CYTOCHROME P450-MEDIATED METABOLISM AND CYTOTOXICITY IN RAT CULTURED HEPATOCYTES

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ABSTRACT

The aim of this investigation was to define the in vitro conditions necessary to support cytochrome P450-mediated metabolism in rat cultured hepatocytes, such that this system could then be used as an in vitro model in the study of cytochrome P450-mediated cytotoxicity.

Maintenance of P450-dependent enzyme activities in culture was not affected by supplementation of culture medium with haem, but was markedly influenced by the age and sex of the hepatocyte donor animal.

Induction in primary culture by phenobarbitone and beta-naphthoflavone was investigated, and found to be quantitatively and qualitatively different to the induction observed in vivo, hepatocytes in culture being particularly refractive to induction by phenobarbitone. The maintenance in primary culture of a range of enzyme activities was determined following treatment of rats in vivo with isoniazid and dexamethasone, in addition to phenobarbitone and beta-naphthoflavone, and in general, there was good maintenance of the induced activities. The activities were chosen as possible selective substrates for the different induced isozymes, with a view to using the activity profiles to characterise different classes of inducer; however, although selective induction was observed with isoniazid, beta-naphthoflavone and dexamethasone, all the chosen activities were induced by phenobarbitone.

The final part of this work involved determining cytotoxicity in vitro, following induction of P450 in vivo with phenobarbitone and

beta-naphthoflavone. Seven known hepatotoxins were investigated, and the results obtained agreed well with available *in vitro* and *in vivo* literature data.

In summary, a range of constitutive and induced enzyme activities were maintained at high levels in hepatocytes cultured for twenty-four hours from adult male rats, and an induction *in vivo*/hepatocyte culture protocol shown to be a viable *in vitro* model for the study of metabolism-mediated toxicity, as an alternative to induction and detection of toxicity *in vitro*.

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ABBREVIATIONS

All abbreviations are defined the first time that they appear in the text, with the exception of the following, deemed acceptable by the Biochemical Journal (257: 1-21,1989) which are used without definition:

AMP	adenosine monophospate
ATP	adenosine triphosphate
CoA	coenzyme A
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis (B-aminoethyl ether) N_1 N_1 N' N'-tetraacetic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
NAD(H)	nicotinamide adenine dinucleotide
NADP(H)	nicotinamide adenine dinucleotide phosphate
mRNA	messenger ribonucleic acid
Tris	tris(hydroxymethyl) aminomethane
UDP	uridine diphosphate

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GENERAL INTRODUCTION

The cytochrome P450 mixed-function oxygenases are involved in the metabolism of a variety of endogenous substrates. However, a considerable number of these enzymes also have the capacity to metabolise a wide range of structurally diverse xenobiotics including dietary constituents, environmental pollutants, insecticides and therapeutically administered drugs. Some of these xenobiotics are noxious to man per se, but many others are biotransformed to reactive highly toxic intermediates, predominantly by the action of cytochrome P450 enzymes. This can lead to cellular dysfunction, carcinogenesis and death. The function of these enzymes is probably to activate compounds such that they can be detoxified by the action of conjugative enzymes, however, the balance of these reactions can be disrupted by many factors. The potential for P450 to generate toxic metabolites has important ramifications in many areas of study e.g. occupational or accidental exposure to industrial chemicals or environmental pollutants, and drug development and therapy.

The cytochrome P450s have been found in most of the organisms in which they have been sought. They are also found in most mammalian tissues, hence the literature available on these enzymes is enormous, and this review cannot adequately cover every aspect of P450 metabolism. The work presented here is concerned with hepatic P450 metabolism and toxicity, and therefore most of this

introduction is confined to the role of cytochrome P450 in hepatic biotransformation, and the development of toxicity in this organ. Where possible, reference is made to drug metabolism in Man; however, much of our understanding of P450 metabolism has been obtained from rodent studies, particularly the rabbit, rat and mouse. This introduction is divided into four main sections; the latter parts deal with regulation of P450 expression and with methods of studying hepatic drug metabolism; the first part of this review deals with the integrated role of P450 in metabolism, particularly in the liver.

1.1 CYTOCHROME P450

1.1.1 Background and history

Cytochromes are electron carrier proteins that contain iron-porphyrin prosthetic groups. Electron transfer is effected by reversible valence changes of the iron during the catalytic cycle. The iron is complexed to the porphyrin ring by co-ordinate bonds (the number of which determine the spin-state of the cytochrome). Most cytochromes, including the mitochondrial respiratory cytochromes and cytochrome P450 contain a haem (iron protoporphyrin IX) prosthetic group (Lehninger, 1975).

In 1958 a membrane-bound cytochrome was described that had an unusual absorption maximum at 450nm on binding carbon monoxide in liver microsomes reduced with dithionite (Garfinkle, 1958; Klingenberg, 1958); this cytochrome was later characterized as a haemoprotein and designated cytochrome P450 (Omura and Sato, 1962).

P450 was later shown to be reduced by both NADH and NADPH, and was rapidly re-oxidised in the presence of oxygen (Omura and Sato, 1964). It is now known that there are multiple isozymic forms of P450; to date, over 50 mammalian gene products have been identified (Nebert *et al.*, 1989). P450s have been found in bacteria, plants and most animal phyla; mammalian P450s are located in the endoplasmic reticulum (ER) of most tissues (Fevold, 1983; Nebert and Gonzalez, 1987), and are also present in the adrenal mitochondria (Nebert and Gonzalez, 1987). Recent work on the topology of P450s suggests that the amino-terminal amino acids, which are predominantly hydrophobic residues, anchor the cytochrome in the ER membrane, the bulk of the molecule sitting on the cytoplasmic side possibly with other regions of the molecule traversing the membrane, and the haem group parallel to, or at a slight angle to the membrane surface (Nelson and Strobel, 1988; Vergères *et al.*, 1989).

P450 is the terminal oxidase component of the microsomal electron transport system (Cooper *et al.*, 1965) which catalyses mixed function oxidation (MFO) of an extensive variety of structurally diverse endogenous and xenobiotic compounds (Table 1.1). These MFO reactions serve to make lipophilic compounds more hydrophilic by the addition of a polar functional group. This is essential in the detoxification process, since the resulting water-soluble derivatives can then be metabolised by conjugative enzymes to readily excretable compounds. Metabolism of xenobiotics falls into two main categories: Phase I, (oxidative or reductive) which adds a

functional group, and Phase II which are generally the true detoxification reactions.

1.1.2 Chemistry and enzymology of MFO reactions

There are three essential components of the MFO system: P450, NADPH-cytochrome P450 reductase and lipid (Lu and Coon, 1968; Lu et al., 1969). NADPH-cytochrome P450 reductase (P450 reductase) is a flavoprotein containing both FAD and FMN prosthetic groups. P450 reductase is also ER-membrane-bound and passes reducing equivalents from NADPH to P450. P450 reductase appears to be a 'transducer' protein, in that the reductase is a two electron acceptor whereas P450 is a one electron acceptor. Electrons are passed sequentially from NADPH + H^+ to FAD to FMN to P450. A heat-stable lipid component is also required for MFO activity and appears to be phosphatidylcholine. Reconstitution studies indicate that the fatty acid composition is also vital for activity, suggesting that the composition of the lipid environment of P450 is a determining factor for MFO activity (Rietjens et al., 1989).

The role of P450 in MFO is to bind both molecular oxygen (O_2) and the substrate, cyclic oxidation/reduction of P450 and P450 reductase resulting in the catalytic conversion of the substrate. Substrate binding to the ferric form of P450 changes the absorbance spectrum of the cytochrome. This is due to alterations in the spin equilibrium.

The spin state is determined by ligand co-ordination. In addition to the four equatorial iron-pyrrole nitrogen bonds there are 1 (penta-co-ordinate) or 2 (hexa-co-ordinate) axial bonds to amino acid side chains. Most P450s exist with the spin equilibrium predominantly in the low-spin state (hexa-co-ordinate), and substrate binding, usually to the protein part of the molecule, alters the conformation of the protein and therefore its ligand interactions with the haem group. This causes a spin-state shift to predominantly high-spin configurations (producing a Type I binding spectrum). Substrate binding to a high-spin form is thought to be via the haem iron itself producing a hexa-co-ordinated low-spin haemoprotein and a Type II binding spectrum (Gibson and Skett, 1986). Substrates were originally classified as to their ability to elicit Type I or II binding spectra (Schenkman et al., 1967), before the existence of multiple isozymes was fully realised.

The overall stoichiometry of MFO reactions is:

$$NADPH + H^+ + O_2 + SH \xrightarrow{P450} NADP^+ + H_2O + SOH$$

where SH is the substrate. A simplified version of the catalytic cycle is shown in Figure 1.1. Briefly;

(i) Substrate binds to P450 causing a shift in spin-state and a change in redox potential which facilitates electron acceptance.

(ii) Two electrons are transferred from $NADPH+H^+$ to P450 reductase, and one electron transferred from P450 reductase to P450.

(iii) Binding of O_2 to the reduced P450 to produce a highly unstable oxy-ferrous-substrate complex.

(iv) Second electron reduction by P450 reductase or cytochrome b5, oxygen insertion (via a peroxy-intermediate?) and product release.

These latter steps are not well characterized, along with the role of cytochrome b5 in the catalytic cycle. Cytochrome b5 can form a covalent complex with P450 that seems to enhance binding of selected substrates, and also the shift in spin-state equilibrium, for certain isozymes (Jansson et al., 1985).

Also, the second electron may be donated either by the reductase or by b5, which can also be reduced by cytochrome b5 reductase, with NADH as the electron donor. These electrons are transferred to stearyl CoA desaturase, but it now seems that they can also be passed from b5 to P450, explaining the observed NADH synergism on NADPH-supported metabolism (Schenkman et al., 1976).

It has also been suggested that b5 increases the 'coupling' of the cycle. During the catalytic cycle, there is an increase in hydrogen

peroxide (H_2O_2) formation, since P450 also has an oxidase activity concomitant with monooxygenation of substrates:

$$NADPH + H^+ + O_2 \longrightarrow NADP^+ + H_2O_2$$

This activity has been viewed as an 'uncoupling' of electron transfer from substrate oxidation. The binding of b5 to P450 may result in tighter coupling due to more efficient/faster electron transfer. It should be noted that P450 isozymes that exist as predominantly high—spin forms i.e. P450nEl, have high endogenous oxidase activity (Gorsky et al., 1984; Ingelman-Sundberg and Johansson, 1984).

1.1.3 Isozymic forms of P450

P450 exists as a superfamily of isozymic forms, which accounts for the observed broad and overlapping substrate specificity. The isozymes have been organised into thirteen families based on the divergent evolution of the P450 genes derived from protein primary sequence alignment data (Nebert et al., 1988; 1989). Families I-IV contain most of the mammalian hepatic catabolic P450s; families XVII, XIX, XXI and XXII comprise the mammalian extrahepatic P450s involved in steroid biosynthesis while family XI contains the mitochondrial P450s that use adrenodoxin for electron transfer. In addition there are the yeast (LI and LII) bacterial (CI and CII) and insect (VI) families (Gonzalez, 1989).

The isozymes have been classified into families and subfamilies based on the percentage similarity of their primary sequences. In general, there is less than 40% similarity between isozymes of different families. Sequences within a family are taken to be greater than 40% similar, and greater than 59% similar for mammalian subfamilies. Also, sequences that are more than 97% similar are assumed to be allelic variants, unless demonstrated to be different gene products. This classification has enabled a standard nomenclature for P450s to be devised. Table 1.2 gives the standard nomenclature for the main hepatic P450s from families I - III. This nomenclature for P450 proteins (e.g. P450IA1) and genes (CYP1A1) will be used here throughout, where possible.

Although the sequence similarity between isozymes from bacteria and mammals is less than 25%, there is sufficient localised sequence identity to classify these isozymes as members of one superfamily e.g. there is a highly conserved 21-residue cysteinyl fragment associated with the haem-binding pocket that is present in all eukaryotic P450s and the prokaryotic P450 C1 (Nebert and Gonzalez, 1987).

There is much speculation as to why there are so many P450 isozymes. Diversification of P450 forms probably occurred as species adapted to new environments. It has been suggested that changes in dietary flora necessitated the development of enzyme systems that could detoxify and/or utilise many of the plant products. In order to be

an evolutionary success, an organism must be able to protect itself from environmental poisons and survive on new energy sources (Gonzalez, 1989).

The P450I family

There are two proteins in this family: P450IA1 is found in most phyla from insects upwards to the mammals, whereas IA2 appears to be expressed only in the liver (Gonzalez, 1989). However, despite the ubiquitous nature of IA1, no endogenous substrate has been identified for this P450. IA1 metabolises planar, aromatic compounds e.g. benzo-[a]-pyrene and 7-ethoxyresorufin; IA2 metabolises predominantly arylamines. Both genes are inducible by polycyclic aromatic hydrocarbons e.g. tetrachlorodibenzo-(p)-dioxin (TCDD), 3-methylcholanthrene (3-MC) and beta-naphthoflavone (BNF; Haugen et al., 1976; Thomas et al., 1983).

The P450II family

The P450II family has five main subfamilies A to E. There are at least three rat genes in the IIA subfamily: CYP2A1 and CYP2A2 are expressed in the liver, and although they exhibit 93% nucleotide sequence similarity, they are differentially and developmentally regulated. IIA1 is a testosterone-7α-hydroxylase, and IIA2 a 15α-hydroxylase. A third gene is expressed in rat lung, although its orthologue in the mouse is expressed in the liver and kidney (Matsunaga et al., 1988; Gonzalez, 1989).

There are at least ten genes in the IIB subfamily, the rat phenobarbitone (PB)-inducible genes being CYP2B1 and 2, the proteins having similar substrate specificities e.g. for benzphetamine and testosterone (Ryan et al., 1979). These genes seem to have diverged from each other recently in the rat, as most rodents do not have orthologues for both these genes (Adesnik and Atchison, 1986).

The IIC subfamily contains mostly constitutive P450s, many of which are developmentally/sex regulated, although some are inducible by PB. The rat has at least five genes and one pseudogene. These include the male-specific IIC11 and 13 and the female-specific IIC7 and 12 isozymes involved in steroid hydroxylations (Waxman et al., 1983; Adesnik and Atchison, 1986). This subfamily also contains three genes (CYP2C8-10) responsible for mephenytoin metabolism (Nebert and Gonzalez, 1988; Gonzalez, 1989).

The IID subfamily is highly polymorphic in the rat and the human, being involved in debrisoquine metabolism. This subfamily appears to have a unique lineage, being less similar to the other subfamilies (Gonzalez et al., 1988; Gonzalez, 1989).

The HE subfamily contains two genes, although, to date only rabbits have been shown to possess both genes, however there is differential tissue expression (Porter et al., 1989). P450IIE1 is inducible by ethanol (Ryan etal., 1986), diabetes and fasting (Ma etal., 1989)

and acetone (Johansson et al., 1988), and substrates include acetone, acetoacetate, halothane and pyridine. This P450 may play a role in gluconeogenesis during fasting (Gonzalez, 1989).

The P450III family.

There are to date six genes in this family: 2 rat, 3 human and 1 rabbit. Of the rat genes, CYP3A1 is inducible by pregnenolone-16α -carbonitrile and dexamethasone (Dex; Heuman et al., 1982), whereas CYP3A2 is constitutive in male rats. Macrolide antibiotics also induce P450IIIA proteins (Wrighton et al., 1978). However, it has been difficult to reconcile interlaboratory data with respect to the multiple isozymes in this family, and thus correspondence of different isozymes from different groups. It may be therefore, that there are several, very closely related isozymes in this family, all of which have not yet been resolved (Halpert, 1988). Both gene products appear to possess testosterone-6β -hydroxylase activity (Nebert and Gonzalez, 1988; Gonzalez, 1989).

The P450IV family.

These constitutive P450s catalyse the -w-hydroxylation of lauric acid and arachidonic acid and are inducible by hypolipidaemic drugs that are also rodent peroxisome proliferators e.g. clofibric acid (Tamburini et al., 1984; Hardwick et al., 1987).

1.2 REGULATION OF P450 EXPRESSION

P450 expression is regulated at two levels. Physiological and environmental factors alter the isozyme complement of an organ by influencing the molecular mechanisms responsible for gene expression, while regulation at the molecular level can be via control of gene transcription, mRNA and/or apoprotein stability and haem biosynthesis/degradation. These will be discussed below (1.2.3). Many factors have been shown to affect P450 expression: there are compounds that induce their own biotransformation and/or the metabolism of other substances, in addition to physiological conditions that affect primarily constitutive P450s. In both cases, alterations in isozyme complement can have profound effects on metabolism/toxicity of P450 substrates, both endogenous and xenobiotic. Induction and control of constitutive expression are discussed in the following sections, and although dealt with separately, are not completely discrete processes.

1.2.1 Constitutive P450 expression

There are many factors that are known to influence constitutive expression of P450, including genotype, hormones and liver disease, as well as infection, starvation and stress (Vesell, 1988).

Genotype Species and strain differences in P450 metabolism in experimental animals have been recognised for a long time (Kato, 1979), but it is now known that there are several examples of genetic polymorphisms for drug metabolism in Man, some with racial

differences in distribution frequency. One of the best characterized is the polymorphic metabolism of the anti-hypertensive drug, debrisoquine (DB). The 4-hydroxylation of DB shows a bimodal distribution between the extensive metabolisers and the poor metabolisers (who excrete no 4-hydroxydebrisoquine), with a low incidence of poor metabolisers. The deficient hydroxylation of DB is an autosomal recessive trait, and is associated with deficient metabolism of several other drugs e.g. sparteine, phenytoin and phenacetin, and is due to the absence of a single P450 isozyme: P450IID1 (Maghoub et al., 1977; Gonzalez et al., 1988). Mephenytoin metabolism is also polymorphic in humans, as well as the non-P450-mediated N-acetylation of many drugs e.g. isoniazid (Levi et a1., 1968; Gonzalez, 1989).

Tissue-specific regulation Many P450s exhibit tissue-specific regulation, although the molecular mechanisms responsible are unknown. P450IIB1 is constitutively expressed in the lung and testis, but not in the liver of untreated rats, whereas IIB2 is only detectable in the liver of untreated animals. P450IA2 also appears to be liver-specific as are IIAl, IIA2 and IICll (Nebert and Gonzalez, 1988; Gonzalez, 1989). Recently, an olfactory-specific P450 was identified which appears to be a member of the II gene family (Nef et al., 1989); the P450IV family, in contrast, may be more ubiquitous, having been found in liver, lung, kidney, placenta, intestine and leukocytes (Gonzalez, 1989).

Hormonal regulation Hormonal influences on P450 expression cover a wide range of conditions including pregnancy and lactation, diabetes and fasting, and is also the basis of age and sex differences in drug metabolism, e.g. 7-propoxycoumarin O-dealkylation (Kamataki et al., 1980), hexachlorobenzene metabolism (Rizzardini and Smith, 1982), phenytoin metabolism (Billings, 1983). Age and sex differences in drug metabolism are related to P450-mediated steroid metabolism which is regulated by growth hormone; the secretion and effects of growth hormone are discussed in detail in Chapter Four and will only be outlined here. Briefly, most P450s are not expressed prenatally: birth, weaning and puberty each influence the P450 profile (Waxman et al., 1985) in particular, neonatal, gonadal androgen production imprints the adult pattern of pituitary growth hormone secretion, which is different in males and females and which then influences hepatic P450 expression, primarily via suppression of P450 synthesis (extensively reviewed by Skett, 1987; 1988).

Thyroid hormones affect P450 expression, although their exact mode of action is unclear. Thyroxine appears to markedly affect the levels of P450 reductase (Kato, 1977; Waxman et al., 1989), and thyroidectomy has been shown to have substrate and sex-specific effects on P450 metabolism, which can be reversed or enhanced by subsequent administration of thyroxine (Skett, 1987). The confusing picture of thyroid effects on P450 metabolism may be a result of the failure in early studies to discriminate the actions of thyroxine and tri-iodothyronine and/or the compounding influence

of age, since thyroid suppression of P450 appears to be particularly marked for neonatally expressed P450s (Yamazoe et al., 1989).

There is a general decrease in drug metabolism in the pregnant rat, thought to be associated with increased progestagen levels (Gibson and Skett, 1986), Decreases in aniline hydroxylation and ethylmorphine N-demethylation were paralleled by changes in P450 content (Kato, 1977). These changes also correlate with the reported decreases in total microsomal phospholipid content and the phosphatidylcholine: phosphotidylethanolamine ratio in pregnancy, along with the decreased proportion of P450 in the high- spin state (Turcan et al., 1981; Symons et al., 1982). The lipid composition of reconstituted systems is known to markedly affect P450 activity (Yamazoe et al., 1988). It has been suggested that sex-differentiated activities as well as the pregnancy-associated inhibition of drug metabolism are due to specific lipid requirements of different isozymes (Skett, 1987).

Diabetes, although a disease state, is a hormonally-based disorder, and its effects on P450 will thus be discussed here. Chemically-induced or spontaneous diabetes mellitus dramatically alters drug metabolism in the male rat, effects in the female being less marked (Kato and Gillette, 1965a; Skett, 1987). In acute chemically-induced diabetes, there is suppression of male-specific and increases in female-specific activities in the male rat, which can be reversed by insulin replacement treatment (Kato and Gillette,

1965a). This is also seen in genetically diabetic rats, where it was also shown that diabetic males had lower serum testosterone levels than the control males (Warren et al., 1983). Castration and diabetes appear to have similar effects on male rats, in that androgens, like insulin, can partially reverse the effects of diabetes. However, the time-courses of changes in drug metabolism and serum testosterone levels do not correlate with each other (Skett, 1987). Despite this, it has been proposed that insulin and androgens may act through a common mediator i.e. growth hormone, this being a feminising factor, and insulin a masculinising factor with respect to P450 metabolism (Skett, 1987).

In addition to effects on sex-differentiated metabolism, diabetes induces a specific P450 isozyme, P450IIE1 (Bellward et al., 1988). This isozyme is also induced by ethanol and isoniazid and is associated with aniline hydroxylase activity (Ryan et al., 1986). Fasting and obesity also induce IIE1 (Hong et al., 1987; Salazar et al., 1988). Fasting appears to exaggerate sex differences in P450 metabolism, with male-type metabolism being depressed, as in diabetes (Kato and Gillette, 1965b; Ma et al., 1989). Fasting and diabetes, however, do not have identical effects on P450 isozyme expression; although IICll and IIAl were decreased by both conditions, fasting decreased and diabetes increased the PCN-E isozyme (a male-specific steroid 6β-hydroxylase); fasting also increased total P450 whereas diabetes had no effect on the total microsomal P450 content (Ma et al., 1989), The induction of IIEl

in diabetes and fasting is associated with ketosis (Honn et al., 1977; Bellward et al., 1988); IIEl is also inducible by acetone and acetoacetate and it has been suggested that IIEl is involved in the gluconeogenic response to glucose deficiency (Gonzalez, 1989). However, there are molecular differences in IIEl induction by acetone and fasting - these will be discussed in section 1.2.3.

Disease Many liver disorders can adversely affect drug metabolism; cirrhosis and chronic viral hepatitis can reduce the number of functional hepatocytes and any disease that affects blood flow through the liver can affect extraction and clearance (Kato, 1977). In general, the impairment of drug metabolism in chronic liver disease correlates with the severity of dysfunction.

Relatively little is known about the effects of the porphyrias on drug metabolism, although clearly deficiencies in haem biosynthesis or increased degradation will reduce the amount of P450-holoenzyme. Regulation of haem biosynthesis and degradation in relation to P450 activity is discussed in Chapter Three. However, it is known that TCDD, oestrogens and hexachlorobenzene can induce hepatic porphyrias in Man and experimental animals (Rizzardini and Smith, 1982), and that acute intermittent porphyria selectively impairs drug metabolism (Anderson et al., 1976).

1.2.2 Induction of P450

Conney *et al.* (1956) first reported the induction of aminoazo dye metabolism by 3-methylcholanthrene (3-MC). The enzyme induced was benzpyrene hydroxylase or aryl hydrocarbon hydroxylase (AHH), now known to be associated with P450IA1. P450IA1, and to a lesser extent, IA2 have been shown to be inducible by the polycyclic aromatic hydrocarbons (PAH), e.g. 3-MC, BNF and TCDD, isosafrole being more selective for P450IA2 induction (Conney *et al.*, 1967; Thomas *et al.*, 1983). The early work on PAH-induction of P450 was facilitated by the discovery of a polymorphism in mice with respect to responsiveness to PAH-induction of AHH. PB and PCN were shown to induce different proteins to BNF and TCDD in responsive mice strains; in non-responsive strains there was no induction of AHH by BNF or TCDD (Haugen *et al.*, 1976). TCDD was shown to be a potent ligand for a receptor whose binding affinity segregated with AHH-responsiveness in mice, and subsequently cytosolic receptor-bound TCDD was shown to be the factor responsible for AHH induction; this receptor is encoded by the *Ah* locus (Poland *et al.*, 1976; Gonzalez, 1989). The TCDD-receptor complex appears to translocate into the nucleus where it acts as a positive transcriptional activation element, possibly binding to regulatory elements that have been identified upstream of the *CYP1A1* gene (Gonzalez, 1989).

Autoregulation of the *Ah* locus by an endogenous repressor has been postulated from work using cell lines with normal receptor levels but

no AHH induction. In these cell lines however there is high constitutive expression of IA1 mRNA, i.e. in the absence of inducer. These mRNAs coded for defective proteins, and it was suggested that the IA1 enzyme metabolises an endogenous compound to a repressor of the Ah locus (Hankinson et al., 1985).

Several other enzymes are associated with the Ah locus including a glutathione transferase, NAD(P)H:menadione oxidoreductase, a UDP-glucuronyl transferase and P450IA2 (Nebert and Gonzalez, 1988; Gonzalez, 1989), suggesting that there is co-ordinate regulation of certain components of Phase I and II metabolism.

In contrast to PAH-induction of P450, *very* little is known about the mechanism of PB induction, other than that PB stimulates de novo synthesis of P450, and there is no evidence for the involvement of a receptor (Adesnik et al., 1981). PB induces proliferation of the ER, and in addition to induction of total P450 and P450s IIB1 and IIB2, levels of P450 reductase, epoxide hydrolase and a UDP-glucuronyl transferase are also increased (Hardwick et al., 1983a). The P450IIIA1/2 proteins and also some members of the IIC subfamily are also inducible by PB (Gonzalez, 1989). Induction of IIB1 and 2 is co-ordinate, but the transcription rate is different to that of the reductase and epoxide hydrolase (Hardwick et al., 1983a). Other inducers of IIB1/2 include SKF-525A, isoniazid, chlordane and Arochlor 1254 (Gonzalez, 1989).

Isoniazid, acetone and ethanol all induce P450IIE1, which is also inducible by fasting and diabetes (see section 1.2.1. above). The mechanism of induction appears to be primarily post-transcriptional and is discussed below.

P450s in the IIIA family are inducible by synthetic glucocorticoids (e.g. DEX) and synthetic steroids (e.g. PCN and betamethasone) as well as hydrocortisone and corticosterone, Progesterones, oestrogens, androgens and mineralocorticoids do not induce these P450s (Schuetz et al., 1984). DEX and PCN both induce the same P450, IIIA1, DEX being the better inducer (Heuman et al., 1982; Hardwick et al., 1983b). DEX/PCN induce the same protein in cultured hepatocytes as in vivo (Schuetz et al., 1984). Induction of P450 does not appear to be mediated by the glucocorticoid receptor: concentrations, time-courses and rank order of potency of the steroids are different for induction of P450 and tyrosine aminotransferase (Schuetz and Guzelian, 1984). PB and organochlorine pesticides also induce one of the IIIA proteins (Heuman et al., 1982; Schuetz et al., 1986).

Clofibrate and other hypolipidaemic agents induce peroxisomal and microsomal proteins involved in fatty acid /3-oxidation, including P450IVA1; this P450 has no immunocrossreactivity with the PAH-, PCN- or PB-inducible P450s (Tamburini et al., 1984; Hardwick *et* al., 1987).

1.2.3 Molecular mechanisms of regulation

Transcriptional activation This is the major regulatory mechanism during developmental induction of P450 activities, and most xenobiotic inducers increase gene transcription to a greater or lesser extent. Induction of P450IA1 by PAHs and IIB1/2 by PB is almost exclusively transcriptional (Adesnik and Atchison, 1986; Gonzalez, 1989). PCN and DEX both transcriptionally activate the CYP3A genes (Hardwick et al., 1983b; Simmons et al., 1987).

Post-transcriptional regulation This can involve stabilisation of mRNA or of protein. Transcription of P450IA2 in vivo is activated two-fold by 3-MC but there is a 30-fold accumulation of protein; similar results were obtained in hepatocyte cultures, suggesting a considerable post-transcriptional component in IA2 regulation (Pasco *et al.*, 1988; Silver and Krauter, 1988). DEX, in addition to transcriptional activation of the CYP3A genes, induces P450s IIB1/2 and P450 reductase via mRNA stabilisation (Simmons et al., 1987). Macrolide antibiotics e.g. triacetyloleandomycin (TAO) have been shown to induce a protein in vitro, indistinguishable from a PCN-inducible protein, in the absence of an increase in mRNA. It was proposed that TAO bound to the P450 inhibiting its degradation, thus stabilising (and inactivating) the P450 (Watkins et al., 1986).

IIEl expression is also induced in the absence of any transcriptional activation. In untreated rats IIEl degradation appears to be biphasic, whereas degradation is monophasic in

pyrazole-treated rats. Dimethyl sulphoxide and ethanol increased IIEl protein 2- to 7-fold, although there was no increase in mRNA levels (Eliasson et al., 1988); in contrast, fasting increased both protein and mRNA levels (Honn et al., 1987). It has been suggested that ligand binding by small organic molecules stabilises IIEl protein by inhibiting degradation whereas fasting and diabetes inhibit RNA catabolism (Eliasson et al., 1988; Gonzalez, 1989).

1.2.4. Inhibition of P450 Induction and inactivation of steroid-inducible P450s by TAO was mentioned above. Other compounds, particularly inhibitors of P450 metabolism, interact with the protein to inactivate it. Inhibitors such as SKF-525A, safrole and piperonyl butoxide form stable, inactive complexes with P450: the inactivation is usually reversible, and these compounds do not destroy the haemoprotein. This type of inactivation requires metabolism of the inhibitor by P450 (Netter, 1980), Safrole and alpha-naphthoflavone selectively inhibit the PAH-inducible P450s (Delaforge et al., 1985), SKF-525A and metyrapone preferentially inactivate PB-inducible P450s (Netter, 1980; Parkinson *et* al., 1982). Metyrapone is a pyridine derivative, and binds at (or near) the haem iron centre, like carbon monoxide, so that the haemoprotein cannot bind oxygen (Netter, 1980). There are also suicide substrates of P450 that are metabolised to intermediates that irreversibly alkylate the P450 haem group, resulting in haem degradation and accumulation of porphyrins e.g. many olefins and acetylenes like allylisopropylacetamide and ethinyloestradiol.

Nitrosamines can also alkylate haem groups (Ortiz de Montellano and Correia, 1983). Metal ions also inhibit P450: cobalt-protoporphyrins decrease total P450 and total haem and also induce haem oxygenase (Galbraith and Jellinck, 1989); zinc, in the form of a zinc-protoporphyrin, inhibits P450 metabolism and decreases P450 content (Matsuura et al., 1988) and may decrease the ability of P450 to interact with substrate and/or reductase (Jeffery, 1982).

Recently it has been shown that P450 is a substrate for cyclic-AMP-dependent kinase (Pyerin et al., 1984) and that phosphorylation inhibits P450 activity: IIB1/2 are markedly phosphorylated, leading to inactivation (Koch and Waxman, 1989). Phosphorylation is also associated with an increase in P420 (Taniguchi et al., 1985). Phosphorylation also inhibited the b5-induced spin-state transition and b5 interaction with P450 (Epstein et al., 1989).

1.3 P450 AND XENOBIOTIC METABOLISM

The P450/MF0 is the major contributory component to Phase I xenobiotic metabolism, however, there are other oxidative enzyme systems including the alcohol and aldehyde dehydrogenases, xanthine oxidase and monoamine oxidase. In addition, the reductases, P450 reductase and menadione:NAD(P)H oxidoreductase can contribute to Phase I metabolism (Gibson and Skett, 1986; Ziegler, 1988). There is also a flavin monooxygenase. This flavoprotein is an integral

microsomal membrane protein with a broad substrate specificity, active towards sulphur, phosphorus and nitrogen centres e.g. thioureas, hydrazines (Ziegler, 1988),

Phase I metabolism produces intermediates which are not necessarily less toxic than the parent compound, and have several possible fates. They can interact with cellular components which may cause toxicity, or they may be further metabolised by the Phase II conjugation enzymes. This usually results in a hydrophilic, readily excretable metabolite. These reactions involve conjugation of reactive intermediates with a variety of molecules e.g. sugars, sulphate and amino acids, these cofactors usually requiring activation prior to reaction (Gibson and Skett, 1986). As with Phase I activities, many endogenous compounds are substrates for Phase II metabolism (Table 1.3).

The major conjugation reaction for detoxification of a range of xenobiotic compounds is glucuronidation. UDP-D-glucuronic acid can be transferred to a variety of functional groups by the UDP-glucuronyltransferases (UDPGTs) to form a glucuronic acid conjugate. These molecules are very polar and easily excretable. The UDPGTs have recently been reviewed (Burchell and Coughtrie, 1989). The liver is the most important site of glucuronidation for many compounds e.g. clofibrate and valproic acid (Howell et al., 1986) and paracetamol (Prescott et al., 1981). The UDPGTs exist as a family of microsomal isozymes, like the P450s, and there is

differential induction of the isozymes, in Man and rats, by the common MFO inducers including PB, 3-MC and clofibrate (Thompson et al., 1982). Often there is simultaneous induction of MFO and conjugation enzymes (Hardwick et al., 1983a).

The sulphotransferases are soluble enzymes that also exist in multiple forms, but in general, sulphation is less important than glucuronidation in Man and rats, due to the limited availability of activated sulphate (phosphoadenosine-5'-phosphosulphate; Gibson and Skett, 1986). Paracetamol and 2 -acetylaminofluorene are two of the many drugs where sulphation is involved in the metabolism (Prescott et al., 1981; Meerman et al., 1987).

The glutathione transferases are another family of soluble isozymes, that are also inducible by MFO inducers. They catalyse glutathione-conjugation e.g. of paracetamol, but glutathione (GSH)can also spontaneously bind to drugs e,g, tetracycline (Gibson and Skett, 1988). GSH-conjugates can be further metabolised by the intestinal flora and the kidney C-S-lyases, to mercapturic acids (Jakoby, 1988). Glycine conjugation, methylation and hydrolysis are other Phase II reactions, but these are relatively minor pathways of metabolism (Jakoby, 1988).

Although Phase I metabolism is often referred to as the activation phase, and Phase II metabolism as detoxification, it is more a case

of Phase I and II metabolism together resulting in detoxification. The interactions between the two, especially under conditions of enzyme induction or limited cofactor availability, will determine the eventual fate and intracellular effects of a potential toxin,

1.3.1 Factors affecting drug metabolism

The main biochemical factors that influence P450 activity are oxygen supply and substrate availability. Oxygen is essential for MFO activity, and under normal circumstances is readily available. However in the centre of an organ like the liver, and under certain pathological conditions oxygen supply may be limiting resulting in a predominance of reductive metabolism, which may markedly alter the metabolic profile of a drug, e.g. halothane can be metabolised by oxidative and reductive P450 pathways, which yield different metabolites with different toxic potentials (Timbrell, 1983). Intra-tissue gradients in oxygen tension may also influence hepatocyte heterogeneity with respect to isozyme activity (see below).

Substrate availability is also important in determining P450 activity: the lipophilicity of a compound will affect its access to the membrane-bound P450s and plasma binding will affect its availability to the hepatocytes. Biliary excretion and enterohepatic recirculation, e.g. of glucuronides, can also affect bioavailability (Gibson and Skett, 1986).

Cofactor supply can limit P450 reactions: NADPH is produced by the pentose phosphate pathway, but is also required for fatty acid biosynthesis and the reduction of oxidized glutathione (Thurman and Kauffman, 1980). Thus NADPH content is determined by prevailing metabolic conditions and may be limiting during oxidative stress. Cofactor supply also influences Phase II metabolism e.g. UDP-glucuronic acid formed from glucose-1-phosphate, which is derived from NAD^+-dependent glycogenic pathways, may be depleted during glucuronidation of xenobiotics e.g. paracetamol (Howell *et al.*, 1986; Hjelle *et al.*, 1986). Maintenance of adequate GSH levels is also important in preventing oxidative stress. The factors that influence drug metabolism have all been extensively reviewed, especially with respect to the liver (Thurman and Kauffman, 1980; Gibson and Skett, 1986).

