pmol); Well 2, cyt. P-452 (32 pmol); Well 3, cyt. P-447 (30 pmol); Well 4, cyt. P-450 B₂ (30 pmol); Well 5, cyt. P-452 (64 pmol), and Well 6, cyt. P-447 (60 pmol).
4.5.2 Western Blot Analysis of Induced Hepatic Microsomal Fractions

The technique of Western blotting was utilised as a means of immunochemical detection in order to identify the presence of induced cytochromes P-450 in hepatic microsomes with time. Because the secondary antibody was not radiolabelled, quantitation of the degree of specific induction was unfortunately not possible. However the results obtained were sufficiently visually comprehensive to indicate where induction had occurred. The specificity of the antibodies in question was also shown with no cross reaction being apparent within the complement of microsomal proteins. Transfer of microsomal proteins from the SDS polyacrylamide gel to its facing nitrocellulose sheet by electrodiffusion was absolute as shown by the following photograph.

Proteins visualised on original, dried, SDS P.A.G.E. gel.

Corresponding proteins transferred to nitrocellulose.

1 = standard microsomal protein markers
2,3 = phenobarbitone-induced microsomes (25, 50 µg, respectively)
4 = purified cytochrome P-450 B₂ (15 pmol)
Following transfer to nitrocellulose, staining of the original SDS P.A.G.E. gel did not detect the presence of any proteins (results not shown). The actual amounts of protein indicated in the photograph were found to be far in excess of that required, due to the sensitivity of this immunochemical detection system. Routinely microsomal protein would be electrophoresed at concentrations of 1-5 µg, and purified cytochromes P-450 at 2-4 pmol. Excess loading just resulted in a far greater degree of non-specific background binding and to a general distortion of the immunoprecipitated bands.

The induction of the phenobarbitone-induced cytochrome P-450s in microsomes was clearly time dependent (figure 4.18). As the antibody raised against the cytochrome P-450 B2 form was also known to recognise the cytochrome P-450 B1 form, it is interesting to note that in the control, 6 and 12 hours time periods following phenobarbitone-induction only the cytochrome P-450 B2 protein was observed. Following this it appears that two proteins which correspond to the cytochrome P-450 B1 and B2 forms were only then recognised. This was particularly apparent when the fractions were reanalysed utilising greater amounts of proteins. Some subsequent background is seen but this is believed to be non-specific background binding. It has been suggested that this could be a function of the age of the quenching pre-immune donkey sera utilised in the washing stages, especially since such binding is seen in purified isoenzyme fractions which are known to be electrophoretically homogeneous. This time difference between the two different isoenzymes could therefore, tentatively, be taken to relate to a different induction control over the two forms by phenobarbitone.
Western Blot Analysis of Phenobarbitone-induced Hepatic Microsomes Reacted Against Anti-Cytochrome P-450 B\textsubscript{2} Antibody

A.

In gel A microsomal protein was 1.5 µg/track; cytochrome P-450 B\textsubscript{1} concentration was 0.58 pmoles/track.

In gel B microsomal protein was increased to 10 µg/track and the amount of purified cytochrome P-450 increased to 3 pmoles/track.

Track 1 = Control Microsomes; Tracks 2,3,4,5 = 6,12,24,72 hour phenobarbitone-induced hepatic microsomes, Track 6 = cytochrome P-450 B\textsubscript{1}, Track 7 = cytochrome P-450 B\textsubscript{2}.

Concentration of primary antibody = 80 µg/30 ml wash

Concentration of secondary antibody = 30 µg/30 ml wash
In a similar experiment BNF and clofibrate-induced microsomes were subjected to screening by the anti-cytochrome P-450 B₂ IgG. In both sets a protein was recognised - the intensity of which slightly increased with time - which comigrated with the marker cytochrome P-450 B₁ isoenzyme. The conclusion reached was that this represented either the steady state level of this protein or possibly a slight increase in its induction.

When clofibrate-induced hepatic microsomes were analysed with anti-cytochrome P-452 antibody a single band was visualised corresponding to authentic cytochrome P-452 (figure 4.19A). Maximal amounts were seen within control, 6 and 12 hour fractions following induction after which an apparent steady state level was observed. However, as pointed out before, this is only a qualitative assessment based on visual inspection and clearly quantitative methods need to be developed to substantiate this tentative conclusion. Renal microsomes were also analysed and were qualitatively shown to contain the cytochrome P-452 isoenzyme. The presence of cytochrome P-452 was also detected within phenobarbitone and BNF-induced microsomes at all time points, albeit at far lower levels within the BNF-induced microsomes (bands very faint, data not shown).

BNF-induced microsomes analysed with anti-cytochrome P-447 antibody show an increase in protein corresponding to homogeneous marker cytochrome P-447 with time (figure 4.22B). Maximal amounts of cytochrome P-447 appeared to be present at 24-72 hours following dosing.
Western Blot Analysis of (A) Clofibrate Induced Microsomes Reacted Against Anti-Cytochrome P-452 Antibody and (B) BNF Induced Microsomes Reacted Against Anti-Cytochrome P-447 Antibody.

A.

Track 1 = standard molecular weight markers, Tracks 2,3,4,5,6 = control, 6,12,24,72 hour clofibrate-induced hepatic microsomes (1 µg/track), Track 7 = purified cytochrome P-452 (2 pmoles), Tracks 8,9,10,11,12 = control, 6,12,24,72 hour clofibrate-induced renal microsomes (15 µg/track).

B.

Tracks 1,2,3,4,5 = control, 6,12,24,72 hour BNF-induced hepatic microsomes (1.5 µg protein), Track 6 = purified cytochrome P-447 (1 pmoles).

Gel A: primary antibody concentration = 100 µg/30 ml wash
Gel B: primary antibody concentration = 80 µg/30 ml wash
Secondary antibody concentration for both gels = 30 µg/30 ml wash.
4.5.3 Antibody Inhibition of Cytochrome P-450 Activity

In reconstitution experiments, both forms of phenobarbitone-induced cytochromes P-450 B₁ and P-450 B₂ were previously shown to support high benzphetamine N-demethylase activity. Subsequent immunological investigations showed that both these forms were immunologically identical when reacted against the anti-cytochrome P-450 B₂ antibody, and therefore the inhibitory effects of this antibody on catalytic activity was investigated. No significant inhibition was observed with pre-immune IgG. However, differences were observed between the 2 isoenzymic forms, with the cytochrome P-450 B₂ form being inhibited at a greater initial rate than the cytochrome P-450 B₁ form (figure 4.20). In liver microsomes (phenobarbitone-induced, 12 hours), inhibition of benzphetamine N-demethylase activity was also evident, but was more refractory as compared to the purified isoenzymes (figure 4.21). This indicated that although cytochromes P-450 B₁ and P-450 B₂ are responsible for a substantial proportion of the activity in microsomes, other isoenzymic forms may also be involved in the N-demethylation. Alternatively these results may be interpreted as a result of incomplete antibody access to the membrane bound forms of cytochrome P-450 B₁ and P-450 B₂. At present, the results do not differentiate between these two possibilities.

When the metabolism of lauric acid by clofibrate-induced microsomes was investigated, it was found that when anti-cytochrome P-452 sera was used, an increase in activity rather than inhibition became apparent (data not shown). The reason for this discrepancy is not clear especially since the specificity of the anti-P-452 antibody has been shown to be absolute. When the experiment was repeated using an IgG fraction raised against cytochrome P-452 (Dr S. Bains personal communication) inhibition was attained. On separation of the metabolites on HPLC, the formation of both the 11- and 12-hydroxy lauric acid metabolites were shown to be inhibited.
Effect of Varying Amounts of Anti-Cytochrome P-450 B₂ Antibody on Benzphetamine N-demethylase Activity Catalysed by Purified Cytochromes P-450 B₁ and P-450 B₂

Values represent the mean ± S.D of 4 determinations. For cytochromes P-450 B₁ and P-450 B₂, the corresponding 100% activity values equal 265 and 212 nmol HCHO/nmol P-450/min respectively.

Effect of Varying Amounts of Anti-cytochrome P-450 B₂ Antibody on Benzphetamine N-demethylase Activity Catalysed by 12 Hour Phenobarbitone-induced Microsomes

Values represent the mean ± S.D of 4 determinations. For the phenobarbitone-induced microsomes (solubilised) 100% control activity equalled 15 nmol/HCHO/nmol P-450/min.
The 12-hydroxylation was shown to be preferentially inhibited to approximately 25% of the original activity, whereas 11-hydroxylation was less sensitive to anti-cytochrome P-452 IgG with the catalytic activity being inhibited to only 65% of the original activity.

The specificity of the anti-cytochrome P-447 antibody was also demonstrated by the inhibition of ethoxyresorufin turnover by the purified cytochrome P-447 isoenzyme (figure 4.22). When the specificity of the antibody against hepatic BNF-induced microsomes (24 hours induction) was determined, the level of inhibition was not complete, indicating that, as was the case seen for the other microsomal fractions, either cross-substrate specificity was being demonstrated by some other isoenzymic cytochrome P-450 form or that antibody access to the cytochrome was limited in the membrane fractions.

4.6 CHARACTERISATION OF MICROSOMAL CYTOCHROME P-450 ISOENZYMES

The hepatic microsomal mixed function oxidase system (M.F.O.) is characterised by several features which makes it unique among mammalian enzymes. Primarily these include the induction of multiple cytochrome P-450 isoenzymes following chemical administration with many having widely different but broad substrate specificities. These differences are dependent not only upon the nature of the inducing agent and its dose, but also, very importantly, on the sex, age and species of the animal involved. Thus it is very important that following induction, one should be able to discriminate whether the levels of pre-existing M.F.O. enzymes are simply elevated (possibly just with minor modifications), or whether novel constitutive forms are evoked to deal with the corresponding chemical challenge.
100% uninhibited activity was equal to 1.0 nmol resorufin/nmol P-450/min. Values represent the mean ± S.D of three determinations.
4.6.1 Changes in the Induction Profile of Hepatic and Kidney Microsomes with Time

Animals were induced with a single dose of phenobarbitone, clofibrate or \( \beta \)-naphthoflavone as described in section 3.1.2. Hepatic microsomes were subsequently isolated (Section 2.3) at different time points in order to investigate the changes occurring within these membrane vesicles formed from the endoplasmic reticulum as a result of chemical administration. This was firstly looked at from the point of view of changes in the specific content of cytochrome P-450 in the microsomes as well as changes in the difference spectra of the CO complexes of the reduced cytochrome P-450. The results in table 4.6 indicate an increase in induction of cytochrome P-450 in all the pretreated animal groups with the absorbance change difference spectra also indicating discrete but subtle changes in the cytochrome P-450 subpopulations. This time course study was also analysed on SDS P.A.G.E. The overall pattern which emerged was that following induction, a general but uniform increase in microsomal proteins was visible with time. This coincided with a specific increase in the induced cytochrome P-450 variant being studied as monitored using the relevant purified cytochrome P-450 isoenzyme as a standard. However, because the analysis was strictly visual on SDS P.A.G.E. no quantitation as such is possible.

4.6.2 Time Course Induction Profiles of Drug Metabolising Activity in Hepatic and Renal Microsomes.

It is fortunate that the majority of cytochrome P-450 isoenzymes isolated to date have relatively specific marker substrates. This has made it possible to correlate the observed induction of drug oxidation activities with selective increases of microsomal cytochromes P-450 and thereby indicate the existence of multiple molecular species of cytochrome P-450. Benzphetamine and ethoxyresorufin are widely accepted marker substrates for cytochromes P-450 and P-447 respectively, and recently lauric acid has been shown to be a suitable marker substrate for cytochrome P-452 (Gibson et al. 1982). Accordingly the turnover rate in
Table 4.6

Induction of Total Cytochrome P-450 Following Administration of
Different Xenobiotics

<table>
<thead>
<tr>
<th>Inducing agent and time of microsomal isolation (hr)</th>
<th>Specific Content (nmol/mg Protein)</th>
<th>Relative Induction (fold)</th>
<th>Absorbance maximum of Fe(^{2+})-CO complex of cytochrome P-450</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td>6</td>
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<td>1.58</td>
<td>450.8</td>
</tr>
<tr>
<td>72</td>
<td>1.25</td>
<td>1.8</td>
<td>450.5</td>
</tr>
<tr>
<td>β-Napthoflavone</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>.845</td>
<td>1.21</td>
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<tr>
<td>Control</td>
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<td>Clofibrate</td>
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<tr>
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<td>.204</td>
<td>1.12</td>
<td>451</td>
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<tr>
<td>72</td>
<td>.267</td>
<td>1.46</td>
<td>449.5</td>
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</table>

Values represent the mean of between 4 to 6 determinations ± S.D from either 2 or 3 separate pools of microsomes of 3 animals.
microsomes for specific substrates was monitored with time following induction in order to assess the response of the M.F.O. system towards these xenobiotics.

A) Benzphetamine-N-demethylase activity

As expected with this marker substrate the highest activity observed was from the hepatic microsomes induced by phenobarbitone. This increase closely followed the increase in specific content of the microsomes. Conversely clofibrate-induced microsomes appear to effect some affinity for this substrates turnover, albeit at lower levels. In BNF-induced microsomes this activity is decreased.

In the renal microsomes a different pattern of induction becomes apparent. There is a large increase in benzphetamine N-demethylase activity for up to 12 hours following which it begins to decline. A similar profile is again apparent with respect to an increase in the microsomal protein levels. The activity of clofibrate and BNF-induced renal microsomes was similar to that seen in the hepatic microsomes. The results are summarised in figure 4.23A and B.

B) Lauric Acid hydroxylation Activity

The total hydroxylation products (11- and 12- hydroxy metabolites) of lauric acid following xenobiotic administration were studied by means of t.i.c. analysis. Subsequently, by the use of H.P.L.C. it was possible to determine the degree of both the 12- and 11- hydroxyl products. The different characteristics of the two liver laurate hydroxylase systems have already been observed (Ellin et al. 1975) and the proposition put forward that separate cytochromes P-450 catalyse the two fatty acid hydroxylation reactions (i.e. the 11- and 12-hydroxylation). Indeed the following results which were obtained from the time course induction profile of the 12- and 11-laurate hydroxylase activities within the differentially induced hepatic and renal microsomes strongly supported the hypothesis that separate cytochromes mediated the two hydroxylation reactions of this fatty acid. The overall values for the total laurate hydroxylation activities are detailed in table 4.7. In both
clofibrate and phenobarbitone-induced microsomes a time dependent response was observed, with the greatest increase being seen following clofibrate-induction. Here a maximal increase in activity was observed after a period of 12 hours after which a decline was observed. Following phenobarbitone-induction a slow but steady increase was observed with time. Treatment of rats with BNF had no stimulatory effect on total laurate hydroxylase activities when compared to control rats.

In the renal microsomes a different pattern of induction appears to emerge with far higher levels of activity seen throughout. Here clofibrate-induced microsomes again exhibited the highest turnover activity with the levels increasing with time. This was also seen with the phenobarbitone-induced microsomes albeit at lower levels. Slight increases in activity were also observed following BNF-induction.

However, the true extent of induction only became fully apparent when the proportion of 11- and 12-hydroxylaurate metabolites were analysed (figures 4.24 A,B,C). No metabolites were formed in the absence of NADPH.

In hepatic microsomes following clofibrate pretreatment 12-hydroxylation of lauric acid was preferentially increased with a maximal 2.5 fold increase at 12 hours. 11-hydroxylation was not affected to any great degree. After 12 hours a decrease in activity was observed. With the kidney microsomes a similar preference was observed with the difference that 11-hydroxylation also increased with time but at a much slower rate. The increase in 12-hydroxylation of lauric acid following a single dose was seen to continue for a period up to 72 hours attaining a maximum level after 24 hours.

Hepatic microsomes from phenobarbitone pretreated animals showed an initial depression of 12-hydroxy activity with a slight but preferential increase in 11-hydroxylauric acid formation. By 72 hrs comparable ratios of the 11-and 12-metabolites became evident. In contrast the renal microsomes catalysed a preferential increase in 12-hydroxylauric acid formation similar to that observed for
Figure 4.23A

Benzphetamine N-demethylase Activity Time Course Profile Following Pretreatment with Different Inducers for Hepatic Microsomes

PB = phenobarbitone-induced, CLO = clofibrate-induced, BNF = β-napthoflavone-induced. a = significantly greater than controls (p<0.001) b = (p<0.01) c = (p<0.05) Student's t-test. Each value represents the mean ± S.D of 4 determinations.
Benzphetamine N-demethylase Activity Time Course Profile Following Pretreatment with Different Inducers for Renal Microsomes

PB = phenobarbitone-induced, CLO = clofibrate-induced, BNF = β-napthoflavone-induced. a = significantly greater than controls (p<0.001), b = (p<0.01), c = (p<0.05) Student's t-test. Each value represents the mean ± S.D of 4 determinations.
Table 4.7
Effect of Clofibrate, Phenobarbitone and 3-napthoflavone on Total 11- and 12-hydroxylauric Acid Formation by Hepatic and Renal Microsomes

<table>
<thead>
<tr>
<th>Fraction</th>
<th>nmol product/mg protein/minute</th>
<th>nmol product/nmol P-450/minute</th>
<th>Percentage of Control</th>
<th>nmol product/mg protein/minute</th>
<th>nmol product/nmol P-450/minute</th>
<th>Percentage of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hepatic Microsomes</td>
<td></td>
<td>Renal Microsomes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.25 ± .2</td>
<td>1.23 ± .092</td>
<td>100</td>
<td>2.94 ± .91</td>
<td>11.08 ± .77</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>1.45 ± .30c</td>
<td>1.37 ± .07</td>
<td>111.3</td>
<td>2.29 ± .48</td>
<td>9.84 ± .15</td>
<td>88.8</td>
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<tr>
<td>Clofibrate 12</td>
<td>4.08 ± .53b</td>
<td>3.1 ± .36a</td>
<td>252</td>
<td>3.64 ± .23</td>
<td>13.62 ± .1</td>
<td>122.9</td>
</tr>
<tr>
<td>induced 24</td>
<td>3.14 ± .52b</td>
<td>2.51 ± .36a</td>
<td>204</td>
<td>3.96 ± .251a</td>
<td>27 ± .29a</td>
<td>243.6</td>
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<tr>
<td>(hours) 72</td>
<td>2.73 ± .22b</td>
<td>1.6 ± .33b</td>
<td>130</td>
<td>5.6 ± 1.17a</td>
<td>30.47 ± .26a</td>
<td>275</td>
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<tr>
<td>6</td>
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<td>90.2</td>
<td>2.74 ± .65</td>
<td>10.36 ± .115</td>
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<tr>
<td>Phenobarbitone 12</td>
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<td>1.36 ± .09</td>
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<td>2.44 ± .55</td>
<td>18.89 ± .28a</td>
<td>170.4</td>
</tr>
<tr>
<td>induced 24</td>
<td>3.46 ± .16b</td>
<td>1.35 ± .02</td>
<td>109.7</td>
<td>3.08 ± .52</td>
<td>21.34 ± .20a</td>
<td>192.5</td>
</tr>
<tr>
<td>(hours) 72</td>
<td>3.47 ± .69</td>
<td>2.06 ± .66</td>
<td>167.4</td>
<td>3.85 ± 1.18</td>
<td>25.75 ± .42a</td>
<td>232.4</td>
</tr>
<tr>
<td>6</td>
<td>1.3 ± .19</td>
<td>1.23 ± .37</td>
<td>100</td>
<td>3.06 ± .66</td>
<td>13.55 ± .16c</td>
<td>122.9</td>
</tr>
<tr>
<td>BNF 12</td>
<td>1.32 ± .08</td>
<td>1.2 ± .34</td>
<td>97.5</td>
<td>2.9 ± .20</td>
<td>14.74 ± .30b</td>
<td>133</td>
</tr>
<tr>
<td>induced 24</td>
<td>1.22 ± .43</td>
<td>1.2 ± .40</td>
<td>97.5</td>
<td>3.58 ± .66b</td>
<td>15.36 ± .94b</td>
<td>138.6</td>
</tr>
<tr>
<td>(hours) 72</td>
<td>1.09 ± .217</td>
<td>0.77 ± .07</td>
<td>62.8</td>
<td>3.89 ± .37</td>
<td>15.6 ± .50a</td>
<td>140.7</td>
</tr>
</tbody>
</table>

The results are from a minimum of two experiments utilising three individual animals for each experiment.

Each value represents the mean ± S.D of four to six determinations.

a = significantly greater than controls (p<0.001)
b = (p<.01)
c = (p<.05), Student's t-test.
Figure 4.24A

Rat microsomal lauric acid hydroxylase time course activities following a single dose of clofibrate

1. Hepatic Microsomes

Open bars represent 11- hydroxylase activities and shaded bars represent 12-hydroxylase activities. Values represent the means ± SD of three determinations.
Rat Microsomal Lauric Acid Hydroxylase Time Course Activities Following a Single Dose of Phenobarbitone

1. Hepatic Microsomes

Open bars represent 11-hydroxylase activities and shaded bars represent 12-hydroxylase activities. Values represent the means ± SD of three determinations.
Figure 4.24C

Rat microsomal lauric acid hydroxylase time course activities following a single dose of β-napthoflavone

1. Hepatic Microsomes

2. Renal Microsomes

Open bars represent 11-hydroxylase activities and shaded bars represent 12-hydroxylase activities. Values represent the means ± SD of three determinations.
<table>
<thead>
<tr>
<th>Fraction (hours)</th>
<th>Hepatic Microsomes</th>
<th>Renal Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol resorufin/mg protein/minute</td>
<td>nmol resorufin/nmol P-450/minute</td>
</tr>
<tr>
<td>0</td>
<td>.105 ± .017</td>
<td>.164 ± .026</td>
</tr>
<tr>
<td>6</td>
<td>.111 ± .017</td>
<td>.196 ± .031</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>.086 ± .007</td>
<td>.176 ± .014</td>
</tr>
<tr>
<td>induced</td>
<td>.094 ± .032</td>
<td>.147 ± .050</td>
</tr>
<tr>
<td>24</td>
<td>.131 ± .003</td>
<td>.186 ± .002</td>
</tr>
<tr>
<td>72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clofibrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>.061 ± .001</td>
<td>.231 ± .021</td>
</tr>
<tr>
<td>induced</td>
<td>.083 ± .002</td>
<td>.245 ± .006</td>
</tr>
<tr>
<td>24</td>
<td>.052 ± .004</td>
<td>.163 ± .013</td>
</tr>
<tr>
<td>(hours)</td>
<td>.060 ± .004</td>
<td>.158 ± .003</td>
</tr>
<tr>
<td>72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BNF</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>.124 ± .028</td>
<td>.351 ± .075a</td>
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<tr>
<td>induced</td>
<td>.358 ± .019a</td>
<td>1.14 ± .005a</td>
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<td>24</td>
<td>.389 ± .021a</td>
<td>1.47 ± .088a</td>
</tr>
<tr>
<td>(hours)</td>
<td>.379 ± .02a</td>
<td>.949 ± .050a</td>
</tr>
</tbody>
</table>

The results are from two experiments utilising three individual animals for each experiment. Each value represents the mean ± S.D of four to six determinations.

a = Significantly greater than controls (p<0.001)
b = (p<.01)
c = (p<.05), Student's t-test
the clofibrate renal microsomes. Again, the 11-hydroxylation of lauric acid was also
induced with time but to a far lesser extent.

Treatment of rats with BNF had no stimulatory effect on either the 11-and 12-
hydroxylase activities within hepatic microsomes when compared to control rats. A
general decrease with time for both metabolite formation was observed. In the
renal microsomes however the induction profile was similar to that for the other
two inducing agents. A preferential increase in 12-hydroxylation was observed with
a corresponding but much smaller increase for the 11-hydroxy products. Although
the general levels of activity were not significantly higher than those seen for
control renal microsomes, a time dependent response was never the less observed.

