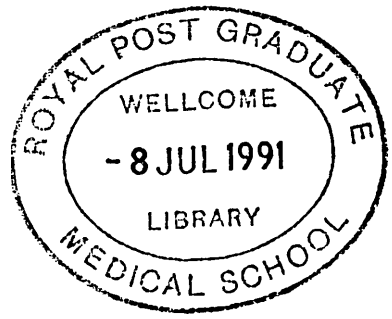
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Immunocytochemical Studies of Cytochromes P450IA and P450IIB

Thesis submitted by

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Abstract

The cell- and tissue-specific expression, development with age and inducibility of 3-methylcholanthrene (MC)-inducible (P450IA) and phenobarbitone (PB)-inducible (P450IIB) isoenzymes have been investigated. Both biochemical and immunological techniques, utilising highly specific and well characterised monoclonal and polyclonal antibodies have been employed. Much of the work involved immunocytochemistry, mainly at the light microscope level, but also to a lesser extent at the ultrastructural level.

Immunocytochemical screening of livers from mouse, rat, rabbit, hamster, guinea-pig and dog indicated that IA1, where expressed, was present in most hepatocytes across the lobule, whereas IA2 was distributed predominately centrilobularly. Treatment with MC led to the induction of both of these isoenzymes in most hepatocytes of rats, rabbits and Balb/c mice but not of the AKR mouse strain.

Cytochromes P450 IA1 and IA2 were constitutively expressed prenatally in rats and rabbits, although distribution patterns were such to suggest that the isoenzymes are differentially regulated. P450IA1 was expressed homogeneously throughout the lobule at all ages, whilst the pattern of P450IA2 expression was very dependent on the stage of development. Treatment with MC led to marked prenatal induction of both isoenzymes, resulting in developmentally advanced livers. However, induction of IA2 occurred several hours or days later than IA1 in rats or rabbits, respectively, and in only a few hepatocytes. The number of cells showing enhanced levels of IA2 gradually increased with age such that in livers of neonates the specific content was equal to or 2-3 times greater than that in adult rats or rabbits, respectively.

The distribution of IIB was found to vary with the strain of rat. Thus, IIB was distributed centrilobularly and/or periportally in Fischer 344 and Lister-Hooded rats, respectively. Expression in Wistar rats was confined to a limited number of cells whilst a more even distribution was observed in livers of Sprague-Dawley rats. Following PB treatment there was an increase in the levels of this isoenzyme in hepatocytes across the whole of the liver acinus in Fischer 344, Sprague-Dawley and Lister-Hooded rats, with a very narrow band of periportal cells remaining negative in the latter two strains. Induction of P450IIB in Wistar rats was greatest in centrilobular cells.

The expression of cytochromes P450 IA1, IA2 and IIB1/2 was shown to vary considerably among different extrahepatic organs and cell types of the rat. Moreover, each isoenzyme was subject to differential induction in either a tissue- and/or age-specific manner. Whereas P450IIB was absent from the kidney, even following pre-treatment of rats with PB, IA1 and

IA2 were constitutively expressed, IA1 predominating in S_3 cells and IA2 in S_2 cells. Isoenzymes from both these families were expressed in the epithelial cells of the intestinal villi, and in the lung. In the latter, IIB was focally concentrated in the Clara cells whilst IA1 was distributed more widely and IA2 not at all. P450IIB was absent from the testis and the pancreas. In contrast, IA1 occurred in acinar cells and in cells lining the inter- and intra-lobular ducts of the pancreas, whilst IA2 was found in the latter. Extrahepatically, IA2 appeared refractive to MC treatment and induction of IA1 in the kidney and small intestine was most significant after parturition. Prenatally, induction of IA1 occurred in interstitial cells of mesenchymal tissue of the lung and in other tissues. Treatment with PB increased levels of IIB in the epithelial cells of the gut only.

Throughout these studies emphasis was placed on the identification of immunoreactive isoenzymes of P450 by utilising two of more antibodies reacting with different epitopes for each tissue, wherever possible.

These results emphasize the complexity of the tissue and age-specific regulation of the expression of the isoenzymes of cytochrome P450. Not only does inducibility depend upon the particular isoenzyme but it also exhibits intra- and inter- tissue differences, which may explain, in part, the tissue and cell-specific toxicity of compounds activated by these isoenzymes.

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Abbreviations

AF	2-aminofluorene
AAF	2-acetylaminofluorene
AHH	aryl hydrocarbon hydroxylase
B	bile canaliculus
ßNF	ß-naphthoflavone
BSA	bovine serum albumin
C	centrilobular
CL	clofibrate
CSA	cyclosporin A
CT	collecting duct
DAB	3,3'-diaminobenzidine tetrahydrochloride
DMBA	dimethylbenz[a]anthracene
DMNA	dimethylnitrosamine
ECDE	ethoxycoumarin O-deethylase
e.l.i.s.a.	enzyme-linked immunosorbent assay
F344	Fischer 344 rat
G	glomerulus
GAM	goat anti-mouse
GAR	goat anti-rabbit
GIII	immature glomerulus
gl	glycogen granules
h	haematopoietic cells
H_2O_2	hydrogen peroxide
IgG	immunoglobulin
IGSS	immunogold-silver staining
INH	isoniazid
IQ	2-amino-3-methylimidazo [4, 5-f] quinoline
ISF	isosafrole
L	Islets of Langerhans
m	macrophage
M	midzonal
MAb	monoclonal antibody
MC	3-methylcholanthrene
MeIQ	2-amino-3, 5-dimethylimidazo [4, 5-f] quinoline
MeIQx	2-amino-3, 8-dimethylimidazo [4, 5-f] quinoxaline

mi	microsomes
M_r	molecular weight
mt	mitochondrion
N	nucleus
NF	nifedipine
NNK	4-(N-methyl-N-nitrosoamino)-1-(3-pyridyl)-1-butanone
OsO_4	osmium tetroxide
P	periportal
PAH	polycyclic aromatic hydrocarbon
PAP	peroxidase anti-peroxidase
PB	phenobarbitone
PBS	phosphate buffered saline
PCN	pregnenolone-16α-carbonitrile
POD	phenacetin O-deethylase
RER	rough endoplasmic reticulum
s	skeletal muscle
S_1	proximal convoluted tubule
S_2	end of proximal convoluted tubule
S_3	proximal straight tubule
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SER	smooth endoplasmic reticulum
Si	sinusoidal cell
TBS	Tris buffered saline
TCDD	2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin

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Aims

Cytochrome P450, a multi-isoenzyme family, is the major system responsible for the detoxication of xenobiotics. Nonetheless, a number of otherwise innocuous compounds are converted to highly reactive intermediates producing toxicity in one organ, whilst others, which are equally accessible, are unaffected. This phenomenon is considered to be a direct consequence of differences in the tissue and, indeed, cellular distribution of isoenzymes of cytochrome P450, as well as that of systems responsible for the detoxication of active species. In addition, induction of specific isoenzymes of P450 can render a tissue more susceptible to such metabolically dependent cytotoxicity. Hence, a knowledge of the organ and cellular distribution of the isoenzymes of cytochrome P450 together with their response to xenobiotics is important in elucidating the consequences of xenobiotic interactions.

Although immunocytochemical studies have allowed the cellular localization of several isoenzymes within various tissues, they have usually involved the use of antibodies that cross-react with a number of isoenzymes. Immunocytochemistry has not previously been used to study the ontogenic expression and inducibility of cytochrome P450 in rodents and few have studied the ultrastructural localization of the isoenzymes. This laboratory has raised and characterized highly specific monoclonal antibodies to several isoenzymes of the P450IA and IIB families. This study describes their application, in addition to others kindly donated from other sources, to further study the inter- and intra-tissue localization of cytochrome P450. Emphasis was placed on assessing:

1) the differential changes in P450IA1, IA2 and IIB1/2 in various tissues of several untreated and/or xenobiotic-treated mammalian species,

2) the effect of age on the development of P450IA1 and IA2 in various tissues of the rat and rabbit and the effect of MC treatment on their normal ontogenic patterns, and

3) the intracellular distribution of P450IA and IIB within the liver at the ultrastructural level.

Chapter one

Introduction

Man is exposed to countless xenobiotic chemicals that are present either naturally in the environment or are the products of human industry, many of which are in use today as drugs, pesticides, general household and industrial products and food additives. The last of these are used to enhance the nutritional value and to improve the stability, safety and acceptability of food stuffs. In addition, many other chemicals can arise unintentionally as a result of the way in which food is prepared, stored or cooked. Other xenobiotics, for example those derived from industrial waste, car exhausts or cigarette smoke, are unintentionally inhaled or ingested as atmospheric or water pollutants, or may even be absorbed after contact with the skin. Most of these compounds are highly lipophilic and would remain in the body indefinitely if it were not for the action of biotransformation (phase I metabolism) and conjugation (phase II metabolism) enzymes. During phase I metabolism, more polar groups (hydroxyl, carbonyl or amino) are introduced into the parent molecule, so providing a substrate for the phase II glucuronyl-, sulpho-, N-acetyl- and glutathione-S- transferases. The balance between these two groups of enzymes often determines susceptibility to chemical toxicity. These enzymes are differentially distributed amongst different tissues and cell types and intracellularly amongst different organelles. Moreover, the activity of an enzyme localized in one compartment may depend upon the availability of a co-factor generated in a different compartment. Although, in general, biotransformation decreases the overall toxicity of xenobiotics, converting them into water soluble conjugates which are then readily eliminated from the body, the products are sometimes chemically reactive. These may interact with protein, RNA or DNA to trigger the events that lead to tissue injury and/or permanent genomic changes that are eventually manifest as cancer.

1.1 Cytochrome P450

Although mitochondrial and flavin-containing monooxygenases, as well as other oxidation-reduction systems, are involved in biotransformation, cytochrome P450, a superfamily of haemoproteins prevalent in the endoplasmic reticulum of the liver, together with its co-enzyme NADPH-cytochrome P450 reductase, represents the major phase I enzyme system. This system is capable of metabolizing thousands of compounds (Yang & Lu, 1989).

Cytochrome P450 is readily distinguishable from other haemoproteins in whole tissue homogenates or microsomal fractions by its pronounced absorption between 447 and 456 nm when the carbon monoxide adduct of the reduced haem protein is formed. Cytochrome P450 has been extensively studied in the liver, from which many isoenzymes have been purified and characterized, and to a lesser extent in extrahepatic tissues (Lu & West, 1980; Waxman, 1986; Black & Coon, 1986; Guengerich, 1987; Masters *et al.*, 1987; Schwab & Johnson, 1987; Guengerich, 1989). Over thirty distinct members of the P450 superfamily have been identified which, based on amino acid sequence homology, have been assigned to one of nine separate gene families. Isoenzymes of this superfamily have been referred to throughout this thesis by the nomenclature recently recommended by Nebert *et al.* (1989) and illustrated in Table 1.1. Within a single species some of these gene families contain multiple members, for example, the IIB family, whereas in other families only a single member has been identified. Members of the P450 families are expressed constitutively at very low levels (Guengerich *et al.*, 1982; Thomas *et al.*, 1983; Christou *et al.*, 1987; Johansson *et al.*, 1988), with the exception of isoenzymes of the P450 III and IV families (Waxman *et al.*, 1985; Hardwick *et al.*, 1987), which is not surprising since these exhibit high catalytic activity towards endogenous chemicals. However, isoenzymes in most families may be considerably induced following pre-treatment with an appropriate xenobiotic. Thus members of the P450IA family can be induced by polycyclic aromatic hydrocarbons (PAHs) and other unrelated compounds, as reported in the mouse, rat, rabbit and hamster (Negishi & Nebert, 1979; Liem *et al.*, 1980; Thomas *et al.*, 1983; Astrom *et al.*, 1986; Raucy *et al.*, 1989); those of the IIB sub-family by phenobarbitone (PB) or polychlorinated biphenyls as reported in the rat, rabbit and dog (Christou *et al.*, 1987; Parandoosh *et al.*, 1987; Duignan *et al.*, 1987); those of the IIE sub-family by apparently unrelated low molecular weight compounds such as isoniazid, acetone or ethanol as seen in the rat and rabbit (Ryan *et al.*, 1985; Koop *et al.*, 1982); those of the P450III family by glucocorticoids and macrolide antibiotics (Elshourbasy & Guzelian, 1980; Schuetz *et al.*, 1984; Wrighton *et al.*, 1985a) and those of the IV family by peroxisome proliferators such as clofibrate (Hardwick *et al.*, 1987).

The isoenzymes of cytochrome P450 exhibit unique but overlapping substrate specificities in the metabolism of xenobiotics. Hence, the relative rates of detoxication and metabolic activation of a foreign compound will depend upon the relative amounts of the different isoenzymes present. The increasing availability of both polyclonal and monoclonal antibodies has enabled the immunoquantification of specific cytochromes P450 in a number of species, and an assessment of their contribution to the metabolism of a variety of compounds in microsomal preparations (Boobis *et al.*, 1981; Guengerich *et al.*, 1982;

Table 1.1: Recommended nomenclature for the members of the cytochrome P450 superfamily

P450 family	Isoenzyme	Trivial name	Species
I	IA1 and IA2	c and d	rat
		P_1 and P_3	mouse, human
		LM_6 and LM_4	rabbit
II	IIA	a	rat
	IIB1 and IIB2	b and e	rat
	IIB4	LM_2	rabbit
	IIC2	K	rabbit
	IIC6	PB-1	rat
	IIC7	f	rat
	IIC11	h	rat
	IIC12	i	rat
	IIC13	g	rat
	IID1	db1	rat
	IID6	db1	human
	IIE1	j and 3a	rat and rabbit
III	IIIA1-3	pcn1, pcn2, HLp	rat, rat and human
IV	IVA1-3	LAω1-3	rat
	IVA5-7	LAω1-3	rabbit
	IVB1	LM5	rabbit
XIA		scc	human
XIB		11ß	cow
XVII		17α	rat, human
XIX		arom	human
XXI		c21	mouse

Gene families are identified by Roman numerals, sub-families by capital letters and individual genes by Arabic numerals (summarised from Nebert *et al.*, 1989)

Thomas *et al.*, 1984a; Thomas *et al.*, 1984b; Reik *et al.*, 1985; Boobis *et al.*, 1985a; Freidman *et al.*, 1986; Thomas *et al.*, 1987a; Barnes *et al.*, 1987). This approach has enabled the identification of substrates which can be used as selective 'markers' for individual isoenzymes, particularly in variously induced animals. For example, the hydroxylation of benzo[a]pyrene (aryl hydrocarbon hydroxylase, AHH) is largely catalysed by IA1 in PAH-treated mouse and rat (Negishi & Nebert, 1979; Guengerich *et al.*, 1982); N-hydroxylation of 2-naphthylamine and 2-acetylaminofluorene (AAF) and the *O*-deethylation of phenacetin are selectively catalysed by IA2 in the liver of many species (Hammons *et al.*, 1985; Boobis *et al.*, 1985a; Yang *et al.*, 1985; Distlerath *et al.*, 1985; Sesardic *et al.*, 1988); N-demethylation of benzphetamine is catalysed largely by members of the IIB family (Guengerich *et al.*, 1982) and the N-demethylation of dimethylnitrosoamine (DMNA) is catalysed by IIE1 (Yang *et al.*, 1985; Levin *et al.*, 1986; Thomas *et al.*, 1987b). A number of both endogenous and exogenous substrates are subject to regiospecific hydroxylation by several isoenzymes of P450, for example, steroid hormones and many PAHs. Thus, cytochromes P450 IIA1, IIC11 and IIB1 specifically hydroxylate testosterone at the 7α-, 2α- and 16ß- positions respectively, whilst many isoenzymes contribute to its hydroxylation in the 16α- position (Waxman, 1988). Cytochrome P450 IIB1 favours the 7-methyl hydroxylation of 7,12 dimethylbenz[a]anthracene (DMBA) whilst IIB2 favours hydroxylation in the 12 position (Wilson *et al.*, 1987).

1.2 Cytochrome P450-mediated toxicity

A wide variety of xenobiotics are converted into reactive metabolites, but whether cytotoxicity occurs depends not only on the substrate but also on the species, strain, organ and age of the animal studied. The common denominator is often cytochrome P450, the isoenzyme profile and levels of which differ in a species- and tissue-specific manner. Table 1.2 lists some examples of tissue- and cell-specific toxicities in which a role for cytochrome P450 has either been established or is implicated. It is apparent from this table that isoenzymes of the P450IA, IIB and IIE sub-families are particularly important in determining mutagenicity, carcinogenicity and cytotoxicity in laboratory species.

Considerable species differences have been observed in susceptibility to carcinogenic nitrosamines which depend upon cytochrome P450 for their activation. Whereas rat, mouse, hamster, guinea-pig and rabbit develop liver carcinogenesis on exposure to DMNA, only the rat produces the same organotrophic effect following administration of diethylnitrosamine (Preussmann & Wiessler, 1987). The carcinogenic effect of this and of

Table 1.2: Some examples of tissue/cell selective toxicities in which cytochrome P450 may be involved

Chemical class and compound	Toxicity[a]	P450 Isoenzyme[b]	References
Alkanes			
n-hexane	neurofilament crosslinking	IIB	Morohashi *et al.* (1983) Toftgard *et al.* (1986)
p-xylene	suicidal destruction of P450 in lung	IIB1, IIB2, IVB1	Smith *et al.* (1982) Pyykkö *et al.* (1987) Philpot & Smith (1984)
Alkenes/alkynes			
Several	suicidal destruction of hepatic P450	IIB	Ortiz de Montellano & Correia (1983)
Halogenated hydrocarbons			
halothane	hepatic necrosis	IIB, IIE	Plummer *et al.* (1988)
benzene	bone marrow aplasia	IIB, IIE, IVB1	Gorsky & Coon (1985) Ingelman-Sundberg *et al.* (1982)
butylated hydroxytoluene	type I alveolar cell damage (lung) and hepatic necrosis	IIB	Nakagawa *et al.* (1984)

Table 1.2 continued:

carbon tetrachloride	centrilobular necrosis of the liver; destruction of P450 Clara cell necrosis (lung)	IIB, IIE	Boyd *et al.* (1980); Noguchi *et al.* (1982);
chloramphenicol	suicidal destruction of P450	IIB	Halpert (1982)
Heteroaromatics			
4-ipomeanol	Clara cell necrosis (lung)	IIB, IVB1	Guengerich (1977); Wolf *et al.* (1982)
Amines			
AAF	hepatotoxicity/hepatocarcinogenicity	IA2	McManus *et al.* (1984)
cocaine	mid-zonal and centrilobular necrosis of the liver	IIB	Shuster *et al.* (1983)
dimethylnitrosamine	hepatocarcinogenicity	IIE	Preussmann & Wiessler (1987) Levin *et al.* (1986)
4-(N-methyl-N-nitrosamine)-1-(3-pyridyl)-1-butanone	lung cancer	IA, IIB1	Devereux *et al.* (1988)
phenacetin/paracetamol	centrilobular necrosis of the liver; renal necrosis	IA2, IIE	Morgan *et al.* (1983) Raucy *et al.* (1989)

Table 1.2 continued:

Thiols and related compounds

parathion	hepatotoxicity	IIB1, IIA1, IVB1	Kamataki & Neal (1976); Guengerich (1977)
thioacetamide	centrilobular necrosis of the liver	IIB1	Dashti *et al.* (1987) Porter & Neal (1978)

[a]For a full explanation of toxicity see Nelson & Harvison (1987) and references therein

[b]This list indicates which isoenzyme(s) may play a role in the formation of either cytotoxins or proximate toxic metabolites as suggested from induction and/or purified enzyme studies, cited under references.

the tobacco-specific nitrosamine, 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is manifest in the lung of hamsters and rats, respectively (Yamamoto *et al.*, 1985; Devereux *et al.*, 1988). The differential susceptibility of these species to carcinogenicity may be a result of the differential expression of the isoenzymes responsible for their activation within these tissues. For example, DMNA is activated by P450IIE1 in rat liver (Yang *et al.*, 1985) whilst NNK is activated by P450IIB1/2 in the lung (Belinsky *et al.*, 1987). Similarly, differences in the toxicity of arylamines may in part be explained by variations in the levels of P450IA2 (Hammons *et al.*, 1985). For instance, the guinea-pig, which is resistant to the hepatocarcinogenic effects of AAF (Gosch *et al.*, 1970; Cardy & Lijinsky, 1980; Matsushima *et al.*, 1980), has very low levels of IA1 and IA2 in the liver, even after treatment of the animals with 3-methylcholanthrene (MC) (Abe & Watanabe, 1982; Thomas *et al.*, 1984a, 1984b), unlike the rat, where levels of IA2 in the liver are relatively high, especially in induced animals (Thomas *et al.*, 1984a, 1984b).

The susceptibility of some organs to chemical carcinogenesis may also, in part be explained by the fact that some xenobiotics, catalysed predominantly by IA2 in the liver, may depend upon IA1 for metabolism extrahepatically since IA2 is absent from these tissues (Goldstein & Linko, 1984). For example, phenacetin *O*-deethylase activity is selectively inhibited by an antibody against IA2 in rat liver but in the kidney activity is inhibited only by an antibody against IA1 (Sesardic *et al.*, 1989). These findings are of particular concern since substrates for each of these isoenzymes, for example, PAHs, aromatic amines and nitrosamines are frequently encountered in our environment and have been associated with human cancer for over a century (Kadlubar & Hammons, 1987).

A number of cytochromes P450 in man have now been purified and characterized, including IA2, referred to as $P450_{PA}$ (Distlerath *et al.*, 1985) and P450IIE1 (Wrighton *et al.*, 1986a). As in animals, both of these isoenzymes catalyse the oxidation of pre-carcinogens ie. N-hydroxylation of aromatic amines and N-demethylation of nitrosamines, respectively. Further, *in vitro* work has shown that microsomes from human liver can catalyse the oxidation of several other toxic and carcinogenic compounds to reactive intermediates, including N-hydroxylation of AAF (Dybing *et al.*, 1979), epoxidation of aflatoxin B_1 (Boobis *et al.*, 1983a), 7,8-dihydrodiol formation from benzo[a]pyrene (Boobis *et al.*, 1978) and the N-hydroxylation of 2-naphthylamine (Hammons *et al.*, 1985). Human liver cytochrome P450 can also activate many nitrosamines to metabolites that are mutagenic in the *Salmonella typhimurium* assay (Shimado & Okuda, 1988). Establishing an unequivocal role for these isoenzymes in cytotoxicity *in vivo* is obviously difficult and the evidence is often based more on association than cause and effect. For example, a

good correlation exists between the basal level of lymphocyte AHH activity and the incidence of bronchogenic carcinoma in cigarette smokers (Kouri *et al.*, 1982). However, a causal relationship has yet to be established. A few groups have also attempted to explain a number of drug-induced cytotoxicities in terms of genetic polymorphisms of drug oxidation. Thus, in man extensive metabolism of debrisoquine by P450IID1 may be associated with lung cancer (Ayesh *et al.*, 1984) although in a recent study, subjects with lung cancer did not metabolise debrisoquine more rapidly than non-cancer patients (Speirs *et al.*, 1990). The incidence of some cancers is closely associated with exposure to pre-disposing factors. For example, primary liver cancer is most likely to occur in those countries in which the staple dietary foods, such as groundnuts, are likely to become contaminated with aflatoxin B_1 (Busby & Wogan, 1984) and oral cancer is most prevalent in India where chewing of tobacco, rich in betel quid (in which nitrosamines are major components), is a widely practised habit (Rivenson *et al.*, 1988).

Whilst a number of isoenzymes of cytochrome P450 have been purified from liver and characterized, relatively few extrahepatic forms have been studied in any detail due to their low specific content. Such studies have been further confounded by the fact that the isoenzymes are localised within specific cell populations. Although much progress has been made in the isolation of specific cell populations, these techniques are not widely used mainly because of their considerable difficulty, time consuming nature, expense and the large numbers of animals often required to produce only a few milligrams of protein (Devereux *et al.*, 1981; Bonkovsky *et al.*, 1985; Domin *et al.*, 1986). Nevertheless, those studies that have been performed on such cell populations have been of considerable value in elucidating those metabolic factors pre-disposing to cell-specific toxicity.

A prime example is found in the lung-specific toxicity of the furan derivative 4-ipomeanol and NNK, both of which produce necrosis of the nonciliated bronchiolar Clara cells of the lower airways (Boyd, 1977; Devereux *et al.*, 1988). Two isoenzymes, both members of the P450IIB family, IIB1 and IIB2, are involved in the oxidation of 4-ipomeanol and probably NNK. Despite the same level of expression of PB-inducible isoenzymes in the liver and lung, the liver is resistant to the toxicity of these two compounds. In the rat, this can be readily explained by the differential expression of the two isoenzymes, IIB2 being preferentially expressed in the liver whilst IIB1 is expressed extrahepatically (Christou *et al.*, 1987). Km values for 4-ipomeanol in these tissues vary by over 10-fold, supporting the view that different isoenzymes are involved in its metabolism. However, this does not appear to be the case in the rabbit, where Km values for 4-ipomeanol do not vary between the two tissues, suggesting that the same isoenzyme might be involved.

However, the production of metabolites that covalently bind to proteins, glutathione or DNA is greater in the lung (Dutcher & Boyd, 1979; Wolf *et al.*, 1982; Devereux *et al.*, 1988). These differences in tissue selective toxicity are consistent with immunocytochemical and autoradiographic studies which showed a high focal concentration of IIB1/2 and adducts of NNK within the Clara cells of the lung (Keith *et al.*, 1987; Serabjit-Singh *et al.*, 1988; Belinsky *et al.*, 1987). Thus, both the tissue-specific expression of IIB1 and IIB2 and their high focal concentration in the cell type of the lung, appear to account for the lung-specific toxicity of these compounds.

Our current knowledge on the relationship between the embryological, foetal and neonatal metabolism of xenobiotics and their toxicity is poor. The major site of bioactivation of drugs and other chemicals in pregnant animals is the maternal liver. Hence, very few reactive metabolites would be expected to reach embryonic tissues because of their high reactivity and short half lives. Only the most stable and least reactive metabolites would be expected to survive export from hepatocytes, transport in the maternal bloodstream, passage across the placenta and transport in the circulation of the conceptus. However, it is becoming increasingly apparent that the metabolism of some chemical teratogens and embryotoxins may be catalysed by foetal cytochrome P450, particularly constitutive isoenzymes (Juchau, 1989). For example, it is suggested that thalidomide, a teratogen in rabbits, monkeys and humans, is activated by an as yet unidentified P450 isoenzyme to an arene oxide metabolite (Gordon *et al.*, 1983; Braun *et al.*, 1986).

Certainly, there is good evidence to suggest that some of the isoenzymes of cytochrome P450 are expressed at an early stage of organogenesis (Galloway *et al.*, 1980; Filler & Lew, 1981; Kimura *et al.*, 1987; Yang *et al.*, 1988). Furthermore, the level of expression of these isoenzymes is far from static and changes considerably during development (Atlas *et al.*, 1977; Guenthner & Nebert, 1978; Klinger *et al.*, 1978; Cresteil *et al.*, 1986). The type and extent of P450-mediated monooxygenase activity present varies with the species and with the phase of development. Moreover, the postnatal development of monooxygenase activities (isoenzymes) is independent of each other. The available evidence suggests that in man foetal and neonatal xenobiotic metabolizing enzymes are regulated in a manner similar to that in animals (Distlerath & Guengerich, 1987). Thus, at each phase of development the foetus or neonate represents essentially a 'unique species' with respect to the isoenzymes of P450 present. The problems that this creates for drug testing are obvious, and thus it is of critical importance to accurately define the ontogenic regulation and expression of the isoenzymes of cytochrome P450. As with extrahepatic tissues, such studies have been limited and this is due, at least in part, to the low levels of

the isoenzymes present which are consequently extremely difficult to measure particularly as the tissue is often excessively contaminated with haem. This necessitates the use of large numbers of animals, making such experiments expensive and demanding of resources. The availability of extremely sensitive, highly specific probes, such as monoclonal antibodies, are beginning, and will continue, to contribute greatly to this field.

1.3 Methods used to assess cytochrome P450 profiles in tissues

Over the past decade, a number of independent groups have utilized polyclonal antibodies in a variety of ways to characterize cytochrome P450 expression in liver and extrahepatic tissues of several species, including man (Table 1.3). These antibodies are useful for quantifying isoenzyme expression by immunoblotting techniques and for assessing the role of individual isoenzymes or families of isoenzymes in the microsomal metabolism of a compound by inhibition studies. However, the results of such studies are only as reliable as the specificities of the anti-P450 antibodies employed. Polyclonal antibodies, by their nature, are heterogeneous containing many immunoglobulins with diverse binding affinities for different epitopes on the antigen against which they are directed. Potential cross-reactivity with uncharacterized P450 forms, or with closely related isoenzymes therefore needs to be considered. For example, IIC7 and IIC6 (P450f and P450PB1) share 50% similarity in nucleotide and amino acid sequences to IIB2 (Kumar *et al.*, 1983; Gonzalez *et al.*, 1986) which in itself shares >97% similarity to IIB1 (Yuan *et al.*, 1983). Isoenzymes of the P450IA family, IA1 and IA2 are also immunochemically related, showing greater than 70% homology (Reik *et al.*, 1982; Thomas *et al.*, 1983).

The development of hybridoma technology (Kohler & Milstein, 1975) has partially solved this problem by making it possible to obtain monoclonal antibodies of high purity directed to a single antigenic site. Amongst the possible properties of such antibodies are their ability to bind to the P450 antigen in enzyme linked immunosorbent assays (e.l.i.s.a), Western blots or radioimmunoassays, or to inhibit enzyme activity catalysed by the antigen of interest. Monoclonal antibodies can also be classified according to their epitope specificity, as measured by their ability to compete with one another for binding to P450 (Thomas *et al.*, 1984a; Reik *et al.*, 1985). Of the monoclonal antibodies produced, many have shown cross-reactivity with related isoenzymes, reflecting interaction with an epitope that is common within a P450 family or sub-family, or across several species (Boobis *et al.*, 1985b; Thomas *et al.*, 1984b; Freidman *et al.*, 1986). Such antibodies can be useful for the detection, immunopurification and partial sequence analysis of immunochemically related P450 isoenzymes in hepatic and extrahepatic tissues, as well as for the

Table 1.3: Characterization of cytochrome P450 profiles in tissues using antibodies

A. Polyclonal antibodies

Technique	P450 Isoenzyme	Species	Tissue	Reference
Immunoquantification/ detection	I, IIB	rat	liver, extrahepatic	Guengerich & Mason (1979)
	IIB	human, rat rabbit	liver	Guengerich *et al.* (1981)
	I, IIB, IIC2	mouse, rat hamster, rabbit, guinea-pig	liver	Thomas *et al.* (1984b)
	I, IIB	rat, rabbit guinea-pig	liver, lung, bladder, aorta	Domin *et al.* (1984)
	I	human	liver	Wrighton *et al.* (1986b)
	IIE	rabbit	nose, kidney	Ding *et al.* (1986)
	I, IIB, III	rat liver	foetal, neonatal and adult	Cresteil *et al.* (1986)
	III	human	foetal and adult liver	Wrighton *et al.* (1988)
	IA1, IIB	rabbit	lung	Domin *et al.* (1986)

Table 1.3 continued:

Inhibition	I, IIB	rat	liver, lung, kidney (*warfarin*)	Kaminsky *et al.* (1979)
	IID6, IA2	human	liver (*bufuralol, POD*)	Distlerath *et al.* (1985)
	IIB1, IIB2	rat	liver, extrahepatic (*DMBA*)	Christou *et al.* (1987, 1989)
	IIB1, IIB2	rat	liver (*DMBA*)	Wilson *et al.* (1987)
	IIB1, IIB2	rabbit	liver, lung (*AF*)	Vanderslice *et al.* (1987)
	III	human	liver (*CSA and NF oxidase*)	Combalbert *et al.* (1989)
	III	human	foetal liver (*erythromycin*)	Kitada *et al.* (1988)
Immunocytochemistry	I, IIB	rabbit	liver, lung, kidney	Dees *et al.* (1982)
	I, IIB, IIC2	rat	liver	Moody *et al.* (1983, 1985, 1986; 1988)
	I, IIB	rat	liver	Wolf *et al.* (1984)
	I, IIB, III	rat, hamster	liver, extrahepatic	Baron *et al.* (1986)
	I, IIB	rat	lung	Keith *et al.* (1987)

Table 1.3 continued:

B. Monoclonal antibodies

Immunoquantification/ detection	I	mouse, rat hamster guinea-pig	liver, kidney, lung	Cheng *et al.* (1984)
	I	rat, rabbit human	liver	Boobis *et al.* (1985b)
	III	human	liver	Beaune *et al.* (1985)
	I	human	placenta, lymphocyte	Song *et al.* (1985)
	IIB	rat	liver	Reik *et al.* (1985)
	I, IIB	rat, rabbit human	liver	Sesardic *et al.* (1986)
	IA2	human	liver	Wrighton *et al.* (1986b)
	III	rat, human	liver	Barnes *et al.* (1987)
	IIB	rat	liver, extrahepatic	Christou *et al.* (1987; 1989)
	III	human	liver	Combalbert *et al.* (1989)
Inhibition	I	human	placenta, lymphocyte (*AHH, ECDE*)	Fujino *et al.* (1982)
	IA2, IIB1	rat, rabbit	liver	Park *et al.* (1982a, 1982b)

Table 1.3 continued:

			(*AHH, ECDE*)	
	I	rat	Reconstituted enzymes (*AHH*)	Thomas *et al.* (1984a)
	IIB	rat	Reconstituted enzymes (*testosterone, benzphetamine*)	Reik *et al.* (1985)
	I, IIB	rat	liver, extrahepatic (*AHH, ECDE*)	Hietanen *et al.* (1987)
	I, IIB	rat	liver, reconstituted enzymes (*testosterone, androstenedione*)	Waxman *et al.* (1987)
	I	rat	liver (*prostaglandins*)	Holm *et al.* (1989)
	IA1	rat	liver (*AHH*)	Sesardic *et al.* (1989)
Immunopurification	I	mouse, rat hamster	liver, kidney, lung	Cheng *et al.* (1984; 1986)
	I	rat	liver, lung	Robinson *et al.* (1986)
	IIE	rabbit	kidney, nose	Ding *et al.* (1986)
cDNA isolation	I, IIB, IIE, III	various	various	reviewed by Gonzalez (1988)

Table 1.3 continued:

Immunocytochemistry	I	rat	liver, extrahepatic	Foster *et al.* (1986)
	I, IIB	rat	liver	Kolyada (1986)
	I	mouse	liver, extrahepatic	Anderson *et al.* (1987)
	III	human	foetal and adult liver	Murray *et al.* (1987)
	IIC2	rabbit	liver, kidney	Finlayson *et al.* (1987)
	IA2	rat hepatocyte cell line	nuclear envelope	Hishinuma *et al.* (1987)
	I	human	placenta	Pasanen *et al.* (1988)
	I, IIB	mouse	liver, lung	Forkert *et al.* (1988; 1989)

Substrates or enzyme activity inhibited is presented in italicised parentheses. POD, phenacetin *O*-deethylase; AHH, aryl hydrocarbon hydroxylase; AF, 2-aminofluorene; CSA, cyclosporin A; NF, nifedipine; ECDE, ethoxycoumarin *O*-deethylase; DMBA, dimethylbenza[a]anthracene.

identification of orthologous isoenzymes in other species (Table 1.3). Inhibitory studies with monoclonal antibodies have further clarified the precise contribution of individual P450 isoenzymes in the metabolism of drugs, carcinogenic chemicals and endogenous compounds, such as steroid hormones (Table 1.3).

Undoubtedly, such antibodies have proved to be powerful tools, with perhaps their most powerful application being in the field of molecular cloning where practically all of the cytochromes P450 in a range of organisms and tissues including isoenzymes previously not known to exist have been identified, isolated and characterized (Gonzalez, 1988). The production of specific cDNA clones has in turn led to the application of *in situ* hybridization (Traber *et al.*, 1988; Dey *et al.*, 1989).

1.4 Immunocytochemistry

The technique of immunocytochemistry originated some 30-40 years ago (Coons *et al.*, 1941) and has since grown from strength to strength as more antibodies have become available and better detection systems have been developed. Immunocytochemistry is the only method readily available whereby changes in the expression and distribution of cytochrome P450 can be studied at the cellular level.

There are four essential requirements for good immunohistochemistry: a highly specific and well characterized antibody, an easily visible label, preservation of antigenicity and method specificity. The application of immunocytochemistry to studying cytochrome P450 expression has been ongoing for the past decade or so, but most information has come from studies using polyclonal antibodies, for which the disadvantages of their use have previously been stated. The past three years has seen the application of more specific monoclonal antibodies for localizing cytochrome P450 in a wide variety of rat and rabbit tissues (Table 1.3). Monoclonal antibodies are specific, reacting with a single sequence of 5-7 amino acids in a protein, which would rarely occur in an unrelated protein. A third type of antibody, gaining popularity in the cytochrome P450 field, is the anti-peptide antibody (Bulinski, 1986). These are polyclonal antibodies raised to synthetic peptides, corresponding to a linear sequence of a limited number of amino acids, usually 6-7, unique to the protein of interest, thus ensuring specificity equivalent to that of a monoclonal antibody in that they react with only a single epitope. Having chosen a sequence of interest, based on a number of criteria including a comparison of the deduced primary sequence of P450 isoenzymes, the peptide is synthesized and coupled to a larger

immunogenic carrier protein such as keyhole limpet haemocyanin against which antibodies, polyclonal or monoclonal, are then produced in the conventional manner. In this way, highly sequence-specific antibodies to members of the IIB and IA families, which do not cross-react with other related forms in the same or other species have been produced (Frey et al., 1985; Edwards *et al.*, 1988, 1989, 1990). A major advantage of such antibodies is the ability to pre-determine the antigenic-epitope. The epitope to which a monoclonal antibody binds is not known in advance and its identification is often difficult. Hence, obtaining monoclonal antibodies that are inhibitory or that are suitable for the immunochemical localization of their antigens in variously fixed tissues, from a panel of 200 or 300 clones, is a tedious task and more often than not fortuitous. The successful application of anti-peptide antibodies in immunocytochemistry has been demonstrated in a single study in which 15 anti-peptide antibodies were used to probe for antigenic sites on the cytoplasmic and luminal sides of rough endoplasmic reticular membranes, as a means of elucidating the transmembrane disposition of cytochrome P450 (De Lemos-Chiarandini *et al.*, 1987).

Reaction of an antibody with its antigen in a tissue section is revealed by utilizing one of several available labels. Those commonly used for light microscopy include peroxidase (Nakane & Pierce, 1967), alkaline phosphatase (Mason & Sammons, 1978), glucose oxidase (Suffin *et al.*, 1979) and highly fluorescent compounds such as fluorescein and rhodamine isothiocyanate (Dees *et al.*, 1982; Valnes & Brandtzaeg, 1982). The end product of some of the enzyme reactions can be made electron dense by reaction with osmium tetroxide, but other electron dense labels such as ferritin (Matsuura *et al.*, 1978) and colloidal gold (Faulk & Taylor, 1971) can also be used to identify immunocytochemical reactions at the ultrastructural level.