The major determinant of the metabolic profile of a compound is the P450 isozyme complement of the organ/cell (and to a lesser extent, the distribution of Phase II enzymes). Differential P450 isozyme expression is probably one of the main explanations for organ-specific toxicity although extra-hepatic toxicity may also be strongly influenced by other factors e.g. solute concentration in the kidney and oxygen tension in the lung; tissue-specific differences in isozyme expression were discussed in 1.2.1. Although the liver is the main site of MFO metabolism and a primary target for MFO-generated toxins, there are well documented examples of non-hepatic MFO-activated toxins e.g. the pulmonary toxin,

4-ipomeanol (Boyd and Burka, 1978); renal toxicity of chloroform and benzene toxicity to bone marrow (Gram et al., 1986),

With respect to the liver itself, it has been proposed that there is metabolic heterogeneity within the functional unit of the liver. The acinus as the functional unit of the liver was first described in 1954(Rappaport et al,), and represents the cellular organisation around the hepatic blood vessels (Figure 1.2), Blood flow is unidirectional and sequential across the acinus from the hepatic portal venules to the hepatic venules, and the acinus is divided into three zones. Zone 1 is the periportal region and zone 3 the perivenous region. Functional differences between the two are thought to relate to composition of the perfusing blood; periportal hepatocytes are exposed to higher concentrations of solutes and oxygen (Gumucio, 1989), Many metabolic processes appear to be regulated by blood composition e.g. the zonal sites of maximal oxygen consumption and carbohydrate metabolism change as the direction of perfusion is changed. Although the oxygen tension of the sinusoidal blood might be expected to affect MFO activity, in fact activity seems to be unaffected by the direction of blood flow and instead appears to be associated with perivenous hepatocytes suggesting a heterogeneous distribution of P450 isozymes (Gumucio, 1989). Many hepatotoxins e.g. paracetamol, carbon tetrachloride, cause predominantly zone 3 necrosis i.e. perivenous, which also suggests that P450s may not be evenly distributed within the acinus. Alternatively, zone 3 hepatocytes may be more susceptible to damage

from P450-generated metabolites e.g. low levels of glutathione peroxidase in perivenous hepatocytes have been reported (Kera et al., 1987), From the work that has been done to date, P450IIE1, IIB1/2 and IA1 appear to be predominantly expressed in the centrilobular region or zone 3 (Gumucio and Chianale, 1988; Ingelman-Sundberg et al,, 1988).

1.4 METHODS OF STUDYING HEPATIC DRUG METABOLISM AND TOXICITY

1.4.1 *In vivo* and perfused organ studies

There are many different techniques available for studying P450-mediated metabolism and toxicity including whole animal studies, intact cells, use of subcellular fractions and purified isozymes. *In vivo* metabolism studies involve sampling of urine, serum or expired air after administration of a drug, followed by analysis of metabolites or biochemical indicators of tissue damage. Thus, the different metabolites of a radiolabelled dose of antipyrine can be recovered from samples, providing information on the extent of metabolism, the relative proportions of the various metabolites and their rate of production and clearance (Poulsen and Loft, 1988), whilst the presence of liver-specific enzymes such as aspartate and alanine aminotransferases in serum samples are sensitive indicators of the extent and severity of hepatic damage (depending on whether the enzyme is cytoplasmic or organellar; Animal Clinical Association, 1988). However, whole animal studies are slow and costly and unsuitable for mechanistic studies or investigations into drug effects at the cellular level. For these reasons investigators have turned to *in vitro* methods of studying metabolism.

The perfused liver has been used as an alternative to *in vivo* studies of drug metabolism. The liver is perfused via the portal vein, but this must be initiated *in situ* to reduce the period of

potentially deleterious anoxia (Thurman and Kaufmann, 1980). The advantages of this system are that it is easy to sample the perfusate, the tissue can be freeze-clamped prior to future analysis and conditions can be carefully controlled, with high doses of drug being administered without the complication of toxicity in a whole animal. This approach has been used to investigate many aspects of drug metabolism including measurement of binding spectra and redox states of P450 (Sies and Brauser, 1970) and the biotransformation of drugs (e.g. valproic acid, Rettenmeier et al., 1985). However, the viability of these perfused organs is limited to a few hours, high flow rates must be used for haemoglobin-free perfusates (which is necessary since the presence of haemoglobin decreases MFO activity, interferes with fluorescence measurements and results in progressive haemolysis) and large volumes of perfusate are required in non-circulating systems (Thurman and Kauffman, 1980).

1.4.2 *In vitro* techniques

Since the P450 system is located in the ER, microsomal fractions have been used to study drug metabolism and toxicity at the subcellular level. The studies include measurement of spectral changes on binding of substrate to P450 (Schenkman et al., 1967) determination of P450-dependent enzyme kinetics (Boobis *et* al., 1981); the metabolic activation of hepatotoxic compounds (Boyd et al., 1978; Ravindranath and Boyd, 1985); the detection of reactive metabolites (Garle and Fry, 1989). However, although microsomal fractions can generate active metabolites, no information is

obtained about possible cytotoxicity of these metabolites. There have been attempts to combine rodent S-9 fractions (9000g supernatant: microsomes and cytosol) with cultured cells to detect cytotoxic metabolites e.g. the activation of bromobenzene by S-9 fractions to metabolites cytotoxic to cultured Chinese hamster fibroblast cells (Horner et al., 1987); however, the use of this approach is not widespread in cytotoxicity studies, although microsomes/S-9 fractions are routinely used as the activation system in in vitro mutagenesis assays (Maron and Ames, 1983).

It is now possible to isolate and purify individual P450 enzymes, from solubilised microsomes and examine their catalytic properties in reconstituted systems (Lu and West, 1980). P450s, like other proteins, can be purified by gel electrophoresis, although resolution of individual isozymes by differential mobility due to molecular weight is difficult, since the molecular weight of most P450s is in the 45,000-60,000 dalton weight range. Purification and characterization of isozymic forms has been facilitated by the use of antibodies, although crossreactivity with closely related isozymes is always possible, even with monoclonal antibodies (Gonzalez, 1989).

However, the use of antibodies, in combination with microsomes and purified P450s in reconstituted systems has allowed both quantitation of different isozymes and estimations of isozymic

contributions to metabolism to be made e.g. an antibody to P450IIA1 that inhibited more than 98% of microsomal testosterone - 7α -hydroxylase activity was used to probe the effects of age, sex and xenobiotics on P450IIA1 expression (Arlotto and Parkinson, 1989); antibodies have been used in conjunction with microsomes and reconstituted systems to identify P450IIC6, IICll and IIBl as the isozymes that are primarily responsible for the activation of cyclophosphamide (Clarke and Waxman, 1989). There are disadvantages to the use of these systems: incorporation of different lipids in the reconsituted systems can affect the measured catalytic rate (Yamazoe et al., 1988); purified P450s may exhibit activities on reconstitution that cannot be inhibited by specific antibodies to the isozyme and vice versa (Waxman et al., 1985; Yamazoe et al., 1988); there is also the problem of crossreactivity of antibody preparations with more than one isozyme.

There are other difficulties associated with biochemical techniques for isolation and purification of P450 isozymes, in that intrinsic membrane proteins are difficult to purify, particularly the minor forms expressed at very low levels (e.g. extrahepatic P450s). There is also the problem of homogeneity, since very similar P450s can have identical SDS-gel mobilities, amino-terminal primary sequences and reactions with antibodies. These problems can be largely overcome by the use of molecular biological techniques. It is possible to generate cDNA (complementary DNA) libraries from mRNAs isolated from untreated or induced animals. Clones of these cDNAs

can then be screened with cDNA probes synthesized from the mRNAs, This method can also be used for low abundance mRNAs, by screening with antibodies. The development of cDNA expression vectors has further aided the isolation of minor P450 isozymes (Gonzalez, 1989).

Purified P450s are particularly useful for investigating substrate specificity and catalytic activity of individual isozymes (assuming that the preparation is homogeneous for a single isozyme). In addition, purified P450s and cDNAs can be used to analyse the primary amino acid and nucleotide sequences of the P450s. This has allowed the evolutionary and structural relationships between different isozymes to be explored (Nebert and Gonzalez, 1987).

The major limitation to the use of subcellular fractions and purified isozymes is in the effects of P450-metabolism on the intact cell, and the assessment of toxic potential. Intact cell systems possess the Phase II enzyme systems in addition to the MFO system, and since the balance between these two systems is crucial for determining the toxicity of many compounds the development of intact cell systems for metabolism studies has received much attention in the last twenty years.

1.4.3 Intact cell systems

The use of intact cells, i.e. isolated hepatocytes, cultured hepatocytes and cell lines, has several advantages over the sub-cellular preparations discussed above, including the presence of

intact intracellular organisation, Phase I and Phase II metabolism and physiological levels of co-factors (Fry, 1982). In addition, hepatocytes can be isolated from other liver cells and maintained in a defined environment free from nervous and hormonal influences. A preparation of phenotypically similar cells can be obtained, and many parameters of cellular function/metabolism can be examined concurrently. Isolated hepatocytes have been used to study many aspects of drug metabolism e.g. the metabolism of menadione (Thor et al., 1982); the effect of tert-butyl hydroperoxide metabolism on the pentose phospate pathway (Rush and Alberts, 1985); the effect of ascorbic acid on conjugation of 4-hydroxybiphenyl (Paterson and Fry, 1983); species differences in paracetamol toxicity (Tee et al., 1987); the effect of induction on the covalent binding of valproic acid (Porubek et al., 1989b). Although the cells are intact, there is disruption of the spatial heterogeneity of the liver (Thurman and Kauffman, 1980); however, the major drawback to the use of isolated hepatocytes is their limited life-span in suspension (several hours). In order to prolong the useful life span of hepatocytes for in vitro studies, methods of primary culture were devised, and these are discussed in the following section.

1.4.4 Hepatocytes in primary culture

In the early seventies, it was reported that a high yield of viable hepatocytes could be isolated from adult rats by collagenase-hyaluronidase perfusion of the liver, and that these cells could be readily cultured (Iype, 1971; Williams and Gunn, 1974). After an initial burst of proliferative activity the cultures became static and many of the cells died. Since the reports of these early studies that established that hepatocytes could survive in primary culture, a large volume of research has ensued to enhance the longevity and improve the maintenance of the phenotype of hepatocytes in culture.

Some of the earliest work investigated the effects of media composition, especially the presence or absence of hormones, on cell survival and biochemistry. It was reported that inclusion of foetal calf serum (FCS) in the medium gave maximal attachment of rat hepatocytes at a concentration of 10%; the presence of insulin ($2mUml^{-1}$) in addition to the FCS increased attachment further with the formation of confluent cultures, although there was no improvement in cell survival in the presence of insulin: 75% survival at 24 hours, around 30% survival at 48 hours (Laishes and Williams, 1976a). Inclusion of DEX (1μM) maintained the morphology of the hepatocytes and increased longevity of the cultures: more than 80% of the cells survived for three days (Laishes and Williams, 1976b). It was later reported that DEX inhibited the production of a neutral protease, and it was suggested that this contributed to

the increased cell survival (Williams et al., 1978). Other workers reported that more than 80% of hepatocytes cultured in Williams' Medium E (WE) containing 10% FCS, insulin (10^{-6}M) and DEX (10^{-5}M) survived for six days in culture, and that, in contrast to freshly isolated cells, protein synthesis could be stimulated by hormones, the induction of tyrosine aminotransferase (TAT) and ornithine decarboxylase could be demonstrated and there was an increase in protein secretion into the medium. It was also shown that the polysomes,which disaggregated in freshly isolated cells, had reaggregated after five days in culture (Tanaka et al., 1978). In the presence of glucocorticoid, insulin and glucagon, the activities of glucokinase, pyruvate kinase and hexokinase were maintained for 2-3 weeks in rat hepatocyte cultures, in addition to albumin secretion and urea synthesis (Dich et al., 1988).

However, an increase in foetal hepatic protein expression, with a concomitant decrease in adult-specific proteins has been reported in primary cultures. As early as 1975, Guguen et al. observed that after seven days in primary culture the predominant form of pyruvate kinase in the cells isolated from adult rats was the K-type. This form is present in foetal liver, placenta and hepatoma cells, but is a minor form in adult parenchymal cells, where the L-type predominates. This form was detectable for five days in culture, but then decreased. Gamma-glutamyl transpeptidase (GGT) and α1-foetoprotein (which are not normally expressed in adults) also increase in rat hepatocytes cultured for more than three days

(Sirica ft al., 1979). GGT levels are normally very low in adult liver and in early hepatocyte cultures, but levels are much higher in foetal and neonatal liver, and in rat hepatoma cells (Edwards, 1982). Although the presence of FCS in the medium suppressed the increase in GGT, DEX actually induced GGT in primary cultures, the effect being particularly marked at higher pH levels (i.e. 7.8; Edwards, 1982). It is not known why this reversion to the foetal phenotype occurs in culture, although the observed changes appear to be associated with less differentiated/ proliferative cellular states; however, these alterations in phenotype would seem to limit the use of hepatocyte primary culture to short-term studies.

In 1977 it was reported that the drug-metabolizing capacity of rat cultured hepatocytes was markedly reduced, compared to the liver in vivo (Guzelian ft al., 1977), The P450 content of non-proliferating primary cultures was 20% of the initial fresh cell value at 24 hours, with 63% of the P450-reductase remaining. Although there was this rapid decrease in P450 levels, no increase in P420 was detected. The maintenance of P450-dependent enzyme activities after 24 hours in culture was variable: aminopyrine-N-demethylase and aniline hydroxylase activities both decreased by more than 70%, and aryl hydrocarbon hydroxylase (AHH) activity fell by more than 50%, whereas nitroanisole demethylase was maintained at 85% of the initial activity. Therefore, it appeared that there was selective loss of P450 activities (and therefore isozymes) in culture. The general decline in total P450 has been reported by

many workers and is not restricted to rat hepatocytes in culture: P450 declines in cultures from other rodent species (Maslansky and Williams, 1982), and in human cultured hepatocytes (Grant et al., 1985), This serious phenotypic alteration is a major obstacle in the use of hepatocytes for metabolism/toxicity studies. For this reason, many different ways of improving the maintenance of P450 in culture have been investigated.

1.4.5 Maintenance of P450 in primary culture

Media composition has received a lot of attention with respect to maintenance of P450 in cultured hepatocytes, Decad et al. (1977) reported that medium supplemented with fatty acids, delta-amino-laevulinate (ALA), alpha-tocopherol and various hormones (including insulin, hydrocortisone and thyroxine) maintained P450 content of rat cultured hepatocytes at 100% of the initial level for 24 hours. A similar medium was shown to significantly improve the maintenance of 7-ethoxycoumarin and 7-ethoxyresorufin O-dealkylases (ECOD, EROD) and biphenyl-4-hydroxylase activities (Dickins and Peterson, 1980), A more recent report suggests that hormone- supplementation alone cannot attenuate the loss of P450 in culture, although the presence of DEX (1μM) stabilised the total P450 content at 65% of initial levels for three weeks (Dich et al., 1988).

Ascorbate (4×10^{-4}M) supplementation partially attenuated the early decline in P450 content, as well as improving the maintenance of cytochrome b5 and P450 reductase (Bissell and Guzelian, 1979). Paine

et al. (1979) demonstrated that the NAD content (NAD^+ and NADH together) of rat hepatocytes after 24 hours in culture was 40% of the fresh cell value, and that nicotinamide supplementation could prevent the loss of NAD. In addition, unphysiological levels of nicotinamide i.e. 25mM could prevent the loss of P450 in culture. Isonicotinamide was more effective in maintaining P450 levels, but had no effect on NAD content. A substituted pyridine, metyrapone (0.5mM) was also effective in preventing the loss of total P450 in rat hepatocyte cultures: more than 95% of the total P450 remained after seven days in culture, compared to only 48% at 24 hours in medium lacking metyrapone (Paine et al., 1982). This medium also maintained ethylmorphine-N-demethylase activity for four days, together with a gradual increase in benzphetamine-N- demethylase to 84% of the initial value over the same time-period. There was also induction of both ECOD and EROD activities over the four days in culture (Lake and Paine, 1982). Therefore, the maintenance of activities observed in culture was not always at the same level as in vivo. A cystine/cysteine-free medium containing ALA maintained total P450 at 96% for 24 hours, however, all enzyme activities were very low (Lake and Paine, 1982).

Nicotinamide, at a lower concentration (5mM) than that reported by Paine et al. (1979) was shown to stabilise ECOD and 7-propoxycoumarin O-dealkylase (PCOD) activities in rat cultured hepatocytes (around 50-60% of fresh cell values) similar to DEX alone, whereas nicotinamide and DEX (1μM) together resulted in large

increases in these activities. However, this effect was still selective for certain P450 forms in that there was no effect on the decline of 7-methoxycoumarin O-dealkylase (MCOD) activity in culture (Warren and Fry, 1988).

Steward ft al. (1985) reported the differential decline of immunoreactive P450 isozymes in primary culture. It was also observed that whereas the total immunoreactive P450 fell to 76% after 72 hours, the total spectrally active haemoprotein was 68% of initial levels, and it was suggested that loss of haem was responsible for a loss of holocytochrome in culture. The same report showed that metyrapone attenuated the loss of immunoreactive P450 over 72 hours, and that nicotinamide had a selective effect with respect to maintenance of immunospecific P450s in culture.

Inclusion of exogenous haem, ALA and selenium has also been reported to maintain total P450 for 72 hours in culture (Engelmann et al., 1985) and 3mM PB has been shown to markedly enhance the long-term survival of hepatocytes in culture, with attenuation of the decline in total P450 (Miyazaki et al., 1985).

Vind et al. (1988) reported that hormonal-supplementation of culture media diminished the loss of P450 over 72 hours only in hepatocytes from adult female rats; there was no effect on hepatocytes from adult males. Croci and Williams (1985) observed little difference in the % maintenance of P450, AHH and benzphetamine-N -demethylase

activities in hepatocytes cultured for 24 hours in hepatocytes from male and female rats, with sex differences in basal activities being maintained in culture. However, glutathione conjugation of l,2-dichloro-4-nitrobenzene declined rapidly in hepatocytes from male rats to around 20% of the fresh cell value; hepatocytes from female rats only lost 66% of this activity.

Thus, media supplementation can profoundly affect the maintenance of P450 and its associated activities. Many supplements appear to exert selective effects on particular isozymes, which are not reflected in measurements of total P450, but are unmasked on immunochemical detection or determination of enzyme activities. Different effects are also observed depending on the concentration (and possible interactions with other supplements) used. This is particularly true for hormonal supplementation. Many different media are employed, depending greatly on personal preference and the aim of the study. However, no medium has yet been formulated that maintains in vivo P450 levels in culture, qualitatively or quantitatively.

Early work involved plating of isolated hepatocytes directly onto plastic culture vessels (e.g. Laishes and Williams, 1976a and b), or onto plastic coated with a thin layer of rat tail collagen (e.g. Bissell and Guzelian, 1980; Dickins and Peterson, 1980) to improve attachment and spreading of the hepatocytes. Other workers have investigated the effects of extracellular matrix components on

hepatocyte function and maintenance of phenotype. Hepatocytes cultured on floating collagen gels have been reported to have a superior induction response of TAT to hydrocortisone (10^{-5}M) after eight days in culture relative to cultures on the usual collagen-coated plates (Michalopoulos and Pitot, 1975); P450 and cytochrome b5 were maintained at detectable levels after ten days of culture on floating collagen gels (Michalopoulos et al., 1976). In a comparison of different extracellular matrix components (Sawada et al., 1987) there were no differences in attachment, longevity or induction of TAT; however, after two weeks in culture GGT activity was highest in cells cultured on collagen and lowest in cells cultured on fibronectin. Matrigel is a mouse tumour cell extract, which has been shown to be superior to collagen as a substratum for hepatocyte culture (Schuetz et al., 1988). More cells survived for eight days on Matrigel than on collagen and there was increased secretion of albumin, transferrin, haemopexin and glutathione peroxidase on Matrigel. Haem oxygenase and P450 reductase gradually declined over the eight day culture period. However, it is still not clear whether the observed changes in gene expression (many of the studies on substrate effects on hepatic function have not involved P450 activities) are sufficiently advantageous to routinely supplant the use of collagen-coated plates, or electrostatically-treated plates ('Primaria' plates, obtainable from Falcon Plastics) which appear to be equivalent with respect to maintenance of hepatocyte cultures.

Another method for maintenance of P450 in culture, which has received a lot of attention in recent years is co-culture of hepatocytes with other hepatic cells. Begue et al. (1984) demonstrated that in hepatocytes co-cultured with rat liver epithelial cells P450 was maintained at initial levels for nine days, together with aminopyrine N-demethylase activity. The P450 content and demethylase activity of conventional cultures declined to negligible levels after four days in culture.

Overall, manipulation of culture media composition appears to be the most successful (and also the simplest) method of maintaining P450 and its associated activities in short-term primary cultures (less than 72 hours) of hepatocytes, although the effects of supplementation are selective for particular isozymic forms. Although different substrata and the presence of other cell types can affect hepatic gene expression and biochemistry, relatively little is known about the effects of these culture components on the drug-metabolizing system of cultured hepatocytes.

1.4.6 Induction of P450 in culture

It was mentioned above that 3mM PB attenuated the loss of P450 in primary cultures of rat hepatocytes; however, P450IIB1, the major PB-inducible isozyme was not immunochemically detectable in these cells (Miyazaki et al., 1985). Other workers have also reported a reduced or negligible response of P450 to inducers in culture, especially the response to barbiturates. Forster et al. (1986)

reported that the aldrin epoxidase activity of rat hepatocytes declined to 55% and 8% of initial levels at 1 and 5 days in culture respectively, and that PB and PCN treatment resulted in statistically insignificant increases in this activity, in contrast to the situation in vivo. Benzanthracene and PB both induced EROD activity in culture (12- and 8-fold respectively), this induction being markedly reduced in the absence of DEX (present in the medium). It was also shown that the time-course of induction depended on the media used, and that P450IA1 increased over five days in control cultures. This anomalous increase in 3-MC-inducible, alpha-naphthoflavone-inhibitible P450s in untreated cultures has also been observed by others (Fry et al., 1980; Turner and Pitot, 1989), although the reason is unknown. PB and BNF induction of ECOD activity is much reduced in culture compared to in vivo, even in the presence of DEX (Warren and Fry, 1988), as is the induction of pentoxyresorufin O-dealkylase by PB in chemically-defined media (Turner and Pitot, 1989). Cultured hepatocytes have also been used to study the metabolism of 2-AAF(McManus et al., 1987), In control cultures, 3-hydroxylation remained constant for 24 hours in culture, whereas the 1-, 5-, 7-, 9- and N-hydroxylations declined rapidly. DEX (0.1μM) did not prevent the initial decline in activity, but after 72 hours in culture the 5- and 3-hydroxylations activities had increased over initial levels and the 7-hydroxylation activity had increased to initial levels (after a decline at 24 hours). There was also some recovery of N-hydroxylation. PCN only induced 7-hydroxylation in

vitro. It was noted that the induction response in vitro was much smaller than that seen in vivo, and also qualitatively different, 3- and 5-hydroxylation being induced by DEX in vitro but not in vivo. Therefore, media composition also affects induction responses in vitro, although in general, P450 induction in vitro is poor, and qualitatively as well as quantitatively different to that seen in vivo. This will be discussed further in Chapter Five.

1.4.7 Metabolism/toxicity studies in cultured rat hepatocytes

In 1977, Decad ft al. demonstrated that aflatoxin B1 could be metabolised by cultured hepatocytes to aflatoxin Ml and various conjugates. It was also shown that a significant amount of radioactivity (after exposure to radioactive aflatoxin) was non-extractable i.e. remained bound to the hepatocytes.

Rat hepatocyte cultures have also been used to demonstrate that substantial acetaminophen covalent binding in the cells occurs before any observable toxicity, and that the covalent binding is mostly in the microsomal fraction (37%) and in the cytosol (25%; Acosta et al., 1987). It has also been shown that cyclophosphamide can be activated to a metabolite stable enough to kill fibroblasts co-cultured with the hepatocytes, and that the toxicity can be inhibited by SKF-525A (Fry and Bridges, 1977).

Cultured hepatocytes have also been used to discriminate between hepatotoxic and non-toxic substances, especially of a related group of compounds e.g. non-steroidal anti-inflammatory drugs (Sorensen and Acosta, 1985). It was demonstrated that indomethacin was the most toxic of the five compounds tested, followed by benoxaprofen and ibuprofen, and aspirin and orpanoxin were non-toxic at the concentrations tested, as judged by viability, lactate dehydrogenase leakage and urea synthesis of the cultures, A more detailed discussion of the parameters used to assess toxicity is given in Chapter Seven.

1.4.8 Human hepatocytes in primary culture

Less work has been done on drug metabolism in human cultured hepatocytes since adult samples are difficult to obtain and it is much harder to get high yields of cells suitable for culture, mainly due to the nature of the samples. However, from the work that has been done, it appears that human hepatocytes in culture de-differentiate slower than their rodent counterparts, and also, that the P450 content declines more slowly: 60% of the spectrally determined P450 remains after four days in culture (Strom et al., 1987). Grant ft al. (1987) demonstrated that P450-dependent activities and glucuronidation were well maintained for 72 hours in human cultured hepatocytes; sulphation however decreased within the first 24 hours, and the P450 reductase had declined to 32% after 72 hours in culture. Begue et al. (1983) investigated the metabolism of an anti-anaphylactic, ketotifen, in human cultured hepatocytes: the main metabolites produced in vitro correlated with the known metabolic pathways in vivo. Also, the rate of metabolism did not alter significantly over the first four days of conventional culture, or seven days for human hepatocytes co-cultured with rat epithelial cells. Thus, human hepatocytes would appear to have several advantages over rodent hepatocytes with respect to maintenance of drug metabolism in vitro.

1.4.9 Use of hepatoma cell lines

It is still not possible to obtain substantial (and reproducible) levels of mitotic activity in adult cultured hepatocytes. Some

work has been done on drug metabolism in hepatoma cell lines, both human and rodent in origin, since a dividing cell line would provide a continuous supply of hepatocytes, obviating the need to isolate and culture cells for every study. Wiebel et al. (1984) examined the P450 activity of six differentiated rat hepatoma cell lines and found that the constitutive activities were very low, and some were undetectable. The expression of MFO sensitive to antibodies to PB-inducible P450s was present in widely differing amounts in the differentiated lines, Benzanthrancene, PB and DEX induction of P450 IA1 and AHH activity and PB and DEX induction of P450IIB1/2 and aldrin epoxidase have also been observed in differentiated cell lines (Wiebel ft al., 1984; Corcos and Weiss, 1988). The human hepatoma, HepG2 has been shown to have levels of glucuronidation and cytochrome reductase similar to those of freshly isolated human hepatocytes although levels of O-dealkylation were reduced (Grant ft al., 1988). Although HepG2 cells remain differentiated in culture, their P450 content was 10-20% that of fresh human hepatocytes, and their P450 activities were 10-fold lower. However, medium composition altered drug metabolism activities (Doodstdar et al., 1988). HepG2 cells also synthesize and secrete the major plasma proteins, and morphologically resemble hepatocytes, although they do possess an abnormal chromosome number (50-56) with a distinctive rearrangement of chromosome 1 (Knowles et al., 1980).

1.4.10 Species differences in metabolism

The preceding sections referred primarily to the use of rodent *in vitro* systems, principally rat hepatocytes, in the study of drug metabolism and mechanisms of toxicity. Increasingly these cells are being used in the development of predictive toxicity tests. In addition to the limitations imposed by the decline of P450 and metabolic capacity *in vitro* discussed above, there are also problems, particularly with toxicity testing, associated with extrapolation both from *in vitro* to *in vivo* and from rodents to Man.

Correlations between drug metabolism studied *in vivo* and *in vitro* have been discussed in the previous sections (1,3.1-5) and the same advantages of *in vitro* techniques with respect to the study of drug metabolism also extend to toxicity studies. Many compounds e.g. heavy metals, mineral fibres and arsenic usually test negative in short-term mammalian cell culture assays, despite the fact that they are known human carcinogens (Lave and Omenn, 1988). In addition, volatile and particulate compounds present special problems. There is also the problem of detection of pro-toxins i.e. the test system must be able to activate known non-toxic precursors to their reactive intermediates. The most successful *in vitro* toxicity assays to date have been those that detect genotoxins by assessing DNA damage e.g. unscheduled DNA synthesis (UDS) and sister chromatid exchange (Grisham and Smith, 1984). The sensitivity of the UDS assay has been improved by use of rat hepatocytes cultured from animals pre-treated with MFO inducers i.e. Arochlor 1254 and PB

(Shaddock ft al., 1989). Hepatocytes from inducer-treated animals have also been used as an activation system for the mouse lymphoma cell mutagenicity assay (Oglesby et al., 1989).

Even when in vitro toxicity information has been obtained, there is still the problem of correlation with the in vivo data, as to which measure of acute toxicity is the most suitable comparison (Fry ft al., 1988). The in vivo data exists in many forms with variations in routes of administration, species tested and assessment of toxicity. It is also difficult to make sensible extrapolations of toxic concentrations from static culture systems to organisms with dynamic elimination systems.

There are also many known species differences in P450 metabolism, reviewed by Kato (1979), which have important implications for (but do not necessarily preclude) the use of experimental animals in the assessment of human metabolism and toxicity. There are species differences with respect to rate of metabolism e.g.hexobarbitone sleep time (Quinn et al., 1958) and the pattern of metabolites produced from a given substrate e.g. 7-hydroxycoumarin is the major metabolite of coumarin in Man, whereas in the rat 3-hydroxylation predominates, with very little 7-hydroxycoumarin being produced (Cohen, 1979). Species differences in P450 composition can result in differences in toxicity due to differing metabolite profiles e.g. the guinea pig, unlike the rat, mouse and rabbit, is resistant to 2-AAF-induced hepatomas. The guinea pig forms very

little N-hydroxylated-AAF, with ring hydroxylations predominating. In contrast, N-hydroxylase activity in the other species correlated well with susceptibility to 2-AAF-induced carcinoma, with hamsters being the least resistant, with the highest N- and lowest ring-hydroxylase activities (Miller et al., 1960; Kato, 1979). Thus, differences in metabolite profiles could be used to predict toxicity in susceptible species. It has also been shown that species differences in susceptibility to paracetamol-induced necrosis correlate with the extent of tissue covalent binding and GSH depletion (Davies et al., 1974). There are also differences in induction and inhibition (Kato, 1979). Another problem is that experimental animals are highly inbred and kept under uniform conditions e.g. diet, and therefore, unlike humans, are *very* homogeneous with respect to response on exposure to xenobiotics (Gregory, 1988). However, the rat is still the most popular model for studying P450 metabolism and toxicity, not least because of the large accessible data base, and the ready availability of animals. Despite the differences in metabolism, vast improvements in predictive toxicology could be made by increased understanding of mechanisms of toxicity, and routes of metabolism in susceptible and resistant species (Grisham and Smith, 1984).

1.5 RATIONALE AND AIMS OF THIS STUDY

The aims of this study were to develop an *in vitro* system for the investigation of hepatic cytochrome P450-mediated toxicity. Cultured rat hepatocytes have the potential for use as a prime *in vitro* model of hepatic drug metabolism and toxicity as has been discussed above (1.4.3 - 1.4.4). However, in order to investigate hepatic metabolism-mediated toxicity, the full range of biotransformation and detoxification enzymes must be present and active in the cultured cells. In this respect, the inability to maintain the P450 isozymes in cultured hepatocytes has proved to be a major drawback to their use in activation/toxicity studies. The large body of work concerned with the maintenance of P450 in culture was reviewed in section 1.4.5, Included in this was the effects of media supplementation of P450 activity *in vitro*. It has been shown that DEX in particular could maintain P450 activities in primary culture, and that DEX (1μM) and nicotinamide (5mM) together improved the maintenance of P450-dependent alkoxycoumarin O-dealkylase activities *in vitro* (Warren *et al.*, 1985; Warren and Fry, 1988). However, this maintenance was selective in that the ECOD and PCOD activities were well maintained for 72 hours, whilst MCOD activity was rapidly lost in culture (Warren and Fry, 1988). In the same study, reduced induction by PB and BNF *in vitro* compared to *in vivo* was reported.

Most of the work presented here was concerned with developing the culture conditions described above to support both the stable maintenance of a wider range of P450 activities and also induction of P450s *in vitro*. The effects of induction *in vivo* on P450 activity and P450-mediated toxicity *in vitro* were also investigated.

TABLES AND FIGURES

TABLE 1.1. ENDOGENOUS AND XENOBIOTIC SUBSTRATES OF CYTOCHROME P_{450}[1].

SUBSTRATE	REACTION
steroids *prostaglandins* *leukotrienes* *arachidonic acid* *vitamin D* *lauric acid* phenobarbitione	HYDROXYLATION (aryl or aliphatic)
arachidonic acid benzo-pyrene bromobenzene	EPOXIDATION
thyroid hormones halothane	DEHALOGENATION
uroporphyrinogen [2] valproic acid[3]	DESATURATION
benzphetamine codeine 6-methyipurine	N-,0- and S- DEALKYLATION
amphetamine	OXIDATIVE DEAMINATION
2-acetylaminofluorene	N-OXIDATION
ethanol	ALCOHOL OXIDATION
azo, nitro compounds halogenated hydrocarbons	REDUCTION

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1 information from Gibson and Skett (1986), except where indicated. Endogenous substrates are in italics, and xenobiotic substrates in plain print.

2 Jacobs *et al.,* 1988

3 Rettie *et al.,* 1988

TABLE 1.2. P_{450} NOMENCLATURE. P_{450} nomenclature has now been standardized by Nebert *et al.*(1987,1989), and this is shown in the first column below. Isozyme designations from different laboratories are shown in subsequent columns, and are based on the summaries by Waxman (1988) and Ryan and Levin (1990).

	LEVIN	GUENGERICH	SCHENKMAN	WAXMAN	KAMATAKI
IA1	c	BNF-B			
IA2	d	ISF-G			
IIA1	a	UT-F	RLM2b	3	
IIA2	m*				
IIB1	b	PB-B	PB RLM5	PB-4	
IIB2	e	PB-D	PB RLM6	PB-5	
IIC6	k	PB-C	RLM5a/PBRLM4	PB-1	
IIC7	f		RLM5b		
iiC11	h	UT-A	RLM5	2c	male
IIC12	i	UT-I	fRLM4	2d	female
IIC13	g		RLM3(g)		
IIE1	j		RLM6		
IIIAI	p				
IIIA2			PCN-E"	2a**	

* Arlotto and Parkinson, 1989.

** It is not entirely clear which of the isozymes isolated in the different laboratories are equivalent to P_{450}IIIA1, and which to P_{450}IIIA2; also, there have been changes in nomenclature of these isozymes within laboratories; the multiplicity of this particular gene family has been discussed by Halpert (1988).

TABLE 1.3. ENDOGENOUS AND XENOBIOTIC SUBSTRATES OF PHASE II METABOLISM[1]

SUBSTRATE	REACTION
steroid hormones	GLUCURONIDATION
bilirubin	
thyroxine	
catecholamines	
acetaminophen	
salicylic acid	
morphine	
steroids	SULPHATION
heparin	
acetaminophen	
2-acetylaminofluorene	
salicylamide	
histamine	N-METHYLATION
catecholamines	
thiouracil	
serotonin	ACETYLATION
Isoniazid	
bile acids	AMINO ACID CONJUGATION
benzoic acids	
bilirubin	GLUTATHIONE
steroids	CONJUGATION
leukotrienes	
vitamin K	
bromobenzene	
acetaminophen	
urethane	

[1] information from Gibson and Skett (1986) and Ziegler(1988). Endogenous substrates in italics, xenobiotics In plain print.

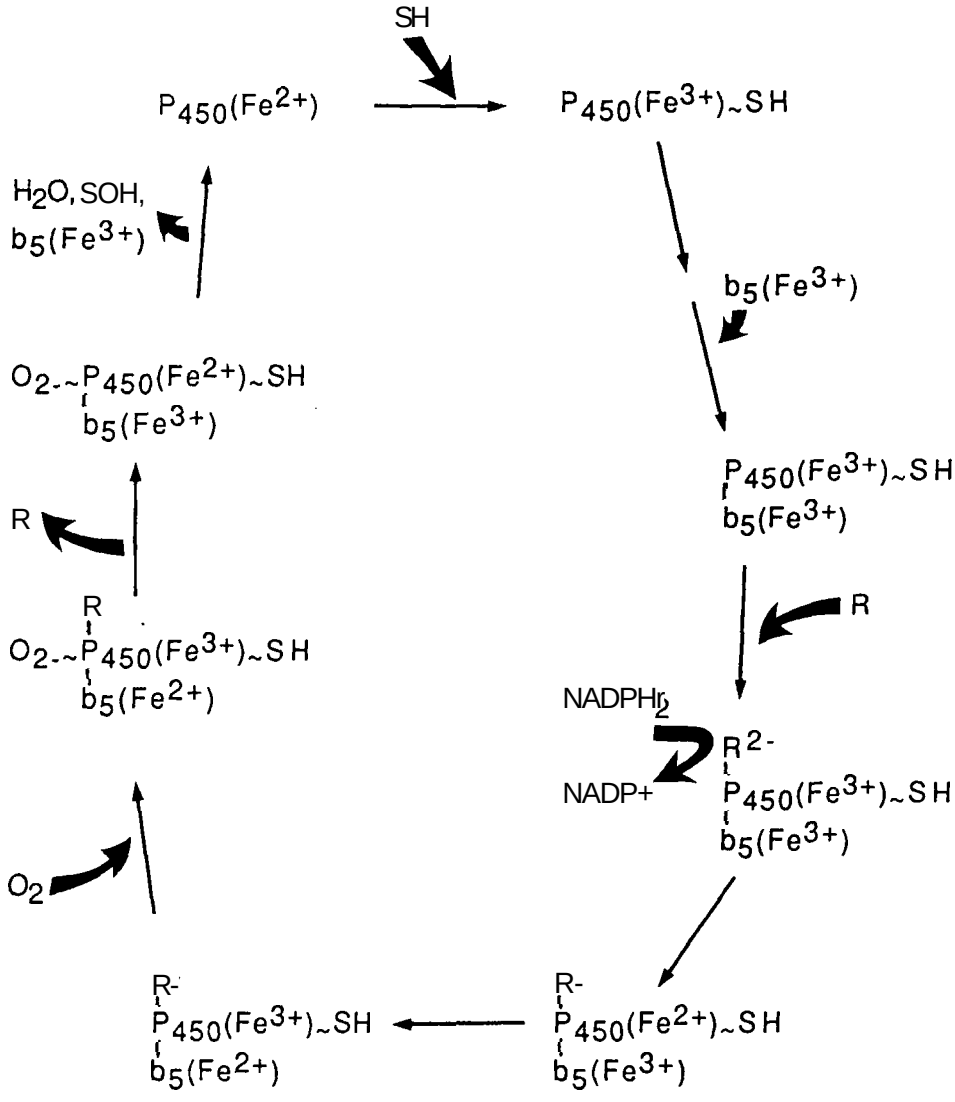


FIGURE 1.1. THE CYTOCHROME P_{450} CATALYTIC CYCLE, Based on information from several sources, including Jansson *et al.,* (1985) and Gibson and Skett (1986).