C) 7-Ethoxyresorufin O-deethylation

The final marker substrate utilised to investigate the inducibility of the
M.F.Q. system was 7-ethoxyresorufin. This is a preferential substrate for
cytochrome P-448 type isoenzymes. The O-deethylation of ethoxyresorufin was
measured as the progressive increase in fluorescence due to the formation of the
product, resorufin, as shown in table 4.8. Activity was detected in both the livers
and kidneys of all the animals induced, with the liver being seen to be by far the
most active tissue. Following both phenobarbitone and clofibrate-induction the
hepatic and renal deethylation of ethoxyresorufin was seen to either marginally
decline or remain at control levels. Only BNF-induction resulted in a significant
increase in activity which was seen to reach a maximum at around 24 hrs following a
single dose in both tissues. These results, in the case of BNF-induction, be they
expressed as activity/mg of microsomal protein or activity/nmol of hemoprotein
clearly indicate a specific increase in synthesis of a cytochrome P-450 that
catalyses the deethylation of ethoxyresorufin.
4.7 IMMUNOQUANTITATION OF CYTOCHROME P-450 ISOENZYMES FOLLOWING XENOBIOTIC INDUCTION

The antibodies raised against each of the different cytochrome P-450 isoenzymes have been shown to be monospecific by criteria described previously and therefore may be used to quantitate the levels of these proteins in hepatic microsomes following xenobiotic induction. However it should be noted that because cytochrome P-450 B1 and P-450 B2 are immunologically indistinguishable in Ouchterlony double diffusion analysis, immunoquantitation will assay both forms. It is important to construct standard curves for each concentration of antibody used due to the marked variation in the degree of immunodiffusion. An example of two such standard curves for purified cytochrome P-450 B2 is shown in figure 4.25A. Figure 4.25B shows the corresponding radial rings from one such analysis, clearly illustrating an increase in ring diameter with increasing amounts of antigen.

The immunochemical quantitation of cytochrome P-450 isoenzymes following induction is detailed in figure 4.26, with the corresponding values expressed as a percentage of the total cytochrome P-450 population present within the liver shown in table 4.9. All three of the major inducers are shown to cause an increase in the specific amounts of the relative cytochrome P-450 isoenzymes with time. This increase is especially rapid in the case of cytochrome P-447 isoenzyme levels within BNF microsomes. At maximal levels, phenobarbitone increased cytochrome P-450 B1 and P-450 B2 22 fold; clofibrate increased cytochrome P-452 2.13 fold and BNF-induction increased cytochrome P-447 26 fold. Following the induction of any major form of cytochrome P-450 it was observed that there was a general depression in the induction of any of the other forms which indicates the specificity of each of the induction processes. The levels of the cytochrome P-450 in the control, phenobarbitone and BNF-induced microsomes was comparable to the data obtained by other authors (Goldstein et al. 1983, Thomas et al. 1983). The concentration of cytochrome P-452 is particularly interesting due to the direct contrast of its concentration in uninduced microsomes compared to that of other well
characterised forms. The fact that the presence of cytochrome P-447 in clofibrate-induced microsomes was not observed is surprising as it was detected-albeit at the extreme levels of detection - by western blotting. This would suggest that although this isoenzyme is present in clofibrate-induced microsomes, its level is so low that it is below the minimum level of immunochemical detection which can be achieved with this technique.
Figure 4.25A

Standard Curves for the Quantitation of Cytochromes P-450 B₁ and P-450 B₂ within Hepatic Microsomes by Single Radial Immunodiffusion Utilising Anti-cytochrome P-450 B₂ Antibody

Figure 4.25B

Single Radial Immunodiffusion Analysis of Cytochrome P-450 B₂ Against Anti-cytochrome P-450 B₂ Sera

track 1

track 2

track 3

track 4

3% anti-

cytochrome P-450 B₂ sera

In track 1 both rings represent 5μl of purified cytochrome P-450 B₂ (3.35 pmol). Tracks 2, 3, 4 represent the radial rings obtained with increasing amounts of purified cytochrome P-450 B₂. This relates to additions of 0.5, 1.2, 3 and 4 μl of purified protein, corresponding to 0.34, 0.67, 1.34, 2.01 and 2.68 pmol cytochrome P-450 B₂ respectively.
Figure 4.26

Immunochemical Quantitation of Cytochrome P-450 Isoenzymes in Hepatic Microsomes following Treatment with Different Inducers

Values represent the mean ± S.D of a minimum of 6 determinations

- Control microsomes, □ phenobarbitone-induced microsomes,
- Clofibrate-induced microsomes, ▪ β-naphthoflavone-induced microsomes.

a = significantly greater than controls (p<0.001)
b = (p<.01)
c = (p<.05), Student's t-test.
Table 4.9

Immunochemical Quantitation of 4 Forms of Cytochrome P-450 in Hepatic Microsomes from Male Rats Treated with Different Inducers

<table>
<thead>
<tr>
<th>Rat treatment</th>
<th>Percent of total cytochrome P-450</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-450</td>
<td>B1/B2</td>
<td>P-452</td>
<td>P-447</td>
</tr>
<tr>
<td>0</td>
<td>1.57</td>
<td>25.7</td>
<td>1.6</td>
<td>71.13</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10.28</td>
<td>6.21</td>
<td>3.5</td>
<td>80.01</td>
</tr>
<tr>
<td>12</td>
<td>20.81</td>
<td>6.86</td>
<td>3.53</td>
<td>68.81</td>
</tr>
<tr>
<td>24</td>
<td>34.3</td>
<td>5.07</td>
<td>2.48</td>
<td>58.15</td>
</tr>
<tr>
<td>72</td>
<td>23.9</td>
<td>3.18</td>
<td>1.66</td>
<td>71.26</td>
</tr>
<tr>
<td>Clofibrate</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8.83</td>
<td>27.84</td>
<td>N/D</td>
<td>63.3</td>
</tr>
<tr>
<td>12</td>
<td>8.09</td>
<td>30.74</td>
<td>N/D</td>
<td>61.17</td>
</tr>
<tr>
<td>24</td>
<td>8.45</td>
<td>42.91</td>
<td>N/D</td>
<td>48.64</td>
</tr>
<tr>
<td>72</td>
<td>7.36</td>
<td>54.72</td>
<td>N/D</td>
<td>37.92</td>
</tr>
<tr>
<td>BNF induced</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4.85</td>
<td>4.37</td>
<td>33.96</td>
<td>56.82</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>3.4</td>
<td>35.65</td>
<td>6</td>
</tr>
<tr>
<td>24</td>
<td>4.28</td>
<td>2.42</td>
<td>40</td>
<td>53.3</td>
</tr>
<tr>
<td>72</td>
<td>3.33</td>
<td>2.84</td>
<td>42.3</td>
<td>51.53</td>
</tr>
</tbody>
</table>

N/D = not detected.
5.1 INTRODUCTION

In the preceding chapter the isolation, purification and characterisation of a number of distinct cytochrome P-450 isoenzymes was reported. In addition, quantitation of these forms in liver microsomes was also determined. It became clear that despite the close similarity of these proteins in terms of their localisation, function and molecular weight range, that these appeared to be distinct isoenzymes under the functional control of specific induction processes. Accordingly, an insight into the molecular basis for this multiplicity was sought by isolation and characterisation of the mRNA's coding for these proteins, and by subsequent immunological analysis of the P-450 peptides synthesised from these mRNAs in vitro.

Messenger RNA molecules can be considered as central components in the expression of eukaryotic structural genes. In a study designed to quantitate specific mRNAs, three experimental parameters must be considered. The first is the extraction of intact functional RNA containing the mRNA of interest from the tissues in which enzyme induction is taking place. As the relative rate of cytochrome P-450 isoenzyme synthesis under maximally induced conditions seldom exceeds 1% of overall protein synthesis this is undoubtedly an important consideration. Secondly, the isolated RNA sample must be capable of accurate translation in an in vitro protein synthesising system capable of using the exogenous mRNA as a template. Finally, the amount of enzyme protein synthesised in vitro in response to the added RNA must be measurable in order to serve as an estimate of the level of translatable enzyme mRNA contained in the RNA extracted.

The results obtained will be described in depth in the following chapter which is concerned with the induction of the cytochrome P-450 isoenzymes in response to
xenobiotic administration. This chapter will deal with the procedures involved, and
describe the inherent technical difficulties encountered in establishing the most
suitable method for analysing the effects of xenobiotic pretreatment on the mRNA
population within rat liver and kidney.

5.2 PURIFICATION AND ISOLATION OF TOTAL RAT LIVER/KIDNEY RNA

In the cell, RNA exists mostly in ribonucleoprotein complexes (ribosomal
subunits, hn RNPs (histone ribonucleoproteins) and mRNPs) which further interact
with extraneous proteins subsequent to cell disruption. The essence of RNA
isolation is therefore highly dependent on a protein denaturation step in conjunction
with extensive ribonuclease inhibition. To achieve this a modification of the
procedures of Chirgwin et al. (1979) and Deeley et al. (1977) was utilised as outlined
in section 3.3. Guanadinium hydrochloride (GHC1) extraction was favoured primarily
because the half life of ribonuclease (RNase) is only 10 seconds in a 4M solution
(Millar and Bolin, 1978), and also because it is a extremely strong denaturant in
which both cationic and anionic forms of GHC1 are potent chaotropic agents.
Reducing agents such as 2-mercaptoethanol and dithiothreitol acting as a disulphide
bond reductant were also included in the homogenisation mixture. Increasing the
ratio of guanadinium hydrochloride stock solution (initial homogenisation volume) -
from 16 (as recommended for the purification of pancreatic RNA) to 22 ml per gram
tissue considerably increased both the yield and purity of the final RNA preparation
(table 5.1). No significant difference was seen between the amount of RNA isolated
per gram tissue for the different inducing agents.

<table>
<thead>
<tr>
<th>GHCI (ml of 7.5M stock solution/gm tissue)</th>
<th>Average RNA yield (mg/g liver)</th>
<th>Liver weight (gm)</th>
<th>Absorbance Ratio (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>13.9</td>
<td>1.35</td>
<td>1.75</td>
</tr>
<tr>
<td>22</td>
<td>10.25</td>
<td>3.4</td>
<td>1.91</td>
</tr>
</tbody>
</table>
Subsequent reprecipitations in GHCl/ethanol followed by sodium acetate/ethanol precipitations further purifies the RNA. These precipitations result in the solubilisation and subsequent removal of all the DNA, tRNA, 5S RNA, and polysaccharides. The RNA preparation was assessed for purity by means of an $A_{260/280}$ ratio reading following which it would be prepared for fractionation on oligo (dT) cellulose. The major disadvantage of this isolation protocol however is that of the presence - although in the minority - of the RNA species of the hn RNAs and also the nuclear precursors of the cytochrome P-450 mRNA sequences (Taylor, 1979). Occasionally if an ultra-pure RNA was required as in the case for cDNA synthesis, then the RNA fraction would be further applied to a CsCl gradient to ensure the removal of all DNA sequences. Routinely $A_{260/280}$ values of $> 2.0$ were obtained for such preparations.

5.2.1 Oligo (dT) Chromatography

The total RNA isolated from the previous extraction step is still grossly contaminated with rRNA and various nuclear RNAs, and as such, further purification is required on oligo (dT) cellulose. Two columns are utilised, the first having a binding capacity of 20-40 $A_{260}$ units of poly (A)/gm cellulose, the second having a higher capacity of 40-60 $A_{260}$ units/gm. The resulting poly (A)$^+$ mRNA from such a purification is highly active translationally in an in vitro protein translation system.

The procedure involves heat denaturation of the secondary structure of the RNA sample (65°C/5 minutes followed by rapid cooling in ice) after which it is hybridised to the column at neutral pH in a high salt concentration (0.5M LiCl). Any unbound nucleic acid is then removed by thorough washing in the same buffer. This tends to remove most of the poly (A)$^+$RNA species. The elution is followed by monitoring $A_{260}$ absorbance. Some rRNA however is still found to be bound with the poly (A)$^+$ RNA fraction due to slight aggregation between the two forms, and also due to a degree of non-specific binding to the column itself. This can be
removed by lowering the salt concentration to 0.1M. Because of the weak interactions of the poly (A)$^+$ mRNA to this specific oligo (dT) column and also because some of the poly (A) segments are too short or blocked in some way, a proportion of the poly (A)$^+$ mRNA is always lost at each stage. Finally the poly (A)$^+$ mRNA is removed from the column by eluting in neutral buffer containing no salt.

This semi-purified poly (A)$^+$ mRNA preparation still has some rRNA and tRNA contamination which can be removed by re-application on a second column of high affinity oligo (dT) cellulose (Bantle et al. 1975). The same column purification steps as before were used. A representative elution profile obtained in the isolation of poly (A)$^+$ mRNA sequences is given in figure 5.1. On analysis of the poly (A)$^+$ mRNA on denaturing formaldehyde gels no significant rRNA contamination was evident (data not shown). These poly (A)$^+$ mRNA preparations were capable of stimulating in vitro translational activity from between 10 to 50 fold above background. Translational activity was also shown to be evident in the non-adsorbed (poly (A)$^-$ mRNA) fractions (figure 5.2). Immunoprecipitatal analysis of these fractions showed that activity was also present for the phenobarbitone-induced cytochromes, albeit at far lower levels than that exhibited by the total RNA and poly (A)$^+$ mRNA fractions. This observation was also reported by Fujii-Kuriyama et al. (1981). Whether or not this represents poly (A)$^+$ mRNA sequences which had bound non-specifically and then been eluted, or else poly(A)$^+$ mRNA sequences which had their tails removed by ribonucleases, or even nuclear precursors of the mRNA sequences which had not yet become polyadenylated is at present unknown. However, because these non-adsorbed fractions represented only a small proportion of the mRNA activity concerned with the cytochrome P-450 isoenzymes, compared with poly(A)$^+$ mRNA fractions, they were not further characterised.

5.2.2 Further Fractionation of Isolated Poly (A)$^+$mRNA Sequences

As a prelude to the synthesis of complimentary DNA (cDNA) the clofibrate-induced poly (A)$^+$ mRNA fraction had to be further purified by means of sucrose
Figure 5.1 Elution Profile of Poly (A)$^+$ mRNA Sequences from Oligo (dT) Cellulose

<table>
<thead>
<tr>
<th></th>
<th>Column One</th>
<th>Column Two</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FIRST COLUMN</td>
<td>SECOND COLUMN</td>
</tr>
<tr>
<td>A260</td>
<td>0.5 M salt</td>
<td>0.5 M salt</td>
</tr>
<tr>
<td></td>
<td>0.1 M salt</td>
<td>0.1 M salt</td>
</tr>
<tr>
<td>Fractions (0.75 ml)</td>
<td>0-10</td>
<td>0-14</td>
</tr>
<tr>
<td>mg RNA/fraction</td>
<td>46.39</td>
<td>1.84</td>
</tr>
<tr>
<td>% yield of total</td>
<td>88.80</td>
<td>2.0</td>
</tr>
<tr>
<td>A$_{260}$/280</td>
<td>1.84</td>
<td>2.34</td>
</tr>
</tbody>
</table>

Data from clofibrate-induced RNA preparation; 52.2 mg total RNA obtained from 2 animals giving a yield of 3.31 mg RNA/gm liver.
Figure 5.2

Translation and Immunoprecipitation of Fractions Obtained Following Oligo (dT) Chromatography of Total RNA Isolated from Phenobarbitone-induced Animals

A. Tracks 1 = standard molecular weight markers, 2 = blank (zero RNA), 3 = total RNA, 4 = 0.5M LiCl wash (column 1 fraction A), 5 = 0.1M LiCl wash (column 1, fraction B), 6 = 0.5M LiCl wash (column 2, fraction D), 7 = 0.1M LiCl wash (column 2, fraction E), 8 = poly(A)+ mRNA (column 2, fraction F). All fractions were translated at an RNA concentration of 0.33 μg/μl.

B. The immunoprecipitation products of the above translated fractions (anti-phenobarbitone-induced cytochrome P-450 IgG) is illustrated directly below the corresponding tracks.
gradient fractionation. Great care has to be taken to avoid ribonuclease contamination, and the procedure utilised is described in section 3.5. This procedure is utilised to attain an enrichment of the mRNA of interest in order to bring the mRNA within cloning range. The ultimate aim is to be able to reduce the cDNA library to a manageable number of clones which can then be screened individually. The procedure involved in this fractionation was essentially the size fractionation of mRNA species. These were then translated in a in vitro translation system and subsequently immunoprecipitated against the anti-cytochrome P-452 antibody. Those fractions which gave positive results were pooled and used in the synthesis of the corresponding cDNA.

The clofibrate poly (A)$^+$ mRNA was solubilised in 99% DMSO, 10 mM Tris-HCL (pH7.5) and then heated before being layered on top of the sucrose gradient. It was found that no more than 300 μg mRNA could be fractionated through a single gradient otherwise aggregation tended to occur. Average values for the recovery of total RNA within this range was in the region of 70%. The separation of the mRNA species was monitored spectrophotometrically with the fractions containing RNA being precipitated by the addition of sodium acetate/ethanol. Because of the very low amounts of RNA present within these fractions, not all of the RNA was recovered and losses of approximately 10% were routine. The profile of the sucrose gradient separation is shown in figure 5.3A. The poly (A)$^+$ mRNA was seen to still contain a fair proportion of rRNA (28s) despite 2 oligo (dT) cycles (data not shown). On translation of the RNA containing fractions, maximal activity was seen predominantly in the 18s region, with the activity for cytochrome P-452 being found in the peak of this area (figure 5.3B). Those fractions which displayed activity for cytochrome P-452 following immunoprecipitation analyses were pooled and concentrated by sodium acetate/ethanol precipitation. This pool was shown to correspond to less than 1% of the total fractionated poly (A)$^+$ mRNA. The $A_{260}/280$ ratio of the enriched fraction was always above two indicating the absence of any significant contaminating protein.
Figure 5.3A.
Fractionation of Cytochrome P-452 mRNA in a Linear 15-30% Sucrose Gradient

After precipitation of RNA in each fraction the RNA was dissolved in water (40μl) and an aliquot (1μl) of each fraction then being assayed for mRNA activity in the in vitro translation system.

Figure 5.3B.
Immunoprecipitation of 18s mRNA Fractions following Gradient Centrifugation with Anti-cytochrome P-452 Antibody

200K
92.5K
69K
46K
30K

STD 14 15 16 17 18 19 20 21
Fraction numbers

The enriched poly (A)

mRNA sequences for cytochrome P-452 were found within fractions 15 (very faint band) to 18.
5.3 OPTIMISATION OF RIBONUCLEIC ACID TRANSLATIONAL ACTIVITY IN THE RABBIT RETICULOCYTE LYSATE SYSTEM.

Throughout the period of this study 3 different *in vitro* translation systems were utilised with the most successful and ultimately adopted system being the commercially available nuclease treated lysate from Bethesda Research Limited (B.R.L.). Of the two others, one was purchased from Amersham International and the other prepared in the laboratory. The main criteria for any translation system are low levels of endogenous mRNA, the requirement for low concentrations of radiolabelled amino acid and most importantly, efficient, correct and reproducible translation of added mRNA. Unfortunately, the 2 latter systems mentioned were not able to satisfy these criteria.

The major problem associated with the lysate from Amersham International was that of a generally low stimulatory translational activity. Translational activity was in general about 5-10 fold above background with a number of different mRNA preparations. In contrast translation with B.R.L.'s lysate for the same mRNA preparations would result in 20 fold increases in activity. These values could also be attained with the home-made lysate preparation, but the major problem found here was that of nuclease contamination. With fresh lysate batches high levels of translational activity were obtainable which unfortunately diminished with time, such that within 4-6 weeks of preparation, translational activity was down to approximately twenty percent of the starting activity. This was undoubtedly associated with the release of the micrococcal nuclease initially used to destroy all the endogenous RNA present. The nuclease is subsequently inactivated with EGTA. This decrease in translational activity is therefore most likely to be due to either the EGTA dissociating itself from the nuclease with time, or alternatively residual calcium interacting with the EGTA which would again result in nuclease activation.

Of primary importance is the optimisation of ion concentrations within the lysate mix to attain maximum activity. These optimisation conditions were shown to significantly vary for both of the commercial lysate preparations reflecting variations in the indigenous proportions of the ions within their respective systems.
When different RNA fractions were translated, i.e. rabbit globin mRNA, rat liver mRNA, the conditions required for optimal activity were not found to significantly differ. The following results, unless otherwise specified, relate to the use of the B.R.L. lysate.

5.3.1 Salt Concentrations

Changes in the ion concentration were shown to significantly influence the degree of activity obtained with the translation system using rat liver RNA (figure 5.4). With $K^+$, the optimal concentration was within the region of 80mM, and for $Mg^{2+}$ about 1mM which is the residual level found within the system. Increasing the $Mg^{2+}$ concentration resulted in inhibition of translational activity. As these conditions were shown to be optimal for the translation of the rat liver RNA fractions isolated throughout the study, they were maintained throughout the rest of the translation experiments.

It was not possible to compare the ion concentration optima between different lysate systems. Generally, these are between 75-150mM for $K^+$ and between 1-2mM for $Mg^{2+}$. Although some of this variation could be attributed to different requirements being needed for different mRNAs this is undoubtedly not the major cause. For example if the same mRNA fractions were translated with the Amersham reticulocyte lysate system totally different ion optima were required. In this case, for $K^+$ this was in the region of 135-140mM and for $Mg^{2+}$ approximately 1.6mM. Such variations emphasise the need for such standardisation experiments in order to ensure maximal translation of all the RNA species present.

5.3.2 Effect of mRNA Concentration on In Vitro Protein Synthesis

The concentration of nucleic acid assayed varied with the purity of the mRNA preparation. Generally, any preparation with $A_{260/280}$ values of less than 1.8 were not capable of high translational activity. Optimisation of the amount of RNA to be translated is also important, as inhibition would be seen to result in incorporation by the reticulocyte lysate system at high concentrations. This is believed to relate to
blocking of the ribosomal binding sites as well as competition for the various initiator/effect factor factors required throughout protein synthesis. A third factor to be considered would also have to be the increased presence of possible inhibitors in the RNA fraction itself, such that wherever possible, the total amount of RNA introduced into the reticulocyte system would not rise above 10% of the total volume. Optimisation profiles for both total RNA and poly (A)+ mRNA are shown in figure 5.5. Increasing the amounts of RNA resulted in a linear increase in translational activity up to a point of saturation. Lower amounts of poly (A)+ mRNA were required in comparable translation activity experiments to total RNA, with this being directly related to the purity of the RNA fraction itself.

It was also possible to enhance the efficiency of translation by a number of heat denaturation steps of the RNA fraction immediately prior to translation. Effectively this reduces the level of aggregation by disrupting the secondary structure of the RNA molecules thereby obtaining a more accurate measure of mRNA activity. This was achieved by heat denaturation at 65°C for 15 minutes in a water bath or else by the addition of methyl mercury hydroxide (Payvar and Schimke, 1979) as outlined in section 3.8. These authors reported on the effects of methyl mercury enhancement of translation and transcription of ovalbumin and conalbumin mRNAs. The effect appears to be mRNA specific with reported increases of 6-10 fold and 2.5 fold being obtained respectively. This is believed to result from an improvement of initiation, elongation and termination of the mRNA or possibly inhibition of degradation. Most certainly, in this study, treatment of phenobarbital-induced poly (A)+ mRNA was affected by methyl mercury denaturation with this resulting in the greatest means of translational stimulation (table 5.2). Activity was almost double that obtained from the standard means of heat denaturation (heating at 65°C/5 min, and then rapid cooling in ice) but ultimately this method was not utilised because of the inherent health dangers of using this toxic chemical. When the translational products were analysed on SDS P.A.G.E. by fluorography, no differences were observed between the polypeptides generated by either of the two procedures (data not shown).
Rat liver poly (A)^+ mRNA was translated at a concentration of 0.037 μg/μl utilising 0.5 μCi ^35^S-methionine/μl. In experiment A, Mg^{2+} concentration was 0.8mM. In experiment B, K^+ concentration was 80mM. In both A and B, the assay mixtures were incubated at 30°C for 90 minutes. For both experiments values represent the average of two separate determinations.
Figure 5.5  

The Effect of mRNA Concentrations and Incubation Time on In Vitro Protein Synthesis

Y axis for all graphs represents $^{35}$S-methionine incorporation (cpm x $10^{-3}$/μl) (a) poly(A)$^+$ mRNA concentration = .02 μg/μl.