The simplest way of staining is by the direct method, where the primary antibody is conjugated directly to the fluorescent probe or marker enzyme (Fig. 1.1a). This method has however, been largely superseded by the indirect method, in which possible degradation of valuable primary antibody (the quantity of which may be limited) during conjugation and purification is avoided and which has general applicability to all primary antibodies from the same species. In this technique, tissue antigen is localized using unlabelled antibody (primary antibody) from a given species (usually mouse or rabbit) which is then reacted with a second antibody directed against the first (Fig. 1.1b). More often than not, and particularly so in studies of cytochrome P450, this second antibody is conjugated to horseradish peroxidase which is then developed in Graham & Karnovsky's (1966) diaminobenzidine (DAB)-H_2O_2 reaction solution. Peroxidase reacts with its specific

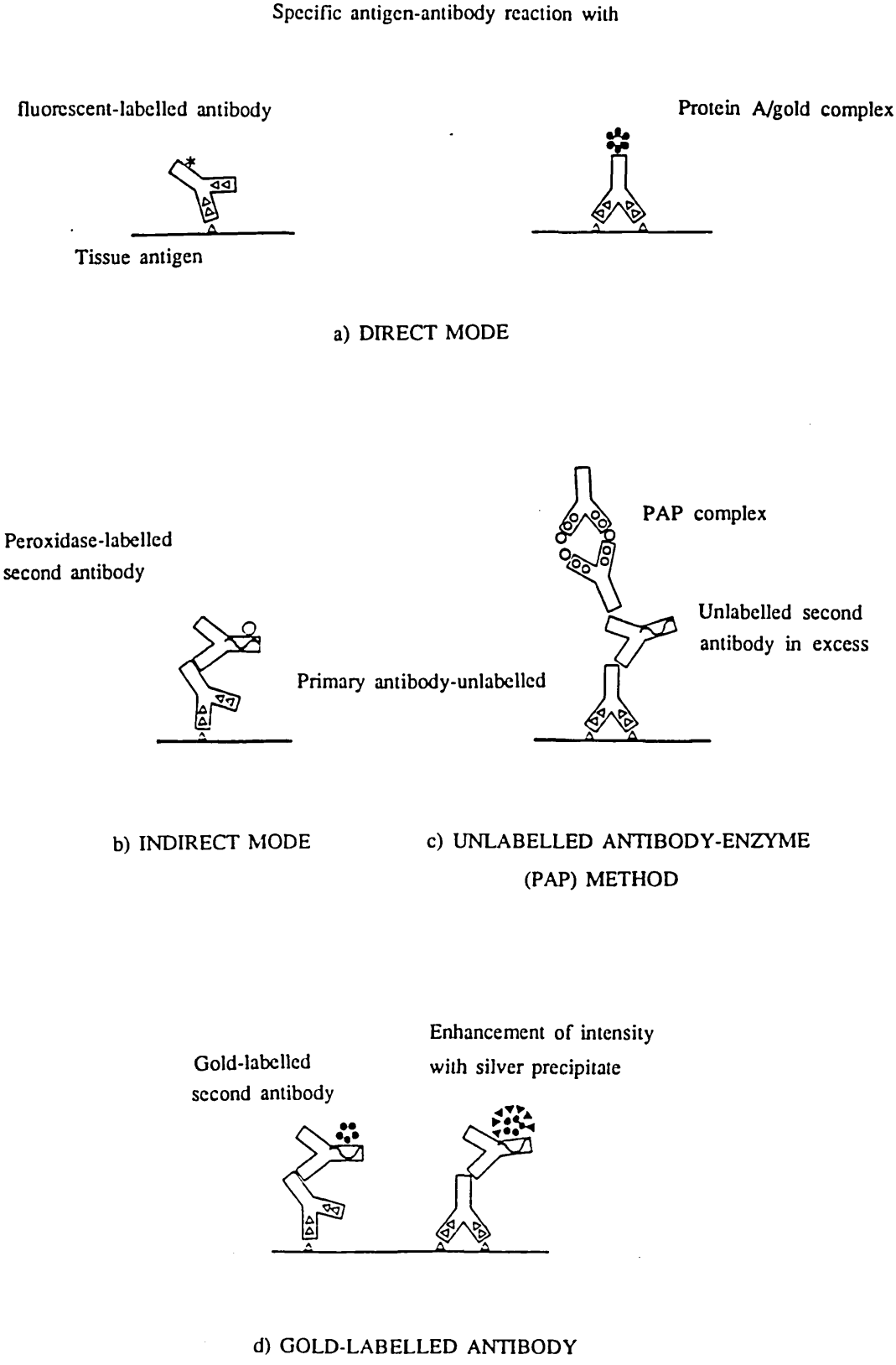


Figure 1.1: An illustration of some immunocytochemical methods of staining (adapted from Polak & Van Noorden, 1987)

substrate, hydrogen peroxide to form a primary product. In the presence of an electron donor, such as DAB, a second product is formed which dissociates to give the highly insoluble, oxidized DAB as a brown precipitate, readily visualized under an ordinary light microscope.

Various methods are available for enhancing the sensitivity of an indirect immunoreaction thus allowing detection of smaller quantities of antigen in the tissue at the same concentration of primary antibody or for increasing the specificity of a reaction by allowing the same quantity of antigen to be detected by a lower concentration of primary antibody. In the latter case, dilution of the antibody often reduces unwanted low affinity reactions, most prevalent with polyclonal antisera. Amongst the most popular methods are unlabelled secondary antibody-enzyme methods such as Sternberger's (1986) peroxidase anti-peroxidase (PAP) system and silver enhancement of immunogold staining (Holgate *et al.*, 1983; Springall *et al.*, 1984). In the former technique, a third layer is introduced, in the form of a stable cyclic complex with three peroxidase molecules to two antibody molecules (Fig. 1.1c). Essentially, more peroxidase ends up at the site of the reaction which in theory, although not always in practice due to the Eng-Bigbee effect, increases sensitivity by up to 100-fold. At higher concentrations of antibody, peroxidase activity can become inhibited, possibly the result of steric hindrance by the antibodies reacting at or near the catalytic site of peroxidase. In the latter method, reduction of gold particles by silver lactate produces a silver deposit, visible as an intense black colour which can increase sensitivity by up to 200-fold (Fig. 1.1d). This technique requires pre-treatment with Lugol's iodine which, as found in this study, can cause deterioration of the morphology of frozen sections and is therefore a disadvantage. Other methods with considerable potential for improving the sensitivity of immunostaining techniques include the use of avidin and biotin (Coggi *et al.*, 1986) and the hapten-sandwich technique in which monoclonal anti-hapten antibodies are used as a bridge (Jasani *et al.*, 1981).

It is conventional to treat fresh or frozen tissue with chemical fixatives, such as formalin or glutaraldehyde to preserve their structure and to prevent tissue components from disintegrating during staining so that the immunoreactive cell or organelle may be identified in context (see Sternberger, 1986). Generally, the greater the degree of protein cross-linking, the better the preservation of morphology but the greater the loss of antigenicity. This may not cause problems where polyclonal antibodies are used, due to the heterogeneity of antibody species present but with mono-specific monoclonal antibodies it can be a major disadvantage. The particular epitope of interest on the antigen molecule may be altered by fixation or processing so that it is no longer recognised by the antibody,

Problem	Remedy
Binding to Fc receptor	Block receptors with normal serum from the species donating the second antibody or inert protein such as BSA.
Hydrophobic and electrostatic binding of Ig to tissue (unfixed frozen sections)	Increase the ionic strength of washing buffers (2.5% NaCl).
Cross-reactivity of one antibody with several antigens	Use a variety of different antisera or region specific antisera to antigen of interest.
Background peroxidase or 'non-specific' staining of macrophages or erythrocytes	Blocking of peroxidase by incubation in 0.3% H_2O_2 in buffer, methanol or ethanol or 1% sodium nitroferricyanide in methanol containing 1% acetic acid.
Binding to active groups in tissue (formaldehyde/glutaraldehyde-fixed tissue)	Reduce aldehyde groups with sodium or potassium borohydride before immunostaining.

Table 1.4: Non-specific staining: problems and remedies
(summarised from Van Noorden, 1986)

resulting in total loss of staining. In fact most monoclonal antibodies will react only with unfixed fresh or frozen material. This may not be a problem with anti-peptide antibodies since epitopes which are not involved in secondary or tertiary structure can be targeted. Nevertheless, other 'fixative' agents can be used which preserve the tissue without crosslinking, for example, alcohols and acetone. However, their reaction with many proteins is reversible, so that when the tissue is rehydrated during staining, there may be some deterioration in morphology. Before performing any immunocytochemical study it is important to assess the effects of different fixatives on the immunoreactivity of the antigen in question.

Positive staining in immunocytochemistry may well be genuine, due to specific antibody-antigen interaction, but the risk of non-specific reaction must be eliminated before any such result can be accepted. Validation of the technique involves a simple series of tests. Substitution of the primary antiserum with buffer, normal serum or an inappropriate antibody should abolish all staining. Any remaining reaction represents background or non-specific staining which may arise from one or more sources (Table 1.4). The specificity of an antiserum for its antigen should also be defined. Immunoreactivity should be abolished by prior adsorption of the primary antibody with excess of its specific antigen, but not by absorption with similar quantities of related substances. Such tests can however sometimes be misleading. For instance, anti-peptide antibodies react with a single sequence, hence a possible source of background, though not non-specific, reactivity is interaction with proteins other than the antigen which contain regions of similar amino acid sequence. Monoclonal antibodies react with one single epitope with uniform affinity. Thus, specificity can be thought of in qualitative terms, synonymous with affinity. Loss of staining from an area of a tissue section upon dilution of the antibody may indicate cross-reactivity with a slightly altered epitope or that there are lower concentrations of specific antigen in these areas. The former might occur when studying tissues in one species with an antibody against the orthologous isoenzyme in another species. Perhaps the best way to validate the specificity of staining with such antibodies, or indeed with any antibody, is to screen with several antibodies, each reacting with a different epitope on the protein of interest. Ideally, each antibody should produce the same pattern of staining.

1.5 Histological organization of tissues in the foetus, neonate and adult

In order to interpret the tissue distribution of isoenzymes of cytochrome P450 within an organ it is essential to consider the cellular composition of the tissues and their morphology during the latter stages of development.

1.5.1 The liver

The embryonic liver arises by proliferation and interstitial invasion of endodermal cells originating from the hepatic diverticulum, derived from the ventral floor of the foregut. There is also a mesodermal contribution brought about by proliferation and migration of mesenchymal cells. Cells from these two sources surround capillaries of the vitelline vein to establish hepatocyte plates or muralium through which the sinusoids run (Du Bois, 1963). It has been proposed that haematopoietic cells develop from the mesenchymal cells just after the appearance of parenchymal cells (Kleihauer, 1978). Thus, the foetal liver consists of two major cell types, the hepatocytes and the haematopoietic cells. Kupffer cells lining the hepatic sinusoids comprise no more than 2% of the total cell number in foetal rat liver (Greengard *et al.*, 1972). In most species studied, the haematopoietic tissue represents a substantial proportion of the liver cells at mid-gestation whilst by term the proportion has fallen dramatically to <15% in the rat, and <5% in the rabbit. The haematopoietic cells during the latter third of gestation are essentially small erythroblasts, with large, densely staining nuclei and granular cytoplasms. The hepatocyte appears as a cell with a modest number of small mitochondria, a large quantity of rough and smooth endoplasmic reticulum, a large nucleus, some peroxisomes and lysosomes and is about a third of the size of mature cells. During development, the liver enlarges by division and growth of its constituent classic lobules. Growth involves both increases in the number and size of hepatocytes, counterbalanced in part by a reduction in the diameter of the sinusoids (MacSween & Scothorne, 1979). Some central veins begin to divide by an enlargement of pre-existing sinusoids under the moulding influence of increased blood flow (Fig. 1.2a). Eventually, division of the lobule follows by re-orientation of hepatocytes into two closely parallel rows, between which connective tissue elements later extend.

Establishment of the typical adult pattern, with hepatic plates one cell thick, a muralium simplex, is a slow process which is not complete until 10 days after parturition in the rat. At this time, hepatic plates are irregular and thick in the periportal region and straight and thin in the region of central veins (LeBouton, 1976). In the 10-20 day old rat, the liver expands its cell population primarily in periportal and midzonal regions whilst overt differentiation occurs in the centrilobular area.

The localization of cytochrome P450 in the liver has been described with respect to the structure of the classic hepatic lobule, a polyhedral prism of hepatic tissue which although clearly defined in the pig is not so in the rodent or man. The lobule is bounded by connective tissue septa (portal canals) or the regular distribution of biliary and vascular

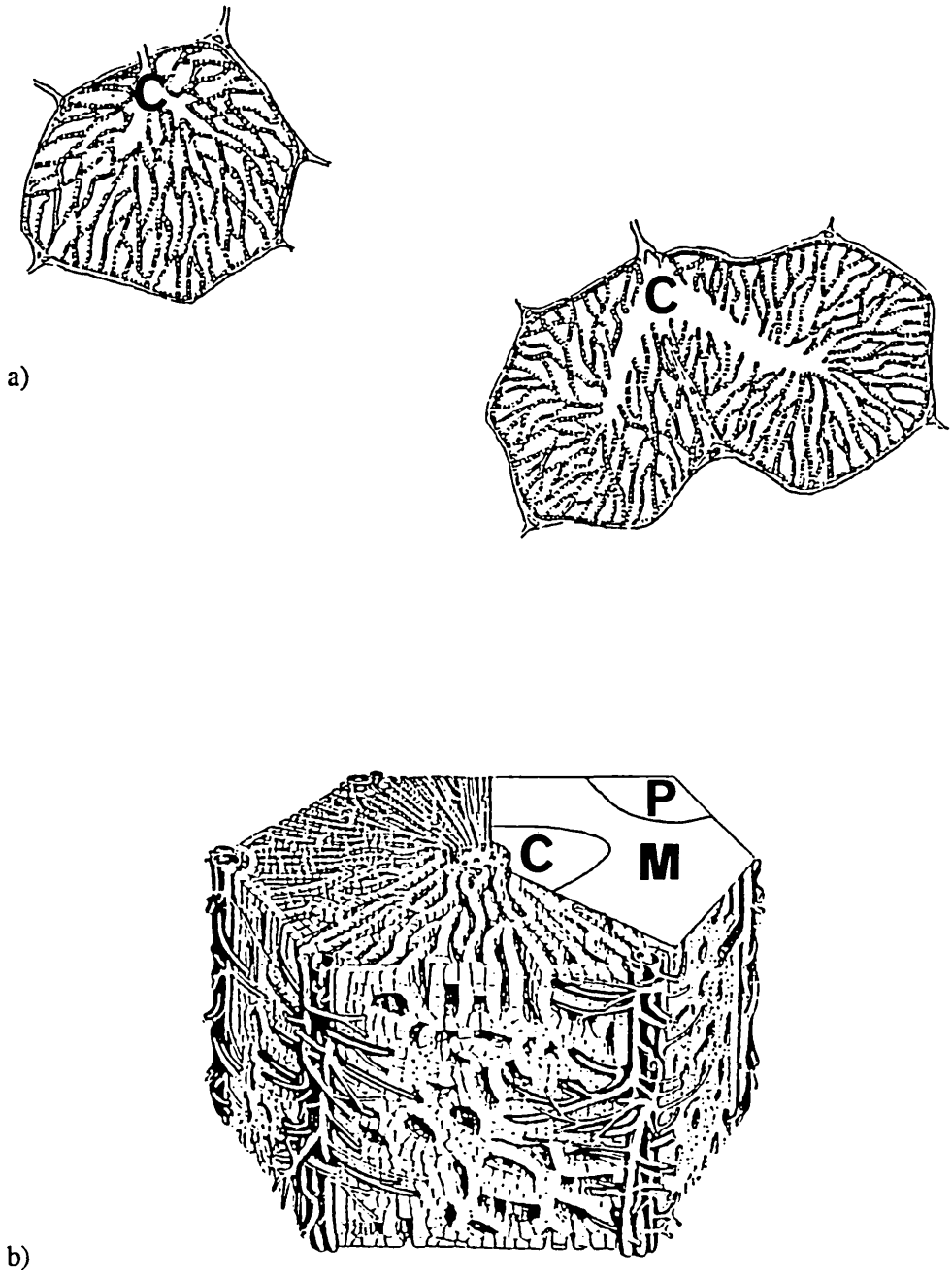


Figure 1.2: Diagrammatic representation of the classic liver lobule

(a) Two stages in the division and growth of a lobule and (b) the zonal arrangement of hepatocytes in the liver. The central vein lies at the centre of the lobule surrounded by anastomosing cords of centrilobular (C) hepatocytes. The lobule is delineated by portal triads consisting of the portal vein, hepatic artery and bile duct. Hepatocytes within a radius of 5-6 cell rows of the triads are described as periportal (P). Cells lying between these two focal points are referred to as midzonal (M).
(redrawn from MacSween & Scothorne (1979) and Jones & Spring-Mills (1988)).

vessels (portal triads) or both (Fig. 1.2b). The centre of the lobule contains the central vein or terminal hepatic venule. Anastomosing plates of hepatocytes, one cell thick, and separated on either side by narrow spaces (lacunae), radiate from the central vein to the portal areas at the periphery of the lobule. Within each lacuna is suspended a sinusoid and specialized capillary lined by a discontinuous layer of fenestrated endothelial, Kupffer and fat-storing cells (Widmann *et al.*, 1972). The lacunae form a continuous labyrinth within each lobule and their varying shape has led to the definition of two basic types of liver (Elias, 1949). There is the saccular liver, such as found in man, where the lacunae are wide and irregularly shaped and the tubular liver, such as in the rabbit, where the lacunae are narrow and almost cylindrical. A type, intermediate between the two, can be seen in the dog, which contains both types of lacunae, as well as transitional forms. Staining patterns in following chapters have been described as centrilobular, mid-zonal or periportal (Fig. 1.2b).

1.5.2 The kidney

The rat kidney consists of a single renal pyramid and is referred to as unipapillate. During the course of development three major phases are recognised: (i) pronephros, where there are only a few pronephric tubules which coalesce to form the pronephric duct; (ii) mesonephros where the kidney enlargens and consists of up to 30 glomeruli-tubular units and (iii) metanephros where nephrogenesis occurs leading to the production of the functional units of the kidney, true nephrons (Wright & Alison, 1984).

Histologically, each of these phases can be sub-divided into four stages (Larsson, 1975). Stage I occurs only in rats younger than 48h. Pronephric tubules form dichotomous branches, whilst other nephrogenic cells form sharply circumscribed masses, renal vesicles. Histologically, these are identified by their oval shape and elongated central lumen and are separated from surrounding tissue by a basement membrane (Fig. 1.3). The differentiating nephron has no primitive glomeruli (glomerular anlages) and is not connected to the collecting tubule. Stage II corresponds to the time at which 'S-shaped' bodies form and differs from stage I in three important ways. The renal vesicle has differentiated into a tubular S-shaped body, the base of which broadens into a double-layered spoon-shaped structure, the renal corpuscle or so called glomerular anlage. Capillaries lie in close proximity to the epithelial cells of the glomerular anlage and the 'S-shaped' body connects to the nephron (Fig. 1.3). Nephrons at this stage of development are common in rats up to 4-5 days of age. Stage III differs from stage II in the shape of the glomerulus and the proximal tubule. The glomerulus is oval or spherical and the capillary loops are more

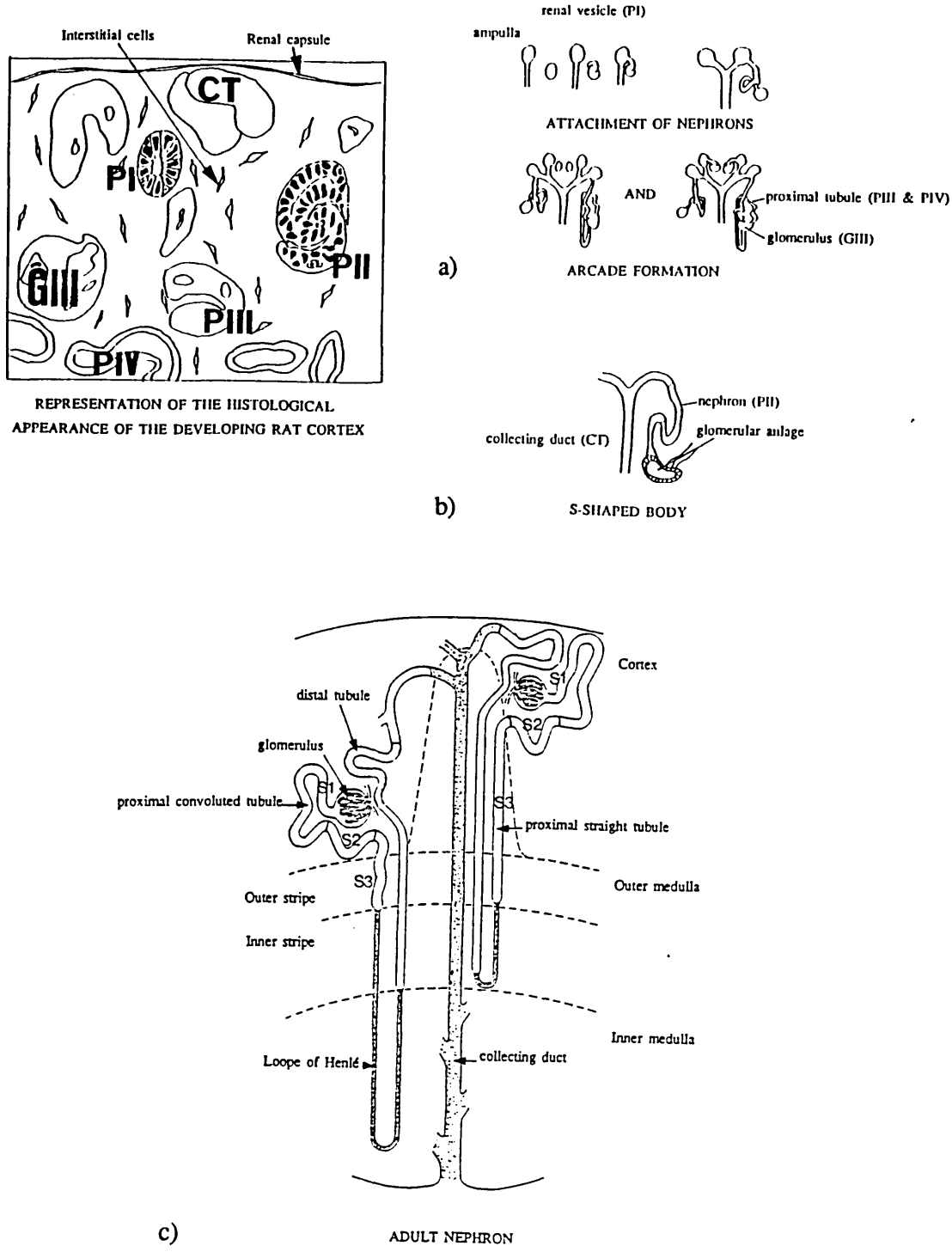


Figure 1.3: Schematic representation illustrating various stages of development of the nephron

(a) The growing end of each collecting duct, the ampulla, attaches to differentiating renal vesicles, which eventually transform into tubular structures shaped like an S (b). The segment at the end of the S broadens into a double-layered, spoon-shaped structure destined to become the glomerulus. The final phase of development is characterized by interstitial growth and continued differentiation of nephrons. (c) In the adult kidney both short- and long-looped nephrons are found. Within the cortex a medullary ray is delineated by a dashed line.

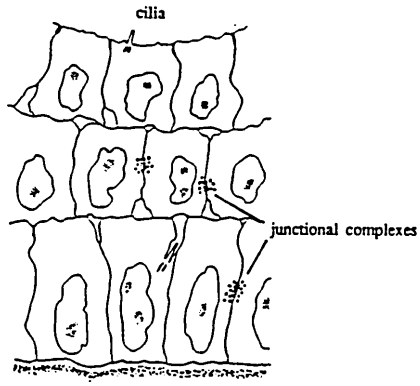
(adapted from Larsson (1975); Wright & Alison (1984) and Kriz & Bankir (1988)).

numerous. Cells lining the tubule are a homogeneous population characterized by their cuboidal shape and lack of a brush border. These nephrons are present in animals up to 5-7 days of age and are located just below the renal capsule (Fig. 1.3). The glomerulus is much larger at stage IV and cells lining the proximal tubules have a distinct brush border. The tubule lumen is also much increased but still no real differences are evident in the cells along the length of the proximal tubule (Fig. 1.3). The loope of Henlé in the nephrons has a thin epithelium much resembling that seen in adults. The final phase of development is characterized by interstitial growth and continued differentiation of nephrons. Thus on examining the kidney at any age, the oldest and most mature nephrons are nearest the medulla.

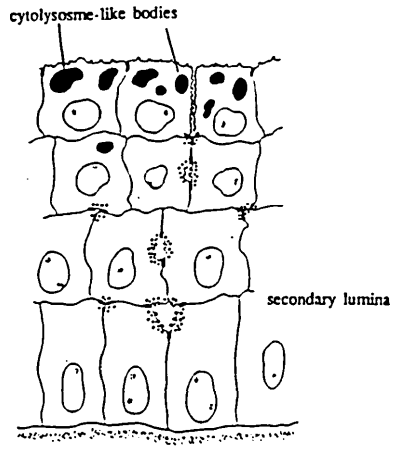
The adult kidney consists of up to 30 000 nephrons the essential components of which are illustrated in Fig. 1.3. There are two types of nephron, those situated in the outer cortex which have short loops of Henlé and those located near to the corticomedullary junction with longer loops of Henlé. As illustrated, the kidney can be subdivided into zones with particular parts of the nephron being found in each zone. Staining within the proximal tubule has been referred to as in S1, S2 or S3 segments. S1 stretches from the beginning to the middle portions of the convoluted tubule where it leads into the S2 segment. The S3 segment follows on from the S2 segment which terminates at the point where the straight part, the pars recta, begins. Generally all proximal tubule segments lying in the outer stripe are S3 segments.

1.5.3 The small intestine and pancreas

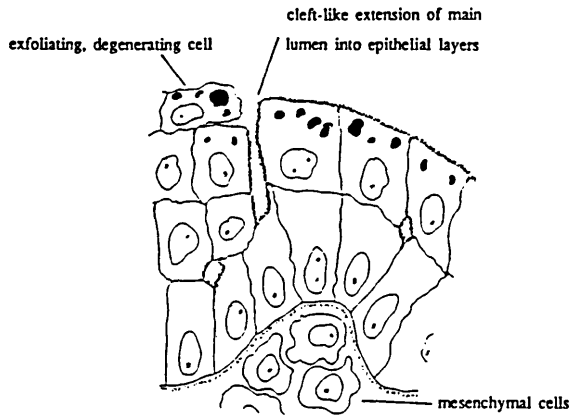
Dramatic growth and differentiation of the small intestine of foetal rats occurs during the last week of the normal 22-day gestational period (Mathan *et al.*, 1976). In the 15 day foetus the duodenal mucosa consists of a circumferential layer of undifferentiated stratified epithelium, two to three cell layers thick, surrounding a tiny lumen (Fig. 1.4). Soon afterwards, there is rapid cell proliferation in all layers and the epithelium thickens to about eight layers. Functional complexes associated with cytoplasmic vesicles and cilia-like structures are visible along the plasma membrane of adjacent cells, and these form secondary lumina over the next few days (Fig. 1.4). Aggregates of mesenchyme invaginate the basal aspect of the epithelial cell layers, which in turn begin to thin by exfoliation processes (Fig. 1.4). With consistent exfoliation and expansion, secondary lumina fuse with each other and the main lumen, and by day 19 of gestation, well-formed, short duodenal villi, lined by a relatively well differentiated single layer of columnar epithelium composed of endocrine and differentiating goblet cells, as well as absorptive



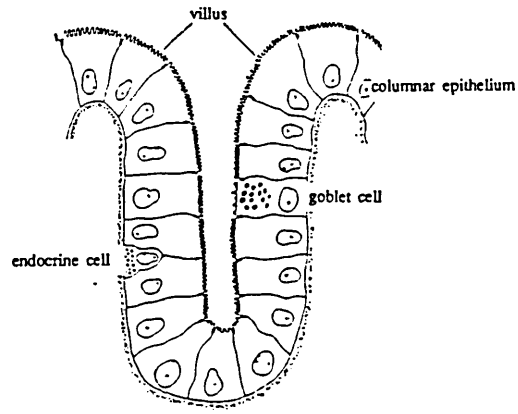
15-16 DAY FOETUS



17 DAY FOETUS



18 DAY FOETUS



19 DAY FOETUS

Figure 1.4: Diagrammatic representation of development of the duodenal mucosa in the rat

(Redrawn from Mathan *et al.* (1976))

cells, are established (Fig. 1.4). Distinct and well formed crypts do not develop in the rat intestine until several days after birth (Dunn, 1967), whereafter they continue to increase in number with increasing age whilst the number of villi remains static (Forrester, 1972).

In the mature villus, which contains up to 5000 cells in the mouse jejunum (O'Connor, 1966), four differentiated cell types are to be found, villus columnar, mucous, entero-endocrine and Paneth cells, all of which originate from a common pool of crypt-base columnar stem cells (Fig. 1.5). As stem cells divide some of the daughter cells remain *in situ* whilst others migrate in the direction of the crypt-villus junction as they undergo differentiation (Fig. 1.5). Division continues until they become non-dividing, mature villus columnar cells. A few acquire mucous globules eventually giving rise to non-dividing goblet cells. A very small proportion of stem cells acquire dense granules and small bundles of filaments to become enteroendocrine cells. Other stem cells become Paneth cells and these remain at the crypt base where they degenerate and die after 2-3 weeks. Columnar, mucous and enteroendocrine cells, connected by junctional complexes and desmosomes migrate in unison upwardly where they are eventually sloughed off into the lumen of the gut to be replaced by an endless cycle of newly produced, upwardly migrating cells. The total turnover time for migrating epithelial cells is estimated at 3-4 days (Cheng & Leblond, 1974).

The pancreas develops as an evagination from the foregut, to invade surrounding mesenchyme. The diverticular tubes undergo branching such that at 15 days of gestation, simple acinar structures are visible (Maylié-Pfenninger & Jamieson, 1980). At this stage rapid cell division occurs and cells synthesize low levels of exocrine and endocrine secretory proteins. By day 17 well demarcated centroacinar lumens are visible and cells enter a secondary transition state, such that at birth acinar cells are grouped into characteristic acini, whose centroacinar lumens are continuous with small collecting ductules lined by centroacinar cells. In the adult, the acinar (exocrine) cells form most of the gland's volume (Jamieson, 1988). These cells are typified by a pyramidal shape with the nucleus situated at the base and the secretory granules at the apex. Each acinus is surrounded by a basal lamina and the entire unit is supported by loose connective tissue. Ducts from groups of acini converge on a larger intralobular duct, forming primary lobules. Groups of lobules in turn are segregated into lobes by connective tissue where interlobular ducts are frequently seen. These ducts and the larger main collecting ducts are lined with a simple cuboidal epithelium. Cells of the endocrine pancreas are arranged together, recognisable histologically, as typical oval masses, known as the Islets of Langerhans, in the parenchyma of the exocrine pancreas (Bauer, 1988).

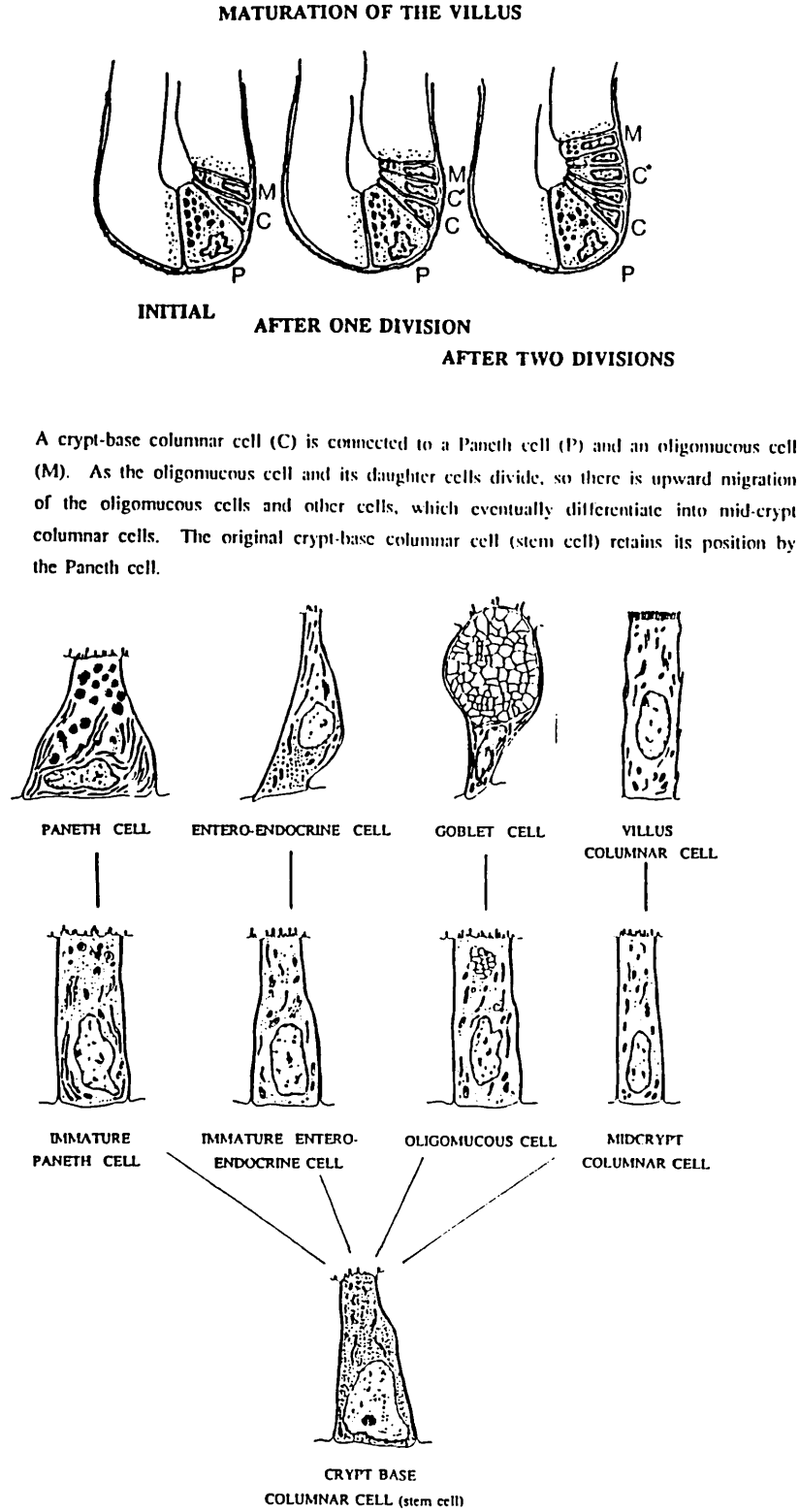


Figure 1.5: The unitarian theory of the origin of the four epithelial cell types of the adult rat small intestine

(Redrawn from Cheng & LeBlond (1974)).

1.5.4 The lung

The lung is populated by cells from two sources, a branching endodermal tube derived from the foregut and a mass of mesenchymal cells. The endodermal tube forms an uninterrupted line of cells from the bronchiolar epithelium to the type I and type II cells of the respiratory units. The mesenchyme provides the endothelial lining of the blood vessels and the interstitial cells associated with the fibrous connective tissues. Throughout the lung's development, a continuous interaction between the endothelial epithelium and the cells of mesenchymal origin plays an important role in morphogenesis (Williams, 1977).

At 16-18 days of gestation in the rat, the lung has a glandular appearance (pseudoglandular stage) where tubes lined with undifferentiated low to cuboidal columnar epithelial cells are separated from mesenchymal elements by a basement membrane layer. Cirrogenesis has begun in developing airway cells at this time and adjacent cells with bulbous or domed apexes are recognized but lack abundant smooth endoplasmic reticulum, typical of Clara cells. At about 19-20 days of gestation, the lung parenchyma consist largely of rosette-like structures, derived from continued division of endodermal tubes (canalicular phase). There is a flattening of the epithelium with consequent distension of the lumen. The mesenchyme differentiates into interstitial tissue and blood vessels including capillaries which lie in close apposition to the terminal respiratory bronchioles. Lamellar bodies begin to appear in some cells, whose presence signals initiation of type II cell differentiation. By 21 days, the lung has assumed an adult-like structure and type I and type II cells can be distinguished in mature areas, marking the beginning of the terminal sac or viable stage, although true alveoli are still absent (Burri, 1974).

At birth the lung is immature and contains no alveoli and no alveolar ducts. Gas exchange occurs in smooth walled channels and saccules, the future alveolar ducts and sacs. Lung structure remains grossly unchanged during the first 4 days of life whereafter, during the following two weeks, an explosive restructuring of the lungs occurs with rapid formation of alveoli. Respiratory bronchioles which were not present at birth are readily observed from day 10 onwards, after which time the other main changes include expansion and thinning of alveolar septa and decreases in the amount of interstitium (Burri, 1974).

In its final differentiated state, the lung is composed of a multiplicity of cells, perhaps as many as 40 different types. The lining of the respiratory airways is predominately pseudostratified, ciliated, columnar or cuboidal epithelium in which all cells are attached to

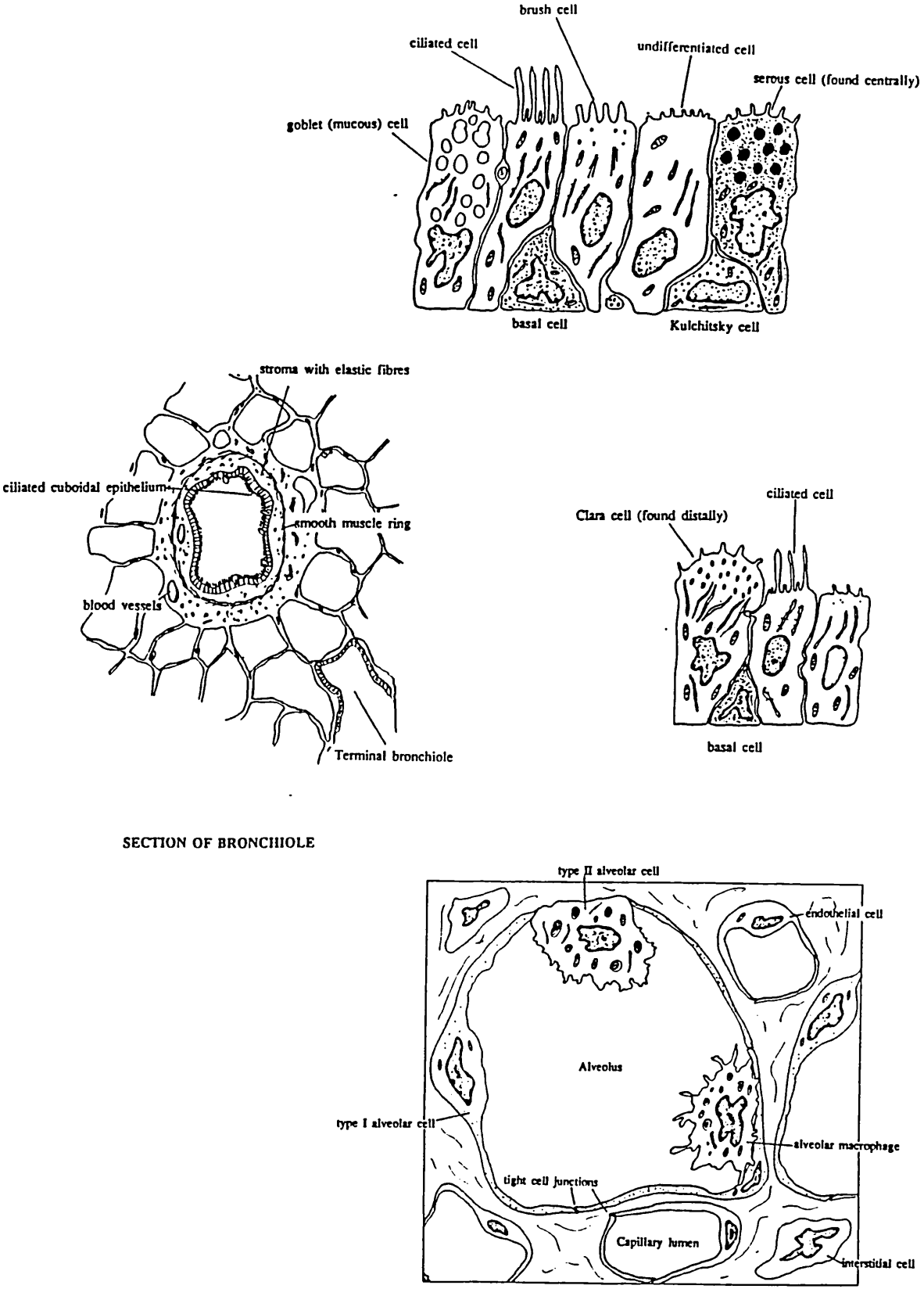


Figure 1.6: Typical cells found in large bronchi, bronchioles and alveolar walls of the adult rat lung

(Redrawn from Netter (1988))

the basement membrane, although not all are exposed at the lumen. Ciliated cells are present in even the smallest airways and respiratory bronchioles, where they are adjacent to alveolar lining cells. The architectural arrangement and major cell types found in the parenchyma of the rat lung are illustrated in Fig. 1.6.