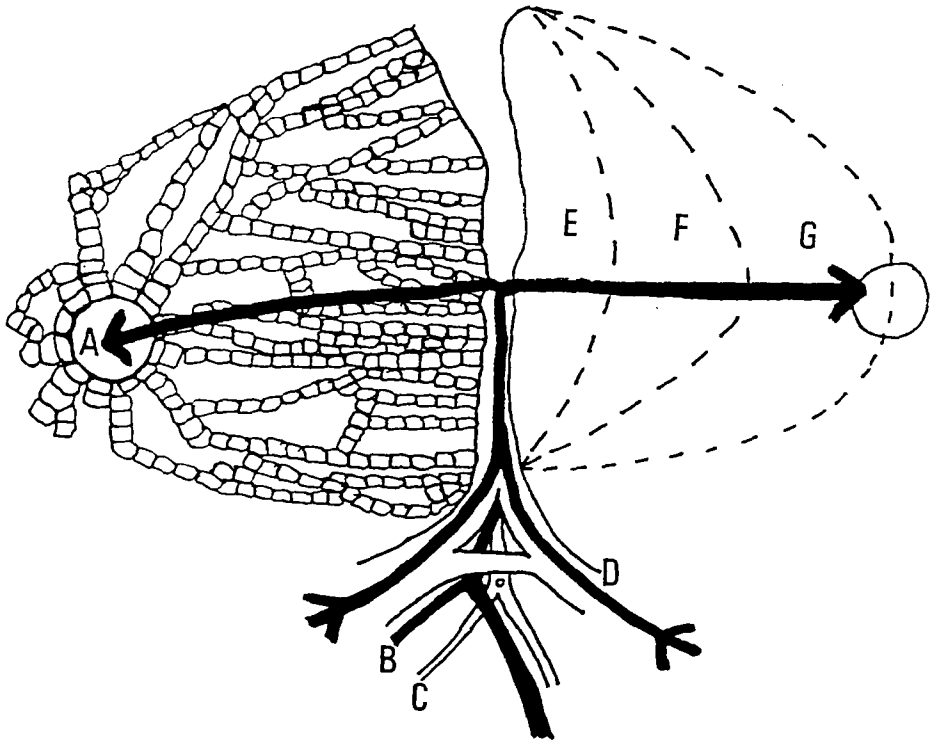


FIGURE 1.2. DIAGRAMMATIC REPRESENTATION OF THE LIVER ACINUS. Adapted from Gumucio and Chianale (1988). The concept of the acinus as the functional unit of the liver was first proposed by Rappaport ef *al.*(1954).

A terminal hepatic venule
B bile ductule
C hepatic arteriole
D terminal portal venule
E zone 1, periportal region
F zone 2, midzonal region
G zone 3, pericentral or perivenous region
Arrows show the direction of blood flow.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

CONTENTS

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

2.1 ANIMALS

The Wistar albino rats used in these studies were obtained from the University of Nottingham Animal Breeding Unit. Male and female rats (70 - 180g) were allowed free access to food and water and were housed at 22°C, with a 12 hour light/dark cycle (light: 08.00 - 20.00), The animals were bedded from birth on the synthetic bedding, Lablit (W.P. Usher, London, U.K.), to minimise enzyme induction from wood shavings (Vesell, 1982; Törrb'nen et al., 1989).

2.2 CHEMICALS AND EQUIPMENT

The suppliers of the chemicals used in these studies were as follows: delta-aminolaevulinate hydrochloride, 7-methoxycoumarin, 2-methylfuran, resorufin and selenious acid were obtained from Aldrich Chemical Co. (Dorset, U.K.); foetal calf serum, glutamine, trypan blue and Williams' medium E, cellulose acetate filters (pore size 0.2μm) and plate sealers were obtained from Flow Laboratories (Irvine, Scotland); collagen type I was obtained from B.C.L. (Sussex, U.K.); phenobarbitone and acetylacetone were obtained from B.D.H. (Dorset, U.K.); fungizone was obtained from Squibb Surgicare Ltd. (Hounslow, U.K.), dexamethasone (Decadron) from Merck, Sharp and Dohme Ltd. (Hoddesdon, U.K.), insulin

(Actrapid MC) from Novo Industri A/S (Denmark); and gentamicin from Roussel Laboratories Ltd. (Uxbridge, U.K.). Ammonium acetate, benzalkonium chloride, benzphetamine, beta-glucuronidase H-I (E.C. 3.2.1.31), beta-naphthoflavone, beta-NADP, bovine serum albumin, butylated hydroxytoluene, collagenase type IV (E,C, 3,4,24,3) Coomassie Brilliant Blue G, dicoumarol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), EDTA,EGTA, erythromycin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase type XI (E,C. 1.1.1.49), glutathione, hemin type 1, HEPES, hyaluronidase type II (E.C. 3.2.1,35), 7-hydroxycoumarin, isoamylalcohol, isoniazid (isonicotinic acid hydrazide), nicotinamide (niacinamide), 4-nitrocatechol, 4-nitrophenol, N,N-dimethylformamide, o-phthalaldehyde, Percoll, precocenes I and II, Rhodamine B, semicarbazide, 6-thiopurine (6-mercaptopurine) and valproic acid were all obtained from Sigma Chemical Co. (Dorset, U.K.). 4-Ipomeanol was a gift from Dr. M.R. Boyd, National Cancer Institute, Bethesda, MD, U.S.A. 7-Ethoxycoumarin, 7-propoxycoumarin and 7-ethoxyresorufin had been synthesized by the method of Prough et al. (1978). "Primaria" culture dishes were obtained from Falcon (Becton Dickinson Ltd., Oxford, U.K.) and 24-well plastic culture dishes from Nunc (Denmark). All other reagents and organic solvents were of analytical reagent grade and obtained from local suppliers.

Low-speed centrifugation was carried out using an MSE Centaur bench centrifuge or an MSE Mistral 6000 refrigerated centrifuge.

A Stuart Tube Rotator TR-2 was used for extraction procedures. Cell suspensions were homogenised using an MSE Soniprep. Spectrophotometric analysis was carried out using a Perkin-Elmer 550S UV-Vis spectrophotometer or a Dynatech plate reader and fluorometric analysis was performed using a Baird RC200 Ratiometric fluorimeter.

METHODS

2.3 STERILIZATION PROCEDURES

Standard sterilization procedures and aseptic techniques were practised throughout these studies, as outlined by Paul (1970) in addition to supplementation of culture media with antibiotics to reduce the risk of microbiological contamination of cell cultures. Water baths and the water tray inside the incubator all contained benzalkonium chloride (0.1%), and the water was changed frequently.

Dry heat

All glassware (openings covered with aluminium foil) and glass pipettes (bunged with cotton wool and placed in metal canisters) were sterilized in a Gallenkamp oven, set at 160-180°C for 2 hours.

Moist heat

Plastic and rubber items, wrapped in aluminium foil, and heat stable solutions were autoclaved in a Cabburn autoclave set at 121°C for a 25 minute cycle.

Filtration

Non-heat stable solutions and small volumes of liquid were sterilized by filtration through cellulose acetate filters with a 0.2µm pore size.

Aseptic technique

All sterile procedures were performed in a laminar flow hood (Intermed, Microflow, Hants., U.K.) regularly swabbed with 70% alcohol. Bottle and glassware openings and pipettes were all regularly flamed during use.

2.4 ISOLATION OF HEPATOCYTES

Hepatocytes were isolated either by the slice method of Paterson and Fry (1983) or by the lobe perfusion method of Reese and Byard (1981).

Slice method

The wash solution was a calcium-, magnesium-free Hanks buffer pH 7.4 (140mM NaCl, 5mM KCl, 0.4mM KH_2PO_4, 0.3mM Na_2HPO_4, 20mM HEPES, 50µM phenol red, 20 ml l^{-1} 0.5N NaOH), to which $NaHCO_3$, glucose, methionine and gentamicin, at 14.8, 5.5, 2mM and 50µgml^{-1} (final concentrations), were added on the day of isolation. The wash solution was pre-gassed with air/CO_2 (95%/5%), by placing in a LEEC incubator throughout the isolation.

The rat was killed by cervical dislocation, the liver lobes removed and placed in 10ml wash solution. The liver was blotted dry and

weighed; 3.0g was used. Subsequent manipulations were carried out in a flow cabinet. Individual lobes were placed on a filter paper on a Perspex sheet and thinly sliced using a degreased razor blade (Wilkinson Sword). Slices in 10ml wash solution were placed in a sterile 250ml conical flask sealed with a sterile rubber bung in a shaking water bath at 37°C, for 10 minutes at 90 oscillations/minute. The solution was then removed and the slices washed twice more. The cells were then washed twice with 10ml wash solution containing 0.5mM EGTA. This chelating solution was removed and replaced with enzyme solution (10ml wash solution containing 1000 units (U) of collagenase Type IV, 4250 U of hyaluronidase and 5mM $CaCl_2$). The flask was then shaken for 45-70 minutes until the slices had disintegrated. The resulting suspension was filtered through a piece of sterile surgical gauze into 20ml wash solution in a sterile beaker. The suspension was centrifuged for 90 seconds at 50g, and the pellet washed twice with fresh wash solution. The final pellet was resuspended in incubation or culture medium (see sections 2.6 and 2.7).

Lobe perfusion method

The apparatus was set up as in Figure 2.1, perfusates being circulated using a Watson-Marlow peristaltic pump. The water bath was kept at 40°C to allow for the 3°C drop in temperature between water bath and lobe (Chenery et al., 1987). Alcohol (70% v/v, 50ml), followed by 100ml sterile water was run through the apparatus prior to perfusion of the lobes. The calcium-, magnesium-free

Hanks buffer containing $NaHCO_3$, glucose, methionine and gentamicin as described above was used to make up the perfusates, which were gassed continuously with O_2/CO_2 (95%/5%). On changing solutions, perfusate was run through the system for 30 seconds before being re-circulated, to flush the previous solution from the system. During perfusion the lobes were covered with a piece of sterile gauze, soaked in Hanks buffer.

The rat was killed by cervical dislocation, and the liver lobes removed and rinsed with Hanks buffer. Care was taken not to damage the lobes, which were cannulated with a 21G x 1½" needle through a vein on an exposed cut surface. The same lobes were routinely used. The lobes were perfused for several minutes with non-circulating Hanks buffer containing 0.5mM EGTA, to flush out the blood. When the lobes were evenly blanched, fresh buffer with EGTA was recirculated for 15-20 minutes. The flow rate was set at 10-15 ml perfusate minute $^{-1}$ cannula^{-1}. The lobes were then perfused with re-circulating buffer containing 100 Uml^{-1} of collagenase Type IV and 5mM $CaCl_2$, for 30 minutes. The lobes were carefully transferred to a beaker containing a small volume of Hanks buffer and minced gently with a pair of scissors. After filtration through a piece of sterile gauze the cell suspension was centrifuged at 50g for 90 seconds and the pellet washed with Hanks buffer. The cells were then resuspended in 5ml culture medium (see 2.6) which was added to 5ml Percoll solution (Percoll diluted 1:10 with concentrated Hanks salt solution: 1.4M NaCl, 50mM KCl, 4mM KH_2PO_4,

3mM Na_2HPO_4, 55mM glucose). Centrifugation at 50g for 10 minutes resulted in a separation of the viable and non-viable cells (Kreamer et al., 1986). The pellet of viable cells was washed with culture medium, followed by centrifugation at 50g for 90 seconds, and finally resuspended in culture medium.

The viability of the final cell suspension obtained from both isolation methods was assessed using trypan blue exclusion (2,5,1). Suspensions of viability less than 85% were rejected. The concentration of the cell suspension was then adjusted (usually to 2,0 X 10^6 cells ml^{-1}) and the cells either used fresh, placed into culture or sonicated to give an homogenate (2,6-2.8).

For the initial kinetic studies (2.10) hepatocytes were isolated by the slice method. In all subsequent experiments lobe perfusion was preferred, since, in combination with the use of larger rats, the yield of hepatocytes per animal was consistently increased whilst the isolation time was decreased. Table 2.1 shows the cytochrome P450-dependent alkoxycoumarin O-dealkylase activities measured in fresh hepatocytes isolated by slicing or lobe perfusion, and the maintenance in culture for 24 hours of these activities. The fresh cell activities are similar, as are the maintenance values, indicating that the method of isolation does not influence the P450 activities measured in fresh or cultured hepatocytes.

2.5 ASSESSMENT OF CELL VIABILITY

2.5.1 Trypan blue exclusion

Viability of hepatocyte suspensions was determined using the trypan blue dye exclusion test, which has been shown to be as sensitive a parameter of viability as lactate dehydrogenase leakage (Jauregui *et al.*, 1981). It is also quicker and simpler to perform, and it enables a quick estimate of the number of viable cells to be made. Cells with an intact plasma membrane will exclude the dye: these cells are judged to be viable. Where membrane integrity is compromised the cells take up the dye and appear blue when viewed under a light microscope. The number of viable and non-viable cells were counted by mixing 0.25ml cell suspension with 0.1ml trypan blue (0.5% dye solution in 85% saline) and placing the mixture onto an improved Neubauer haemocytometer (depth 0.1mm). The percentage viability was then calculated.

2.5.2 Reduction of MTT

In experiments where compounds were screened for toxicity to cultured hepatocytes the number of viable cells remaining after treatment was determined by assessment of mitochondrial function. The mitochondrial enzyme, succinate dehydrogenase, can reduce the yellow tetrazolium salt, MTT, to a blue formazan product which absorbs at 560-570nm. Only cells with viable mitochondria can reduce the MTT and the amount of formazan produced is proportional to the number of viable cells (Figure 2.2). The method was modified from that of Denizot and Lang (1986), for use with hepatocyte

cultures. WEC containing $0.2 mg ml^{-1}$ MTT was placed on the cells. After a 3 hour incubation at 37°C, the medium was removed and the formazan that precipitated in the cells solubilised in isopropanol. Absorbance was determined spectrophotometrically at 560nm or at 570nm using a Dynatech plate reader.

2.6 USE OF FRESH CELLS

For all enzyme assays in freshly isolated hepatocytes, cells were resuspended at 2.0×10^6 cells ml^{-1} in culture medium or Hanks buffer (2.4) containing 20mM HEPES. Incubations were performed in 25ml conical glass flasks shaken continuously in a 37°C water bath. Incubation times of 20-30 minutes were routinely used, the cells being used immediately after isolation. There was no loss of viability of the cells over these time periods.

2.7 PRIMARY CULTURE OF HEPATOCYTES

2.7.1 Culture medium

Hepatocytes were cultured in Williams' medium E, the composition of which is shown in Table 2.2. To 445ml single strength Williams' E was added: glutamine 2mM, fungizone $2.5 \mu g ml^{-1}$, gentamicin $50 \mu g ml^{-1}$, foetal calf serum 10% v/v, insulin $10 mU\ ml^{-1}$, nicotinamide 5mM and dexamethasone $1 \mu M$. All concentrations are final concentrations. This medium is referred to as WEC, and has been shown to maintain certain P450-dependent activities (Warren and Fry, 1988).

2.7.2 Procedure

Hepatocytes were cultured either on 60mm diameter "Primaria" petri dishes at 2.5 x 10^6 cells $dish^{-1}$, or on collagen-coated plastic 24-well plates at 2.5 x 10^5 cells $well^{-1}$. Collagen was diluted down to 250µgml^{-1} with sterile 0.1M acetic acid and spread over each well to give a coating of 5µgcm^{-2}. "Primaria" dishes are electrostatically treated to promote attachment and survival of cells. Both the "Primaria" dishes and the multiwell plates were pre-incubated with WEC, before hepatocytes were plated onto them. After allowing 1-2 hours for attachment, monolayers were rinsed with Hanks buffer and fresh WEC was added. Medium was replaced every 24 hours.

2.8 PREPARATION OF CELL HOMOGENATES

Fresh cell suspensions were centrifuged at 50g for 90 seconds, and the pellet resuspended in ice-cold Tris-sucrose-EDTA buffer, pH 7,4 (20mM - 0.25mM - 5.4mM final concentrations respectively) at approximately 10 x 10^{fi}cells $ml^{"-1}$. Cultured hepatocytes were scraped from the culture dishes, the contents of 5 "Primaria" plates being pooled into a final volume of 1.5ml ice-cold buffer. The resulting suspensions were sonicated using a Soniprep probe at an amplitude of 15 for two 15 second pulses. This achieved maximum disruption with minimum enzyme damage. Homogenates were either used fresh, or frozen in liquid nitrogen and stored until required (always less than 1 week from preparation).

2.9 DETERMINATION OF PROTEIN

The protein content of fresh cell samples, cultures and homogenates was determined using the Bradford assay (Bradford, 1976). Samples were dissolved in 0,5N NaOH and diluted to approximately $100\mu g ml^{-1}$. NaOH extract (100µl) was mixed with 1.0ml dye reagent (100mg Brilliant Blue G in 50ml absolute ethanol added to 100ml 85% orthophosphoric acid, made up to 1 litre with water) and left to stand for 5 minutes. Absorbance at 595nm was determined and the protein content derived by interpolation from a standard curve, constructed with bovine serum albumin. Protein content of hepatocytes was shown to be linear with respect to cell number (Figure 2.3).

2.10 ALKOXYCOUMARIN O-DEALKYLASE ACTIVITIES

2.10.1 Kinetics

The O-dealkylation of alkoxycoumarins are P450-dependent activities that have been shown to have biphasic kinetics in rat liver microsomes (Boobis *et al.*, 1981; Paterson *et al.*, 1984), preliminary experiments in isolated hepatocytes confirming these observations. The kinetics of 7-methoxy- and 7-ethoxycoumarin O-dealkylation (MCOD, ECOD), determined in freshly isolated hepatocytes are shown in Figures 2.4 and 2.5 respectively. The kinetics were analysed using Hanes plots (see Appendix:A2.1). The two activities are clearly biphasic in the freshly isolated cells. Similar kinetic studies in hepatocytes cultured for 24 hours showed that whereas ECOD remained biphasic in culture (Figure 2.7), MCOD became

monophasic (Figure 2.6).

Estimations of the kinetic parameters, Km and Vmax, are presented in Table 2.3. The Km of MCOD activity measured in the cultured hepatocytes is similar to that of the total activity in the fresh cells. This suggests that it is the low affinity form of the activity that is retained, albeit at a reduced level (reduced Vmax), whereas the high affinity form is lost completely. Both components of ECOD activity were present in cultured hepatocytes (similar Km values), although the activity was reduced (lower Vmax values).

This information suggests that different populations of P450 are Involved in the high and low affinity components of these activities, a suggestion which is reinforced by the differential response of the substrates to P450 inducers (Matsubara et al., 1983; Okuno et al., 1989). Therefore, the alkoxycoumarins provide a series of substrates for the investigation of a range of P450s, since multiple isozymes appear to be involved in the O-dealkylase activities. In the studies reported here, O-dealkylase activities have been used in this way as markers for different populations of P450 isozymes, those responsible for the high and low affinity components of each activity. For this reason, activities are measured at two substrate concentrations: 10 or 20μM for the high affinity form (MCOD activity could not be accurately and reproducibly determined at substrate concentrations lower than 20μM) and 500μM for the total activity. This two substrate concentration

approach has been suggested previously by Boobis et al. (1986).

2.10.2 Determination of activity

Alkoxycoumarin O-dealkylase activities were determined by the well characterised method of Fry and Bridges (1980) in which the 7-hydroxycoumarin (7-HC) produced by O-dealkylation is assayed fluorometrically. Substrate (the alkyl ether) in N,N-dimethylformamide (DMF) at $2\mu l ml^{-1}$ medium was added to freshly isolated or cultured hepatocytes. Fresh hepatocytes were incubated with substrate for 20 minutes at 37°C in a shaking water bath; cultured hepatocytes were Incubated with substrate for 4 hours at 37°C. No toxicity of the substrates to the cells was observable after these Incubations. The reaction was stopped by adding the incubate (medium + fresh cells or medium alone from the cultures) to 4ml ice-cold water/0.2M acetate buffer (3:1 v/v). 320 U of beta-glucuronidase H-1 (in 1ml acetate buffer) was added to each sample to deconjugate the glucuronide and sulphate derivatives of 7-HC prior to extraction and assay; Incubation with enzyme was for 2 hours or overnight at 37°C. Under these conditions more than 97% of the conjugates were released with no hydrolysis of the sulphate (Paterson ft al., 1984). The 7-HC was then extracted into 4ml diethylether (containing isoamylalcohol 1.5% v/v). Organic phase (1ml) was then extracted into 4ml 0.05M glycine-NaOH buffer, pH 10.4, and the fluorescence determined at an excitation wavelength of 370nm (ex 370nm) and an emission wavelength of 450nm (em 450nm). The 7-HC content was then derived by interpolation from a standard

curve. The production of 7-HC under these conditions was shown to be linear with respect to time and cell number.

2.11 DETERMINATION OF CYTOCHROME P450 CONTENT

The P450 content of hepatocyte homogenates was determined using the method of Estabrook et al. (1972). Homogenates were diluted in 0.1M Tris buffer (pH 7.4), containing 20% v/v glycerol, to the equivalent of 1-2 X 10^6 cells ml^{-1}. Carbon monoxide was bubbled through the suspension for 1 minute, at 1-2 bubbles $second^{-1}$. The sample was split equally between 2 cuvettes and scanned between 390 and 510nra. Sodium dithionite was added to one cuvette, which was mixed well, and re-scanned. The P450 content was calculated, using the absorbance difference between 450 and 490nm, and the extinction coefficient of $91mM^{-1}$ cm^{-1}.

2.12 ANALYSIS OF ENZYME ACTIVITIES IN CELL HOMOGENATES

Enzyme activities in cell homogenates were determined using an Incubation mix similar to that described by Paterson and Fry (1983) for microsomal Incubations. Determination of enzyme activities in homogenates has also been described previously (Grant et al., 1985). The 1ml incubation mix contained 5mM magnesium sulphate, $1Uml^{-1}$ glucose-6-phosphate dehydrogenase, and cofactors (0.5mM NADP and 5mM glucose-6-phosphate, final concentrations) in 0.2M phosphate buffer, pH 7.4. Cell homogenates were routinely used at concentrations of 0.5-1.0mg protein ml^{-1} incubation mix.

Activities were determined from standard curves of the amount of product. Substrate was also included in blank and standard samples. Reactions were determined to be linear at the chosen substrate concentrations over the incubation period at the required homogenate protein concentration, in homogenates from control and Inducer-treated animals.

2.13 DETERMINATION OF 7-ETHOXYRESORUFIN O-DEALKYLASE ACTIVITY

7-Ethoxyresorufin is dealkylated in microsomes to a fluorescent product, resorufin (Pohl and Fouts, 1980). The incubation mix used was a slight modification of that described in 2.12, in that homogenate was used at 0.1 mg protein ml^{-1} and bovine serum albumin (1mg ml^{-1}) was included in the phosphate buffer, to prevent product inhibition, and maintain linearity of the reaction respectively. Prior to assay with substrate, dicoumarol (final concentration 10μM) was added to the incubation mix, which was then incubated at 37°C for 10 minutes. The dicoumarol inhibits microsomal reductases that can reduce the unstable resorufin (Lubet et al., 1985). 7-Ethoxyresoruf1n (final concentration 1.7μM in DMSO) was then Incubated with the samples for 30 minutes. When homogenates from BNF-treated rats were assayed, the incubation period was reduced to 10 minutes. The reaction was terminated by the addition of 2ml methanol. The fluorescence of the resorufin in the cleared supernatant was then determined. The product, resorufin, is relatively unstable, therefore Rhodamine B was used as a standard. The fluorescence of Rhodamine 8 at ex 550nm and em 585nm was

determined to be twice that of resorufin.

2.14 DETERMINATION OF NITROPHENOL HYDROXYLASE ACTIVITY

4-Nitrophenol hydroxylase activity was assayed by determination of the amount of 4-n1trocatechol produced. The method used was essentially that of Reinke and Moyer (1985). Nitrophenol (100µM final concentration) was Incubated in 1ml standard incubation mix (2.12) for 30 minutes at 37°C. TCA (15%, 0.1ml) was used to stop the reaction. After centrifugation to remove the precipitated protein, 0.1ml 10N NaOH was added to the supernatant. The absorbance of the nitrocatechol was determined at 546nm.

2.15 DETERMINATION OF N-DEMETHYLASE ACTIVITY

N-Demethylase activity was measured by determining the amount of formaldehyde produced from benzphetamine or erythromycin (final concentration 50µM). The formaldehyde was trapped with semicarbazide (10mM final concentration in incubation mix). Samples were incubated for 30 minutes at 37°C, and then stopped with 15% TCA. Fresh Nash reagent (30g ammonium acetate + 0.4ml acetyl acetone in 100ml water, 1ml) was added to 1ml protein-free supernatant, and incubated at 37°C for 45-50 minutes. Samples were centrifuged if necessary to clear the supernatant. The formaldehyde was then measured spectrophotometrically at 412nm or fluorometrically at ex 410nm em 510nm (Nash, 1953; Belman, 1963).

Structures of P450-dependent enzyme substrates and products are shown in Figure 2.8.

2.16 DETERMINATION OF CELLULAR GLUTATHIONE

Cellular glutathione was determined by a modification of the method of Hissin and Hilf (1976), in which o-phthalaldehyde forms a fluorescent derivative with reduced glutathione. Hepatocyte suspensions were treated with 15% TCA, and 0.1ml supernatant added to 3ml 0.4M K_3PO_4 buffer, pH 8.2. o-Phthaladehyde solution (0,1ml; 1mg ml^{-1} in methanol) was added to each sample, with thorough mixing. The fluorescence was then measured exactly 15 minutes later at ex 350nm, em 420nm. The glutathione content was determined from a standard curve.

APPENDIX

A2.1 Kinetic analysis of enzyme activity

The kinetics of enzyme-catalysed reactions can be described by the Michaelis-Menten equation:

$$V = \frac{Vmax[S]}{Km + [S]} \quad \ldots \quad [1]$$

where v is the rate of reaction at substrate concentration [S], Vmax is the maximum rate at saturating substrate concentrations and Km is the Michaelis constant, commonly defined as the substrate concentration at which $v = \frac{Vmax}{2}$. The Km is thus a measure of the enzyme's affinity for the substrate, and the Vmax gives an indication of the amount of active enzyme present. Equation [1] describes a rectangular hyperbola which can be linearly transformed in several ways to enable graphical estimations of Km and Vmax to be made (Roberts, 1977),

The kinetics of MCOD and ECOD activities were described in 2.10 by use of a Hanes plot, in preference to the double-reciprocal Lineweaver-Burk plot. For a Hanes plot (Hanes, 1932) [S]/v is plotted against [S], re-arrangement of equation [1] giving:

$$\frac{[S]}{v} = \frac{1}{Vmax} .[S] + \frac{Km}{Vmax} \quad \ldots . \quad [2]$$

A plot of [S]/v against [S] of a biphasic activity will give two components, each described by equation [2], from which the Km and Vmax can be calculated. The component with the lower Km is the

high affinity form and the component with the higher Km represents the total activity. Subtraction of the high affinity form from the total will give the contribution of the low affinity component.

With regard to estimations of the kinetic parameters Vmax and Km, statistical considerations indicate that of the graphical determinations of Vmax and Km more accurate subjective estimates can be obtained from Hanes plots than from the Lineweaver-Burk, assuming a homogeneous variance of v (Wilkinson, 1961).

The Lineweaver-Burk plot becomes vastly inferior to the Hanes when applied to unweighted linear regression lines, since the smaller (and less accurate) determinations of v become inordinately important in determining the position of the line. If the error in V is assumed to be small, the Hanes plot gives reasonably reliable estimates of Km and Vmax for unweighted lines. This means that Km and Vmax values can be graphically estimated, especially if the aim is to compare values from two similar experiments where the bias of the unweighted data will be similar, rather than to obtain values for the true Vmax and Km (Dowd and Riggs, 1965). Thus, in the kinetic experiments presented in 2.10, Km and Vmax values were estimated from the unweighted linear regression lines obtained from Hanes plots of the data. The Km and Vmax values obtained from the graphs were used to compare the isozymic populations present in fresh and cultured hepatocytes, and should not be taken as accurate measures of the true values of the kinetic parameters.

A2.2 Statistical analyses

Standard statistical tests were applied to the data presented in this thesis, which are described in detail in general statistics texts (Bailey, 1959). Briefly, linear regression analysis was applied to linear, graphical data, to obtain the line of best fit. Student's t-test was used to compare two group means where only one variable was being studied; for multiple comparisons analysis of variance (ANOVA) was employed. If the ANOVA detected differences between groups Dunnett's test (Winer, 1971) was used to compare the treated groups to the control group. The important point regarding this test is that the significance level applies to the collective data - the test is not Independent for each group v control test (i.e. groups x and y but not group z *are* significantly different to the control group at a level of $p<0,05$). The Mann-Whitney U-test (Campbell, 1967) is a distribution-free test which can be used on transformed data, e.g. culture maintenance data which is expressed as a percentage of, or relative to, a fresh cell value. It assesses the overlap of two population distributions, differing in the location of their median value by ranking the data.

TABLES AND FIGURES

TABLE 2.1a. ALKOXYCOUMARIN O-DEALKYLASE ACTIVITIES IN HEPATOCYTES ISOLATED BY THE SLICE AND LOBE PERFUSION METHODS,

SUBSTRATE CONCENTRATION (μM)	ACTIVITY (pmol $min^{-1} mg^{-1}$) SLICE	PERFUSION
7-MC 20	18±2	17± 4
7-MC 500	106±10	91± 9
7-EC 10	15±2	19± 4
7-EC 500	293±44	309± 9
VIABILITY	89± 1%	91± 1%

Values are the mean ± SEM activity where n = 6 (slice) and n = 4 (perfusion).

TABLE 2.1b. MAINTENANCE OF ACTIVITIES AFTER 24 HOURS IN CULTURE.

SUBSTRATE CONCENTRATION (μM)	MAINTENANCE SLICE	PERFUSION
7-MC 20	0.17	0.21
7-MC 500	0.27	0.54
7-EC 10	0.35	0.49
7-EC 500	0.22	0.40

Activities in the fresh and cultured cells were determined in separate experiments, therefore each value is the mean activity at 24 hours relative to the mean fresh cell activity, which = 1.0. Slice: n = 4; perfusion: n = 4.

TABLE 2.2. COMPOSITION OF WILLIAMS' MEDIUM E[1]

Component	mg L^{-1}
INORGANIC SALTS	
$CaCl_2$ (anhydrous)	200.0000
$CuSO_4.5H_2O$	0.0001
$Fe(NO_3)_3.9H_2O$	0.0001
KCl	400.0000
$MgSO_4$	400.0000
$MgSO_4.7H_2O$	200.0000
$MnCl_2.4H_2O$	0.0001
NaCl	6800.0000
$NaHCO_3$	2200.0000
$NaH_2PO_4.H_2O$	140.0000
$ZnSO_4.7H_2O$	0.0002
AMINO ACIDS	
L-Alanine	90.0000
L-Arginine	50.0000
L-Asparagine.H20	20.0000
L-Aspartic acid	30.0000
L-Cysteine	40.0000
L-Cystine	20.0000
L-Glutamic acid	50.0000
Glycine	50.0000
L-Histidine	15.0000
L-Isoleucine	50.0000
L-Leucine	75.0000
L-Lysine HCl[2]	87.5000
L-Methionine	15.0000
L-Phenylalanine	25.0000
L-Proline	30.0000
L-Serine	10.0000
L-Threonine	40.0000
L-Tryptophan	10.0000
L-Tyrosine	35.0000
L-Valine	50.0000

(continued over)

[1] From Williams and Gunn (1974)

[2] The original formula lists lysine at 70.00 mg L^{-1}

TABLE 2.2.(continued)

VITAMINS	
Ascorbic acid	2.00
Biotin	0.50
D-Ca2+pantothenate	1.00
Choline chloride	1.50
Ergocalciferol	0.10
Folic acid	1.00
i-Inositol	2.00
Menadione sodium bisulphate	0.01
Nicotinamide	1.00
Pyridoxal HCl	1.00
Riboflavin	0.10
α-Tocopherol phosphate, disodium	0.01
Thiamine HCl	1.00
Vitamin A acetate	0.10
Vitamin B_{12}	0.20
OTHER COMPONENTS	
D-Glucose	2000.00
Glutathione	0.05
Methyl linoleate	0.03
Phenol red	10.00
Sodium pyruvate	25.00

TABLE 2.3. ESTIMATED KINETIC PARAMETERS OF MCOD AND ECOD ACTIVITIES,

ENZYME ACTIVITY		HIGH AFFINITY		TOTAL	
		K_{m1}	V_{max}[1]	K_{m2}	V_{max2}
MCOD	fresh	33	53	270	167
	24 h	-	-	210	42
ECOD	fresh	35	77	160	200
	24h	36	33	135	77

Kinetic parameters were graphically determined (see Appendix 2.1).

1 Units of K_m are μM

2 Units of V_{max} are pmol min^{-1} mg^{-1}.

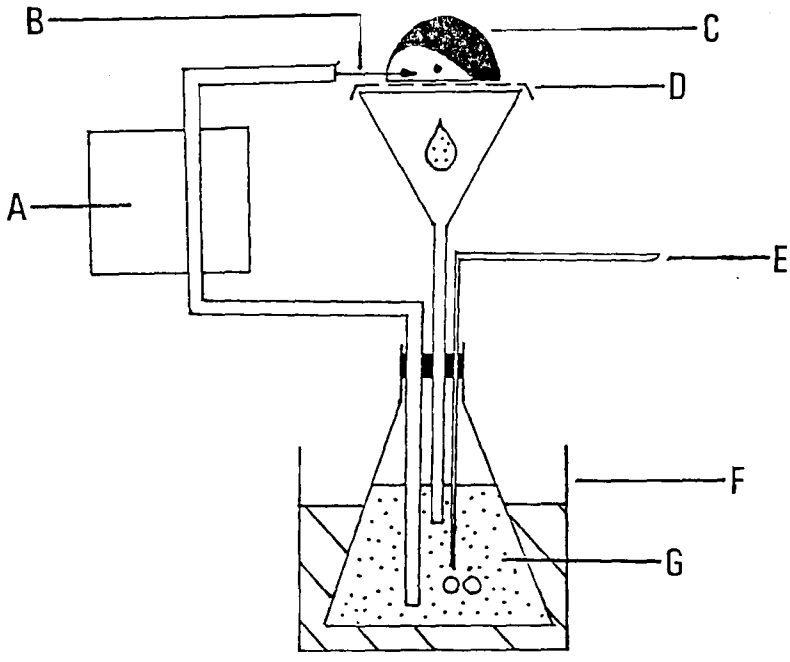


FIGURE 2.1. APPARATUS FOR THE PERFUSION OF INDIVIDUAL HEPATIC LOBES. Method modified from Reese and Byard, (1981); for details, see section 2.4.

A peristaltic pump

B 21G×1.5" needle inserted into an exposed vein

C lobe of liver

D wire mesh, to support lobe

E 95% O_2 : 5% CO_2

F water bath maintained at 40°C

G re-circulated perfusate

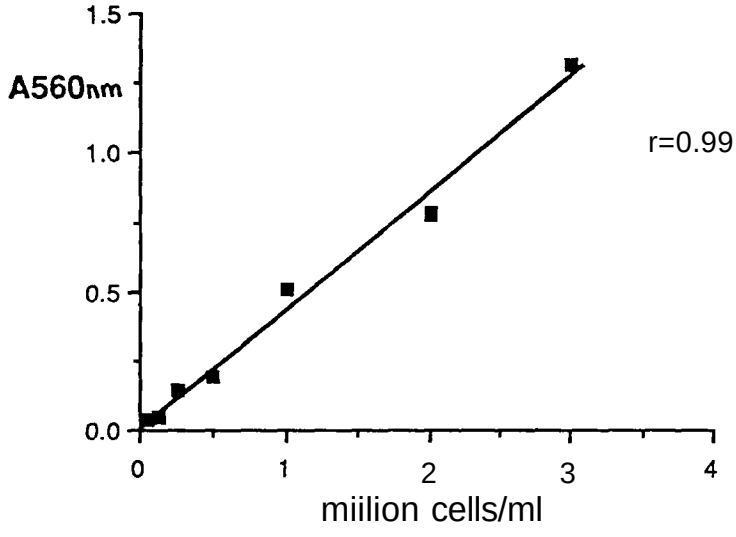


FIGURE 2.2, RELATIONSHIP OF FORMAZAN ABSORBANCE TO HEPATOCYTE NUMBER, The absorbance of the reduced MTT at 560nm was determined in hepatocyte suspensions (n=4; SE<0.01), as described in section 2.5.2.

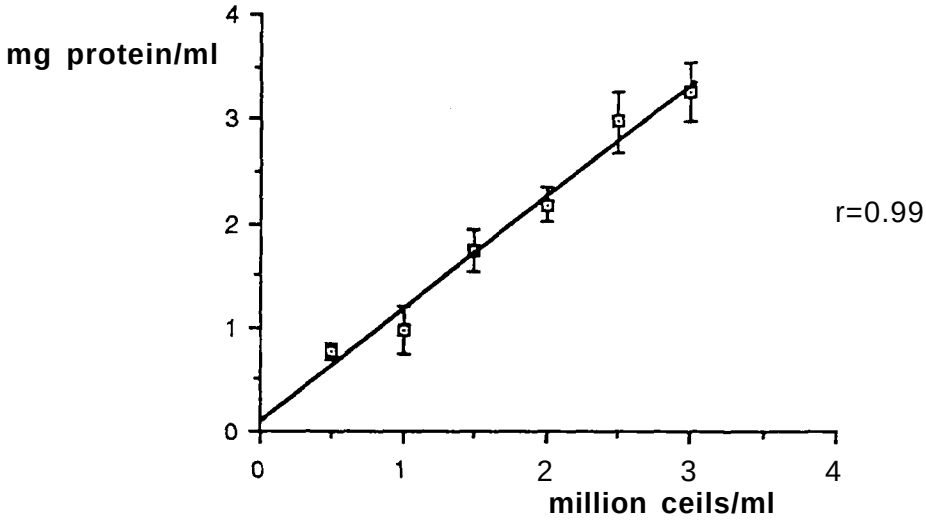


FIGURE 2.3. RELATIONSHIP OF CELL PROTEIN TO CELL NUMBER. Protein content of hepatocyte suspensions was determined as described in section 2.9. ($\bar{x} \pm SEM, n=5$).