All values represent the means from two separate determinations.
Table 5.2
The Effect of Different Denaturation Procedures on the Translation of Poly (A)$^+$ mRNA In Vitro

<table>
<thead>
<tr>
<th>mRNA fraction pretreatment</th>
<th>$^{35}$S-Methionine incorporation cpm/µl</th>
<th>Fold enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3,359</td>
<td>-</td>
</tr>
<tr>
<td>Heat denaturation</td>
<td>4,338</td>
<td>1.29</td>
</tr>
<tr>
<td>Methyl mercury hydroxide (1mM)</td>
<td>7,162</td>
<td>2.13</td>
</tr>
</tbody>
</table>

Phenobarbitone-induced poly (A)$^+$ mRNA was translated at a concentration of 0.03 µg/µl using $^{35}$S-methionine (0.5 µCi/µl) as the radioactive tracer for 90 mins in the in vitro translation system. Values represent the means from a minimum of two determinations.

5.3.3 Time Course for Translation of Different mRNAs

Characterisation of the in vitro reticulocyte lysate system indicated that at optimal conditions a linear increase in translational activity with time was observed (figure 5.5). Accordingly all translations were carried out for (a period of) 90 minutes to ensure maximal translation of all available mRNA species. This is especially relevant in the case where high molecular weight polypeptides are being synthesised, as ribosomes require approximately 30-40 minutes to assemble a polypeptide of 250,000 daltons, such that a high percentage of the ribosomes would still be carrying uncompleted chains after 1 hour. One of the features of the lysate is that it tends to stop initiating new chains before it loses the ability to elongate and complete chains which have already started, so that longer incubation times predispose to higher yields of full length translation products. The in vitro translation was carried out at 30° (and not 37°C), for at 37°C although the initial reaction is faster there is greater tendency for the system to shut down earlier, resulting in the synthesis of only low molecular weight polypeptides. In addition if
the mRNA has become contaminated by ribonucleases or proteases, incubation at 30°C reduces the risk of degradation.

In the analysis of translation products, $^{35}$S-methionine was utilised at a concentration of 0.5-0.75 μCi/μl. In the case of immunoprecipitation analysis, this was increased to 1.0 μCi/μl in order to increase sensitivity of analysis. Although greater stimulation could have been attained by increasing further the amount of radiolabel this was not carried out because of the expense of the compound. The only disadvantage with using $^{35}$S-methionine as a label is the occasional presence of a non-specific background polypeptide band which has an apparent molecular weight of approximately 48,000. The presence of this band was a nuisance as it can interfere with the interpretation of gel patterns when the added mRNA translation products have molecular weights in the region of 50,000 (the cytochrome P-450 region). The precise reason for its presence is unknown but was definitely related to the age of the radioactive amino acid precursor as it was never observed with fresh batches of $^{35}$S-methionine. One suggestion is that it arises from a tRNA-dependant but ribosome - independent addition of methionine to a pre-existing protein and that its appearance can be circumvented by storing the $^{35}$S-methionine in 50mM tricine (pH7.4) (N.E.N. technical bulletin). Here however, problems are also encountered in that the long-term stability of the label within this medium is diminished.

5.3.4 Fluorographic Analysis of Translation Products

On analysis of in vitro translation products by SDS P.A.G.E. it was found that weak β-particles such as those emitted by $^{35}$S are adsorbed within the gel itself, and as such are recorded inefficiently by film. This adsorption can be overcome by impregnating the sample with a scintillator to achieve maximum contact between the isotope and the scintillator. In this way even weak emissions can transfer their energy to scintillator molecules which can emit ultra violet light. Provided the sample is translucent and colourless, the light can travel further than the original β-particle to form an image on the adjacent film. This process is called fluorography.
The use of such scintillators has to be looked at not only from the point of view of efficiency but also cost-effectiveness with the three different types utilised all having their own corresponding advantages as well as disadvantages. Procedures for their use are as described in section 3.10.

The most satisfactory scintillator utilised was Enhance which provided not only a corresponding increase in sensitivity but also gave clear, precise and definitive images of the polypeptide bands which had been translated. Exposure to X-ray film was carried out at -70°C because at this temperature, increased light production from the fluor and increased sensitivity of the X-ray film to low intensities of light dramatically increases resolution (Luthi and Waser, 1965). In addition the duration of exposure was an important factor with corresponding increases in time resulting in increased image resolution. This is quite forcefully illustrated in figure 5.6 where the translation products from globin mRNA and RNA from control, phenobarbitone and clofibrate-induced rats are shown. Illustrations A and B show the respective products of both total RNA and poly (A) mRNA directed translations following 1 weeks exposure against X-ray film. Illustration C (total RNA) and D (poly A mRNA) show the same fractions but after two weeks exposure. Correspondingly when just the analysis of total translation products was required a period of 1 weeks exposure against film was utilised. In the case of immunoprecipitation analysis this was increased to 2 weeks, not only to get better resolution of the immunoprecipitated product but also to ensure that no other cross recognition had occurred between the antibody and heterologous translation products.

Sodium salicylate (Chamberlain, 1979) was also used as a fluorographic enhancer but generally this was not found satisfactory because of poor resolution due to mottled images. This made individual band analysis more difficult such that its use was limited only to those experiments where this was not a major factor of concern. Amplify (Amersham International) was also used with its advantages being its safety (non-DMSO based) and the speed of application. Qualitatively however it
The Influence of Exposure Period on the Translation of Dissimilar Total RNA and Poly (A)$^+$ mRNA Fractions

Fractions: 1 = standard molecular weight markers, 2 = zero RNA blank, 3 = globin RNA 4 = clofibrate RNA, 5 = phenobarbitone RNA, 6 = control RNA.

Illustrations A, C = translations of total RNA fractions (0.16 µg/µl) exposed for 1 and 2 weeks respectively.
Illustrations B, D = translations of poly (A)$^+$ mRNA fractions (0.037 µg/µl) exposed for 1 and 2 weeks respectively.
was not judged as efficient as Enhance which ultimately became the fluorographic scintillator of choice.

5.4 USE OF IMMUNOADSORBENT COMPLEXES IN TRANSLATIONAL IMMUNOPRECIPITATION ANALYSIS

Quantitation of the mRNA species coding for specific cytochromes P-450 can be achieved by the immunoprecipitation of their corresponding translation products. It is therefore very important not only to obtain total immunoprecipitation of the specific cytochrome P-450 but also to be able to accurately quantitate it.

5.4.1 Specificity of Immunoadsorbent Complexes in Translational Immunoprecipitation Analysis

In the course of developing an assay for specific protein synthesis within in vitro rabbit reticulocyte lysate translation systems three different immunoprecipitation procedures were investigated (section 3.9). Essentially the immunoprecipitation of antigens involves two main steps. First, specific antibody is added to a detergent-treated cell lysate containing the radiolabelled antigen. Next, the complexes of labelled antigen or antibody are bound with a second agent that provides sufficient mass to facilitate precipitation. The size of this precipitate can be further enhanced by the addition of cold carrier antigen. Traditionally this second agent has been anti-immunoglobulin antibody directed against the first antibody but alternative approaches now include the use of chemically fixed, protein A bearing strains of Staphylococcus aureus bacteria (Kessler, 1975) which have an extremely high affinity for IgG.

In the analysis of the double antibody technique phenobarbitone IgG (specific against cytochrome P-450 forms B₁ and B₂) was directed against the translation products coded for by phenobarbitone-induced poly(A)⁺ mRNA fractions. The experimental procedure could be considered a success in that immunoprecipitation
was apparent with there being minimal non-specific background. On analysis of the immunoprecipitated products by gel electrophoresis three major bands were seen in the cytochrome P-450 region followed by two further bands at approximately 50K and 25K respectively. These last 2 bands are believed to correspond to the heavy and light chains of the precipitating IgG, where because there was such an excess of antibody, some $^{35}$S label may have been trapped within the immune complexes themselves.

The addition of PMSF (0.01M) - a protease inhibitor - in the washing procedures had no effect on the appearance of these bands, indicating that their presence was not a result of proteolytic or other degradative influences. To a certain extent, the presence of these bands could be significantly reduced by increasing the detergent concentration in the final washing steps but this also resulted in a concomitant reduction in the intensity of the immunoprecipitated polypeptide. The final and possibly major drawback seen with this system was that because analysis is performed by gel electrophoresis the large amount of precipitating IgG utilised results in gross protein overloading and distortion of resolution within the region of the IgG chains. This makes the quantifiable analysis of immunoprecipitated polypeptides both difficult and not wholly reliable. To a certain extent this could be circumvented, but this required that quantitative antigen recoveries be sacrificed in order to obtain lower levels of background radioactivity. These drawbacks necessitated a different immunoprecipitation method to be developed. Towards this end the use of S. aureus ghosts and protein A were investigated.

The advantage of protein A usage is its high binding specificity to the Fc regions of many IgG subclasses (Goding, 1978). It behaves as an immunoabsorbent possessing a high adsorbation capacity and rapid binding which permits the specific recovery of antigen from cell lysates simply by centrifugation. Differential separation of the protein A fractions from the antigen/antibody complexes can then be obtained by heat denaturation and chemical reduction just prior to
In both S. aureus cell and protein A aided immunoprecipitations, a pre-precipitation reaction as well as a number of subsequent washing cycles were found to be essential in order to remove material within the lysate extract which could bind non-specifically to immunoglobulins or staphylococci. If this was not carried out, a high background of non specific bands was observed. The amount of proteins which were adsorbed in this way was quite considerable (figure 5.7A) and consisted primarily of lower molecular weight proteins (<50K). This latter gel illustrates the pre-clearing precipitate obtained with pre-immune IgG and protein-A sepharose slurry. The major band visible at 50K represents the heavy chain of IgG. No major proteins appear to be adsorbed in the cytochrome P-450 region.

If the lysate supernatant was also assayed following specific IgG immunoprecipitation with immune antibodies, then again no major peptides are visible within this region, indicating the specificity by which the corresponding antigenic determinants are removed by this procedure (figure 5.7B). When S. aureus cells were used in the preclearing step the addition of pre-immune IgG was shown not to be required with sufficient adsorbtion being obtained by the cells alone. There was some variation in the proteins adsorbed, although again they were primarily of low molecular weight. When the pre-clearing fractions obtained using either S. aureus cells or pre-immune IgG - protein A sepharose slurry were analysed by immunoprecipitation no evidence of cytochrome P-450 presence was seen to be evident (figure 5.8).

The antigen-antibody complexes were still contaminated with a fair degree of non-specific background proteins and their removal necessitated further resuspension and centrifugation through a detergent containing wash buffer. This was especially true in the analysis of the phenobarbitone-induced cytochrome P-450 isoenzymes where an additional centrifugation step through a 0.3M sucrose/detergent cushion was an absolute necessity. The sucrose supernatant
Preclearing was achieved using pre-immune IgG with protein A sepharose slurry.

Track 1 = standard, Track 2 = precleared fraction from zero mRNA blank, Tracks 3-7 = precleared fractions from phenobarbitone poly (A)$^+$ mRNA translations.

All tracks represent the supernatant from an in vitro translation mix coded for by phenobarbitone-induced poly (A)$^+$ mRNA following immunoprecipitation with anti-phenobarbitone-induced cytochrome P-450 antibody.
fractions following such centrifugation were also immunoprecipitated against the anti-phenobarbitone-induced cytochrome P-450 antibody. No precipitation was evident indicating no dissociation of the antigen from the immunoprecipitation complex throughout this step. This rigorous washing procedure was only required in the analysis of the phenobarbitone-induced cytochromes P-450. If cytochrome P-452 (and to a lesser extent cytochrome P-447) immunoprecipitated polypeptides were subjected to this washing procedure then complete disruption of the antigen-antibody complex was obtained with the immunoprecipitation not succeeding. Gentle washing with a non-detergent buffer was sufficient for removing any non-specifically bound proteins.

The effects of the addition of cold carrier antigen to the in vitro translated polypeptide mix just prior to immunoprecipitation with an aim to increase the yields was also investigated (figure 5.9). Increasing the amount of antibody itself increased the amount of immunoprecipitated cytochrome P-450. However addition of cold homogeneous cytochrome P-450 B2 resulted in lower amounts of the polypeptide being precipitated. Undoubtedly competitive binding between the translated polypeptides and the cold protein was the major factor, and as such this aspect of immunoprecipitation was not pursued any further.

On translation of clofibrate and BNF-induced RNA preparations, immunoprecipitation analysis of the translation products indicated the presence of a single polypeptide band which was shown to co-migrate with the corresponding homogeneous cytochrome P-450 when analyzed by SDS-PAGE. This was not the case however with immunoprecipitated phenobarbitone-induced cytochrome P-450 isoenzymes following translation of the corresponding RNA fraction. Here a group of 3-4 proteins were immunoprecipitated, two of which corresponded to the B1 and B2 cytochrome P-450 forms. The same pattern was observed when either S. aureus cells or protein A sepharose was used to pull down the antigen-antibody complex. Of this group of precipitated polypeptides, the fourth (being the lowest having an apparent molecular weight of 50,000) was eventually shown to be an artifact as a
Figure 5.8 Analysis and Immunoprecipitation of S. aureus Cell and Pre-immune Antibody-protein A Sepharose Aided Preclearing Fractions

Tracks 3,4 illustrate the precleared proteins removed by protein A with corresponding immunoprecipitations by immune antibody shown in tracks 5,6. Tracks 7,8 represent S. aureus precleared fractions with immunoprecipitation analysis shown in tracks 9,10.

Figure 5.9 The Effect of Carrier Protein on the Immunoprecipitation of Phenobarbitone-Induced Cytochrome P-450 Isoenzymes

Track 1 = standard molecular weight proteins, Tracks 2 = zero mRNA blank. Tracks 3-10 represent the total precleared proteins following translation of clofibrate induced poly (A)+ mRNA. Tracks 3,4 illustrate the precleared proteins removed by protein A with corresponding immunoprecipitations by immune antibody shown in tracks 5,6. Tracks 7,8 represent S. aureus precleared fractions with immunoprecipitation analysis shown in tracks 9,10.
non-specific binding. Its presence was shown to be dependent not only upon the result of amount of precipitating matrix utilised but also upon the degree of washing of the immunoprecipitated complex. It was for this reason that the final sucrose/detergent wash of the isolated phenobarbitone directed RNA precipitate was carried out. This is illustrated in figure 5.10 which illustrates the effect of increasing background with increasing amounts of immunoprecipitating S. aureus cells. No precipitation is observed for the preclearing stage, or with S. aureus cells alone, as well as with control pre-immune IgG on the total translation products. Two major proteins are immunoprecipitated whereas a third underlying protein is seen to become evident when greater amounts of S. aureus cells are used to bring down the antigen-antibody complex.

The sensitivity of antigen detection by immunoprecipitation is therefore limited by such background radioactivity. The primary cause is believed to be various polypeptides primarily in the form of glycolipids associating non-specifically with the immunoprecipitates (Kessler, 1981). Most certainly throughout the course of this study, it was observed that S. aureus cells - despite being stored at -70°C and washed prior to use to remove any dead cells - had an increased tendency to bind such "sticky proteins" with time. Thus a balance always had to be maintained between the possibility of a low intrinsic background and the sensitivity of antigen detection attainable.

5.4.2 Quantitation of Antibody Concentrations Required for Optimum Immunoprecipitation of Cytochrome P-450 Apoproteins.

The final parameter which had to be determined for each antibody was the optimal concentration required for antigen isolation. This varied between the different antibodies and although a molar excess of antibody to antigen was always used care also had to be exercised to ensure that subsequent resolution on SDS P.A.G.E. was not affected. To overcome the possibility that not all the antibody specified antigen was being extracted, immunoprecipitations were carried out
Figure 5.10 Analysis of S. aureus Aided Immunoprecipitation
of Phenobarbitone-induced Poly (A)+ mRNA Directed Translation

Track 1 = standard molecular weight proteins
Phenobarbitone-induced poly (A)+ mRNA was translated and subsequently
immunoprecipitated under a variety of conditions.
Track 2: immunoprecipitation with pre-immune IgG.
Track 3: precipitation with S. aureus cells alone (no antibody)
Track 4: Immunoprecipitation of precipitate from preclearing stage with 20μg anti-
cytochrome P-450 antibody and 100μl S. aureus cells.
Tracks 5, 6, 7: Immunoprecipitation with 20μg anti-cytochrome P-450 antibody
utilising 20, 80 and 200μl of S. aureus respectively to bind the antigen/antibody
matrix.

(S. aureus cells were prewashed and used as a 10% (w/v) suspension)
sequentially such that a minimum of two immunoprecipitations were employed. The majority of any antigen present would be extracted following the first immunoprecipitation, with the remainder being removed in the second. Subsequent immunoprecipitations occasionally indicated the presence of further antigen, but this was rare and the amounts negligible (figure 5.11). Subsequent isolations were shown to result in increased background which relates to the S. aureus cells themselves. No differences were observed in the immunoprecipitation of polypeptides when either S. aureus cells or protein A was used as the immunological matrix.

Both unfractionated antisera and IgG were used for antigen recovery. Apart from the fact that the IgG fractions could be used at far lower concentrations no specific differences were observed throughout the immunoprecipitation procedure (data not shown). A slightly higher level of background radioactivity was observed with whole antisera but this was countered by the more stringent washing procedures.

With anti-cytochrome P-452 antibody the optimal amount of antibody was approximately in the range 250-500 µg anti-sera per fraction immunoprecipitated (figure 5.12), although as little as 10 µg antisera was sufficient to pull down the antigen. In the case of the antibody against the phenobarbitone-induced cytochromes P-450, maximal efficiency of immunoprecipitation was seen with 20 µg IgG (data not shown). For this antibody fraction immunoprecipitation was attainable with as little as 0.01 µg indicating its high specificity for these forms. For the immunoprecipitation of BNF-induced polypeptides an anti-sera fraction was utilised at a concentration of 500 µg/assay (data not shown).
Clofibrate total RNA was isolated at 6,12,24,72 hours following induction, translated, and subsequently immunoprecipitated against anti-cytochrome P-452 antibody (tracks 4,5,6,7). Tracks 1 = standard molecular weight proteins, 2 = zero mRNA blank, 3 = 72 hour clofibrate RNA immunoprecipitated against pre-immune IgG. Four consecutive, identical immunoprecipitations were then carried out on the same fractions with the products then being analysed on SDS P.A.G.E. (Illustrations A,B,C,D).
Figure 5.12 Determination of the Antibody Titre of Unfractionated Sera in the Immunoprecipitation of Cytochrome P-452 Coded for by Clofibrate-induced Poly (A)$^+$ mRNA Within an in vitro Translation System.

Track 1 = standard molecular weight proteins.
Track 2 = zero mRNA blank.
Track 3 = pre-immune IgG immunoprecipitation (500µg).
Tracks 4-9 = immunoprecipitations with increased amounts of anti-P452 antibody utilising 1, 10, 100, 250, 500 and 1,000 µg/track respectively.
(from the original X-ray film, immunoprecipitation is evident from a concentration of 10 µg anti-P-452 antibody. Clofibrate poly (A)$^+$ mRNA was isolated following 18.5 hours induction, and translated at a concentration of 0.037µg/µl, 1.0 µCi $^{35}$S-methionine/µl).

<table>
<thead>
<tr>
<th>Antibody Concentration (µg)</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>250</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage cytochrome P-452 immunoprecipitated of total translation products.</td>
<td>0.28</td>
<td>0.41</td>
<td>0.72</td>
<td>0.89</td>
<td>0.40</td>
</tr>
</tbody>
</table>
All samples to be analysed by SDS P.A.G.E. analysis were boiled in sample buffer containing SDS and 2-mercaptoethanol reductant for 5 minutes. This effectively elutes any bound protein from the staphylococci so that only the immunoprecipitated peptides were analysed. If the resulting staphylococci pellet following such treatment was reuspended in buffer, recounted and analysed on SDS P.A.G.E. no trace of immunoprecipitated protein was evident, indicating that the separation of bound protein to S. aureus cells was absolute.

Quantitation of the immunoprecipitated complex was achieved by excising the region within the gel containing the immunoprecipitated protein. The gel slice was then rehydrated and the corresponding radioactivity leached out of the gel matrix by means of a gel solubiliser (Protosol, section 3.9.3). A corresponding area of the gel (within the 50-55,000 migratory molecular weight region) in which no radioactivity was present was also excised with every analysis, to serve as a blank control of background counts. Typically values for such blanks would be in the region of 1-200 counts/minute with this value being subtracted from all the immunoprecipitation obtained data. The manufacturers (NEN) estimate that greater than 80% recovery of radioactivity from gel slices is obtained with this protocol utilising Protosol.

5.5 SYNTHESIS OF THE COMPLEMENTARY DNA (cDNA) FROM PURIFIED POLY (A)+ mRNA DERIVED FROM CLOFIBRATE-INDUCED RAT LIVER

Poly (A)+ mRNA was isolated from rat liver by oligo (dT) chromatography undergoing subsequent fractionation on a linear sucrose gradient as described earlier in this chapter. This is to remove other RNA sequences present especially rRNA. Subsequent immunoprecipitation results in even further purification of the mRNA such that the final preparation was highly enriched for the clofibrate poly (A)+ mRNA sequence.

Because of the importance of cDNA, considerable effort has gone into defining conditions which optimise its synthesis. Unfortunately amongst all the different laboratories employing this technique no standardised procedure appears to be
available, no doubt reflecting the fact that different mRNAs are copied into DNA with different efficiencies. As such, the optimisation for the yield of cDNA from an RNA must be established for each analysis. In this section such optimisation for the synthesis of cDNA to clofibrate-induced mRNA is described.

5.5.1 First Strand Synthesis

The ultimate aim of this work was to determine those conditions required in order to get maximal cDNA yield and length, which would subsequently be used in cloning experiments. The first and most important parameter to be considered is that of the use of reverse transcriptase (RT). The ratio of reverse transcriptase to mRNA template as well as the time of incubation was found to be critical, as was the presence of different RNase inhibitors. Although the Avian myeloblastosis virus RT is supplied in a highly purified state inhibitors of RNase are still required. Both vanadyl-ribonucleoside complexes and sodium pyrophosphate were employed and shown to be effective in preventing the degradation of the mRNA template. To begin transcription of its template, RT requires an oligonucleotide primer with a free 3'-OH group. This was provided by using short oligo (dT) molecules 12-18 nucleotides in length which bind to the 3' end of the poly (A)+ mRNA. On subsequent initiation of the cDNA synthesis reaction the ratio of RT/µg RNA could then be determined. In this case an optimum time period of 90 minutes utilising 50 units RT/µg RNA was used (table 5.3).