Chapter Two

Materials and Methods

2.1 Materials

Sources of materials are listed by their manufacturer or UK distributor.

2.1.1 Chemicals

Amersham International Plc, Amersham, Bucks	Nitrocellulose (Hybond C)
BDH Chemicals Ltd, Poole, Dorset	Acetone, counterstains, CNP30 (stabilised mixture of 1,1,1-trichloroethane and trichloroethylene-based clearing agent), dichlorodifluoromethane (Arcton), Folin and Ciocalteu's phenol reagent, Formvar, hydrogen peroxide (H_2O_2), hydroquinone, synthetic mounting medium containing resin in xylene (Pertex), TissueTek II embedding medium, sodium dithionite and general laboratory solvents and reagents
Bio-Rad Laboratories Ltd, Watford, Hertfordshire	Amido black 10B, 4-chloro-1-naphthol
Fluka Chemicals Ltd, Glossop, Derbyshire	Silver lactate
ICN Biomedicals Ltd, High Wycombe, Bucks	Anti-mouse (GAM) and anti-rabbit (GAR) IgG conjugated to either horseradish peroxidase or 5, 10 or 30 nm colloidal gold
May and Baker Ltd, Dagenham, Kent	Euthatal (sodium pentobarbitone, 200 $mg.ml^{-1}$), Sagatal (sodium pentobarbitone, 60 $mg.ml^{-1}$)
MacArthy's, London	sodium phenobarbitone (PB)

National Diagnostics, Aylesbury, Bucks	Acrylamide, bis-acrylamide, sodium dodecyl sulphate (SDS), ammonium persulphate
Pelling & Cross, London	Kodak Unifix
Polysciences Ltd, Moulton Park, Northampton	Lowicryl K4M
Sigma Chemical Co. Ltd, Poole, Dorset	All other biochemicals not listed individually, which were of the highest grade available
Taab Laboratories Ltd, Reading, Berks	Araldite resin and basic copper and nickel grids

2.1.2 Animals

A. Tuck & Son Ltd, Battlesbridge, Essex	Balb/c and AKR male mice (20-30 g), Dunkin Hartley male guinea-pigs (300-400 g)
Consort Ltd, Harwood End, Hereford	Golden Syrian male hamsters (90-110 g)
Mrs. Cook, Moseley, Middlesex	Greyhound male dogs (≈28 kg; but not specifically for this study)
Froxfield Farms (UK) Ltd, Petersfield, Hampshire	6-8 day old and 14-18 day old New Zealand White male rabbits (120-190 g and 180-270 g, respectively)
Harlan Olac Ltd, Bicester, Oxon	Wistar, Sprague-Dawley, Lister-Hooded and Fischer 344 (F344) male rats (180-250 g)
ICI Plc, Alderley Park, Cheshire	Alderley Park Wistar-derived 5 and 15 day old male and female neonatal (6-8 g and 20-25 g respectively) and sexually mature female and male rats (300-350 g). Adult rats were mated overnight and the next day was considered day one of pregnancy. Foetuses were obtained at days 17/18 and 18/19 of gestation

Interfauna UK Ltd, Cambridge, Cambs	Sexually mature female New Zealand White rabbits (3-4 kg) each primed with an intravenous injection of 25 iu of Profasi (chorionic gonadotrophin) and sexually mature male and weanling rabbits (1.2-2 kg). Primed rabbits were artificially inseminated 21 days following treatment with Profasi according to ICI's standard operating procedures. Foetuses were obtained on days 26/27 and 28/29 of gestation
Labsure Animal Products, Poole, Dorset	PRD diet for mice, rats, and hamsters; RGP diet for guinea-pigs and R14B diet for rabbits

Human tissues were from the Human Tissue Bank maintained in the Department and were used with the approval of the local Research Ethics Committee

2.1.3 Specialised laboratory equipment

Cambridge-Optical, UK	2040 Autocut-Reichert-Jung microtome, Reichert-Jung Ultracut E/GA/E-82/10 microtome
Perkin-Elmer Ltd, Beaconsfield, Bucks	Model 555 split-beam scanning spectrophotometer with microprocessor controlled background correction
Pharmacia LKB Biotechnology, Central Milton Keynes, Bucks	LKB 2178 Knifemaker II, Ultroscan XL enhanced laser densitometer

2.1.4 Antibodies to the PB-inducible isoenzymes of cytochrome P450

The production and purification of two monoclonal antibodies (MAb) to rat cytochrome P450IIB1/2, 1/4 and 10/1 was as described (Sesardic *et al.*, 1986). A polyclonal antibody that reacted with both P450IIB1 and P450IIB2 together with other minor related forms, in the rat, was a kind gift from Dr. Alan Paine, St. Bartholomew's Hospital Medical School, London.

2.1.5 Antibodies to the PAH-inducible isoenzymes of cytochrome P450

A monoclonal antibody to rat cytochrome P450IA1 and P450IA2 (12/2/3/2) that has been described (Sesardic *et al.*, 1986) was used. In addition, monoclonal antibodies specific to rat

Table 2.1: A summary of the specificity of the antibodies used in this study

Antibody	P450 Isoenzymes Recognised	Mammalian species
10/1	IIB1/2	rat
1/4	IIB1/2	rat
anti-PB-P450	IIB1 and IIB2 and other variants	rat
3/4/2	IA1	mouse, rat
	IA1 and IA2	rabbit, human
12/2/3/2	IA1 and IA2	rat
107	IA2	rabbit
C8	IA1	rat
1-7-1	IA1 and IA2	mouse, rat
anti-$d_{290\text{-}296}$	IA2	mouse, rat
anti-d(-c)	IA2	rat
anti-P450IA	IA1 and IA2	mouse, rat, rabbit
B3A3	none, raised against bovine serum albumin	
Control IgG	none, pre-immune rabbit serum	

cytochrome P450IA1, C8 (Thomas *et al.*, 1984b), 3/4/2 (Boobis *et al.*, 1985b) and 1-7-1 (Cheng *et al.*, 1984) and two monospecific polyclonal antibodies to rat cytochrome P450IA2, anti-d(-c) (Reik *et al.*, 1982) and anti-$d_{290\text{-}296}$, an antibody raised against a peptide sequence (Ser-Glu-Asn-Tyr-Lys-Asp-Asn) unique to cytochrome P450IA2 in the rat (Edwards *et al.*, 1988; Edwards *et al.*, 1990) were employed in this study. A monoclonal antibody, 107, raised against rabbit P450IA2 (Boobis *et al.*, 1981) and a polyclonal antibody (referred to as anti-P450IA) recognising IA1 and IA2, a kind gift from Dr. J. Hardwick, Argonne National Laboratories, Argonne, were used to monitor the distribution and expression of these isoenzymes in the rabbit.

The specificity of the antibodies described in Sections 2.1.4 and 2.1.5, in addition to B3A3, a monoclonal antibody raised against bovine growth hormone, which was used as a negative control, is summarised in Table 2.1.

2.1.6 Miscellaneous

The following items were kind gifts from the persons or institutitions indicated

G. D. Searle, Skokie, Illinois	Pregnenolone-16α-carbonitrile (PCN)
ICI Plc, Pharmaceuticals Division, Macclesfield, Cheshire	Clofibrate (CL)
Dr. R. J. Edwards, RPMS, London	Enriched rat cytochrome P450IA2 preparation, 5 nmoles.ml^{-1} and enriched rabbit cytochrome P450IA2 preparation, 1.5 nmoles.ml^{-1}.
Dr. S. Baker, RPMS, London	Purified rat cytochrome P450IA1, 14 nmoles.mg^{-1} protein.
Dr. A. Paine, St. Bartholomew's Hospital Medical School, London	Enriched rat cytochrome P450IIB1 preparation, 10.5 nmoles.mg^{-1} protein
Dr. C. R. Wolf ICRF, Oncology Unit, Edinburgh	Enriched cytochrome P450IIB2 preparation, 20.2 nmoles.mg^{-1} protein

2.2 Methods

Adult mice, hamsters, rats, guinea-pigs and rabbits were housed in groups of 1-24, 1-3, 1-5, 1-3 or singly per stainless steel grid-bottomed cage, respectively. Pregnant rats or rabbits were housed singly with nesting facilities and dams with their litters. All animals were subject to a controlled 12 h light and 12 h dark lighting regimen and had free access to food and water.

2.2.1 Treatment of animals with inducing compounds

2.2.1.1 Treatment of rats with PB

Male Wistar, Sprague-Dawley, Lister-Hooded and F344 rats were treated daily with PB in saline (8 $mg.ml^{-1}$), 80 $mg.kg^{-1}$ body weight, for 4 days by intraperitoneal (i.p.) injection. Animals were killed 24 h after the last dose.

2.2.1.2 Treatment of rats with MC

Non-pregnant adult female, adult male and neonatal (3 and 13 day old) Alderley Park, Wistar-derived rats were injected i.p. with MC dissolved in corn oil (8 $mg.ml^{-1}$), 80 $mg.kg^{-1}$ body weight, 48 h prior to being killed. Groups of pregnant rats received similar doses on a kg^{-1} body weight basis on days 16/17, 17/18 and 18/19 of gestation. Foetuses/newborns were killed 48 h later, after caesarian section if necessary.

2.2.1.3 Treatment of rats with isosafrole (ISF)

Male Wistar rats were treated daily with ISF dissolved in corn oil, 160 $mg.kg^{-1}$ body weight, by i.p. injection for 4 days and were killed 24 h after the last dose.

2.2.1.4 Treatment of rats with isoniazid (INH)

Rats received INH in their drinking water, at a concentration of 0.1% (w/v) and adjusted to pH 7.4 with 100 mM HCl, for 11 days. They received a further single i.p. injection of INH in saline, 50 $mg.kg^{-1}$ body weight, 3 h prior to being killed.

2.2.1.5 Treatment of rats with CL

Male Wistar rats were treated daily with CL in saline, 200 $mg.kg^{-1}$ body weight, for 3 days by i.p. injection and were killed 24 h after the last dose.

2.2.1.6 Treatment of rats with acetone

Male Wistar rats were treated daily with acetone as a 25% (v/v) solution, 5 $ml.kg^{-1}$ body weight, intragastrically 22 h prior to being killed.

2.2.1.7 Treatment of rats with PCN

Male Wistar rats were treated daily with PCN dissolved in corn oil, 100 $mg.kg^{-1}$ body weight, for 3 days by i.p. injection and killed 24 h after the last dose.

2.2.1.8 Treatment of rabbits with MC

Non-pregnant adult female, adult male, neonatal (4, 8 and 16 day old), male and female weanling New Zealand White rabbits received a single i.p. injection of MC in corn oil, 100 $mg.kg^{-1}$ body weight, 48 h prior to killing. Groups of pregnant rabbits received similar doses on a kg^{-1} body weight basis on days 25/26, 27/28 and 29/30 of gestation. Foetuses/newborns were killed 48 h later, after caesarian section if necessary.

2.2.2 Preparation of frozen tissue blocks

With the exception of 9-21 day old, weanling and adult rabbits, all animals were killed by stunning and/or decapitation. Neonatal/weanling and adult rabbits were killed by overdosing with Sagatal or Euthatal, administered by i.p. injection or via a marginal ear vein, respectively.

One or more of the following organs were immediately removed from the animals when dead: livers, some of which were perfused *in situ* with ice-cold 0.9% NaCl, kidneys, 1-2 cm lengths of proximal upper small intestine with pancreas attached and testes. Slices of approximately 0.5 cm thickness were cut from the left lateral or median lobes of the liver and from the other organs, centred onto cork discs and rapidly frozen in melting Arcton (at -156°C; pre-cooled in liquid nitrogen). Lungs were inflated with a 1:1 (v/v) solution of TissueTek embedding medium in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4) via the trachea and rapidly frozen as above in liquid nitrogen. Slices were then cut from the whole organ at -20°C in a cryostatic chamber and embedded onto cork discs in TissueTek embedding medium prior to sectioning. Rat foetuses and neonates were cut open down the ventral midline and slowly frozen whole in liquid nitrogen.

2.2.3 Preparation of paraffin-embedded tissue blocks
(Voigt *et al.* 1985)

Tissues were fixed for 1.5-3 h maximum, in 95% (v/v) ethanol containing 1% (v/v) acetic acid. Lungs were inflated with the same solution and, after securing of the trachea, placed in approximately 100 ml of fixative. Tissues were then taken through absolute alcohol (1 h), toluene (1 h) and molten wax at 60°C (1 h) and were allowed to harden overnight prior to embedding. For embedding, samples were replaced in wax (1 h) at 60°C and taken through two further wax baths (30 min each) and finally incubated in wax under vacuum for 30 min to remove any air bubbles. Samples were then 'blocked' in wax and allowed to harden.

2.2.4 Fixation, dehydration and high temperature embedding of tissues for electron microscopy

2.2.4.1 Millonig's osmium tetroxide (OsO_4) fixative
(Millonig, 1961)

To 25 ml of 4% (w/v) OsO_4 was added 1.69 g NaH_2PO_4, 0.54 g glucose, and 48.2 ml 0.2 M NaOH. The solution was diluted to 100 ml and stored at 4°C and discarded when it turned black.

2.2.4.2 Preparation of 2% *p*-formaldehyde containing 0.2% glutaraldehyde

A 10% (w/v) solution of *p*-formaldehyde was prepared by dissolving in distilled water heated to 60°C and then adding a few drops of 1N NaOH until the solution cleared. The final fixative solution was prepared by adding 1 ml of 25% (v/v) glutaraldehyde to 20 ml of 10% (w/v) *p*-formaldehyde and bringing to a final volume of 100 ml with 0.1 M sodium phosphate buffer, pH 7.4.

2.2.4.3 Araldite resin

Solution	**ml**
Agar 100	25
Araldite CY212 or CY219	15
Dodecanyl succinic anhydride	55
Di-butyl phthalate	2
DMP 30	1.5

Araldite resin was prepared by mixing the above reagents together well and using within 12 h.

The lateral or median lobe of the liver was removed, chopped finely into strips, no thicker than 1-2 mm, and fixed immediately in either 4% (v/v) glutaraldehyde in PBS for 1-2 h followed by post-fixation in Millonig's OsO_4 for 1 h or 2% (w/v) *p*-formaldehyde containing 0.2% (v/v) glutaraldehyde for 30-45 min with continuous agitation. Tissue was dehydrated by sequential incubation (1 h each) in an ascending series (50, 70, 90 and 100 % (v/v)) of acetone solutions. Tissue was washed in absolute acetone twice (1 h) and resuspended and incubated in 50% (v/v) Araldite resin in absolute acetone overnight. Tissue was further incubated in increasing concentrations of resin (70 and 90 % (v/v)) for a minimum of 1 h in each and finally resuspended and incubated in two changes of absolute resin. Specimens were finally embedded in fresh resin by polymerization at 60°C for 24-36 h. Blocks destined for immunocytochemical studies were slightly under 'cured', polymerization taking place for no more than 18 h.

2.2.5 Fixation, dehydration and low temperature embedding of tissues for electron microscopy
(Robard & Sleytr, 1985)

2.2.5.1 Lowicryl K4M Resin

This resin consisted of 2.7 g crosslinker, 17.3 g monomer B and 0.1 g initiator C, which were mixed well, care being taken not to introduce air bubbles, just prior to use.

Strips of liver were prepared and fixed as described in Section 2.2.4. Tissues were dehydrated by sequential incubation (1 h each) in an ascending series of ethanol solutions (50, 70, 90 and 100 % (v/v)). With the exception of fixation and incubation in 50% (v/v) ethanol, all stages were performed at -20°C. The tissues were transferred through two further changes of absolute ethanol (1 h each) before being incubated (1 h minimum) in increasing concentrations of Lowicryl K4M resin in absolute ethanol (50, 70 and 90 % (v/v)). After two changes in absolute resin, tissue pieces (≈1-2 mm^3) were placed into gelatin capsules and embedded in absolute resin by polymerization by indirect UV light for 2-3 days at -20°C.

2.2.6 Cryostat sectioning of frozen tissue blocks

2.2.6.1 Preparation of poly-L-lysine-coated slides
(Huang *et al.*, 1983)

Glass slides were cleaned with acetone and smeared with a drop of poly-L-lysine (M_r >150 000; 10 $\mu g.ml^{-1}$). A smooth film, thin enough for interference colours to be seen, was produced. This film dried rapidly at which point the slides were ready to use. Poly-L-lysine was stored in vials at 1 $mg.ml^{-1}$ at -20°C until required for use.

Sections of 7-10 µm thickness were cut from the frozen tissues, on a Slee cryostatic microtome at a temperature dependent on the organ ie. liver was cut at -14°C, kidney at -18°C and other organs containing more connective tissue at -22 ± 2°C. Sections tended to detach from normal slides during incubations and were therefore mounted onto slides coated with poly-L-lysine as described above and air dried overnight at room temperature. Slides were then placed back to back, wrapped in foil and stored with desiccant in sealed polythene bags, at -80°C until required for immunocytochemistry.

2.2.7 Sectioning of wax-embedded tissue blocks

Blocks were prepared for sectioning by trimming on a 2040 Reichert-Jung microtome and then placed on ice blocks face upwards for 30 min. Sections (5 µm) cut from blocks, previously cooled face downwards on ice just prior to sectioning, were floated onto 30% (v/v) alcohol and then onto water in a thermostatically controlled water bath at 50-60°C, containing approximately 0.5 g each of potassium dichromate and leaf gelatin. Flattened sections were picked up onto slides, allowed to air dry for 5-10 min at room temperature and then overnight at 37°C. Thereafter sections were stored in racks at 4°C until required for immunocytochemistry.

2.2.8 Ultrathin sectioning of Araldite/Lowicryl-embedded tissue blocks

2.2.8.1 Preparation of glass knives

Glass knives (45° angle) were made using an LKB 2178 Knifemaker II. Strips of glass were washed thoroughly with detergent, rinsed and dried just prior to use.

Excess resin was trimmed away from the edge of specimens with a razor blade. Blocks were fixed in the chuck of a Reichert-Jung UK Ultracut microtome and aligned with the knife (knife angle 3-6°, depending on the resin type). After cutting down into the block (advance feed set at 0.5-1 µm) and reaching the specimen, gold coloured sections (90-120 nm) were cut (advance feed automatic, speed 2-3 $mm.sec^{-1}$) and mounted onto either uncoated or Formvar-coated copper or nickel grids (Hayat, 1970).

2.2.9 Preparation of microsomal fractions

2.2.9.1 Procedure 1
(Atlas *et al.*, 1977)

This procedure was used for the preparation of microsomal fractions from tissues of most

animals, with the exception of the rats used for the ontogenic study. Livers, kidneys and lungs from foetal and neonatal rabbits were pooled by litter for each experimental group and age point. Kidneys and lungs from groups of 9-11 day old and 17-21 day old rabbits were also pooled. A 20% (w/v) homogenate in 0.25 M potassium phosphate buffer, pH 7.25, containing 1 mM EDTA and 0.15 M KCl, was prepared by chopping the tissues with scissors and then homogenizing for 3 x 30 sec with a Polytron tissue homogenizer (Kinematica) at setting 6. Samples were centrifuged at 10 000 x g for 20 min at 4°C. Microsomes were harvested from the 10 000 g supernatants by centrifugation at 105 000 x g for 75 min. The microsomal pellets were resuspended in 0.25 M potassium phosphate buffer, pH 7.25 containing 30% (v/v) glycerol (≈1 volume.g^{-1} wet weight of liver and 0.5 volume for extrahepatic tissues), by hand homogenization with a Potter-Elvehjem Teflon-glass homogenizer. Aliquots of microsomal fractions (15-20 mg protein.ml^{-1}) were frozen at -80°C until required.

2.2.9.2 Procedure 2
(Cresteil *et al.*, 1979)

Livers and lungs from foetal and neonatal rats were each pooled by litter, there being a minimum of 6 animals per litter; kidneys from all litters for a given time point and treatment were pooled for microsome preparation. For pups of 5 and 15 days old, microsomes were prepared from a pool of 2 livers; a pool of 4-8 pairs of kidneys and a pool of 2 lungs. A 10% (w/v) homogenate in 0.25 M sucrose containing 1 mM Tris-HCl buffer, pH 7.4 and 1 mM EDTA, was prepared and subjected to differential ultracentrifugation as described in Section 2.2.9.1. Microsomal pellets were resuspended in 150 mM Tris-HCl buffer, pH 7.4 containing 30% (v/v) glycerol, also as described in Section 2.2.9.1.

This protocol has been shown to give the best recovery of microsomes, with little contamination from mitochondria, from foetal and neonatal rat tissues (Fig. 2.1). The addition of 1 mM EDTA prevents the aggregation of ribosomes in polysomes and the aggregation of microsomes amongst themselves or with mitochondria.

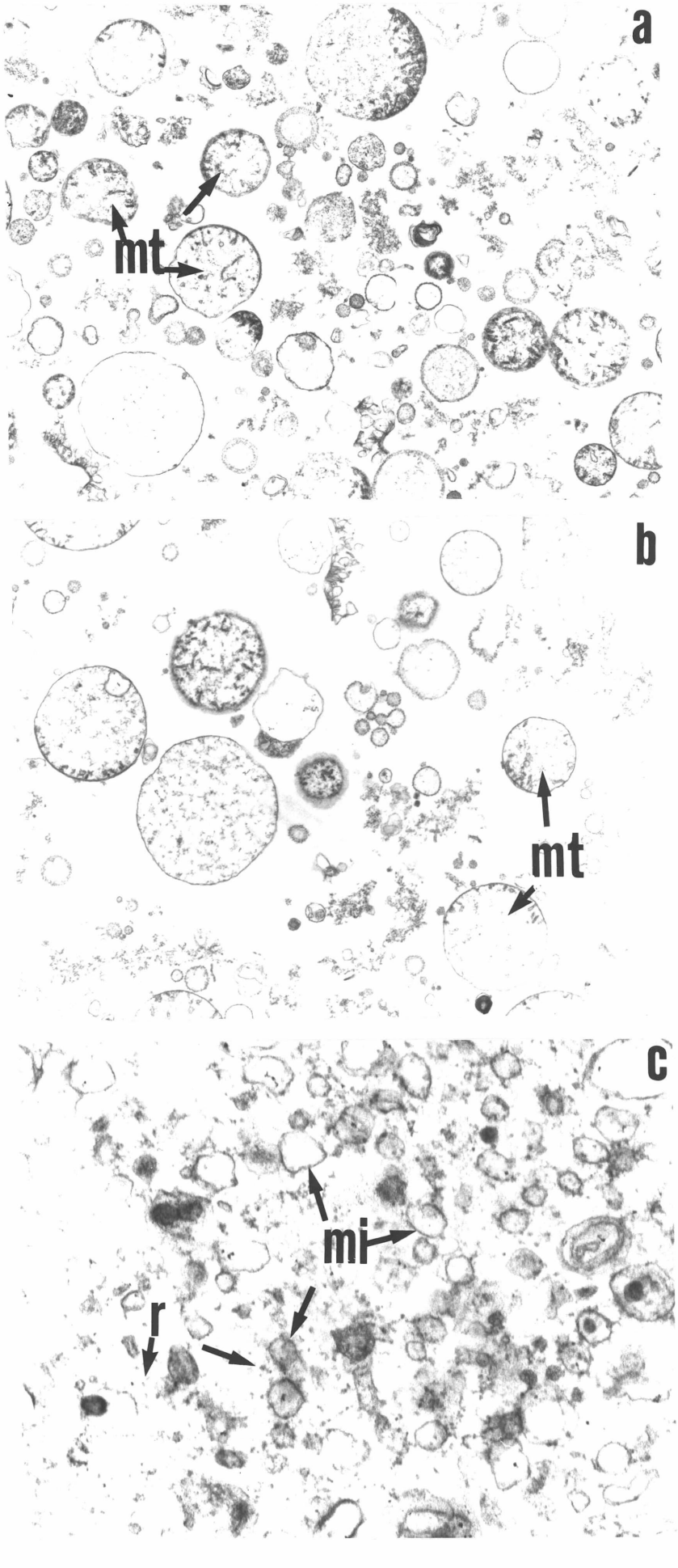
2.2.10 Protein Assay
(Lowry *et al.*, 1951)

A 1ml aliquot of 3% (w/v) Na_2CO_3 in 0.1 M NaOH containing 0.01% (v/v) copper sulphate and 0.02% (v/v) potassium tartrate was added to 0.2 ml of sample (containing 75-400 µg protein) and mixed thoroughly. After 10 minutes, 0.1 ml of Folin and Ciocalteu's phenol reagent (diluted 1:1.5 (v/v) with distilled water) was added and the solution mixed vigorously. Colour was allowed to develop for 40 min and the absorbance determined at 660 nm. The

Figure 2.1: Electron micrographs of (a) whole homogenate, (b) 10 000g and (c) 105 000g pellets prepared from livers of MC-treated 19/20 day old foetuses

mt, mitochondria; mi, microsomes; r, ribosomes

Magnification, (a, b) x9 900 and (c) x42 750.


a
mt
b
mt
c
mi
r

amount of protein was estimated by reference to a standard curve prepared using bovine serum albumin (BSA; 50-350 $\mu g.ml^{-1}$).

2.2.11 Spectroscopic determination of cytochrome P450

2.2.11.1 Procedure 1
(Omura & Sato, 1964)

Microsomes were diluted in 0.25 M potassium phosphate buffer, pH 7.25, containing 30% (v/v) glycerol, at 4°C to a final volume of 3 ml, such that the final protein concentration ranged between 0.5-1.0 $mg.ml^{-1}$. The suspension was reduced by the addition of a few mg of sodium dithionite and 1 ml added to each of two cuvettes. After 3 min a baseline spectrum was recorded between 400 and 500 nm and the contents of the sample cuvette then bubbled with carbon monoxide for 1 min. A second scan over the same range was recorded with correction for the background. The content of cytochrome P450 was determined from the absorbance difference between 450 and 490 nm, assuming an extinction coefficient of 91 $mM^{-1}cm^{-1}$. The monochromator was calibrated using a holmium crystal filter (Perkin Elmer) each time the instrument was used.

2.2.11.2 Procedure 2
(Johannesen & DePierre, 1978)

Microsomes, prepared from foetal livers and all extrahepatic tissues, were diluted as in Section 2.2.11.1. The suspension was then bubbled for 1 min with carbon monoxide and 1 ml added to each of two cuvettes. A baseline spectrum was recorded as in Section 2.2.11.1 and then a few mg of sodium dithionite added to the sample cuvette, and mixed well. After 3 min a second scan over the same range was recorded with background correction and the cytochrome P450 content determined as described in Section 2.2.11.1, assuming an extinction coefficient of 105 $mM^{-1}cm^{-1}$.

2.2.12 Solid-phase enzyme-linked immunosorbent assay (e.l.i.s.a.)
(Engvall, 1980)

Wells of polystyrene microtitre plates (Dynatech, UK Ltd) were coated with microsomal fractions prepared from untreated and xenobiotic-treated rats, diluted to a protein concentration of 100 $\mu g.ml^{-1}$ (100 $\mu l.well^{-1}$) or with partially purified or apparently homogeneous preparations of cytochrome P450, diluted to a concentration of 40 $pmol.ml^{-1}$ (100 $\mu l.well^{-1}$) with 50 mM sodium carbonate buffer, pH 9.5. After incubation at 37°C for 1 h non-specific binding sites were blocked by incubation with 3% (w/v) BSA (100 $\mu l.well^{-1}$) for 1 h.

Primary antibody, over a range of 0.1-10 μg.well^{-1}, was applied for 1 h, removed by washing, followed by horseradish peroxidase conjugated to either rabbit anti-mouse or GAR IgG (1:2000 dilution, 100 μl.well^{-1}) as appropriate to the primary antibody in the presence of 1 % (v/v) normal rat serum (to reduce non-specific binding), again for 1 h. After each stage of the assay, plates were washed extensively with PBS containing 0.05% (v/v) Tween 20 (3 x 5 min). Development was with 100 μl of a solution of 2.8 mM 1,3-phenylenediamine dihydrochloride, 4.4 mM H_2O_2 in a buffered solution containing 50 mM citric acid and 100 mM Na_2HPO_4, pH 5.0. After 10 min the absorbance of the coloured product was determined at 492 nm using an automatic colorimetric plate reader.

2.2.13 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemlli (1970) as described by Boobis *et al.* (1985b), using 9% polyacrylamide as the running phase and 4% polyacrylamide for the stacking phase. Gels were run at 40 mA through the stacking gel and 100 mA through the running gel until the dye front reached the bottom edge of the gel.

2.2.13.1 Sample Preparation

Microsomal samples were diluted to a final protein concentration of 2 mg.ml^{-1} in 62.5 mM Tris-HCl buffer, pH 6.8 containing 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 1% (w/v) SDS and 0.002% (w/v) bromophenol blue (Sample buffer). Prior to use they were placed in boiling water for 2 min and then onto ice to cool. Samples were loaded into prepared wells (5-60 μg microsomal protein or 1-10 pmoles purified cytochrome P450).

2.2.13.2 Molecular weight markers

A mixture of marker proteins was also applied to gels. Pre-stained molecular weight marker proteins (α_2-macroglobulin, M_r 180 000, 5 μg; ß-galactosidase, M_r 116 000, 10 μg; fructose-6-phosphate kinase, M_r 84 000, 10 μg; pyruvate kinase, M_r 58 000, 10 μg; fumarase, M_r 48 500, 10 μg; lactate dehydrogenase, M_r 36 500, 15 μg and triosephosphate isomerase, M_r 26 600, 15 μg, dissolved in Sample buffer to a final volume of 2 ml) were applied to gels (10 μl.well^{-1}) that were to be transferred to nitrocellulose paper. Unstained molecular weight marker proteins were applied to gels destined for Coomassie blue staining.

2.2.13.3 Coomassie blue staining of gels

Gels were stained in 0.2% (w/v) Coomassie Brilliant Blue R250 dissolved in 50% (v/v) methanol containing 10% (v/v) acetic acid (filtered prior to use through Whatman No.1) and

then destained in 50% (v/v) methanol containing 10% (v/v) acetic acid and cleared with 7% (v/v) acetic acid.

2.2.14 Electrophoretic transfer of proteins to nitrocellulose (Western blotting)
(Burnette, 1981)

Microsomal proteins were subjected to SDS-PAGE as described in Section 2.2.13. At the end of the running period gels were equilibrated in blotting buffer (25 mM Trizma base containing 192 mM glycine and 20% (v/v) methanol which had a pH of 8.3) for 10-15 min. Nitrocellulose sheets were treated similarly. The gel and a nitrocellulose sheet were sandwiched between sheets of filter paper and foam pads and inserted into an electrophoresis tank filled with blotting buffer. Blotting was carried out in a standard field (60 mV) at room temperature for 1 h. At the end of the electrophoretic transfer period nitrocellulose sheets were removed and immunostained with appropriate antibodies.

2.2.15 Preparation of dot blots

Dot blotting was performed using a Bio-Dot microfiltration apparatus from Bio-Rad Laboratories. The samples were diluted appropriately in PBS, pH 7.4 and aliquoted directly onto the nitrocellulose membrane through the wells by applying suction with a vacuum pump. Several identical blots were prepared, one being stained for protein with Amido black and the other(s) immunostained with appropriate antibodies.

2.2.16 Immunostaining of proteins on nitrocellulose

Nitrocellulose sheets containing transferred electrophoresed protein (Western blots) or dot blots were washed in PBS for 2-5 minutes. Non-specific binding sites were blocked by incubating in 3% (w/v) BSA in 0.9% (w/v) NaCl for 1 h. Following 2 x 5 min washes in PBS/0.05% (v/v) Tween 20, the nitrocellulose sheets were incubated with primary antibody for 3 h at 25°C. The nitrocellulose sheets were washed as before and then incubated with horseradish peroxidase conjugated to either rabbit anti-mouse or GAR IgG (diluted 1:2000) as appropriate for 90 min at room temperature. Following sequential washes (4 x 5 min in PBS/0.05 % (v/v) Tween 20 and 2 x 5 min in PBS alone) specific antibody binding was revealed by development in PBS containing 1 mM 4-chloro-1-naphthol and 0.01% (v/v) H_2O_2 for 15 min. The reaction was quenched with running tap water.

2.2.17 Amido black staining
(Towbin *et al.*, 1979)

Blots were stained with 0.1% (w/v) amido black, dissolved in 40% (v/v) methanol containing 10% (v/v) acetic acid, for 2 min. They were subsequently destained in 40% (v/v) methanol containing 10% (v/v) acetic acid. The limit of detection using this stain was 0.05 µg protein.

2.2.18 Indirect immunostaining of sections for light microscopy
(Polak & van Noorden, 1987)

Frozen tissue sections, warmed to room temperature, were fixed for 15-20 minutes in cold acetone and then rehydrated in PBS, pH 7.4. Wax sections were de-paraffinized by bringing to water by sequential incubations (1 min each) in two changes of CNP-30, absolute alcohol, 90% (v/v) alcohol, 70% (v/v) alcohol, 30% (v/v) alcohol and finally water. Non-specific binding sites were blocked by incubation in PBS containing 3% (w/v) BSA for a minimum of 10 min. Excess blocking solution was drawn off with a tissue, care being taken not to let the sections dry. To each section was applied a drop (≈20-30 $\mu l.cm^{-2}$ section) of polyclonal (stock protein > 20 $mg.ml^{-1}$) or monoclonal or anti-peptide (stock protein = 1 $mg.ml^{-1}$), antibodies using a range of dilutions. Sections were incubated overnight in a humidified chamber at 4°C. The secondary antibody, horseradish peroxidase conjugated to either rabbit anti-mouse or goat anti-rabbit IgG as appropriate, diluted 1:200 in PBS containing 5% (v/v) normal rat serum, was applied for 90 min at room temperature. Sections were washed, 3 x 5 min in PBS following antibody incubations. Specific antigen-antibody complexes were visualized by development in 0.025% (w/v) DAB in PBS, pH 7.4 containing 0.03% (v/v) H_2O_2 for 20 min. The reaction was quenched by rinsing slides in running tap water for 5 min. Nuclei were lightly counterstained (1-2 min) with Cole's haematoxylin and sections were then dehydrated through an ascending series of alcohols, cleared in two changes of CNP-30 and mounted in Pertex.

The inducibility of cytochrome P450 in each tissue examined was assessed semi-qualitatively from the changes in the staining intensities of the sections when the antibodies were used at varying dilutions ie induction of cytochrome P450 in a tissue results in binding of the antibody at lower concentrations (higher dilutions) than in tissues from untreated animals. A minimum of 5 sections for each tissue were examined, there being at least 3 animals per group. At no time were interindividual differences in staining within a group greater than those between groups.

2.2.19 Immunogold-silver staining of sections
(Springall *et al.*, 1984)

Frozen sections were fixed in acetone as described in Section 2.2.18. Slides were then immersed in Lugol's iodine (1% (w/v) iodine and 2% (w/v) potassium iodide) for 5 min, rinsed briefly in running water and de-colourized in 2.5% (w/v) sodium thiosulphate solution. After thorough washing (≈10 min) in running water, sections were equilibrated in PBS, pH 7.4 (2 x 5 min). Undiluted normal goat serum was applied to sections and drained off after 10 min. Sections were then exposed to appropriately diluted primary antibody overnight at 4°C as described in Section 2.2.18. They were subsequently washed in 2 x 10 min changes of PBS, pH 7.4 and 2 x 10 min changes of Tris buffered saline (TBS 8.2: 50 mM Tris-HCl, pH 8.2, containing 2.5% (w/v) NaCl). Normal goat serum was applied as before, sections drained of serum and incubated with GAM IgG adsorbed to 5 nm colloidal gold, diluted 1:100 in TBS 8.2 containing 0.8% (w/v) BSA, for 60 min. Sections were washed thoroughly in 3 x 10 min changes of TBS 8.2 and 6 x 5 min washes in distilled water with agitation. This washing had to be very thorough to ensure that all halide was removed prior to silver development.

Slides were then transferred to the physical silver development solution of Moeremans *et al.* (1984) and allowed to develop in the dark for 5 min. Sections were then washed in photographic fixer, Kodak Unifix, diluted 1:4, for 1-2 min followed by 10 min in running tap water. Sections were then counterstained with Cole's haematoxylin and 0.1% (w/v) yellowish eosin or haematoxylin alone, dehydrated through an ascending series of alcohols, cleared in two changes of CNP-30 and mounted in Pertex.

2.2.20 Elemental silver development solution

All glassware was washed in 5% (v/v) nitric acid prior to use.

Stock solutions

i)Citrate buffer	23.5 g trisodium citrate dihydrate and 25.5 g citric acid dissolved in 850 ml distilled water
ii)Reducing agent	850 mg hydroquinone, dissolved in 85 ml of citrate buffer just prior to use.
iii)Silver lactate	110 mg silver lactate dissolved in 15 ml distilled water just prior to use.

The reducing agent and silver lactate solution were mixed together in subdued light, when required.

2.2.21 Cole's haematoxylin
(Stevens, 1977)

Haematoxylin (3 g) was dissolved in 25 ml alcohol and further diluted with 500 ml water. To this was added 100 ml 1% (w/v) iodine in 95% (v/v) alcohol. To this cocktail was further added 1400 ml of saturated aqueous ammonium alum (iron ammonium sulphate). The haematoxylin solution was artificially ripened by bringing to the boil. After cooling, the solution was bottled and stored in the dark. The solution was filtered prior to use.