FIGURES 2,4-2,7, These figures show the data obtained for ECOD and MCOD activities measured in freshly isolated and cultured hepatocytes as Hanes plots.The details of this kinetic analysis are given in Appendix A2.1. For all figures, [S]/v was determined using the mean activity at each substrate concentration of 7 separate preparations, each from a different animal.

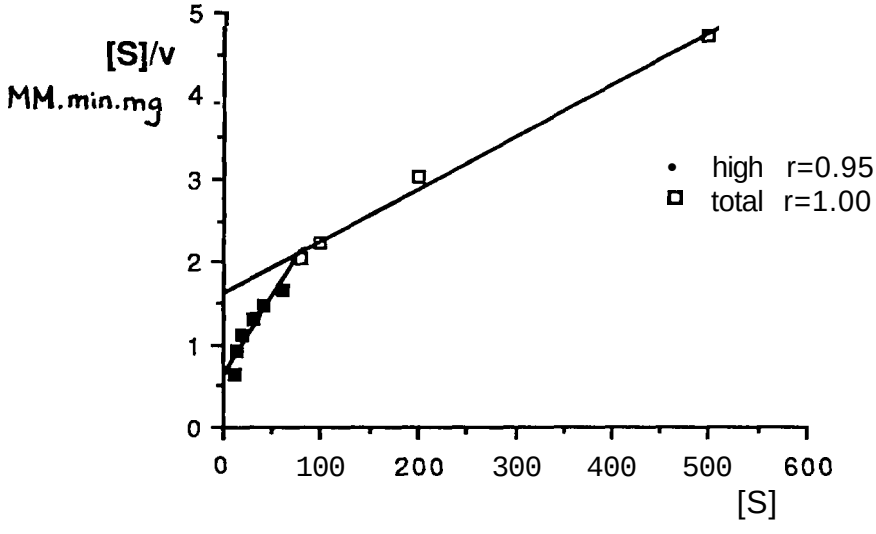


FIGURE 2.4. HANES PLOT OF MCOD ACTIVITY MEASURED IN FRESHLY ISOLATED HEPATOCYTES.
See Appendix 2.1 for details of kinetic analysis.

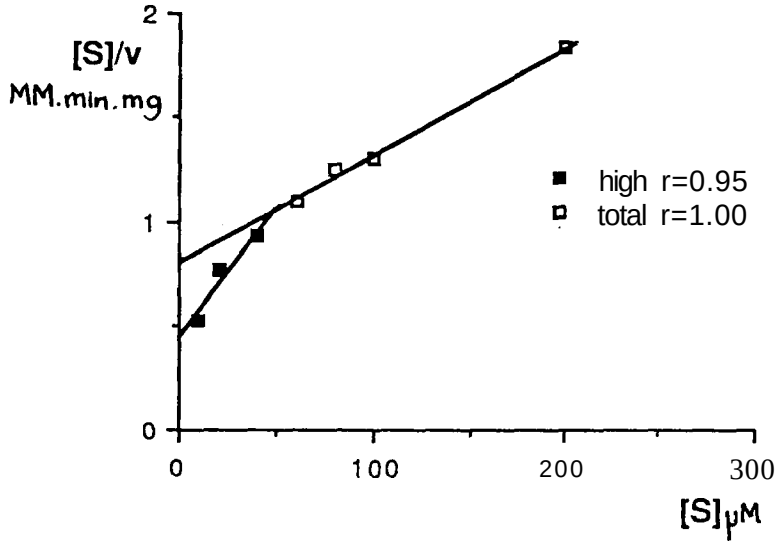


FIGURE 2.5, HANES PLOT OF ECOD ACTIVITY MEASURED **IN FRESHLY ISOLATED HEPATOCYTES,**
See Appendix 2.1 for details of kinetic analysis.

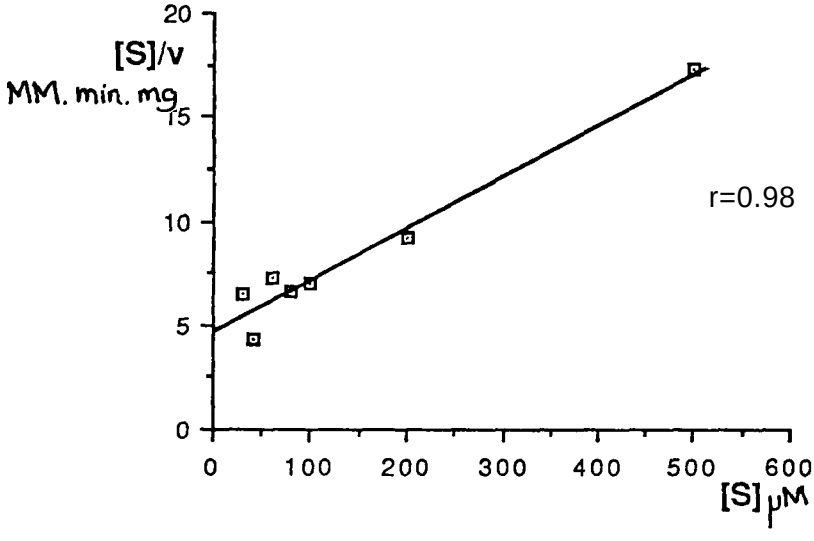


FIGURE 2,6, HANES PLOT OF MCOD ACTIVITY MEASURED IN HEPATOCYTES CULTURED FOR 24 HOURS,
See Appendix 2.1 for details of kinetic analysis.

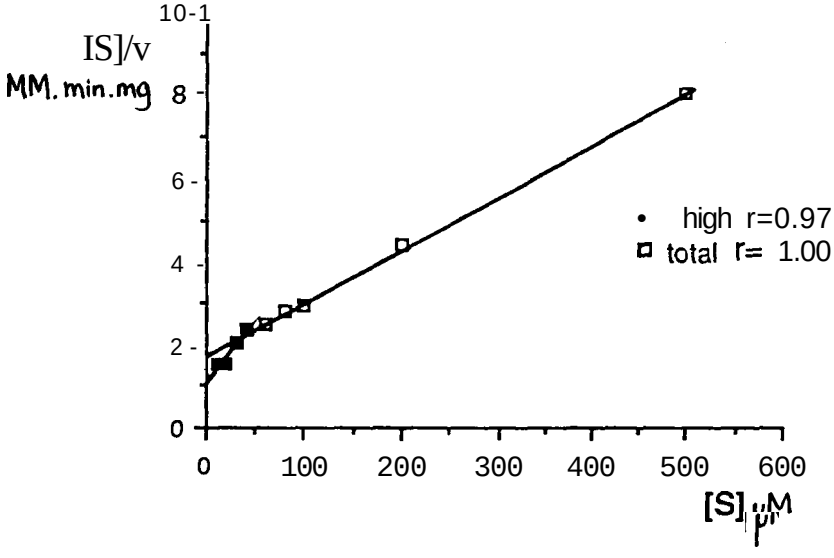
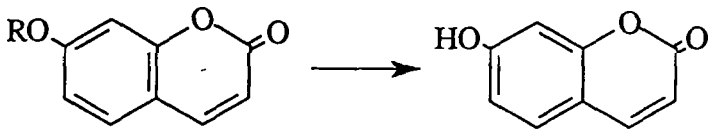


FIGURE **2.7.** HANES PLOT OF ECOD ACTIVITY MEASURED IN HEPATOCYTES CULTURED FOR 24 HOURS.
See Appendix 2.1 for details of kinetic analysis.

A. 0-DEALKYLATION OF ALKOXYCOUMARINS. Method: 2.10.

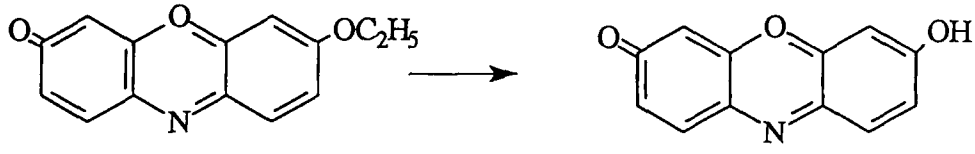
ALKOXYCOUMARIN 7-HYDROXYCOUMARIN



R= methyl, ethyl or propyl group

B, O-DEETHYLATiON OF **7-ETHOXYRESORUFIN.**Method: 2.13

7-ETHOXYRESORUFIN RESORUFIN



CHYDROXYLATION OF P-NITROPHENOL,Method: 2.14

P-NITROPHENOL P-NITROCATECHOL

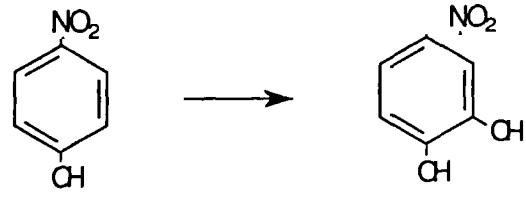
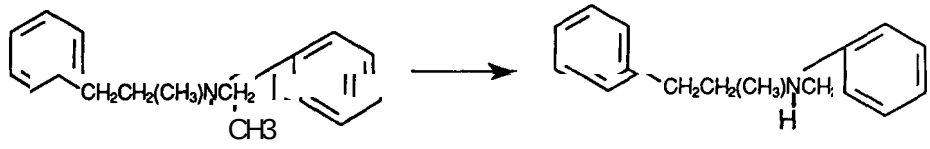


FIGURE 2.8. SUBSTRATES AND METABOLIC PRODUCTS OF THE P450-DEPENDENT ASSAYS USED IN THIS **INVESTIGATION,** For details of the relevant methods see pages 70-75.

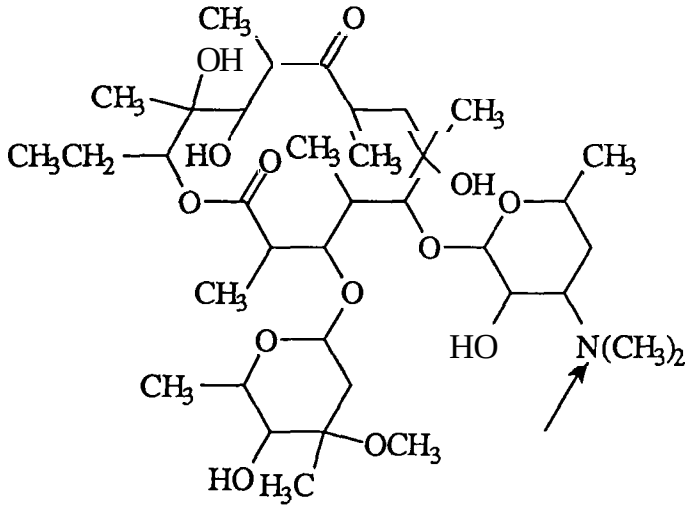
D, N-DEMETHYLATION OF BENZPHETAMINE AND ERYTHROMYCIN, Method: 2,15.

BENZPHETAMINE



\+ formaldehyde

ERYTHROMYCIN



reaction as above; arrow denotes site of action.

FIGURE 2.8. CONTINUED. SUBSTRATES AND METABOLIC PRODUCTS OF P450-DEPENDENT ACTIVITIES.

CHAPTER 3

SUPPLEMENTATION OF CULTURE MEDIUM WITH EXOGENOUS HAEM: INFLUENCE ON THE MAINTENANCE OF P450-DEPENDENT ENZYME ACTIVITIES

CHAPTER 3

CONTENTS **PAGE**

CHAPTER 3

3.1 INTRODUCTION

A variety of methods have been employed in attempts to attenuate the loss of cytochrome P450 that occurs in hepatocyte cultures, including manipulation of culture medium composition (reviewed in Chapter One). In 1985 Engelmann *et al.* reported that addition of exogenous haem to culture medium maintained the P450 content of rat cultured hepatocytes at more than 80% of the level in freshly isolated cells for 72 hours; there was no effect on the haem content of the hepatocytes. The rationale of this supplementation centres on haem as the prosthetic group of mammalian haemoproteins.

Haem (or iron (II)-protoporphyrin IX) is synthesized in the hepatocyte for incorporation into mitochondrial cytochromes, catalase and tryptophan pyrrolase as well as the microsomal cytochromes; however, on induction up to 70% of newly synthesized haem can be incorporated into cytochrome P450s (Muller-Eberhard and Vincent, 1985). It is known that deficiencies in haem biosynthesis can result in selective deficiencies in drug metabolism (Anderson *et al.*, 1976). Also, Steward *et al.* (1985) showed that although total P450 declined in culture over 72 hours, there was a selective maintenance of certain apoproteins. They concluded that loss of the haem moiety was responsible for the overall loss of holoenzyme in culture. This, coupled with the shorter turnover of haem relative to apoprotein (Sadano and Omura, 1983), suggests that some deficiency, block or inhibition of haem biosynthesis is responsible

for the loss of P450 in culture.

It is thought that haem is extracted from an Intracellular haem pool (or pools), fed both exogenously and endogenously, for synthesis of microsomal haemoproteins (Muller-Eberhard and Vincent, 1985), although the mechanism of regulation of this pool is uncertain. Haem itself exerts feedback repression on the mitochondrial enzyme delta-aminolaevulinate synthase (ALAS), which is the rate limiting enzyme in haem biosynthesis (Granick, 1966), outlined in Figure 3.1. Haem also Induces haem oxygenase which degrades free haem from P450s to biliverdin (Kutty *et al.*, 1988). The administration of delta-aminolaevulinate (ALA) in vivo can also induce haem oxygenase in the rat, presumably by stimulating haem synthesis, resulting in an expansion of the haem pool (Anderson et al., 1981). However, Paine and Legg (1978) could find no correlation between induction of haem oxygenase activity and loss of P450 in hepatocyte cultures, making increased degradation of haem unlikely as a cause of P450 loss. Evarts et al. (1984) reported that although the P450 content fell rapidly within the first 24 hours, hepatic ALAS remained elevated throughout four days of culture; therefore the biosynthetic pathway does not appear to be repressed although the sustained Increase in ALAS suggests a demand for haem in the cultured hepatocyte.

Addition of exogenous haem, both in vivo and in vitro, has been shown to have a sparing effect on allylisopropylacetamide-induced

destruction of P450, presumably by promoting incorporation of haem into newly synthesized apoproteins (Liem et al., 1983). This protection against degradation following allylisopropylacetamide administration appears to be specific for certain isozymes, favouring those predominating at the time of treatment (De Matteis et al., 1983; Bornheim et al., 1984), Exogenous haem also seems to act directly, as a positive modulator of P450 gene transcription, via the nuclear haem pool (Bhat and Padmanaban, 1988).

Selenium is an essential trace element that is an inducer of both ALAS and haem oxygenase (Maines and Kappas, 1976). It is not porphyrinogenic, thus the Induction of both enzymes appears to maintain a steady state. At concentrations greater than 0.5μM the element is an Inhibitor of ALAS,

In this experiment, the effect of culture medium supplemented with haem, ALA and selenium on the maintenance of P450-dependent dealkylase activities was investigated, these conditions being similar to those described by Engelmann et al. (1985).

3.2 METHODS

Hepatocytes were isolated from 150g male rats by lobe perfusion. MCOD and ECOD (both total and high affinity components) were measured in hepatocytes cultured for 24 hours and in freshly Isolated cells from the same animals; hepatocytes were cultured on "Primaria" dishes in either WEC or in WEC supplemented further with

haem (1μM), ALA (200μM) and selenious acid (Se; 0.1μM). Haem was dissolved in a bovine serum albumin solution (1mgml^{-1} 0.1N NaOH) to enable dilution into culture medium as described by Engelmann et al. (1985). This medium is designated WEC+H. Protein content of fresh cells and cultures was determined and all activities expressed as pmol 7-HC produced min^{-1} mg protein^{-1}. Maintenance of activities in culture is expressed as a percentage of the fresh cell activity, and calculated for each animal.

3.3 RESULTS

3.3.1 Maintenance of activities in culture

Figure 3.2 shows the activities measured in fresh cells, and in hepatocytes cultured for 24 hours in WEC or WEC+H. It is clear that the activities in the cultured hepatocytes are similar regardless of the medium. The WEC+H conferred no advantage over the unsupplemented WEC. However, the WEC (and also WEC+H) appeared to be adequate for maintenance of three of the activities measured. However, total MCOD was not maintained, falling to 46% of the fresh cell activity.

Table 3.1 presents the maintenance data for all four activities as percentages of the fresh cell data, for both media. The mean values and ranges were *very* similar for the two media, and although only three animals were used, the maintenance of total and high affinity ECOD and high affinity MCOD is Indisputable. This maintenance of activity, however, is in contrast to that of the

kinetics experiments (Chapter 2.10, Table 2.4) where none of the four activities were maintained, using the same WEC medium. The maintenance data from the two experiments are presented together in Table 3.2 for comparison, and it is necessary to consider the possible reasons for the inconsistency of these results. The most obvious causes of discrepancies in maintenance data are differences in the viabilities and/or fresh cell activities of the initial fresh cell suspensions.

3.3.2 Comparison of dealkylase activities and viabilities

The activities and initial viabilities of the cell preparations used in this and the former experiments (taken from Table 2.1a) are presented in Table 3.3. The viabilities were 1dentical(91 $\pm$ 1%), and the ECOD activities were *very* similar. However, the MCOD activities were very different. Total MCOD activity in this experiment was twice that measured previously whereas the high affinity form had around half the activity of the former determination. These differences in MCOD activity, whilst requiring an explanation, do not account for the observed differences in maintenance between the two experiments.

Thus supplementation of WEC with haem, ALA and Se did not improve maintenance of dealkylase activities over those obtained with WEC alone, but this maintenance in WEC of three of the four activities *over* the 24 hour culture period was completely unexpected given the previous maintenance data for the same activities in similar culture conditions.

3.4 DISCUSSION

The use of WEC supplemented with haem did not improve maintenance of P450-dependent enzyme activities in hepatocyte cultures, but then again there was good maintenance over twenty four hours in the unsupplemented medium. There was a selective loss of MCOD activity in this experiment, and supplementation of WEC with haem, ALA and Se did not prevent this loss. Engelmann et al. (1985) reported an overall maintenance of P450 content in contrast to a 25% loss at 24 hours in medium minus haem. They used adult female rats, for which haem supplementation of media may have been more effective, since hepatocytes from female rats appear to be more susceptible to haem loss (Evarts et al., 1984).

Total MCOD activity of hepatocytes fell by 50% during 24 hours in culture and whereas this is consistent with data from the preliminary kinetic experiments, the maintenance of the other three activities, at more than 90% of the fresh cell value in WEC, is not. Also, the MCOD activities in the fresh cells were different to those measured previously. Those experiments had ascertained that cell preparations isolated by slice or perfusion were equivalent with respect to dealkylase activity and maintenance. A major difference between the experiments, however, is the size, and therefore age, of the hepatocyte donor animal. For the initial kinetics and perfusion experiments 70g male rats were used, but in this study larger 150g rats were used. This made cannulation of the lobes easier, as well as increasing the yield of hepatocytes per rat.

The male Wistar rats used as donors are sexually mature at 150g (6 - 8 weeks old) unlike the 70g rats (4 weeks old).

It is known that changes in some P450-dependent activities occur during post-natal development in the rat, especially at the onset of puberty (Gram et al., 1969). It is possible that MCOD activity is dependent on a developmentally regulated P450(s) which accounts for the observed differences in MCOD activity. It is also possible that the age of the animal Influences the maintenance of P450 activities in culture.

It has been reported that the haem moiety of P450 undergoes biphasic turnover (Levin et al., 1975), with both a fast and a slow component. The ratio of fast : slow turnover forms decreased with increasing age in the male rat (ratio of 4.4 in immature males and 1.9 in adult males). Parkinson et al. (1983) investigated turnover of both the haem and apoprotein moieties for specific P450 Isozymes and reported biphasic turnover for the total apoprotein. However, only one purified isozyme showed biphasic turnover of haem (and of apoprotein). This was P450IIA, now known to exist as at least two very similar isozymes IIAl and IIA2 which are differentially regulated. P450IIA2 is adult male-specific; P450IIA1 is expressed only in females and pre-pubertal males (Matsunaga et al., 1988). It is possible that the rats of Parkinson et al., used at 4-6 weeks of age, contained both forms of P450IIA, and that one is a fast and one a slow turnover form.

Other workers have only found monophasic turnover kinetics for a number of purified P450s (Shiraki and Guengerich, 1984) but it is possible that changes in rate of turnover are associated with the developmentally regulated P450s like P450IIA1, which is a steroid hydroxylase, under hormonal control (Arlotto and Parkinson, 1989). Also, adult females, like immature males, appear to have predominantly fast turnover P450s (Levin et al., 1975). Thus, an Increase in slow turnover forms of P450 at puberty in the male rat could explain the improved maintenance of P450-dependent activities in this experiment relative to the kinetics experiment, due to the differences in age of the animals used for the hepatocyte isolations. It could also explain why haem supplementation of medium had no effect on maintenance, in contrast to the results of Engelmann *et al.* (1985), who used hepatocytes from female rats, in which fast turnover P450 forms predominate (Levin et al., 1975).

In summary, these results are not consistent with hepatocyte haem deficiency being responsible for the loss of P450 activities in culture. They do Indicate that hepatocytes isolated from adult males may show better maintenance of selected activities in our chosen medium than those from immature males. The effects of age and sex of donor animal on P450-dependent activities is examined in more detail in the following chapter.

TABLES AND FIGURES

TABLE 3.1. MAINTENANCE OF ALKOXYCOUMARIN O-DEALKYLASE ACTIVITIES IN CULTURE FOR 24 HOURS: EFFECT OF MEDIUM SUPPLEMENTED WITH HAEM.

SUBSTRATE	MAINTENANCE (% fresh cell value)	
CONCENTRATION (μM)	WEC	WEC+H
7-MC 10	96 (57-150)	93 (91-113)
7-MC 500	46 (36-50)	51 (48-53)
7-EC 10	101 (63-126)	84 (66-111)
7-EC 500	91 (85-104)	69 (59-79)

Values are means with ranges in brackets, for 3 animals.

TABLE 3.2. MAINTENANCE OF ALKOXYCOUMARIN O-DEALKYLASE ACTIVITIES IN CULTURE FOR 24 HOURS: COMPARISON OF DATA FROM TWO DIFFERENT EXPERIMENTS.

SUBSTRATE	MAINTENANCE IN WEC (% fresh cell value)	
CONCENTRATION (μM)	I	II
7-MC 20	96	21
7-MC 500	46	54
7-EC 10	101	49
7-EC 500	91	40

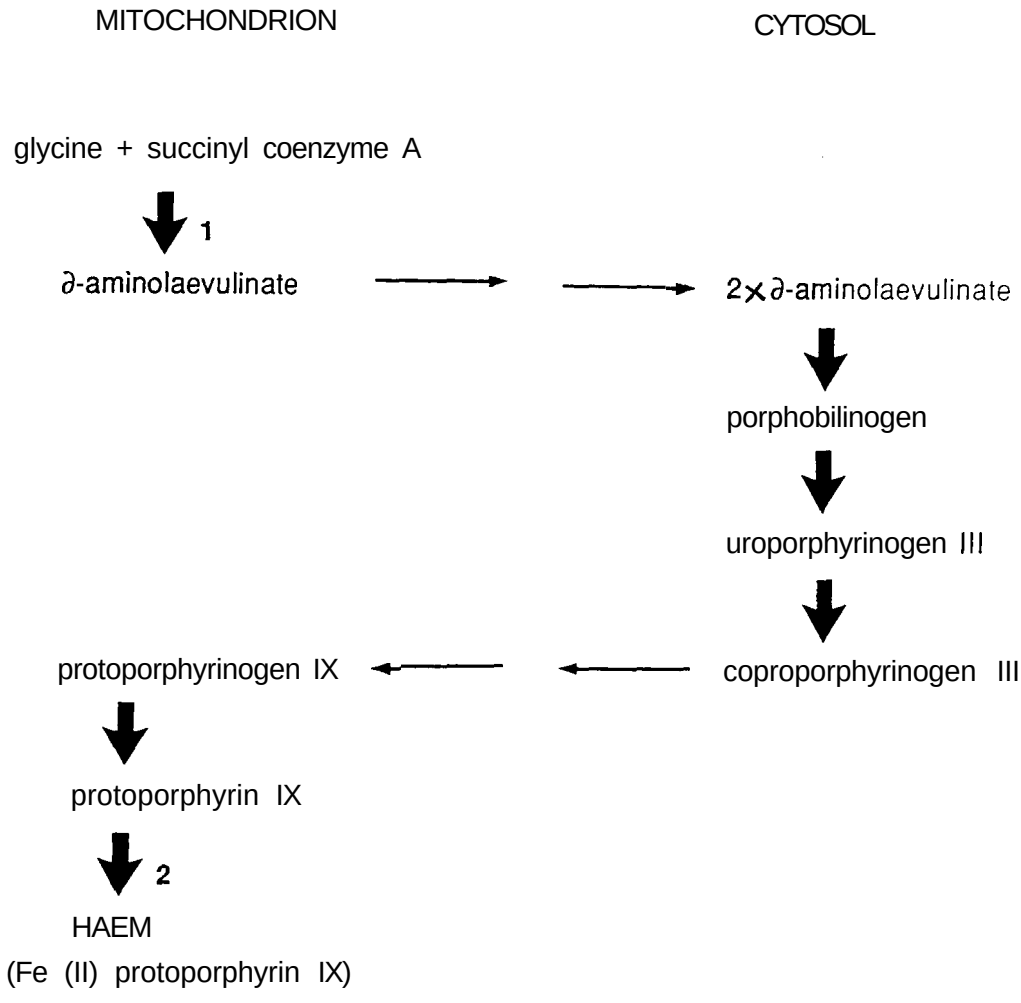
I data from Table 3.1.
II data from Table 2.1b.

TABLE 3.3. ALKOXYCOUMARIN O-DEALKYLASE ACTIVITIES IN FRESHLY ISOLATED HEPATOCYTES FROM TWO DIFFERENT EXPERIMENTS.

SUBSTRATE CONCENTRATION (μM)	ACTIVITY (pmol min^{-1} mg protein^{-1}) I	II
7- MC 20	10±1	17±4
7-MC 500	195±43	91±9
7-EC 10	19±1	19±9
7-EC 500	277±45	309±92
Viability of preparation	91±1%	91±1%

Values are means ± SEM for 3(I) or 4(II) animals.
I data from Figure 3.1.
II data from Table 2.1a.

FIGURE 3.1. HAEM BIOSYNTHESIS
(adapted from Maines and Kappas, 1977)



1 ∂-Aminolaevulinate synthase
2 Iron (II)-ferrochelatase

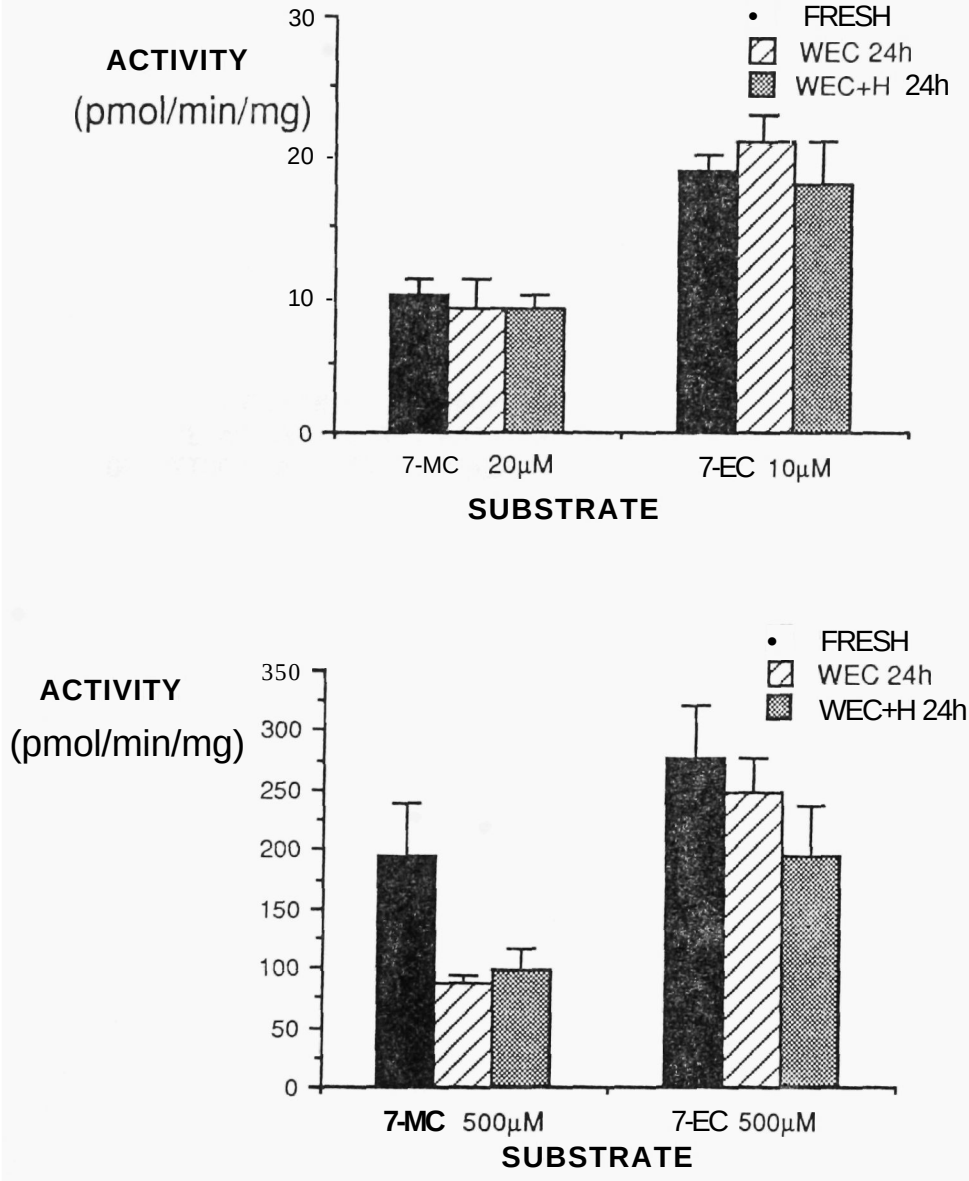


FIGURE 3.2. MCOD AND ECOD ACTIVITIES IN FRESHLY ISOLATED HEPATOCYTES AND IN CELLS CULTURED FOR 24 HOURS IN THE PRESENCE OR ABSENCE OF EXOGENOUS HAEM. Hepatocytes were cultured for 24 hours in either **WEC** medium (see 2.7.1.) or in WEC+H (see 3.2.).

CHAPTER 4

INFLUENCE OF DONOR AGE AND SEX ON THE ACTIVITY, AND MAINTENANCE IN CULTURE OF CYTOCHROME P450-DEPENDENT ENZYME ACTIVITIES

CHAPTER 4

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CHAPTER 4

INFLUENCE OF DONOR AGE AND SEX ON THE ACTIVITY, AND MAINTENANCE IN CULTURE OF CYTOCHROME P450-DEPENDENT ENZYME ACTIVITIES

4.1 INTRODUCTION

To date, at least fourteen different Isozymes of cytochrome P450 are known to exist in rat liver (Nebert and Gonzalez, 1987). The isozyme profile changes with age in both male and female rats, although the total P450 content remains relatively constant. These changes in Isozyme profile are reflected in changes in enzyme activity (Gram et al., 1969; Waxman et al., 1985). Major alterations occur during post-natal development and sexual maturation, and again at the onset of old age, the changes being particularly marked in male rats. This is due to the development of a distinct sex difference in P450 activities at puberty (Waxman et al., 1985), which disappears in older rats (Kamataki et al., 1985), This is probably caused by changes in the levels of the sex-specific P450 forms (Kamataki et al., 1982; Maeda et al., 1984). These age and sex differences in P450 forms are related to changes in constitutive steroid hydroxylase activities (Waxman et al., 1985).

A summary of the various sex-specific P450 forms and their associated activities is presented in Table 4.1.

It is now known that these hepatic sex differences are initiated and maintained by androgens and pituitary growth hormone (extensively reviewed by Skett, 1987; 1988). The pattern of secretion of growth

hormone is different in male and female rats, although the mean level is similar. In males there is a pulsatile secretion with high peak values every 3 - 4 hours and a very low basal secretion during the intervening periods, whilst in females there is a more continuous secretion, but at a higher baseline level (Edén, 1979). Hypophysectomy or continuous growth hormone (GH) infusion in male rats essentially feminises hepatic P450 metabolism, whereas intermittent infusion of GH in hypophysectomised animals restores the male pattern of metabolism (Waxman, 1988), Neonatal exposure to androgens imprints or programmes the pattern of adult male P450 steroid metabolism by imprinting the pulsatile pattern of growth hormone secretion (Jansson et al., 1985; Waxman *et* al., 1985; Waxman, 1988). This imprinting appears to function via the oestrogen receptor (Reyes and Virgo, 1988). Androgens are also required in adult life to maintain a male pattern of activity (Skett, 1988; Waxman, 1988); interruption of the hypothalamic-pituitary axis by cisplatin in adult male rats reduces the amount of circulating androgen, resulting in a feminisation of P450 metabolism (LeBlanc and Waxman, 1988).

The male-specific IICll (Nebert et al., 1989; named as P450-2c by Waxman et al., 1985) is dependent on pulsatile secretion of GH for its expression, whilst the female-specific IIC12 form (named as 2d by Waxman et al., 1985) requires a more constant secretory pattern (Waxman, 1988). The male-specific 2a form does not depend on pulsatile GH secretion, as it is constitutively expressed in male

rats, and in immature females (Waxman et al., 1985). However, P450-2a appears to be repressed by high levels of GH (Waxman et al., 1988), and this accounts for the suppression of this form in adult females, when GH is secreted at a higher, although still continuous, baseline level than in the immature male and female animals. It is possible that synthesis of P450-2a in the adult male is continually suppressed and de-repressed as the GH concentration fluctuates (Waxman et al., 1988).

In addition to sex differences in metabolism due to direct hormonal influences on P450 levels, it has been reported that hypophysectomy and/or continuous GH supplementation lower the amount of hepatic P450 reductase, (which is lower in female than male rats), but that this (and steroid hydroxylase activities) can be raised again by addition of thyroxine (Waxman et al., 1989).

Results presented in the previous chapter suggested that hepatocytes cultured from adult male rats may maintain P450-dependent enzyme activities better than hepatocytes from younger immature males, and that differences in P450 turnover may be the reason. Adult females also have a greater proportion of rapid turnover P450s, like Immature males (Levin et al., 1975), and therefore if turnover is affecting maintenance, hepatocytes from female rats should maintain activities at a similar level to the younger male rats. However, Vind et al. (1988) reported that P450 and demethylase activities were maintained better in hepatocyte cultures from female rats. It

is likely that the *in vivo* hormonal regulation of P450 expression, which changes with age and sex in the rat, is disrupted or lost *in vitro*, and it is this that affects the differential maintenance of P450 in hepatocytes from male and female rats of different ages. The maintenance of alkoxycoumarin O-dealkylase activities was therefore investigated in hepatocytes from immature and adult males, and adult females to assess the relative stabilities of P450-dependent activities in hepatocyte cultures.

4.2 METHODS

The rats used in this study were immature males (IM) and young adult male (AM) and female rats (AF). The adult animals were 8 weeks old and the immature males were 4 weeks old. Puberty occurs 4-6 weeks after birth in the male rat (Gram *et al.*, 1969; Levin *et al.*, 1975). Dealkylase activities were determined in fresh cells and hepatocytes cultured for 24 and 72 hours from each animal. Hepatocytes were cultured on "Primaria" dishes in WEC. In addition to MCOD and ECOD, 7-propoxycoumarin O-depropylase (PCOD), which is known to be a male-specific activity (Kamataki *et al.*, 1983; 1985), was also measured at two substrate concentrations in the same manner as ECOD and MCOD (Chapter 2.10). Protein content of fresh cells and cultures was also determined and all activities expressed as pmol 7-HC produced min^{-1} $mg\ protein^{-1}$. Statistical analysis on mean fresh cell activities was by unpaired t-tests. Maintenance at 24 and 72 hours was calculated as a percentage of the fresh cell value for

each animal and expressed as mean ± range; statistical analysis was by the Mann-Whitney U-test.

4.3 RESULTS

Viabilities of initial cell preparations were very similar: AM 93 ± 1%, AF 93 ± 1%, IM 96 ± 1%.

4.3.1 Dealkylase activities in hepatocytes from male rats

Dealkylase activities measured in freshly isolated cells from AM and IM rats are presented in Table 4.2. Previous studies confirmed the biphasic nature of MCOD and ECOD in IM cells (2.10), the high affinity component representing less than 25% of the total activity. The data in Table 4.2 is consistent with these activities, as well as PCOD, being biphasic in cells from AM rats.

ECOD activities of IM and AM cells were very similar. Total PCOD activity was significantly less in AM cells together with a numerical but non-significant decrease in the activity of the high affinity component relative to IM cells. Total MCOD was significantly greater and the high affinity activity significantly less in AM cells compared to IM cells (as observed in the previous chapter). Therefore, total PCOD and both components of MCOD activity appear to change with age in the male rat, implying some developmental regulation.