Because the amounts of cDNA synthesised are so small (<1 µg) great care has to be taken to ensure all losses from the subsequent manipulations are kept to a minimum. This is especially true in the case of sephadex chromatography and phenol extraction. The sephadex column is used to remove all the unincorporated dNTP's. The elution profile is visualised as 2 peaks, the first, representing the as cDNA, the second being by far the largest, the remaining unincorporated nucleotides. Here a large column (10-12 inches, sephadex G-75) was shown to be a necessity as anything smaller resulted in poor separation. The phenol extractions
were carried out in the presence of 8-hydroxyquinoline which is a potent RNase inhibitor. On analysis of the organic phase following phenol extraction no ss cDNA was shown to be present. The ss cDNA is then ethanol precipitated, and sized on a alkaline agarose gel. A smear of radioactivity was observed (figure 5.13) ranging in size up to the largest species in the RNA preparation, indicating successful cDNA synthesis. For the clofibrate poly (A)$^+$ mRNA, the yield of the first strand reverse transcriptase reaction was generally between 25-50% of the weight of the poly (A)$^+$ RNA added, with the product having an approximate size of six to eight hundred base pairs. This is illustrated in the following autoradiograph of a 1.2% alkaline agarose gel in which 2 fractions of ss cDNA from different preparations are analysed.

(conditions of isolation were as previously described).
As indicated on the gel, reproducibility of synthesis of cDNA strands was obtained. The fractions were then utilised for the synthesis of the corresponding second strand.

5.5.2 Synthesis of Double Stranded cDNA (ds cDNA).

As before, the extent of ds cDNA synthesis can be monitored either by measuring the incorporation of label into TCA precipitable material or else by monitoring its decrease in electrophoretic mobility.

Essentially the ss cDNA is suspended in a buffered synthesis media (section 3.12.1) containing the Klenow fragment of E. coli DNA polymerase I (30 units per original µg RNA). The reaction mix is incubated at 15°C for 20 hrs. It is essential to ensure that the Klenow fragment concentration does not exceed 10-15% of the total reaction mix, otherwise synthesis can be inhibited by the glycerol and phosphate present within the storage buffer. The reaction temperature is kept low to minimise the possibility of DNA synthesising back upon itself, with the long time period allowing the enzyme to find the first strand cDNA molecules with hairpin loops at their 3' ends. This is because it is believed that these structures are both unstable and transient with the enzymes having to wait for and catch molecules in this unlikely configuration in order to begin synthesis of the second DNA strand (Maniatis et al. 1982). It is also advantageous to use the Klenow cleavage fragment of DNA polymerase I rather than DNA polymerase I itself because of the former's lack of 5' to 3' exonuclease activity. Both possess 3' to 5' nuclease activity essential for generating a suitable hairpin primer.

At this stage however, the length of the ds cDNA sequence is still unlikely to be complete because of the presence of strong-stop sequences in the cDNA which cause incomplete sequences within the synthesised second strand. To a certain extent this can be overcome by the subsequent use of reverse transcriptase which responds to different sequences than the Klenow polymerase. As such, a greater yield of full-length ds cDNA can be obtained. This is visualised in figure 5.13 which
Table 5.3

Dependance of Reverse Transcriptase and Incubation Time, upon ss cDNA Synthesis,
(as determined from nucleotide incorporation).

Reverse Transcriptase (AMV) Units.

<table>
<thead>
<tr>
<th>Assay 1</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td>Time (minutes)</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>0</td>
<td>.028</td>
<td>2.8</td>
<td>.023</td>
<td>2.23</td>
<td>.035</td>
<td>3.5</td>
<td>.017</td>
</tr>
<tr>
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<td>.258</td>
<td>25.8</td>
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<td>38</td>
<td>.154</td>
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</table>

Assay 2

<table>
<thead>
<tr>
<th>Time (minutes)</th>
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<th>B</th>
</tr>
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<tbody>
<tr>
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<td>.94</td>
</tr>
<tr>
<td>30</td>
<td>.195</td>
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<td>.266</td>
<td>26.6</td>
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<tr>
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<td>.322</td>
<td>32.2</td>
</tr>
<tr>
<td>120</td>
<td>.289</td>
<td>28.9</td>
</tr>
</tbody>
</table>

In both assays the mRNA template was highly purified clofibrate poly (A)^+ mRNA.

Column A represents μg ss cDNA synthesised per μg mRNA, column B represents percentage yield expressed as μg of product per μg of template. In assay 1 vanadyl ribonucleoside complex, and in assay 2 sodium pyrophosphate were utilised as RNase inhibitors. In assay 2, 50μg RT/μg RNA was used.
Figure 5.13  Optimisation of the Conditions Required for
the Synthesis of Full Length ds cDNA

Track 1 = ss cDNA (600 cpm)
2 = ds cDNA following Klenow fragment addition (700 cpm)
3 = ds cDNA following RT synthesis (1,350 cpm)
4 = Total ds cDNA (4700 cpm)

Analysis was carried out on a 1.2% alkaline agarose gel. The gel was exposed to X-ray film for 12 hours prior to developing.
shows the changes in size, as judged by electrophoretic mobility, of the ds cDNA following synthesis from the ss cDNA as obtained using the aforementioned techniques.

That the synthesis of the second strand was successful is shown in the following figure 5.14 which compares ss and ds cDNA on a 1.4% alkaline agarose gel. The length of the ds cDNA on the basis of the gel was seen to be approximately double that of the first strand with the size distribution of the ds cDNA being in the region of 1,200 to 1,500 base pairs. This is a result of the first and second strands being covalently joined by the hairpin loop which is subsequently cleaved with S1 nuclease.

Figure 5.14
Size Distribution of ss and ds cDNA Complimentary to Clofibrate-induced Poly (A)+ mRNA.

Tracks 1,2 represent the ss cDNA derived from clofibrate-induced poly (A)+ mRNA. Track 3 represents the corresponding ds cDNA sequence.
Having prepared the ds cDNA the next stage would be the digestion of the hairpin loop by $S_1$ nuclease activity followed by the generation of cohesive ends of the ds cDNA for ultimate insertion into a cloning vector. However it was decided to terminate the study at this point for a number of reasons. Firstly, by this stage experimental time remaining was a critical factor such that cloning analysis would just not have been possible. Secondly the direct objective of this study had only been the development and optimisation of methods for the synthesis of ds cDNA which would eventually be used for cloning analysis.

5.6 DISCUSSION

The primary aim of this chapter was to describe the methods developed and the results obtained in the course of characterising the RNA species coding for cytochromes P-450.

The first objective therefore was the isolation of RNA in an undegraded and active form. A typical mammalian cell contains about $10^{-5}$ µg of RNA of which 80-85% is ribosomal (chiefly 28s, 18s and 5s) with a further 10-15% consisting of a variety of low molecular weight species (tRNAs, hn RNAs etc). In contrast, the mRNA only makes up between 1-5% of the total RNA and as it is this form which is directly responsible for the assembly of amino acids into proteins a high yield is preferable. The success of the extraction, is ultimately governed by the degree of ribonuclease activity and as such the use of guanidinium hydrochloride was judged to be the most efficient means of isolating functional mRNA. The use of phenolic solvents to extract poly (A) containing RNA was not undertaken because of the greater potential for loss of the poly (A) tail from the RNA species during extraction (Perry et al, 1972). Animals were not starved prior to death. This was primarily because starvation has been reported to result in significant release of polyribosomes from the endoplasmic reticulum into the cytoplasm (Yap et al, 1978) but also because of the concomitant changes in glycogen, lipoprotein and hormonal levels. It was found to be critical to excise and homogenise the tissues with GHCl
as rapidly as possible as any delay resulted in accompanying RNA degradation. Subsequent GHCl/ethanol reprecipitations, were followed by 3M sodium acetate/ethanol precipitation and resulted in a successful deproteinisation of the RNA complex as judged by A$_{260/280}$ ratios. The RNA was occasionally further purified on CsCl gradients, although at this stage occasional problems of aggregation were encountered. This could be avoided to a large extent by heat denaturation of the secondary structure of the RNA prior to centrifugation. The precise reason for this is not known but is believed to relate to the interactions of the adenine-uracil rich regions of the molecules involved (Glisin et al. 1974). Further purification of the RNA was still required and this was achieved by two cycles of oligo (dT) chromatography. The resulting poly (A)$^+$ mRNA fractions were shown to be translationally active and only minimally contaminated with 28s ribosomal RNA.

It is of interest to note that all of the poly (A)$^-$ containing fractions isolated from the oligo (dT) purification of phenobarbitone-induced mRNA displayed not only total translational activity, but also activity coding for the phenobarbitone-induced cytochromes P-450. Undoubtedly this is representative of both competitive chromatographic binding and weak interactions of the RNA species for the oligo (dT) itself, as well as some mRNA chains having such short poly (A) segments that they are just not able to bind to the oligo (dT) adsorbent. In addition because the total RNA represents both nuclear and cytoplasmic species the poly (A)$^-$ fractions could conceivably contain RNA species not yet capped with a polyadenylate tail awaiting transfer across the nuclear membrane. Indeed, although the precise role of the poly A tail has still not been elucidated, it is known to confer resistance for the mRNA species against degradation during such transfer, although the tail itself has been shown not to be required for the efficiency of mRNA species promoting total polypeptide synthesis (Brawerman, G. 1981). Finally this could also be a result of ribonuclease action against the completed mRNA sequences but this is less likely due to the presence of RNase inhibitors throughout all the stages involved in the
extraction of poly (A)$^+$ mRNA.

Translation of the purified poly (A)$^+$ mRNA sequences allows elucidation of the correspondingly coded polypeptides. These \textit{in vitro} translation systems are highly prone to external influences (eg RNase contamination) however, and as such optimisation of the correct conditions for efficient, reproducible activity must be carried out. Excluding the possibility of RNase contamination, the most important parameters are those of energy requirement and salt concentration. The energy requirement is overcome by supplementing the system with an excess of creatine phosphate to catalyse the $\text{ADP} \rightarrow \text{ATP}$ reaction. (All told, it takes the equivalent of four high energy phosphate groups split to make one phosphate bond, half of this total being required to charge tRNA with amino acids, the other half being used for reactions occurring on the ribosome). The salt optimisation within the translation system is similarly an important factor. This is particularly important because minor variations from the optimum ion concentration can result in an increased missense error frequency (Pettersson et al. 1980) and in extreme cases total inhibition.

As only very small quantities of protein are produced by \textit{in vitro} translation of mRNAs, with it being extremely rare to be able to detect any corresponding biological activity it is therefore essential that an amino acid of very high specific activity is utilised. The label chosen was $^{35}\text{S}$-methionine, which although not the most predominant amino acid present within the amino acid composition of cytochromes P-450 does not present any problems in terms of overall incorporation into the synthesised polypeptides. This is because, (A) the label is always utilised in excess, (B) the methionine content of all the cytochromes P-450 sequenced to date is very similar (table 5.4).
### Table 5.4
Comparison of Methionine Amino Acid Composition of Different Cytochromes P-450.

<table>
<thead>
<tr>
<th>Species and form.</th>
<th>Number of Methionine residues per molecule</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat/Hepatic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-450b</td>
<td>11</td>
<td>Fujii-Kuryama et al. (1982)</td>
</tr>
<tr>
<td>P-450d</td>
<td>8</td>
<td>Kawajiri et al. (1984)</td>
</tr>
<tr>
<td>Rabbit/Hepatic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-450 LM&lt;sub&gt;2&lt;/sub&gt;</td>
<td>8</td>
<td>Tarr et al. (1983)</td>
</tr>
<tr>
<td>Rat/Mitochondrial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-450 (SCC)</td>
<td>13</td>
<td>Ogishima et al. (1983)</td>
</tr>
<tr>
<td>P-450 (11β)</td>
<td>13</td>
<td>&quot;</td>
</tr>
<tr>
<td>Rat/adrenal cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-450 (C-21)</td>
<td>11</td>
<td>&quot;</td>
</tr>
<tr>
<td>P-450 (17α,lyase)</td>
<td>8</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

The final constraint of the translation system which has to be considered is that of the assumption that all the mRNA species coding for their respective proteins are being translated with the same efficiency. This is very important because analysis of the translation products reveals the nature of the mRNA and the relative quantity of any particular kind of mRNA. Such translational competition has been reported in the synthesis of the liver proteins hemopexin, ferritin and albumin as analysed by quantitative immunoprecipitation of the corresponding mRNA coded translation products (Kaempfer et al. 1983). Here the relative translation yield of these proteins changed as a function of the amount of mRNA present, revealing the existence of translational competition between individual species of mRNA from the liver. Analysis indicated that this was due to competitive binding by the different RNA species towards an initiation factor (eIF-2). This functions in the indispensable role of binding the Met-tRNA-eIF complex to 40s ribosomal subunits during initiation, as well as possessing mRNA
binding properties and being able to recognise the ribosome binding site sequences in mRNA. Kaempfer et al (1983) further suggested that a relationship exists between the competitive strength of a given mRNA species and the degree of differentiation accompanying expression of the gene that encodes it. Whether or not this is the case for the translation of all mRNA species in vivo is not known, but should still be taken into consideration when analysing mRNA coded in vitro translated products.

The immunological characterisation of peptides synthesised by different cytochrome P-450 mRNAs has enabled the relationship between these proteins and their mRNAs as well as their subsequent characterisation to be undertaken. This relationship is based upon the recognition that specific antibodies have for distinct antigenic determinants. As such, a number of criteria have to be followed to ensure that the immunoprecipitated product is indeed a cytochrome P-450 including:

1. RNA extracted from a different source (i.e. globin mRNA), which does not synthesise cytochrome P-450, does not stimulate amino acid incorporation into a peptide that is immunoprecipitable.

2. The immunoprecipitated material is the same size as the corresponding authentic homogenous cytochrome P-450.

3. The immunoprecipitated material is specific for anti-cytochrome P-450 antibody. This criteria was fulfilled by immunoprecipitating the translation products of phenobarbitone coding poly (A)+ mRNA with anti-albumin IgG and a human γ-globulin fraction (isolated from my own sera). In both cases (data not shown) there was no evidence of any immunoprecipitated radioactivity. However when the total in vitro translation products from differently induced mRNA fractions were immunoprecipitated with different anti-cytochromes P-450 antibodies some cross reaction might be expected. This is because the cytochromes P-450 have many structural and functional characteristics in common, possibly including their substrate specificities, their heme binding sites and their binding sites for NADPH-cytochrome P-450 reductase. As such, it would be feasible to expect certain peptides having some antigenic
features in common, even though they are distinct peptides derived from different mRNAs. This subject is discussed in detail in the following two chapters.

The final analysis to be discussed here is that of the synthesis of cDNA to clofibrate-induced poly (A)$^+$ mRNA. The ultimate advantage of such a cDNA probe is that it can be used to quantitate the absolute levels of the corresponding mRNA within control or drug-induced animals. The first step in the isolation of a ds cDNA was that of enriching the mRNA population coding for cytochrome P-452. This was achieved by fractionation on a sucrose gradient where despite 2 passes through oligo (dT) cellulose considerable rRNA contamination was evident. On immunoprecipitation of the 18s fraction, only a very small percentage was shown to have activity coding for cytochrome P-452. It was this fraction which was used as a template for cDNA synthesis. A ss cDNA molecule of approximately 600-800 bp was synthesised whose size was doubled on synthesis of the second strand. Although reports (Payvar et al. 1979) have shown that fuller length transcripts can be obtained by first denaturing the mRNA with methyl mercury hydroxide this was not carried out. The length of the ds cDNA is relatively small but then again it is not known what size fragments would be obtained following $S_1$ nuclease digestion. Generally, reported values of cDNA sizes coding for the cytochromes P-450 range from 200-2000 bp. (Philips et al. (1983), Fujii-Kuriyama et al. (1981). Also on cloning it would be unlikely that a full length cDNA insert coding for cytochrome P-452 would be obtained. Amongst other things this is because 15-20 nucleotides corresponding to the extreme 5'-terminal region of the mRNA are invariably lost when the hairpin loop is digested with $S_1$ nuclease. At present, no full length cDNA copy for any cytochrome P-450 isoenzyme has yet been isolated, with the elucidation for the gene structure of those cytochromes P-450 now determined being achieved by overlapping cDNA sequences (Mizukami et al. 1983).
INDUCTION OF CYTOCHROME P-450 ISOENZYMES IN RESPONSE TO XENOBIOTIC ADMINISTRATION.

6.1 INTRODUCTION

In chapter 4, the induction and characterisation of hepatic microsomal cytochrome P-450 isoenzymes was undertaken. Moreover, this analysis was extended so that the same parameters were also investigated for specific purified cytochromes P-450. The results clearly indicated differences amongst the isoenzymic population on the basis of spectrophotometric and physical properties, peptide patterns generated by partial proteolysis, immunological reactivities and substrate specificity. Consequently the study was expanded towards the analysis of the mRNA fractions coding for the cytochromes P-450, in order to analyse what degree of coordinate induction exists in male Wistar rats following induction by a single dose of xenobiotic.

Such elucidation of the molecular events leading to an accumulation of cytochromes P-450 was achieved by using the techniques of in vitro translation with subsequent immunoprecipitation of the translated proteins. In this way it was possible to quantitate the level of functional cytochrome P-450 mRNA in response to single dose induction with time. Similar analysis of the induction of different cytochromes P-450 have been carried out by other research groups, but generally the results obtained exhibit significant variation concerning the time of maximum mRNA level and the extent of mRNA increase. Undoubtedly these differences are a reflection of different dosing regimens, species and strain differences and means of quantitation of the mRNA itself. This study hopes to circumvent some of these problems by standardising (as far as possible) the protocol for such quantitation following induction for all three inducers previously described in one rat strain.
Figure 6.1

Fluorographic Analysis of Translated mRNA Fractions following
A) Single Dose Phenobarbitone and, B) Single Dose Clofibrate Pretreatment.

Tracks 1 = standard molecular weight proteins, 2 = zero mRNA blank, 3 = uninduced mRNA (zero time point), 4,5,6,7 = mRNA fractions isolated following 6,12,24 and 72 hrs pretreatment with A) phenobarbitone, B) clofibrate respectively. All fractions were translated at a mRNA concentration of 0.156 µg/µl with approximately $2.5 \times 10^4$ acid precipitable cpm being loaded per track.
6.1.1 Time Course Induction Profile of Hepatic mRNA following Pretreatment with Phenobarbitone

Total RNA fractions were isolated at times 0, 6, 12, 24, and 72 hours following induction. These fractions were subsequently translated in vitro and the corresponding polypeptides analysed on SDS P.A.G.E. (figure 6.1). Analysis of the translation products of these differentially induced mRNA fractions indicated clear variations in the polypeptides synthesised. However, qualitative visualisation of specific individual polypeptides with time was more difficult. This was primarily a reflection of length of exposure of the SDS polyacrylamide gel against X-ray film, but also of the many translated products present (figure 5.6).

Fluorographic analysis of the translation of phenobarbitone-induced, compared to uninduced (zero time point) mRNA fractions, indicated prominent differences in polypeptides synthesised with apparent molecular weights in the region of 25 - 30K, 52 - 55K, 60K, and 77K with time (figure 6.1A). This in itself is not really surprising considering the potent inductive effect of barbiturates upon integral membrane proteins of the endoplasmic reticulum. Phenobarbitone itself is known to be responsible not only for the induction of specific cytochromes P-450, but also of NADPH-cytochrome P-450 reductase (Hardwick et al. 1983), glutathione-S-transferases (Pickett et al. 1982) and epoxide hydrolase (Pickett et al. 1981). The lower molecular weight polypeptide bands visualised are believed to represent the glutathione-S-transferase subunits. Here an increase in intensity of the polypeptide bands is seen with time with the lower molecular weight bands (25-30K) being the most markedly enhanced. The origin of the 60K band is unknown.

The above data is consistent with the previously determined phenobarbitone time course induction of glutathione-S-transferase B (Pickett et al. 1982). These workers showed that this particular isoenzymes exists as a heterodimer comprised of a Ya subunit (M.W. approximately 27K) and a Yc subunit (M.W. approximately 29.5K). Translatable hepatic glutathione-S-transferase B mRNA levels were shown to be maximally induced at 16 to 24 hours after a single injection of phenobarbitone.
to rats, with the Ya subunit being markedly enhanced in comparison to the Yc subunit.

The translated polypeptides in the molecular weight region of 52-55K represent the major phenobarbitone-induced cytochromes P-450. Within this area 2 bands are seen in the translation products of uninduced mRNA, with this number increasing to 3 possibly 4 polypeptides, by 24 hours following phenobarbitone induction. A subsequent decrease in the intensity of the synthesised polypeptides is seen thereafter. A similar, although much less intense pattern of induction is seen with the 77K molecular weight polypeptide. This is believed to represent the corresponding NADPH-cytochrome P-450 reductase protein as it migrates in the same position with the purified protein on SDS-PAGE (data not shown).

6.1.2 Time Course Induction Profile of Hepatic mRNA following Clofibrate Pretreatment

Analysis of the clofibrate time course translation products (figure 6.1B) revealed an overall similar but not identical complement of synthesised polypeptides. The presence of the proposed glutathione-S-transferase subunits was observed but at intensities not rising above that seen in the uninduced mRNA fraction, suggesting that no induction had occurred. The 60K polypeptide seen following 12 and 24 hours phenobarbitone-induction was not observed in any of the clofibrate time course fractions. Cytochrome P-450 isoenzyme induction was evident amongst the polypeptides of apparent M.W. 51-55K. Between 3-5 polypeptides are maximally induced within this region above basal levels, although as before visual qualification is difficult because of the extremely close proximity of these proteins.

6.1.3 Time Course Induction Profile of Hepatic mRNA following BNF pretreatment

BNF-pretreatment increased the translation of a polypeptide of apparent M.W. 55K, which corresponds to the major BNF-inducible cytochrome P-447 isoenzyme
isolated as described previously. A rapid induction of this isoenzymes level is seen within 6 hours of dosing with optimal levels being attained at approximately 12 hours, after which a decline is observed (figure 6.2). Within the cytochrome P-450 molecular weight region only one other polypeptide is visualised with an approximate molecular weight of 52K. The level of this polypeptide is seen to steadily decrease with time following dosing but at different rates than that observed for the cytochrome P-447 isoenzyme following BNF-pretreatment. This would appear to indicate that the mRNAs for these 2 polypeptides were under some different form of temporal control. A decrease in the intensity of polypeptide bands believed to represent the glutathione-S-transferase subunits was also evident following BNF-pretreatment.

6.2 TIME COURSE IMMUNOPRECIPITATION ANALYSIS OF HEPATIC mRNA TRANSLATIONS FOLLOWING PHENOBARBITONE PRETREATMENT

Quantitation of the expression of mRNAs coding for the cytochromes P-450 was achieved by their translation and subsequent immunoprecipitation. Fluorographic analysis enabled detection of the immunoprecipitated peptides to be made, which could then be extracted and quantitated. It should be noted that for such analysis the fluorographic exposure is far longer than required for total translation products, and as such in certain gels minor immunoreactive species may be visible. These are believed to represent non-specific background binding and are not a reflection upon antigen-antibody specificity.

Figure 6.3 shows the polypeptides immunoprecipitated by anti-cytochrome P-450 B2 IgG from the translation products of phenobarbitone-induced rat liver fractions. That the specificity of the antibody in the immunoprecipitation of these isoenzymes was absolute was confirmed by binding competition analysis with unlabelled purified cytochrome P-450 B2 (section 5.4.1). The radioactivity contained in the immunoprecipitated peptides was lowered as larger quantities of
Figure 6.2  Fluorographic Analysis of mRNA Fractions following Single Dose β-naphthoflavone Pretreatment

Tracks 1 = standard molecular weight proteins, 2 = zero mRNA blank, 3, 4, 5, 6 = mRNA fractions isolated following 6, 12, 24 and 72 hours BNF-pretreatment respectively.

All fractions were translated at a mRNA concentration of 0.156 μg/μl with approximately $3.5 \times 10^4$ acid precipitable cpm being loaded per track for SDS P.A.G.E. analysis.
Table 6.1  Induction of Rat Liver Cytochrome P-450 B<sub>1</sub>/B<sub>2</sub> mRNA by Phenobarbitone following Single-dose Pretreatment

<table>
<thead>
<tr>
<th>Treatment (hours)</th>
<th>Percentage of translatable mRNA activity immunoprecipitated with anti-cytochrome P-450 B&lt;sub&gt;1&lt;/sub&gt;/B&lt;sub&gt;2&lt;/sub&gt; antibody (a)</th>
<th>Increase (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.034</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>0.0504</td>
<td>1.48</td>
</tr>
<tr>
<td>12</td>
<td>0.113</td>
<td>3.32</td>
</tr>
<tr>
<td>24</td>
<td>0.245</td>
<td>7.2</td>
</tr>
<tr>
<td>72</td>
<td>0.205</td>
<td>6.02</td>
</tr>
</tbody>
</table>

Conditions for quantitation are as described in figure 6.3.