2.2.22 Immunocytochemistry of ultrathin sections
(Varndell & Polak, 1984)

All solutions used in this technique were filtered prior to use through 0.45 µm Millipore filters. Sections from Araldite-embedded tissues only were 'etched' by placing grids face down onto 50 µl droplets of 10% (v/v) H_2O_2 for 10 min and then washed briefly in distilled water (3 x 1 min) prior to staining. Excess aldehyde groups were blocked by placing grids onto droplets of 20 mM glycine in PBS, pH 7.4, for 10-15 minutes. After two washes in PBS, non-specific binding sites were blocked by incubation with normal goat serum, diluted 1:30 in PBS, for 30 min. Sections were then placed face down onto 10 µl droplets of varying dilutions of primary antibody, diluted in PBS containing 0.1% (w/v) BSA (PBS/BSA) and left overnight at room temperature in a humidified atmosphere. Sections were thoroughly washed on 200 µl droplets of PBS/BSA (3 x 15 min), followed by 3 x 15 min changes on 200 µl droplets of 50 mM Tris-HCl buffer, pH 8.2. They were then placed face down onto droplets of GAR IgG conjugated to either 10 or 30 nm colloidal gold, diluted 1:50 in 50 mM Tris-HCl buffer, pH 8.2 containing 1% (w/v) BSA (TBS/BSA), for 60 min. The secondary antibody solution was centrifuged at 2 000 g x 20 min prior to use to remove any aggregates. Sections were washed thoroughly (3 x 10 min) in TBS/BSA buffer then briefly in several changes of distilled water and allowed to dry before counterstaining with uranyl acetate and lead citrate.

2.2.23 Counterstaining of ultrathin sections

2.2.23.1 Reynold's Lead Citrate
(Lewis & Knight, 1977)

Lead citrate (1.33 g) and sodium citrate (1.76 g) were added to 30 ml distilled water. In

order to complete the conversion of lead nitrate to lead citrate, the mixture was shaken vigorously at intervals for about 30 min in a 50 ml volumetric flask. To this milky suspension was added 8 ml 1 N NaOH. The solution was diluted to 50 ml and mixed by inversion until the lead citrate had completely dissolved. The pH of the solution was 12.

Grids were placed face upwards in saturated, methanolic uranyl acetate for 10-15 minutes, washed briefly in several changes of water, filtered through 0.45 μm Millipore filters prior to use, and then floated face down onto droplets of lead citrate, sitting on wax strips in a petri dish, for 2-3 min. Sections were washed thoroughly in large volumes (≈100 ml) of distilled water and allowed to dry before viewing under an electron microscope.

Chapter Three

A comparison of the distributions of isoenzymes of the cytochrome P450IA sub-family in the liver and some extrahepatic tissues of various mammalian species

3.1 Introduction

The PAH-inducible family of cytochrome P450, P450I, comprises a single sub-family containing two major isoenzymes, IA1 and IA2 (Nebert *et al.*, 1989). Other common names often used to describe these two isoenzymes in the liver are listed in Table 3.1. Both isoenzymes are coincidently, but differentially induced, in a non-co-ordinate manner by a number of xenobiotics of which MC, ß-naphthoflavone (ßNF), 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3, 4, 5, 3', 4',5'-hexachlorobiphenyl and isosafrole are but a few (Thomas *et al.*, 1983; Kelley *et al.*, 1987). Genetic studies in the mouse suggest that induction of both proteins is controlled by the Ah receptor at the level of transcription. However, several inducers also form complexes with IA2 protein which may prevent its degradation and lead to increases in its specific content (Voorman & Aust, 1988). The molecular biology and regulatory mechanisms controlling the expression of these isoenzymes in mouse, rat and rabbit have been the topic of interest of many laboratories over the past 30 years and have been well reviewed (Ioannides & Parke, 1987; Gonzalez, 1988 and references therein). As a consequence, these aspects will not be reiterated here except to note that in a given species the isoenzymes share >70% homology of their primary amino acid sequences and that there is 93% homology between the rat and mouse orthologues of each isoenzyme. In man, there are also two isoenzymes of P450 which share 80% homology with each other and 70-80% homology with IA1 and IA2, respectively, in mice, rats and rabbits (Jaiswal *et al.*, 1985; Quattrochi *et al.*, 1985; Distlerath *et al.*, 1985; Kawajiri *et al.*, 1986; Wrighton *et al.*, 1986b).

In spite of the homology between these two isoenzymes, they do exhibit differences in their minimum molecular weights on SDS-PAGE, peptide maps and substrate specificities (Guengerich *et al.*, 1982; Thomas *et al.*, 1984a; Watanabe *et al.*, 1987; Schwab & Johnson, 1987 and references therein). Typically, IA1 is associated with activation of PAHs (Thorgeirsson & Nebert, 1976) and IA2 with activation of carcinogenic aromatic amines (Kamataki *et al.*, 1983) including potent promutagens derived from the action of heat on meat and fish (Kato & Yamazoe, 1987). There is evidence which strongly implicates a causal relationship between P450IA1 expression and the production of cancer in animals.

Table 3.1: Trivial names for orthologous products of the P450IA1 and P450IA2 genes

P450IA1	P450IA2	Species	Reference
c	d	rat	Ryan *et al.* (1982)
ßNF-B	ISF-G	rat	Guengerich *et al.* (1982)
448-L	448-H	rat	Kamataki *et al.* (1982)
MCII	MCI	hamster	Watanabe *et al.* (1987)
MC_2	MC_1	rat	Wolf *et al.* (1984)
P_1450	P_3450	mouse	Negishi & Nebert (1979)
LM_6	LM_4	rabbit	Johnson & Muller-Eberhard (1977)
P_1	PA	man	Jaiswal *et al.* (1985); Distlerath *et al.* (1985)
HLc	HLd	man	Wrighton *et al.* (1986b)

Principally the administration of various PAHs, which lead to induction of IA1, was found to produce more tumours and toxicity in Ah-responsive mice (reviewed Nebert, 1981). Moreover, the site at which the tumour/toxicity occurred was directly related to the route of application. For example, topical or subcutaneous application led to development of epidermal carcinoma or ulceration whereas intraperitoneal administration caused hepatic necrosis. Although there is less evidence for such a role in man, a correlation between P450IA1 inducibility and cancer amongst cigarette smokers has been suggested (Kouri *et al.*, 1982). Human cytochrome P450IA2 plays an important part in the genotoxic activation of IQ (2-amino-3-methylimidazo [4, 5-f] quinoline), MeIQ (2-amino-3, 5-dimethylimidazo [4, 5-f] quinoline), MeIQx (2-amino-3, 8-dimethylimidazo [4, 5-f] quinoxaline) and 2-acetylaminofluorene (AAF) (Shimado & Okuda, 1988). Thus, the specific content of these isoenzymes within a tissue may be a major determinant in individual susceptibility to cancer as well as in target organ toxicity upon exposure to xenobiotics.

The use of specific antibodies has established or confirmed the similarity, not only of different forms of cytochrome P450 but also of orthologous forms in different species, including man (Thomas *et al.*, 1984b; Song *et al.*, 1985; Wrighton *et al.*, 1985b; Boobis *et al.*, 1985b; Beaune *et al.*, 1986; Pelkonen *et al.*, 1986; Sesardic *et al.*, 1986; Wrighton *et al.*, 1986b). In particular, monoclonal antibodies, characterized by their high specificity for a single epitope of only a few (perhaps 4-7) amino acids in length, have proven very effective as probes in detecting the presence of their epitope in other proteins in other species. In this respect, Boobis and co-workers have produced two monoclonal antibodies and one anti-peptide antibody that are specific for isoenzymes of the P450IA family (Boobis *et al.*, 1981; Boobis *et al.*, 1985b; Sesardic *et al.*, 1986; Edwards *et al.*, 1990). The first of the monoclonal antibodies reacts with orthologues of IA1 and/or IA2 in a wide variety of species. It is referred to as 3/4/2. The binding of 3/4/2 to hepatic microsomal proteins varies amongst mammalian species, as revealed by e.l.i.s.a., and can be expressed as follows: Wistar, F344 and DA rat $\geq$ Balb/c and AKR mouse and guinea-pig > rabbit > Syrian hamster, pig, baboon and human (Boobis *et al.*, 1985b). Interestingly, 3/4/2 does not react with IA2 in rat or mouse, but does recognise both IA1 and IA2 in hepatic microsomal fractions from MC-treated rabbits.

The second of these monoclonal antibodies, 107, was one of several raised against rabbit liver cytochrome P450IA2 (Boobis *et al.*, 1981). This antibody reacts with a discontinuous epitope so it cannot be used for Western blotting. It does not recognise rabbit IA1 in e.l.i.s.a. but it does inhibit >99% of the N-hydroxylation of AAF by rabbit liver microsomes, a reaction catalysed exclusively by IA2 in this species. Also it did not inhibit

benzo[a]pyrene hydroxylase activity of reconstituted IA1, confirming that it does not interact with this isoenzyme. Subsequent characterization of the antibody by D. Sesardic (RPMS) showed that it does not bind to isoenzymes of the P450 IIB or IIE families in the rabbit nor to P450IA1 or IA2 in the rat. Further, following immunopurification of solubilised hepatic microsomal fractions, from rabbits treated with MC, with 107, a single band of M_r 54 000 was obtained. This band co-migrated with an enriched preparation of rabbit P450IA2 (B. Murray, also of this laboratory). Additionally, activation of MeIQx in the *Salmonella typhimurium* mutagenicity assay by microsomes from MC-treated rabbits, could be inhibited by more than 94% by this antibody (Murray *et al.*, 1988). Together, these studies verify the specificity of 107 for rabbit IA2.

The third antibody was raised against a synthetic peptide sequence (Ser-Glu-Asn-Tyr-Lys-Asp-Asn) at positions 290-296, of cytochrome P450IA2, believed to be unique to this isoenzyme in the rat (Edwards *et al.*, 1988; Edwards *et al.*, 1990). Further characterization of this antibody by R.J. Edwards (RPMS), has shown that it also cross-reacts with IA2 in the mouse. This was not unexpected as it contains the identical sequence at these positions to that in rat IA2 (Kimura *et al.*, 1984; Sogawa *et al.*, 1985). However, the antibody has no detectable interaction with any proteins in microsomal fractions of human liver, as predicted from the sequence of the immunizing peptide (Edwards *et al.*, 1990).

The group of W. Levin, P.E. Thomas and colleagues have prepared mono-specific polyclonal antibodies and panels of monoclonal antibodies to a variety of cytochromes P450 (Reik *et al.*, 1982; Thomas *et al.*, 1984a; Reik *et al.*, 1985). Samples of two of these antibodies, anti-d(-c) (polyclonal) against rat IA2 and C8 (monoclonal) against rat IA1 were generously provided by W. Levin (Hoffman La Roche Inc, Nutley, New Jersey) and these have been used in conjunction with those antibodies described above, as a means of verifying the identity of isoenzymes within some tissues.

The purpose of the following investigation was to characterize the immunocytochemical distribution of isoenzymes of the cytochrome P450IA sub-family within the liver and some extrahepatic organs of various mammalian species. The availability of human tissues dictated which rat extrahepatic tissues were included in this part of the study.

3.2 Results

3.2.1 Immunostaining of Western blots of liver microsomes

Liver microsomal proteins from untreated, PB-treated and MC-treated rats, untreated and MC-treated mice (Balb/c and AKR strains), untreated guinea-pig, hamster, rabbit, dog and human were analysed by immunoblotting with MAb 3/4/2 for IA1 (Fig. 3.1). A single weakly reacting band, of M_r 52-53 000, could be discerned in liver samples from untreated hamsters. A single band of M_r 56-57 000 was clearly recognised with relatively strong immunoreactivity in a human liver microsomal fraction (Fig. 3.1a). A weakly reactive band was also visible in microsomes from untreated rabbit (Fig. 3.1b). No immunoreactive bands could be detected in microsomes from untreated rat, guinea-pig, AKR or Balb/c mice or dog. Similarly, no immunoreactive bands could be detected in liver microsomal fractions from four different strains of untreated rats or rats pre-treated with PB (Fig. 3.1b). Following MC-treatment, a single immunoreactive band of M_r 57 000 was detected in liver microsomal fractions from Wistar rats and Balb/c mice, that co-migrated with a preparation of IA1 purified from MC-treated rats (Fig. 3.1a). Total, spectrally determined cytochrome P450 content increased from 433 ± 83 (5) to 1708 ± 24 (3) and from 457 to 1434 pmoles.mg^{-1} protein, respectively in these species. No immunoreactive band was detected in hepatic microsomes from MC-treated AKR mouse. Total, spectrally determined cytochrome P450 content increased from a relatively lower value of 255 pmoles.mg^{-1} to 555 pmoles.mg^{-1} protein following MC treatment of this strain. In hepatic microsomes from MC-treated rabbit, two bands of M_r 54 000 and 57 000, respectively, reacted moderately with 3/4/2. Based on their relative mobilities these corresponded to forms IA1 and IA2 (Fig. 3.1b). Similarly, in this species total spectrally determined cytochrome P450 content in liver increased 3-fold following MC treatment.

3.2.2 Distribution of P450IA1 and P450IA2 in rat liver

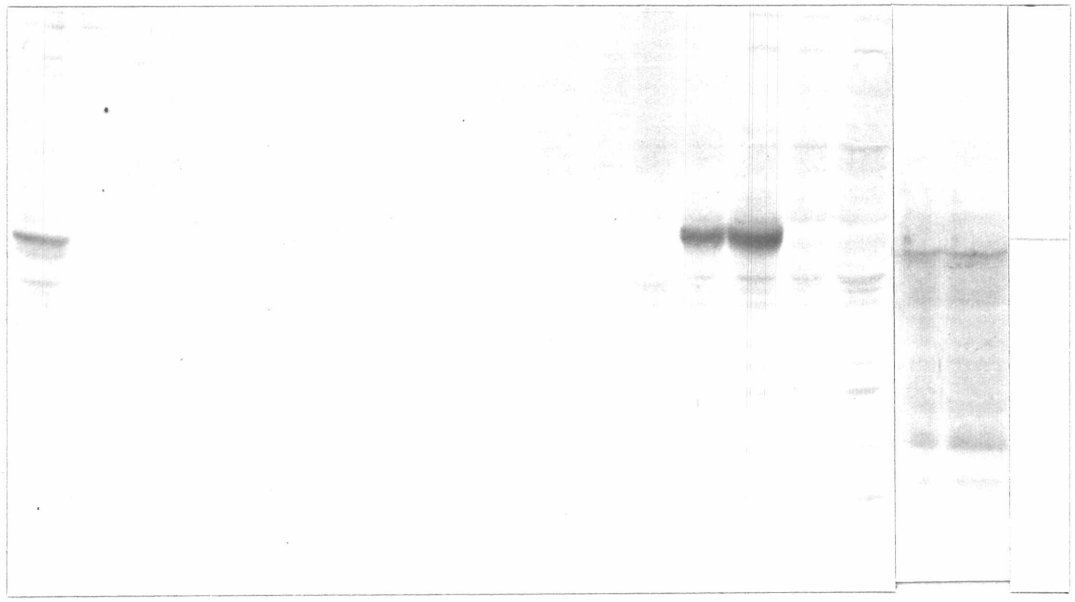
In liver from untreated animals, specific immunostaining was detected in all hepatocytes, following incubation with either 3/4/2 or C8 (which recognise IA1), at dilutions of 1:50 or less (Table 3.2). Immunoreactivity was most intense in a narrow band of cells radiating out from the central vein when using immunoperoxidase detection (data not shown). An alternative technique, immunogold silver staining, was also used to study the distribution of P450IA isoenzymes. This method is much more sensitive (≈200-fold) than immunoperoxidase staining, at least in paraffin sections (Holgate *et al.*, 1983). The extent to which sensitivity was increased over that with immunoperoxidase detection in the present

Figure 3.1: Western blotting of hepatic microsomal fractions from different mammalian species

Microsomal proteins were separated by SDS-PAGE and subjected to Western blotting as described in Sections 2.2.13 and 2.2.14. Immunoblots were immunostained with 3/4/2, 1 $\mu g.ml^{-1}$. (a) Hepatic microsomal fractions were as follows: lane 1, MC-treated Balb/c mouse, 40 µg; lanes 2 and 3, untreated Balb/c mouse, 20 µg and 40 µg; lanes 4 and 5, MC-treated AKR mouse, 20 µg and 40 µg; lanes 6 and 7, untreated AKR mouse, 20 µg and 40 µg; lanes 8 and 9, guinea-pig, 20 µg and 40 µg; lanes 10-13, dog, 40 µg; lanes 14 and 15, MC-treated rat, 20 µg and 40 µg; lanes 16 and 17, untreated rat, 20 µg and 40 µg; lanes 18 and 19, hamster, 20 µg and 40 µg and lane 20, human, 30 µg. (b) Hepatic microsomal fractions (30 µg) were as follows: lanes 1 and 20, purified P450IA1, 1 and 2 pmoles; lane 2, untreated and lanes 3-5, PB-treated Wistar rat; lane 6, untreated and lanes 7-9, PB-treated Sprague-Dawley rat; lane 10, untreated and lanes 11-13, PB-treated Lister-Hooded rat; lane 14, untreated and lanes 15-17, PB-treated F344 rat; lane 18, untreated rabbit and lane 19, MC-treated rabbit.

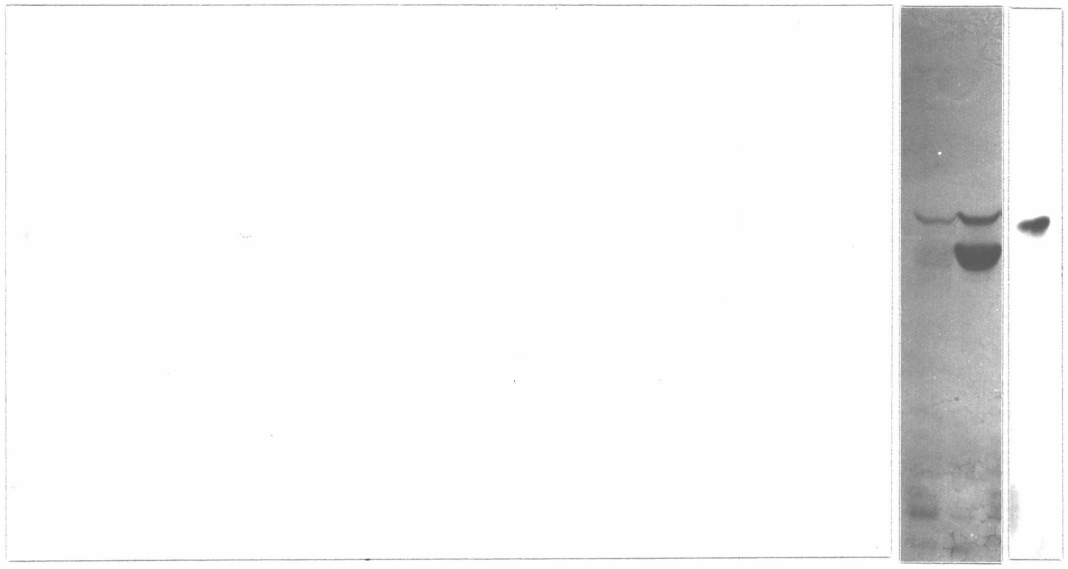
Immunoblots were aligned by comparison of R_f values of molecular weight standards (not shown in figure).

a



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

b



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Table 3.2: Immunotitration of the binding of antibodies to sections from adult rat liver

			Distribution of staining			
			Centrilobular		Periportal and Mid-zonal	
Strain	Treatment	Antibody/	Antibody dilution			
			1:50	1:200	1:50	1:200
Wistar	None	3/4/2	++	+/-	+	-
		C8	++	+/-	+/-	-
		anti-$d_{290-296}$	+++	++/+	+	-
		anti-d(-c)	+++	+++	+	-
		'Control IgG	-	-	-	-
	PB	3/4/2	++	+	+	+/-
		C8	++	+	+	+/-
	MC	3/4/2	+++	++	+++	++
		C8	+++	++	+++	++
		anti-$d_{290-296}$	+++	+++	+++	+++
		anti-d(-c)	+++	+++	+++	+++
		Control IgG	-	-	-	-
Sprague Dawley	None	3/4/2	++	+/-	+/-	-
		C8	+	+/-	+/-	+/-
	PB	3/4/2	++	+	+	+
		C8	++	+	+	+/-

Table 3.2 continued:

Lister Hooded	None	3/4/2	++	+/-	++	+/-
		C8	++/+	-	+/-	-
	PB	3/4/2	++	+	+	+/-
		C8	++	+/-	+	+/-
F344	None	3/4/2	++	+	+	+/-
		C8	+	+/-	+	+/-
	PB	3/4/2	++/+	+	+	+
		C8	++/+	+/-	+	+/-

Intensity of staining: +++ intense, ++ moderate, ++/+ moderate to weak, + weak, +/- weak to none, - none.

*Control IgG = Pre-immune rabbit serum.

study, was estimated using liver sections from either untreated rats or rats pre-treated with PB. Whereas, staining for cytochrome P450, with either 3/4/2 or C8 using immunoperoxidase detection was apparent only at low antibody dilutions (<1:50), a relatively strong immunoreaction was obtained with immunogold silver staining at dilutions of 3/4/2 or C8 of 1:500. Moreover, a wider band of centrilobular hepatocytes was stained, although the degree of silver precipitation within individual cells varied, from very heavy in some to just perceptible in others (Fig. 3.2a and Fig. 3.2b). Thus, this method appears to increase the limits of detection in frozen cryostat sections, by at least 10-fold and was therefore useful for enhancing weak antigen-antibody interactions.

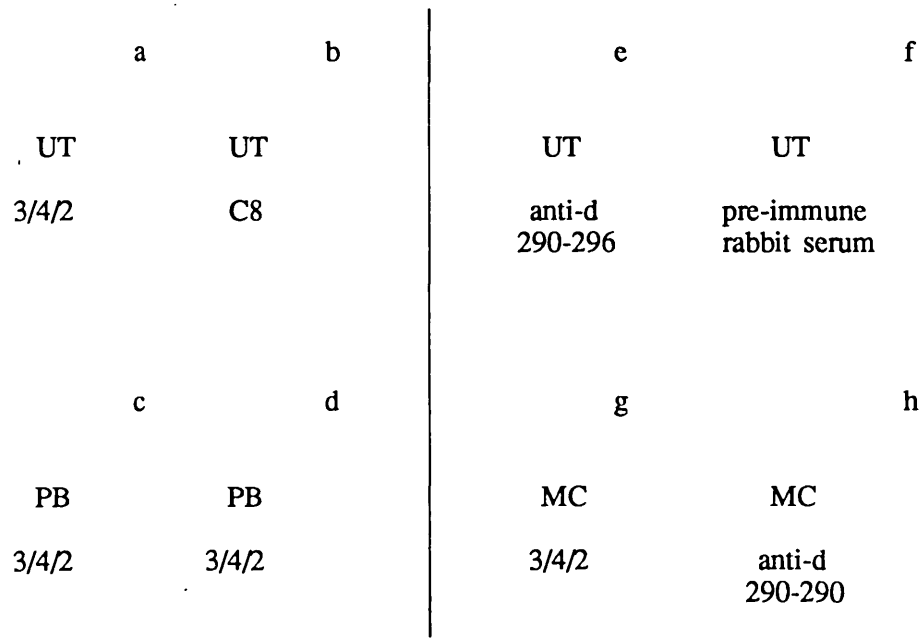
With either antibody, periportal and mid-zonal hepatocytes were stained only weakly, and the diffuse immunoreactivity disappeared upon dilution of the antibodies (Table 3.2). This preferential centrilobular localization for cytochrome P450IA1 was observed in livers from Wistar, Sprague-Dawley and F344 strains, although in Sprague-Dawley and F344 strains, very intense staining was also seen in single cells, distributed throughout this area, but only with higher titres of the antibodies. In contrast, in the Lister-Hooded strain, a more even distribution of immunoreactive protein was observed throughout the lobule particularly with 3/4/2 (Table 3.2).

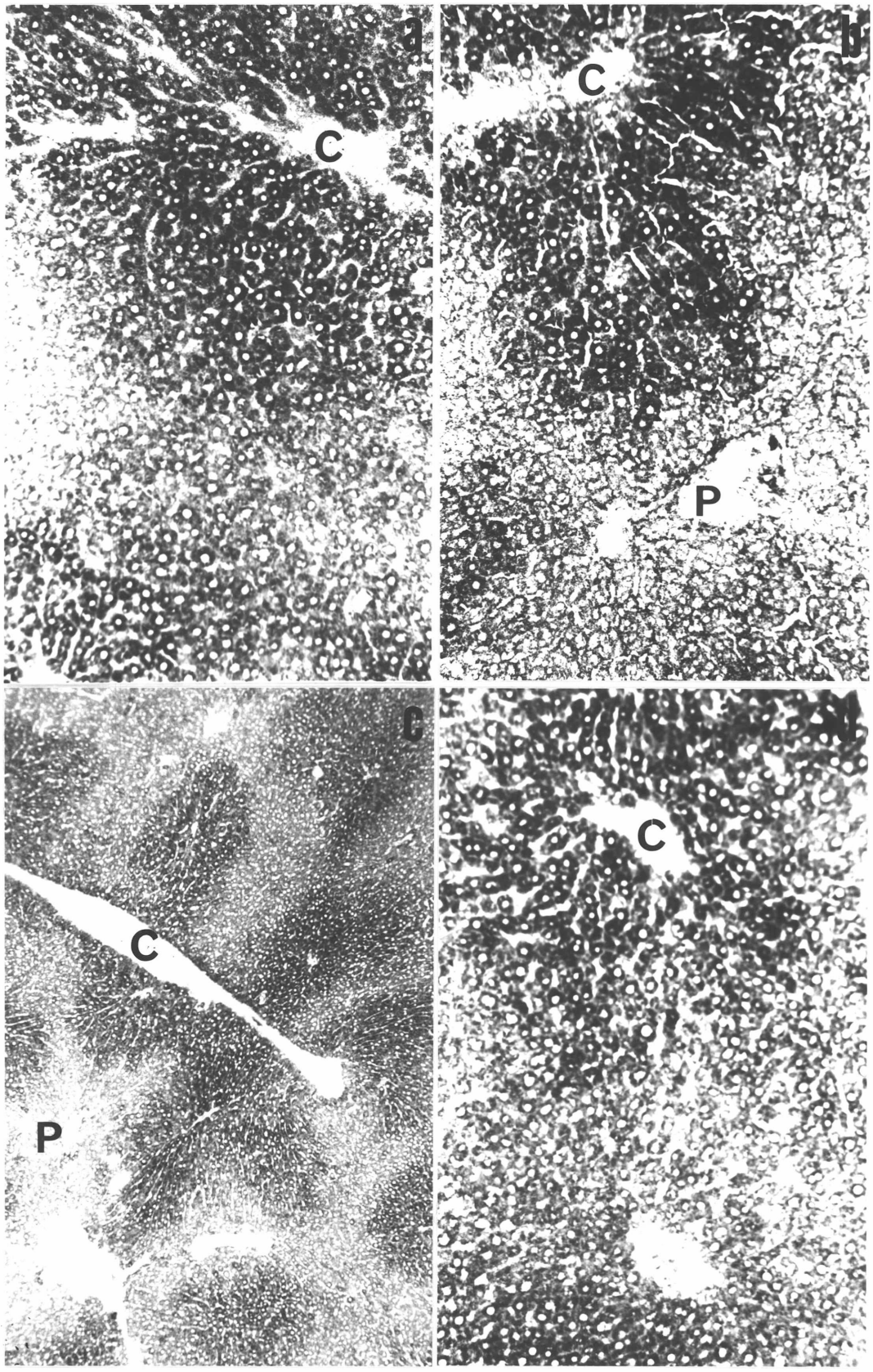
No change in the intensity or distribution of staining with 3/4/2 was apparent in livers following PB-treatment of rats (Fig. 3.2c and Fig. 3.2d; Table 3.2). Serial liver sections exposed to C8 showed the same pattern of staining as with 3/4/2 (Table 3.2). There was some variability in the intensity of the immunoreaction amongst sections from different animals, which probably reflects variation in the levels of the isoenzyme to which these antibodies bind.

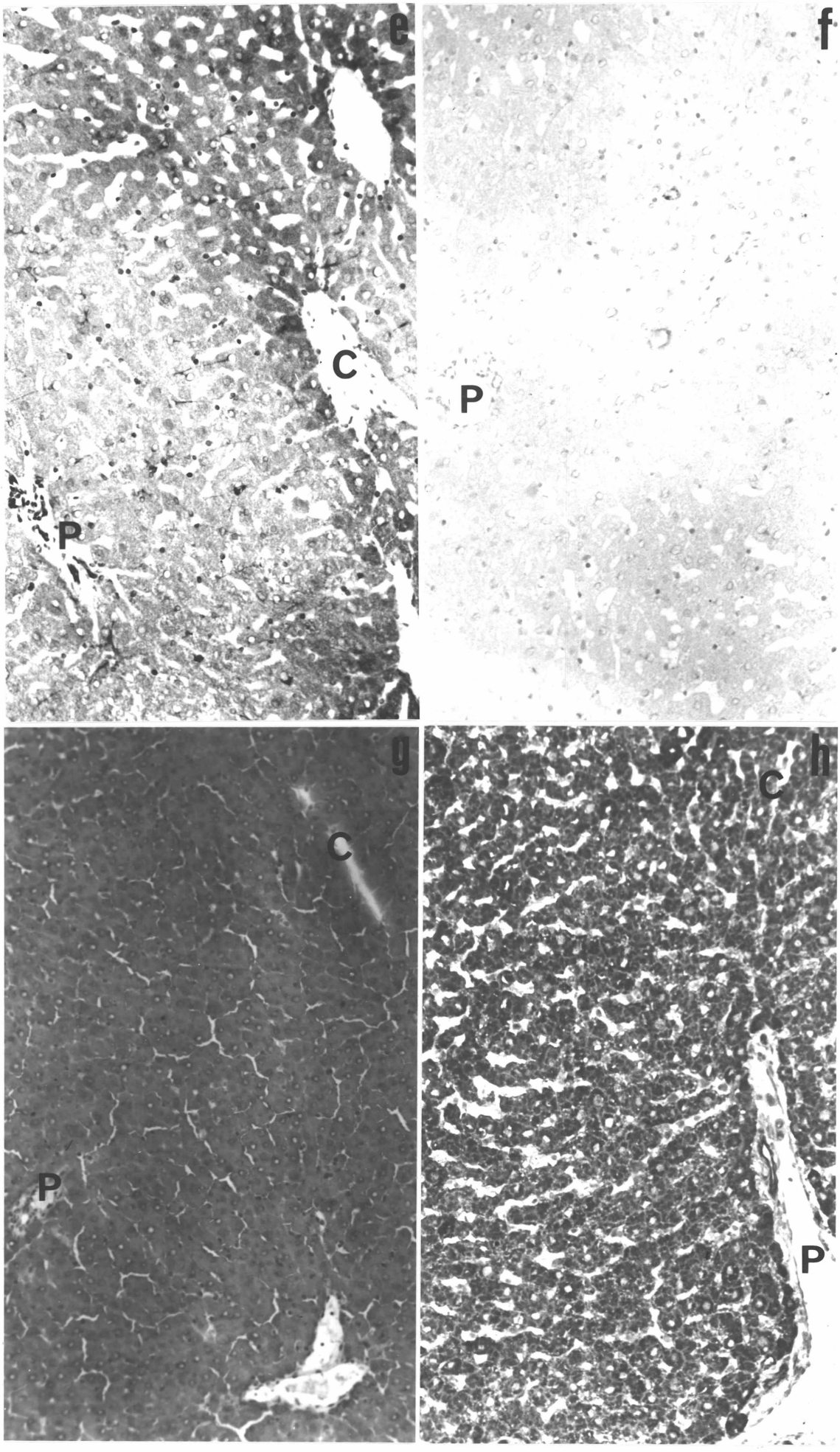
Incubation of sections with antibodies directed against P450IA2 showed that the highest levels of this isoenzyme were present in centrilobular regions. There was a gradual decrease in the intensity of staining of hepatocytes moving out from the central vein, and periportal cells were very weakly stained (Fig. 3.2e; Table 3.2).

Following MC-treatment of rats, a very strong immunoreaction was apparent in all hepatocytes across the lobule, with no preferential staining of hepatocytes in any one area of the lobule, by either 3/4/2 or C8 (Fig. 3.2g). Whereas staining was apparent in sections from untreated animals only with antibody dilutions less than 1:50 (Wistar strain), following MC-treatment, immunoreactive protein was detectable at dilutions as high as 1:1000, indicating considerable induction of this isoenzyme (Table 3.2). Incubation of sections with

Figure 3.2: Immunocytochemical localization of P450IA1 and P450IA2 in rat liver
Sections from untreated (a, b, e, f), PB-treated (c, d) or MC-treated (g, h) rats were incubated with either 3/4/2 (a, c, d, g) or C8 (b), each at a dilution of 1:50 or anti-$d_{290\text{-}296}$ (e, h) or pre-immune rabbit serum (f) each at a dilution of 1:100. (a-d) specific immunoreaction was detected using GAM conjugated to 5 nm colloidal gold and the signal enhanced with silver enhancement solution. (e-h) indirect immunostaining using IgG conjugated to HRP. C, central vein; P, portal tract. Magnifications, (a, b, d) x135, (c) x50 and (e-h) x170.




C
C
P
C
P
C


e
C
P
f
P
g
C
P
h
C
P

anti-$d_{290-296}$ or anti-d(-c) revealed a marked increase in the staining intensity of most hepatocytes across the lobule relative to that in untreated animals (Fig. 3.2h). The intensity of this immunoreaction was maintained at higher titres of antibodies, down to dilutions of 1:500. Less strongly stained hepatocytes were apparent in some periportal regions, particularly following immunostaining with the anti-peptide antibody at dilutions of 1:500 or less. The reaction product obtained on incubation with this antibody was not only distributed homogeneously within the cytoplasm but also appeared to be 'clustered' within cells giving them a stippled appearance.

Following replacement of any of these antibodies with an inappropriate antibody, 107, or pre-immune rabbit serum, respectively, there was a complete lack of staining of liver sections from either untreated or MC-treated rats (Fig. 3.2f; Table 3.2).

3.2.3 Distribution of P450IA1 and P450IA2 in mouse liver

A moderate to weak immunoreaction was observed in livers of untreated Balb/c and AKR mice, following incubation of sections with 3/4/2, at dilutions of 1:10 (Table 3.3). Immunostaining was diffusely distributed in hepatocytes across the whole of the lobule and was slightly less intense in the AKR strain (not shown). In contrast, on incubation of sections with anti-$d_{290-296}$ antibody, staining of hepatocytes was apparent only in centrilobular areas of the lobule and was not evident in periportal cells (Figs. 3.3a and 3.3b). There was a gradation in staining, hepatocytes close to the central vein being most intensely stained, whereas those nearer mid-zonal areas were less intensely stained. There were no significant differences between the two strains in the staining characteristics of this antibody.

Following MC-treatment of mice, there was an increase in staining intensity by 3/4/2 observed only in sections from the Balb/c strain (Table 3.3). MC-treatment of this strain increased the levels of cytochrome P450IA1 in nearly all hepatocytes except in a narrow band of cells (4-5 rows) around portal tracts which remained weakly stained, the intensity of which was identical to that in untreated animals (not shown). Within an area of the liver, for example the centrilobular area, there was no variation in the intensity of staining amongst hepatocytes. Immunoreactive protein appeared to be confined to, and was homogeneous within, the cytoplasm of hepatocytes, there being no detectable staining of nuclear components. P450IA1 was not induced in the non-responsive AKR strain, as apparent from the lack of any change in either the intensity or distribution of staining of this isoenzyme following MC-treatment (Table 3.3).

Table 3.3: Immunotitration of the binding of antibodies to mouse liver

Strain	Treatment	Antibody	Antibody dilution 1:10	1:50	1:100	1:500
Balb/c	None	3/4/2	++	++/+	-	
		anti-$d_{290-296}$		+++	+++	++/+
		B3A3	-	-	-	
		[a]Control IgG		+/-	-	-
	MC	3/4/2	+++[b]	+++	+++	
		anti-$d_{290-296}$		+++	+++	++/+
		B3A3	-	-	-	
		Control IgG		-	-	-
AKR	None	3/4/2	+	-	-	
		anti-$d_{290-296}$		+++	++	+
		B3A3	-	-	-	
		Control IgG		-	-	-
	MC	3/4/2	+	-	-	
		anti-$d_{290-296}$		+++	++	-
		B3A3	-	-	-	
		Control IgG		-	-	-

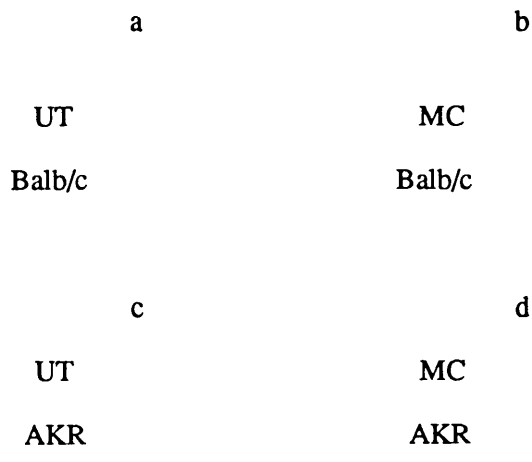
Intensity of staining: +++ intense, ++ moderate, + weak, - negative.

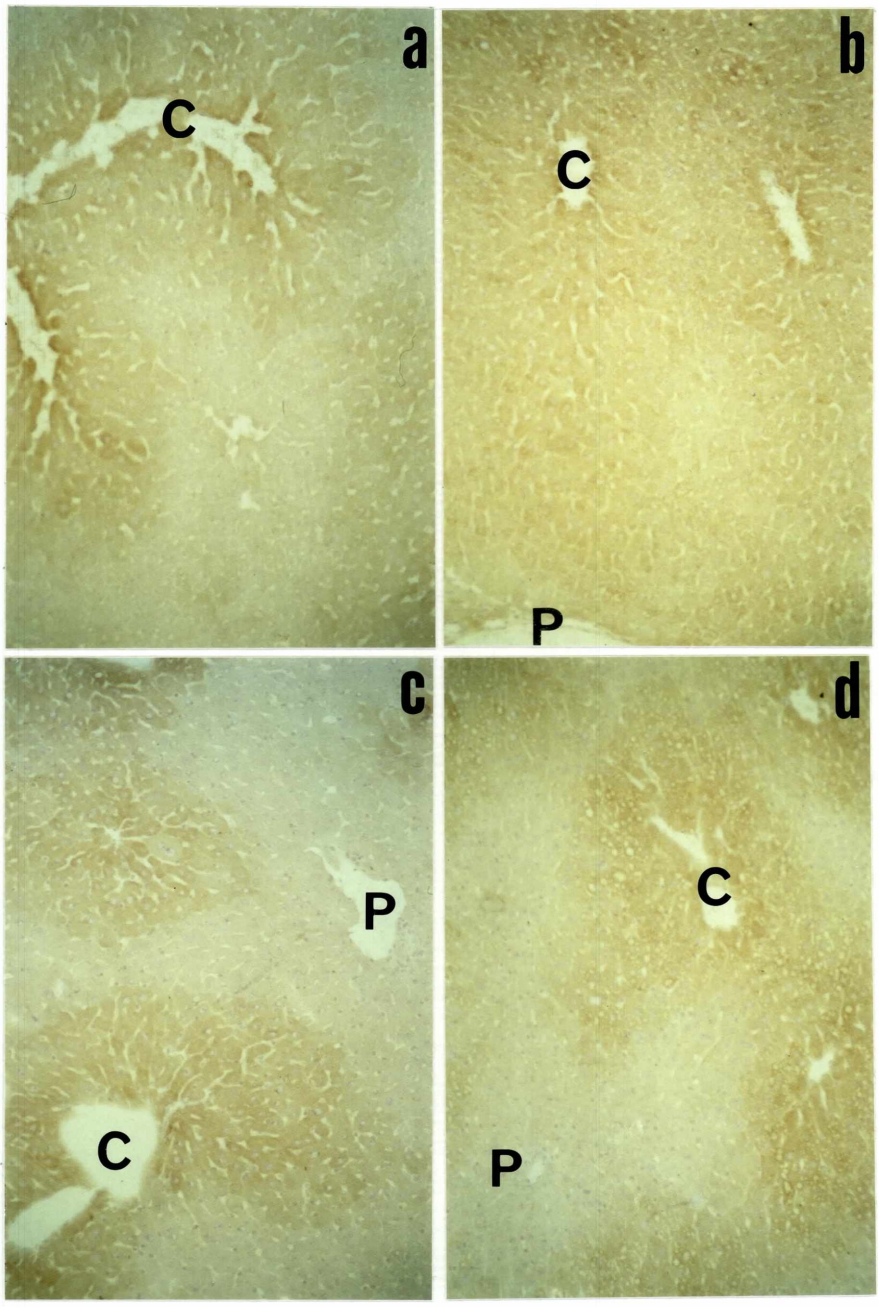
[a]Control IgG = Pre-immune rabbit serum.