4.3.2 Dealkylase activities in hepatocytes from male and female rats

The dealkylase activities in fresh cells from female rats, together with those from AM rats are presented in Table 4.3. The high affinity components of both MCOD and ECOD represented less than 25% of the total activity, indicating that these activities are probably also biphasic in hepatocytes from AF rats. However, PCOD activity measured at 10μM accounted for 60% of the total activity in AF cells; from this, and the known involvement of a male-specific form of P450 in PCOD activity (Kamataki et al., 1985), it is suggested that PCOD is a monophasic activity in AF cells, existing only as a high affinity form. Consequently, data on the maintenance of PCOD in cultures from AF rats are reported only for the high affinity form.

Total MCOD activity was significantly less in AF cells relative to AM cells and was very similar to that measured in IM cells (AF 71 ± 5 pmol min^{-1} mg^{-1}; IM 88 ± 17 pmol min^{-1} mg^{-1}). High affinity MCOD in AF cells was not significantly different to that in AM cells. Total ECOD was markedly and significantly less in AF cells, whereas high affinity ECOD was slightly increased. The high affinity PCOD activity in AF cells was not significantly different to that of AM cells and, like total MCOD, was very similar to the activity in IM cells (AF 24 ± 5 pmol min^{-1} mg^{-1}; IM 25 ± 6 pmol min^{-1} mg^{-1}). Therefore, there appear to be sex differences as well as age differences in several of these activities.

4.3.3 Maintenance of activities in culture

(a) MCOD

Maintenance of MCOD activities in culture is shown in Figure 4.1. Both components of MCOD activity in IM cells declined to 50 - 60% of the fresh cell value at 24 hours. Kinetic studies (2.10) demonstrated that MCOD becomes monophasic in IM cultures with loss of the high affinity component; this explains the similarity in the magnitudes of the loss of activity at the two substrate concentrations. There was a similar decline in cultures from AF rats, suggesting the loss of the high affinity form. The MCOD activity declined further by 72 hours in IM and AF cells. In contrast, MCOD remained biphasic in AM cultures, the high affinity form being maintained (83% fresh activity) alongside a decline in the total activity (44% of fresh activity). These results for the AM rats are similar to those reported in the previous chapter. Both components fell to less than 20% of the fresh activity after 72 hours in culture.

(b) ECOD

Figure 4.2 shows the maintenance of ECOD activity in hepatocyte cultures. ECOD appeared to remain biphasic over the 72 hour culture period in all three donor cell types. High affinity ECOD activity was maintained for 72 hours in hepatocytes from IM and AM rats, whilst the activity fell to around 50% of fresh in cultures from AF rats.

Total ECOD was significantly reduced at 24 hours in cultures from IM rats but was retained in AM cells. By 72 hours the activity had declined to the same level as in cells from IM rats. In cultures from AF rats the activity fell to around 80% at 24 hours and fell slightly further by 72 hours.

(c) PCOD

Maintenance of PCOD activity in culture is shown in Figure 4,3, High affinity PCOD was maintained at high levels over 72 hours in cultures from IM and AM rats; the equivalent activity fell to around 50% in AF cells, PCOD also remained biphasic in cultures from male rats, although, in contrast to the high affinity form, total PCOD fell rapidly within 24 hours in culture, and declined further by 72 hours.

Overall, these dealkylase activities were maintained at higher levels in cells from AM rats than in cells from either IM or AF animals. This maintenance was significantly greater for four out of the six activities, with a further activity being maintained in both AM and IM cells. All three enzymes remained biphasic in cultures from AM rats. Although total MCOD and total PCOD fell to less than 70% of the fresh activity after 24 hours in culture the other four activities were maintained at high levels, with the high affinity forms of ECOD and PCOD being maintained for 72 hours.

4.4 DISCUSSION

The results of this study have demonstrated that the maintenance in culture of rat hepatic P450-dependent alkoxycoumarin O-dealkylase activities is dependent upon the choice of coumarin used as substrate, its concentration and the age and sex of the hepatocyte donor animal. It is highly likely that selective maintenance of particular P450 enzymes accounts for these variabilities, but the exact nature of these forms remains to be established. The general conclusion is one of better maintenance of activity over 24 hours in culture in hepatocytes Isolated from AM rats, but that such selectivity for AM cells is lost by 72 hours in culture. This is consistent with the idea that maintenance is linked to differential turnover of P450, as discussed in the previous chapter. To recapitulate briefly: AM rats have a lower fast:slow turnover ratio for haemoproteins than IM or AF rats, which implies a more stable population of P450 forms in the AM rat, and also improved maintenance in culture. The lack of selectivity for AM cells after 72 hours in culture is also consistent with this, given the reported half-life of 41-44 hours for the slow turnover component (Levin *et al.*, 1975). If changes in turnover do influence P450 maintenance in culture, this would suggest that total MCOD and total PCOD activities are fast turnover forms, since they are the least well maintained (at 40-60% of fresh activity at 24 hours), even in AM cells.

Total MCOD activity is 50% greater in AM cells than in IM or AF cells, indicating a possible adult male-specific component in this activity. PCOD activity has already been described as male-specific (Kamataki et al., 1983; 1985), and the results from this study imply that it is the low affinity form(s) that is male-specific, and that this is absent in AF cells. This agrees with the work of Kamataki et al., who also measured PCOD activity at a 500μM substrate concentration. However, the P450-male isozyme reported to be responsible for PCOD activity (Kamataki et al., 1983), and equivalent to Waxman's P450-2c (1985), is sharply Induced (20-fold) at puberty in the male rat, and the PCOD activities presented here do not fit with this pattern of expression. The total PCOD activity measured in this study does agree with the expression of P450-PB-2a (also known as 2a, Waxman et al., 1985; 1988), which is similar in both AM and IM rats but suppressed in AF rats. This Isozyme has also been shown to catalyse more than 85% of microsomal testosterone 6β-hydroxylase activity in the male (Waxman et al., 1985), and recently Yamazoe et al. (1988) demonstrated that male-specific PCOD activity correlated with 6β-hydroxylase activity in microsomes. They also showed that whereas antibodies to P450-male (P450-2c) inhibited PCOD activity in a reconstituted system, they did not inhibit PCOD activity at all in microsomal preparations. With regard to the maintenance of this P450, the content of P450-2a (measured immunochemically) was not reduced during culture of AM rat hepatocytes for 72 hours in medium containing glucocorticoid and nicotinamide (Steward et al., 1985), whereas the low affinity

(total) PCOD activity measured here, is markedly reduced by this time in culture. These workers also proposed that loss of haem rather than apoprotein accounted for much of the loss of active P450 in culture. Supplementation of media with haem, reported in the previous chapter, had no effect on maintenance of activities; nevertheless, it seems that the apparent anomaly in maintenance of PCOD activity and the putative isozymes involved can be rationalized in terms of selective maintenance of apoprotein, but not holoenzyme.

It appears then that the male-specific PCOD activity is mediated by the P450 involved in constitutive 6β-steroid hydroxylation. This isozyme/activity is regulated by growth hormone (GH, Waxman et al., 1988; Yamazoe et al., 1988), continuous high level secretion of GH suppressing its synthesis. It was also suggested (Waxman et al., 1988) that the Isozyme may be suppressed in pre-pubertal males, as well as in adult females, the same or a *very* similar Isozyme being subsequently re-introduced at puberty. This situation would be analogous to that reported for P450IIA1 and IIA2, where P450IIA1 is expressed in IM rats and IIA2 (a *very* closely related Isozyme) in adult males (Matsunaga et al., 1988). This would also explain the significantly lower total PCOD activity in the AM cells in this study.

It could also be argued from this Investigation that the developmentally regulated P450s are the most susceptible to decline in culture, being dependent for expression on GH and androgen levels

(Skett, 1988). The addition of GH to hormone-free culture medium causes a feminisation of the P450 phenotype, similar to that seen in hypophysectomised male rats (Guzelian et al., 1988), and recent reports have implicated the thyroid hormones, thyroxine and tri-iodothyronine, in addition to the effects of GH, in suppression of liver-specific P450IIA2, the male-specific 6β-hydroxylase and the female-specific 7α-hydroxylase (Arlotto and Parkinson, 1989; Yamazoe et al., 1989). Both GH and thyroxine are present at significant levels in calf serum (Honn et al., 1975), and it is possible that these serum constituents suppress liver-specific functions in vitro (Enat et al., 1984; Silver and Krauter, 1988), The WEC used also contains insulin, which can mimic the effects of GH on the hepatocyte, but is also essential for culture of hepatocytes under most conditions (Guzelian et al., 1988).

Pulsatile secretion of GH, with peaks *every* 3-4 hours that suppress selective P450 synthesis, could be envisaged to maintain relatively constant levels of rapid turnover P450s (ti 6-7h, Levin et al., 1975), whereas slow turnover P450s (regulated by GH) would tend to accumulate. It is plausible (if speculative) that GH/thyroid hormones regulate rapid turnover P450s to maintain constant levels of constitutive P450s, in the adult at least.

It has been demonstrated in this study that donor age and sex Influence both the activity and maintenance in culture of hepatic P450-dependent alkoxycoumarin O-dealkylase activities. The best

maintenance of a range of activities was achieved in hepatocytes from AM rats, cultured for 24 hours in WEC,

TABLES AND FIGURES

TABLE 4.1. SEX-SPECIFIC CYTOCHROME P450 ISOZYMES AND THEIR ASSOCIATED STEROID HYDROXYLASE ACTIVITIES.

P450 FORM [1]	ACTIVITY	SPECIFICITY
P450IIA1	testosterone 7α-hydroxylase[2]	female, immature male
P450IIA2	testosterone 15α-hydroxylase[3]	adult male
P4501IC11	testosterone 16α-hydroxylase[4]	adult male
P450IIC12	steroid 15ß-hydroxylase[5]	female, immature male
P450IIC13	progesterone 16α-,6ß-hydroxylase[6]	adult male
P450IIIA2	testosterone 6ß-hydroxylase[7]	adult male, immature male, female

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1 **Nebert et al.,(1989)**

2 **Arlotto and Parkinson, (1989)**

3 **Matsunaga et al., (1988)**

4 **Waxman et al., (1985)**

5 **Waxman et al., (1985)**

6 **Swinney et al.,(1988)**

7 **Waxman et al,, (1985); Yamazoe et al.,(1988).**

TABLE 4.2. ALKOXYCOUMARIN O-DEALKYLASE ACTIVIES IN HEPATOCYTES ISOLATED FROM IMMATURE (IM) AND ADULT (AM) MALE RATS.

SUBSTRATE CONCENTRATION (μM)		ACTIVITY (Pmol $min^{-1} mg^{-1}$) IM	AM
7-MC	500	88±17	157±16* *
7-MC	**20**	19±2	11± 2*
		(21.6)	(7.0)
7-EC	500	247±34	228±17
7-EC	10	19±4	13± 1
		(7.7)	(5.7)
7-PC	500	297±32	215± 8**
7-PC	10	25±6	15± 1
		(8.4)	(7.0)

Values are mean± SEM (n=4).
Figures in brackets are the mean activities at 10 or 20μM (the high affinity form) expressed as a percentage of the mean activity at 500μM (the total activity).
AM values significantly different to IM values at *$p < 0.05$ or **$p < 0.01$.

TABLE 4.3. ALKOXYCOUMARIN 0-DELAKYLASE ACTIVITIES IN HEPATOCYTES ISOLATED FROM ADULT MALE (AM) AND ADULT FEMALE (AF) RATS.

SUBSTRATE CONCENTRATION (μM)	ACTIVITY (pmol min^{-1} mg^{-1}) AM	AF
7-MC 500	157±16	71± 5 **
7-MC 20	11±2	16± 2
	(7.0)	(22.5)
7-EC 500	228±17	67±12* * *
7-EC 10	13±1	16± 1 *
	(5.7)	(23.9)
7-PC 500	215± 8	40± 6 ***
7-PC 10	15±1	24± 6
	(7.0)	(60.0)

Values are mean ± SEM (n+4).

Figures in brackets are the mean activities at 10 or 20 μM (the high affinity form) expressed as a percentage of the mean activity at 500 μM (the total activity).

AF values significantly different to AM values at *p < 0.05; * *p < 0.01; * ** p < 0.001.

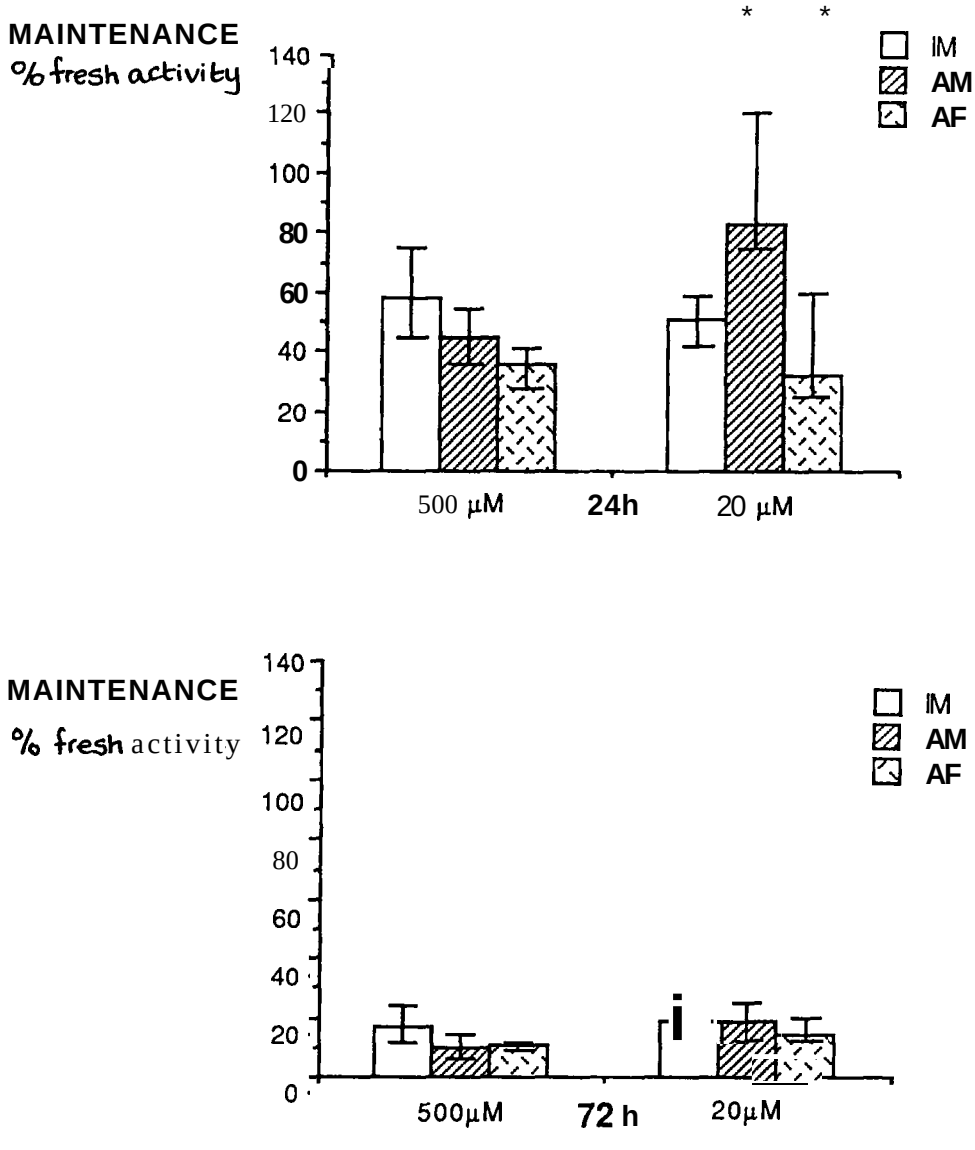
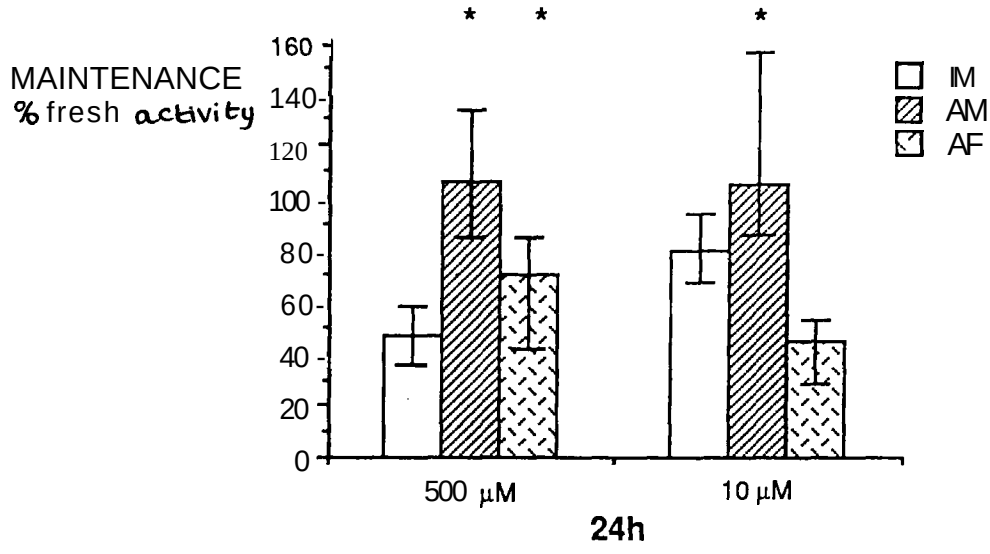


FIGURE 4.1. MAINTENANCE OF MCOD ACTIVITY IN HEPATOCYTES CULTURED FOR 24 AND 72 HOURS FROM IM, AM AND AF RATS. Values shown are the mean maintenance values (n=4) with the ranges.

* AM significantly different to to IM, and AF significantly different to AM values, $p<0.05$ (Mann-Whitney U-test).



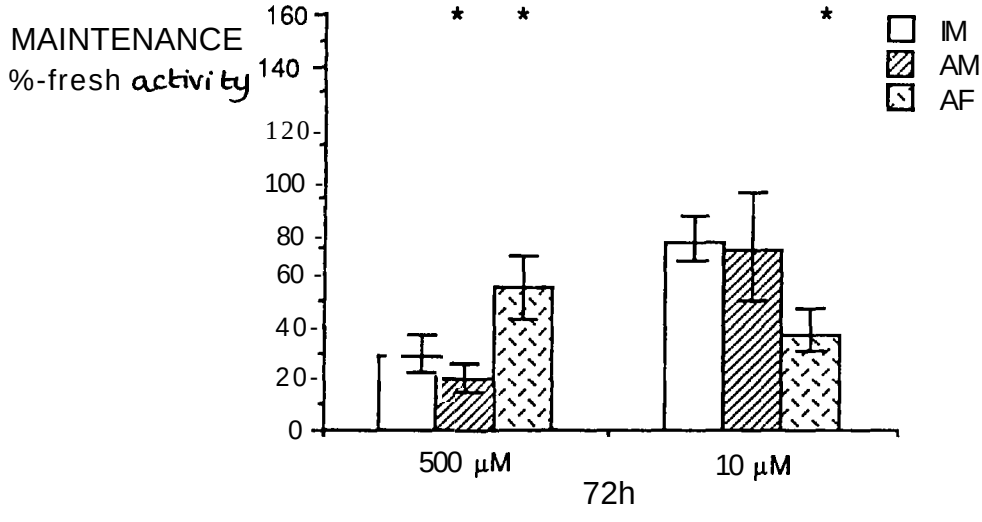


FIGURE 4.2, MAINTENANCE OF ECOD ACTIVITY IN HEPATOCYTES CULTURED FOR 24 AND 72 HOURS FROM IM, AM AND AF RATS, Values shown are the mean maintenance values (n=4) with the ranges.

* AM significantly different to to IM, and AF significantly different to AM values, $p<0.05$ (Mann-Whitney U-test).

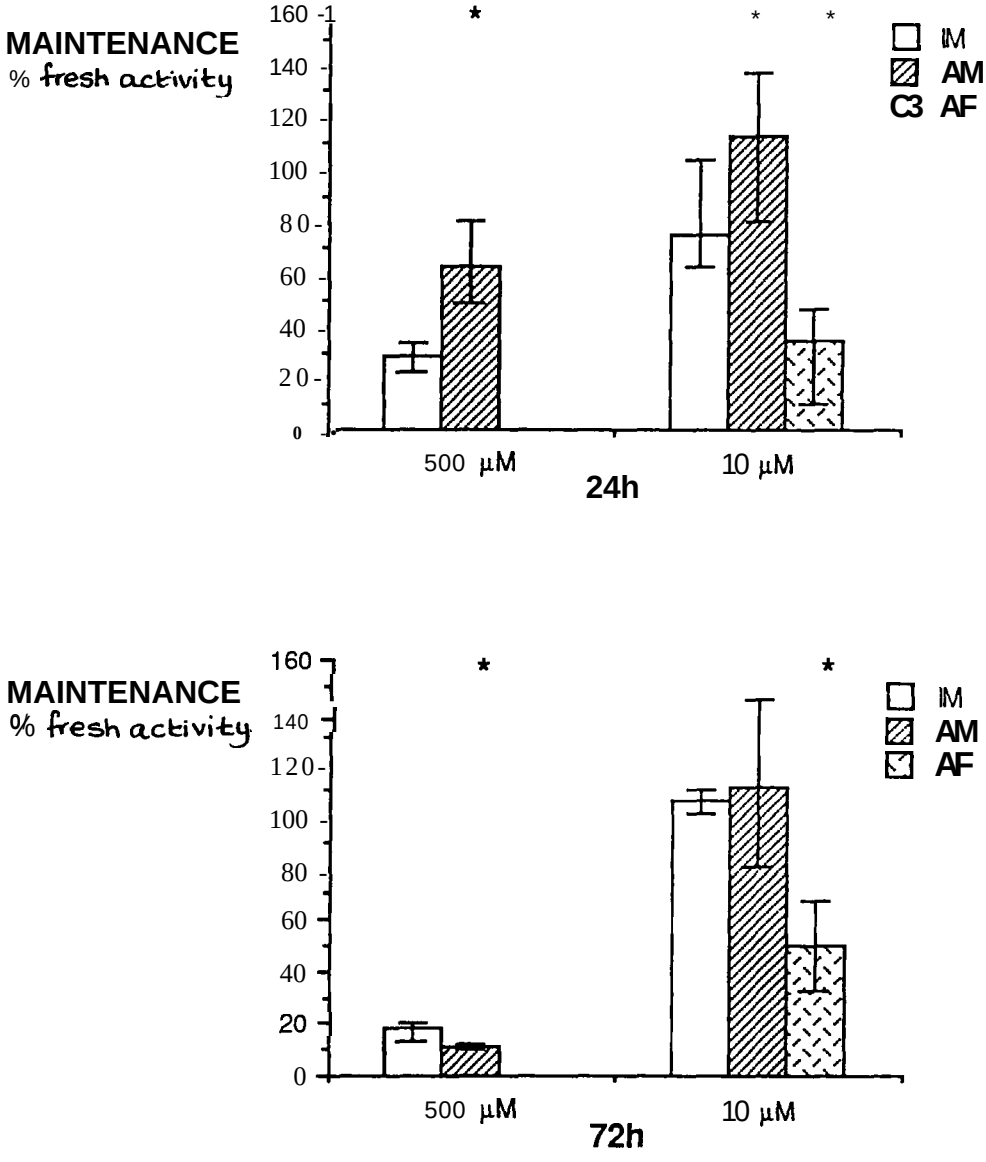


FIGURE **4.3.** MAINTENANCE OF PCOD ACTIVITY IN HEPATOCYTES CULTURED FOR 24 AND 72 HOURS FROM IM, AM AND AF RATS, Values shown are the mean maintenance values (n=4) with the ranges.

* AM significantly different to to IM, and AF significantly different to AM values, $p<0.05$ (Mann-Whitney U-test).

CHAPTER 5

INDUCTION OF ALKOXYCOUMARIN O-DEALKYLASE ACTIVITIES *IN VIVO* AND *IN VITRO*

CHAPTER 5

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CHAPTER 5

INDUCTION OF ALKOXYCOUMARIN O-DEALKYLASE ACTIVITIES *IN VIVO* AND *IN VITRO*

5.1 INTRODUCTION

Many xenobiotics that are metabolised by cytochrome P450 Induce their own biotransformation and/or the metabolism of other compounds, endogenous and exogenous. This induction of P450 activity can have a profound effect on the metabolism and toxicity of xenobiotics, since increased activation as well as increased detoxification can occur. The different classes of inducer have already been discussed in Chapter One.

Induction of hepatic P450s can be studied in microsomes and hepatocytes isolated from animals that have been treated with inducers. The changes in P450 and its associated activities can be easily measured in these systems. The alkoxycoumarin O-dealkylases have been used to Investigate Induction *in vivo* since the enzymes have different induction profiles. Microsomal MCOD activity (measured at 500μM) is Induced by PB, and ECOD (at 500μM) by PB and BNF (Matsubara *et al.*, 1983; Paterson *et al.*. 1984). This pattern of induction has also been reported for total MCOD and ECOD activities measured in hepatocytes Isolated from animals Induced *in vivo* with PB and BNF (Warren and Fry, 1988). Boobis *et al.* (1986) showed that the total and high affinity components of microsomal ECOD activity were differentially Induced by 3-MC, total ECOD activity being induced 9-fold more than the high affinity form. PB

induced the total and high affinity forms 5- and 2-fold respectively. The differential Induction of the components of this biphasic activity is further evidence for the Involvement of multiple P450 forms, making the alkoxycoumarins good substrate markers for a range of hepatic P450 isozymes.

More recently, researchers have tried to Induce P450 activities in cultured hepatocytes, with little success in reproducing the observed in vivo induction in the in vitro situation. The induction in vitro of total ECOD activity by 3-MC (and 3-MC-like compounds) has been demonstrated to be similar to that seen in vivo (Fry et al., 1980; Edwards et al., 1984), and the Induction and expression of P450IA1 and IA2 mRNAs in response to 3-MC in cultured hepatocytes has also been reported (Silver and Krauter, 1988). However, the anomalous expression of P450IA1 in untreated and in PB-treated cultures has been noted by several workers (Fry et al., 1980; Steward et al., 1985; Turner and Pitot, 1989). In contrast to the situation in vivo, total MCOD activity is not induced by PB in hepatocyte cultures (Warren and Fry, 1988), and the poor (and altered) response of P450 in culture to PB is now a well recognised phenomenon (Edwards et al., 1984; Forster et al., 1986). Interestingly, it is known that selenium deficiency leads to a reduced induction response to barbiturates (Burk and Masters, 1975), although the reason for this is not known; it has also been reported that selenium is necessary for PB induction of P450 in cultured hepatocytes (Engelmann et al., 1985).

Although the two components of microsomal ECOD activity were shown to be differentially induced by 3-MC and PB (Boobis *et al.*, 1986), only the induction of total MCOD activity has been reported, (Matsubara *et al.*, 1983; Warren and Fry, 1988). Also, the previous Induction *in vitro* study on MCOD and ECOD was performed with substrate concentrations of 500μM, representative only of the total activity (Warren and Fry, 1988). Therefore, in this study, the *in vitro* induction of both components of MCOD and ECOD activities by PB and BNF was determined, and compared to that measured in fresh hepatocytes isolated from animals Induced *in vivo*. The effect of Se on PB induction of MCOD activity *in vitro* was also investigated.

5.2 METHODS

5.2.1 Induction *in vivo*

Adult male Wistar rats (150g) were injected i.p. with PB (80 $mgkg^{-1}$, saline), BNF (80 $mgkg^{-1}$, oil of arachis), saline or oil of arachis, once a day for 3 days. Hepatocytes were then isolated by lobe perfusion, and alkoxycoumarin O-dealkylase activities determined in the freshly Isolated cells, as described in 2.10, except that incubation with substrate was for 5 minutes only (over which period the production of 7-HC was linear in cells from inducer-treated animals).

5.2.2 Induction *in vitro*

Hepatocytes from untreated adult male rats (150g) were cultured for 72 hours, in WEC, in the presence or absence of inducer, which was added after the 2 hour attachment period. Fresh medium containing inducer was placed on the cells after 24 and 48 hours in culture. After 72 hours, O-dealkylase activities were determined as described in 2.10 except that incubation of Inducer-treated cultures with 500μM 7-EC was for 1 hour only. MCOD activity (at 500μM substrate concentration) was also determined in PB-treated hepatocytes cultured in WEC and WEC containing haem (1μM), ALA (200mM) and Se (0.1μM) as described in 3.2. PB was used at 3.0mM (dissolved in saline) and BNF at 50μM (dissolved in DMF). These concentrations were ascertained, from initial experiments, to give maximal Induction in the absence of any observable toxicity (before and after incubation of the induced cells with substrate). Cultured hepatocytes were exposed to inducer for 3 days to compare directly the induction *in vitro* with that *in vivo*.

5.2.3 Expression of Results

O-Dealkylase activities are expressed as pmol 7-HC min^{-1} $mg\ protein^{-1}$, and induction of activity is expressed relative to the control activity (which is given the value 1.0). Statistical analysis was by use of t-tests and ANOVA, as appropriate.

5.3 RESULTS

5.3.1 Induction *in vivo*

The viabilities and yields of the cell preparations from the groups of treated rats were compared. These values from a group of untreated rats were also included in the comparison. The results are presented in Table 5.1. There were no significant differences in yield (as judged by ANOVA), although the yield from BNF-treated rats was lower than in the other groups. There were also no differences in viability. The O-dealkylase activities of the two control groups, saline and oil of arachis treated, were compared with those obtained from untreated animals (data from Table 4.3). Again, there were no significant differences between the three groups. Thus, treatment of animals with Injection vehicle does not affect these activities, the cells from vehicle-treated animals being equivalent to untreated control animals (Table 5.2).

Induction of activities

The activities determined in fresh cells isolated from the control and inducer-treated groups are shown in Table 5.3. PB significantly induced all four O-dealkylase activities. Both components of MCOD activity were Induced to the same extent *in vivo* (6.8-and 6.0-fold relative to the saline controls). High affinity and total ECOD activities were induced 12.9- and 5.3-fold respectively relative to saline controls. BNF-treatment resulted in a non-significant Increase in MCOD activity (3.4- and

1.8-fold Increases relative to oil-treated controls). However, there was a significant induction of both components of ECOD activity: 8.4- and 8.7-fold increases over control. It should be noted that some of the standard errors in these activities are quite large, especially amongst the PB and BNF-treated groups. This degree of variability probably reflects differences in the success of administration of the i.p. injections in addition to Inter-individual variation in response to the Inducers.

5.3.2 Induction *in vitro*

The activities determined in hepatocytes cultured for 72 hours in the presence or absence of inducer are shown in Table 5.4, After 72 hours in culture, all the activities in the control cultures are low, as expected from the data presented in Chapter Four, In the PB-treated cultures there is a slight increase in MCOD activity but this is only 1,5- to 2,5-fold relative to the control cultures. There was a significant induction of ECOD activity in culture, the Induction of total ECOD activity relative to control being 6.7, and of the high affinity form 3.6. In BNF treated cultures there was a slight Increase in MCOD activity (1.5- to 2.6-fold relative to control) and a significant induction of ECOD. Induction of the high affinity and total activity form was 7.2- and 20-fold respectively relative to control. The *in vitro* Induction data is summarised in Table 5.5. As was observed with the *in vivo* induction, there is a wide range of response to the Inducers. Since the culture conditions were uniform, this must reflect either inter-individual

variation in response which is retained in vitro, or differences in the cell populations obtained from the isolation process.

5.3.3 Comparison of induction in vivo and in vitro

The induction of activity relative to control in vivo and in vitro by PB and BNF is shown in Figures 5.1 and 5.2. It can be seen from Figure 5.1 that PB Induction is blunted 1n vitro, with MCOD activity being almost totally refractory to the Inducer, in contrast to the situation in vivo. Only total ECOD activity is induced in vitro to the same extent as in vivo. BNF did not Induce MCOD in vivo or in vitro (Figure 5.2). Induction of high affinity ECOD in vitro was similar to that seen in vivo whilst the induction of total ECOD in vitro was much greater than that seen in vivo. Therefore, there are not only differences between induction in culture and in vivo with respect to the magnitude of the effect, but the pattern of Increase of the four chosen activities is also altered.

5.3.4 Effect of selenium on PB induction in vitro

Table 5.6 shows the effects of haem, ALA and Se supplementation of WEC (i.e. WEC+H) on PB Induction of total MCOD activity. WEC+H was used as the sole control medium since MCOD activity in WEC and WEC+H has already been demonstrated to be equivalent (3.3.1). Total MCOD was chosen since it is the least well maintained, and most refractory to PB induction in culture. There was a numerical, but non-significant, induction (3.0-fold relative to control) of MCOD activity in PB treated cultures. The magnitude of induction was

greater than in the previous experiment (5.3.2), but whereas the induction in 5.3.2 represents the mean of 5 animals, in this experiment all the cultures used were derived from the same animal. This result confirms the observations on heterogeneity of response to Inducer *in vitro*. Nevertheless, the increase is only 50% of that seen *in vivo*, PB induction of MCOD activity in WEC+H was not significantly different to that in WEC (3,0- and 3,5-fold respectively). Therefore, the presence of Se (haem and ALA) in the medium did not Improve PB induction of MCOD *in vitro*,

5.4 DISCUSSION

The pattern of MCOD and ECOD induction was as expected: PB Induced both MCOD and ECOD; BNF induced only ECOD activity. Whereas both components of MCOD activity were induced to the same extent *in vivo*, PB induced high affinity ECOD more than the total activity and BNF Induced the total more than the high affinity form. BNF also induced only ECOD activity *in vitro*. PB failed to induce MCOD *in vitro* although there was good induction of total ECOD activity, and a modest induction of the high affinity component. The Inclusion of haem, ALA and Se in the culture medium did not improve PB induction of MCOD activity *in vitro*. This contrasts with the work of Engelmann *et al*. (1985) in which induction of P450 content by PB was only observed in medium containing exogenous haem and Se. However, these workers used PB at a concentration of 1mM, which did not Induce O-dealkylase activity in initial experiments performed in this study. The Se may possibly be potentiating an effect at this

lower concentration of inducer. It is also possible that the Se is increasing the total P450 Induced by PB, but that these P450s are not PB-specific Isozymes. This would explain the lack of effect of Se on PB Induction of MCOD activity, and would be consistent with other work on PB induction *in vitro*.

PB-inducible P450s have been reported previously to be refractory to induction in hepatocyte culture (Forster *et al.*, 1986; Warren and Fry, 1988), but it also appears that the isozymes Induced by the compound *in vivo* and *in vitro* are different. Edwards *et al.* (1984) determined that the proportion of PB-inducible P450s that could be inhibited by metyrapone after *in vitro* induction was different to that after *in vivo* Induction, suggesting that the populations of P450 Induced in the two conditions were not identical. In 1985, Miyazaki *et al.* observed that although PB effectively arrested the rate of decline of total P450 in hepatocyte cultures and preserved the cell morphology, the main Isozyme induced by PB, P450IIB1, was not detectable immunochemically in the cultures.

Thus, the poor induction by PB *in vitro* is probably a function of altered regulation. It is known that the transcription rates of other PB-inducible proteins are different to that of P450IIB1 (Hardwick *et al.*, 1983a), however there have been no reports on the induction *in vitro* of non-P450 PB-inducible proteins. It may be that changes in the regulation of PB induction reflect alterations in other factors necessary for PB-inducible gene expression.

Although no receptor for PB has been identified, it appears that protein synthesis is essential for Induced expression of the CYP2B1 and CYP2B2 genes (Chianale et al., 1988), and the Involvement of trans-acting regulatory factors has been suggested. Alterations in turnover/regulation of transcription factors in vitro could result in reduced gene expression.

Although PB did not induce MCOD in vitro, ECOD activity was induced, ECOD activity must involve a different population of isozymes, since the activity is also induced by BNF, The Induction of ECOD by PB may Involve PB Induction of BNF-inducible P450s, The anomalous expression of P450IA1 in untreated and PB-treated cultures has been reported previously and seems to be more marked in hormone-free culture medium (Fry et al., 1980; Turner and Pitot, 1989). Also, good expression of both P450IA1 and IA2 seems to require serum-free medium. It is therefore possible that these P450s are normally repressed (e.g. by a hormone) but that this repression is disrupted in vitro. Despite the unusual expression of P450IA1 in control and PB-treated cultures, the induction of BNF-inducible forms by BNF in vitro is similar to that seen in vivo.

It was suggested that the variability in response to Inducer was a result of inter-individual variation and/or variation in administration of inducer, these variations being retained on placing hepatocytes into culture. However, it is also known that hepatocyte heterogeneity exists with respect to distribution of P450

isozymes, as well as carbohydrate metabolism (Gumucio, 1989). The perivenous hepatocytes (in the distal part of the liver acinus) contain more PB-and 3-MC-inducible isozymes in the uninduced state, and whereas PB Induction predominates in perivenous hepatocytes (Baron *et al.*, 1981), 3-MC Induction seems to predominate in the periportal regions (Tazawa et al., 1988). It has been shown that the direction of liver perfusion during hepatocyte Isolation influences the relative proportions of proximal and distal acinar hepatocytes obtained in the final preparation (Gumucio et al., 1986). It is possible then, that selective isolation and/or selective survival in culture of hepatocytes with different P450 isozyme profiles and different metabolic activities may also be a factor in altered inducibility of P450s in vitro.

In summary, the induction of alkoxycoumarin O-dealkylase activities in vitro by PB and BNF was not representative of the induction observed in vivo. It is likely that P450 induction in vivo will only be successfully reproduced in culture when the mechanisms of induction are fully understood. However, in the meantime, an alternative approach for studying induced P450s in hepatocyte cultures would be to induce the isozymes in vivo, and then culture hepatocytes from the treated animals. The feasibility of this is investigated in the following chapter.

TABLES AND FIGURES

TABLE 5.1. YIELD AND VIABILITY OF CELL PREPARATIONS FROM UNTREATED AND INDUCER-TREATED ADULT MALE RATS.