(a) - determined by dividing total immunoprecipitated activity (cpm) by the total amount of acid precipitable activity utilised in each immunoprecipitation analysis.
Figure 6.3A.
Time Course Induction by Phenobarbitone of Translatable P-450 mRNA following Single Dose Pretreatment

200K
92.5K
69K
46K
30K

1 2 3 4 5 6

Track 1 = standard molecular weight proteins,
Track 2 = zero mRNA blank. Tracks 3, 4, 5, 6 = mRNA fractions isolated at 6, 12, 24 and 72 hours following pretreatment. Immunoprecipitation was achieved with anti-cytochrome P-450 B1/B2 antibody. All preparations were translated and immunoprecipitated in duplicate. Photograph illustrates polypeptides immunoprecipitated in the first precipitation analysis.

Figure 6.3B.
Importance of Multiple Immunoprecipitations of In Vitro Translation Products for the Accurate Quantitation of Induced mRNA Cytochrome P-450 Levels

The above data is representative of the average determinations in the quantitation of cytochrome P-450 B1/B2 mRNA levels of following immunoprecipitation. A minimum of 5 x 10^5 cpm was utilised per translation assay using a concentration of 0.16 μg mRNA/assay.
unlabelled cytochrome P-450 was added (figure 5.9). This result confirmed the specificity of the immunoprecipitation reaction, and excluded significant non-specific adsorption of radioactivity on the immunoprecipitate.

At present, no inductive quantitation of the mRNA species coding for cytochromes P-450 in Wistar rats has been undertaken, thereby making comparison to other results impossible.

From the data obtained in this study, phenobarbitone pretreatment of Wistar rats resulted in a maximal increase in the mRNA's coding for cytochromes P-450 \( B_1/B_2 \) at 24 hours. At this point a 7 fold induction was evident with this level subsequently declining with time (table 6.1). A total of 3 peptides are immunoprecipitated, and as far as it is possible to qualitatively ascertain from SDS P.A.G.E. analysis it appears that they are equally induced with time. All three proteins are evident at 6 hours following pretreatment (bands very faintly discernable on photograph) with the upper two bands being believed to represent cytochromes P-450 \( B_1 \) and \( B_2 \). These immunoprecipitated polypeptides co-migrate with their homogeneous counterparts when analysed on SDS P.A.G.E. (data not shown). The third band possibly represents the product of a constitutive cytochrome P-450 mRNA and this will be discussed in the following chapter. It should also be noted that the presence of this latter protein was not detected in phenobarbitone-induced microsomal fractions when analysed by Western blotting.

The need to do multiple immunoprecipitations of the in vitro translation products should also be stressed (figure 6.3B), otherwise erroneous values in the quantitation of mRNA's could result due to incomplete polypeptide determination.

6.2.1 Time Course Immunoprecipitation analysis of hepatic mRNA following clofibrate pretreatment

The molecular pattern for clofibrate induction appears to be of a different nature to that observed following phenobarbitone and BNF-pretreatment. In the
Tracks 1 = standard molecular weight proteins, 2 = zero mRNA blank, 3 = 72 hour induced clofibrate mRNA immunoprecipitated with pre-immune IgG, 4,5,6,7 = mRNA fractions isolated at 6,12,24 and 72 hours following clofibrate administration immunoprecipitated with anti-cytochrome P-452 antibody. Translations were carried out using RNA concentrations of 0.16 µg/µl.

Values represent the average from two separate mRNA determinations. Cytochrome P-452 mRNA represents 0.068% of total uninduced mRNA.
Immunoprecipitation analysis by Anti-cytochrome P-452 antibody on the translation products of Uninduced and Clofibrate Pretreated (16 hours, single dose) mRNA Fractions.

Tracks 1 = standard molecular weight proteins, 2 = zero mRNA blanks. In illustration A, tracks 3,4 and tracks 5,6 represent the immunoprecipitated peptide from uninduced and clofibrate pretreated poly (A) mRNA fractions respectively. In illustration B, tracks 3,4 represent the immunoprecipitated peptides from uninduced and clofibrate pretreated poly (A) mRNA fractions respectively. The combined immunoprecipitation products of these different mRNAs is shown in track 5. All fractions were translated at a concentration of 0.037 μg/μl.
latter cases pretreatment stimulates the synthesis of corresponding mRNA's with a concomitant increase in protein synthesis. In the case of clofibrate pretreatment however, an initial drop in the level of mRNA activity coding for cytochrome P-452 is observed which does not regain basal levels until 24 hours following pretreatment. Even at 72 hours the level of coding cytochrome P-452 mRNA is only just slightly increased (figure 6.4). This is in direct contrast to the data obtained following single radial immunoquantitation of hepatic microsomes (table 4.11), which showed that despite the fact that cytochrome P-452 is present within uninduced hepatocytes at extremely high levels, a linear increase in the level of this cytochrome is observed with time (2 fold increase at 72 hours) upon pretreatment. This immunologically derived quantitative data however, does not correlate with the metabolic activity data of these microsomal fractions. In the case of lauric acid hydroxylation - a preferential substrate of cytochrome P-452 - an increase in metabolic activity is seen to increase only for a 12 hour period following pretreatment, after which a steady decline is observed.

One important consideration was to ensure that the immunoprecipitated peptide from the translation products of clofibrate-induced RNA fractions was the same as that obtained upon immunoprecipitation of uninduced RNA. Separate analysis, in which the immunoprecipitated products from clofibrate and uninduced RNA fractions were compared to the unlabelled cytochrome P-452 isoenzyme by migration on SDS P.A.G.E., indicated that this appeared to be the case. However when these immunoprecipitated products were analysed side by side on SDS P.A.G.E. (figure 6.5A), and also upon combination within the same track (figure 6.5B), this indication became slightly more tentative. The SDS P.A.G.E. data indicates that there is a possibility that in fact the two immunoprecipitated polypeptides are separate isoenzymes of nearly identical molecular weight, both being recognised by the anti-cytochrome P-452 antibody. At this point it should be stressed that the data from which this conclusion is derived from is not specific enough to be able to definitely say whether this is or is not the case. Its inclusion is important however,
Figure 6.6 Induction of Rat Liver Cytochrome P-447 by BNF following Single Dose Pretreatment

Table 6.2 Quantitation of mRNA Activity for Cytochrome P-447 and Unknown Higher Molecular Weight Peptide in BNF-induced mRNA Fractions

<table>
<thead>
<tr>
<th>Treatment (hours)</th>
<th>Percentage of translatable mRNA coding for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A) Cytochrome P-447</td>
</tr>
<tr>
<td></td>
<td>B) Higher molecular weight peptide</td>
</tr>
<tr>
<td>0</td>
<td>0.0101</td>
</tr>
<tr>
<td>6</td>
<td>0.078</td>
</tr>
<tr>
<td>12</td>
<td>0.132</td>
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<tr>
<td>16</td>
<td>0.112</td>
</tr>
<tr>
<td>24</td>
<td>0.106</td>
</tr>
<tr>
<td>72</td>
<td>0.041</td>
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<td>N.D.</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>0.062</td>
</tr>
</tbody>
</table>

N.D. = not determined

mRNA was translated at concentrations of 0.156 µg/µL.
because of the contrasting parameters which have already been observed during the induction process by clofibrate. Whether or not these factors are indicative of subtle heterogeneic changes within the cytochrome P-450 subpopulation is not known. All these factors will be brought together and discussed in detail in the following discussion chapter.

6.2.2 Time Course Immunoprecipitation Analysis of Hepatic mRNA following β-napthoflavone Pretreatment

Following BNF pretreatment an extremely rapid induction of mRNA activity coding for the major inducible BNF cytochrome P-450 is observed. Increased mRNA synthesis could be demonstrated as early as six hours after BNF administration with a maximum increase at between 12-16 hours (figure 6.6). Immunoprecipitation of the in vitro translation products of the time course mRNA fractions indicates the presence of one major protein which comigrates in SDS P.A.G.E. with the corresponding homogeneous protein purified from BNF-pretreated rats (data not shown). A second polypeptide of approximate molecular weight 70K is also immunoprecipitated by the anti-cytochrome P-447 antibody. Its induction appears to be independant and opposite to that of the mRNA coding for cytochrome P-447 (table 6.2). Induction of this peptide was seen to decrease when there was an accompanying increase in cytochrome P-447 mRNA activity and vice versa.

Visualisation of the immunoprecipitated cytochrome P-447 from uninduced mRNA translation products was not possible even with the aid of fluorography. Quantitation was achieved by excising the region of the SDS polyacrylamide gel which corresponded to the area of immunoprecipitation, as determined by using purified unlabelled cytochrome P-447 as a marker. Quantitation of the mRNA activity coding for the higher molecular weight polypeptide within uninduced RNA was not determined. The significance or identification of this protein is not known.
6.3 ANALYSIS OF DIFFERENTIALLY-INDUCED mRNA SEQUENCES CODING FOR SPECIFIC CYTOCHROMES P-450 WITHIN THE LIVER FOLLOWING PRETREATMENT BY DIFFERENT XENOBIOTICS

In the previous section quantitation of induced mRNA coding for distinct cytochromes P-450 following xenobiatic administration was determined. Induction by these compounds resulted in an increase (with time) of polypeptides synthesised which were recognised by corresponding antibodies raised against these forms. No immunoprecipitation was observed with pre-immune IgG or formalised Staph. aureus cells (Fagan et al. 1983). The specificity of the antibody directed immunoprecipitation of differentially induced mRNA in vitro translation products was extremely high as shown in figure 6.7 A,B,C. The first illustration shows the total acid-precipitated protein - which would include traces of Staph. aureus cells, sera, non-specifically bound proteins etc. - analysed on SDS P.A.G.E. for subsequent fluorographic analysis. The second illustration portrays the corresponding immunoprecipitated polypeptides, with their relative position in the original gel being shown last. The reasoning that the immunoprecipitated polypeptides represent the corresponding homogeneous proteins is based on several experimental factors. Firstly their co-migration was indistinguishable in electrophoretic mobility from corresponding purified cytochromes P-450 on SDS P.A.G.E. analysis (data not shown). Secondly the polypeptide is quantitatively removed from the translation products by precipitation with the corresponding antibody and finally, because the addition of an excess of non-radioactive purified cytochrome P-450 blocks only the immunoprecipitation of the corresponding radiolabelled protein.

This section is directed towards extending this analysis in order to determine whether or not the administration of a specific xenobiotic which is known to induce a cytochrome P-450 isoenzyme, also induces other mRNA species coding for other distinct cytochromes P-450. In these analysis, all the poly (A)+ mRNA fractions were isolated at 16 hours following xenobiatic pretreatment. Less stringent detergent washing procedures of the immunoprecipitated complex were employed to
Staphylococcus aureus Aided Immunoprecipitation Analysis of Uninduced and Clofibrate Pretreated Poly (A)^+ mRNA Coding In Vitro Translation Products

Figure 6.7A

Figure 6.7B

Figure 6.7A represents the total acid-precipitable fractions of the antigen/antibody/S. aureus complex loaded onto SDS P.A.G.E. as analysed by coomassie blue staining. Track 1 = zero mRNA blank, tracks 2,3 and 4 are representative of the translation products of uninduced mRNA; tracks 5,6 and 7 are representative of the clofibrate mRNA translation products, track 8 = standard molecular weight proteins.

Figure 6.7B represents the antibody immunoprecipitated products of the total translated fractions which were visualised in figure 6.7A. In figure 6.7B, track 1 = standard molecular weight proteins; track 2 = zero mRNA blank, tracks 3,4 and 5 are representative of the translation products of uninduced mRNA immunoprecipitated with anti-cytochromes P-450 B_1/B_2, P-447 and P-452 antibodies respectively, tracks 6,7 and 8 are representative of the translation products of clofibrate mRNA immunoprecipitated with anti-cytochromes P-450 B_1/B_2, P-447 and P-452 antibodies respectively.
Figure 6.7C illustrates the position of the immunoprecipitated peptides (figure 6.7B) in relation to the total acid-precipitable translation products (figure 6.7A), thereby illustrating the specificity of both the antibodies and technique in question.

Track 1 = standard molecular weight proteins; track 2 = zero mRNA blank, tracks, 3,4 and 5 are representative of the translation products of uninduced mRNA immunoprecipitated with anti-cytochromes P-450 B₁/B₂, P-447 and P-452 antibodies respectively; tracks 6,7 and 8 are representative of the translation products of clofibrate-induced mRNA immunoprecipitated with anti-cytochromes P-450 B₁/B₂, P-447 and P-452 antibodies respectively.
maximise the degree of immunoprecipitation. In some cases this resulted in an increase in background non-specific binding.

6.3.1 Uninduced Poly (A)$^+$ mRNA

Primary immunoprecipitation analysis of uninduced poly (A)$^+$ mRNA translation products with antibodies directed against cytochromes P-450 B$_1$/B$_2$, P-452 and P-447 is shown in figure 6.8A. Only one peptide is immunoprecipitated, of approximate M.W. 51,000, with the anti-cytochrome P-452 antibody. No visual immunoprecipitate was observed with the anti-cytochrome P-447 antibody. Occasionally the antibody directed against the phenobarbitone-induced cytochromes P-450 B$_1$/B$_2$ immunoprecipitated a peptide with an approximate molecular weight in the region of 48-50K (figure 6.8B). Subsequent analysis indicated that the immunoprecipitation of this peptide amongst different mRNA fractions translated was only associated with the cytochrome P-450 B$_1$/B$_2$ antibody. Its presence was shown to relate to the fact that the strict detergent washing protocol for the S. aureus/antigen/antibody complex which was found necessary for the immunoprecipitation of the cytochrome P-450 B$_1$/B$_2$ phenobarbitone peptides was not employed (section 3.9). If such washing procedures were utilised then the presence of this band was not apparent, indicating that it is highly likely that the presence of this peptide is the result of non-specific binding.

Because no visual immunoprecipitation was observed for peptides corresponding to cytochromes P-447 and P-450 B$_1$/B$_2$ this is indicative that either there is no mRNA present coding for these cytochromes P-450 within uninduced hepatic mRNA or alternatively that the amounts are too low for detection by this technique. Quantitation of the basal levels of these cytochromes P-450 was achieved by excising that portion of the SDS P.A.G.E. gel which corresponded to the unlabelled purified protein. This is in agreement with data obtained by other workers (Phillips et al. 1981, Gozukara et al. 1984). (N.B. a background gel slice
Figure 6.9A,B

Separate Immunoprecipitation of Uninduced and Clofibrate-Induced Poly (A)⁺ mRNA Translation Products by Antibodies Directed Against Cytochromes P-452, P-450 B₁/B₂ and P-447

In both gels tracks 1 = standard molecular weight proteins, 2 = zero mRNA blank. Gel A is representative of the immunoprecipitated products (first precipitation) of separately translated fractions of uninduced mRNA (tracks 3,4,5), and clofibrate mRNA (tracks 6,7,8). Immunoprecipitation was achieved with antibodies directed against cytochrome P-452 (tracks 3,6), cytochrome P-447 (tracks 4,8) and cytochrome P-450 B₁/B₂ (tracks 5,7). Gel B shows immunoprecipitated products obtained on a second immunoprecipitation with the same antibody on the same translated fraction. Tracks 3,4,5 represent uninduced mRNA translated fractions; 6,7,8 clofibrate mRNA translated fractions immunoprecipitated with anti-cytochrome P-450 B₁/B₂, P-447 and P-452 antibodies respectively.
count is determined and subtracted from all quantitations). Secondary immunoprecipitation of the same fractions with the corresponding antibodies showed further cytochrome P-452 immunoprecipitation (figure 6.8B). As before, the presence of any immunoprecipitated cytochrome P-447 was not detected.

6.3.2 Clofibrate-induced Poly (A)$^+$ mRNA

Clofibrate induction is seen to maximally express the cytochrome P-452 peptide, as was also identified in the uninduced mRNA translation (figures 6.8A,B; 6.9). No immunoprecipitable activity is seen with the anti-cytochrome P-447 antibody. As before the anti-cytochrome P-450 B$_1$/B$_2$ antibody recognises the 50K peptide.

It is apparent from the results obtained from this analysis that the induction of cytochrome P-452 is relatively unique as compared to that of the other cytochromes P-450 investigated. On clofibrate pretreatment, only the mRNA coding for this peptide is expressed with the level of mRNA coding for the other cytochromes P-450 remaining at or near that seen in the uninduced state. This would suggest - making the assumption that all mRNAs are being equally translated - that the genes transcribing for these particular isoenzymes are not being activated. Analysis of the mRNA population following phenobarbitone and BNF-pretreatment however, still shows the presence of actively translating mRNA species coding for cytochrome P-452 (figure 6.9). At 16 hours, relative to the uninduced mRNA level coding for cytochrome P-452 a corresponding decrease of 12 and 71 per cent was seen for this mRNA in phenobarbitone and BNF-induced fractions respectively. These differences would again suggest subtle differences in genetic control.
Figure 6.9

Immunoprecipitation Analysis of the Translation Products of Uninduced, Phenobarbitone, Clofibrate and β-napthoflavone-Induced Poly (A)$^+$ mRNA by Anti-Cytochromes P-450 B$_1$/B$_2$ and P-452 Antibody

Tracks 1 = standard molecular weight proteins, 2 = zero mRNA blank. The following tracks represent the immunoprecipitated products coded for by:-

3,7 = uninduced poly (A)$^+$ mRNA  
4,9 = phenobarbitone-induced poly (A)$^+$ mRNA  
5,8 = clofibrate-induced poly (A)$^+$ mRNA  
6,10 = BNF-induced poly (A)$^+$ mRNA.

Immunoprecipitations were carried out with anti-cytochrome P-450 B$_1$/B$_2$ antibody for fractions in tracks 3-6, and with anti-cytochrome P-452 antibody for fractions in tracks 7-10. Poly (A)$^+$ mRNA was translated at a concentration of 0.037 µg/µl.
6.3.3 Phenobarbitone-induced Poly(A)^+ mRNA

Following phenobarbitone-induction, immunoprecipitation analysis of the translation products shows the presence of 3-4 polypeptide bands when reacted against anti-cytochrome P-450 B1/B2 antibody. The lowest band (the 50K protein) was believed to result from the non-specific adsorption of the protein onto the immunoprecipitation complex, as its presence was easily removed by a detergent wash. The 2 upper bands represent the cytochrome P-450 B1 and B2 isoenzymes with the cytochrome P-450 B1 peptide being immunoprecipitated to the greatest extent (figure 6.10). The nature of the lower band - which has a molecular weight of approximately 50-51,000 - and which was shown to be present in all the RNA fractions isolated for the determination of the phenobarbitone mRNA time course induction profile, is unclear (figure 6.9) although the tenous possibility that this represents cytochrome P-452 cannot be entirely discounted (figures 6.10, 6.11A,B).

The fact that immunoprecipitation of the mRNA fractions isolated at 16 hours following phenobarbitone pretreatment with anti-cytochrome P-452 antibody, should reveal the presence of actively translating mRNA coding for cytochrome P-452, is not in itself surprising. This is for a number of reasons. First, data obtained during this study showed that cytochrome P-452 was immunologically detectable at high levels in an uninduced state, and even though phenobarbitone-induction resulted in a decrease of these levels, they were still detectable 72 hrs after single dose pretreatment (table 4.10, 4.11). This was also manifested in the metabolic activity profile of the induced microsomal fractions. This would suggest that phenobarbitone exerts an influence at the cytochrome P-452 genome level, in that although it does not appear to totally repress the gene coding for cytochrome P-452 it certainly possesses a modulatory role.
Figure 6.10 Immunoprecipitation Analysis of the In Vitro Translation Products of Liver Poly (A)$^+$ mRNA Isolated from Phenobarbitone-treated Animals

Tracks 1 = standard molecular weight proteins, 2 = zero mRNA blank, 3,4,5,6 = phenobarbitone-induced poly (A)$^+$ mRNA immunoprecipitated with pre-immune IgG, anti-cytochromes P-450 B$_1$/B$_2$, P-452 and P-447 antibody respectively. Poly(A)$^+$mRNA was translated at a concentration of 0.037 µg/µl with immunoprecipitation being achieved with Staphylococcus aureus cells.
Minimal washing of the immunoprecipitated complexes (figure 6.11A,B) was carried out in order to get maximal visualisation of precipitated proteins. Track 1 = standard molecular weight proteins; tracks 2,3 = immunoprecipitated products from phenobarbitone-induced poly (A)^+ reacted against cytochrome P-450 B_1/B_2 antibody; track 4 = immunoprecipitated products from phenobarbitone-induced poly (A)^+ mRNA reacted against anti-cytochrome P-452 antibody. The immunoprecipitated products visualised in this gel (figure 6.11A) are derived from the first antibody immunoprecipitation of the mRNA fraction concerned.
Sequential Immunoprecipitation Analysis of Liver Poly (A)+ mRNA following Phenobarbitone and Clofibrate-induction

The immunoprecipitated products visualised in this gel (figure 6.11B), unless otherwise indicated, are derived from secondary, identical antibody immunoprecipitations of the fractions concerned. Track 1 = standard molecular weight proteins. Track 2 = zero mRNA blank. Track 3 = immunoprecipitated products from phenobarbitone-induced mRNA reacted against anti-cytochrome P-452 antibody (corresponds to second immunoprecipitation of track 4 fraction, figure 6.11A). Tracks 4,6,8 represent the secondary immunoprecipitation products of phenobarbitone-induced mRNA reacted against anti-cytochrome P-450 B1/32 antibody (tracks 6,8 represent the secondary immunoprecipitated products of tracks 2,3, figure 6.11A). Track 5 (first immunoprecipitation), track 8 (second immunoprecipitation of mRNA fraction utilising track 5 of this assay), represent the immunoprecipitation products from clofibrate-Induced mRNA reacted against anti-cytochrome P-452 antibody.
The possibility that this peptide represents cytochrome P-452 is based on its migration within SDS P.A.G.E. in comparison to immunoprecipitated cytochrome P-452 from both phenobarbitone and clofibrate-induced RNA fractions (figures 6.11A,B). As such this experimental observation must be regarded as tenous and will be amplified upon in the following discussion chapter.

6.3.4 β-napthoflavone Induced Poly (A)+ mRNA

Analysis of the immunoprecipitation products derived from BNF-induced mRNA fractions indicates the presence of translatable mRNA with the capability of expressing all four forms of cytochromes P-450 analysed (figure 6.12). The peptides corresponding to cytochromes P-450 B and P-450 B are just visible in figure 6.12, with there also being precipitated a much larger amount of the 50K peptide. With anti-cytochrome P-447 antibody, 2 peptides are precipitated with the lower one corresponding to the cytochrome P-447 isoenzyme.

6.3.5 Expression of Hepatic mRNA Sequences Coding for Specific Cytochromes P-450 Subsequent to Xenobiotic Induction

With the development of the in vitro translation system in conjunction with immunoprecipitation utilising cytochrome P-450 specific antibodies, it has been possible to measure the levels of translatable cytochrome P-450 mRNAs and thereby characterise the temporal induction profile by phenobarbitone, clofibrate and β-napthoflavone in Wistar rats.

The data determined from the last part of this study detailing the levels of mRNA coding for the specific cytochromes P-450 as well as the overall fold increases of the mRNA over that seen in the uninduced state is given in table 6.3.