[b]not in periportal cells which were ++/+.

Figure 3.3: Immunocytochemical localization of P450IA2 in mouse liver

Sections from untreated (a, c) and MC-treated (b, d) Balb/c (a, b) or AKR (c, d) mice, were incubated with anti-$d_{290-296}$ at a dilution of 1:50. C, central vein; P, portal tract. Magnification x65.




a
C
b
C
P
c
P
C
d
C
P

Following MC-treatment, the distribution of staining with anti-$d_{290-296}$ in liver sections from AKR mice was the same as that in the untreated animal (Figs. 3.3c and 3.3d; Table 3.3). However, in Balb/c mice, the immunoreaction in the majority of hepatocytes was more intense, with no gradation in the intensity of staining throughout the lobule. However, in centrilobular areas, strongly reacting hepatocytes were interspersed with moderately to weakly reacting cells, resulting in a heterogeneously stained population of cells in this area (Figs. 3.3c). Immunoreactive protein was distributed not only homogeneously within the cytoplasm of hepatocytes but also appeared stippled. In the non-responsive AKR strain, there appeared to be a slight increase in the intensity of staining of a few hepatocytes scattered throughout the centrilobular area, although overall, the immunotitration curve was very similar to that for untreated livers (Table 3.3).

Following replacement of these antibodies with an inappropriate monoclonal antibody, B3A3 against bovine growth hormone or with pre-immune rabbit serum, respectively, there was a complete lack of staining of hepatocytes in liver sections from either untreated or MC-treated mice (Table 3.3). There was, however, some staining of smooth muscle and bile duct epithelial cells by B3A3 (not shown).

3.2.4 Distribution of P450IA in hamster liver

Following incubation of liver sections from untreated hamsters (n=3) with 3/4/2 at dilutions of 1:50 or less, a relatively strong immunoreaction (+++) was evident in most hepatocytes across the lobule (Fig. 3.4a). The cytoplasm of the cells was evenly stained, with no staining of nuclei apparent; nor was there any staining of smooth muscle or bile duct epithelial cells. In some sections, those hepatocytes radiating out from central veins appeared to be slightly more intensely stained.

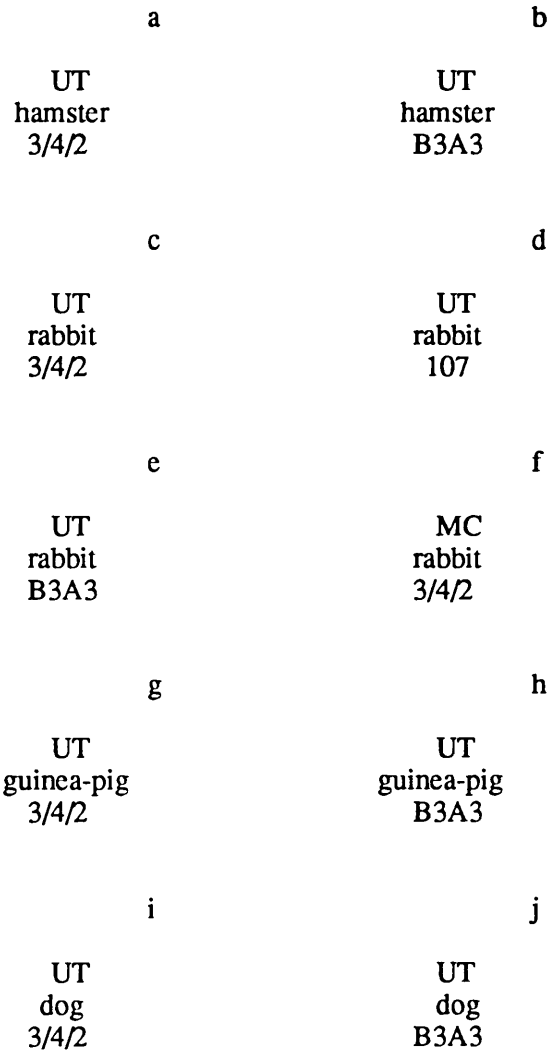
Following replacement of 3/4/2 with B3A3, there was a total lack of staining of hepatocytes (Fig. 3.4b). However, as in mice, this antibody did produce some staining of smooth muscle and bile duct epithelial cells (not shown).

3.2.5 Distribution of P450IA1 and P450IA2 in rabbit liver

Moderate to strong immunostaining was apparent in all hepatocytes across the lobule in livers of untreated animals, following exposure to either 3/4/2 or 107 at dilutions of less than 1:100 or 1:1000 respectively (Table 3.4). It was apparent that the cytoplasm of these hepatocytes was homogeneously immunostained (Fig. 3.4c and 3.4d).

Figure 3.4: Immunocytochemical localization of hepatic P450IA in some mammalian species

Sections from untreated hamster (a, b), untreated rabbit (c-e), MC-treated rabbit (f), untreated guinea-pig (g, h) and untreated dog (i, j) were incubated with either 3/4/2 (a, c, f, g, i) or B3A3 (b, e, h, j), each diluted 1:10, except for (f) where a 1:100 dilution was used. (d) a section incubated with 107 at a dilution of 1:100. C, central vein; P, portal tract. Magnifications, (a, b) x175 and (c-j) x90.



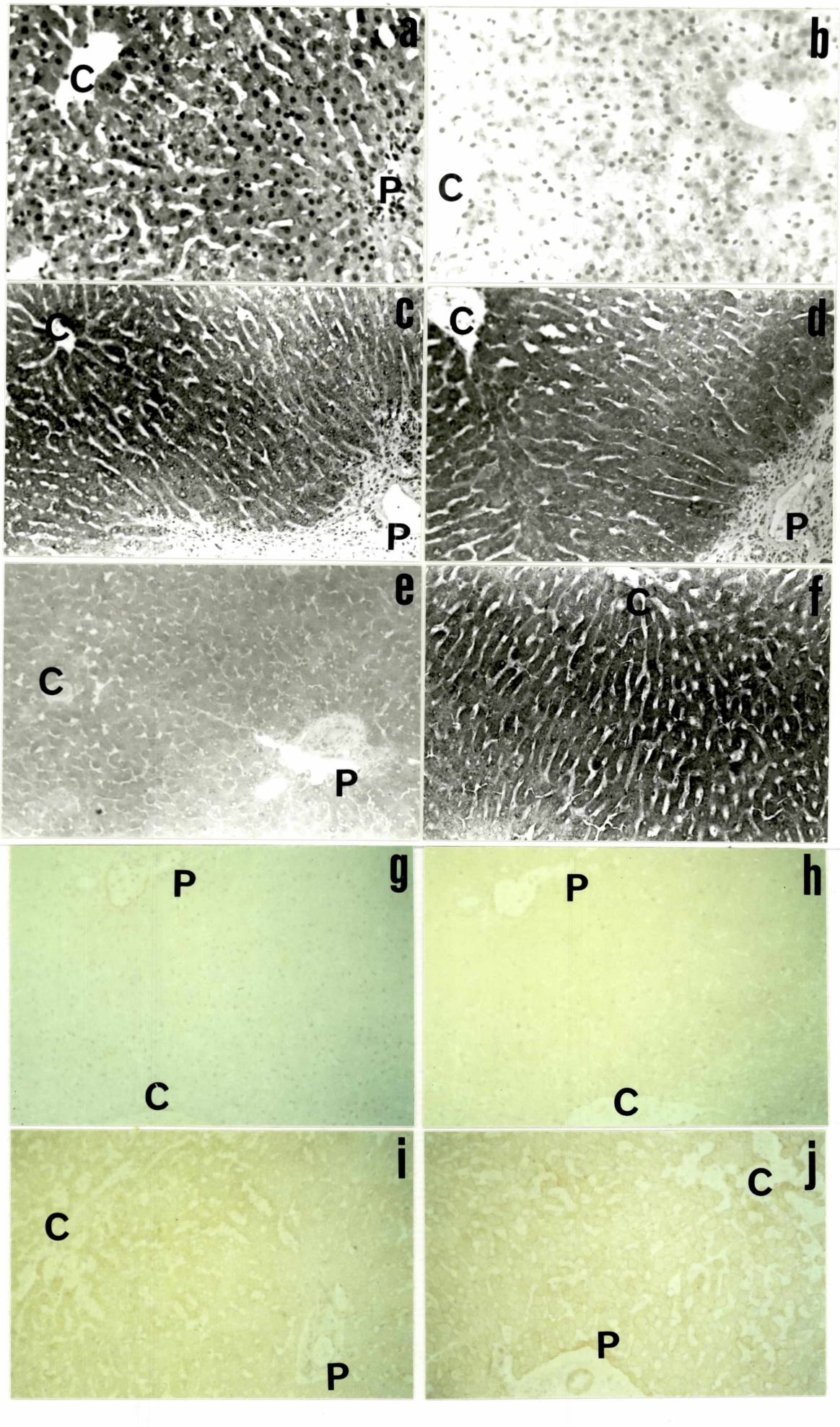

a
C
P
b
C
c
C
P
d
C
P
e
C
P
f
C
g
P
C
h
P
C
i
C
P
j
C
P

Table 3.4: Immunotitration of the binding of antibodies to adult rabbit liver

Treatment	Antibody	Antibody dilution			
		1:100	1:500	1:1000	1:5000
None	3/4/2	++	++	+	-
	107	+++	+++	+++	++/+
	B3A3	-	-		
MC	3/4/2	+++	+++	+++	++/+
	107	+++	+++	+++	+++
	B3A3	-	-		

Intensity of staining: +++ intense, ++ moderate, ++/+ some sections moderate others weak, + weak, - negative

The distribution of staining within rabbit liver did not change following MC-treatment of the animals (Fig. 3.4f) but immunotitration showed that induction of isoenzymes of the cytochrome P450IA family had occurred (Table 3.4). Immunotitration with 107 resulted in a sharp cut-off at high titres of antibody, below which no staining was evident. Coupled with the undoubtedly high affinity of this antibody for its epitope, induction of IA2 was more difficult to assess.

There was no staining of hepatocytes when either of these antibodies was replaced with B3A3 or buffer alone (Fig. 3.4e).

3.2.6 Distribution of P450IA in guinea-pig liver

No specific immunostaining of hepatocytes was evident following incubation of liver sections from untreated guinea-pigs (n=2) with 3/4/2, even at dilutions of 1:10 (Fig. 3.4g). Although there was some immunostaining of smooth muscle, staining of these cells was also apparent in sections incubated with B3A3 (Fig. 3.4h).

3.2.7 Distribution of P450IA in dog liver

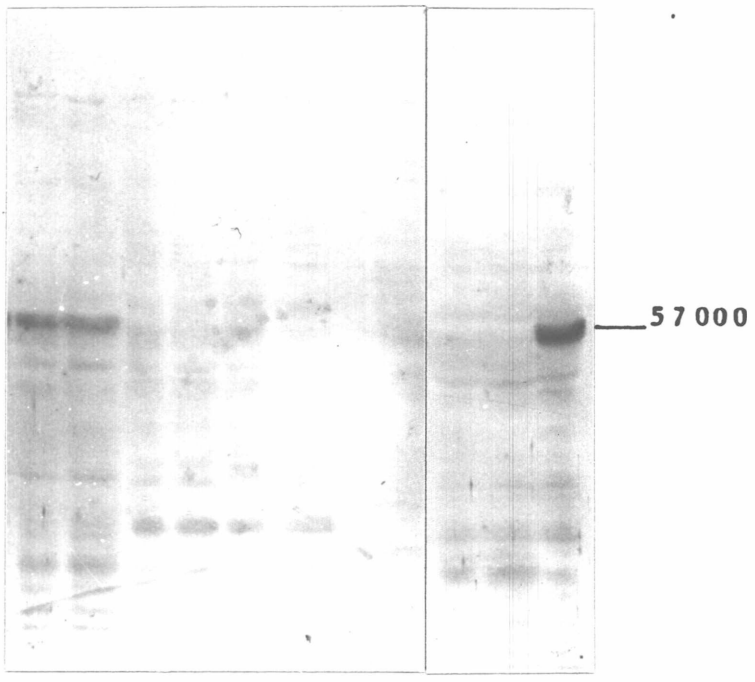
In sections from two of four dog livers, very weak (+) immunostaining with 3/4/2 could be discerned in hepatocytes throughout the lobule, with a slightly more intense immunoreaction in centrilobular cells, but only at antibody dilutions of 1:10 (Fig. 3.4i). However, no significant staining of hepatocytes was apparent in liver sections from the other two dogs. Monoclonal antibody 3/4/2 also produced intense staining of bile duct epithelial cells and of smooth muscle cells of blood vessels. However, replacement of this antibody with B3A3 also resulted in staining of these structures (Fig. 3.4j). B3A3 did not produce any significant staining of hepatocytes, although staining between hepatocytes was apparent.

3.2.8 Immunostaining of Western blots of kidneys

Microsomal samples of kidney from untreated and MC-treated rat, and Balb/c and AKR mice and human were analysed for their content of cytochrome P450IA after immunostaining of blots with 3/4/2. Only a single band was observed in kidneys from MC-treated rat and Balb/c mouse (as a faint band on the original gel), which co-migrated with IA1 in MC-treated rat liver microsomes (Fig. 3.5). No immunoreactive band was detected in the other microsomal samples.

Figure 3.5: Western blotting of kidney microsomal fractions from some mammalian species

Microsomal proteins were separated by SDS-PAGE and subjected to Western blotting as described in Sections 2.2.13 and 2.2.14. Immunoblots were immunostained with 3/4/2, 1 $\mu g.ml^{-1}$. Microsomal fractions were as follows: lanes 1 and 2, MC-treated rat; lanes 3 and 4, untreated rat; lanes 5 and 6, MC-treated Balb/c mouse; lanes 7 and 8, MC-treated AKR mouse and lanes 9 and 10, human kidney, all 20 µg and 40 µg respectively. Lane 11, MC-treated rat liver, 30 µg.


57000
1 2 3 4 5 6 7 8 9 10 11

3.2.9 Distribution of P450IA1 and P450IA2 in rat kidney

Following incubation of kidney sections from untreated rats with either MAb 3/4/2 or C8, moderate to weak staining of cells of the S_3 portion of the proximal tubule of juxtamedullary nephrons, which predominate in the inner cortex and outer medulla, was evident (Fig. 3.6a). The distribution of staining was the same with the two antibodies. Following MC-treatment of rats, immunotitration of the binding of the antibodies showed that there was an increase in the content of P450IA1. Whereas moderate staining in kidneys from untreated animals was evident only at dilutions of 1:10, following MC-treatment intense staining was observed with dilutions as high as 1:500 (Table 3.5). This was accompanied by an increase in the distribution of staining, with cells throughout the entire length of the proximal tubule of both cortical and juxtamedullary nephrons showing an intense immunoreaction (Fig. 3.6c).

The content and distribution of IA2 in the kidney was investigated by incubation of sections with polyclonal antibody, anti-$d_{290\text{-}296}$ or anti-d(-c). The intensity and distribution of staining produced by these antibodies was the same in sections from untreated as from MC-treated rats (Table 3.5). The distribution of this isoenzyme, however, did vary from that of IA1. There was a relatively strong, specific immunoreaction localized in the S_2 portion of the proximal convoluted tubule of cortical nephrons, which predominate within the outer cortex (Fig. 3.6b and Fig. 3.6d). A weaker immunoreaction was evident throughout the remainder of the cells of the proximal tubule.

Glomeruli, distal tubules of the cortex and medullary structures were not stained in sections from either untreated or MC-treated rats with most of these antibodies, except C8 which bound strongly to glomeruli, especially at higher concentrations (Fig. 3.6a). When the antibodies were replaced with pre-immune rabbit serum there was no staining of any structure of the kidneys from either untreated and MC-treated rats (Fig. 3.6e).

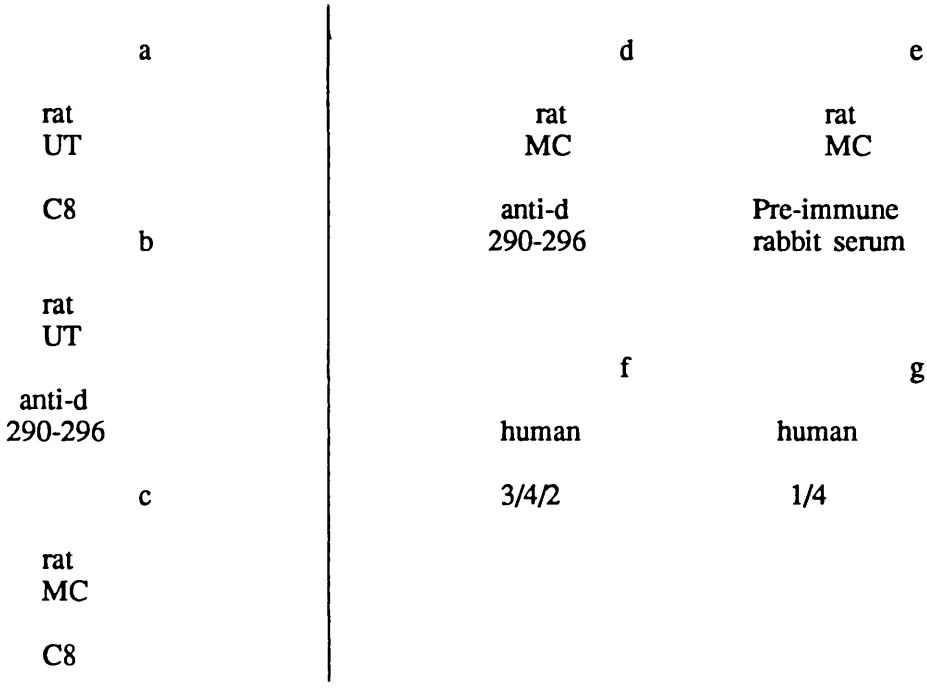
3.2.10 Distribution of P450IA in human kidney

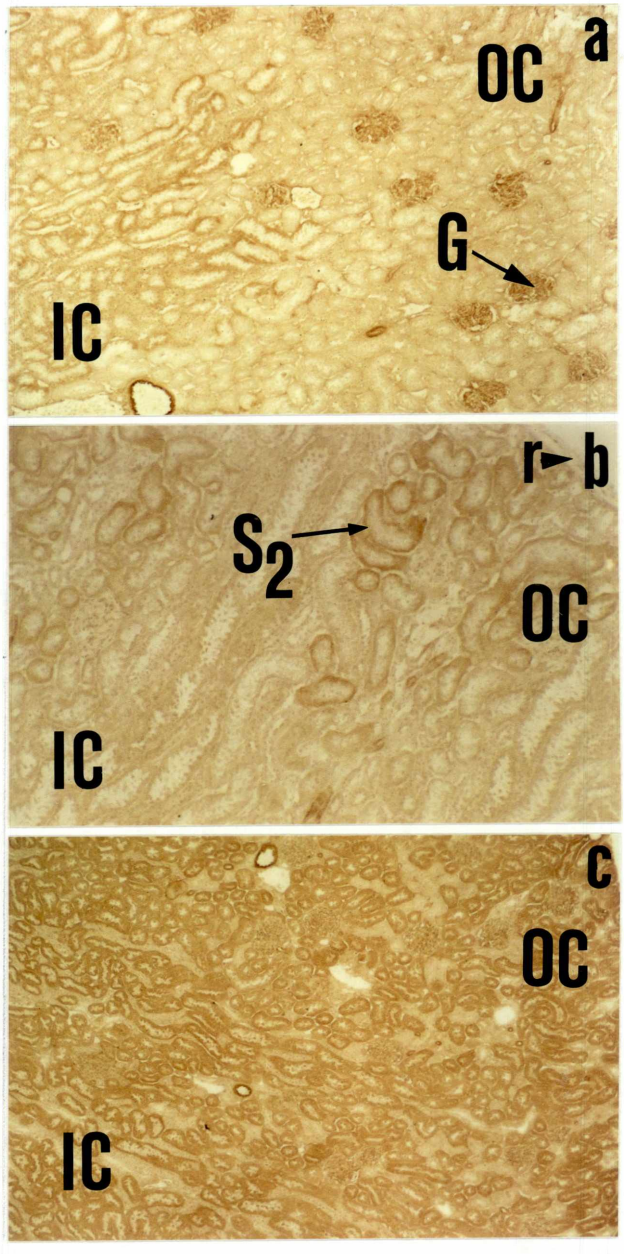
Sections were cut from a sample of female human kidney which had been maintained at -80°C for approximately 18 months. The patient was born 7/9/24 and died of apparently natural causes.

Exposure of sections of this kidney to high concentrations of 3/4/2 (< 1:10 dilution) resulted in a weak, specific immunostaining of the proximal tubules of the cortex (Fig. 3.6f). There

Figure 3.6: Immunocytochemical localization of P450IA in kidney

Sections from untreated (a, b) or MC-treated (c-e) rat were incubated with either C8, at a dilution of 1:10 (a, c), anti-$d_{290\text{-}296}$ at a dilution of 1:100 (b, d) or pre-immune rabbit serum at a dilution of 1:100 (e). Sections from human (f, g) were incubated with monoclonal antibodies 3/4/2 or 1/4, respectively, each diluted 1:10. r, renal capsule, OC, outer cortex; IC, inner cortex, S_2, proximal convoluted tubule; S_3, proximal straight tubule; G, glomerulus. Magnifications (a, c) x32, (b) x40, (d, e) x170 and (f, g) x120.




a
OC
G
IC
r
b
S_2
OC
IC
c
OC
IC

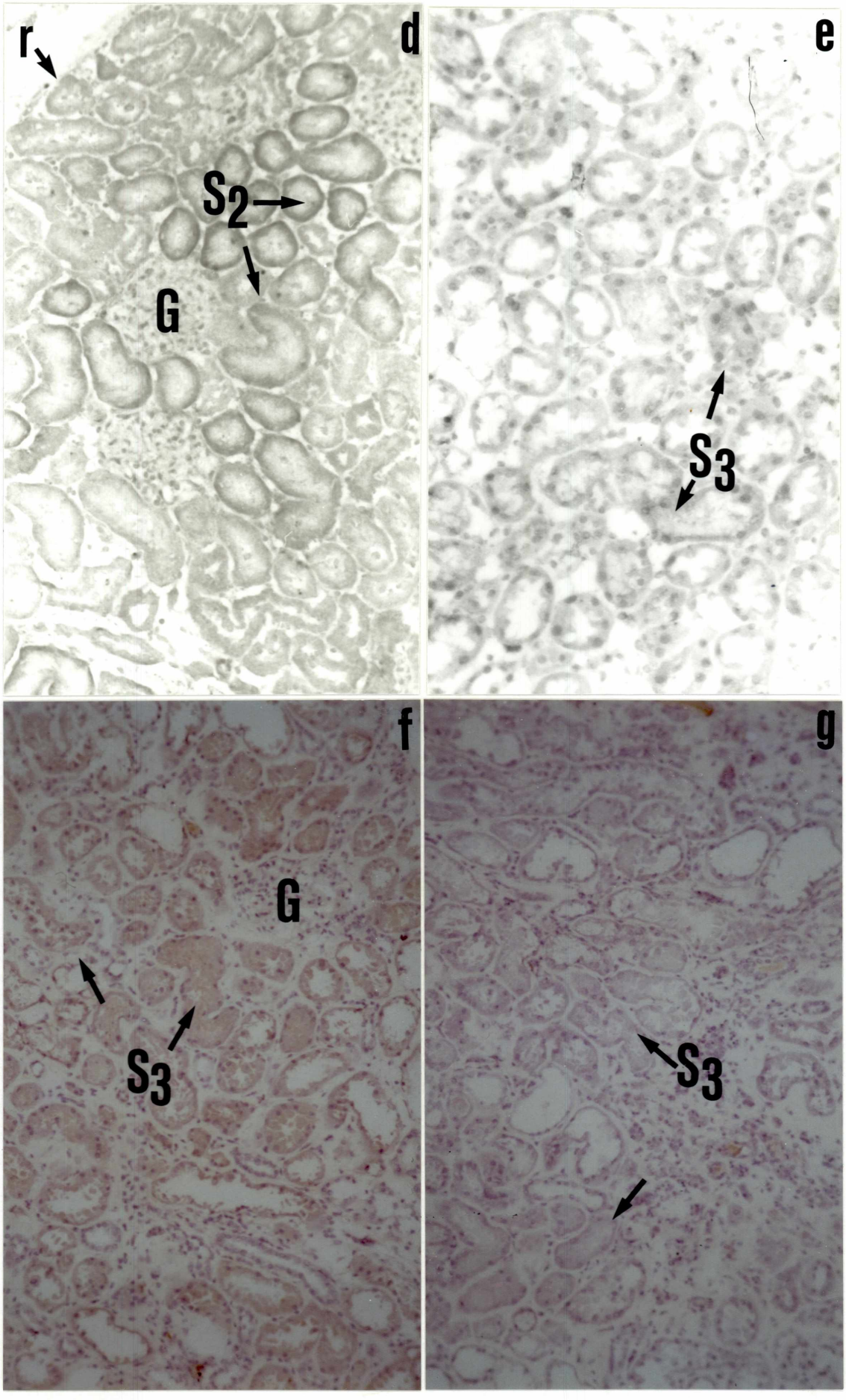

r
d
S2
G
e
S3
f
G
S3
g
S3

Table 3.5: Immunotitration of the binding of antibodies to adult rat kidney

Treatment	Antibody	Antibody dilution 1:10	1:50	1:100	1:500
None	3/4/2	+	+/-	-	-
	C8	+	+/-	-	-
	anti-$d_{290-296}$		++	+	
	anti-d(-c)		++	+	
	[a]Control IgG		+	-	
MC	3/4/2	+++	+++	+++	+++
	C8	+++	+++	+++	+++
	anti-$d_{290-296}$		++	+/-	
	anti-d(-c)		++	+	
	Control IgG		+	-	

Intensity of staining: +++ intense, ++ moderate, + weak, +/- weak to negative, - negative.

[a]Control IgG = Pre-immune rabbit serum.

was no significant staining of glomeruli, distal tubules or structures of the inner medulla. When 3/4/2 was replaced with an inappropriate antibody, 1/4 specific for IIB1/2 in the rat, or diluent alone, there was a complete lack of staining of any of the structures of the kidney (Fig. 3.6g).

3.2.11 Distribution of P450IA in rat pancreas

Immunoreactive cytochrome P450IA1 was detected in the pancreas from untreated rats (n=2) following incubation with either 3/4/2 or C8, but only at antibody dilutions of 1:10. The intensity of staining was strong (+++) in epithelium of inter- and intra-lobular ducts and weaker (++) in acinar cells of the exocrine pancreas. There was some intralobular variation in the distribution of strongly reacting cells, these being more prominent at the periphery of lobules. No staining of cells in the Islets of Langerhans was apparent (Fig. 3.7a and 3.7b). Following MC-treatment of rats, the intensity of immunostaining for P450IA1 was significantly increased in acinar cells. The intensity of the immunoreaction observed in epithelium of ducts was also increased, although not as markedly. In contrast, following incubation with anti-$d_{290\text{-}296}$ or anti-d(-c) antibody, cells of the exocrine pancreas were unstained in sections from both untreated and MC-treated rats. Both of these antibodies, however, did produce some moderate to weak staining of epithelium of ducts, the intensity of which did not change appreciably following pre-treatment with MC (Fig. 3.7c).

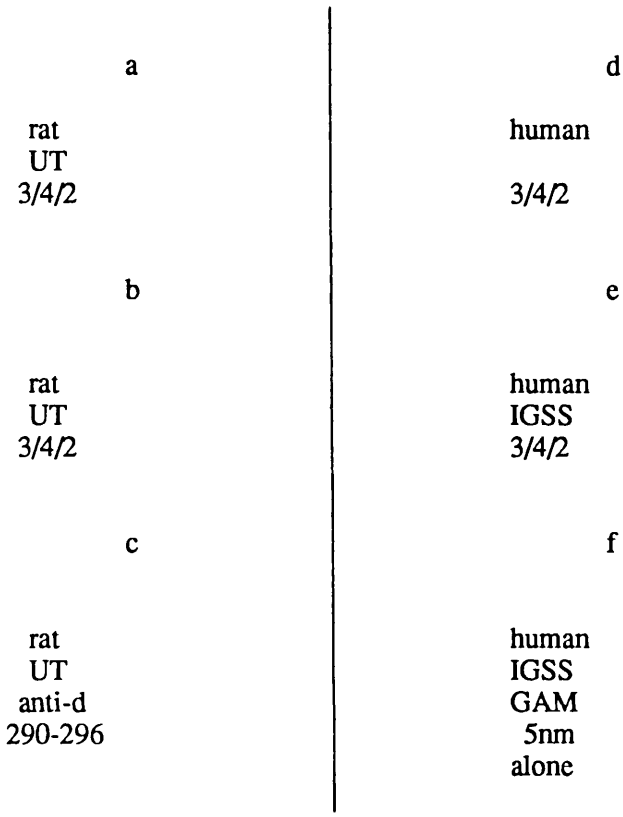
Relatively weak immunohistochemical staining of acinar cells was observed when the above antibodies were replaced with pre-immune rabbit serum or diluent alone. Strong immunoperoxidase staining was apparent in smooth muscle cells of blood vessel walls which was due, presumably, to non-specific adsorption of horseradish peroxidase conjugated antibodies.

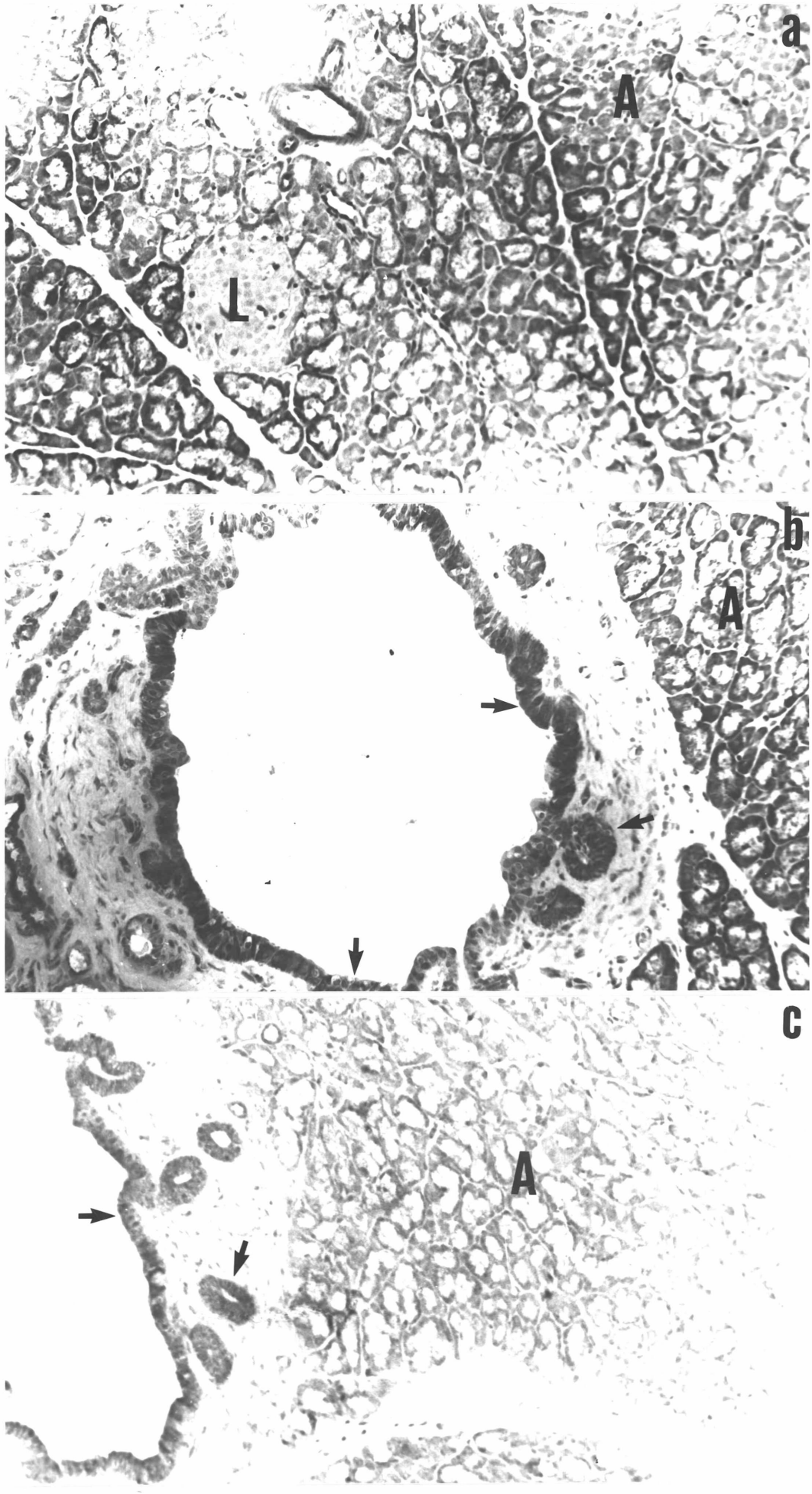
3.2.12 Distribution of P450IA in human pancreas

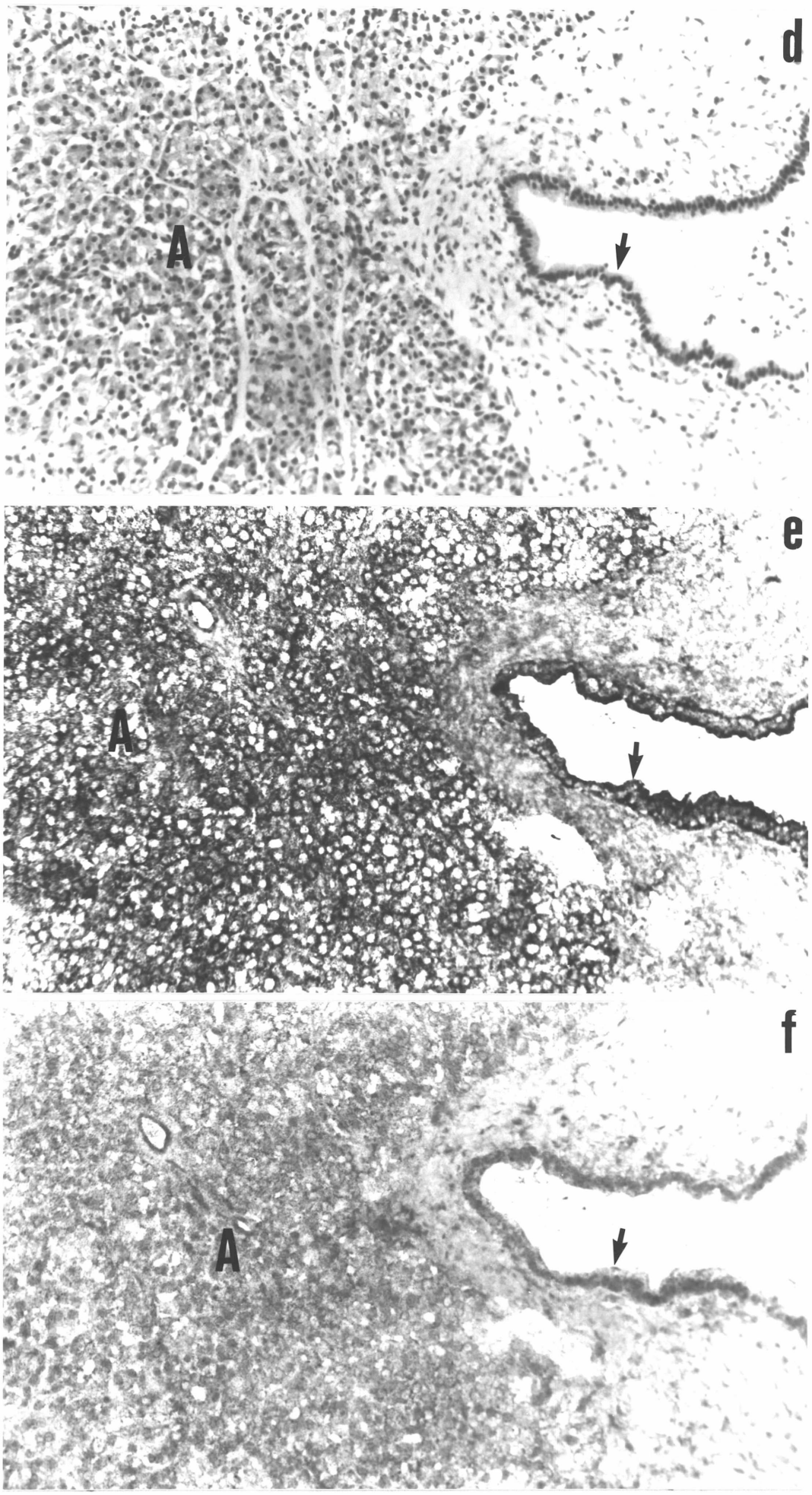
When sections of human pancreas exposed to 3/4/2 were developed with immunoperoxidase no staining was detectable (Fig. 3.7d). However, when immunogold silver detection was used in place of HRP, specific staining of epithelial cells lining ducts was apparent (Fig. 3.7e). There was quite high background staining in this tissue, due to non-specific adsorption of the colloidal gold labelled antibody but this was never as intense as that observed with 3/4/2 (Fig. 3.7f). Despite the high background, with 3/4/2 a slightly heavier immunoprecipitation of silver could be discerned in the acinar cells of the exocrine pancreas.

Figure 3.7: Immunocytochemical localization of P450IA in pancreas

Sections from untreated rat were incubated with either 3/4/2, at a dilution of 1:10 (a, b) or anti-$d_{290\text{-}296}$ at a dilution of 1:50 (c). Sections from human were incubated either with 3/4/2 at a dilution of 1:10 and developed by immunoperoxidase (d) or immunogold-silver staining (IGSS) (e) or with GAM conjugated to 5 nm colloidal gold alone (f). A, acinar cells; L, Islets of Langerhans; → cuboidal cells lining interlobular ducts. Magnifications, (a-c) x175 and (d-f) x65.




a
A
L
b
A
c
A


d
A
e
A
f
A

3.3 Discussion

Immunohistochemical localization of cytochrome P450 has been performed utilizing monoclonal and polyclonal antibodies specific to individual isoenzymes. There was a clear consistency in the distribution of the two isoenzymes of the cytochrome P450IA family in the liver of various mammalian species.