GROUP	YIELD (million cells)	PERCENTAGE VIABILITY
UNTREATED	44± 3	92± 1
SALINE	40± 1	91 ± 1
PB	46± 4	91± 2
OIL OF ARACHIS	45±16	91 ±1
BNF	18±2	92± 2

Values are mean ± SEM (n=4).
ANOVA : no significant differences between the five groups.

TABLE 5.2. ALKOXYCOUMARIN O-DEALKYLASE ACTIVITIES IN FRESHLY ISOLATED HEPATOCYTES FROM CONTROL GROUPS OF ADULT MALE RATS.

SUBSTRATE	CONCENTRATION (μM)	ACTIVITY (pmol $min^{-1} mg^{-1}$) UNTREATED	SALINE	OIL OF ARACHIS
7- MC	20	11±2	8±2	13± 1
7-MC	500	157±16	138±16	125± 6
7-EC	10	13±1	9±2	17± 1
7-EC	500	228±17	294±37	314±19

Values are mean ± SEM (n=4).
ANOVA : no significant differences between the three groups.
Untreated group: data taken from Table 4.3.

TABLE 5.3. ALKOXYCOUMARIN O-DEALKYLASE ACTIVITIES IN HEPATOCYTES ISOLATED FROM CONTROL AND INDUCER-TREATED ADULT MALE RATS: INDUCTION *IN VIVO.*

SUBSTRATE CONCENTRATION (μM)		ACTIVITY (pmol min"" m g^{-1}) SALINE	PB	OIL OF ARACHIS	BNF
7-MC	20	8±3	54± 11*	13± 1	44± 7
7-MC	500	138±16	834±169*	125± 6	219± 34
7-EC	10	9±2	116± 26*	17± 1	143± 21**
7-EC	500	294±37	1554±296*	314±19	2740±208**

Values are mean ± SEM (n=4).

* PB significantly different to saline control $p < 0.05$;

* * BNF significantly different to oil of arachis control $p < 0.05$; unpaired t-tests.

TABLE 5.4. ALKOXYCOUMARIN O-DEALKYLASE ACTIVITIES IN HEPATOCYTES CULTURED IN THE PRESENCE OF INDUCER: INDUCTION *IN VITRO.*

SUBSTRATE CONCENTRATION (μM)	ACTIVITY (pmol $min^{-1} mg^{-1}$) PB	CONTROL	BNF
7-MC 20	1.710.3	0.710.2	1.810.6
7-MC 500	11.012.0	7.311.0	12.013.0
7-EC 10	7.512.0	2.110.5*	14.013.0*
7-EC 500	110138	21.0±3.0*	3641112*

Values are mean ±SEM (n=5, 7-MC; n=4, 7-EC).

* control significantly different to PB and BNF significantly different to control $p < 0.05$; paired t-test.

TABLE 5.5. ACTIVITY AFTER INDUCTION *IN VITRO* RELATIVE TO CONTROL.

SUBSTRATE CONCENTRATION (μM)	RELATIVE INDUCTION PB	BNF
7-MC 20	2.5(1.9-3.3)	2.6 (1.0-5.2)
7-MC 500	1.5(1.1-1.9)	1.5 (0.8-2.3)
7-EC 10	3.6(2.8-5.2)	7.2 (3.9-11.0)
7-EC 500	6.7(1.8-13.0)	20.0 (5.6-37.0)

Induced activity relative to control was determined for each animal and the values above are the mean induced activities relative to control, with ranges (n=4 or 5).

TABLE 5.6. EFFECT OF MEDIUM SUPPLEMENTATION ON PB INDUCTION OF TOTAL MCOD ACTIVITY,

	MEDIUM		
	WEC+H	WEC PB	WEC+H PB
pmol $min^{-1} mg^{-1}$	12± 1	36± 7	421 3
relative induction	1.0	3.0	3.5

Values are mean ± SEM (n=4 plates).
WEC PB not significantly different to WEC+H; WEC+H PB not significantly different to WEC PB; paired t-test.

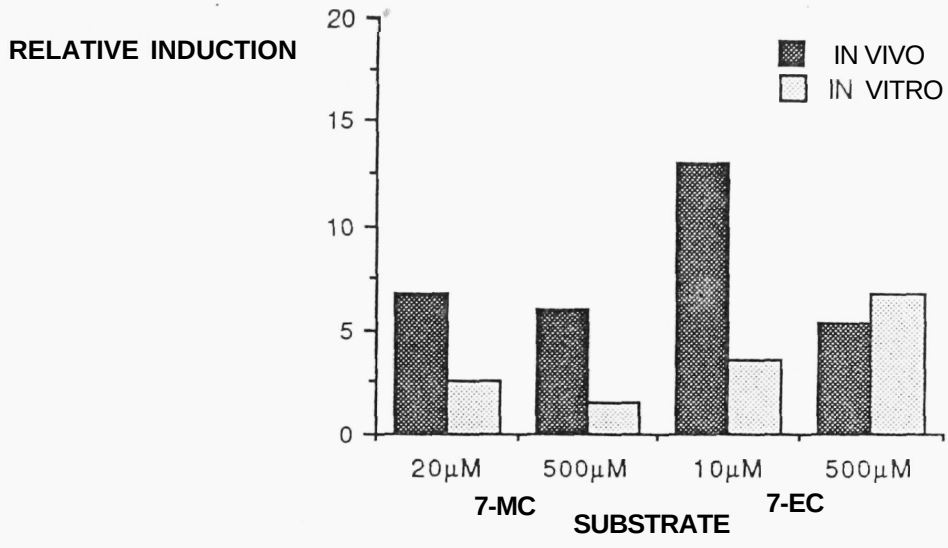

FIGURE 5.1. INDUCTION OF MCOD AND ECOD ACTIVITIES BY PB *IN VIVO* AND *IN VITRO*. Activities were determined in freshly isolated cells from inducer-treated animals, or in hepatocytes cultured for 72 hours in the presence of inducer. Activities are expressed relative to the control activity, i.e. from the untreated cells or animals.

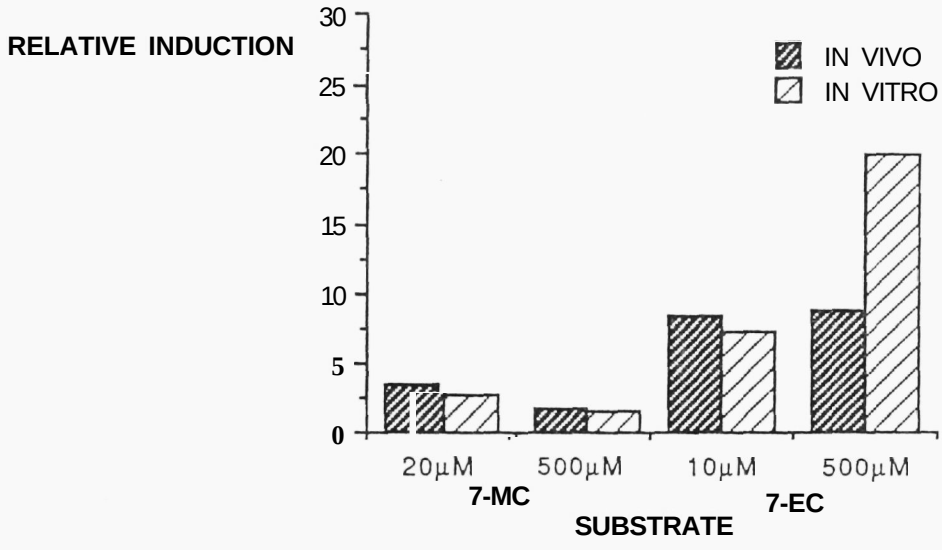

FIGURE 5.2. INDUCTION OF MCOD AND ECOD ACTIVITIES BY BNF *IN VIVO* AND \N *VITRO.* Activities were determined in freshly isolated cells from inducer-treated animals, or in hepatocytes cultured for 72 hours in the presence of inducer. Activities are expressed relative to the control activity,i.e. from the untreated cells or animals.

CHAPTER 6

INDUCTION OF P450 AND ENZYME ACTIVITIES *IN VIVO* AND MAINTENANCE IN RAT HEPATOCYTE CULTURES

CHAPTER 6

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CHAPTER 6

INDUCTION OF P450 AND ENZYME ACTIVITIES *IN VIVO* AND MAINTENANCE IN RAT HEPATOCYTE CULTURES

6.1 INTRODUCTION

Exposure to Inducers of P450 can profoundly alter the metabolism and toxicity of xenobiotics by altering the isozyme population. Different Isozymes have different substrate specificies, which is reflected by the differential induction of an activity on exposure to a range of Inducers (Okuno *et al.*, 1989). It should be possible, therefore, to measure enzyme activities selective for different isozymes, resulting in an activity/isozyme profile for each inducer. This has been attempted using stereospecific hydroxylation products of testosterone (Darby *et al.*, 1986), and the dealkylation of a series of alkoxyresorufins (Burke *et al.*, 1985), measured in microsomes from animals treated with P450 inducers.

It was therefore decided to measure several enzyme activities which it was hoped would prove to be selective for different Induced P450 Isozymes. This was done in hepatocytes Isolated from animals treated *in vivo* with prototypic inducers of the main P450 gene families. The inducers chosen were β-naphthoflavone (BNF), phenobarbitone (PB), isoniazid (ISO) and dexamethasone (DEX) which induce P450s in the IA, IIB, IIE and IIIA families respectively. Prospective selective substrates for the different induced isozymes were chosen. Microsomal 7-ethoxyresorufin O-deethylation (EROD) is selectively induced by 3-MC and BNF (Burke *et al.*, 1985);

benzphetamine demethylation (BZDM) has been reported to be catalysed by P450IIB1 (Ryan et al., 1979); p-nitrophenol hydroxylase (PNPH) is induced in microsomes from ethanol-fed rats (Reinke and Moyer, 1985), ethanol and isoniazid both inducing the same isozyme (P450IIE1) in the rat (Ryan et al., 1986), DEX and PCN both induce the same microsomal P450 (Heuman et al., 1982), DEX being a better Inducer. The macrolide antibiotics also induce this family of isozymes, inducing their own metabolism at the same time (Danan et al., 1981; Watkins et al.. 1986), and therefore erythromycin N-demethylation (EMDM) was chosen as the fourth enzyme activity. The enzyme/activity profile for each inducer was then examined and the maintenance of the activity profile in culture determined.

6.2 METHODS

6.2.1 Animals

Adult male rats (6-8 weeks old) were treated i.p. with dexamethasone sodium phosphate (100 $mgkg^{-1}$ in saline) for 4 days or BNF (80 $mgkg^{-1}$ in arachis oil) for 3 days. PB and ISO (0.1%) were administered in drinking water for 5 and 10 days respectively.

6.2.2 Procedures

Hepatocytes were isolated by lobe perfusion, and half of the final cell suspension was sonicated to give an homogenate of fresh cells as described in Chapter 2.8. The other half of the suspension was plated out on "Primaria" dishes in WEC. After 24 hours in culture

the monolayers were washed with saline and homogenates prepared. P450, protein content and enzyme activities were determined in the cell homogenates as described in Chapter 2.11 - 2.13.

6.2.3 Presentation of results

Enzyme activities were calculated as nmol or pmol product min^{-1} mg protein^{-1} (the "activity") and as nmol or pmol product min^{-1} nmol P450^{-1} (the "specific activity"). Induction of activity relative to control indicated the actual change in activity, whereas changes in specific activity relative to control indicated the involvement of induced P450 Isozymes in the activity. Statistical analysis was by ANOVA and Dunnett's tests for the induction of fresh activity relative to control, and by use of paired t-tests for the maintenance data.

6.3 RESULTS

6.3.1 Induction of enzyme activities

Table 6.1 shows the P450 content and enzyme activities measured in homogenates from hepatocytes isolated from untreated and treated adult male rats. P450 content and BZDM activity were both significantly Induced by PB, DEX and BNF but not by ISO. EMDM was Increased by PB and DEX; PNPH was induced by PB, DEX and ISO. EROD was significantly Induced only by BNF. The 5,6-fold induction by PB was not significant; this was probably because the 320-fold induction of EROD by BNF, being so much greater than that by PB (and

the other Inducers), weighted the Dunnett's test (by yielding a very high estimate of the residual mean square value in the ANOVA).

Table 5.2 shows the specific enzyme activities. PB did not significantly increase any of the specific activities, whereas DEX significantly induced EMDM, ISO significantly induced PNPH and BNF significantly Induced EROD.

6.3.2 Induction **relative** to control

Figures 6.1 - 6.4 show the changes in P450 content, activity and specific activity relative to those measured in untreated rats (controls = 1.0).

PB (Figure 6.1a) induced BZDM, EMDM and EROD activities to the same extent as the P450 content (7.4-, 5.9-, 5.6- and 6.6-fold respectively), and accordingly the relative specific activities (RSA; Figure 6.1b) of these enzymes approximated to 1.0 (1.3, 0.8 and 0.8), In contrast, PNPH activity was Induced 2.4-fold byPB although the RSA was only 0.3. The demethylase results agree with microsomal data for the PB-inducible aminopyrine demethylase, in that the activity was Induced to the same extent as the P450 content (Fry, 1981). Also, the induction of PNPH and EROD determined here from whole cell homogenates were similar to the Inductions of the same activities determined in microsomes (1.7-fold induction of PNPH, Reinke and Moyer, 1985; 6-fold induction of EROD, Burke et al., 1985).

DEX (Figure 6.2) induced P450 and BZDM activity 5.6-fold, the RSA of BZDM being 1.1, as seen with PB Induction. EMDM activity was induced 11.8-fold, whereas the RSA was 2.0. Thus, only half of the observed induction of activity could be attributed to the increase in total P450. However, this was the only activity measured that was selectively induced by DEX; the RSA of PNPH and EROD were less than 0.6.

Only PNPH activity was increased by ISO as seen in Figure 6.3 (7.4-fold increase, similar to the 6.1-fold increase observed in microsomes from ethanol-fed rats, Reinke and Moyer, 1985). The RSA was 3.9, indicating selective induction of this activity, although the total P450 content did not increase significantly after ISO treatment.

EROD was selectively Induced by BNF (Figure 6.4; RA 320, RSA 32), and again, this was much greater than the observed induction of P450 (9-fold). The RSAs of the other enzymes were less than 0.6.

It would appear that BNF selectively Induced EROD, ISO selectively induced PNPH and DEX selectively induced EMDM, whereas PB induced all three demethylase activities to more or less the same level.

6.3.3 Maintenance of activities in culture

The maintenance of the activities after 24 hours in culture, in hepatocytes from untreated and treated animals is shown in Figures

6.5-6.9. Activity at 24 hours relative to that of the fresh cells (RA24) and relative specific activity at 24 hours (RSA24) are both shown. An RA24 of 1.0 and an RSA24 greater than or equal to 1.0 for a particular enzyme indicates maintenance of that particular activity and loss of total P450; an RA24 and an RSA24 less than 1.0 indicates a specific loss of activity whilst the bulk of the P450 is maintained.

In cultures from untreated rats (Figure 6.5) P450 content fell by 31%; there was a selective loss of BZDM (RSA24 0.46) and a loss of EROD activity. The loss of EROD in culture has been reported previously (Grant *et al.*, 1985), as has the loss of P450 and of certain PB-inducible activities (Warren et al., 1985).

In cultures from PB-induced rats (Figure 6.6) there was a significant loss of P450; there was a selective loss of EMDM and maintenance of BZDM, in contrast to the control cultures.

In cultures from DEX-induced rats (Figure 6.7) the P450 content fell by 43% and unlike the control cultures BZDM was maintained, with total loss of PNPH and some loss of EMDM activity, although the RSA was maintained.

In cultures from ISO-treated animals (Figure 6.8) both P450 and EROD were maintained, as well as EMDM and PNPH activities; there was a loss of BZDM, similar to that seen in control cultures.

In cultures from BNF-treated animals (Figure 6.9) the P450 content fell by 47% and EROD activity was selectively lost. (However, EROD activity in these cultures was still 7-fold greater than that measured in control cultures). The other activities were maintained.

In general, P450 declined by 30-50% in hepatocytes after 24 hours in culture and EROD activity was the least stable. The activities selectively induced (as determined in the fresh cell homogenates) were maintained in culture over 24 hours, although some of the other forms were lost.

6.4 DISCUSSION

The selective induction of enzyme activities was demonstrated in this study, using four of the prototypic P450 inducers. ISO only Induced PNPH activity and specific activity; BNF selectively induced EROD (i.e. increase in RA and RSA) and DEX selectively induced EMDM. The latter two inducers also increased at least one other enzyme activity, but not the relevant specific activity. PB, in contrast, induced all the activities but was completely non-selective (for the activities measured in this study).

The fold-induction of specific activities by BNF, ISO and DEX was greater than the fold-induction of total P450 in each case, and there are several explanations for these apparent discrepancies. Firstly, a small increase in an isozyme with a high affinity for the

substrate would increase the observed activity (especially at low substrate concentrations), but if the total P450 was induced or remained unchanged a *very* low RSA value would be obtained. This could explain PB induction of PNPH, where the RA was 2,4 and the RSA only 0,3, Secondly, a constitutive isozyme present at low levels could be dramatically Increased on induction, but still account for a small proportion of the total P450: this could result in an RSA value much higher than the increase in total P450, This probably accounts for the induction of PNPH by ISO, The RA and RSA of this Induced activity were 7,4 and 3,9 respectively, but, in contrast to the other Inducers, there was no increase in the total P450 content. Other workers have reported 6-fold inductions of P450IIE1 and PNPH in the absence of any increase in the total microsomal P450 (Ryan et al., 1985).

With respect to substrate specificity/affinity there were problems associated with choice of substrate in this study. There was a problem of specificity: PB did not significantly induce any RSA value. However, this may reflect the limited number of enzyme activities studied and/or the choice of activities e.g. the induction of pentoxyresorufin O-dealkylase (PROD), as opposed to BZDM, may be more specifically induced by PB (Burke et al., 1985).

In addition to the choice of substrate, it has been shown recently that DEX but not PCN (pregnenolone-16α- carbonitrile) induces the main PB-inducible forms in addition to the P450III proteins (Namkung

et al., 1988), and also that PB can Induce the CYP3A1 gene (Nebert and Gonzalez, 1987). Also, PCN does not induce the PB-inducible PROD activity (Burke *et* al., 1985). This probably explains the similar inductions of EMDM and BZDM by PB and DEX. In this study DEX was chosen over PCN because of its greater magnitude of induction. However, in the light of the work by Namkung et al. (1988) PCN would appear to be a more selective Inducer of the P450III family. It is also difficult to find selective substrates for this gene family that are relatively easy to assay. The macrolide antibiotics such as triacetyloleandomycin and erythromycin can present problems, since they are capable of binding to the cytochrome forming an Inactive complex (Danan et al., 1981). For this reason Namkung et al. (1988) attempted to find alternative substrates; separation of different testosterone metabolites by HPLC was the most successful assay that they tried, with respect to selectivity.

In general, the selectively Induced enzyme activities were maintained for 24 hours in hepatocytes cultured from the treated rats, although the total P450 content declined by 30-50% over this time period. This again demonstrated selective stability of isozymic forms, in addition to selective induction.

In hepatocytes cultured from ISO-treated animals, there was a general maintenance of both P450 and enzyme activities, although there was selective loss of BZDM (RA24 and RSA24 of less than 0.5),

similar to that seen in cultures from untreated rats. In these control cultures BZDM activity declined by more than 70%, whereas the total P450 fell by only 31%. The other activities were maintained in control cultures although there was some loss of EROD activity. This activity declined to some extent in all the cultures studied (except those from ISO-treated animals), however, constitutive BZDM appeared to be particularly labile in hepatocyte culture, unlike the induced activity.

It therefore appears that the induced form of BZDM, and also induced EMDM, are not identical to the constitutive forms present in control cultures. The induced EMDM was lost more rapidly than the constitutive form. This is consistent with the rapid degradation of P450s Induced by DEX (Watkins et al., 1986), In contrast, the BZDM Induced by PB and DEX was maintained in culture for 24 hours, whereas the constitutive activity was lost. It has been reported that the main PB-inducible P450 in the liver, P450IIB1 has a high specificity for BZDM and the 16α-hydroxylation of testosterone (Ryan *et al.*, 1979). This latter activity is catalysed by P450IIC11 in untreated rat liver (Waxman et al., 1985). It is also possible that the poor maintenance of constitutive BZDM reflects the poor maintenance of liver-specific Isozymes, especially in medium containing serum (Enat, 1984). This has been discussed in detail in previous chapters. It would appear then, that induced P450IIB1/2 Isozymes can be maintained in culture for 24 hours, and this also suggests that the inability to detect PB-induction of P450IIB1/2 in

vitro is an induction- mechanism defect, not a maintenance problem.

This study has shown that it is possible to use selective marker activities to monitor changes in Isozyme profile on induction. Induced activities can be maintained in cultured hepatocytes for 24 hours. It should be possible to extend this approach to other Inducing agents, and also to compare profiles from compounds that induce the same P450 Isozymes. These diagnostic profiles could then be used to investigate new compounds for their ability to induce P450s.

SUMMARY

In the previous four chapters an hepatocyte culture system has been defined and characterised with respect to maintenance of P450 and its associated enzyme activities and the induction of these activities. The conditions resulting in the best maintenance of a range of activities seem to Involve hepatocytes from young adult male rats, cultured for 24 hours in WEC medium. At this time point, the P450 content is 50-70% that of the freshly isolated cells, with a range of enzyme activities being maintained at 70% or more of the fresh activity. The liver specific forms appear to be less well maintained, so there are further modifications that could be made to this system, including re-appraisal of the use of serum-containing medium. It is also possible to induce activities *in vivo* and maintain them for 24 hours in the cultured hepatocytes;

this approach was more successful than Induction *in vitro*. The next chapter reports on investigations into P450-mediated toxicity using this culture system.

TABLES AND FIGURES

TABLE 6.1. CYTOCHROME P450 CONTENT AND ENZYME ACTIVITIES IN SUBCELLULAR FRACTIONS OF FRESHLY ISOLATED RAT HEPATOCYTES FROM UNTREATED AND TREATED ADULT MALE RATS.

	TREATMENT OF ANIMALS				
VARIABLE	CONT	PB	DEX	ISO	BNF
P450 content[1]	0.16±0.02	1.25±0.11*	0.29±0.18*	0.29±0.04	1.50±0.29*
BZDM[2]	0.84±0.04	2.50±0.80*	1.90±0.20*	0.89±0.05	7.80±0.80*
EMDM[2]	0.17±0.01	1.20±0.08*	0.20±0.20*	0.28±0.28	0.28±0.09
PNPH[2]	0.24±0.04	0.38±0.05*	0.81±0.07*	1.1±0.10*	0.80±0.09
EROD[3]	5.0 ± 0.8	28±5	7. ± 0.5	5.6 ± 0.6	1500±400*

Values are mean ± S.E.M. of 3-8 animals.

* treated groups significantly different to control groups, $p < 0.05$.

1 nmol mg protein^{-1}

2 nmol product min^{-1} mg protein^{-1}

3 pmol product min^{-1} mg protein^{-1}

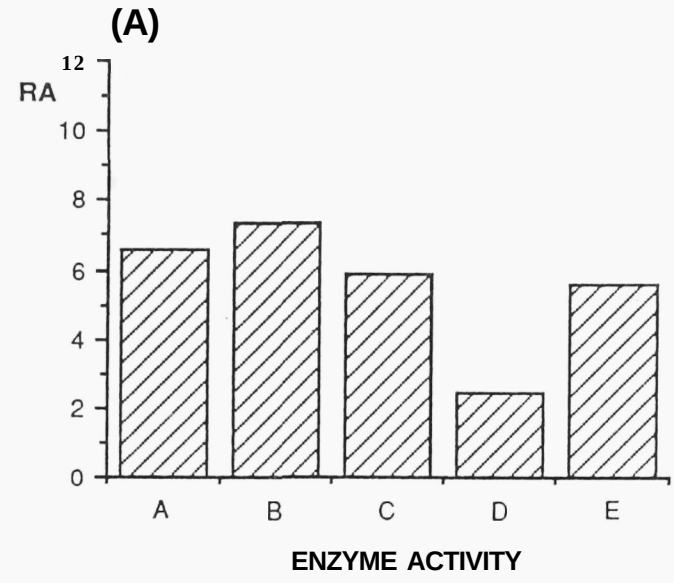
TABLE 6.2. SPECIFIC ENZYME ACTIVITIES MEASURED IN HOMOGENATES OF FRESHLY ISOLATED HEPATOCYTES FROM UNTREATED AND DUAL TREATED ADULT MALE RATS.

	TREATMENT OF ANIMALS				
ACTIVITY[1]	CON	PB	DEX	ISO	BNF
BZDM	0.9±0.0	0.8± 0.3	0.9±0.0	0.6±0.4	1.1±0.0
EMDM	1.0±0.1	1.2± 0.1	0±1.3 *	0.88±0.18	0.20± 0.07
PNPH	17± 0.4	85610 .04	7.0±0.1	8.6±1.1*	0.90± 0.08
EROD	84±4	0 ±5	0 ±1	0 ±2	1 10011 00*

Values are mean ± SEM for 8-8 animals.

* Treated groups significantly different to control groups, p < 0.05 ANOVA and Dunnett's tests.

[1] Activities are nmol (pmol) ERODP product min[1] mg protein[1].



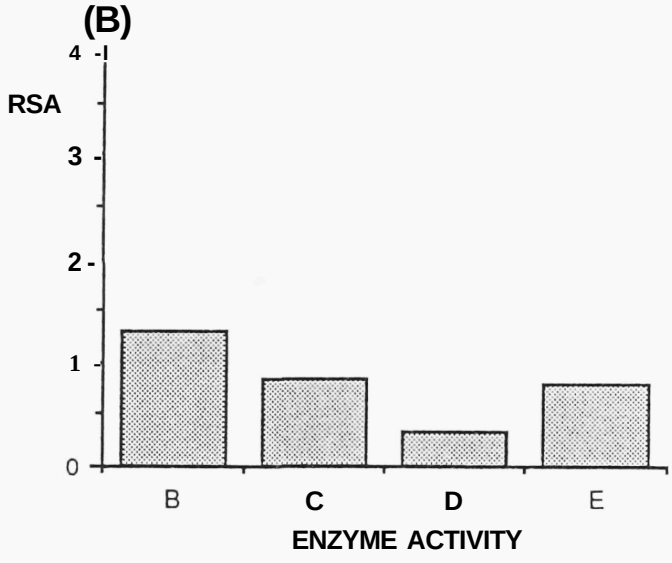


FIGURE 6.1. INDUCTION *IN VIVO* OF P450 AND ENZYME ACTIVITIES BY PB. Each value is the mean activity (A), or mean specific activity (B) measured in freshly isolated hepatocytes from treated animals relative to that of the untreated animals.

A; P450 content B: BZDM C: EMDM D:PNPH E:EROD

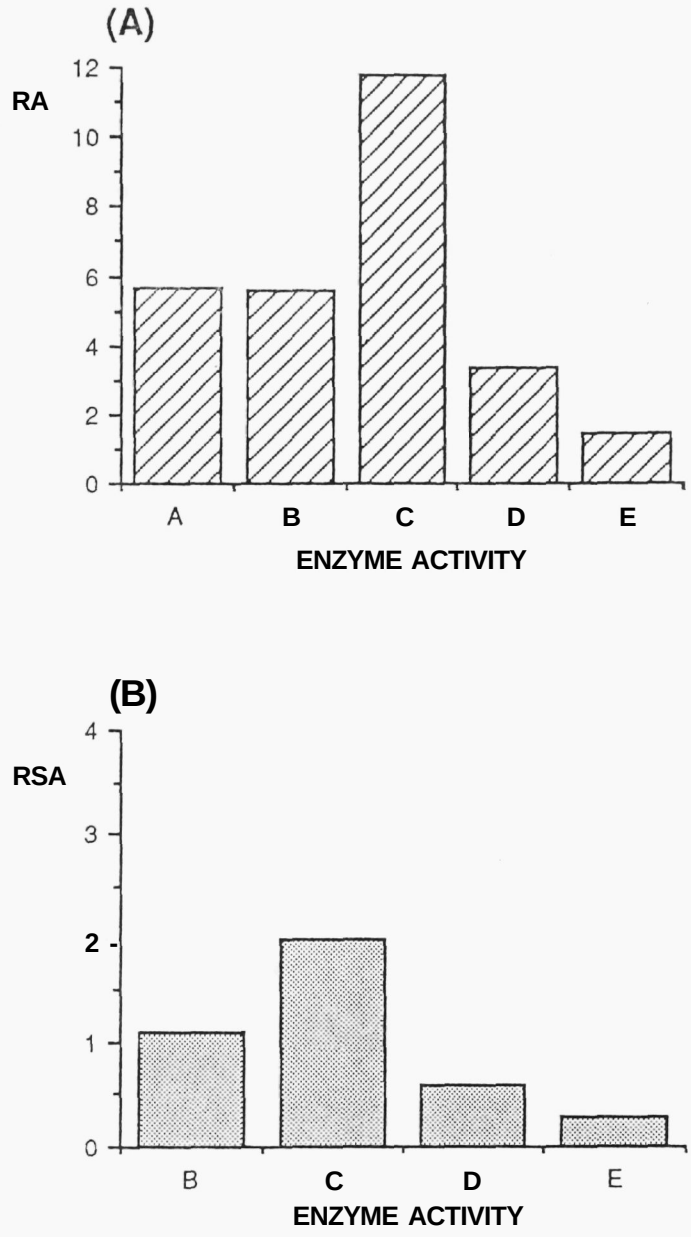


FIGURE 6.2. INDUCTION *IN VIVO* OF P450 AND ENZYME ACTIVITIES BY DEX. Each value is the mean activity (A), or mean specific activity (B) measured in freshly isolated hepatocytes from treated animals relative to that of the untreated animals.

A: P450 content B: BZDM C: EMDM D:PNPH E:EROD

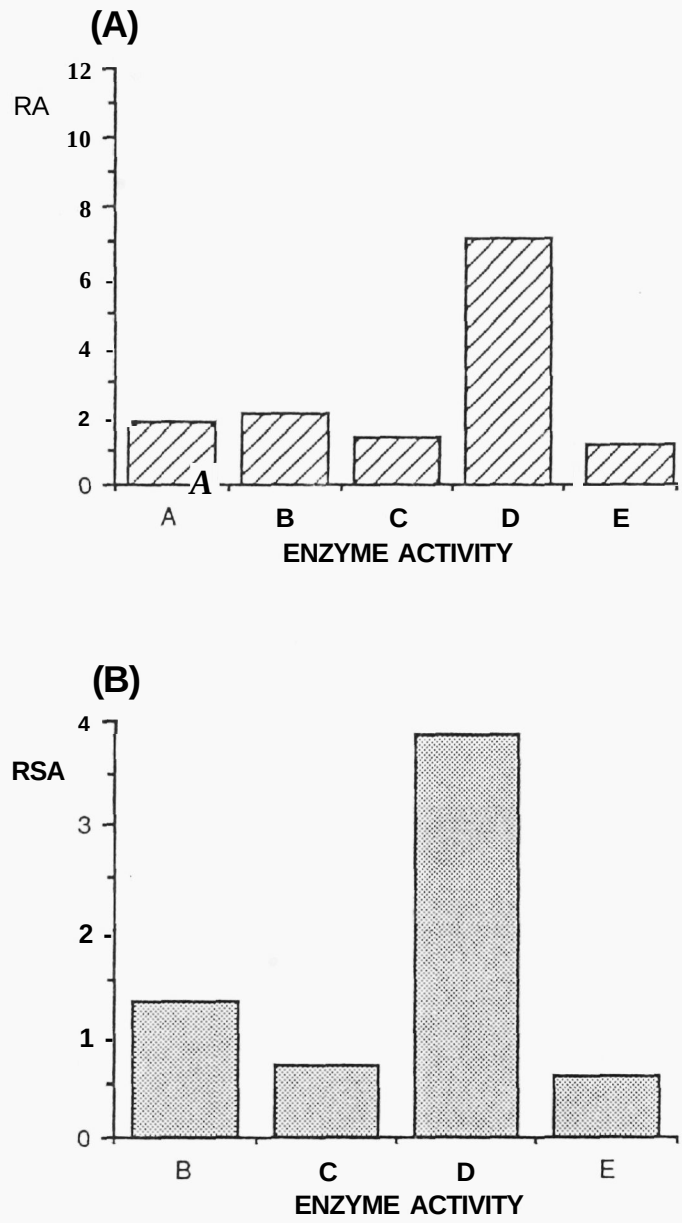


FIGURE 6.3. INDUCTION *IN VIVO* OF P450 AND ENZYME ACTIVITIES BY ISO. Each value is **the** meanactivity (A), or mean specific activity (B) measured in freshly isolated hepatocytes from treated animals relative to that of the untreated animals.

A: P450 content B: BZDM C: EMDM D:PNPH E:EROD

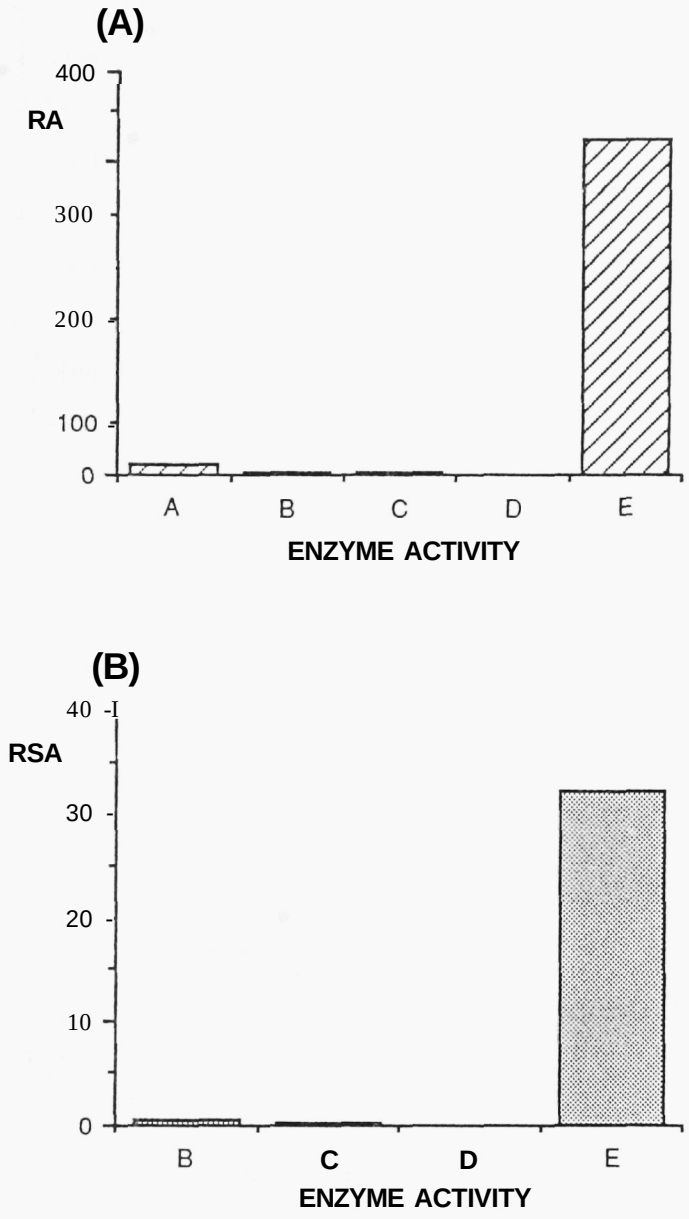


FIGURE 6.4. INDUCTION *IN VIVO* OF P450 AND ENZYME ACTIVITIES BY BNF. Each value is the mean activity (A), or mean specific activity (B) measured in freshly isolated hepatocytes from treated animals relative to that of the untreated animals.