This data, in conjunction with the immunological and metabolic data obtained from the analysis of the pretreated microsomal fractions (chapter 4) will be analysed together, in detail, in the following discussion chapter.
Figure 6.12

Separate Immunoprecipitation Analysis of Liver Poly (A)$^+$ mRNA following Single Dose β-naphthoflavone Pretreatment

Track 1 = standard molecular weight proteins, 2 = zero mRNA blank. The following tracks represent β-naphthoflavone poly (A)$^+$ mRNA fractions separately immunoprecipitated against pre-immune (track 3), anti-cytochrome P-450 B$_1$/B$_2$ (track 4), anti-cytochrome P-452 (track 5) and anti-cytochrome P-447 (track 6) antibodies respectively. BNF-poly (A)$^+$ mRNA was translated at a concentration of 0.037 μg/μl.
Table 6.3  
Expression of mRNA Sequences Coding for Specific Cytochromes P-450 in Differentially Induced Liver mRNA Populations

<table>
<thead>
<tr>
<th>Hepatic mRNA isolated from animals treated</th>
<th>Cytochromes</th>
<th>Cytochrome</th>
<th>Cytochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P-450 B₁/B₂</td>
<td>P-452</td>
<td>P-447</td>
</tr>
<tr>
<td>Uninduced</td>
<td>0.034&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.068</td>
<td>&lt;sup&gt;b&lt;/sup&gt;0.0101&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>0.155 (4.56)</td>
<td>0.06 (0.88)</td>
<td>&lt;sup&gt;b&lt;/sup&gt;0.023 (2.27)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>N/D</td>
<td>0.061 (0.90)</td>
<td>0.0105 (1.03)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-naphthoflavone</td>
<td>0.016 (0.47)</td>
<td>0.024 (0.35)</td>
<td>0.107 (10.6)</td>
</tr>
</tbody>
</table>

All poly (A)<sup>+</sup> mRNA fractions were isolated at 16 hours following xenobiotic pretreatment. Percentage mRNA levels are calculated as the percentage of immunoprecipitated peptide of the total translation products (cpm). Numbers in parenthesis represent the increase (fold) of mRNA levels relative to the uninduced state. (a) - represents values which could only be maintained by excising the gel regions corresponding to the homogeneous proteins as all values are the mean of duplicates, except those marked b, in which only 1 determination was made.
6.4 Inductive Response of Kidney mRNA Populations following Specific Cytochrome P-450 Inducer Administration

Preliminary investigation into the inductive response of mRNA species coding for cytochromes P-450 in the kidney was also carried out. Some caution has to be shown in the analysis of the experimental data as it was found that the amounts of translational activity (in terms of acid-precipitable cpm) significantly affected the extent of peptide immunoprecipitation. This was due in part to the relatively low amounts of translational cpm utilised per fraction per assay ($1-5 \times 10^5$) as compared to the amounts used in the immunoprecipitation of liver mRNA fractions ($0.5 - 1.5 \times 10^6$ cpm). This was not believed to affect the immunoprecipitation of the cytochrome P-450 species preferentially expressed, but only those in which the level of expression is very low.

Figure 6.13 shows the translation products of total RNA isolated from rat liver and kidney pretreated with clofibrate for 16 hours. Full length peptides are visualised within the range of the SDS polyacrylamide gel indicating functional translational integrity of the isolated mRNA.

On immunoprecipitation of the translation products ($2 \times 10^5$ cpm) in uninduced RNA by preimmune and anti-cytochromes P-450 B1/B2, P-452 and P-447 the major peptide precipitated throughout was the 50K protein. Only anti-cytochrome P-452 precipitated any other peptide which could be visualised, with this corresponding to cytochrome P-452. Even here, the image obtained was extremely faint (data not shown). Quantitation as such, in order to assess the basal levels of any possible precipitated peptide was achieved by excising that area of the gel corresponding to the homogenous protein. The results of all the data, expressed as an an increase (fold) of mRNA over uninduced levels, found with the kidney mRNA species upon induction is shown in table 6.4.
Figure 6.13  Fluorographic Analysis of In Vitro Translation Products
from Liver and Kidney Total RNA Isolated from
Clofibrate-pretreated Animals

Tracks 1 = standard molecular weight proteins, 2 = zero mRNA blank, 3 = total liver RNA, 4 = total kidney RNA. Total RNA fractions were translated at a concentration of 0.156 µg/µl.
When kidney RNA isolated from clofibrate-pretreated animals was subjected to immunoprecipitational analysis (1 x 10^5 cpm), only the cytochrome P-452 peptide was precipitated (by anti-cytochrome P-452 antibody). This precipitated peptide comigrated with the corresponding precipitated peptide from liver RNA of clofibrate-pretreated animals. (figure 6.14A). However on increasing the number of translational counts for immunoprecipitation (4 x 10^5 cpm) the presence of both cytochrome P-450 B_1 and cytochrome P-447 could be detected when precipitated with the specific antibodies (figure 6.14B). The second precipitation also pulled down the 50K protein which had not been observed in the first precipitation analysis. The presence of cytochrome P-450 B_2 upon immunoprecipitation with the antibody directed against the phenobarbitone-induced isoenzymes was not visualised. With anti-cytochrome P-447 antibody, the upper 70K fragment which was seen upon the immunoprecipitation of cytochrome P-447 coding mRNA in liver, was also apparent.

Sequential immunoprecipitation of kidney RNA following phenobarbitone-induction was also undertaken (figure 6.15). The 50K band was immunoprecipitated by all the antibodies utilised, including pre-immune antisera, further suggesting that this does indeed represent a non-specifically adsorbed protein. No immunoprecipitation was observed with the anti-cytochrome P-450 B_2 antibody although this could be a reflection of the counts utilised in the translation assay (2 x 10^5). Anti-cytochrome P-452 antibody however, immunoprecipitated the cytochrome P-452 peptide indicating the presence of the mRNA coding for this isoenzyme in kidney following phenobarbitone-induction. The fact that this peptide was immunoprecipitated in contrast to the other isoenzymes, would also suggest that this is the predominant cytochrome P-450 isoenzyme present in this induced state.
Table 6.4

Increase in mRNA Induction over Uninduced Levels Coding for Cytochromes P-450 in Kidney RNA following Xenobiotic Pretreatment

<table>
<thead>
<tr>
<th>Kidney RNA Isolated from animals pretreated with:</th>
<th>Cytochromes P-450 B$_1$/B$_2$</th>
<th>Cytochrome P-452</th>
<th>Cytochrome P-447</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clofibrate</td>
<td>0.62</td>
<td>0.7</td>
<td>0.58</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>2.3</td>
<td>1.03</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Increase (fold) relates to relative increase in mRNA levels compared to those in the uninduced state.
Although only the presence of cytochrome P-450 B$_1$ was detected following immunoprecipitation with anti-cytochrome P-452 on clofibrate kidney RNA, the presence of the cytochrome P-450 B$_2$ form cannot be precluded.
Immunoprecipitation Analysis of Kidney Total RNA Fractions Isolated from Wistar Rats following Single Dose Clofibrate Pretreatment

In this assay the relative translational activity (cpm) per fraction approximated $1 \times 10^5$ cpm.

Tracks 1 = standard molecular weight proteins, track 2 = zero mRNA blank, track 3 = clofibrate-induced hepatic poly (A)$^+$ mRNA immunoprecipitated against anti-cytochrome P-452 antibody. Tracks 4, 5, 6, 7 represent the separate translation products of clofibrate total kidney RNA fractions immunoprecipitated against pre-immune (track 4), anti-cytochrome P-450 B$_1$/B$_2$ (track 5), anti-cytochrome P-452 (track 6), and anti-cytochrome P-447 (track 7) antibodies respectively.
For this assay the relative translational activity (cpm) per fraction was increased to approximately $4 \times 10^5$ cpm.

Track 1 = standard molecular weight proteins, Track 2 = zero mRNA blank. Tracks 3,4,5 represent the separate translation products of clofibrate total kidney RNA fractions immunoprecipitated against anti-cytochromes P-450 $B_1/B_2$ (track 3), anti-cytochromes P-452 (track 4) and anti-cytochrome P-447 (track 5) antibodies respectively. Total RNA fractions utilised in both assays (figures 6.14A,B) were isolated at 16 hours following clofibrate pretreatment.
Figure 6.15

Immunoprecipitation Analysis of Kidney RNA Isolated from Phenobarbitone-induced Wistar Rats with Pre-immune, Anti-Cytochrome P-450 B₁/B₂, P-452 and P-447 Antibodies

Track 1 = standard molecular weight proteins, track 2 = zero mRNA blank. Tracks 3,4,5,6 represent the separate translation products of phenobarbitone-induced (16 hours) total kidney RNA fractions immunoprecipitated against preimmune (track 3), anti-cytochrome P-450 B₁/B₂ (track 4), anti-cytochrome P-452 (track 5), and anti-cytochrome P-447 (track 6) antibodies respectively.
Chapter 7

DISCUSSION

The cytochrome P-450 mediated mixed-function monooxygenases of liver and kidney microsomal membranes are of central importance in the metabolism of a wide variety of endogenous and exogenous compounds. Our research groups interest has evolved from initial studies on the contribution of these distinct inducible cytochrome P-450 isoenzymes towards drug metabolism to an overall analysis of the biochemistry and biophysics of purified cytochrome P-450 catalysis. The study was initiated with a viewpoint to developing a purification protocol for the major phenobarbitone-inducible form(s) of cytochrome P-450. These isoenzymes were to be characterised and then contrasted to the already purified cytochrome P-450 isoenzymes (P-452 and P-447) available in this laboratory, with emphasis being placed on their mode(s) of induction. The resultant data obtained for both the development of the techniques utilised and the results relating to the induction and characterisation of these isoenzymes, was presented in the last three chapters. Accordingly the aim of this chapter is to analyse the complete data set in an attempt to provide a greater understanding of the processes involved in cytochrome P-450 induction.

7.1 PHENOBARBITONE INDUCTION

In recent years the molecular biology of cytochrome P-450 induction has been extensively studied, and evidence suggests that the major influence of phenobarbitone induction is to augment transcription of specific genes (Gonzalez et al. 1982). To date, a number of distinct phenobarbitone-induced hepatic cytochromes P-450 have been isolated from Sprague-Dawley rats (Waxman et al. 1982), Long-Evans rats (Ryan et al. 1982) and Wistar rats (this study). These purified hemoproteins can be distinguished from each other by minor structural differences although they share several common properties. The presence of these
isoenzymes - in unique combinations - is now known to be a characteristic of different strains and colonies of rats (Vlasuk et al. 1982). The two major forms isolated-designated cytochromes P-450<sub>B</sub> and P-450<sub>E</sub> (Ryan et al. 1982) - have now undergone successful cloning analysis such that the primary structure of these cytochromes P-450 has been elucidated (Fujii-Kuriyama et al. 1982, Yuan et al. 1983).

The cytochrome P-450<sub>E</sub> gene was shown to span at least 13Kb pairs and contain at least 9 exons separated by 8 introns. Except for the ninth exon (568-569 base pairs), all the exons are more or less similar in size, varying from 150 bp for the third exon to 201 bp for the first one. The sizes of the intron sequences are much more variable, ranging from 0.3 to 3.2 Kb. Comparison to the cytochrome P-450<sub>B</sub> cDNA clone indicated only 40 base substitutions in a sequence of approximately 1,900 bases, with 15 of them resulting in 14 amino-acid replacements. These replacements occurred in relatively limited regions of the gene sequences with most being found within exons 6,7,8 and 9 (Mizukami et al. 1983). Most of the amino acid replacements between the two isoenzymes were relatively conservative changes although four of the substitutions were thought likely to contribute either to structural changes or to charge differences between the two isoenzymes (Mizukami et al. 1983). Further analysis of the rat genome has suggested the presence of a further four genes or gene-like sequences which hybridise to phenobarbitone-induced cytochrome P-450 cDNA. The significance of these sequences is unknown. No protein product has been isolated from any of these sequences and they may in fact be pseudogenes (Mizukami et al. 1983).

Also of interest is the fact that the genes coding for these isoenzymes encode a cysteine-rich containing tridecapeptide segment which has also been observed in two dissimilar forms of rabbit cytochrome P-450 and a bacterial cytochrome P-450<sub>cam</sub> of Pseudomonas putida (Ozols et al. 1981, Fujii-Kuriyama et al. 1982). One of the rabbit liver cytochromes P-450 was a constitutive form (LM-3b), the other, a form induced by phenobarbitone (LM-2). Although a tempting speculation,
it is not possible to say at present whether or not compatible genes for corresponding cytochromes P-450 exist across species.

Cytochromes P-450 $B_1$ and P-450 $B_2$ were purified from the hepatic microsomes of phenobarbitone-induced Wistar rats in the current study. On extensive characterisation they were shown to be distinct isoenzymes (chapter 4) although still possessing many similar properties suggestive of cytochromes P-450$_b$ and P-450$_e$ isolated from other rat strains. However despite much compelling evidence these forms can not be conclusively shown to represent the aforementioned forms although the slight variations observed could be explained in terms of microheterogeneity across species (Vlasuk et al. 1982). Amongst the most important evidence for this degree of homology was that obtained from immunological studies. The antibodies utilised throughout this work had been extensively characterised and shown to be highly specific, therefore uniquely suited as probes to identify and discriminate between the functionally similar proteins such as the cytochrome P-450 isoenzymes. None of the antibodies prepared against the corresponding purified cytochromes P-450 cross-reacted with their heterologous antigens, with the only cross reactivity being seen between the cytochrome P-450 $B_1$ and P-450 $B_2$ forms. Both these findings are in agreement with the results obtained by other research groups involved in the purification of cytochromes P-450$_b$ and P-450$_e$ from other strains of rat (Ryan et al. 1982). Ouchterlony double diffusion analysis (figure 4.19) indicates that the cytochromes are immunochemically identical as determined by the lack of spurs at the junction of the immunoprecipitin bands. Both isoenzymes also form a line of identity with liver microsomes of rats treated with phenobarbitone. This cross specificity curtailed any individual quantitation of the specific phenobarbitone-induced isoenzymes by immunological means. This antibody specificity was also apparent in the antibody inhibition studies which were set up with a view to determining the contributions of these two isoenzymes to the benzphetamine N-demethylase activities of phenobarbitone-induced microsomes. Using the purified proteins it became apparent that at low antibody concentrations
the cytochrome P-450 B$_2$ hemoprotein was preferentially inhibited (30% of control activity as compared to 50% for the cytochrome P-450 B$_1$ form). This degree of inhibition however decreased with increasing antibody concentration such that equal rates of inhibition were observed for both isoenzymes. At this antibody level (5mg IgG/nmol P-450) approximately 20 per cent of control activity is observed, and the earlier differences thought to relate to the specificity of antibody binding to the corresponding antigenic epitope. In phenobarbitone-induced microsomes only fifty per cent inhibition could be observed at comparable antibody concentrations. This low degree of inhibition - considering that the antibody is known to recognise both phenobarbitone-induced isoenzymes which have a very high activity for benzphetamine turnover (table 4.6) - could reflect insufficient access to the antigen within the endoplasmic reticulum by the antibody. Alternatively, also far more likely is that this is indicative of the presence of other forms of cytochrome P-450 which also possess this ability for this substrates turnover. This antibody cross-specificity for the two phenobarbitone-induced isoenzymes has to date been found in all the different rat strains from which these isoenzymes have been purified.

Another similarity which these two cytochrome P-450 isoenzymes (B$_1$ and B$_2$) have in common with the phenobarbitone-induced isoenzymes from other strains is that of their interaction with metyrapone. It is now known that the selectivity of metyrapone in binding to hemoproteins parallels its selective inhibitory action on those monooxygenase activities which are phenobarbitone-inducible. Of the purified hemoproteins analysed, only the cytochromes P-450$_b$ and P-450$_e$ (this includes any microheterogeneous isoenzyme variation) have been shown to effectively bind this ligand (Luu-The et al. 1980). Values obtained for the apparent spectral dissociation constant (Ks) for the major purified cytochrome P-450 form have ranged from 1.09, 1.2 to 1.8 µM in Holtzman, Long-Evans and Sprague-Dawley strains respectively (Ryan et al. 1982a, Luu-The et al. 1980). In this study utilising Wistar rats a Ks value of 1.01 µM was reported for the major cytochrome P-450 B$_1$ form which is comparable to the above reported values. However for the cytochrome P-450 B$_2$
form a far lower value was obtained \((K_s = 5.56)\), suggesting differences in the active site of the two hemoproteins, resulting in a decreased affinity for ligand binding. When phenobarbitone-induced microsomes were analysed a \(K_s\) value of 0.69 \(\mu M\) was determined which is similar to that determined for phenobarbitone-induced microsomes isolated from Sprague-Dawley rats \((K_s = 0.6 \, \mu M, \text{Mitani et al. 1982})\). An inhibition profile of phenobarbitone-induced microsomes isolated at different time points was also carried out. Maximal inhibition was seen at a period of 24 hours following pretreatment at which point one also observes maximal induction of the phenobarbitone-induced cytochromes P-450.

However, despite these various parameters suggesting that cytochrome P-450 \(B_1\) represents P-450\(_b\) and cytochrome P-450 \(B_2\) represents P-450\(_e\) some anomalies in characterisation have been shown to exist. These relate to the cytochrome P-450 \(B_2\) form, with the first relating to its apparent sub-unit molecular weight. In this study the size of cytochrome P-450 \(B_2\) was shown to be 2000 \(K\) larger than the corresponding P-450 \(B_1\) form. In other strains the relative differences in size only tend to differ by about 500 \(K\) (Ryan et al. 1982). These differences could simply be the result of anomalous behaviour on SDS PAGE, with such difficulties associated with this isoenzyme having already been reported (Levin et al. 1984). The second difference was that of catalytic activity in that cytochrome P-450\(_e\) has been reported to exhibit only 15-20 per cent of the activity of cytochrome P-450\(_b\) (Ryan et al. 1982). For the isoenzymes isolated from Wistar rats a level of 80 per cent activity is attained. Whether these differences are simply due to a microheterogeneous variation across different strains or alternatively whether or not these proteins do in fact represent distinct and unrelated isoenzymes cannot be fully ascertained at present.

The molecular mechanisms governing, phenobarbitone-induction of cytochrome P-450 is still not fully understood (Rees, D., 1979) although it has been suggested that activation of gene transcription plays an important role (Hardwick et al. 1983). This conclusion is based on the observations that mRNAs coding for epoxide
Table 7.1
Summary of the Reported Data on Determinations for mRNA Coding for Phenobarbitone-inducible Forms of Cytochrome P-450

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>System</th>
<th>Phenobarbitone dose (mg/kg body weight)</th>
<th>Peak of cytochrome P-450 synthesis or of cytochrome P-450 mRNA Time (hours)</th>
<th>Increase (fold)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprague Dawley</td>
<td>In Vitro (polysome)</td>
<td>75</td>
<td>12-18</td>
<td>5</td>
<td>Colbert et al., 1979.</td>
</tr>
<tr>
<td>Sprague Dawley</td>
<td>In Vitro (RNA)</td>
<td>40</td>
<td>24</td>
<td>10</td>
<td>Gozukara et al., 1984.</td>
</tr>
<tr>
<td>Sprague Dawley</td>
<td>In Vitro (RNA)</td>
<td>75</td>
<td>12</td>
<td>16</td>
<td>Omiecinski et al., 1981.</td>
</tr>
<tr>
<td>Sprague Dawley</td>
<td>Hybridisation (filter)</td>
<td>75</td>
<td>12</td>
<td>15</td>
<td>Omiecinski et al., 1983.</td>
</tr>
<tr>
<td>Long Evans</td>
<td>Hybridisation (RNA)</td>
<td>100</td>
<td>20</td>
<td>30</td>
<td>Adesnik et al., 1981.</td>
</tr>
<tr>
<td>Sprague Dawley</td>
<td>In Vitro (RNA)</td>
<td>100</td>
<td>20-30</td>
<td>8</td>
<td>Morohashi et al., 1984.</td>
</tr>
<tr>
<td>Sprague Dawley</td>
<td>Hybridisation (dot)</td>
<td>100</td>
<td>20-30</td>
<td>10</td>
<td>Morohashi et al., 1984.</td>
</tr>
<tr>
<td>Wistar</td>
<td>In Vitro (RNA)</td>
<td>125</td>
<td>24</td>
<td>7</td>
<td>Present study.</td>
</tr>
</tbody>
</table>

In Vitro means cell free translation using various RNA sources such as shown in parenthesis. Hybridisation indicates DNA-RNA hybridisation analysis by the method indicated in parenthesis. The table lists the time when a maximum level of phenobarbitone-inducible cytochrome P-450 mRNA as well as the extent of increase was attained after phenobarbitone injection.
hydratase, NADPH-cytochrome P-450 reductase and cytochrome P-450\textsubscript{b} are rapidly elevated after xenobiotic pretreatment (Gonzalez et al. 1981) and that this elevation is blocked by the RNA synthesis chain terminator 3'-deoxyadenosine. In addition, prior to the appearance of elevated levels of the major phenobarbitone-induced cytochrome P-450\textsubscript{b} mRNA in the cytoplasm, a marked increase in intranuclear pre mRNA species is noted (Gonzalez et al. 1982). After phenobarbitone-pretreatment, the cytochrome P-450\textsubscript{b} gene was transcriptionally activated within 60 min, with maximal transcription (23 fold) being attained at 3 hours after dosing. The corresponding increase in cytochrome P-450\textsubscript{b} observed paralleled the appearance of increased mRNA within the cytoplasm. The transcription of the gene continues for a period of up to 24 hours, at which time point a 14 fold increase in activity over basal levels is still observed (Hardwick et al. 1983).

In this study, analysis of the \textit{in vitro} translation products of isolated hepatic mRNA sequences at different time points, indicated that phenobarbitone-induction (and clofibrate and β-naphthoflavone) did not seem to significantly alter the template activity of total liver mRNA. This is in agreement with other studies (Phillips et al. 1981), although selective specific increases in the level of translatable mRNA for several polypeptides is apparent. The quantitation for the expression of these different mRNAs was determined by immunoprecipitation analysis. This technique has been shown to accurately reflect the increase of induction of mRNAs in that comparable results have been obtained utilising techniques in which analysis is both at the RNA and DNA level (table 7.1).

Immunoprecipitation analysis of these time course fractions indicated that maximal induction of the mRNAs coding for the cytochromes P-450 B\textsubscript{1}/B\textsubscript{2} occurred at 24 hours following single dose phenobarbitone-pretreatment. With pretreated Sprague-Dawley rats comparable values of between 16-24 hours have also been reported (Dubois et al. 1981, Gozukara et al. 1984). With Wistar rats, immunoprecipitation analysis indicated that three polypeptides were recognised by
the anti-cytochrome P-450 B₂ antibody. The lowest band which was induced to the least extent - possibly represents a constitutive from of cytochrome P-450, with the upper 2 bands representing cytochromes P-450 B₁ and P-450 B₂ respectively. The reasoning behind this conclusion is that this lower band represents a constitutive cytochrome P-450 form - possibly cytochrome P-452 - is only based upon co-migratory evidence within SDS P.A.G.E. (figure 6.10), and as such has to be considered speculative. Its presence is difficult to explain however, in that although clearly evident at the induced mRNA level, it is not recognised by the same antibody in the microsomal fractions following phenobarbitone pretreatment upon Western blot analysis. This cannot simply relate to its relatively low level of induction - as judged by the fact that its presence was only seen during a first immunoprecipitation analysis (figures 6.11A,B) - in that the sensitivity of Western blot analysis against microsomal fractions is extremely high.