Cytochrome P450IA1 is found in all hepatocytes across the lobule of rats, mice, hamsters and probably rabbits, albeit at low levels with slightly greater isoenzyme content in centrilobular regions of some species. In contrast, P450IA2 is predominately localized in centrilobular hepatocytes of rats and is not detectable at all in periportal regions of mice. In dog and guinea-pig, staining is very weak and/or negligible, respectively, a reflection of either reduced affinity with which 3/4/2 binds to its epitope in these tissues or the absence of the orthologous isoenzyme. Nevertheless, when present, immunoreactive protein is more predominant in centrilobular hepatocytes.

Similar patterns of distribution to those found in the present study have been reported by others for constitutively expressed members of the P450IA sub-family in mouse, rat and rabbit. Members of the P450IA sub-family have been reported to predominate in the centrilobular hepatocytes of mice, regardless of strain (Forkert *et al.*, 1988). In contrast, Anderson and co-workers (1987) could not detect any staining in livers of untreated mice, despite using the same antibody, 1-7-1, and the same strains of mice as Forkert *et al.* (1988). However, the two groups used different fixation regimens and a reduction in antigenicity by 10% formalin could well account for this difference. Anderson *et al.* (1987) also appeared to have consistent problems with high background levels of staining which, in spite of their efforts to take account of, may have influenced the interpretation of their results. High background staining was initially encountered with mouse liver in the present study. However, a systematic investigation revealed that this was, in fact, due to non-specific binding of the second antibody and could be abolished by increasing the concentration of NaCl in the washing buffers. Contrary to the results of Forkert *et al.* (1988) it was found that the distribution of staining differed between IA1 and IA2 in the two strains examined here, IA1 being weakly expressed in all hepatocytes of the lobule whilst IA2 is localized centrilobularly.

In previous studies of rat liver with monoclonal antibodies, cytochrome P450IA1 was found to be relatively evenly distributed across the lobule (Foster *et al.*, 1986), as found in the present study. However, when polyclonal antibodies have been used a slightly higher

concentration of cytochrome P450 was found in the centrilobular region which may be due to strain-related differences (Baron *et al.*, 1981; Wolf *et al.*, 1984). However, polyclonal antibodies bind to more than one epitope and will recognise IA2 as well as IA1, and as shown here IA2 has a predominantly centrilobular distribution in this species. In contrast, IA1 and IA2 were found in hepatocytes throughout the lobule of untreated rabbits when using either 3/4/2, which recognises both isoenzymes, or 107, which recognises only IA2. Dees *et al.* (1982), however, found these isoenzymes to be distributed preferentially in centrilobular and midzonal hepatocytes of untreated rabbits, although immunofluorescence was very weak whereas following TCDD treatment, each isoenzyme was strongly expressed throughout the lobule.

The effect of PB treatment on the distribution of IA1 in the rat was assessed using 3/4/2 and C8. Both these antibodies are highly specific for PAH-inducible isoenzymes (Boobis *et al.*, 1985b; Thomas *et al.*, 1984a). No immunoreactive bands were detected in microsomal fractions from livers of Wistar, Sprague-Dawley, Lister-Hooded or F344 strains of either untreated or PB-treated rats in this study. Thus, it was assumed that staining with 3/4/2 would reflect the expression of only IA1 in these livers. To confirm this hypothesis, a comparison of the distribution of staining was made with that of C8. It is important to note that C8 differs from 3/4/2 in that it is inhibitory and must therefore interact with a different epitope. Indeed, both 3/4/2 and C8 produced identical staining patterns in untreated rats which did not change following treatment with PB, namely immunoreactive protein was confined to a narrow band of centrilobular cells. Thus, treatment of rats with PB leads to a down regulation in the expression of P450IA1 in both midzonal and periportal hepatocytes.

Pretreatment of rats and Balb/c mice with MC induced IA1, manifest as an increase in immunostaining, which was homogeneous throughout the lobule. In contrast, the population of hepatocytes reported by other groups to respond to PAH-treatment of rats is smaller and concentrated in periportal regions (Wolf *et al.*, 1984; Foster *et al.*, 1986). The latter group used the same monoclonal antibody as that employed here. However, these groups used ßNF as the inducing agent rather than MC. Thus it appears that MC is a more potent inducer of IA1 expression than ßNF, which may be related to the pharmacokinetics of the two compounds. This is not the case for C57BL/6 mice in which following treatment with the two agents, the patterns of distribution of IA1 are indistinguishable (Forkert *et al.*, 1988), suggesting the same potency of induction. The considerable inducibility of IA1 by MC in the responsive, Balb/c mice is not observed in the AKR strain. This is consistent

with the findings of numerous previous investigations in which lack of inducibility in certain strains of mice such as DBA/2 and AKR, by MC has been established (Negishi & Nebert, 1979; Anderson *et al.*, 1987; Forkert *et al.*, 1988). Inducibility of IA1 in mice is regulated by a high affinity, cytosolic Ah receptor which is deficient in non-responsive strains. Only a low affinity Ah receptor is present in the latter strains, the levels of which can also be very low (Nebert, 1986; Okey *et al.*, 1988). This might in turn lead to a limited expression of IA1 which could explain the slight reduction in the intensity of staining for IA1 observed in livers of untreated AKR mice.

In the rat, induction of IA2 occurs in a large number of hepatocytes throughout the lobule, although there was more variation amongst cells. In addition, unlike P450IA1 which was uniformly dispersed throughout the cytoplasm of hepatocytes, IA2, as demonstrated with the anti-peptide antibody, also appeared to be more highly concentrated within discrete areas of the cytoplasm, often giving cells a stippled appearance. A similar phenomenon was found in livers from Balb/c mice and intensely stained cells were also interspersed with moderately stained cells, emphasizing the heterogeneous nature of hepatocytes within any one region of the lobule and the specific association of IA2 with certain organelles, a factor which was investigated further in studies at the electron microscope level (see Section 6.2.4).

These results also reveal a difference in the way that individual hepatocytes respond to PAH treatment. Obviously, since the liver is stained homogenously for IA1 after MC-treatment, induction of this isoenzyme is greatest in those hepatocytes which were constitutively deficient, namely, periportal and/or midzonal cells. However, whereas IA1 is distributed homogeneously throughout the lobule, IA2 is more heterogeneously distributed. Intensely staining cells are interspersed with moderately stained cells although all cells were induced and hence the former cells must be more 'responsive' to MC-treatment. It is unlikely that this increased responsiveness is related to transcriptional regulation at the level of the Ah receptor since IA1 does not show such variation in its expression. The post-transcriptional events regulating IA2 might be involved. For example, increased levels of protein can result from decreased degradation, which may be a consequence of ligand binding (Voorman & Aust, 1988). However, why cells should vary in this response is not known.

Less information is available concerning the expression of isoenzymes of the P450IA sub-family in hamster, guinea-pig and dog liver. The data reported here, together with those of Thomas and co-workers (1984b), suggest that the level of P450IA in untreated guinea-pig liver is very low. Indeed, this species is resistant to a number of carcinogens dependent upon metabolic activation (Cardy & Lijinsky, 1980) and is refractory to induction of AHH

and ethoxyresorufin *O*-deethylase by MC (Abe & Watanabe, 1982; Rush *et al.*, 1982). Nevertheless, Abe & Watanabe (1983) purified an isoenzyme (referred to as $P450_{IIA}$) from hepatic microsomal fractions of MC-treated guinea-pigs, that had a minimum M_r slightly greater than that of rat IA2 (54 000 vs. 52 000) and low catalytic activity both towards benzo[a]pyrene and AAF when compared to the rat. Hence, this protein may represent a variant member of the P450I family.

In the hamster, as in the rat there is evidence that the constitutive expression of IA1 is low or absent whereas IA2 is expressed at readily detectable levels (Thomas *et al.*, 1984b; Raucy *et al.*, 1989). In Western blots this protein has a molecular weight of 52 000 (Thomas *et al.*, 1984b). A band of similar mobility was recognised by 3/4/2 in immunoblots of hepatic microsomal fractions from this species. Hence, in the hamster, as in the rabbit, 3/4/2 appears to recognise IA2 as well as IA1. Thus, staining of sections from untreated hamster liver with this antibody will reflect the distribution of the orthologue of IA2 rather than that of IA1.

To date, no MC-inducible isoenzymes have been purified from dog. The present study suggests that members of this family are normally very low or absent.

Clearly, these studies emphasize the value of highly specific antibodies for characterizing the expression and distribution of orthologous isoenzymes in different species. The unavailability of purified human cytochrome IA1 or a tissue other than the placenta, in man highly responsive to induction by PAHs has made the conclusive identification of this isoenzyme in man difficult. Jaiswal *et al.* (1985), using a cDNA probe for mouse IA1, isolated a clone, from a human breast carcinoma cell cDNA library, which had greater than 80% sequence homology with mouse IA1. Cytochrome P450 isoenzymes responsible for AHH activity with antigenic sites common to those of rat P450IA have been identified in lymphocytes and some samples of human liver (Fujino *et al.*, 1982). Recently, by using more specific methods such as Northern blotting, mRNA to IA1 has been identified in the placentas of women who smoked, where ethoxyresorufin *O*-deethylase activity is high. Interestingly, no induction of the IA2 gene was observed (Pasanen *et al.*, 1989). More recently, a human orthologue to rat P450IA2 was isolated from liver by immunoadsorption (Wrighton *et al.*, 1986b) and has been identified as the major enzyme responsible for the high affinity O-deethylation of phenacetin (Distlerath *et al.*, 1985) the role of which has recently been confirmed (Sesardic *et al.*, 1988).

In studies from this laboratory, yet to be published, it was shown that 3/4/2 binds to an

isoenzyme in human liver which has a centrilobular localization. In immunoblotting of microsomal fractions of human liver this antibody recognises a single protein which has the same molecular weight as rat IA2. The intensity of this band shows a positive correlation with cigarette smoking (Sesardic *et al.*, 1988). The centrilobular localization of staining resembles that of IA2 in the rat and mouse. In fact, several additional lines of evidence support the view that IA2 is the only isoenzyme expressed, to any significant extent in human liver. Enzyme activities associated with this isoenzyme are elevated in livers from cigarette smokers (Sesardic *et al.*, 1988) and also appear to be increased on ingestion of charbroiled meats (Boobis & Davies, 1984). Whilst AHH activity (IA1) is greatly increased in the placentae of women who smoke, activity in the liver is unaffected (Pelkonen *et al.*, 1986).

The specific localization of cytochrome P450 in the proximal tubule of rat and human kidney is consistent with that reported in other immunohistochemical and biochemical studies (Wattenburg & Leong, 1962; Fowler *et al.*, 1977; Dees *et al.*, 1982; Foster *et al.*, 1986). In these studies, products of the P450IA genes or evidence of their activity (fluorescent PAH metabolites) were localized in the proximal convoluted tubule of the kidney, expression being greatest in the renal cortex and outer stripe of the medulla, following PAH pre-treatment of the animals. In the present study, a differential distribution of IA1 and IA2 in rat kidney was demonstrated for the first time. P450IA2 was concentrated in the S_2 portion of the proximal tubule whilst IA1 was confined to the S_3 segment in untreated animals. Moreover, whereas renal IA1 is inducible following administration of MC, IA2 is totally refractory to such treatment, consistent with the results of Western blotting (Sesardic *et al.*, 1990).

It is not clear with which isoenzyme orthologue 3/4/2 interacts in human extrahepatic tissues. However, it is currently believed that IA2 is generally expressed only in the liver whereas IA1 is expressed in extrahepatic tissues (Guengerich, 1989). Certainly, in these studies the pattern of staining in human kidney (proximal tubules) and pancreas resembles that of IA1 in the rat. Namely, IA1 was markedly expressed in the cuboidal epithelium lining inter-and intra-lobular ducts of the exocrine pancreas. Acinar cells and those of the Islets of Langerhans were relatively weakly stained. Data on the expression of these isoenzymes in pancreas are limited with only one report in which weak staining of acinar cells of the exocrine pancreas was found (Kawabata *et al.*, 1984). These authors did not detect any immunoreactive cytochrome P450IA in inter- or intra-lobular ducts. It is of interest that epidemiological investigations suggest a causal relationship between exposure to environmental chemicals and human exocrine pancreatic cancer (Wynder, 1975). Moreover,

pancreatic ductular adenocarcinomas can be induced by PAHs and nitrosamines (Bockman *et al.*, 1978; Flaks *et al.*, 1982) providing support for the localization of P450 isoenzymes in these structures.

Chapter four

Ontogenic development of cytochromes P450IA1 and P450IA2 in the rat and rabbit

4.1 Introduction

A large number of studies have been performed on the development of drug-metabolizing systems in the foetus and neonate particularly during the 1970's (for comprehensive reviews see Neims *et al.*, 1976; Klinger *et al.*, 1978; Dutton, 1982; Pelkonen, 1982; Klinger, 1987). Due to the lack of availability of antibodies against specific isoenzymes of drug metabolism, most of these earlier studies concentrated on the development of drug metabolism reactions and attempted to correlate these with the occurrence and/or change in specific electrophoretic bands (Atlas *et al.*, 1977; Guenthner & Nebert, 1978). In a very small number of studies the presence of specific isoenzymes of cytochrome P450 was verified with monoclonal and polyclonal antibodies (Cresteil *et al.*, 1986; Murray *et al.*, 1987; Pacifici *et al.*, 1988; Wrighton *et al.*, 1988).

In rodents, the monooxygenase system appears to be present from very early in embryonic development, as demonstrated by the appearance, and its inducibility by TCDD, of AHH activity in preimplantation mouse embryos, at the time of blastocyst formation (Filler & Lew, 1981). Measurement of P450IA1 and IA2 mRNA levels indicated that the P450IA1 gene was constitutively expressed in 7 day old mouse embryos and in differentiating F9 teratocarcinoma cells (a malignant cell line similar in many respects to the inner cell mass of the early mouse embryo) whilst that of P450IA2 occurred near to the time of birth (Tuteja *et al.*, 1985; Kimura *et al.*, 1987). By day 12 of gestation, benzo[a]pyrene hydroxylation is readily detectable in the rat (Galloway *et al.*, 1980). This period, of organogenesis, also represents the earliest time point at which other biotransformation reactions can be detected (Yang *et al.*, 1988). Using specific antibodies and selective inhibitors and inducers this group demonstrated that at least four different P450 isoenzymes, one of which was identical to or highly homologous with rat P450IA1, are involved in the oxidation of a series of phenoxazone ethers. As a consequence of such reports it was postulated that P450IA1 may serve an unidentified developmental function.

Clearly, embryos are able to metabolize a limited number of xenobiotics, greater metabolizing capacity coinciding with the maturation of the principle organs, such as the liver, lung, kidneys and intestine, a few days prior to parturition. Thus, it is not until after

birth that marked increases in benzo[a]pyrene hydroxylase, 7-ethoxyresorufin O-deethylase, biphenyl-4-hydroxylase, acetanilide 4-hydroxylase and N-acetylarylamine N-hydroxylase activities occur (Guenthner & Mannering, 1977; Atlas *et al.*, 1977; Guenthner & Nebert, 1978; Klinger *et al.*, 1978; Cresteil *et al.*, 1986; Pacifici *et al.*, 1988). Immunochemical studies have further shown that members of the P450IA family could not be detected until two weeks after birth (Cresteil *et al.*, 1986). Also, a monoclonal antibody, 1-7-1, which recognises IA1 inhibited no more than 30% of ethoxyresorufin-O-deethylase activity (Pacifici *et al.*, 1988). Cresteil *et al.* (1986) could not find any correlation between certain monooxygenase activities (for example, benzphetamine-N-demethylase, benzo[a]pyrene hydroxylase and 11- and 12-lauric acid hydroxylase) and the appearance of specific isoenzymes of P450, known to catalyze these reactions in adult animals, in hepatic microsomal preparations from untreated rats (as determined by Western blotting) with increasing age.

Nevertheless, many of the isoenzymes of P450 are induced by perinatal exposure to foreign chemicals, in some instances correlating with the development of specific isoenzymes (Cresteil *et al.*, 1986). For example, the ontogeny of inducible AHH and acetanilide 4-hydroxylase activities in the rat corresponds well with increases in electrophoretic bands of 56 000 and 54 000 molecular weight, respectively (Guenthner & Nebert, 1978). This was also true for the 2- and 4-hydroxylation of biphenyl respectively, in the rabbit (Atlas *et al.*, 1977). The electrophoretic band of 57 000 molecular weight induced transplacentally by TCDD in the rabbit was identified as P450 LM6 (IA1) by comparison of peptide fingerprints with those of purified adult P450 LM6 (Norman *et al.*, 1978). In later experiments, using Western blot analysis with polyclonal antibodies, the isoenzymes induced in foetal and neonatal rat were identified as P450IA1 and IA2, respectively (Cresteil *et al.*, 1986). Whereas AHH (IA1) is strongly inducible prenatally, the onset of induction of biphenyl-4-hydroxylase (IA2) is delayed by at least 5 days and occurs only following parturition, suggesting that an age dependent factor controls the development of IA2 inducibility (Atlas *et al.*, 1977; Cresteil *et al.*, 1986).

Some cytochrome P450 isoenzymes have been detected in extrahepatic tissues, but at levels 10 to 100-fold less than those found in liver. Nevertheless, P450IA1 appears to be ubiquitous in the body, evidence from many laboratories, using Northern blotting, enzymatic, electrophoretic and immunochemical techniques, supporting its expression in a wide variety of tissues of different mammalian species at least after PAH treatment (Atlas *et al.*, 1977; Guengerich & Mason, 1979; Klinger *et al.*, 1978; Liem *et al.*, 1980; Dees *et al.*, 1982; Domin *et al.*, 1984; Goldstein & Linko, 1984; Kawabata *et al.*, 1984; Tuteja *et*

al., 1985; Baron *et al.*, 1986; Kimura *et al.*, 1986; Foster *et al.*, 1986; Finlayson *et al.*, 1987; Anderson *et al.*, 1987; Keith *et al.*, 1987; Baron *et al.*, 1988; Söderkvist *et al.*, 1988; Näslund *et al.*, 1988; Sesardic *et al.*, 1990). In contrast, IA2 is virtually absent and is refractive to induction by MC or TCDD, expression being at the limits of detection of the assays employed (Tuteja *et al.*, 1985; Kimura *et al.*, 1986; Söderkvist *et al.*, 1988; Sesardic *et al.*, 1990).

Of these many reports only in three was the ontogeny of development of these isoenzymes in extrahepatic tissues examined, one by the method of Northern blotting (Tuteja *et al.*, 1985) and the others by measurement of AHH activity (Atlas *et al.*, 1977; Klinger *et al.*, 1978). All of the studies indicated that the extent of induction by MC varied with the age of the animal and that in the rabbit kidney was associated with an increase in an electrophoretic band of 57 000 M_r, most probably IA1. In contrast, only a moderate induction of AHH activity by MC was observed in lung microsomes from 3 day old rabbits (Atlas *et al.*, 1977). No increases in the levels of IA2 mRNA or of monooxygenases catalyzed by this isoenzyme was reported in these tissues. From these studies, it was concluded that at least three different tissue-specific modes of regulation for P450IA1 exist.

The use of one monooxygenase activity such as AHH as a probe for a single isoenzyme, such as in many of the studies above, is not valid because of the overlap in substrate specificity of the isoenzymes of cytochrome P450. Further, densitometric scanning of a band on an SDS polyacrylamide gel may well result in mis-leading conclusions, as a number of unrelated microsomal proteins may have very similar monomeric molecular weights, which might also change in response to the xenobiotic. Clearly there is a need for more specific techniques/probes in the study of the ontogeny of specific isoenzymes of cytochrome P450. The age-related variation in the relative distributions of different isoenzymes of P450 not only between tissues but also within tissues, which are, more often than not, composed of heterogeneous cell populations has yet to be determined. The utilization of immunochemical techniques has been extended in these studies to monitor the expression and patterns of distribution of P450IA1 and IA2 in various tissues from untreated and MC-treated rat and rabbit, with highly specific monoclonal, 3/4/2, C8, 1-7-1, 12/2/3/2 and 107 and well characterized polyclonal, anti-$d_{290\text{-}296}$ and anti-d(-c), antibodies.

4.2 Results

4.2.1 Ontogeny of P450IA1 and P450IA2 in rat liver

4.2.1.1 Total cytochrome P450 content of liver microsomes

Cytochrome P450 was below detection in microsomal homogenates of untreated rat foetal livers (Table 4.1). Within 24 h of parturition, total cytochrome P450 content rose to 20% of the adult value and thereafter to 50% and 80% of adult values by 5 and 15 days, respectively. Following MC-treatment of pregnant rats, P450 could be detected as early as 24 h pre-partum, when levels were less than 6% of the value in MC-treated adult (Table 4.1). Thereafter, levels rose gradually to reach 75% of adult values in 15 day old rats. Maximum induction of total P450 content by MC occurred sharply 24 h pre-partum, with a >25-fold increase relative to those in untreated animals. There was no induction of cytochrome P450 in 1 day old rats but levels were almost doubled in microsomal homogenates of 5 day old rats. Thereafter, significant (about 1.5-fold) induction by MC continued into adulthood (Table 4.1).

4.2.1.2 Immunostaining of Western blots of liver microsomes

Western blots containing liver microsomal proteins from both untreated and MC-treated rats were probed with either a monoclonal antibody, 12/2/3/2, which recognises both IA1 (P450c) and IA2 (P450d) in the rat (Sesardic *et al.*, 1986) or the polyclonal antibody, anti-$d_{290\text{-}296}$ which recognises only IA2. No immunoreactive proteins could be detected in microsomal samples from untreated adult rats. In samples from MC-treated adults two bands could be detected with 12/2/3/2 (Fig. 4.1a) but only one band with anti-$d_{290\text{-}296}$ (Fig 4.1b). These specifically reacting bands were identified as P450IA1 (P450c, Mr 57 000) and IA2 (P450d, Mr 54 000), respectively, by their co-migration with purified preparations of these isoenzymes.

At the very high protein levels used to try to detect low levels of these isoenzymes in hepatic microsomal samples from foetal and perinatal rats, there was some staining of high (M_r > 75 000) and low molecular weight bands (M_r < 48 000). However, these same bands were detected in Western blots probed only with the secondary antibody, either anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase, and thus are thought to represent non-specific binding of the second antibody (data not shown). In general, levels of IA1 were below detection in untreated animals at most ages, although a weak band

Age/days	Treatment	Total P450	Immunoreactive P450IA1	Immunoreactive P450IA2
		pmoles P450.mg^{-1} protein		
-3	None	bd	11 ± 6 (4)	74
	MC	bd	bd	85
-1	None	bd (3)	bd (3)	88 ± 19 (8)
	MC	36 ± 11* (3)	bd (3)	114 ± 20* (5)
1	None	84 ± 3 (3)	bd (3)	97 ± 15 (8)
	MC	79 ± 16 (3)	bd (3)	93 ± 25 (6)
5	None	194 ± 27 (4)	8	75 ± 21 (13)
	MC	347 ± 42* (4)	39 ± 11* (6)	110 ± 23* (14)
15	None	351 ± 70 (4)	bd (4)	132 ± 49 (9)
	MC	475 ± 87 (4)	97 ± 18* (6)	219 ± 60 (11)
Adult	None	433 ± 83 (5)	2 ± 3 (4)	72 ± 4 (3)
	MC	1710 ± 200 (3)	815	190 ± 51 (5)

Table 4.1: Ontogenesis of cytochromes P450IA1 and P450IA2 in rat liver

Results are mean ± standard deviation, where shown, with the number of determinations in parentheses. A minimum of two preparations were used at each time point. * MC values significantly different from untreated values at P≤0.05.

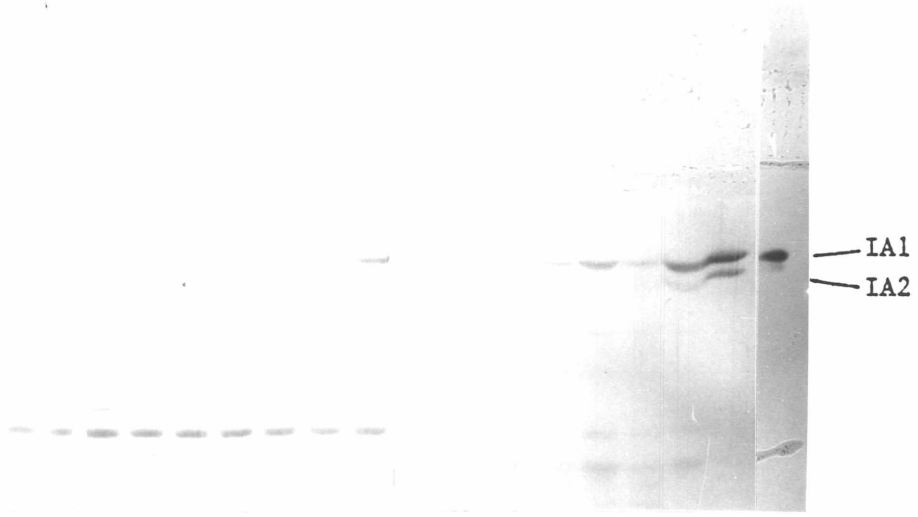
bd below detection. Limit of detection for P450IA1 and P450IA2, 1.25 pmole.mg^{-1} protein.

Figure 4.1: Ontogenic expression of P450IA1 and P450IA2 in hepatic microsomes of rats determined by immunoblotting

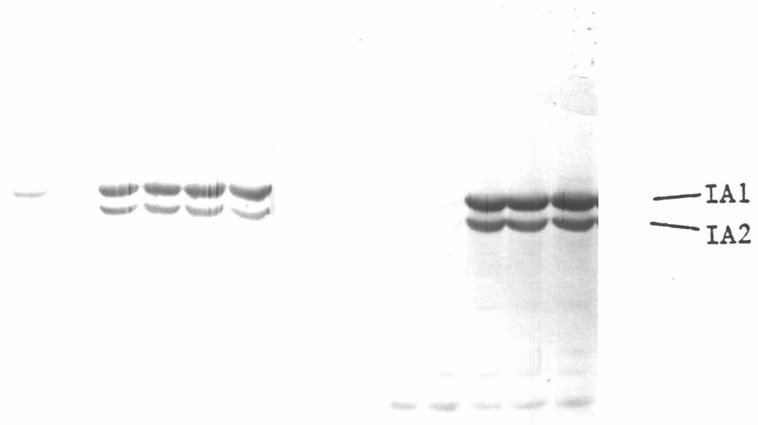
Microsomal proteins were separated by SDS-PAGE and subjected to Western blotting as described in Sections 2.2.13 and 2.2.14. Immunoblots were stained with (a) MAb 12/2/3/2, 1 $\mu g.ml^{-1}$ or (b) anti-$d_{290-296}$ antibody, 3.5 $\mu g.ml^{-1}$. Fractions were as follows: (a) Lane 1, untreated (UT) -3 day old, 60 µg; lanes 2 and 3, MC-treated -3 day old, 60 µg; lanes 4-6, UT -1 day old, 60 µg; lanes 7-9, MC-treated -1 day old, 60 µg; lanes 10-12, UT 1 day old, 60 µg; lanes 13-15, MC-treated 1 day old, 60 µg; lane 16, MC-treated pregnant mother to -3 day old foetuses, 20 µg; lane 17, MC-treated pregnant mother to -1 day old foetuses, 20 µg; lane 18, purified rat P450IA1, 2 pmoles; lane 19-22, UT 5 day old, 20 µg; lanes 23-26, MC-treated 5 day old, 20 µg; lane 27-30, UT 15 day old, 20 µg; lanes 31-33, MC-treated 15 day old, 20 µg.

(b) As (a) except lane 10 and 18 purified P450IA2, 2 and 1 pmole respectively.

a

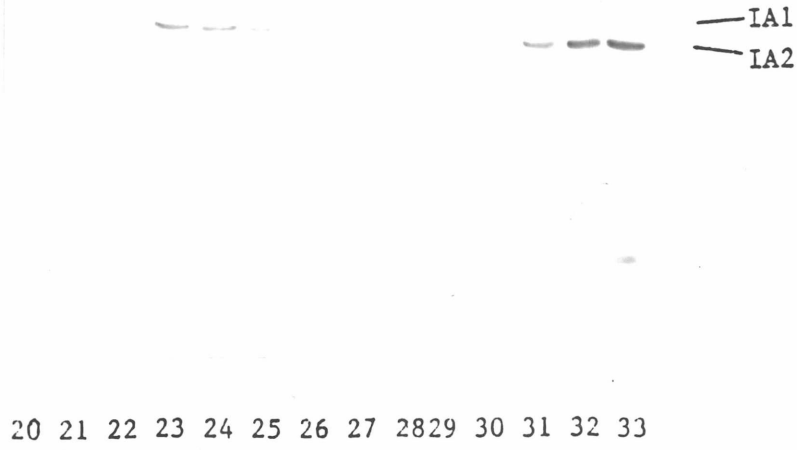


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18



19 20 21 22 23 24 25 26 27 2829 30 31 32 33


IA1
IA2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18


IA1
IA2
19 20 21 22 23 24 25 26 27 2829 30 31 32 33

could be detected in some samples of 5 day old rat livers (Fig. 4.1a). In contrast, no immunoreactive band co-migrating with purified IA2 could be detected in microsomal samples from untreated rats at any age (Fig. 4.1b).

Following MC-treatment of rats, an immunoreactive band co-migrating with IA1 could be detected in microsomal samples from as early as 24 h pre-partum (Fig. 4.1a). This band increased in intensity with increasing age. It was not until 5 days after parturition that a band co-migrating with IA2 could be detected on MC-treatment, the intensity of which increased with increasing age (Fig 4.1b).

4.2.1.3 Immunoquantification of P450IA1 and P450IA2 in liver

Dot blots, probed with antibodies specific to either IA1, 3/4/2 or IA2, anti-$d_{290\text{-}296}$, were used to quantify the changes in the levels of these two isoenzymes observed in Western blots. There was a linear relationship between the amount of protein applied to the nitrocellulose sheets and the density of the dot, both for purified preparations of IA1 and IA2 and for the microsomal preparations studied, over the range of concentrations used ($r \geq 0.91$). The gradients of the concentration-optical density lines were the same before and after treatment of the animals with MC. The limits of detection for each of these isoenzymes was 125 fmoles of cytochrome P450.

Constitutive levels of IA1 represented less than 5% of the total cytochrome P450 pool at any age whilst those of IA2 were higher but variable, ranging from 17-40% (Table 4.1). Following MC-treatment of rats, levels of IA1 increased 5-fold in 5 day old rats but >100-fold in 15 day old and adult rats. This isoenzyme was still below the limits of detection in foetal and perinatal livers. Although detectable constitutively, cytochrome P450IA2 was refractive to MC treatment in foetal and perinatal livers. It was not until 5 days after parturition that levels increased significantly (Table 4.1). At 15 days, IA2 was increased 2-fold approaching levels of induction similar to that seen in adults.

4.2.1.4 Effect of age on the localization of P450IA1 in liver

A specific immunoreaction was detected in sections from livers of 72 h pre-partum rats following incubation with all three of the MAbs, 3/4/2, C8 and 1-7-1 but only when the antibodies were used at low dilutions (Table 4.2). Most hepatocytes across the lobule were intensely stained with 3/4/2, although some cells did not stain (Fig. 4.2a).

Table 4.2: Immunotitration of the binding of antibodies to rat liver

Degree of staining: +++ intense, ++ moderate, ++/+ some sections moderate others weak, + weak, +/- some sections weak others negative, - negative.

[a] Control IgG= pre-immune rabbit serum

[b] some intensely stained cells, interspersed with moderately to weakly stained cells

[c] a few scattered cells, not indicated in the table, were intensely stained

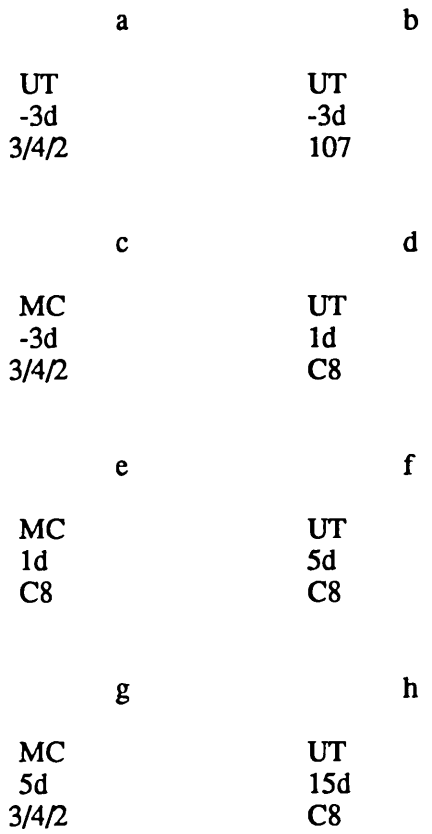
[d] intensely stained cells interspersed with moderate to weakly reacting cells

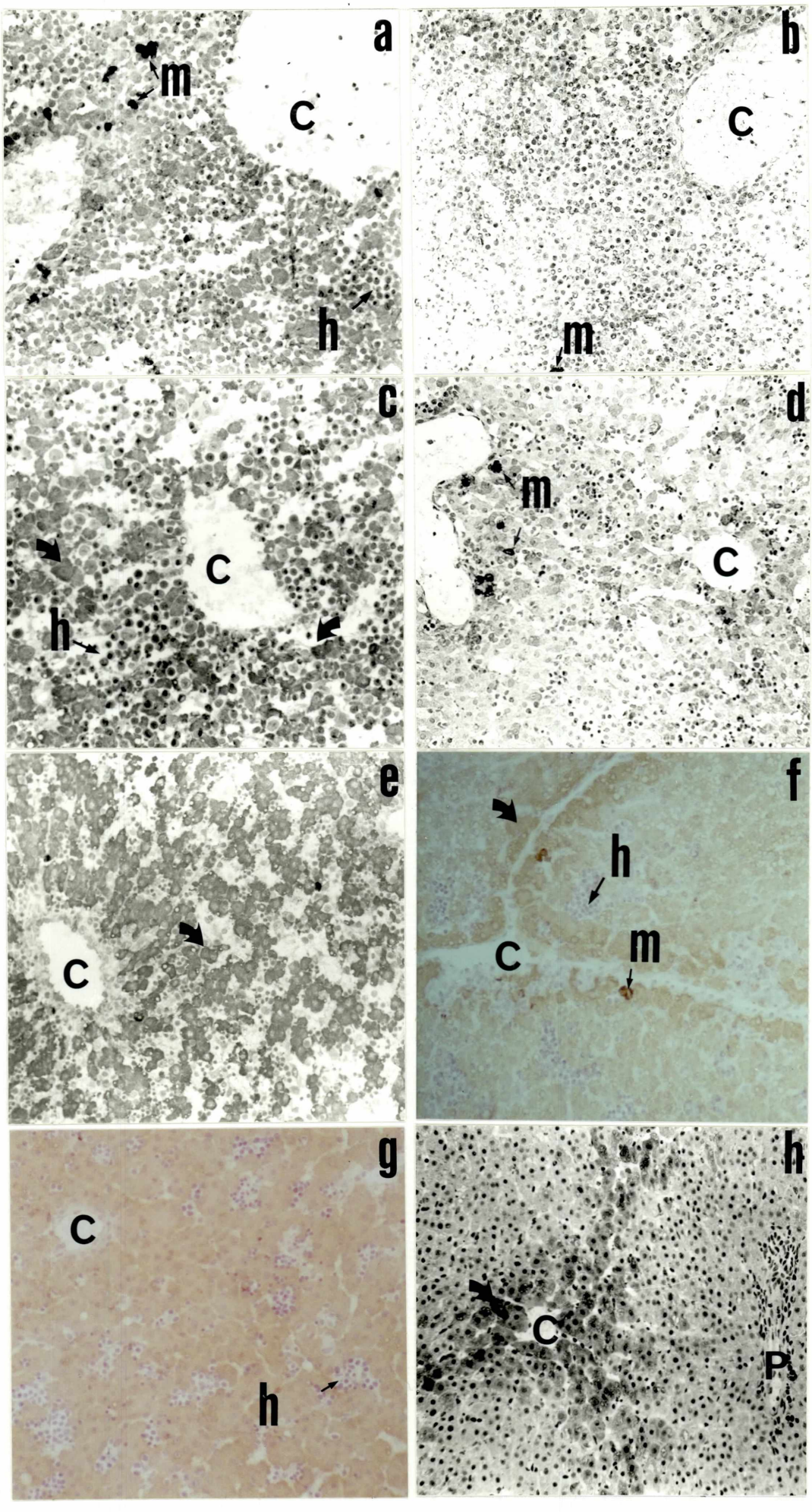
[e] staining confined to a narrow band of centrilobular cells; others weak to negative.

Age/days	Treatment	Antibody	Antibody dilution 1:10	1:50	1:100	1:500
-3	None	3/4/2	++	++	+	-
		C8	+	+/-	-	
		1-7-1	+	+/-	-	
		anti-$d_{290-296}$		++	++/+	+/-
	MC	3/4/2	+++	+++	++	+
		C8	+++	++	++	+
		1-7-1	+++	++	++	+
		anti-$d_{290-296}$		++	+	+/-
-1	None	3/4/2	+/-	+/-	-	-
		C8	+/-	-	-	-
		1-7-1	+/-	-	-	
		anti-$d_{290-296}$		+++	++	+/-
		anti-d(-c)		++	++/+	+/-
		Control IgG[a]		-	-	-
	MC	3/4/2	+++	+++	+++	+++
		C8	+++	+++	+++	+++
		1-7-1	+++	+++	+++	++
		anti-$d_{290-296}$		+++	++	++
		anti-d(-c)[b]		+++/++/+	++/+/-	++/+/-
		Control IgG		-	-	-
1	None	3/4/2	+/-	+/-	-	-
		C8	+	+/-	-	-
		1-7-1[c]	+/-	-	-	
		anti-$d_{290-296}$		++	+	+/-
		anti-d(-c)		+	-	
		Control IgG		-	-	
	MC	3/4/2	+++	+++	++	++
		C8	+++	+++	++	++
		1-7-1	+++	+++		
		anti-$d_{290-296}$		+++	++	++
		anti-d(-c)[b]		+++/++/+	+++/++/+	+++/++/+
		Control IgG		-	-	-
5	None	3/4/2	++	++/+	+	
		C8	++	++/+		
		anti-$d_{290-296}$		++	++/+	+
	MC	3/4/2	+++	+++	++	++/+
		C8			+++	++
		anti-$d_{290-296}$[d]		+++/++/+	+++/++/+	++/+/-
15	None	3/4/2[e]	+++	+++	++/+	-
		C8[e]	+++	+++	+++	++
		anti-$d_{290-296}$		++	+	-
	MC	3/4/2	++	++		++
		C8	++	++		+
		anti-$d_{290-296}$		+++	+++	++

Figure 4.2: Ontogeny of immunocytochemical localization of P450IA1 in rat liver

Sections from untreated -3 day old (a, b), 1 day old (d), 5 day old (f) or 15 day old (h) rats or MC-treated -3 day old (c), 1 day old (e) or 5 day old (g) rats were incubated with monoclonal antibodies 3/4/2 (a, c, g), C8 (d-f, h) or 107 (b) at dilutions of 1:10 (a, b, d) or 1:100 (c, e-h). C, central vein; P, portal tract; m, tissue macrophages containing non-specifically reacting peroxidases; h, haematopoietic cells; ➨ most intensely stained hepatocytes. Magnifications, (a, b) x138; (c-e, h) x110 and (f, g) x164.