A: P450 content B: BZDM C: EMDM D:PNPH E:EROD

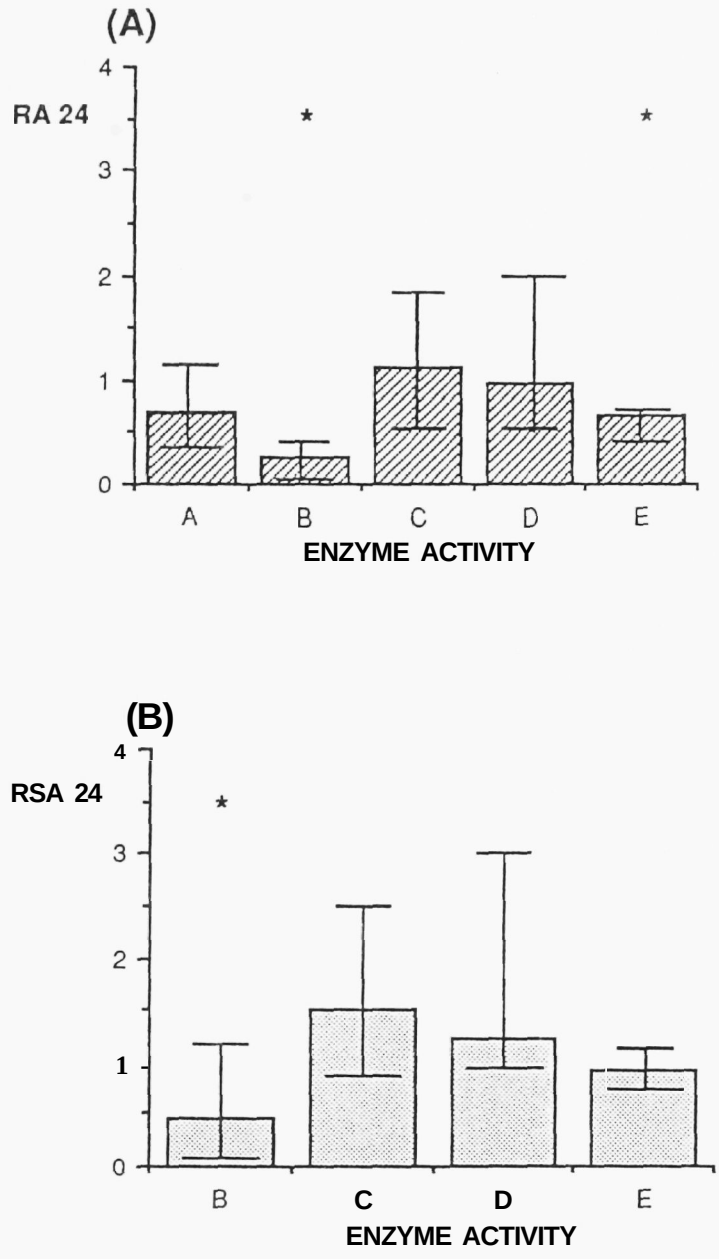


FIGURE 6.5. MAINTENANCE OF P450 AND ENZYME ACTIVITIES AT 24 HOURS IN HEPATOCYTES CULTURED FROM UNTREATED RATS. Each value is the mean activity (A), or mean specific activity (B), with range, relative to the fresh cell value. * $p<0.05$, {paired t-test). A; P450 content B: BZDM C: EMDM D:PNPH E:EROD

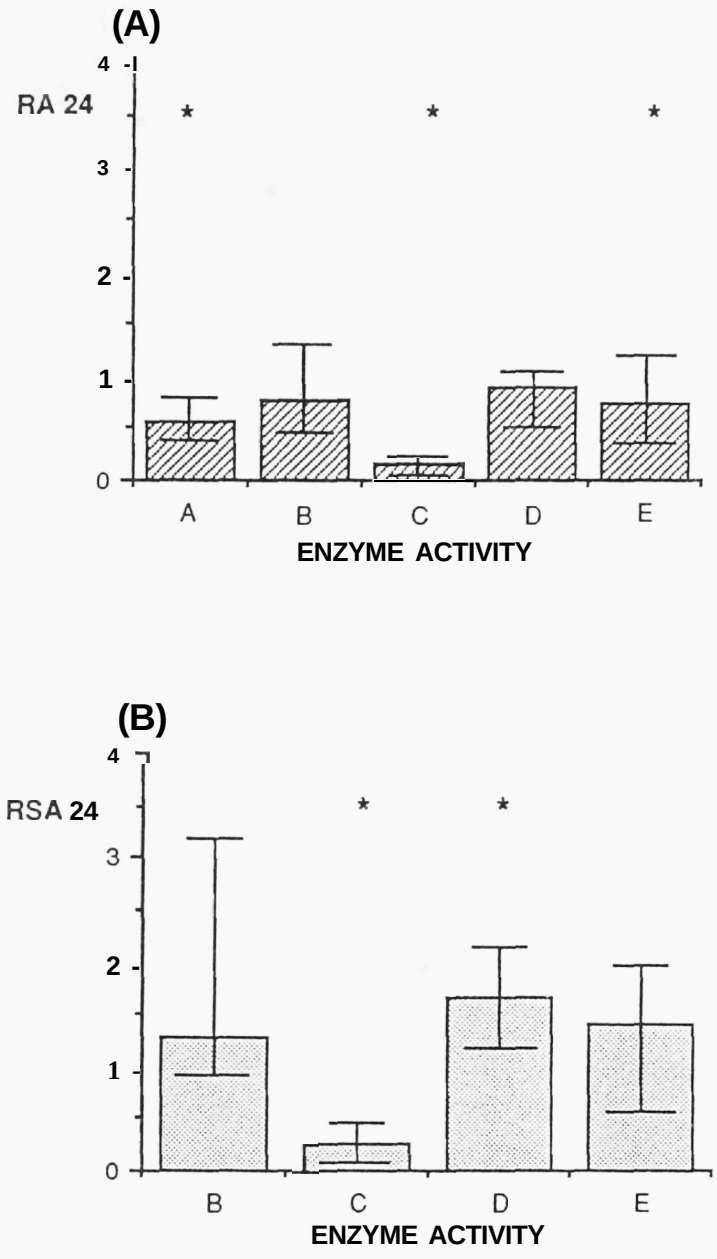


FIGURE 6.6. MAINTENANCE OF P450 AND ENZYME ACTIVITIES AT 24 HOURS IN HEPATOCYTES CULTURED FROM PB-TREATED RATS. Each value is the mean activity (A), or mean specific activity (B), with range, relative to the fresh cell value. * $p<0.05$, (paired t-test).
A: P450 content B: BZDM C: EMDM D:PNPH E:EROD

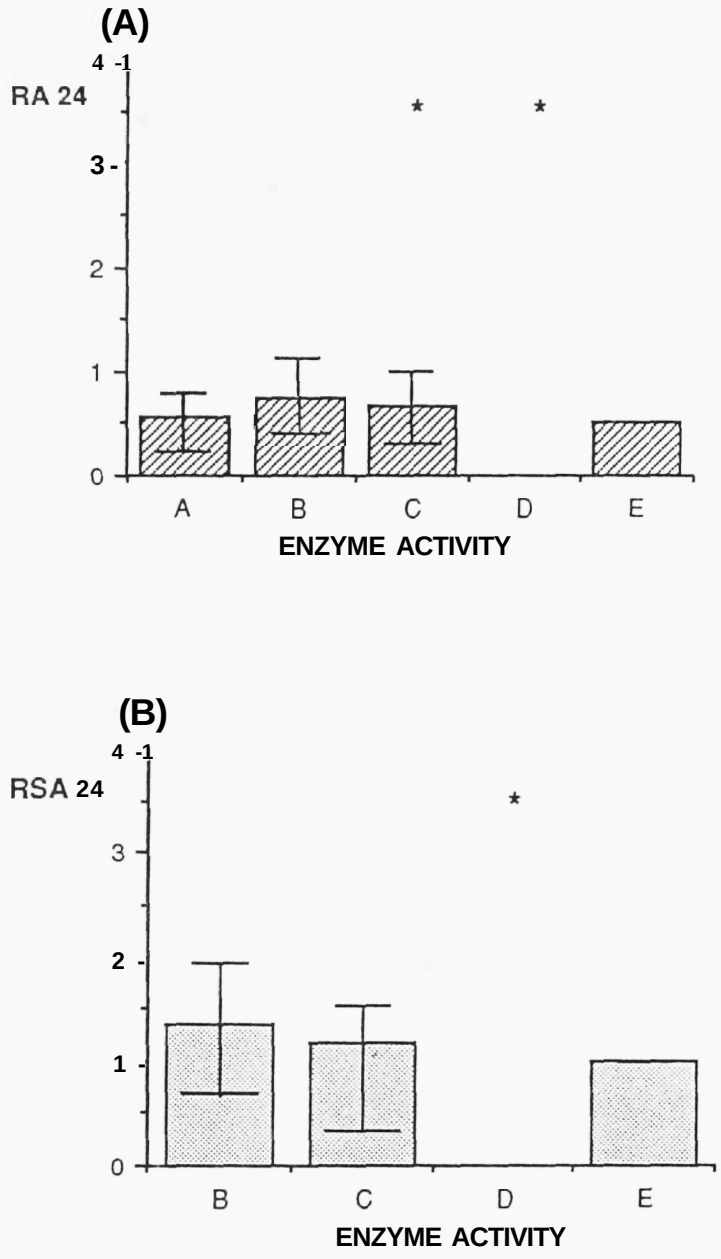
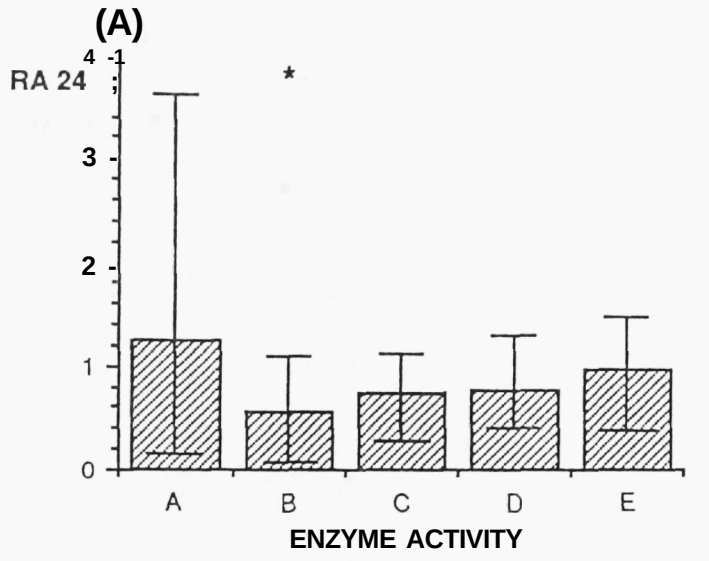


FIGURE 6.7. MAINTENANCE OF P450 AND ENZYME ACTIVITIES AT 24 HOURS IN HEPATOCYTES CULTURED FROM DEX-TREATED RATS. Each value is the mean activity (A), or mean specific activity (B), with range, relative to the fresh cell value. * $p<0.05$, (paired t-test).
A: P450 content B: BZDM C: EMDM D:PNPH E:EROD



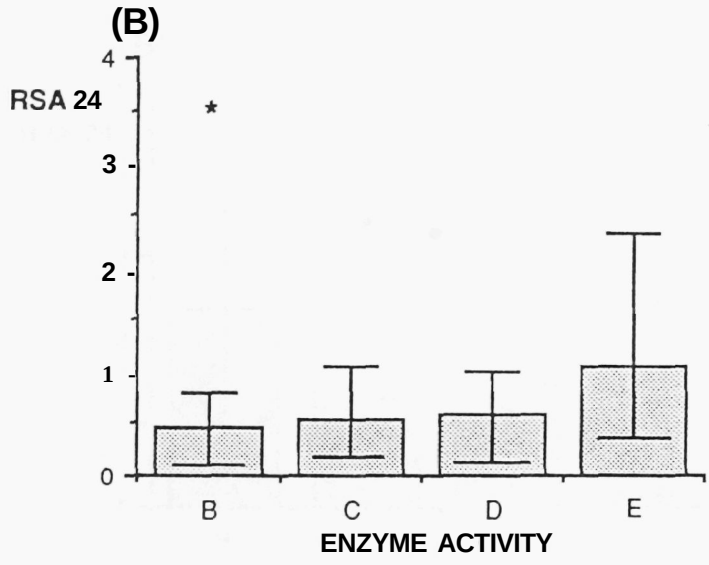
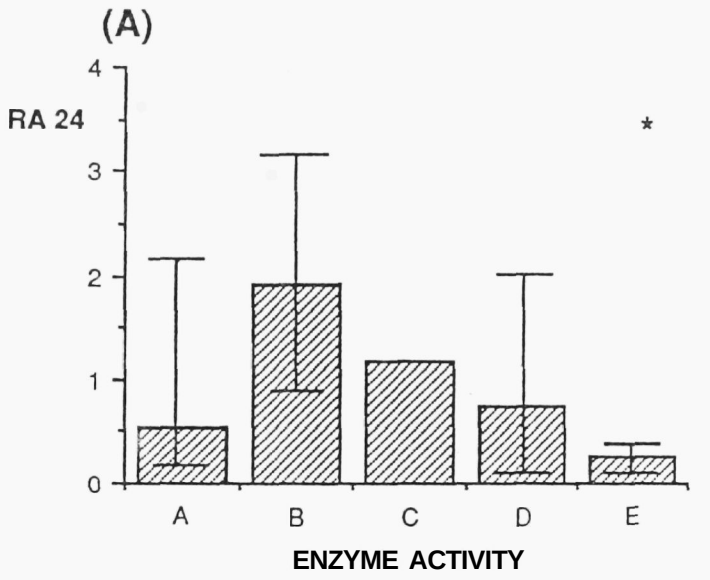


FIGURE 6.8. MAINTENANCE OF P450 AND ENZYME ACTIVITIES AT 24 HOURS IN HEPATOCYTES CULTURED FROM ISO-TREATED RATS. Each value is the mean activity (A), or mean specific activity (B), with range, relative to the fresh cell value. * $p<0.05$, (paired t-test).
A: P450 content **B: BZDM C: EMDM** D:PNPH E:EROD



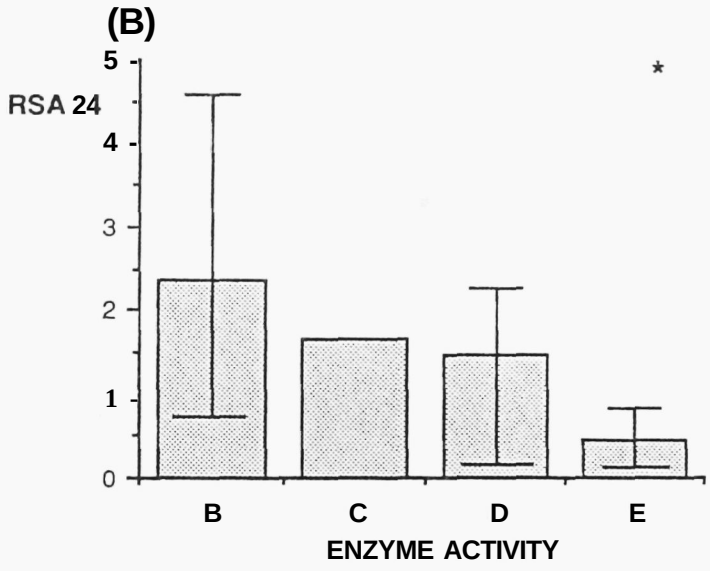


FIGURE 6.9. MAINTENANCE OF P450 AND ENZYME ACTIVITIES AT 24 HOURS IN HEPATOCYTES CULTURED FROM BNF-TREATED RATS. Each value is the mean activity (A), or mean specific activity (B), with range, relative to the fresh cell value. * $p<0.05$, (paired t-test).
A: P450 content B: BZDM C: EMDM D:PNPH E:EROD

CHAPTER 7

TOXICITY STUDIES IN CULTURED HEPATOCYTES

CHAPTER 7

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CHAPTER 7

TOXICITY STUDIES IN CULTURED HEPATOCYTES

7.1 INTRODUCTION

The use of *in vitro* systems for toxicity testing has been reviewed in Chapter One. In recent years, increasing use has been made of hepatocyte cultures in the investigation of mechanisms of toxicity. As outlined in Chapter One, cultured hepatocytes offer several advantages over other cellular and subcellular preparations for this kind of study: they are intact cells containing both Phase I and II biotransformation enzymes that can be maintained in a defined environment free from hormonal and other physiological influences; they survive longer than hepatocyte suspensions and comprise a metabolically stable cell population, also unlike suspensions. There is evidence that freshly isolated cells and hepatocytes that are Initially placed into culture are in a state of metabolic flux, with increased catabolism, but that by 24 hours in culture the cells have recovered from alterations in metabolism sustained during isolation and have reached a more stable state (Tanaka *et al.*, 1978; López *et al.*, 1988). It has also been reported that the toxicity observed when hepatocytes are exposed to a toxic compound during the first 18 hours of culture is greater than when exposure is after 24 hours in culture; there is also greater variability in the observed toxic response (Tolman *et al.*, 1989). This is probably a reflection of a heterogeneous cell population with respect to metabolic stability in addition to changing P450 levels. Therefore, although the P450

content has declined to 50-70% of the fresh level at 24 hours (Chapter Six), in the studies described in this chapter the hepatocytes were cultured for 24 hours prior to exposure to the xenobiotic.

Cultured hepatocytes have been used in several different toxicological investigations. They have been used to assess the role of metabolic activation in the toxicity of xenobiotics (e.g. Acosta et al., 1987); to elucidate the temporal relationships of biochemical alterations (e.g. Long and Moore, 1988; Berger et al., 1989); to compare the pattern of metabolites produced in vivo and in vitro (e.g. McManus et al., 1987), and to investigate carcinogen activation (e.g. Butterworth et al., 1989).

There are also a number of methods available for assessing the toxic effects of xenobiotics. Determination of plasma membrane permeability is often used as an indicator of cell viability, since damaged membranes will allow access/egress of bulky, charged particles. These assays Include trypan blue exclusion, fluorescein diacetate inclusion, ion leakage and enzyme leakage (Tyson and Green, 1987; Cook and Mitchell, 1989). However, membrane damage is not always the Initial lesion and early toxicity can thus be underestimated. Also, loss of membrane integrity is disastrous for the cell and therefore not particularly sensitive. Enzyme leakage assays are particularly vulnerable in this respect due to problems of compartmentalisation, high leakage in control samples and

inactivation of the enzyme by the toxin (Tyson and Green, 1987). Also, leakage does not account for cells that detach from the monolayer (Chao et al., 1988), unless there is some assessment of latency.

Other indicators of toxicity are based on determination of cellular function e.g. DNA or protein synthesis; urea synthesis; ATP content or mitochondrial function (Tyson and Green, 1987; Cook and Mitchell, 1989). However, not all of these are suitable for routine use with large numbers of samples, and although the assay may involve measurement of an intrinsic cellular activity, there may still be wide inter-individual variations in activity. In addition, there are cell proliferation assays that assess colony formation and plating efficiency (Cook and Mitchell, 1989) but these cannot be used in static monolayer cultures.

There *are* also non-specific Indicators of cell injury which can demonstrate a potential for toxicity. These include cellular glutathione content and covalent binding (Tyson and Green, 1987). The depletion of reduced glutathione is not cytotoxic in itself and is usually reversible. However, a sustained depletion suggests that normal cellular regeneration/synthesis mechanisms have been inhibited/overwhelmed. Cell proteins can bind activated molecules, and this covalent binding is used as a measure of toxicity although the exact relationship of cell death to covalent binding remains to be established. It may be that the reactive metabolites bind to

critical cell proteins i.e. enzymes, inhibiting their function.

The above are all used as indicators or measures of cytotoxicity, but do not distinguish *per se* between directly-acting toxins and those that require metabolic activation by P450. The latter can be demonstrated using P450 inducers and inhibitors (Mitchell *et al.*, 1973) and by showing a dependence on the presence of cofactors in microsomal incubations (Garle and Fry, 1989).

In this study, the effect of seven hepatotoxins (thought to be activated to toxic species by cytochrome P450) on hepatocyte viability was determined, using mitochondrial function (MTT reduction) as the parameter of viability (2.5.2). The effect of induction of P450-mediated activity on the toxicity of the compounds was also determined, by culturing hepatocytes from inducer-treated animals, as for the study reported in the previous chapter. A report on acetaminophen toxicity in cultured hepatocytes (Kyle *et al.*, 1989) utilised a similar induction approach. The seven compounds used, with summaries of the available information on their hepatotoxicity are presented in Table 7.1. In Chapter Five it was shown that treatment of hepatocyte cultures with P450 inducers resulted in reduced and altered Induction of enzyme activities, relative to *in vivo* treatment with inducers. This suggested that *in vitro* induction would alter the observed toxicity of xenobiotics to cultured hepatocytes, and so toxicity/viability assessments were made after induction *in vitro* and compared to the results obtained after induction *in vivo*.

7.2 METHODS

7.2.1 *In vivo* Induction, and toxicity

Hepatocytes were isolated from untreated, and PB or BNF-treated young adult male rats and placed into culture. Hepatocytes were cultured on 24-well plates coated with collagen (as described in Chapter 2.7.2) for 24 hours. Cultures were then exposed to WEC containing the test chemical, at various concentrations, for a further 24 hours. The number of viable cells remaining was then determined using the MTT assay (2.5.2). In some experiments an inhibitor of P450-med1ated activity, SKF525-A, was added at the same time as the test chemical.

7.2.2 *In vitro* induction, and toxicity

Hepatocytes were isolated from untreated male rats and cultured on collagen-coated 24-well plates for 72 hours in the presence or absence of inducer (3mM PB or 50μM BNF, as described in Chapter 5.2.2). Exposure to the test chemical was for a further 24 hours followed by assay of viability by MTT.

7.2.3 Addition of compounds and inhibitors

Test chemicals were diluted into WEC from 1M stock solutions in methanol (DMSO for 6-thiopur1ne and SKF525-A). Solvent concentration did not exceed 0.1%. Initial dose response experiments were performed with 0.1-10mM concentrations. However, with the exception of 4-ipomeanol (4-IP) and 2-methylfuran (2-MF),

1mM was found to be the maximum working concentration due to increased acidity of the medium (VPA, 5-TP) or precipitation out of the medium (precocenes, BHT). Plate sealers were used with 2-MF to prevent possible loss due to volatility. However, similar results were obtained with plates incubated with or without the plate sealers, so volatility was probably not an important consideration. In some experiments SKF525-A was added to the cultures; the maximum, non-toxic concentration was found to be 10μM.

7.2.4 Cellular glutathione and toxicity

Cellular glutathione (GSH) was determined (as described in 2.16) in hepatocytes cultured on "Primaria" dishes. GSH content at different time-points after exposure of 24 hour hepatocytes to precocene II (PII) was determined, together with the viability, measured by MTT reduction.

7.2.5 Analysis of results

Dose-response curves for each compound were derived from the viability data. The percentage survival at each dose (or time point) was plotted, and the concentration at which 50% of the cells survived, the ID50, was determined from the curve. The ID50 was determined for each compound in cultures from at least four animals, and the mean $\pm$ range quoted in the results. The range of ID50 values gives an indication of inter-individual variability in response, which is an important consideration in assessing toxicity of xenobiotics.

7.3 RESULTS

Figures 7.1-7.7 show representative dose-response curves for each compound, as determined in cultured hepatocytes from control, PB- and BNF-treated rats. Table 7.2 presents the mean ID50 value with the range for each compound, in each condition.

7.3.1 Toxicity to hepatocytes from control rats

VPA, BHT, PII and PI were all moderately toxic to hepatocytes from untreated rats (Figures 7.1-7.4 respectively) with mean ID50 values between 516-643μM. The ID50 range determined for BHT was very narrow (less than 100μM), with respect to the other three compounds (ID50 ranges around 300μM). Also, it can be seen from the dose response curves that there was a sharp decrease in viability from 80 to 20%, a difference of around 450μM with BHT, whereas with VPA and the precocenes the same loss occurred over a difference of 600-800μM.

6-TP and 2-MF were less toxic to control hepatocytes (Figures 7.5 and 7.6), the viability at 1mM being around 40%, the mean ID50 values being 720 and 800μM. 4-IP was toxic to control hepatocytes only at high concentrations (ID50 3.4mM).

7.3.2 Toxicity to cultures from inducer-treated rats

The seven compounds fall into three groups with respect to the effects of induction on toxicity.

BHT and VPA

PB treatment enhanced the toxicity of VPA and BHT to cultured hepatocytes (Figures 7.1 and 7.2), the mean ID50 values being 232 and 106μM respectively, with similar ID50 ranges of around 200μM (Table 7.2). However, the loss of viability from 80 to 20% is much steeper with BHT, a difference of only 150μM, as opposed to around 900μM with VPA. BNF did not significantly alter the toxicity of either compound (as judged by ID50) relative to the control cultures, the dose-response curves being *very* similar in the hepatocytes from control and BNF-treated rats.

Precocenes I and II

Both PB and BNF treatment induced the toxicity of the precocenes to cultured hepatocytes, PB having the most marked effect (Figures 7.3 and 7.4, Table 7.2). BNF produced a five-fold and PB a 14-fold increase in toxicity of precocene II and a 29-fold Increase of precocene I toxicity, with similar ID50 ranges.

6-TP, 2-MF and 4-IP

These three compounds, that had minimal toxicity in control cultures, were all toxic to cultures from PB- and BNF-treated animals. 6-TP and 2-MF were the most toxic to cultures from PB-treated rats (Figures 7.5 and 7.6) with 10- and 25-fold Increases in response respectively. BNF and PB had similar effects on 4-IP toxicity: 13 to 14-fold Increases with ID50 values around 250μM.

Table 7.3 summarises these results. Induction of P450 had the least effect on VPA toxicity; BHT toxicity was potentiated by PB, which markedly increased the toxicities of the precocenes, 2-MF, 4-IP and 6-TP. BNF had the greatest effect on 4-IP toxicity with moderate effects on 2-MF, 6-TP and the precocenes.

7.3.3 Variability in observed toxicity

In addition to the wide range of ID50 values obtained for VPA, 6-TP and 2-MF, there was also variability in response of the cells to these compounds, in that concentrations up to 1mM did not always elicit a sufficient loss of viability for an ID50 to be determined. In the control cultures this may be simply because the compounds are only moderately toxic, the upper range of the ID50 being around 1mM. However, this variability (i.e. toxic or not toxic) was also observed in hepatocytes from inducer-treated animals. The practical considerations that could be responsible include instability of the compound/stock solution, volatility of 2-MF and intrinsic inter-animal differences. Use of fresh dilutions of fresh stock solutions had no effect on the observed variability, and the plate sealers used with 2-MF were judged to function adequately since the gradation of any effect was uniform across the plate. In any case, volatility was probably not crucial here, since incubation without plate sealers gave similar results to sealed plates. It has already been observed that there are inter-individual differences in response to Inducers (Chapter Five), and this can affect the extent of any toxicity that is due to metabolism. For

this reason it is Important to use hepatocytes from different animals, and not replicate dishes from single animals, in assessing the extent of a compound's effects. However, VPA, 6-TP and 2-MF are all more markedly toxic to Induced animals, and a complete lack of effect in some cultures is somewhat unexpected, and is unlikely to be due to poor induction, in which case a reduced effect would be more probable. Therefore the possible roles of other intrinsic factors have to be considered.

7.3.4 Induction *in vitro* and toxicity

Table 7.4 compares ID50 data from the *in vivo* induction experiments with those observed after induction *in vitro*, for PII and 4-IP. These compounds were chosen since there were marked (and easily reproducible) changes in toxicity on induction *in vivo*. However, after treatment of hepatocytes *in vitro* with PB and BNF there was no toxicity of 4-IP following PB, and only a slight increase due to BNF. PB and BNF *in vitro* had similar effects on PII toxicity (mean ID50 values of 463 and 459μM respectively), but this response was much reduced compared to that obtained with induction *in vivo*. Also, no toxicity of PII to control hepatocytes was observed. This is probably due to the decline in P450 over the 72 hour culture period, and suggests that the toxicity to 24 hour hepatocytes is (in part) P-450 mediated. This failure to Induce toxicity *in vitro* is at least consistent with the failure to Induce P450 enzyme activities *in vitro* (Chapter Five) and the observed decline in some P450 activities in 72 hour cultures (Chapter Four).

7.3.5 Further studies with the precocenes

Figures 7.8 and 7.9 show the effects of SKF 525-A *in vitro* upon precocene-induced toxicity in hepatocytes from control and PB-treated rats. SKF 525-A did not inhibit precocene II toxicity in control cultures, although there was a slight shift in the precocene I ID50 range: Table 7.5. However, in hepatocytes from PB-treated rats there was a marked inhibition of PII toxicity (a 4-fold Increase in ID50 - Table 7.5), but not of PI toxicity. This was rather unusual, in that the reported effects of these two compounds are *very* similar. A further preliminary experiment showed that exposure of hepatocytes cultured from PB-treated animals to PII resulted in a depletion of cellular glutathione that was sustained over 24 hours, and which preceded the loss of cellular viability, as judged by MTT reduction (Figure 7.10).

7.4 DISCUSSION

The results presented in this chapter demonstrate that induction of P450 *in vivo* can potentiate the toxicity of compounds in cultured hepatocytes. A certain amount of Information on the extent and degree of toxicity can be determined from the data.

BHT was moderately toxic to hepatocytes from control and BNF-treated rats and PB significantly increased the toxicity. In all cases, the dose-response curves fell steeply between 80 ands 20% viability. This suggests that BHT may have one or a few *very* specific sites of toxic assault within the cell, and that once a critical

concentration is achieved cell death occurs rapidly. It has been reported that the cytotoxicity of BHT in control hepatocytes at concentrations up to 750μM is not P450-mediated, but a direct effect upon the mitochondria. There is a rapid loss of ATP, followed by dissipation of the membrane potential (Thompson and Moldéus, 1988). This would account for the narrow ID50 range in controls and the steep dose-response curve (since mitochondrial function is vital for cell survival). It would also Indicate that BHT toxicity has a direct effect in hepatocytes on induction with BNF. PB increased BHT toxicity in hepatocytes. This agrees with reported data on BHT: that PB, but not 3-MC, increased the formation of a microsomal BHT-GSH conjugate (Tajima et al., 1985) and that PB increased serum transaminase levels in vivo, in BHT-fed rats (Nakagawa, 1987).

VPA, like BHT, was also toxic to hepatocytes from PB-treated rats, the toxicity in hepatocytes from control and BNF-treated rats being *very* similar. However, in contrast to BHT, the VPA dose-response curves were much flatter, the toxic effects being manifest over a much wider range of concentrations. This probably reflects more variability in inter-cellular response, and/or more than one site of action of VPA. It is thought that there are two major, competing pathways of VPA metabolism in the hepatocyte. VPA is metabolised by P450 to an unsaturated metabolite, Δ^4-VPA, and this reaction 1s Increased by PB in vitro (Rettie et al., 1988). A^4-VPA can exist as 2 enantiomers: the (R) form is metabolised further by P450 to 4,5-dihydroxy-VPA-γ-lactone, and the (S) form is metabolised by

mitochondrial /3-oxidation to a 3-oxo derivative which can then inhibit i3-ox1dation, production of the (R) enantiomer being favoured (Porubek et al., 1989a). The Inhibition of /3-oxidation would eventually be toxic to the hepatocyte, and would explain the observed steatosis in VPA-treated rats (Kesterson et al., 1984). VPA and Δ^4-VPA have both been reported to be hepatotoxic, but the data are confusing. PB-treatment increases the overall rate of metabolism but not the ratio of enantiomer production (Porubek et al., 1989a); PB does not Increase VPA or A^4-VPA covalent binding in hepatocytes, although clofibrate, an Inducer of fatty acid oxidation, does (Porubek et al., 1989b); the reported toxicity of VPA and Δ^4-VPA to hepatocyte cultures is unconvincing on critical evaluation of the presented data (Kingsley et al., 1983). Thus, it appears that the P450 system is not solely responsible for VPA toxicity (and may also be Involved in detoxification reactions) and overall, it is probably the balance of the effects of VPA metabolites produced by /3-oxidation and P450 that determine the toxicity, and that this dual effect accounts for the broad concentration range of the toxic effects and the resistance of some animals/cell cultures to VPA. In any case, the mixed in vitro data obtained with VPA mirrors the in vitro situation with respect to incidence of VPA toxicity and severity of effect, which would also be consistent with the idea that some individuals may be physiologically more prone to VPA toxicity, especially in conjunction with other drugs.

6-TP also gave some conflicting results. Both PB and BNF increased 6-TP toxicity, although toxicity was not always observed. PB (but not 3-MC) has been reported to Increase the covalent binding of 6-TP to microsomal protein, although PB treatment decreases 6-TP covalent binding in vivo (Hyslop and Jardine, 1981a; 1981b). The authors suggest that this discrepancy is due to PB induction of conjugation enzymes, which results in Increased detoxification in vivo, which are absent in microsomal preparations. If this is correct, then it implies that the Phase II reactions are inhibited/less active in hepatocyte cultures. It has been reported that UDP-glucuronyl transferases and some of the glutathione transferases are reasonably stable over 24-72 hours of hepatocyte culture, although there was a rapid depletion of UDP-glucuronic acid (UDPGA) during isolation (Croci and Williams, 1985; Grant and Hawksworth, 1986). This may account for the in vivo/in vitro discrepancy in PB Induction of 6-TP hepatotoxicity with regard to the variability in the 6-TP results. It is also possible that P450 is not the only enzyme system Involved in 6-TP metabolism. A major route of 6-TP transformation is via xanthine oxidase to thiouric acid; however, a xanthine oxidase inhibitor, allopurinol, did not inhibit the covalent binding of 6-TP (Hyslop and Jardine, 1981a). Also, 6-TP contains several nitrogen atoms and a sulphur group, which would make it a good substrate for the flavin monoxygenase system (FMO), which predominantly forms N,S and P-oxides (Gibson and Skett, 1986). The FMO appears to be fairly stable in hepatocyte cultures (Sherratt and Damani, 1989), but compared to the MMO, little work has been performed on the role

of the FMO in drug metabolism and this makes it difficult to assess the relative contributions of the two systems.

4-IP and 2-MF both contain a furan group, which has been implicated in 4-IP toxicity (Boyd *et al.*, 1983). Both PB and BNF Increased the toxicity of these furans to cultured hepatocytes. 4-IP is analogous to 6-TP in that PB increases the covalent binding of 4-IP in microsomes, but *in vivo* it is reduced (Boyd and Burka, 1978; Boyd *et al.*, 1978). Statham and Boyd (1982) reported the increased urinary excretion of a 4-IP-glucuronide on PB induction *in vivo*, and therefore, as suggested above, it may be a depletion of UDPGA in cultured hepatocytes that accounts for the discrepancies in the *in vivo* and *in vitro* data. The increased toxicity *in vitro* of 4-IP on induction with BNF is consistent with the *in vivo* data. PB has been shown to increase the covalent binding of 2-MF in microsomes, whereas 3-MC did not (Ravindranath *et al.*, 1985). This does not agree with the results presented here although 2-MF has been shown to deplete GSH in microsomes from BNF-treated rats (Garle and Fry, 1989).

Both of the precocenes were toxic in control hepatocytes, induction markedly increasing their toxicity. The bigger increase in toxicity was with PB. Precocene I has been shown to deplete GSH *in vivo* and lead to an increase in serum transaminase levels (Halpin *et al.*, 1984; Ravindranath *et al.*, 1987). Both precocenes can deplete GSH, 80-90% of the dose being rapidly conjugated with GSH

(Foureman *et al.*, 1989). PB has been shown to Increase, and SKF525-A to decrease, covalent binding of PII (Hsia *et al.*, 1981). This agrees with the data presented here, that SKF525-A can inhibit PII toxicity, and that a sustained depletion of GSH precedes loss of cellular viability. That GSH depletion and loss of viability do not occur concurrently, suggests that GSH depletion is one of the first steps in the pathology of PII toxicity. Depletion of GSH is not in Itself toxic; the inability of the cell to synthesize/regenerate GSH however would be more serious. Maintenance of GSH levels is linked to GSSG, NADPH, NADH and ATP levels. It would appear that whilst a toxic dose produces a sustained GSH depletion, a non-toxic dose results in a transient depletion, which suggests that GSH depletion is an early reversible event, not the cause of toxicity, and that some other biochemical change can precipitate a sustained loss of GSH e.g. NADPH depletion, inhibition of glutathione reductase.

Overall, the approach of *in vivo* Induction followed by hepatocyte culture was successful in this study in detection of hepatotoxicity, the results agreeing or complementing the *in vivo* and the microsomal data. P450 Induction has been shown to increase toxicity in culture, and inhibition of P450 was also demonstrated. This method of investigating the role of P450-mediated toxicity of xenobiotics is probably more applicable to interaction and mechanism studies rather than to screening of compounds of unknown toxicity, since at present the system needs constant evaluation with respect to the toxicity *invivo*.

TABLES AND FIGURES

TABLE 7.1. REPORTED TOXIC EFFECTS OF THE COMPOUNDS USED IN THIS STUDY[1].

VALPROATE (VPA) anticonvulsant

in vivo steatosis ± necrosis; inhibition of mitochondrial ß-oxidation[2]; increased covalent binding[3].

in vitro PB increases rate of metabolism; covalent binding of VPA and A[4]-VPA to protein in cultured hepatocytes[4].

BUTYLATED HYDROXYTOLUENE (BHT) antioxidant

in vivo necrosis; PB increases serum transaminase levels[5].

in vitro PB increases formation of BHT-GSH conjugate;conjugate formation inhibited by CO,metyrapone and SKF 525-A[6].

PRECOCENE II (PII) insecticide, from *Ageratum houstonianum*

in vivo centrilobular necrosis;

in vitro PB increases covalent binding; SKF 525-A inhibits covalent binding[7].

PRECOCENE I (PI) insecticide, from *A. houstonianum*

in VIVO necrosis; GSH depletion; increases in serum transaminase levels[8].

in vitro PB increases rate of metabolism[9].

6-THIOPURINE (6-TP) antineoplastic and immunosuppressive

in vivo PB increases the covalent binding[10].

in vitro PB increases, GSH and SKF 525-A inhibit, covalent binding[11].

2-METHYLFURAN {2-MF) constituent of coffee and cigarette smoke

in vitro PB increases, piperonyl butoxide inhibits, covalent binding; metabolism to acetylacrolein[12].

4-IPOMEANOL (4-IP) from *Ipomea batatas* infected with *Fusarium solanae*

in vivo PB decreases covalent binding and lethality[13];

in vitro PB increases the covalent binding[14].

1 *in vivo:* rat liver;*in vitro:*rat hepatic microsomes, unless stated.

2 Kesterson *et al.,*1984

3 Porubek *et al.,* 1989a

4 Porubek *et a*/., 1989b

5 Nakagawa,1987

6 Tajima *et al.,* 1985

7 Hsia *et a*/.,1981

8 Ravindranath et *al.,*1987

9 Halpin ef *al.,*1984

10 Hyslop and Jardine, 1981b

11 Hyslop and Jardine, 1981a

12 Ravindranath and Boyd, 1985

13 Boyd and Burka, 1978

14 Boyd et *a*/., 1978

TABLE 7.2. EFFECT OF INDUCTION *IN VIVO* ON TOXICITY *IN VITRO.*

	mean ID_{50} value with range (μM)		
compound	CON	PB	BNF
VPA	643 447-708	232 100-281*	704 501-1000
BHT	516 468-537	106 33-224*	447 422-473
PII	550 437-794	40 16-60*	124 56-300*
PI	546 447-708	19 7.5-38*	114 56-168*
6-TP	720 501-794	76 10-251*	134 30-141*
2-MF	800 668-944	32 10-45*	84 10-211*
4-IP	3400 1400-6300	243 178-316*	259 96-335*

Values are mean ID_{50}s, with ranges, determined from dose-response curves for 4-7 animals.