Alternatively, it may simply represent another member of the phenobarbitone-induced cytochrome P-450 isoenzyme family possessing similar epitopic determinants (such as seen for the cytochromes P-450 B₁/B₂) which makes it accessible to recognition by the anti-cytochrome P-450 B₂ antibody. The fact that its presence is not seen in the microsomal fractions after induction and Western blotting analysis could be explained in a number of ways. Analysis of the NH₂-terminal residue of cytochrome P-450ₐₐ (Haugen et al. 1982, Heinemann et al. 1983) has shown it to be rich in methionine, and as this was the radioactively labelled amino acid utilised during the translation experiments one would expect the corresponding radiolabelled apoprotein to be easily detected after immunoprecipitation and fluorographic analysis. One possibility therefore, could be that removal of the transient signal sequence from the translated apoprotein occurs, which is essential for the co-translational insertion of the cytochrome P-450 into the endoplasmic reticulum (Bar-Nun et al. 1980). This transient signal sequence present in a vast number of nascent secretory peptides tends to be rich in hydrophobic amino acid residues and is located usually at the NH₂-terminal end.
(Blobel et al. 1975). Its removal - possibly by some form of proteolytic degradation process - would result in the particular isoenzyme not being able to insert itself into the endoplasmic reticulum environment required for its activity. Subsequent breakdown within the cytoplasm would then occur. Alternatively, it could be that there is an absence of a signal recognition particle which is a ribonucleoprotein complex required in addition to the transient signal sequence for the co-translational insertion of the cytochrome P-450 into microsomal membranes (Walter et al. 1984). The importance of such a complex has been demonstrated for the co-translational insertion of the major phenobarbitone-inducible form of rabbit liver microsomal cytochrome P-450 into heterologous microsomal membranes (Sakagucki et al. 1984). A final possibility could also be that the insertion of the cytochrome P-450 isoenzyme into the endoplasmic reticulum results in the blocking of the isoenzymes antigen binding site(s) which is being recognised by the anti-cytochrome P-450 B2 antibody.

Monitoring the time course of mRNA induction coding for the phenobarbitone-induced isoenzymes shows an identical profile to that of the immunochemically quantitated isoenzymic levels (figure 7.1). In both cases maximal induction is attained at 24 hours following pretreatment, at which stage a gradual decline is observed. This induction was reflected within microsomes by a corresponding increase in benzphetamine N-demethylase activity which is a preferential substrate for the phenobarbitone-induced cytochrome P-450 isoenzymes. Immunochemical quantitation of these isoenzymes indicates an extremely rapid induction with time with the levels attained being similar to those found within other rat strains upon induction. Following pretreatment, a greater than 6-fold increase in mRNA translation is observed within 6 hours. This rapid rise (table 4.10) coupled with the data now known about the rapid increase in transcription of specific cytochrome P-450 genes (Hardwick et al. 1983) followed by translation of the induced mRNA's (table 6.2) is strongly indicative of a inducer-receptor complex being associated with phenobarbitone-induction. However, no hard evidence has emerged to support this
At present there is no available method to differentiate quantitatively the level of cytochrome P-450\textsubscript{b} in rat liver microsomes from that of cytochrome P-450\textsubscript{e}. Western blotting analysis (figure 4.21) suggests that at basal levels, the cytochrome P-450 B\textsubscript{2} form is the major isoenzyme present, with equal levels of the isoenzymes only being seen at between 6-12 hours following pretreatment. At this onwards point qualitative visualisation suggests that the cytochrome P-450 B\textsubscript{1} isoenzyme is the major form present. This is in contrast to the observed induction of these isoenzymes within rat liver microsomes of Sprague-Dawley rats. Here it has been suggested (Vlasuk et al. 1982) - on the basis of SDS P.A.G.E. analysis -that regardless of changes in the absolute level, the ratio of these two isoenzymes remains relatively constant. It is striking though, that these two isoenzymes of apparent immunochemical identity, peptide mapping patterns and amino-acid sequences, still appear to be synthesised from independent mRNA molecules, not appearing to undergo significant post-translational modifications being expressed as co-dominant alleles as determined from mating experiments (Walz et al. 1982, 1982a).

Monitoring the metabolic activity of the microsomal fractions shows an initial drop in benzphetamine turnover activity up to 6 hours, with the turnover only increasing above basal levels after this period of time. This difference suggests that the association of heme with the apoprotein may be a rate-limiting event, a conclusion also speculated upon by other research groups (Dubois et al. 1979). The continuing increase in specific content (24 to 72 hours) as compared to a gradual decline in the level of immunologically detectable cytochrome P-450 (radial immunodiffusion) and mRNA levels probably reflects the continued induction of other M.F.O. enzymes (Gozukara et al. 1984). The fact that there is still an increase in metabolic activity at 72 hours despite the decrease in mRNA levels, and to a certain extent immunologically detectable cytochrome P-450 B\textsubscript{1}/B\textsubscript{2} (figure 7.1), is believed to relate to the in vivo turnover of both the apoprotein and heme
Figure 7.1

The Induction of Hepatic Cytochromes P-450 B₁/B₂ following Single-dose Phenobarbitone Pretreatment

(A) represents increase (fold) of immunoprecipitable mRNA activity coding for cytochromes P-450 B₁/B₂.

(B) represents increase (fold) of cytochromes P-450 B₁/B₂ in phenobarbitone-induced microsomes as determined by single radial immunodiffusion assay.

(C) represents increase (fold) in benzphetamine N-demethylase activity within phenobarbitone-induced microsomes.

All values represent increases above basal levels. (Data from chapters 4 and 6).
moieties of the cytochrome P-450 isoenzymes, as well as the contribution of other cytochromes P-450 towards benzphetamine demethylation. The turnover of hepatic cytochromes P-450\textsubscript{b} and P-450\textsubscript{e} within Long-Evans rats has been shown to correspond to half lives of 37 and 28 hours for the apoprotein and heme moieties respectively (Parkinson et al. 1983). The involvement of other isoenzymic cytochromes P-450 in benzphetamine turnover has already been shown both by the antibody-inhibition data (section 4.5.3) as well as reconstitution analysis with the purified cytochrome P-450 isoenzymes (table 4.6).

The inhibition data showed that the cytochromes P-450 B\textsubscript{1}/B\textsubscript{2} were responsible for an important part of the benzphetamine N-demethylase activity in phenobarbitone-treated rat microsomal fractions, but that there were also other isoenzymic forms present with the potential for such activity. This is in contrast to studies in Long Evans rats where 100% inhibition of benzphetamine N-demethylase activity was obtained with the anti-cytochrome P-450\textsubscript{b} antibody (Thomas et al. 1976). The precise reason for this discrepancy is unclear, but is not believed to relate to non-antibody specificity especially since other homogeneous cytochromes P-450 were also shown to possess this metabolising activity (table 4.6), albeit at far lower amounts.

Interestingly, on the basis of the single radial immunodiffusion and metabolism data (chapter 4), it is possible to conclude that phenobarbitone acts not only at the molecular level for the genes coding for cytochromes P-450 B\textsubscript{1}/B\textsubscript{2}, but also has the capability for regulating other cytochrome P-450 isoenzymic gene activity (figure 7.2). Analysis of the mRNA sequences within animals pretreated for 16 hours with phenobarbitone (table 6.5) indicates that the mRNA sequences coding for cytochrome P-452 remain at or near basal levels, whereas those coding for cytochrome P-447 have undergone a 2-fold increase. These results however, are not entirely consistent with the metabolic and immunochemical quantitation data (figure 7.2) suggesting that other regulatory mechanisms may be involved.

The level of immunoquantitatable cytochrome P-452 is seen to rapidly
diminish (24% of basal levels following 6 hours pretreatment), and although a slight increase is observed at 12 hours the isoenzymic levels further decrease after this period. Cytochrome P-452 is preferentially induced following clofibrate pretreatment, and has been shown to be involved in the \( \omega \) and \((\omega-1)\)-hydroxylation of saturated fatty acids such as lauric acid within the liver (Gibson et al. 1982). Purified cytochrome P-452 preferentially hydroxylates lauric acid at the (12-) position. The presence of cytochrome P-452 within the complement of mRNA species induced by phenobarbitone-pretreatment has also been conclusively shown for the first time by immunoprecipitation analysis (figure 6.9). However despite the fact that there is a relative decrease in immunologically detectable cytochrome P-452, there is a corresponding increase in total 11- and 12-lauric acid hydroxylation activity with time (figure 7.2). This would strongly support the hypothesis put forward that separate cytochromes P-450 catalyse the two fatty acid-hydroxylation reactions in liver microsomes (Björkhem et al. 1970, Okita et al. 1980).

Phenobarbitone-induction results in a maximal 1.5 fold increase in 11- and 12-hydroxylation of lauric acid, a value in agreement with other authors (Orton et al. 1982). HPLC analysis (figure 4.11B) indicated that following pretreatment with phenobarbitone there was a decrease in 12-hydroxylaurate production and a preferential increase in 11-hydroxylation for a period up to 24 hours. This is also in agreement with other studies, which have shown that phenobarbitone causes a small but preferential increase in 11-hydroxylauric formation (Orton et al. 1982). By 72 hours however, this product ratio was reversed in that metabolism was preferentially occurring at the 12-hydroxy position. This reversibility further lends credence to the belief that two different isoenzymes are induced, with both being under independent genetic control.

Phenobarbitone does not appear to exert any specific effect on the gene coding for cytochrome P-447. Although a slight increase in the expression of its corresponding mRNA was observed, the relative immunologically detectable proportion of the isoenzyme stayed at basal levels. Some metabolic activity (figure
Figure 7.2

The Induction of Hepatic Cytochrome P-452 (A) and Cytochrome P-447 (B) within the Microsomal Fractions of Rats Pretreated with a Single Dose of Phenobarbitone

(A) Cytochrome P-452

(B) Cytochrome P-447

(1) Represents the quantitative determination (single radial immunodiffusion) of the corresponding cytochrome P-450 isoenzyme following phenobarbitone-pretreatment.

(2) Represents the metabolic profile of the cytochrome P-450 isoenzymes with their preferred substrates following phenobarbitone-pretreatment. For cytochrome P-452 the substrate was lauric acid (total 11- and 12-hydroxylation) and for cytochrome P-447 7-ethoxyresorufin.

All values represent increases above basal levels. (Data taken from chapter 4).
7.2) was observed but this decreased with time and is more likely to be a reflection of cross-substrate specificity amongst different isoenzymes.

In conclusion, it was demonstrated in this study that the purified cytochrome P-450 preparation from male Wistar rats is comprised of two major polypeptides. Immunoprecipititational analysis of the induced mRNA species corroborates this finding and further suggests the possible presence of one other minor form of phenobarbitone-induced cytochrome P-450. The levels of mRNA coding for these phenobarbitone-induced isoenzymes represent 0.034% of the total hepatic poly (A)$^+$ RNA isolated from control animals. This is in close agreement to the levels (0.029%) found in male Sprague-Dawley rats (Shephard et al. 1982). After induction the two predominant cytochrome P-450 isoenzymes become major microsomal polypeptides. This is further corroborated by metabolic and immunological data. All three polypeptides are the products of distinct mRNAs. These mRNAs are at very low levels in untreated rats but accumulate rapidly in response to the acute administration of phenobarbitone.

This data is consistent with the studies of Ades et al. (1981) and Phillips et al. (1985). These groups utilised cDNA clones to quantitate the level of cytochrome P-450 mRNA as a function of phenobarbitone-induction. The induction of the phenobarbitone isoenzymes was shown to be mediated by an induction of the specific mRNAs, with this being accounted for almost entirely by an increase in the transcription of the phenobarbitone cytochrome P-450 genes.

7.2 CLOFIBRATE INDUCTION

Clofibrate has been administered to human patients as a hypolipidaemic agent, although its precise mechanism of action still remains unknown. Within rats it has been shown to cause hepatomegaly and marked proliferation of peroxisomes in liver cells (Moody et al. 1978). The peroxisomal proliferation is almost always associated with an increase in peroxisomal enzyme activities including the fatty acid $\beta$-oxidation system (Reddy et al. 1984), as well as increasing a number of mixed
function oxidase enzyme activities (Orton et al. 1982). Of these, the major cytochrome P-450 isoenzyme induced appears to represent a form termed cytochrome P-452 (Gibson et al. 1982). This form has since been extensively characterised and shown to be representative of a unique cytochrome P-450 isoenzyme (Tamburini et al. 1984). Evidence obtained in this laboratory is also highly suggestive that cytochrome P-452 represents a constitutive cytochrome P-450 isoenzyme, and that many hypolipidaemic agents function as inducers of constitutive hemoprotein isoenzymes.

Although considerable understanding of the action of clofibrate at a biochemical level has now been attained (Cohen et al. 1981, Reddy et al. 1984), very little or no work has been carried out at the molecular level.

The induction profile following clofibrate pretreatment was the most complex of the three xenobiotic inducers studied. This was primarily because the molecular pattern appears to be of a different nature to that observed following phenobarbitone and BNF-pretreatment. In these latter cases pretreatment was shown to stimulate the synthesis of corresponding mRNAs with a concomitant increase in protein synthesis. In the case of clofibrate-induction however, an initial decrease in the level of mRNA coding for cytochrome P-452 was observed concomitant with an increase in both immunologically detectable cytochrome P-452 and an increase in lauric acid hydroxylation (figure 7.3).

Single radial immunoquantitation (table 4.11) showed that the basal levels of cytochrome P-452 in hepatic microsomes was high (25% of total cytochrome P-450). Upon clofibrate-induction a 2-fold increase in cytochrome P-452 was seen. This high level of cytochrome P-452 in uninduced microsomes is in direct contrast to the amounts of other well characterised isoenzymic forms, and for this reason is believed to represent a constitutive form. For example, cytochromes P-450\textsubscript{b\,\,}, P-450\textsubscript{c} and cytochrome P-450\textsubscript{c} only occur to the extent of approximately 1-2% in uninduced microsomes (Thomas et al. 1983), with these levels being increased to 55% and 71% respectively of the total cytochrome P-450 population upon
Figure 7.3 Induction of Hepatic Cytochrome P-452 following Single Dose Clofibrate Pretreatment

A) Represents increase (fold) of immunoprecipitable mRNA translational activity coding for cytochrome P-452.

B) Represents increase (fold) of cytochrome P-452 within clofibrate induced microsomes as determined by single radial immunodiffusion assay.

C) Represents increase (fold) in total 11- and 12- lauric acid hydroxylation activity in clofibrate-induced microsomes.

All values represent increases above basal levels. (Data is from pertinent result sections, chapters 4 and 6).
corresponding xenobiotic induction. That this immunoquantitation is an accurate reflection, is based entirely upon the specificity of the antibody utilised. The specificity is believed to be absolute however; not only on the basis of Ouchterlony analysis and the presence of single radial immunodiffusion rings, but also from the data obtained from Western blot analysis of the induced microsomal fractions. In a different study (Bains et al. 1985) clofibrate induction (clofibrate was administered as 0.4% (w/w) of standard powdered laboratory diet for 14 days) was also shown to induce similar levels of the cytochrome P-452 isoenzyme (57% of the total cytochrome P-452). Comparable values to this study were also observed within uninduced hepatic microsomes.

This induction however was not correlated to a corresponding increase in lauric acid hydroxylation activity (figure 7.2). An initial rapid increase was seen which subsequently declined at between 12-24 hours following pretreatment. A maximal 2.5 fold increase in activity was seen, which is comparable to the levels observed within the aforementioned study also using Wistar rats (Bains et al. 1985). The time differences observed with respect to the amount of immunologically detectable isoenzyme and metabolic activity between the two studies are believed to relate entirely to the different dosing regimens utilised and therefore bioavailability of the drug.

The discrepancy arises from the fact that a fall in metabolising activity is observed at a time when the immunologically detectable levels of cytochrome P-452 are rising. The reason for this contradiction is at present unknown.

That clofibrate pretreatment resulted in the induction of a specific isoenzyme was also conclusively shown by analysis of the hydroxylated lauric acid metabolites by HPLC (figure 4.11A). This showed that the increase was due to specific induction of the 12-hydroxylase, with the 11-hydroxylase activity not being significantly affected at all. This constrasts with phenobarbitone (figure 4.11B) which was shown to cause an initial small, but preferential increase of 11-hydroxylase activity.

It should also be mentioned that recent work carried out in this laboratory in
fact suggests that lauric acid serves only as a model substrate for a physiologically more relevant endogenous substrate. This is based on the observation that only trace amounts of this fatty acid are found in hepatic microsomal membranes, and also because of the ability of cytochrome P-452 to metabolise arachidonic acid (Bains et al. 1985). Absolute inhibition of this activity can be obtained with the anti-cytochrome P-452 antibody.

A further puzzling observation was that of the temporal expression of the mRNA sequences coding for cytochrome P-452 on induction. An apparently different trend of induction to that expected is again observed (figure 7.2). An initial drop in mRNA activity coding for the induced cytochrome P-452 isoenzyme is seen which continues for a period of 12 hours, following which the levels begin to rise. At 72 hours following pretreatment the mRNA levels are only just above those present in the uninduced state. This drop in activity would appear to suggest either a repressive or some form of modulatory mechanism operating on the gene coding for the cytochrome P-452 isoenzyme, or possibly even the switching on of a different gene altogether. It has also been speculated that hypolipidaemics exert their inductive effect by binding to a cellular receptor protein (Lalwani et al. 1983), in a manner analogous to that seen with the Ah locus (Nebert et al. 1981). As yet however, this work has not been corroborated and further study in this area is required.

These contrasting parameters make it difficult to specify the precise nature of clofibrate induction. One possible explanation which encompasses all the data, is indicative of a subtle heterogeneity occurring within the cytochrome P-450 sub-population, as follows.

The analysis of clofibrate-induced mRNA isolated at 16 hours following pretreatment indicated a substantially lowered level of expression for the cytochrome P-452 mRNA (60%) as compared to that observed in uninduced mRNA species. That one does not see a large increase in the level of mRNA expressed upon induction need not necessarily be surprising. Firstly, this is because of the high
level of this mRNA being present in the uninduced state (table 6.5) which no doubt is representative of the very high constitutive levels of cytochrome P-452. Secondly, upon induction a maximal 2-fold increase only, is seen for the cytochrome P-452 isoenzyme and as such this increase could be more related to an increased processing and/or stabilisation of the mRNA itself. The actual mechanism(s) remains to be determined.

Upon translation and subsequent immunoprecipitation of uninduced and clofibrate pretreated (16 hours) mRNA fractions, a single polypeptide band was observed which appeared to migrate with authentic unlabelled cytochrome P-452 on analysis on SDS P.A.G.E. (figure 6.5A). However if the two immunoprecipitates were combined and analysed together on SDS P.A.G.E. then the possible presence of two polypeptides stacked immediately on top of each other was just discernable (figure 6.5B). This discrepancy is based upon a tentative observation especially because the differences in size between these two possibly immunoprecipitated polypeptides is so small. If one accepts that this is not an artifact however, then the SDS P.A.G.E. analysis indicates that the polypeptide visualised upon immunoprecipitation of the uninduced mRNA fraction represents the top band (i.e. higher molecular weight), and the lower polypeptide represents the induced polypeptide.

A possible explanation for this observation is that the polypeptide which is visualised within the uninduced mRNA state represents the precursor form of cytochrome P-452 with the mature form only being expressed upon induction. A number of workers have shown that certain proteins are synthesised as precursor forms (table 7.2) although this does not extend to other enzymes of drug metabolism, including certain cytochrome P-450 isoenzymes (Bresnick et al. 1981), NADPH-cytochrome P-450 reductase (Gonzalez et al. 1980), epoxide hydratase (Gonzalez et al. 1980a), and the glutathione-S-transferases (Pickett et al. 1982). These latter workers have shown that the above proteins are synthesised as the complete, mature polypeptide. The only problem with this supposition however, would be the extremely short length of the precursor segment.
A more likely explanation is that some different form of control over gene activity is emerging following induction. This could involve a decrease in expression of the cytochrome P-452 gene in uninduced animals with a concomitant increase in activity of a highly related gene coding for a similar cytochrome P-452. Certainly from the results presented, a decrease in mRNA activity is observed followed by a subsequent increase after 12 hours. This data taken in conjunction with the induction metabolism profile (figure 7.3) would support this hypothesis. If this is indeed the case then the fact that the anti-cytochrome P-452 antibody recognises both related isoenzymes is also not surprising (consider the antibody recognition for cytochromes P-450 B₁/B₂). The fact that one does not get a decrease in total cytochrome P-452 level (with respect to the single radial immunodiffusion data and increase in microsomal specific content with time) could also be explained by a corresponding balance between the levels of the constitutive and induced isoenzymic form.

The possibility of such a regulatory role over gene activity is now emerging (Mizuno et al. 1984), with it appearing that short RNA molecules made when one gene is active having the capability of binding to a mRNA of a different but
Figure 7.4  The Induction of Hepatic Cytochromes P-450 B$_1$/B$_2$ (A) and Cytochrome P-447 (B) in the Microsomal Fractions of Rats Pretreated with a Single Dose of Clofibrate

(1) Represents the quantitative determination (single radial immunodiffusion) of cytochromes P-450 B$_1$/B$_2$ with time, following clofibrate pretreatment.

(2) Represents the metabolic profile of the cytochrome P-450 isoenzymes with their preferred substrates following clofibrate pretreatment. For cytochromes P-450 B$_1$/B$_2$ the substrate was benzphetamine, and for cytochrome P-447 7-ethoxyresorufin.

All values represent increases above basal levels. (Data is from Chapter 4).
intimately linked gene thereby modulating its activity (Simons et al. 1983). In this manner the activity of any one gene is capable of increasing or decreasing the protein production of another.

From the immunological and metabolic data, it also becomes apparent that clofibrate functions as a highly specific inducing agent, not acting at the genome level coding for the phenobarbitone or BNF-induced cytochrome P-450 isoenzymes (figures 7.4). Although there is immunological evidence for the increased presence of cytochromes P-450 B1/B2 above uninduced levels (table 4.II) upon clofibrate pretreatment, this is not coupled to an increase in benzphetamine N-demethylase activity. Similarly upon immunoprecipitation of clofibrate-induced mRNA (16 hours pretreatment) no evidence of expression for the phenobarbitone-induced isoenzymes was found. In the case of cytochrome P-447 no evidence for immunological presence was found within clofibrate microsomes. This corresponded to the metabolic turnover of 7-ethoxyresorufin which stayed at or near basal levels (figure 7.4).

This specificity of clofibrate for the induction of cytochrome P-452 has also been suggested across other species and strains by means of hybridisation techniques. Stupans et al. (1984) have presented data which indicates that there is no cross-hybridisation between clofibrate-induced mRNA and either mouse D2 strain cytochrome P-450b cDNA or mouse cytochrome P1-450 cDNA clones. At the level of hybridisation stringency utilised in the Northern analysis, there was no cross-hybridisation between the phenobarbitone-induced mouse or Sprague-Dawley rat cytochrome P-450β and the cytochrome P1-450 cDNA probe. This was also the case when hybridisation was carried out between the 3-methylcholanthrene-induced mouse or rat cytochrome P1-450 and the cytochrome P-450b cDNA probe.

The same results were obtained when total liver poly (A)† from clofibrate-induced Wistar rats (this study) was analysed with a probe coding for the major BNF-induced cytochrome P-450 (Phillips, I. 1984, personal communication).

In conclusion, with the data obtained throughout this study it is not possible to
precisely define the mode of induction mediated by clofibrate-pretreatment. The
specificity of clofibrate induction for cytochrome P-452 was also determined, and
this coupled with the hybridisation data discussed above, strongly suggests that
clofibrate pretreatment involves activation of cytochrome P-450 gene(s) not
present in the multigene families induced by either phenobarbitone or the polycyclic
aromatic compounds.