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Sections from livers of 24 h pre- and post-partum rats, did not show any appreciable immunostaining following exposure to 3/4/2, C8 or 1-7-1. However, in one of the 24 h post-partum livers a strong reaction with C8 and 1-7-1 was observed in a few, scattered cells throughout the lobule (Fig. 4.2d).

A moderate immunoreaction was observed in liver sections from 5 day old rats following exposure to either 3/4/2 or C8 at dilutions of 1:100 or less (Table 4.2). This was most intense in a narrow band of hepatocytes (1-3 cell rows) lining bifurcating central veins (Fig. 4.2f). Hepatocytes surrounding other central veins and across the rest of the lobule were weakly and evenly stained.

At 15 days of age, the cross sectional anatomy of the liver is more representative of that seen in the adult and very few haematopoietic cells remain. Following exposure of sections to either 3/4/2 or C8, a specific immunoreaction was observed in a narrow band of cells (3-4 cell rows) radiating out from central veins (Fig. 4.2h). The intensity of this reaction was maintained at high antibody titres (Table 4.2). A weak immunoreaction was evident in the other hepatocytes across the lobule, but only following exposure to high concentrations of 3/4/2 (1:10). In sections from adult livers, a moderate to weak, specific immunoreaction was evident in most hepatocytes across the lobule, with stronger staining in a narrow band of centrilobular cells (see Section 3.2.1).

Following MC-treatment, a strong, specific immunoreaction was observed in liver sections from rats of all ages, with both 3/4/2 and C8, even at dilutions as low as 1:500, indicating considerable induction of IA1 (Table 4.2). The immunoreactive product was homogeneous throughout the cytoplasm of the hepatocytes (Fig. 4.2c, 4.2e, 4.2g). In perinatal livers (1 day old) the intensity of the immunoreaction varied between hepatocytes throughout the lobule, although there was no difference between the overall staining intensity of centrilobular and periportal cells (Fig. 4.2e).

Replacement of either of these antibodies with an inappropriate antibody, 107, or normal rat serum, resulted in a total lack of staining of hepatocytes in sections from both untreated and MC-treated rats of all ages (e.g. Fig. 4.2b).

Haematopoietic cells were clearly evident in liver sections from rats up to 5 days of age. No staining of such cells was observed with these antibodies. However, extremely intense staining of other non-parenchymal cells, present in clusters around portal tracts and scattered throughout other areas of the lobule was noticeable in these sections (Fig. 4.2a,

4.2d, 4.2f). Serial sections probed with MAb 107 or normal rat serum also showed intense staining of these cells (Fig. 4.2b). Sections were not pre-incubated in H_2O_2, hence staining probably represents residual endogenous peroxidase activity of tissue macrophages, eg Kupffer cells. Interestingly, only a few of these cells were present in liver sections from 72 h pre-partum rats and their numbers appeared to gradually decrease with increasing age.

4.2.1.5 Effect of age on the localization of P450IA2 in liver

Exposure of liver sections from untreated 72 h pre-partum foetuses to anti-$d_{290\text{-}296}$ antibody or anti-d(-c) antibody, resulted in specific staining of most hepatocytes. Immunoreactive protein was homogeneously distributed within the cytoplasm of cells (Fig. 4.3a).

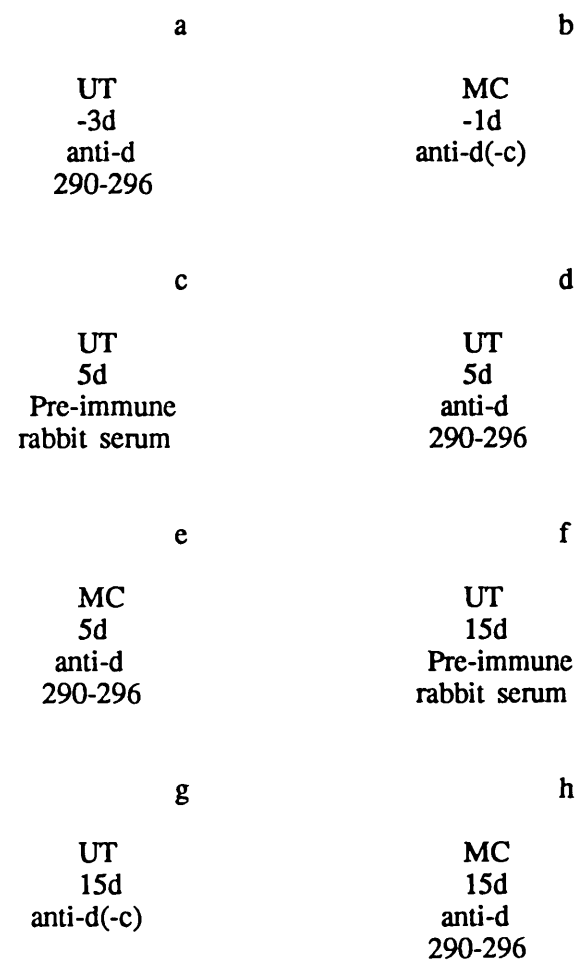
The distribution and intensity of the immunoreaction produced by these antibodies did not change through parturition and for the first 5 days after birth (Fig. 4.3d). However, by 15 days, the immunoreaction to both antibodies was slightly more intense in centrilobular cells (3-5 cell rows) (Fig. 4.3g). The intensity of the immunoreaction in the other hepatocytes was moderate to weak. The same pattern of staining with the anti-$d_{290\text{-}296}$ antibody in sections from adult livers was the same as in these livers, but extended to more centrilobular hepatocytes (see Section 3.2.1). With the anti-d(-c) antibody, staining in adult livers was more even across the lobule.

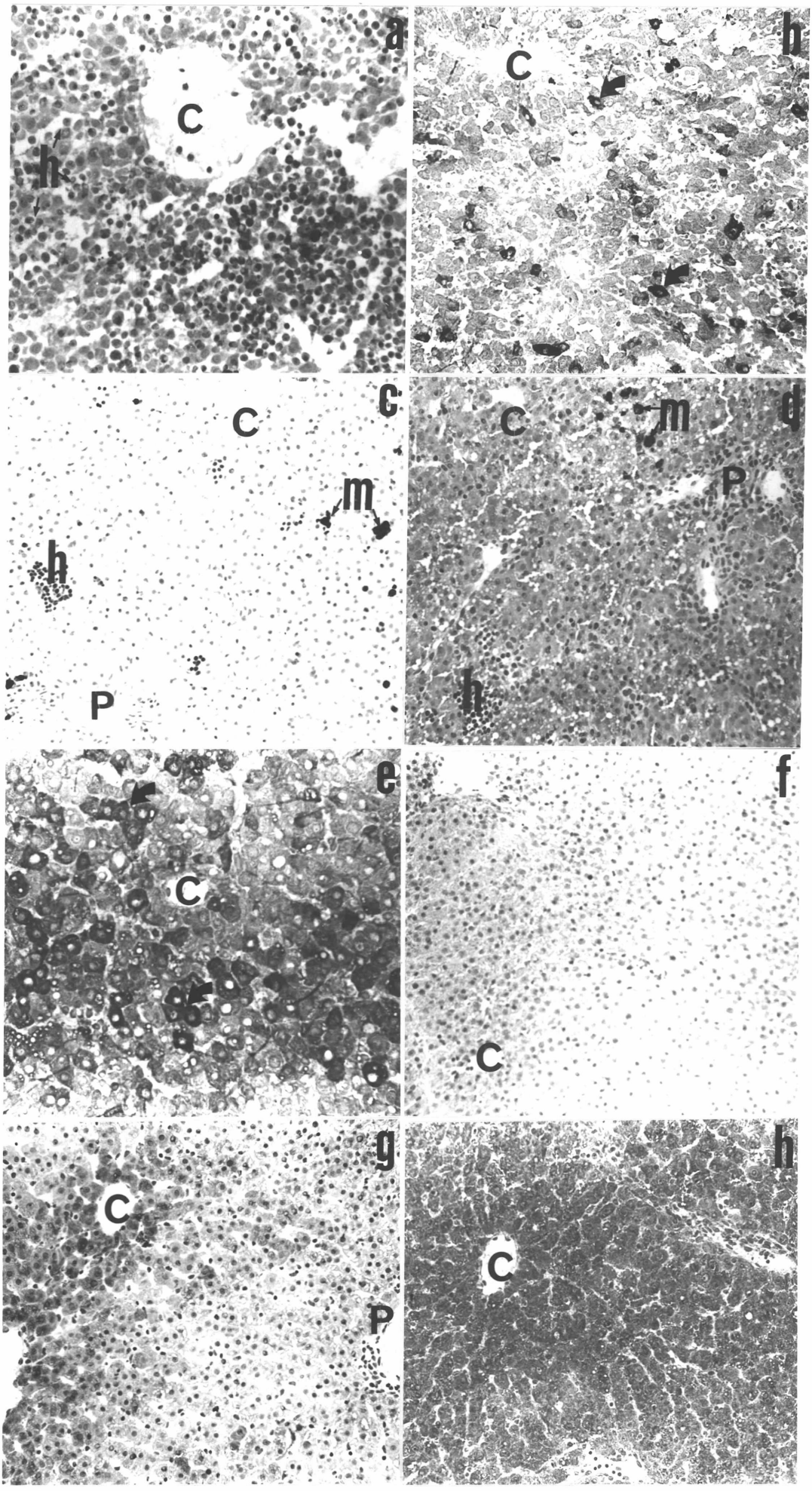
The distribution and intensity of staining of cytochrome P450IA2 in the liver of 72 h pre-partum rats were not affected by MC-treatment. This was also true for two of four 24 h pre-partum livers examined (Table 4.2). However, in the other two 24 h pre-partum livers, strong immunostaining with anti-d(-c) antibody was observed in single hepatocytes, randomly scattered across the lobule (Fig. 4.3b). This was also true following incubation with anti-$d_{290\text{-}296}$, but the intensity of the immunoreaction was slightly weaker, and against more intense staining of the other hepatocytes, was more difficult to see.

Following MC-treatment, intense immunostaining of single hepatocytes scattered throughout the lobule was also observed in livers from 24 h post-partum rats. Immunostaining of the other hepatocytes was the same as that of hepatocytes in the respective untreated livers (Table 4.2). In 5 day old rats, MC-treatment caused a dramatic increase in the intensity of staining of a large number of hepatocytes (Fig. 4.3e). Mosaics of moderate to strongly reacting hepatocytes were observed around central veins and in other parts of the lobule. However, small foci of cells in midzonal and periportal regions were less intensely stained,

Figure 4.3: Ontogeny of immunocytochemical localization of P450IA2 in rat liver

Sections from either untreated -3 day old (a), 5 day old (c, d) or 15 day old (f, g) rats or MC-treated -1 day old (b), 5 day old (e) or 15 day old (h) rats were incubated with anti-$d_{290\text{-}296}$ (a, d, e, h) or anti-d(-c) (b, g) or pre-immune rabbit serum (c, f), all at a dilution of 1:100. C, central vein; P, portal tract; h, haematopoietic cells; m, tissue macrophages containing non-specifically reacting peroxidases; ➦ intensely stained hepatocytes. Magnifications, (a, e) x156; (b) x138 and (c, d, f-h) x110.




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resembling more those of untreated livers. The intensity and distribution of this immunoreaction was maintained even at higher dilutions of antibody (Table 4.2).

In livers from 15 day old and adult rats treated with MC, a very strong specific immunoreaction was observed in most hepatocytes throughout the lobule with both antibodies (Fig. 4.3h) even at high dilutions (Table 4.2). The immunoreaction was not entirely homogeneous within the cytoplasm of individual hepatocytes, but appeared punctate or stippled (see Section 3.2.1). Moreover, the absolute intensity of the immunoreaction within individual cells varied in an apparently random fashion in livers of 15 day old rats.

There was no specific immunostaining of haematopoietic cells by these antibodies at any age. However, at low antibody dilutions (1:50) some staining of megakaryocytes was apparent which disappeared upon further dilution of the antibody. Replacement of either of the antibodies with pre-immune rabbit serum or normal rat serum, resulted in a total lack of staining of hepatocytes (Table 4.2; Figs. 4.3c and 4.3f).

4.2.2 Ontogeny of P450IA1 and P450IA2 in rabbit liver

4.2.2.1 Total cytochrome P450 content of liver microsomes

Total cytochrome P450 content was less than 70 pmoles.mg^{-1} protein in livers of untreated -4 day old rabbit foetuses, representing 10% or less of that in microsomal samples from adult livers (Table 4.3). Levels gradually increased with age, to a plateau of approximately 600 pmoles.mg^{-1} protein in weanling and adult rabbits. MC-treatment of rabbits caused significant increases in total cytochrome P450 content at most ages, except perinatally (1 day of age). Maximum induction occurred in 9-11 day old rabbits, in which levels were 4-fold greater than those of untreated rabbits (Table 4.3).

4.2.2.2 Immunostaining of Western blots of liver microsomes

Western blots of liver microsomal proteins from untreated and MC-treated rabbits were probed with antibodies which recognise both rabbit IA1 and IA2; namely, 3/4/2 or a polyclonal antibody raised against a purified preparation of rat cytochrome P450IA2 (P450d). This antibody has been referred to as anti-P450IA in this study. Monoclonal antibody 3/4/2 had a much lower affinity for isoenzymes of the IA sub-family in the rabbit than for IA1 in the rat, as evident from the weak signal obtained in immunostained blots

Table 4.3: Ontogeny of cytochrome P450IA2 in rabbit liver

Age/days	Treatment	Total P450	Immunoreactive P450IA2
		pmoles P450.mg^{-1} protein	
-4	None	63	bd (3)
	MC	223 ± 75 (3)	bd (3)
-1	None	108 ± 13 (3)	bd (3)
	MC	151	bd (3)
1	None	155 ± 35 (3)	2 ± 1 (3)
	MC	113 ± 27 (3)	3 ± 1 (3)
6	None	187 ± 19 (3)	2 ± 1 (4)
	MC	264 ± 47 (3)	40 ± 13 (6)
9-11	None	286 ± 61 (3)	9 ± 2 (9)
	MC	1216 ± 62 (3)	721 ± 136 (5)
17-21	None	499 ± 65 (3)	40 ± 12 (14)
	MC	1744 ± 212 (3)	1049 ± 148 (13)
Weanling	None	597 ± 64 (6)	42 ± 10 (5)
	MC	1801 ± 465 (5)	333 ± 72 (4)

Results are mean ± standard deviation from at least three separate preparations, with the number of determinations in parentheses. In rabbits over 6 days of age, MC values significantly different from untreated values at $P \leq 0.001$. bd, below detection. Limits of detection for P450IA2 1.5 pmole.mg^{-1} protein.

(Section 3.2.1). Anti-P450IA recognized orthologues of both IA1 and/or IA2 in mouse ($P_3$450), rat (P450s c and d) and rabbit (forms 4 and 6) with relatively high affinity when compared with 3/4/2 (Fig. 4.4a). Hence, this antibody was particularly useful for screening livers from rabbits of different ages, for the presence of these isoenzymes. No immunoreactive bands were detectable in microsomes from untreated, PB-, PCN-, or INH-treated rats, but an immunoreactive band, co-migrating with IA2 was recognized in microsomes from rats pre-treated with isosafrole and both IA1 and IA2 were detected in microsomes from MC-treated rats.

No IA1 or IA2 could be detected in Western blots of microsomal samples from untreated rabbits, 6 days old and younger (Fig. 4.4b). A band co-migrating with purified rabbit IA2 (M_r 54 000) was detected in all livers from rabbits 9-11 days old and above, the intensity of which increased with age. A weak band (approximate M_r 57 000) corresponding to IA1, based on its electrophoretic mobility, was detected only in livers from rabbits 9-11 days old and above (Fig. 4.4b).

P450IA2 in liver was still below detection after MC-treatment of rabbits 1 day old and younger. In contrast, IA1 was induced in livers from 96 h pre-partum foetuses as revealed by the presence of an immunoreactive band of M_r 57 000 (Fig. 4.4b). The intensity of this band in MC-treated animals decreased with increasing gestational age, being barely detectable in livers from 24 h pre-partum foetuses. In livers from rabbits 6 days old and above, there was a marked increase in both IA1 and IA2, the inducibility of which increased as a function of age (Fig. 4.4b).

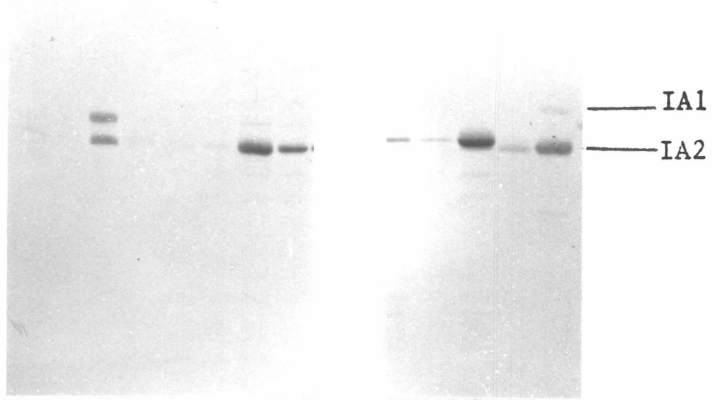
4.2.2.3 Immunoquantification of P450IA2 in liver microsomes

In the rabbit, only IA2 could be immunoquantified by the dot blot method, as no antibody specific for only rabbit IA1 was available. Thus, dot blots were probed with MAb 107, specific to IA2, which recognises only undenatured protein, although with very high affinity. This was reflected in the fact that the intensity of blots stained with very high titres of this antibody (1:10 000) were identical to that of blots stained with dilutions of 1:1000. There was a linear relationship between the amount of protein applied to the nitrocellulose sheets and the density of the dot, both with partially purified IA2 (15-500 fmole.well^{-1}) and with the microsomal preparations studied, over the range of concentrations used ($r \geq 0.95$). The gradient of the concentration-optical density lines did not change following treatment of rabbits with MC. However, immunoquantification of IA2 in hepatic microsomal samples by MAb 107, which does not interact with IA1, will

Figure 4.4: Ontogenic expression of P450IA1 and P450IA2 in rabbit liver

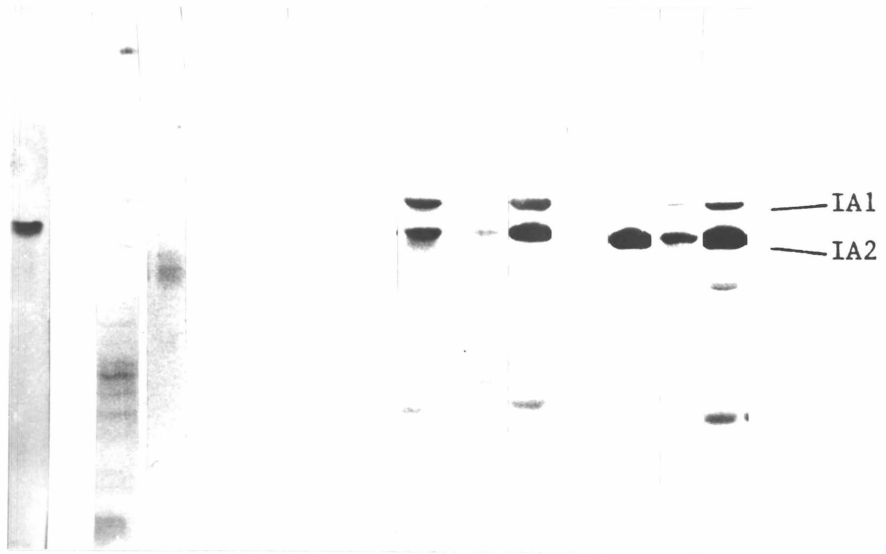
Microsomal proteins were separated by SDS-PAGE and subjected to Western blotting as described in Sections 2.2.13 and 2.2.14. Immunoblots were stained with anti-P450-IA, diluted 1:1000. (a) Microsomal samples (20 μg) were as follows: Lane 1, untreated rat; lane 2, PB-treated rat; lane 3, MC-treated rat; lane 4, acetone-treated rat; lane 5, PCN-treated rat; lane 6, INH-treated rat; lane 7, ISF-treated rat; lane 8, purified rat P450IA2, 4 pmoles; lane 9, MC-treated AKR mouse; lane 10, untreated Balb/c mouse; lane 11, MC-treated Balb/c mouse; lane 12, untreated rabbit and lane 13, MC-treated rabbit. (b) Microsomal samples were as follows: lane 1, rabbit P450IA2, 2 pmoles; lane 2, untreated and lane 3, MC-treated -4 day old, 50 μg; lane 4, untreated and lane 5, MC-treated -1 day old, 50 μg; lane 6, untreated and lane 7, MC-treated 1 day old, 50 μg; lane 8, untreated and lane 9, MC-treated 6 day old, 50 μg; lane 10, untreated and lane 11, MC-treated 9-11 day old, 50 μg; lane 12, untreated and lane 13, MC-treated 17-21 day old, 25 μg; lane 14, untreated and lane 15, MC-treated weanling, 25 μg.

a



1 2 3 4 5 6 7 8 9 10 11 12 13

b



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

be slightly underestimated since the preparation of IA2 against which staining was standardized, was contaminated with small amounts of IA1 as revealed when >4 pmoles was loaded on to Western blots (data not shown).

Cytochrome P450IA2 could not be detected in microsomal fractions from either untreated or MC-treated foetuses (Table 4.3). Very low levels (<1% of adult values) were observed in livers within 24 h of parturition. Thereafter, levels increased gradually, such that in livers from 17-21 day old and weanling rabbits they were still <30% of those in adult livers (143 ± 44 pmole.mg^{-1} protein).

This isoenzyme was not inducible by MC-treatment of rabbits until between 1 and 6 days after parturition. In 6 day old animals IA2 content increased 20-fold representing >15% of the total cytochrome P450 pool (Table 4.3). The most dramatic increase in this isoenzyme occurred in livers of 9-11 day old rabbits (>80-fold induction), such that levels of IA2 represented >60% of the total cytochrome P450 pool. Thereafter, in livers from weanling and adult rabbits, the fold-induction was not as great (4-8-fold) and the contribution of IA2 to the total cytochrome P450 pool was <40% (Table 4.3).

4.2.2.4 Effect of age on the localization of P450IA1 in liver

Haematoxylin and eosin staining of sections of liver from rabbits at most ages revealed that the hepatocytes had a 'foamy' appearance, particularly in untreated animals. This was due to a high content of glycogen and fat in these cells, which varied with the diet of the animal. During acetone fixation, these components were removed from the cytoplasm so that there appeared to be considerably less cytoplasm than in rat hepatocytes.

From the results of the dot and Western blot studies, immunostaining with 3/4/2 was assumed to reflect the distribution of IA1 in the liver of untreated rabbits up to 9-11 days of age and thereafter of both IA1 and IA2. Following MC-treatment of rabbits, localization of 3/4/2 immunostaining can be assumed to reflect that of IA1 alone only in rabbits 6 days old and younger.

Moderate to strong immunostaining of all hepatocytes across the lobule was observed on exposure of sections from 96 h pre-partum rabbits to 3/4/2 at dilutions of less than 1:100 (Table 4.4). Immunoreactivity was homogeneous within the cytoplasm of the cells and was evenly distributed throughout the lobule (Fig. 4.5a). The intensity of immunostaining with 3/4/2 did not change with age, with a consistently moderate immunoreaction being

Table 4.4: Immunotitration of the binding of antibodies to rabbit liver

Age/days	Treatment	Antibody	Antibody dilution						Distribution
			1:10	1:50	1:100	1:500	1:1000	1:10 000	
-4	None	3/4/2	+++	+++	++	+	-		WL
		107	-	-	-				
		B3A3	-	-	-				
	MC	3/4/2	+++	+++	++	+	-		WL
		107	+++	+++	+++	++	+		Odd CL cell
		B3A3	-	-	-				
-1	None	3/4/2	+++	++	++/+	+	+/-		WL
		107	++/+	++/+	+				Odd cell, WL
		B3A3	-	-	-				
	MC	3/4/2	+++	+++	++	++	+[a]		WL
		107	+++	+++	+++	++			More cells, WL
		B3A3	-	-	-				
1	None	3/4/2	+++	++	+/-	-	-		WL
		107	+++/++/-	+++/++/-	++/+/-				Random cells, WL
		B3A3	-	-	-				
	MC	3/4/2	+++	+++	++	+	-		WL
		107	+++	+++	+++	+++	++		More random cells, WL
		B3A3	-	-	-				
6	None	3/4/2	++	++/+	+	-	-		WL
		107	+++	+++	+++	+++	+++	++	CL
		B3A3	-	-	-				

Table 4.4 continued:

6	MC	3/4/2	+++	+++	+++	++	+		WL
		107	+++		+++		++	-	CL, MZ, some PP cells
		B3A3	-	-	-				
9-11	None	3/4/2	+++	++	+	-	-		WL
		107	+++	+++	+++	+++	++		CL
		B3A3	-	-	-				
	MC	3/4/2	+++	+++	+++	++	+		WL
		107			+++	+++	+++	++	WL
		B3A3	-	-	-				
17-21	None	3/4/2	+++	+++	++	+	+/-		WL
		107			+++	+++	++	-	CL
		B3A3	-	-	-	-			
	MC	3/4/2	+++	+++	++	++	++		WL
		107			+++	+++	+++	++	WL
		B3A3	-	-	-	-			
Weanling	None	3/4/2	+++	+++	+++	++	+		WL
		107			+++	+++	+++	++/+	WL
		B3A3	-	-	-	-			
	MC	3/4/2	+++	+++	+++	+++	+++		WL
		107			+++	+++	+++	+++	WL
		B3A3	-	-	-	-			

Intensity of staining: +++ intense, ++ moderate, +++/++/- a mixture of intense and moderately stained cells amongst negative ones, ++/+ some sections moderate others weak, + weak, +/- some sections weak others negative, - negative.

[a] overall weak but some intensely stained cells as seen with 107

WL whole lobule; CL centrilobular; MZ midzonal; PP periportal

observed at dilutions less than 1:100, through to adulthood (Table 4.4; Fig. 4.5h and Fig. 4.5p). The apparent distribution of IA1 did not change following MC-treatment of rabbits 6 days old and younger (Fig. 4.5c); nor was there any change in the distribution of staining in older animals. Immunotitration of 3/4/2 indicated that livers from foetal and neonatal rabbits appeared to be refractive to MC-treatment, with no increase in the intensity of staining evident in livers at lower dilutions of monoclonal antibodies, until rabbits were at least 6 days of age (Table 4.4).

4.2.2.5 Effect of age on the localization of P450IA2 in liver

Immunostaining of livers with MAb 107, even at dilutions of 1:10, confirmed that there was no detectable IA2 in 96 h pre-partum livers (Fig. 4.5b; Table 4.4). However, in livers of 24 h pre-partum rabbits a few hepatocytes distributed around major vessels were intensely stained for this isoenzyme (Fig. 4.5f). In livers from 1 day old rabbits, IA2 was also found in many more cells scattered throughout the lobule. The intensity of the immunoreaction varied amongst these cells whilst most hepatocytes were still negative.

By 6 days of age this isoenzyme was expressed only in a narrow band of centrilobular cells (1-2 cell rows) (Fig. 4.5g). Strongly reacting hepatocytes were interspersed with moderate to weakly stained cells. The intensity and distribution of this immunoreaction did not change, even at high titres of antibody (Table 4.4). No staining of other hepatocytes across the lobule was apparent.

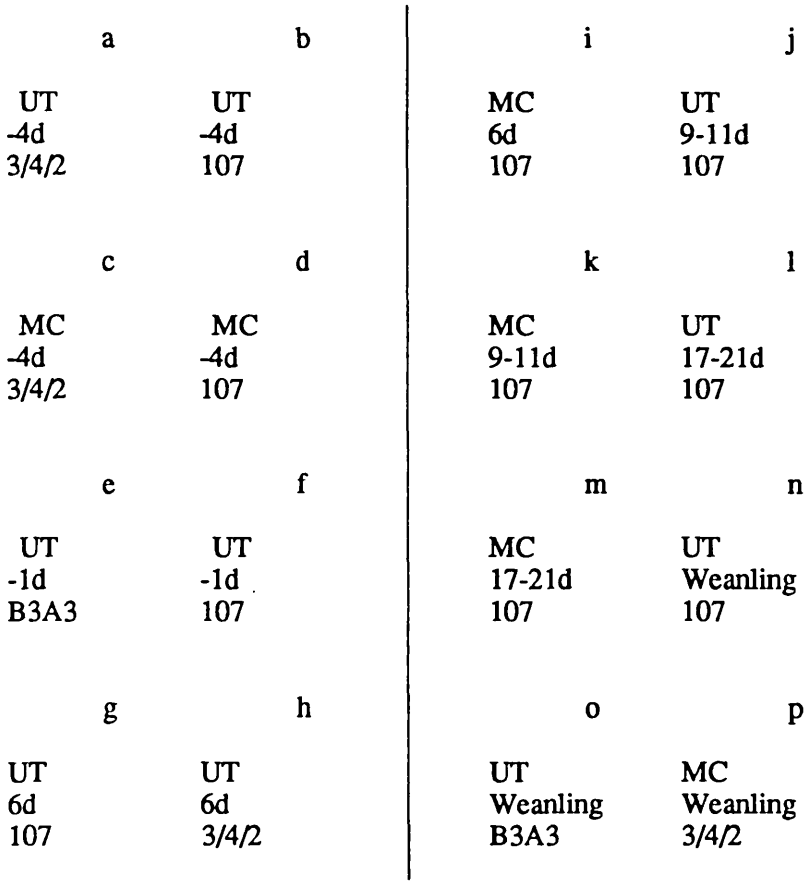
The distribution of IA2 remained the same in livers from 9-21 day old rabbits, except it was expressed in progressively more centrilobular hepatocytes with increasing age (Fig. 4.5j, Fig. 4.5l and 4.5n). However, in 17-21 day old rabbits, staining of some periportal hepatocytes was evident (Fig. 4.5l). The intensity of the immunoreaction varied amongst individual cells, as in 6 day old rabbits.

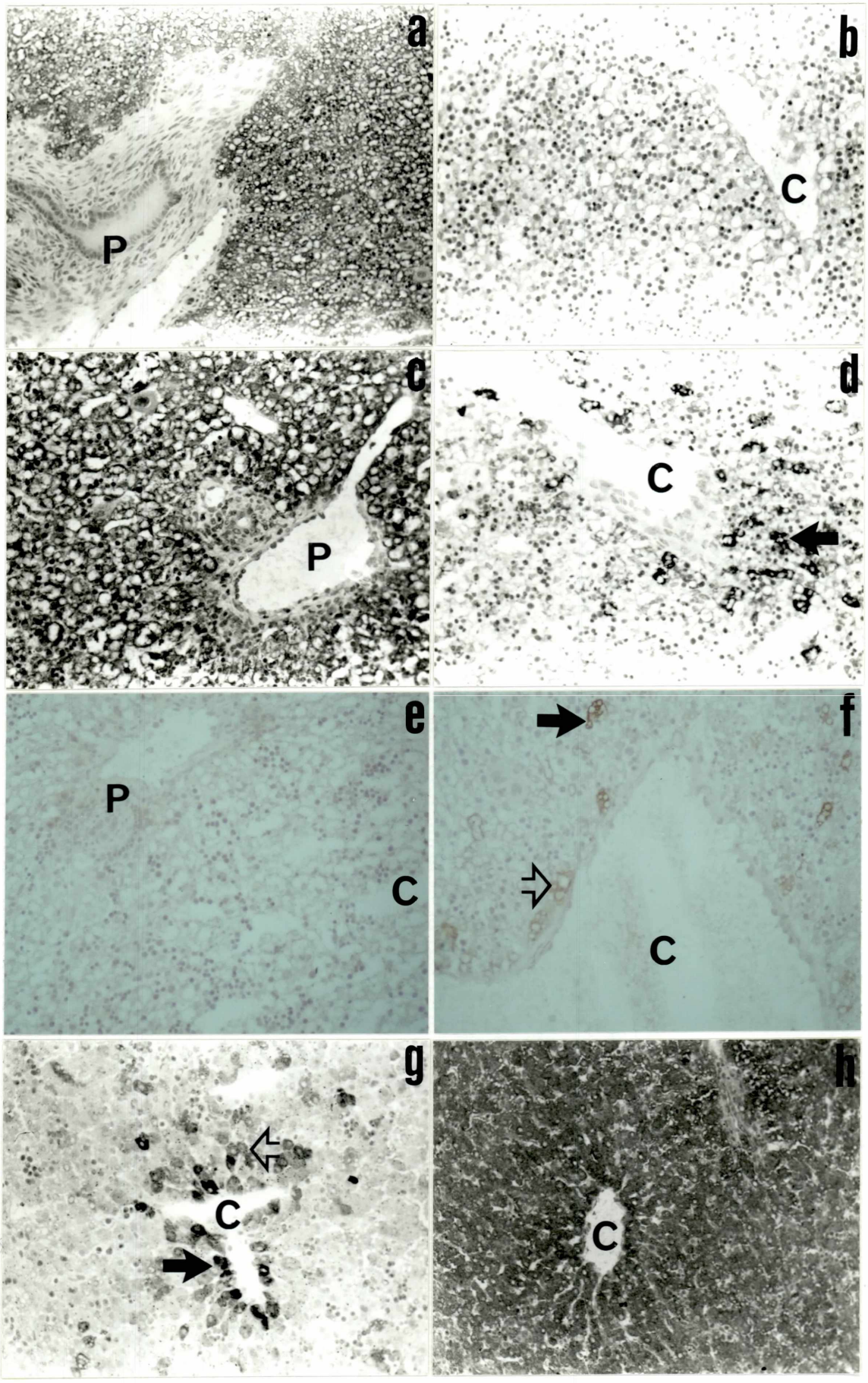
A strong, specific immunoreaction was seen in all hepatocytes throughout the lobule in sections from weanling livers exposed to MAb 107, even at dilutions of 1:10 000 (Table 4.4; Fig. 4.5n). In sections from some livers, the immunoreaction was slightly more intense in centrilobular areas than around portal tracts.

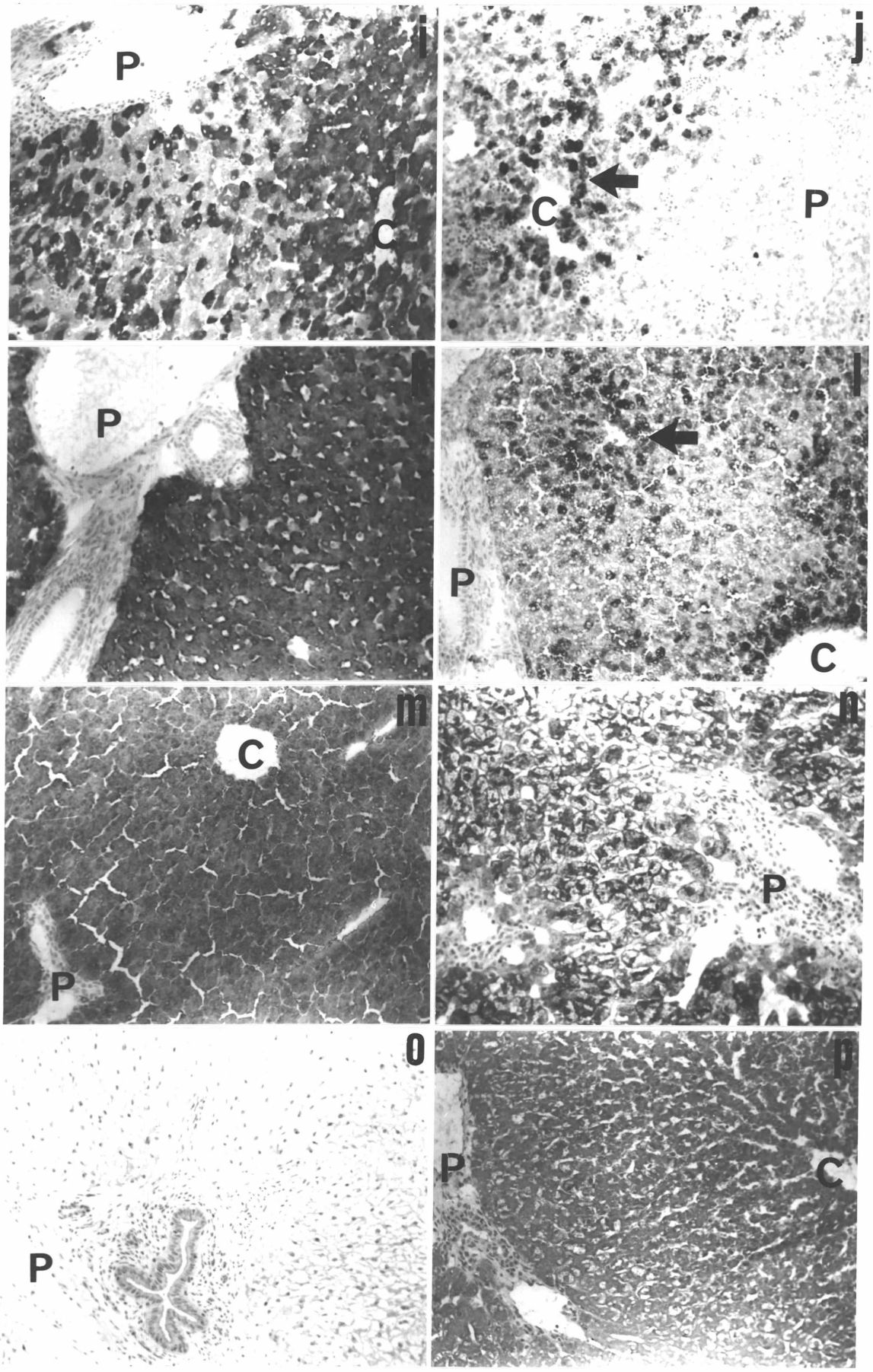
Following MC-treatment of dams, in 96 h pre-partum foetuses, specific immunostaining of IA2 was evident in a few cells indicating inducibility of this isoenzyme (Fig. 4.5d).

Figure 4.5: Effect of age on the localization of P450IA1 and P450IA2 in rabbit liver

Distribution of isoenzymes was examined in livers from -4 day old (a-d), -1 day old (e, f), 6 day old (g-i), 9-11 day old (j, k), 17-21 day old (l, m) or weanling rabbits (n-p). Sections from untreated (a, b, e-h, j, l, n, o) or MC-treated (c, d, i, k, m, p), were incubated with 3/4/2 at a dilution of 1:100 (a, c, h, p) or 107 at a dilution of 1:10 (b, d, f, g) or 1:1000 (i-n) or B3A3 at a dilution of 1:10 (e) or 1:1000 (o). C, central vein; P, portal tract; ➡ intensely stained hepatocytes; ⇨ moderately stained hepatocytes. Magnifications, (a, e-p) x100 and (b-d) x 156.




a
P
b
C
c
P
d
C
e
P
C
f
C
g
C
h
C


i
P
C
j
C
P
l
P
l
P
C
m
C
P
n
P
o
P
p
P
C

Increasing numbers of hepatocytes were stained specifically with MAb 107 in liver sections from 24 h pre- and post-partum rabbits, following MC-treatment of the dams. However, in sections from 6 day old rabbits, the number of cells staining specifically for IA2 increased dramatically (Fig. 4.5i). All centrilobular cells were evenly stained, whereas the immunoreaction varied in intensity amongst hepatocytes of the mid-zonal and periportal areas, some staining strongly and others weakly.