* significantly different to control, Mann-Whitney U-test, $p < 0.05$.

TABLE 7.3. SUMMARY TABLE OF THE EFFECTS OF INDUCTION *IN VIVO* ON TOXICITY *IN VITRO.*

compound	CONTROL[1]	PB[2]	BNF[2]
VPA	+	3	0.9
BHT	+	5	1.2
PII	+	14	5.0
PI	+	29	5.0
6-TP	-	10	5.0
2-MF	-	25	9.0
4-IP	-	13	14

1 indicates toxic (+) or non-toxic (-) to control cells.

2 fold-induction of toxicity over control (control value = 1.0). Values greater than 1.0 represent an induction of toxicity, which is a decrease in ID_{50}.

TABLE 7.4. THE EFFECTS OF *IN VIVO* AND *IN VITRO* INDUCTION ON THE TOXICITY OF PRECOCENE II AND 4-IPOMEANOL TO CULTURED RAT HEPATOCYTES.

condition	mean ID_{50} and range (μM) *in vivo*	*in vitro*
4-IPOMEANOL		
CON	3400 1400-6300	>1000
PB	243 178-316	>1000
BNF	259 96-335	848 708-944
PRECOCENE II		
CON	550 237-794	>1000
PB	40 16-60	463 355-562
BNF	124 56-300	459 300-562

Each value is the mean ID_{50} and range of 3-5 animals.

TABLE 7.5. EFFECT OF SKF 525-A ON THE TOXICITY OF PRECOCENE I AND II IN HEPATOCYTES FROM CONTROL AND PB-TREATED RATS.

condition		mean ID_{50} and range (μM) -SKF 525-A	+SKF 525-A
PI	CON	546 447-708	767 708-841
	PB	39 4-71	69 56-89
PII	CON	550 237-794	701 525-708
	PB	57 33-75	234 178-300 *

* range is significantly different to control, $p<0.05$ Mann-Whitney U-test.The values are the mean ID_{50} values determined for 4 animals,the toxicity in the presence and absence of inhibitor being determined in cells from each animal.

FIGURES 7.1-7.7.Hepatocytes were isolated from control and inducer-treated animals and maintained in culture for 24 hours prior to exposure to the test compound. In the following figures the data presented are from individual, but representative rats. The mean ID_{50} values are shown in Table 7.2.

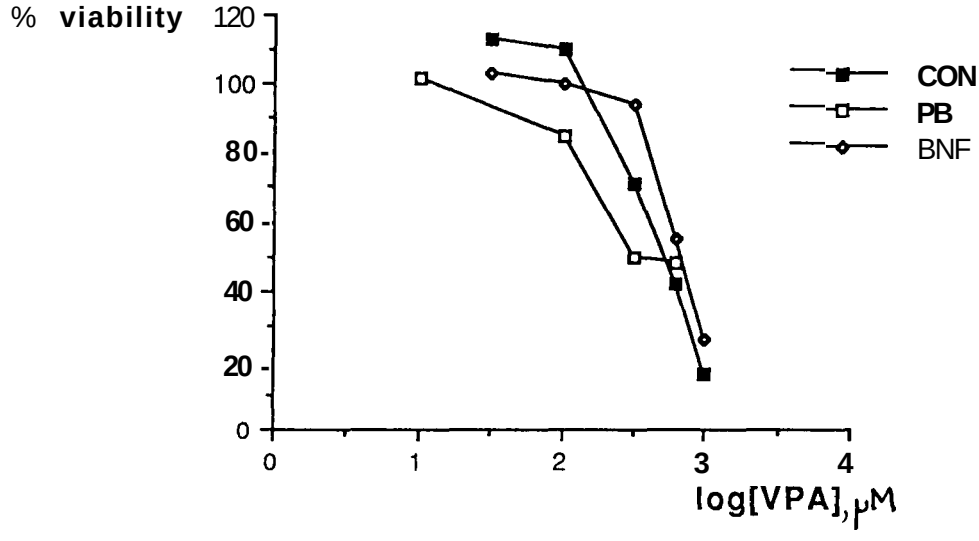

FIGURE 7.1. REPRESENTATIVE DOSE-RESPONSE CURVES FOR VALPROIC ACID.

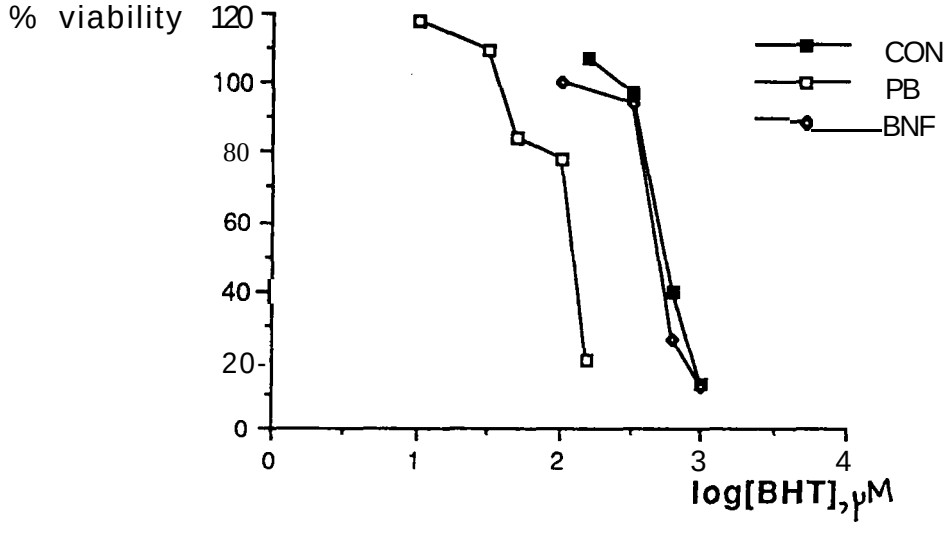

FIGURE 7.2. REPRESENTATIVE DOSE-RESPONSE CURVES FOR BUTYLATED HYDROXYTOLUENE.

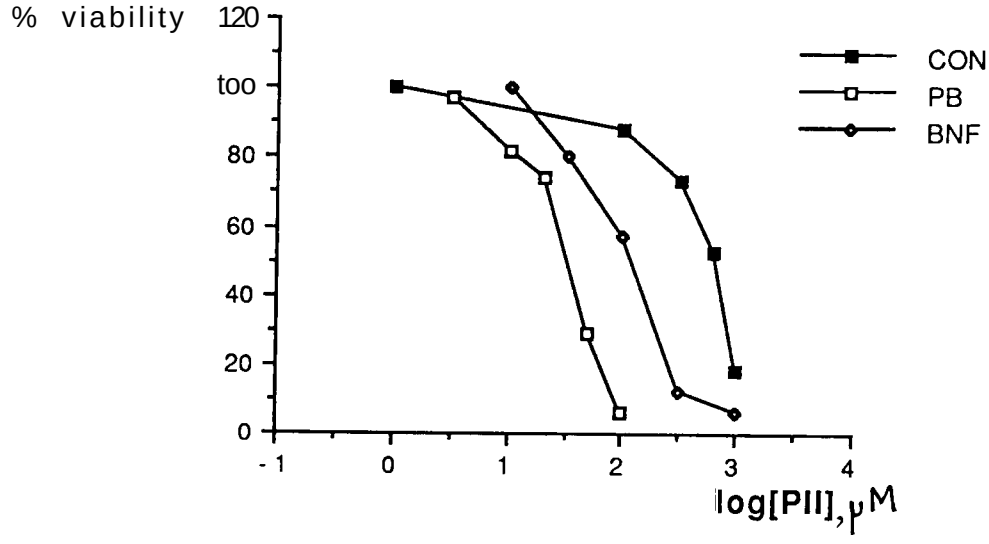

FIGURE 7.3. REPRESENTATIVE DOSE-RESPONSE CURVES FOR PRECOCENE II.

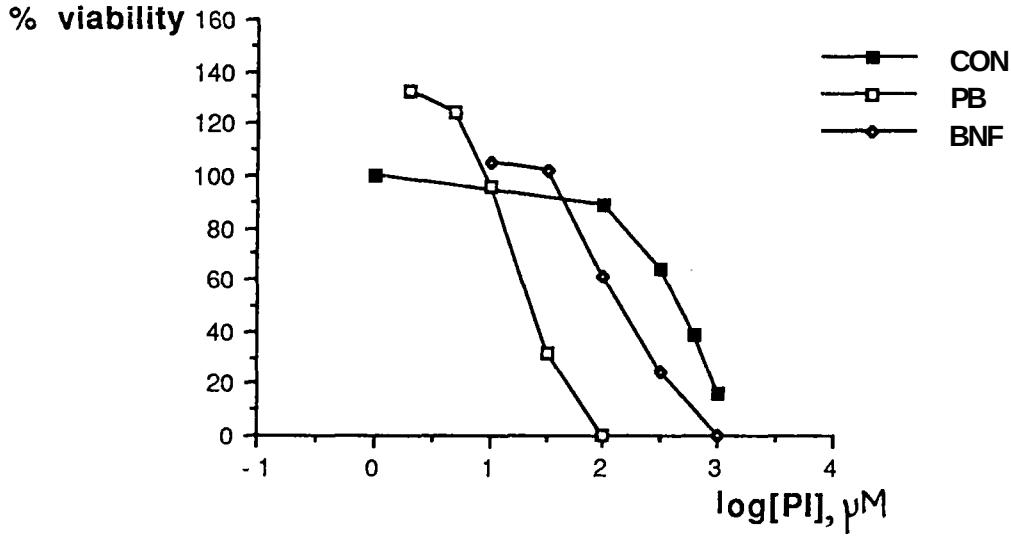

FIGURE 7.4. REPRESENTATIVE DOSE-RESPONSE CURVES FOR PRECOCENE I.

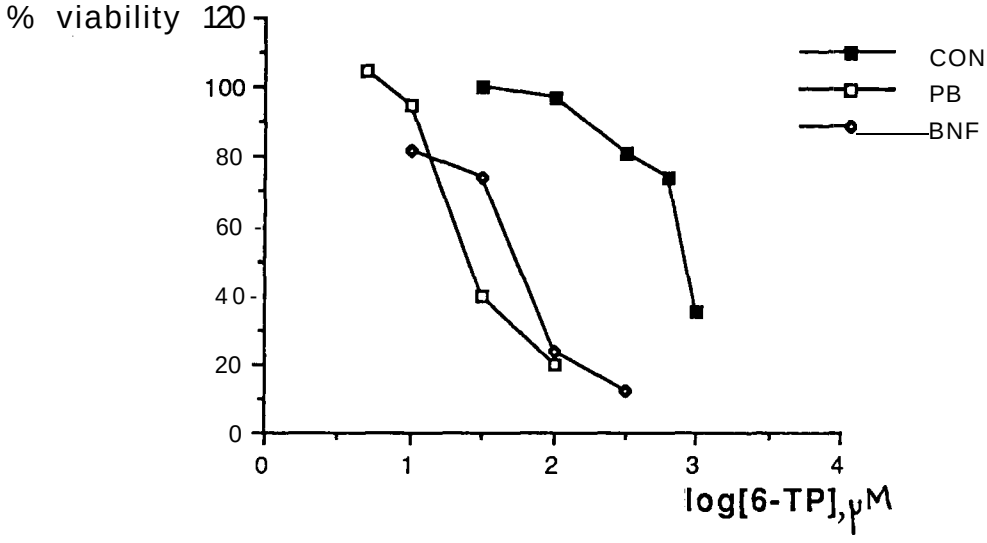

FIGURE 7.5. REPRESENTATIVE DOSE-RESPONSE CURVES FOR 6-THIOPURINE.

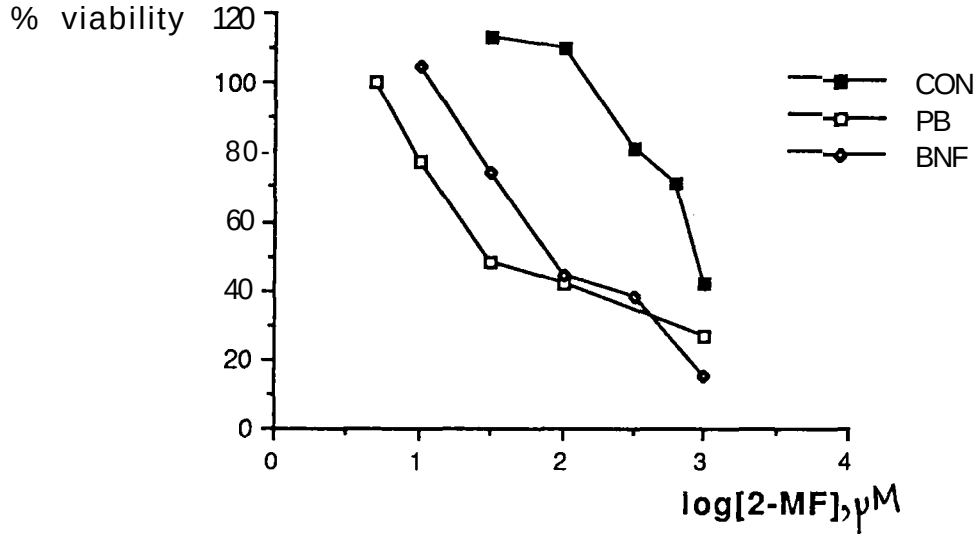

FIGURE 7.6. REPRESENTATIVE DOSE-RESPONSE CURVES FOR 2-METHYLFURAN.

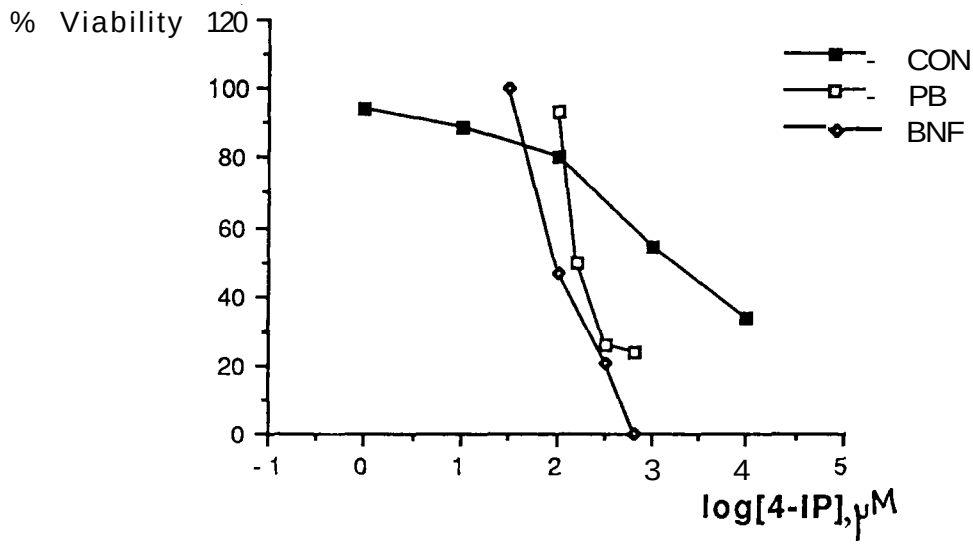

FIGURE 7.7. REPRESENTATIVE DOSE-RESPONSE CURVES FOR 4-IPOMEANOL.

FIGURES 7.8 AND 7.9. Hepatocytes were cultured from control and PB-treated rats. After 24 hours the cells were exposed to PII ± SKF-525A (10μM). The curves shown are from individual, but representative rats. Mean ID_{50} values are presented in Table 7.5.

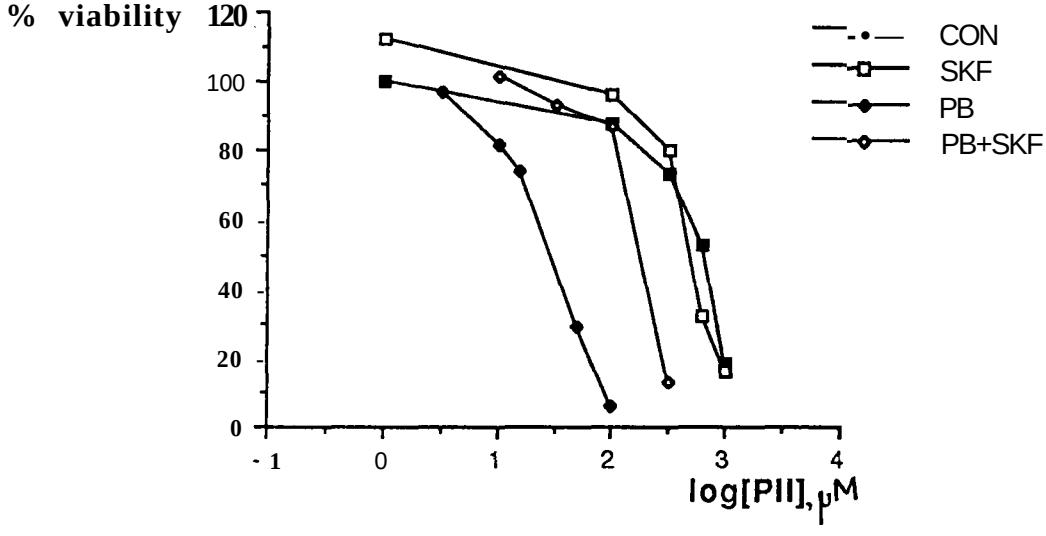

FIGURE 7.8. EFFECT OF SKF-525A ON THE TOXICITY OF PRECOCENE II TO CULTURED HEPATOCYTES.

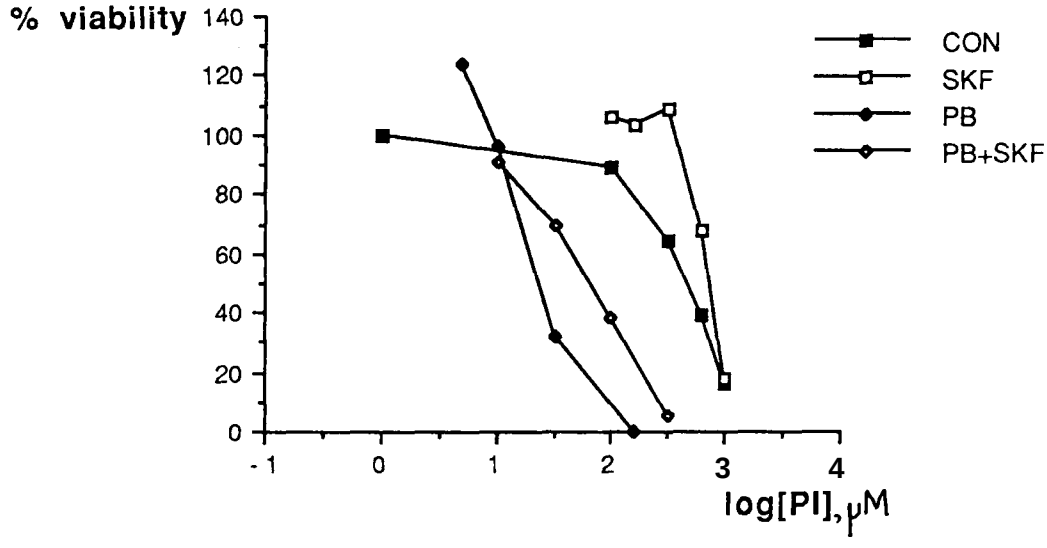

FIGURE 7.9. EFFECT OF SKF-525A ON THE TOXICITY OF PRECOCENE I TO CULTURED HEPATOCYTES.

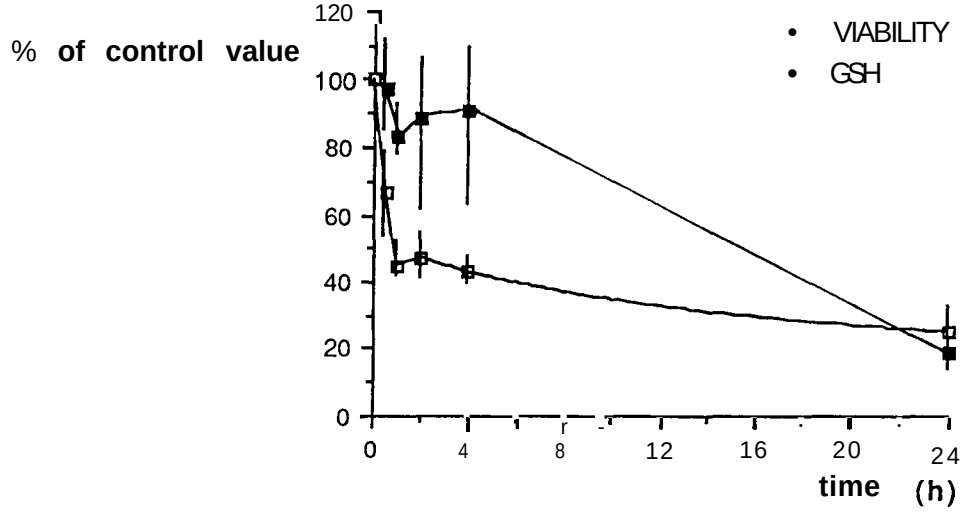

FIGURE 7.10. TIME COURSE OF GLUTATHIONE DEPLETION AND LOSS OF VIABILITY ON EXPOSURE TO PRECOCENE II. Hepatocytes were cultured from PB-treated rats and exposed to 100μM precocene II. Values are mean ±range of 4 animals.

CHAPTER 8

DISCUSSION

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GENERAL DISCUSSION

The aims of this Investigation were to develop and characterise a system for the use of rat cultured hepatocytes in metabolism-mediated toxicity studies. This necessitates conditions in which constitutive P450 levels/activities can be maintained, and in which the in vivo induction of P450 isozymes can be reproduced. Initial studies were performed on the kinetics of alkoxycoumarin O-dealkylase activities to confirm the biphasic nature of these activities and to determine their maintenance in cultured hepatocytes. These enzymes were chosen as indicators of P450 activity because they are biphasic, the separate components representing different populations of P450 isozymes (Chapter 2.10). The results of these preliminary experiments indicated that although both MCOD and ECOD activities were biphasic in freshly isolated hepatocytes, MCOD activity became monophasic in hepatocytes cultured for 24 hours, with complete loss of the high affinity form; the maintenance of the other activities was poor: 55% or less of the fresh cell activities. The medium used was Williams' Medium E, developed specially for hepatocyte culture (Williams and Gunn, 1974), supplemented further with insulin, dexamethasone and nicotinamide. This medium (WEC) has been shown to attenuate the loss of P450 activities in primary culture (Warren and Fry, 1985).

The studies comprising this investigation were concerned initially with improving the maintenance of P450 activities: the effects of

further supplementation of culture medium and the influence of the age and sex of the hepatocyte donor animal were determined. Subsequent experiments compared induction *in vitro* to that seen *in vivo*, and the maintenance of induced enzyme activities was also ascertained. Finally, the metabolic activation of seven known hepatotoxins was investigated in the rat hepatocyte cultures, under the previously defined conditions.

8.1 AGE AND SEX OF HEPATOCYTE DONOR: P450-DEPENDENT ACTIVITIES AND MAINTENANCE

It was suggested in Chapter Three that the maintenance of three of the four alkoxycoumarin O-dealkylase activities at 70% or more of their fresh activities (in contrast to the results of the initial kinetic experiments, in which none of the activities were maintained) was due to the increased age of the donor animals. The results presented in Chapter Four confirmed this suggestion.

There were differences in fresh cell activities between immature and adult male rats and between male and female rats, with respect to MCOD and PCOD activities, indicating that there are developmental/gender differences in the P450-isozyme complement and/or the regulation of these activities. The low affinity form of PCOD activity, which is absent in adult female rats, appears to correspond to the male-specific, steroid 6β-hydroxylase activity, with total MCOD activity displaying an adult male-specific component (Chapter Four). It is thought that these age and sex differences in P450 metabolism are due to the effects of hormones, especially

growth hormone (and possibly the thyroid hormones), on the hepatocyte. These differential hormonal effects may also be linked with the lower P450-reductase levels in female rats, which Influences the rate of metabolism (Skett, 1988; Waxman et al., 1989).

The differences in metabolism extend to maintenance in primary culture. All the activities measured were maintained at higher levels in hepatocytes cultured for 24 hours from adult male rats with all 3 alkoxycoumarin O-dealkylase activities remaining biphasic in culture. Overall, this selectivity for adult male rats was lost by 72 hours in culture; only the high affinity forms of ECOD and PCOD were maintained at this time point. This is consistent with the proposal that P450 activities are maintained in hepatocytes from adult males because of the reported predominance of slow turnover P450s, compared to females and Immature males (Levin et al., 1975). It was also observed that the least well maintained activities were those for which developmental regulation, and therefore hormonal regulation, was indicated, which implies that these P450s are the rapid turnover forms described previously (Chapter 4.4).

Thus, although a range of P450-dependent activities were maintained better in hepatocytes from adult male rats, the differences in hepatic P450 metabolism and maintenance due to age and gender demonstrated here are important per se, not only in making inter-laboratory data comparisons but also in assessing inter-individual susceptibility to iatrogenic hepatic injury. It

is often assumed that the observed sex differences in drug metabolism in the laboratory rat have little relevance to Man, where sex differences are less marked. It is possible though that it is just more difficult to demonstrate any differences in Man, due to the Influences of many varied exogenous factors. However, it is well-known that neonates and young children (as well as the elderly) are more susceptible to drug-induced toxicity than adults (Hurwitz, 1969; Mirkin, 1970).

8.2 DIFFERENTIAL MAINTENANCE OF P450 ACTIVITIES IN CULTURE

As described above, there was a selective loss of P450-dependent O-dealkylase activities in hepatocytes cultured for 24 hours. In Chapter Six it was shown that this selectivity in maintenance extends to other enzyme activities. In control cultures the total P450 fell by 31% but EMDM and PNPH activities were well maintained over 24 hours, and EROD activity declined slightly. However, there was a 54% loss of BZDM activity. In this respect BZDM is similar to total MCOD and PCOD activities in that it is poorly maintained in culture. Induced enzyme activities, however, were well maintained for 24 hours.

It was suggested in the preceding section, and in Chapter Four, that it is the developmentally/hormonally regulated P450s that are the most susceptible to decline in culture, possibly because they are fast turnover forms of P450. It was also suggested that the loss of these forms in culture could be a result of suppression of

synthesis due to the presence of hormones in the serum that is added to the medium (Chapter 4.4). Foetal calf serum contains significant, but also variable amounts, of growth hormone and thyroid hormones (and also androgens and oestrogens) which are known to have important regulatory effects on hepatic P450, e.g. growth hormone and tri-iodothyronine have both been shown to suppress the male-specific 6β-hydroxylase (Yamazoe et al., 1989). Other workers have reported the expression of liver-specific P450s in vitro, only in medium lacking serum e.g. P450IA2 (Silver and Krauter, 1988). The presence of serum could effectively 'feminise' the P450 metabolism of the hepatocytes, in a manner similar to that seen with growth hormone supplementation (Guzelian et al., 1988). Serum is routinely Incorporated into cell culture medium since most cell types, including hepatocytes do not attach and/or grow well without it. However, it now appears that 'blanket' addition of hormones to culture medium, in the form of serum, may be responsible for at least some of the alterations in phenotype observed on placing cells into culture. In order to maintain constitutive expression in vitro it may be necessary to replace serum-containing media with hormonally-defined media. The development of appropriate media will be facilitated by further research into the hormonal regulation of the different P450 isozymes.

8.3 EFFECT OF EXOGENOUS HAEM ON MAINTENANCE OF P450

Addition of exogenous haem, ALA and Se did not affect the maintenance of alkoxycoumarin O-dealkylase activities in primary

culture (Chapter Three). Engelmann et al. (1985) reported a maintenance of total P450 in medium supplemented with haem, ALA and Se, but they did not measure any P450-dependent activities. In their unsupplemented cultures, the P450 content fell to 75% of the fresh cell value, equivalent to the P450 level reported in Chapter Six of this study (70% of Initial levels in control cultures at 24 hours). However, although the effect of haem-supplemented medium on total P450 levels was not determined in Chapter Three, it did not prevent the selective loss of MCOD activity in culture. This suggests that exogenous haem may boost total P450 levels, but that this masks alterations in the isozyme population.

Engelmann et al. also reported that haem, ALA and particularly Se were essential for induction of P450 by PB in vitro. Again, they measured total P450 content. In an experiment reported in Chapter Five, no induction of total MCOD activity by PB was observed in vitro, in medium containing haem, ALA and Se, whereas this activity is induced 6-fold by PB in vivo. The Induction of total P450 by PB in vitro was not measured. Therefore, it seems that although haem, ALA and Se may increase PB induction of total P450 in vitro, this is not necessarily an Increase in PB-specific isozymes. This also Infers that determination of total P450 levels, in the absence of any activity and/or immunochemical data, is not particularly informative. The results presented in Chapters Three and Five suggest that a reduced haem supply does not contribute to the selective loss of P450 activity and Inducibility seen in primary culture.

8.4 INDUCTION OF P450: METABOLISM AND TOXICITY

a. Induction *in vivo*

In Chapters Five, Six and Seven the induction of P450 and its effects on metabolism and toxicity were investigated. Induction of the different families and subfamilies of P450 was demonstrated using four enzyme activities (Chapter Six). DEX, ISO and BNF each produced a characteristically different profile of enzyme activities in hepatocytes isolated from rats treated *in vivo*, with specific Induction of EMDM, PNPH and EROD respectively. PB, however, induced all the chosen activities to some extent. The non-selectivity of PB in this study was probably related to the choice of substrates and/or inducers, as discussed in Chapter 6.4. This approach should be refined and extended to other inducers (and in the case of PB, other enzyme activities) to obtain diagnostic profiles for the different classes of inducer (and possibly different inhibitors) which could be a useful tool in drug development for the prediction of possible drug interactions, as well as identification of novel inducers/inhibitors.

The selective induction of P450 activities by different inducers indicates the involvement of specific isozymes. These Induced Isozymes may be responsible for changes in toxicity due to metabolism. The potential *in vitro* toxicity of seven compounds, reported to be hepatotoxic, after induction *in vivo* with PB and BNF was also investigated (Chapter Seven). Of the four compounds that were toxic to hepatocytes cultured from untreated rats, two were

more toxic to hepatocytes from PB-treated rats and two were more toxic to hepatocytes from PB- and BNF-treated rats. Treatment with each inducer produced significant toxicity in vitro with the three remaining compounds, which were judged to be relatively non-toxic to cultures from untreated rats. Overall, the results of the study reported in Chapter Seven agreed well with available literature data on the seven compounds, although there were some discrepancies, particularly with respect to the in vivo data where involvement of non P450-mediated metabolism, Including Phase II metabolism, has been implicated as a determining factor in the toxic response. It was observed in Chapter Seven that for some compounds there were large variations in response of the cultured hepatocytes from individual animals (e.g. VPA), and for other compounds there was very little variation (e.g. BHT, in hepatocytes from control and BNF-treated animals). This is not necessarily a problem, since inter-individual variability in response to inducers and drugs is an important consideration in metabolism and toxicity studies. If the variability can be reproduced in vitro, the source of the variability can be elucidated, whether it be due to P450 isozyme content (which varies with age, sex, Induction etc.) or competing P450 and intermediary metabolic pathways (VPA). Induction can also affect Phase II metabolism, which can Influence the fate of products of P450 metabolism (e.g. the effect of glucuronidation on 4-IP toxicity). This study has shown that toxicity observed in vivo can be reproduced in an in vivo induction-hepatocyte culture system, and indicates that, despite the problems that remain to be resolved,

hepatocyte cultures do have an important role in toxicological studies, particularly in mechanistic studies which are *very* difficult to perform in vivo.

b. Induction in vitro

Unfortunately, attempts to induce both P450-dependent activities and P450-med1ated toxicity in culture, were unsuccessful. There were qualitative and quantitative differences on administration of PB and BNF in vitro. BNF did Induce ECOD activity in vitro, but the induction of total ECOD activity was greater than that observed in vivo (Chapter Five). In contrast, with the exception of total ECOD activity, Induction of activities by PB in vitro was *very poor.* This suggests that whereas the high affinity component comprises PB- and BNF-inducible forms the isozymes Involved in total ECOD activity may be BNF-inducible forms that can also be Induced by PB in vitro. This would explain why total ECOD activity was the only activity induced by PB in vitro. In Chapter Six it was shown that BZDM and EROD activities were induced by PB in vivo to the same extent (around 7- and 6-fold) but BNF only induced EROD activity. This is also consistent with the existence of PB- and BNF-specific isozymes, in addition to BNF-inducible Isozymes that also respond to PB in vitro. It has been reported that P450IA1, the major BNF-inducible Isozyme, increased in untreated hepatocyte primary cultures and that PB Induced this form further (Turner et al., 1988; Chapter 5.4). Therefore, an increase in P450IA1 basal levels coupled with P450IA1 responsiveness to PB in culture, could account for PB Induction

of total ECOD activity *in vitro* in the absence of MCOD induction by PB, and also for the increase in BNF induction of total ECOD activity *in vitro* over that seen *in vivo*. Also, constitutive EROD activity may be inducible by PB whereas P450IA1 induced *in vivo* is not.

In addition to the anomalous induction of P450IA1 in culture, it is now known that, although total P450 can be induced in culture by PB, there is no specific induction of P450IIB1/2. Thus, the regulation of PB induction *in vitro* appears to fail. New evidence is being accumulated on regulation of PB induction: the hepatic levels of P450IIB1 and 2 can be suppressed by growth hormone and by tri-iodothyronine (Yamazoe *et al.*, 1989); phosphorylation of PB-inducible P450s inhibits their activity, and phosphorylation via cAMP is under hormonal control (Bartlomowicz *et al.*, 1989; Koch and Waxman, 1989). It may be that, like the reduced expression of constitutive, liver-specific P450s in serum-containing medium, the induction of P450s IIBl and 2 is suppressed by serum. Another factor could be altered regulation of transcription *in vitro*. The initial decrease of protein synthesis *in vitro* (Tanaka *et al.*, 1978) may affect synthesis of transcription factors necessary for PB induction. Also, as discussed in Chapter Five, there appear to be intrinsic differences within the hepatocyte population with respect to induction, and these differences are retained in culture (Gumucio, 1989; Bars *etal.*, 1989).

Another problem with induction *in vitro* is that cultures must be exposed to inducer for several days, over which time period the total P450 is declining. This leads to reduced metabolic, toxic and Induction responses. In Chapter Seven, Induction *in vitro* was Investigated for precocene II and 4-1pomeanol. However, in contrast to 24 hour control hepatocytes, PII was not toxic to the 72 hour control cells.

8.5 TOXICITY STUDIES *IN VITRO*

Some of the problems associated with the use of cultured hepatocytes for metabolism studies have been discussed in the preceding sections. It is clear, then, that careful thought must be given to the development of protocols for the investigation of toxicity *in vitro*.

The choice of time in culture prior to exposure to xenobiotic is a crucial decision, since the metabolic stability of the cells must be balanced against the decline in P450. In this study, hepatocytes were cultured for 24 hours prior to exposure to allow for recovery from the isolation procedure and the establishment of a homogeneous cell population. By 24 hours, all the viable cells have flattened onto the dish, and other workers have suggested that the cells are in a metabolically stable state (Tanawa *et al.*, 1978; López *et al.*, 1988).

It was also clear from the results presented in Chapters Five, Six and Seven that inter-individual differences in toxicity and response to inducers persist in primary culture. This implies that cells from a number of animals should be used, rather than many cultures from the same animal, to assess the toxic effects of xenobiotics and particularly the effects of Induction on toxicity. This is less Important, however, in more mechanistic studies. It is also more Informative to present the results as a range of ID50 values or ID30-50-70 values rather than a single numerical value.

The toxicity results presented in Chapter Seven agree well with other literature data on the seven compounds used in this study, which suggests that an in vivo Induction-hepatocyte culture approach is potentially very useful for the Investigation of the role of P450 in hepatotoxicity, particularly since many aspects of cell function can be assessed concurrently e.g. viability and cellular glutathione.

8.6 GENERAL CONCLUSIONS

This study has shown that it is possible to use cultured hepatocytes in metabolism and toxicity studies. It has been shown that:

- a range of P450 activities , both constitutive and induced, can be maintained at high levels in hepatocytes cultured for 24 hours .
- differences in P450 activity and in maintenance of these activities with age and sex of hepatocyte donor exist.

- P450 activities are maintained better in hepatocytes cultured from adult male rats.
- P450 activities can be used to profile the different classes of Inducer.
- induction *in vivo* coupled with hepatocyte culture is a useful approach for investigating metabolism-mediated toxicity.
- toxicity *in vivo* can be reproduced in an hepatocyte culture system.

The main problems that were identified Involve the behaviour of hepatocytes in culture, and the alterations in phenotype that occur. One of the most important issues that must be addressed is the effect of the variable (and often unphysiological) levels of hormones that are added to culture medium in the form of serum. A more rational use of hormonally-defined media may enhance maintenance of developmentally regulated forms, in addition to improving both the overall maintenance of the constitutive P450 population *in vitro* and induction of P450s *in vivo*. Also, P450 metabolism *in vitro* cannot be investigated in isolation, particularly with respect to its role in toxicity. This became clear from the results presented in Chapter Seven, in which some of the discrepancies with the *in vivo* data may be due to alterations in Phase II metabolism *in vitro*. Less is known about the behaviour of non-MFO enzyme systems in hepatocyte culture e.g. Phase II metabolism, FMO. The balance of the activities of all these systems is crucial in determining whether a compound is detoxified or activated.

Overall, cultured hepatocytes would seem to have a useful role to play in the investigation of metabolism-mediated toxicity, in the evaluation of potential and actual toxicity, and, particularly, in elucidation of mechanisms of hepatotoxicity. Further research into the regulation of P450 Induction and of hepatic metabolism in general should greatly enhance the development of a culture system which mirrors the situation in vivo. Meanwhile, much information can be obtained by the use of carefully planned studies with due consideration of the present limitations governing the use of primary hepatocyte cultures.

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