7.3 β-NAPTHOFAVONE INDUCTION

The induction of drug metabolising enzymes by foreign chemicals such as
3-methylcholanthrene (3MC), β-napthoflavone and TCDD has been extensively
studied within the mouse and is now known to be controlled by the Ah locus (Nebert
et al. 1981). The induction response is the result of an increase in several gene
products associated with the metabolism of drugs and carcinogens chemically
related in structure to the inducers. These gene products include cytochromes
P$_1$-450 (Negishi et al. 1979), P$_2$-450 (Ohyama et al. 1984), P$_3$-450 (Negishi et al.
1979) and UDP-glucuronosyltransferase (Owens, I. 1977). The presence of such a
cytosolic high affinity Ah receptor has been shown to be strain specific (Hannan et
al. 1981) with inheritance of this receptor following Mendelian genetics (Nebert et

Within a number of different rat strains pretreatment with 3MC has been
shown to result in the induction of three separate cytochrome P-450 isoenzymes,
termed P-450$_a$, P-450$_c$ and P-450$_d$ (Ryan et al. 1979, 1980). Of these forms, the
major cytochrome P-450 isoenzyme is the P-450$_c$ form with this being shown to
correspond to the same major isoenzyme induced upon pretreatment with β-
napthoflavone. (Lau et al. 1982).

Following the chromatographic purification of microsomes isolated from BNF-
pretreated Wistar rats a single major isoenzyme corresponding to the major
isoenzymic cytochrome P-450$_c$ form found in Sprague-Dawley rats was isolated.
Immunological characterisation (figure 4.20) indicated that there was no cross-
reactivity between this form and the other cytochrome P-450 isoenzymes analysed.
Figure 7.5

The Induction of Hepatic Cytochrome P-447 following
Single Dose β-napthoflavone Pretreatment

(A) represents increase (fold) of immunoprecipitable mRNA translatable activity
coding for cytochrome P-447.

(B) represents increase (fold) of cytochrome P-447 within β-napthoflavone-
induced microsomes as determined by single radial immunodiffusion assay.

(C) represents increase (fold) in 7-ethoxyresorufin activity within β-napthoflavone
induced microsomes.

All values represent increases above basal levels. (Data is from pertinent result
sections, chapters 4 and 6).
This is in agreement with other authors who have also shown no immunological cross-reactivity amongst the major homogeneous cytochrome P-450 isoenzymes purified to date (Thomas et al. 1981).

Analysis of the Wistar rat microsomal fractions following single dose pretreatment indicated an increase in microsomal specific content with time (table 4.3). This also manifested itself in the qualitative visualisation of cytochrome P-447 within microsomes as visualised by Western blotting (figure 4.22B), and also by quantitative determination carried out by single radial immunodiffusion analysis (table 4.10). With respect to the Western blotting analysis one major band was visualised which was shown to correspond to the authentic cytochrome P-447 isoenzyme. A similar pattern was obtained upon immunoprecipitation of mRNA fractions isolated at different time points following BNF pretreatment (figure 6.12). This is in contrast to the results obtained by other workers who have been studying similar parameters in different rat strains. Immunoprecipitation analysis of mRNA fractions from 3MC-pretreated animals with anti-cytochrome P-450_c antibody, has revealed the presence of two polypeptides within both Sprague-Dawley and Long-Evans rat strains. (Fagan et al. 1983, Lippman-Morville et al. 1983). The upper higher molecular weight polypeptide was shown to correspond to cytochrome P-450_c and the lower polypeptide to cytochrome P-450_d which is also the major isoenzymic form induced following isosafrole pretreatment (Ryan et al. 1980). The reason for this cross-reactivity is the sharing of common antigenic determinants, although both isoenzymes can be distinguished on the basis of spectral properties, catalytic activities and the N- and C-terminal amino acid sequences (Reik et al. 1982). The complete nucleotide sequences for the 3MC-inducible cytochrome P-450_c (Sogawa et al. 1984) and P-450_d (Kawajiri et al. 1984) genes have been determined and although similarities exist, the genes cannot be considered identical.

This would suggest that the mode of induction by 3MC and BNF is different either at the strain level or alternatively at the level of immunological identity between the two isoenzymes. Analysis of the total translation products of the
mRNA fractions isolated at different time points (figure 6.2) indicated the presence of an induced polypeptide with time of approximate molecular weight 52K. On the basis of mobility upon SDS P.A.G.E. this could represent cytochrome P-450\(_d\). A further factor also to be considered is the dosing regimen. It has been reported that on consecutive dosing the ratio of cytochrome P-450\(_c\) to cytochrome P-450\(_d\) is far greater than obtained on single dosing (Yabusaki et al. 1984). Possibly within Wistar rats the expression of the mRNA for cytochrome P-450\(_d\) is much lower than that seen within other rat strains. At the moment the available data is not sufficient to rule out or support any of these possibilities.

In the present study, subsequent to BNF pretreatment, a rapid induction in the expression of the mRNA coding for cytochrome P-447 was observed. This expression was observable as early as 6 hours following pretreatment attaining a maximum at between 12-16 hours (figure 7.5). After this period the mRNA coding for cytochrome P-447 was seen to diminish, although at 72 hours following dosing was still higher than that seen in uninduced animals. This time period for maximal induction is in agreement with other authors who have shown that in Sprague-Dawley pretreated animals (both 3MC and BNF pretreated), the corresponding optimal increases were at 15 hours (Bresnick et al. 1981) and 16 hours (Dubois et al. 1980) respectively.

Previous work (Kumar et al. 1980) has suggested that cytochrome P-450\(_c\) is first synthesised as a precursor molecule with a molecular weight 6000 greater than the mature protein. This study using the in vitro translation system assay was unable to confirm the existence of a precursor segment of the size suggested, and it would appear that the cytochrome P-447 protein was synthesised in the mature form. Evidence amongst different strains has been presented both for (Fagan et al. 1983), and against (Bresnick et al. 1981) the presence of this precursor segment.

Coupled with the rapid induction of the mRNA coding species is a rapid increase in the rate of metabolism of the substrate 7-ethoxyresorufin. A maximum is achieved at 24 hours post-dosing with the levels decreasing thereafter in line
with the decrease in mRNA levels (figure 7.5). On immunological quantitation an even greater increase is seen in the level of immunologically detectable cytochrome P-447 with levels 25 fold over that seen in uninduced animals being observed. This immunochemical induction profile is similar to that observed in Sprague-Dawley rats (Harada et al. 1981), except that at the maximally determined levels the percentage of cytochrome P-447 of total cytochrome P-450 present within the hepatic microsomes of Wistar rats was approximately 40-50% of that seen in pretreated Sprague-Dawley animals (Harada et al. 1981, Goldstein et al. 1984). One possible reason for this discrepancy could be that with Sprague-Dawley pretreated animals the anti-cytochrome P-450\textsubscript{c} antibody recognises both the P-450\textsubscript{c} and P-450\textsubscript{d} isoenzymes (Reik et al. 1982), which according to the results from this study does not appear to be the case in Wistar rats. This possibility is further strengthened by the fact that the ratio of the contents of cytochromes P-450\textsubscript{c} and P-450\textsubscript{d} in BNF pretreated Sprague-Dawley rats is about 3:1 (Thomas et al. 1983). Taking this value into consideration would therefore suggest that the degree of induction of cytochrome P-447 would then be approximately the same across the two strains.

The fact that this was indeed the major cytochrome P-447 peptide induced was also ascertained by means of antibody inhibition analysis (figure 4.23). Comparable values for the inhibition of ethoxyresorufin O-deethylase activity was obtained within a reconstituted enzyme system utilising both the purified protein and microsomal fractions.

It was also interesting to note how quickly mRNA coding for cytochrome P-450 following BNF pretreatment was expressed. This was attained at approximately 12 hours following dosing and contrasts sharply to the comparable time period following phenobarbitone pretreatment. This difference could possibly relate to the effect of various xenobiotics upon the integrity of RNA polymerases which are essential components in the transcription of mRNA. 3MC pretreatment was shown to increase RNA polymerase I and II activity by 70% and 30% respectively, resulting in increased transcription of both ribosomal and mRNA
Figure 7.6  The Induction of Hepatic Cytochromes P-450 B₁/B₂ (A) and Cytochrome P-452 (B) in the Hepatic Microsomes of Rats Pretreated with a Single Dose of β-naphthoflavone

(1) Represents the quantitative determination (single radial immunodiffusion) of the corresponding cytochrome P-450 isoenzyme with time following β-naphthoflavone pretreatment.

(2) Represents the metabolic profile of the cytochrome P-450 isoenzymes with their preferred substrates following β-naphthoflavone pretreatment. For cytochromes P-450 B₁/B₂ the substrate was benzphetamine, and for cytochrome P-450 lauric acid, (values represent total 11- and 12- hydroxy laurate metabolites).

All values represent increases above basal levels. (Data is from chapter 4).
molecules (Liberator et al. 1981). Phenobarbitone on the other hand, exerted no effect on either RNA polymerase I or II activity (Liberator et al. 1981). This factor is probably fundamental in explaining the differences observed in time for maximal induction by the two xenobiotics. Inhibitors of RNA synthesis or RNA polymerase activity totally prevent the induction of cytochrome P-450 mediated reactions (Cutroneo et al. 1973, Jacob et al. 1974).

Evidence was also presented (figure 7.6) that BNF also exerted an effect on the genes expressing cytochromes P-450 B<sub>1</sub>/B<sub>2</sub> and P-452. Immunological quantitation of BNF pretreated microsomes showed the presence of slightly elevated levels of cytochromes P-450 B<sub>1</sub>/B<sub>2</sub> above that seen within the uninduced state. These levels decreased with time, ranging from 2-3% of the total cytochrome P-450 population. Similar values as high as 5% have been reported within Sprague-Dawley rats pretreated with 3MC (Harada et al. 1981). The presence of these isoenzymes could also be detected in BNF pretreated microsomes by means of Western blotting, although visualisation was at the minimum level of detection. There was no increase in benzphetamine N-demethylase activity above basal levels indicating the lack of inducibility of metabolically active cytochromes P-450 B<sub>1</sub>/B<sub>2</sub>. The reason for the discrepancy between the presence of actively metabolising holoenzyme and immunologically detectable protein is unclear, although the possibility that the antibody is recognising some other form of cytochrome P-450 cannot be discounted. Analysis of the mRNA species coding for the phenobarbitone-induced isoenzymes indicates that there is a 53% drop in activity relative to the uninduced state following BNF pretreatment (16 hours). This is in very close agreement with that observed in Sprague-Dawley rats where the level was shown to be decreased by 55% upon BNF pretreatment (Phillips et al. 1983a). These results in the Wistar rats would therefore concur with previous findings in the Sprague-Dawley rat (Phillips et al. 1983a). These results also indicated that the decrease in specific phenobarbitone cytochrome P-450 upon BNF pretreatment was not the result of passive dilution effects as a result of microsomal membrane proliferation but more
The effect of β-napthoflavone upon the cytochrome P-452 gene is even more pronounced in that almost complete repression is observed. Immunological quantitation reveals that the cytochrome P-452 isoenzyme is decreased by up to 90% of its level as compared to its uninduced state. This corresponds to a 65% decrease in the cytochrome P-452 mRNA activity following β-napthoflavone pretreatment. Although some lauric acid hydroxylase activity is still observed following pretreatment (figure 7.6), HPLC analyses indicates that this is primarily 11-hydroxylase activity. Hydroxylation at the 12-position is depressed to the greatest extent (figure 4.11C). This would be expected, as it has already been shown (chapter 4) that hydroxylation at the 11-position is primarily attributed to phenobarbitone-type isoenzymic activity and hydroxylation at the 12-position as a result of clofibrate-induced cytochrome P-452 activity. This suggests that the lauric acid hydroxylation activity observed within BNF pretreated microsomes is therefore possibly being mediated by the cytochrome P-450 B1/B2 isoenzymes still present - albeit at very low levels - within the microsomal membranes.

In summary, it has been shown that BNF pretreatment of Wistar rats results in a very rapid increase in expression of mRNA molecules coding for cytochrome P-447. This is concomitantly reflected in an increase in metabolic activity (ethoxyresorufin O-deethylase) and immunologically detectable protein. Despite the overall increase of total cytochrome P-450 within the liver microsomal membranes however, β-napthoflavone was shown to decrease the amounts of both cytochromes P-450 B1/B2 and P-452 as well as their corresponding translatable mRNAs. The gene(s) coding for cytochrome P-452 appeared to be affected to the greatest extent. This indicates that induction of cytochromes P-450 by xenobiotics is not an indiscriminate process, but involves both the expression and repression of gene activity for various cytochrome P-450 variants.
7.4 INDUCTION OF RENAL CYTOCHROMES P-450 FOLLOWING XENOBIOTIC PRETREATMENT

Whereas cytochrome P-450 dependent monooxygenases in liver microsomes have been extensively studied, only a limited amount of information is available from kidney microsomes. Homogeneous cytochrome P-450 has been isolated from the pig kidney cortex and shown to be active in the 11- and 12-hydroxylation of prostaglandins A₁, E₁ and lauric acid (Okita et al. 1981). Two other forms have been purified (Ogita et al. 1982) - and a further form speculated upon (Kusunose et al. 1981) - from the kidneys of New Zealand white rabbits. Within this species pretreatment with 3MC (Ogita et al. 1982) resulted in the isolation of one form active in the 11- and 12-hydroxylation of fatty acids and the other in the preferential hydroxylation of benzo(a)pyrene. In both species, the cytochrome P-450 was primarily distributed within the proximal tubule (Masters et al. 1981; Endou, 1983). Additional work utilising rat kidney microsomes (Sprague Dawley) has also led to the suggestion that the kidney cytochrome P-450 system has a very high substrate specificity which is primarily directed towards fatty acid hydroxylation (Ellin et al. 1972, 1975).

Single-dose clofibrate pretreatment resulted in an initial decrease, followed by an increase attaining a maximum at 24 hours, of specific microsomal content (table 4.3). This pattern was also reflected in Western blot analysis with anti-cytochrome P-452 antibody of the pretreated renal microsomal fractions (figure 4.22A). Here a single polypeptide band was visualised which was shown to co-migrate on SDS P.A.G.E. with homogenous hepatic cytochrome P-452. Catalytic analysis of total lauric acid metabolites showed a linear increase with time, with this being further evidence for cytochrome P-452 induction. HPLC analysis (figure 4.11A) of the metabolites indicated that activity was preferentially at the 12-position although lower levels of activity were also seen at the 11-position. It is interesting to note that 11-hydroxylation was linear in activity with time, whereas 12-hydroxylation began to very slowly decrease after 24 hours. This change which is reflected in the
12- to 11-hydroxylation ratios (table 7.3) could suggest that two hydroxylases were involved. This is in contrast to the observed effects of Sprague-Dawley rats where it is believed that only one hydroxylase is present (Ellin et al. 1975).

**Table 7.3**
The Effect of Xenobiotic Pretreatment Upon Renal Lauric Acid Metabolites Hydroxylation Ratios

<table>
<thead>
<tr>
<th>Pretreatment time (hours)</th>
<th>Clofibrate pretreatment</th>
<th>Phenobarbitone pretreatment</th>
<th>β-naphthoflavone pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.04</td>
<td>3.04</td>
<td>3.04</td>
</tr>
<tr>
<td>6</td>
<td>2.55</td>
<td>2.88</td>
<td>3.34</td>
</tr>
<tr>
<td>12</td>
<td>3.14</td>
<td>3.12</td>
<td>3.3</td>
</tr>
<tr>
<td>24</td>
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</tr>
<tr>
<td>72</td>
<td>3.25</td>
<td>4</td>
<td>3.15</td>
</tr>
</tbody>
</table>

Other differences between the two species are also apparent. Firstly in Sprague-Dawley rats the cytochrome P-450 hemoprotein population involved in laurate oxidation has an absorption peak in the CO-complexed form at 453-454 nm (Ellin et al. 1972). Within uninduced Wistar rats this value is 450.5 nm which increases to 451 nm upon clofibrate pretreatment. Secondly, using lauric acid as a substrate, the ratio between the 12- and 11-hydroxylated products was approximately 2:1 (Ellin et al. 1973), whereas in Wistar rats this value was closer to 3:1. The final difference relates to the overall levels of lauric acid turnover. With Sprague-Dawley rats (Jakobsson et al. 1970, Ellin et al. 1973) the level of lauric acid hydroxylation approximated that seen with the liver. Within Wistar rats however a 20-30 fold higher level of overall activity was observed (nmol product/nmol P-450/min). This difference was not related to the dietary state of the animals as the animals were not starved prior to death, an event known to increase the level of fatty acid hydroxylation activity (Ellin et al. 1971).
On the basis of metabolic activity reconstitution experiments, clofibrate pretreatment did not appear to induce either cytochromes P-450 B\textsubscript{1}/B\textsubscript{2} or P-447 in terms of the metabolic turnover of benzphetamine and 7-ethoxyresorufin. This was also proved by immunoprecipitation analysis of renal mRNA species isolated at 16 hours following clofibrate pretreatment. Similarly the levels of mRNA coding for cytochromes P-450 B\textsubscript{1}/B\textsubscript{2} and P-447 (table 6.6) were shown to be depressed as compared to basal levels. Despite this, visual precipitation of these peptides was still possible (figure 6.14B) which is to an extent surprising as this was not seen to be the case on analysis of the hepatic mRNA complement following clofibrate pretreatment. The reason for this difference is unclear but may be representative of a different mode of induction between the two organs.

On quantitating the mRNA levels coding for cytochrome P-452 these were seen to be just lower than those observed within the liver. Because of the very high lauric acid hydroxylation rates observed within the kidney this could suggest that a for more efficient expression, or alternatively an increase in the stability of the cytochrome P-452 coding mRNA population is apparent. Unfortunately the available data is not sufficient to discriminate between these possibilities.

Phenobarbitone pretreatment resulted in the induction of cytochrome P-450 B\textsubscript{1} as determined by Western blot analysis and in a increase in benzphetamine N-demethylase activity with time. It is not possible to conclude from the results of this study whether or not there is any cytochrome P-450 B\textsubscript{2} present within renal microsomes or alternatively whether it is present in such low amounts that the techniques utilised are not sufficiently sensitive. Immunoprecipitation of mRNA directed translation products indicated that a 2.3 fold increase in its mRNA coding complement for this isoenzyme occurred. This is approximately half the level observed within the hepatic complement at 16 hours dosing. As the values observed for benzphetamine N-demethylase activity are similar to that seen within the liver, then the possibility exists that the cytochrome P-450 B\textsubscript{1} coding mRNA is being influenced in the same manner as that seen for the clofibrate mRNA.
Immunoprecipitation analysis of the mRNA complement clearly indicated the presence of cytochrome P-452. Quantitation of these levels indicated that they were approximately the same as that seen within the uninduced state, which is a similar finding to that observed within the hepatic mRNA complement. This increase was also reflected in an increased level of lauric acid hydroxylation with the 12-hydroxylase being preferentially expressed (table 7.3, figure 4.11B). It should be noted here that with hepatic microsomes the 11-hydroxylase is primarily induced. This increase in induced renal lauric acid hydroxylase activity is again different to that observed in Sprague-Dawley rats (Jakobsson et al. 1970), where no measurable effects on either the cytochrome P-454 level or ω-oxidation activity was observed. This difference is believed to relate to the involvement of the induced cytochrome P-452 - as is seen within the liver - and not due to any of the induced phenobarbitone isoenzymes. The fact that a linear increase in 12-hydroxylation with time is observed however, in contrast to clofibrate pretreatment where maximal activity occurs at 24 hours post-dosing, cannot preclude the possibility that other forms of cytochrome P-450 are involved.

No expression of the cytochrome P-447 gene is indicated, in that both the levels of mRNA coding for cytochrome P-447 and the rate of metabolic turnover of 7-ethoxyresorufin are decreased by 60%.

Only limited analysis was carried out upon the effects of β-naphthoflavone induction upon renal microsomes. Upon single-dose pretreatment a very rapid increase in the turnover of 7-ethoxyresorufin O-deethylase activity was observed with this attaining a maximum at 24 hours. The rate of cytochrome P-447 induction was much higher within the renal than hepatic microsomal complement, although the maximal rate (9 fold) and trend of induction were the same in both cases.

The presence of cytochrome P-452 could also be detected within the BNF-induced microsomes by Western blot analysis. This was also reflected in a slight increase in 12-hydroxylase activity (table 7.3, figure 4.11C). The levels of cytochrome P-452 coding mRNA within the total RNA complement induced by β-
napthoflavone was not determined. However the limited increase in lauric acid hydroxylation would suggest that as within the liver, β-napthoflavone acts to repress cytochrome P-452 gene activity. As no activity towards the demethylation of benzphetamine was observed a similar premise can be applied towards the cytochrome P-450 B$_{1}$/B$_{2}$ gene.

From the limited results obtained upon analysis of the renal fractions, one can conclude that clear differences emerge between the mode of xenobiotic induction in the liver and the kidney as well as across strains (Wistar and Sprague-Dawley rats). Cytochrome P-452 appears to be present in all of the xenobiotically pretreated microsomal fractions suggesting that as within the liver it is present in high levels as a constitutive form. Pretreatment resulted in the specific induction of corresponding cytochrome P-450 isoenzymes which was reflected in increases in the metabolism of marker substrates. This is in contrast to the Sprague-Dawley strain (Hodgson et al. 1980) where the kidney cytochromes P-450 were shown to be relatively inactive in xenobiotic oxidation.

The fact that such high levels of ω-hydroxylation are observed lends support to previous observations that renal cytochrome P-450 mixed function oxidase activity is directed towards the ω- and (ω-1)-hydroxylation of fatty acids.

The evidence presented also strongly suggests that only one hydroxylase is induced, with this being preferentially stimulated by all the xenobiotics studied. This cannot be considered absolute however as the relative rates of induction of both hydroxylases was shown to differ with time upon clofibrate pretreatment. The precise reason for this is unclear, but could possibly be explained by the consideration that the constitutive renal cytochrome P-452 form is preferentially responsible for the ω-hydroxylation of fatty acids. On pretreatment with clofibrate, this activity is enhanced with a further isoenzymic variant of cytochrome P-452 catalysing the (ω-1)-hydroxylation of fatty acids also being induced.
Suggestions for Further Work

In the last few years tremendous progress in the understanding of gene structure and gene expression with its concomitant regulation of the drug metabolising enzymes has been achieved. It is anticipated that this progress will continue, and that the techniques of molecular biology will be increasingly used to probe the existence and induction of the isoenzymic groups involved in xenobiotic biotransformation in experimental animals.

With a view to this study the intriguing pattern of the clofibrate-induced cytochrome P-450 isoenzyme merits further study. This is further justified by the indication that it is present as a major constitutive isoenzymic form with a wider substrate activity than previously thought. Although its induction parameters were analysed, questions still remain, and the next stage should be directed towards the cloning analysis of the cytochrome P-452 isoenzyme(s). At the same time immunoquantitation in parallel with metabolic activity profiles for this isoenzyme should also be investigated within other organs (especially the kidney).

The ultimate goal of any such study should be the eventual isolation of well characterised cDNA animal probes directed against the different cytochromes P-450 in order to type human liver. This will provide a far more detailed understanding of the drug-metabolising enzymes within humans, such that animal experimentation data could provide a more accurate prediction of potential toxicity of any compound within man.

This could be achieved by the isolation and subsequent characterisation of highly purified drug metabolising enzymes. The subsequent preparation of monospecific antibodies against these variants could then be used to immunoquantitate these forms amongst different species, including man. In conjunction with these studies, molecular biology techniques could be utilised in order to enrich for the specific mRNAs from animal livers from various sources in order to construct the corresponding cDNAs. These would be utilised to identify the gene
structure coding for these isoenzymes, which could then be compared and contrasted to that present within the human genome.

Aside from the greater understanding of genetic regulation upon xenobiotic pretreatment which would be attained, the possibility of improved clinical/toxicological diagnosis has to be considered. Indeed such genetic characterisation could well result in it being possible to predict clinically, individuals who are at increased risk for developing certain types of chemically induced cancers or who would suffer deleterious effects after certain forms of drug therapy due to a patient's underlying pharmogenetic disorder.
REFERENCES