In sections from rabbits of 9-11 days of age and older, all of the hepatocytes throughout the lobule were specifically and evenly immunostained (Fig. 4.5k and 4.5m), although the intensity of the immunoreaction was somewhat weaker than that in most hepatocytes of 6 day old livers. However, immunotitration indicated that the isoenzyme had been induced (Table 4.4).

Replacement of MAb 107 or 3/4/2 with an inappropriate MAb, B3A3, raised against bovine growth hormone, at the same protein concentrations, resulted in a total lack of staining of hepatocytes (Fig. 4.5e and Fig. 4.5o). However, there was significant staining of epithelial cells of portal tracts (not shown).

4.2.3 Ontogeny of P450IA1 and P450IA2 in extrahepatic tissues of the rat

4.2.3.1 Immunostaining of Western blots of kidney microsomal samples

The expression of IA1 and IA2 in kidney microsomal samples from both untreated and MC-treated rats, was investigated by Western blotting with 12/2/3/2. A very weak immunoreactive band co-migrating with purified IA1, was seen in a microsomal sample from one of the untreated mother kidneys (Fig. 4.6a). Following MC-treatment of rats, weakly immunoreacting IA1, could be detected in kidney samples at all ages. The intensity of this band in 24 h pre-partum rats, was weak, but it increased in intensity with age. No significant bands, co-migrating with IA2 could be seen in any of the preparations from untreated or MC-treated animals.

Expression of these isoenzymes was similarly studied in kidney microsomal fractions of rabbits. No immunoreactive bands, migrating in the 50-58 000 molecular weight region could be detected in untreated rabbits, with the anti-P450IA antibody. Following MC-treatment, a very weak band of approximately 57 000 molecular weight, corresponding to IA1, was discernable in samples from 9-11 day old, 17-21 day old, weanling and adult rabbits (Fig. 4.6b).

Figure 4.6: Ontogenic expression of P450IA1 and P450IA2 in kidney from rat and rabbit

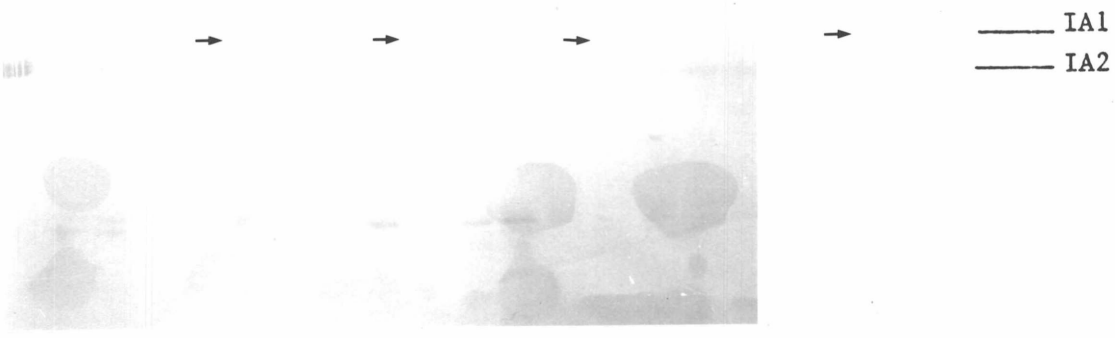
Microsomal samples were separated by SDS-PAGE and subjected to Western blotting as described in Sections 2.2.13 and 2.2.14. Immunoblots containing microsomal fractions from rat kidneys were stained with (a) 12/2/3/2, 1 $\mu g.ml^{-1}$ and those containing microsomal fractions from rabbits were stained with (b) anti-P450-IA diluted 1:1000. (a) Microsomal samples (60 µg) were as follows: lane 1, untreated and lane 2, MC-treated -1 day old; lane 3, untreated and lane 4, MC-treated 1 day old; lane 5, untreated and lane 6, MC-treated 5 day old; lanes 7 and 8, untreated 15 day old; lanes 9 and 10, MC-treated 15 day old; lane 11, untreated and lane 12, MC-treated mother to -1 day old foetuses; lane 13, untreated and lane 14, MC-treated mother to 1 day old; lane 15, MC-treated rat liver, 20 µg and lane 16, purified rat P450IA1, 2 pmoles. (b) Microsomal samples were as follows: lane 1, enriched preparation of rabbit P450IA2, 1 pmole; lane 2, MC-treated and lane 3, untreated 1 day old rabbit; lane 4, MC-treated and lane 5, untreated 6 day old rabbit; lanes 6 and 7, MC-treated 9-11 day old rabbit; lanes 8 and 9, untreated 9-11 day old rabbit; lanes 10 and 11, MC-treated 17-21 day old rabbit; lanes 12 and 13, untreated 17-21 day old rabbit; lanes 14 and 15, MC-treated weanling rabbit; lanes 16 and 17, untreated weanling rabbit; lanes 18 and 19, untreated adult rabbit and lanes 20 and 21, MC-treated adult rabbit.
→ indicates weakly stained band co-migrating with IA1.

a



1 2 3 4 5 6 7 8 9 10 11 1213 14 15 16

b



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

4.2.3.2 Effect of age on the localization of P450IA1 in rat kidney

Immunostaining of kidney sections from 72 h and 24 h pre-partum rats, with 3/4/2, C8 or 1-7-1, at dilutions of 1:10, resulted in very weak to negligible staining of stage I nephrons ie, renal vesicles (Fig. 4.7a). Even 24 h after birth, when nephrons are beginning to elongate and there is some association with glomerular anlage and connection to collecting ducts, very little IA1 could be detected (Table 4.5). However, by 5 days of age, weak staining of proximal tubules in the stage IV nephrons situated at the cortico-medullary junction could be detected with 3/4/2 at dilutions of 1:10 (Fig. 4.7e). Nephrons at stages II and III of development, situated at the periphery of the cortex, were only very weakly stained. Somewhat anomalously, no significant immunoreaction was evident in serial sections exposed to C8 at similar dilutions (Table 4.5). By 15 days, when all nephrons have reached stage IV of development, with both 3/4/2 and C8, specific immunostaining was evident throughout the nephron, being slightly more intense at the cortico-medullary junction (Table 4.5; Fig. 4.7g). However, the intensity of the immunoreaction varied amongst animals. Staining of sections from adult kidneys with these antibodies has been described in Section 3.2.9.

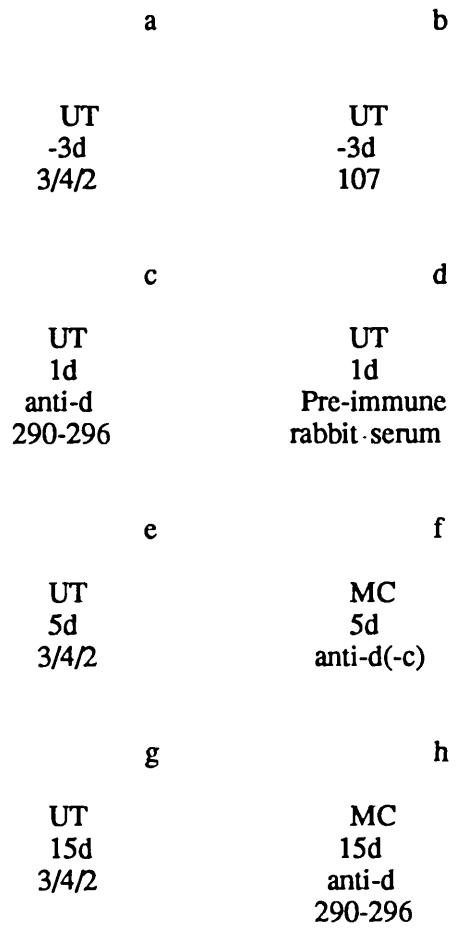
MC-treatment of dams had no effect on either the intensity or distribution of staining of foetal kidney sections with 3/4/2, C8 or 1-7-1 (not shown). However, immunotitration of staining with 3/4/2 or C8, of kidneys from 1 day old rats, suggested a small increase in the content of IA1 (Table 4.5). At both 5 and 15 days of age, MC-treatment resulted in a very strong immunoreaction, with both 3/4/2 and C8, in all cells lining the proximal tubule, IA1 also being expressed in the more immature stage II and III nephrons (Table 4.5).

The glomerular anlage, glomeruli and distal tubules of the cortex and structures within the medulla were not stained in kidney sections from rats at any age, either before or following MC-treatment (Fig. 4.7a and 4.7e).

Replacement of these antibodies with an inappropriate antibody, MAb 107, or normal rat serum, resulted in a total lack of staining of proximal tubules in sections from either untreated or MC-treated rats (Table 4.5; Fig. 4.7b).

Figure 4.7: Ontogeny of immunocytochemical localization of P450IA1 and P450IA2 in rat kidney

Sections from either untreated -3 day old (a, b), 1 day old (c, d), 5 day old (e) or 15 day old (g) rats or MC-treated 5 day old (f) or 15 day old (h) rats were incubated with monoclonal antibodies 3/4/2 (a, e, g) or 107 (b), each diluted 1:10 or anti-$d_{290\text{-}296}$ (c, h), or anti-d(-c) (f) or pre-immune rabbit serum (d), each diluted 1:100. r, renal capsule; PI, renal vesicle; PII, S-shaped nephron; S3, proximal convoluted tubule; IC, inner cortex; OC, outer cortex; G, glomerulus. Magnifications, (a) x138; (b-d) x110; (e, f) x110 ; (g) x60 and (h) x32.



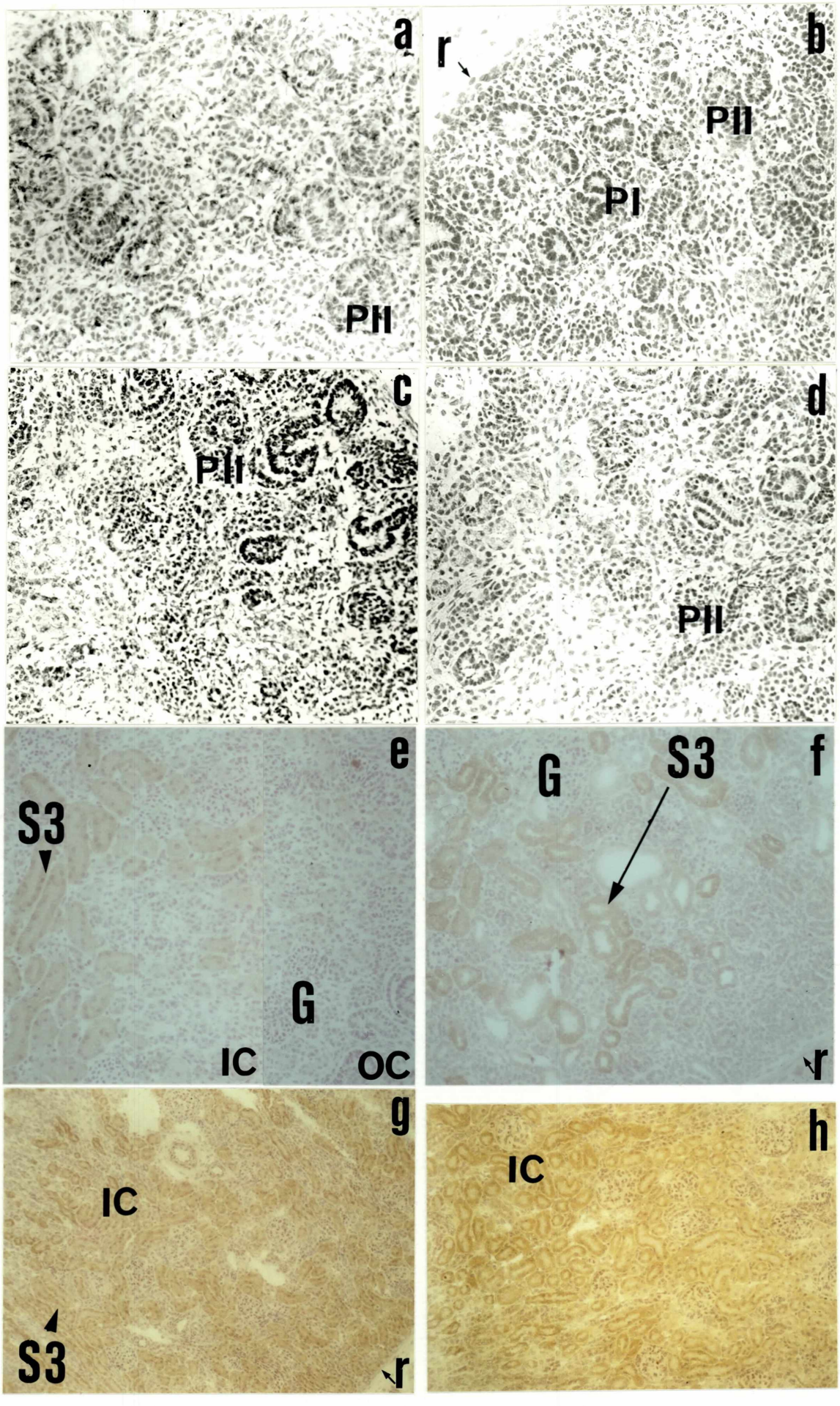

a
PII
b
r
PII
PI
c
PII
d
PII
e
S3
G
IC
OC
f
G
S3
r
g
IC
S3
r
h
IC

Table 4.5: Immunotitration of the binding of antibodies to rat kidney

Age/days	Treatment	Antibody	Antibody dilution 1:10	1:50	1:100	1:500
-3	None	3/4/2	+/-	-	-	
		C8	-	-	-	
		1-7-1	-	-	-	
		anti-$d_{290-296}$		-	-	
		Control IgG[a]		-	-	
	MC	3/4/2	+/-	-	-	
		C8	+/-	-	-	
		1-7-1	-	-	-	
		anti-$d_{290-296}$	-	-	-	
		Control IgG	-	-	-	
-1	None	3/4/2	+	-	-	
		C8	+	-	-	
		1-7-1				
		anti-$d_{290-296}$		++	+	-
		anti-d(-c)	++	+	-	-
		Control IgG	-	-	-	-
	MC	3/4/2	+	-	-	-
		C8	+	+/-	-	-
		1-7-1		+	-	-
		anti-$d_{290-296}$		++	+	+/-
		anti-d(-c)		++	+	+/-
		Control IgG	+/-	-	-	
1	None	3/4/2	-	-	-	-
		C8	-	-	-	-
		anti-$d_{290-296}$		++/+	+/-	-
		anti-d(-c)		-	-	-
		Control IgG		-	-	-
	MC	3/4/2	++/+	+/-	+	+
		C8	+	+/-	-	
		anti-$d_{290-296}$		++/+	+/-	-
		anti-d(-c)		++	-	-
		Control IgG		-	-	
5	None	3/4/2	++/+	+	-	
		C8	-	-		
		anti-$d_{290-296}$		++	+	-
		anti-d(-c)				
		Control IgG		-	-	-
	MC	3/4/2	+++	+++	++	+
		C8	++	+++	+++	++
		anti-$d_{290-296}$		++	+	-
		anti-d(-c)		++	+	+/-
		Control IgG		-	-	

Table 4.5 continued:

15	None	3/4/2	++	++	+/-	-
		C8	+	+/-	-	-
		anti-$d_{290-296}$		++	++	+
		anti-d(-c)		++	++	
		Control IgG		-	-	-
	MC	3/4/2	+++	+++	+++	+++
		C8	++	++	++	++
		anti-$d_{290-296}$		++	++	+
		anti-d(-c)		+++	+++	++
		Control IgG		-	-	-

Intensity of staining: +++ intense, ++ moderate, ++/+ some sections moderate, others weak, + weak, +/- some sections weak others moderate, - weak.

*Control IgG= pre-immune rabbit serum.

4.2.3.3 Effect of age on the localization of P450IA2 in rat kidney

Nephrons in kidney sections from untreated foetal, perinatal and neonatal rats, were weakly to moderately stained by anti-$d_{290-296}$ or anti-d(-c) antibodies, at dilutions of less than 1:500. Although the intensity of staining of IA2 was greater than that of IA1 the distribution of the two isoenzymes was very similar (Table 4.5; Fig. 4.7c). Incubation with anti-$d_{290-296}$, however, did result in a more overall, weak to moderate staining, of most other structures of the kidney, particularly at dilutions of 1:50.

The intensity and distribution of staining by anti-$d_{290-296}$ and anti-d(-c) antibodies was not affected at any age by treatment of rats with MC (Table 4.5; Fig. 4.7f and 4.7h).

Replacement of these antibodies with pre-immune rabbit serum resulted in a total lack of staining of nephrons in kidney sections from both untreated and MC-treated rats, of all ages (Table 4.5; Fig. 4.7d).

4.2.3.4 Immunostaining of Western blots of lung microsomal samples

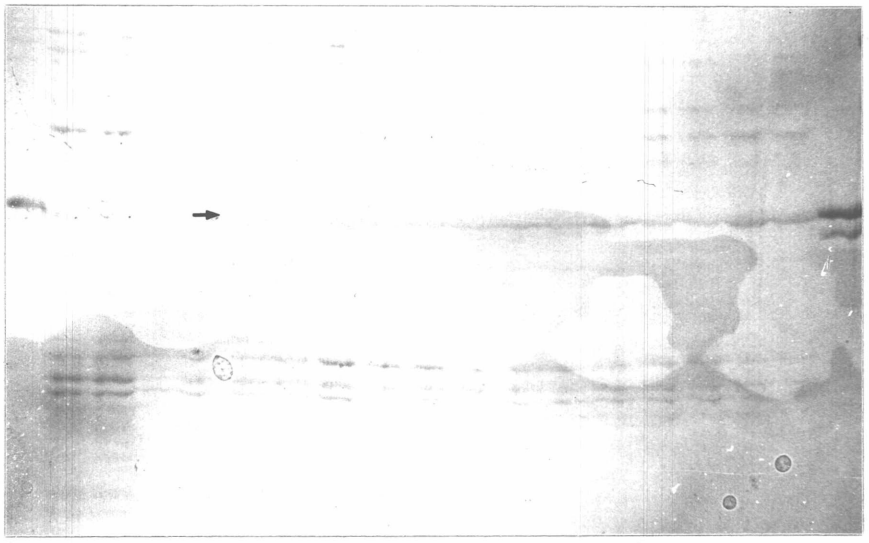
The expression of IA1 and IA2 in microsomal samples from lungs of untreated and MC-treated rats and rabbits was investigated. Western blots of rat samples were immunostained with 12/2/3/2 or 107 (as a negative control) and those of rabbit samples with anti-P450-IA. Although the blots were poorly stained, a very weak, specific immunoreactive band, co-migrating with IA1 could be discerned in lungs from all post-partum rats, with the exclusion of adults, there being no apparent difference in its intensity between untreated and MC-treated rats (Fig. 4.8a). At the high protein loads necessary to detect low levels of isoenzymes, various high and low molecular weight bands were weakly stained, but this was non-specific as they were also seen in blots immunostained with MAb 107 (Fig. 4.8b). There was, however, no staining in the 50-60 000 M_r region of these blots. Moreover, no IA2 could be detected, even after MC-treatment.

No immunoreactive bands, corresponding to IA1 or IA2, were detected in microsomal samples from either untreated or MC-treated weanling or younger rabbits (Fig. 4.8c). However, a relatively weak band, co-migrating with hepatic P450IA1 was detected in microsomes of lungs from untreated and MC-treated adult rabbits (Fig. 4.8c). A strongly reacting band was also present which migrated just below IA2.

Figure 4.8: Western blotting of lung microsomal samples from rats and rabbits of various ages

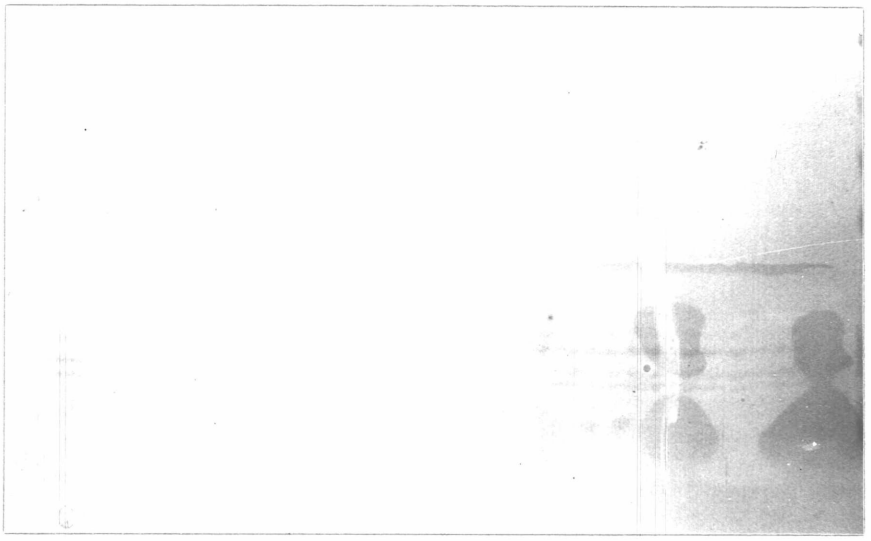
Microsomal samples were separated by SDS-PAGE and subjected to Western blotting as described in Sections 2.2.13 and 2.2.14. Immunoblots were stained with (a) 12/2/3/2, (b) 107, both 1 $\mu g,ml^{-1}$ or (c) anti-P450-IA diluted 1:1000. (a and b) Microsomal fractions from rats (60 µg) were as follows: lane 1, purified rat P450IA1, 2 pmoles; lanes 2 and 3, untreated -3 day old; lane 4, MC-treated -3 day old; lanes 5-7, untreated -1 day old; lanes 8-10, MC-treated -1 day old; lane 11, untreated 1 day old; lane 12, MC-treated 1 day old; lane 13, untreated 5 day old; lane 14, MC-treated 5 day old; lane 15 and 16, untreated 15 day old; lanes 17 and 18, MC-treated 15 day old and lane 19, MC-treated rat liver microsomes, 30 µg. (c) Microsomal fractions from rabbits (50 µg) were as follows: lanes 1 and 14, rabbit P450IA2, 1 and 4 pmoles, respectively; lane 2, untreated and lane 3, MC-treated -1 day old; lane 4, untreated and lane 5, MC-treated 1 day old; lane 6, untreated and lane 7, MC treated 6 day old; lane 8, untreated and lane 9, MC-treated 9-11 day old; lane 10, untreated and lane 11, MC-treated 17-21 day old; lane 12, untreated and lane 13, MC-treated weanling; lanes 15 and 16, untreated adult and lanes 17 and 18, MC-treated adult.
→ indicates weakly stained band co-migrating with P450IA1.
The high molecular weight immunoreactive band in microsomal preparations from rabbit lung may represent dimeric P450IA1/2. This may have arisen due to insufficient boiling of the preparations prior to being applied to SDS polyacrylamide gels.

a



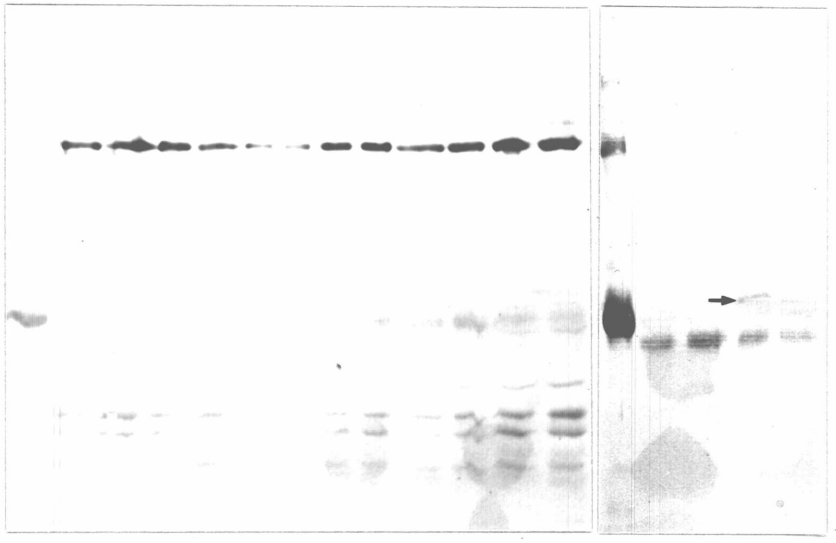
1 2 3 4 5 6 7 8 9 10 11 12 1314 15 16 17 18 19

b



1 2 3 4 5 6 7 8 9 10 11 12 1314 15 16 17 18 19

c



1 2 3 4 5 6 7 8 9 10 11 12 1314 15 16 17 18

4.2.3.5 Effect of age on the localization of P450IA1 and P450IA2 in rat lung

Neither 3/4/2 nor C8 produced any significant staining of specific cell types in lung sections from untreated rats of any age (Table 4.6; Fig. 4.9a). However, with MAb 1-7-1, there was specific immunostaining of bronchiolar epithelial cells, all other cell types being weak to negative (not shown).

Following treatment of dams with MC, strong, specific staining of interstitial cells lining the cuboidal cells of endodermal tubes of the lung parenchyma was observed with all three of the monoclonal antibodies (3/4/2, C8 and 1-7-1), in lungs from -3 day, 1 day and 5 day old rats (Fig. 4.9c and Fig. 4.9e). Serial sections probed with 107, used as a negative control, showed no staining of these cells, indicating that considerable induction of IA1 must have occurred (Fig. 4.9b). All other cells of the lung were only weakly stained for this isoenzyme. However, 1-7-1 did stain epithelial cells lining bronchioles of lungs from both untreated and MC-treated rats (Fig. 4.9d). The intensity of this immunoreaction did not change on MC-treatment of the dams. In sections from MC-treated neonatal and adult rats, immunostaining was evenly distributed throughout most cells of the lung and although no direct comparison can be made with that in lungs from 5 day old untreated rats, it did appear to be more intense after MC-treatment (Table 4.6). In these sections, type II cells were intensely stained (Fig. 4.9e). In adult rats, there was no preferential staining of Clara cells, but there was a slightly more intense reaction in an additional alveolar cell, this being either a type I or type II pneumocyte (not shown).

The intensity and distribution of staining produced by anti-$d_{290-296}$ and anti-d(-c) antibodies were such as to suggest little or no expression of IA2 in lungs of either untreated or MC pre-treated rats (Table 4.6; Fig. 4.9f). Although there was some staining of alveolar cells this was also observed with pre-immune rabbit IgG, and hence presumably represents that of residual endoperoxidases in alveolar macrophages (Fig. 4.9g).

4.2.3.6 Effect of age on the localization of P450IA1 and P450IA2 in rat small intestine

A weak, specific immunoreaction was observed in cells lining villi in the duodenal area of the small intestine of untreated rats, but only following parturition and only at low dilutions of antibodies (Table 4.7). Immunoreactive P450IA1 was detectable only in rats of 1 day of age and older and was evenly distributed throughout epithelial cells of villi (Fig. 4.10a). Crypts, which were not present in guts from younger animals were unstained in the adult.

Table 4.6: Immunotitration of the binding of antibodies to rat lung

Age/days	Treatment	Antibody	Antibody dilution 1:10	1:50	1:100	1:500	Distribution
-3	None	3/4/2	-	-	-		
		C8	-	-	-		
		anti-$d_{290-296}$		-	-	-	
		Control IgG[a]		-	-	-	
	MC	3/4/2	+++	++	+		Interstitial cells
		C8					
		anti-$d_{290-296}$		-	-	-	
		Control IgG		-	-	-	
-1	None	3/4/2	+/-	-	-		Throughout
		C8		-	-		
		1-7-1	++	++	+	+	Bronchioles
		anti-$d_{290-296}$		+	-	-	Throughout
		anti-d(-c)	+/-	-	-	-	
		Control IgG	+	-	-	-	Throughout
	MC	3/4/2	+++	+++	++	++	Interstitial cells
		C8	+++	+++	+++	++	Interstitial cells
		1-7-1	+++	+++	++	++	Interstitial cells, bronchioles
		anti-$d_{290-296}$		-	-	-	
		anti-d(-c)		-	-	-	
		Control IgG	+	-	-	-	

Table 4.6 continued:

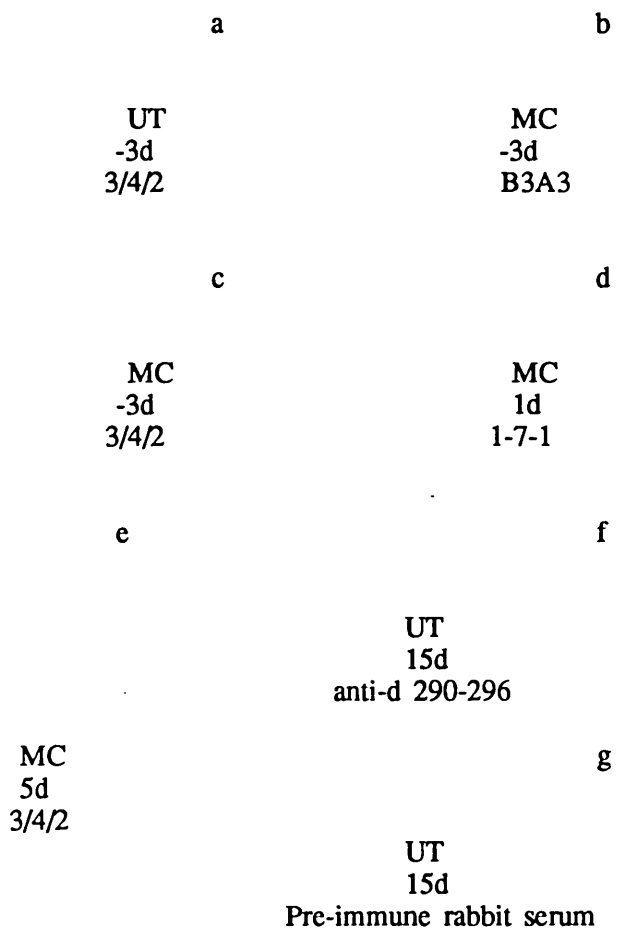
1	None	3/4/2	++/+	+/-	-		Throughout
		C8	++/+	+/-	-		Throughout
		1-7-1	++	++	+	+/-	Bronchioles
		anti-$d_{290-296}$		+	+/-	-	
		anti-d(-c)		+	-	-	
		Control IgG		+/-	+/-	-	
	MC	3/4/2	+++	+++	++	+	Interstitial cells
		C8	+++	++	++	++/+	Interstitial
		1-7-1	+++	+++	+++	++	Bronchiolar and interstitial cells
		anti-$d_{290-296}$		++	+	+/-	Throughout
		anti-d(-c)		-	-	-	
		Control IgG	+	+/-	-	-	Throughout
5	MC	3/4/2	+++	+++	++		Throughout
		C8	+++	+++	++		Throughout
		anti-$d_{290-296}$		++	+		Throughout
		anti-d(-c)		++	+		Throughout
		Control IgG	+	+/-	-		Throughout
Adult	None	3/4/2	+	+/-	-		Throughout
		C8	+	+/-	-	-	Throughout
		anti-$d_{290-296}$	+/-	-	-	-	+++, type II
		anti-d(-c)	+/-	-	-	-	+++, type II
		Control IgG		+	+/-	-	+++, type II
	MC	3/4/2	++	++/+	+		Throughout, +++ type II
		C8	+++	++	+/-		Throughout, +++ type II
		anti-$d_{290-296}$		-	-	-	+++, type II
		anti-d(-c)		+/-	-	-	+++, type II
		Control IgG		+/-	-	-	+++, type II

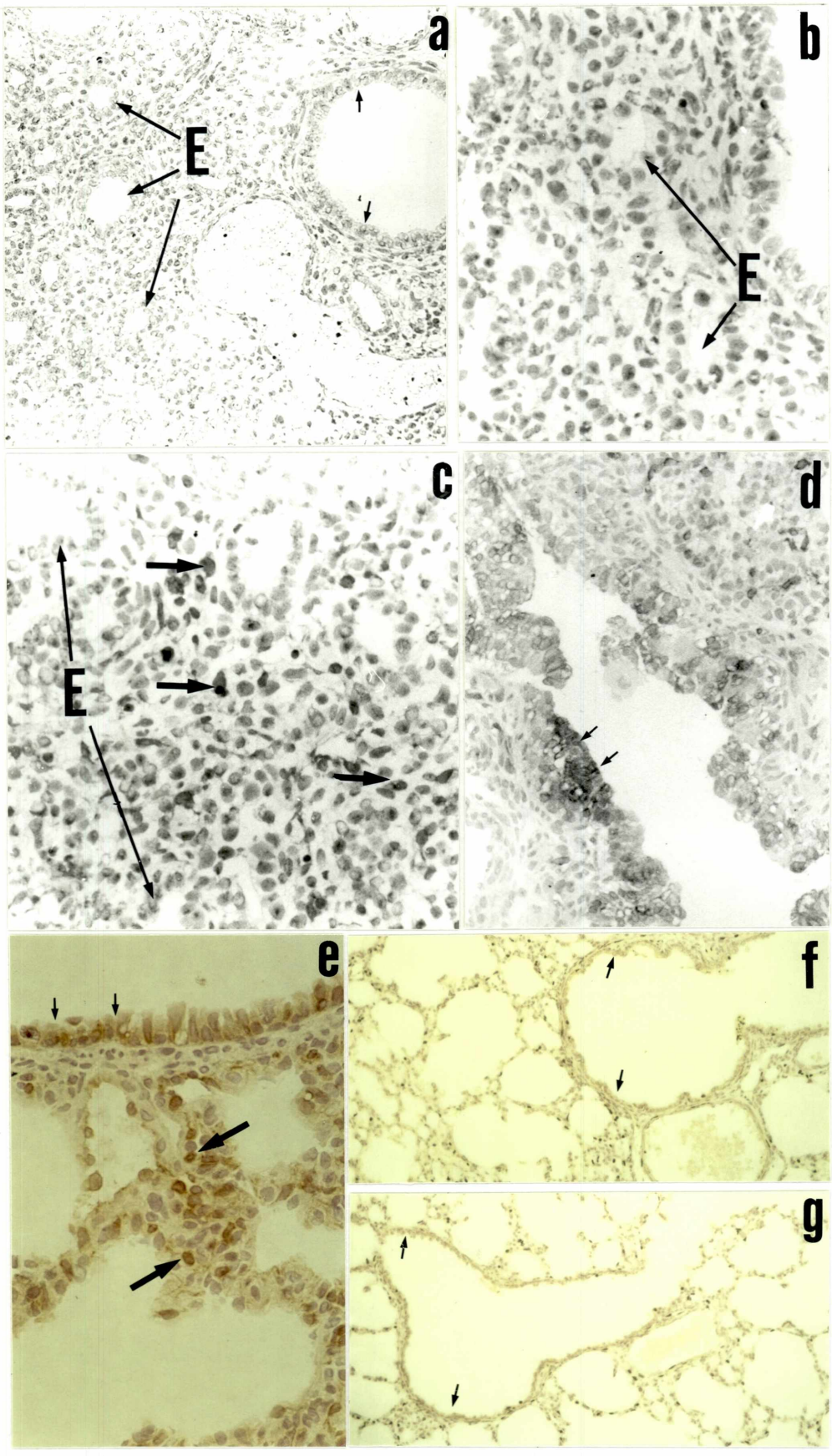
Intensity of staining: +++ intense, ++ moderate, ++/+ some sections moderate others weak, + weak, +/- some sections weak others negative, - negative.

*Control IgG= pre-immune rabbit serum

Figure 4.9: Effect of age on the localization of P450IA1 in rat lung

Sections from either untreated -3 day old (a) or 15 day old (f, g) rats or MC-treated -3 day old (b, c), 1 day old (d) or 5 day old (e) rats were incubated with monoclonal antibodies 3/4/2, at a dilution of 1:10 (a, c, e), B3A3 at a dilution of 1:10 (b) or 1-7-1 at a dilution of 1:500 (d) or anti-$d_{290\text{-}296}$ (f) or pre-immune rabbit serum (g), each diluted 1:100. E, endodermal tubes; → bronchiolar cells; ➡ intensely stained interstitial cells. Magnifications (a) x110; (b, c) x230; (d) x210; (e) x240 and (f, g) x88.




a
E
b
E
c
E
d
e
f
g

Following MC-treatment of dams there was still no specific staining of foetal intestinal cells (Table 4.7), nor was there any significant increase in the intensity of staining in small intestinal epithelial cells in sections from 1 day old rats. However, in 5 day old MC-treated rats there was a very intense immunoreaction in epithelial cells lining the villi of the small intestine. Interestingly, cells at the base of the villi were more intensely stained than those at the tip, with both 3/4/2 and C8 (Fig. 4.10c). Whether this is a true reflection of selective induction in these cells could not be determined as no untreated animals were available for comparison. In the adult, whereas in untreated animals staining was apparent only at antibody dilutions of 1:10, following MC-treatment, there was intense staining at antibody dilutions as high as 1:100, indicating considerable induction of P450IA1 (Table 4.7). Cells lining the villi and within the crypts were positively stained, but the vacuolated goblet cells and the lamina propria were negative (not shown).

Sections of foetal small intestine were not stained by antibodies against P450IA2, even after pre-treatment of dams with MC (not shown). Following parturition, immunoreactive protein could be detected in the epithelial cells of the small intestine, with no preferential staining of cells in any one part of the villus (Fig. 4.10b). Immunotitration of the staining with anti-$d_{290\text{-}296}$ and anti-d(-c) antibodies showed that MC had little or no effect on the level of expression of P450IA2 in these animals (Table 4.7). In contrast, the distribution of this isoenzyme in the villi of small intestine from 5 day old rats was markedly different from that of IA1. Staining was most intense in the upper half to two thirds of the villi, with only weak to negative staining of immature basal epithelial cells (Fig. 4.10d). The intensity and distribution of staining for this isoenzyme in small intestine from both untreated and MC-treated adult rats were very similar to those in 5 day old MC-treated rats. There was no significant increase in staining intensity following MC-treatment of adult animals (Table 4.7).

4.2.3.7 Localization of P450IA in other extrahepatic tissues of perinatal rats

Excluding the tissues considered previously, in general, most other well defined structures in whole foetal and perinatal sections were unstained following incubation with any one of the antibodies. Hence, no immunoreactive P450IA1 or IA2 was detectable in cartilage of limbs, ribs or spine, skeletal muscle, heart, spleen, stomach or skin. No staining for cytochromes P450IA1 or IA2 was apparent in pancreatic cells in sections from either untreated or MC-treated perinatal rats. However, very strong staining with anti-d(-c) was apparent in the centroacinar cells lining primary ducts of the pancreas of MC-treated 5 day old rats, with weaker staining of other acinar cells (Fig. 4.11a). In contrast, weak staining

Table 4.7: Immunotitration of the binding of antibodies to rat small intestine

Age/days	Treatment	Antibody	Antibody dilution 1:10	1:50	1:100	1:500
-3	None	3/4/2	-	-	-	
		C8	-	-	-	
		1-7-1	-	-	-	
		anti-$d_{290-296}$		-	-	-
		Control IgG[a]		-	-	-
	MC	3/4/2	-	-	-	
		C8	-	-	-	
		1-7-1	-	-	-	
		anti-$d_{290-296}$		-	-	-
		Control IgG		-	-	-
-1	None	3/4/2	+/-	-	-	
		C8	-	-	-	
		1-7-1	-	-	-	
		anti-$d_{290-296}$		+	+/-	-
		anti-d(-c)	++	+	-	-
		Control IgG	-	-	-	-
	MC	3/4/2	+	+/-	-	-
		C8	+	+	-	-
		1-7-1	+	+	-	-
		anti-$d_{290-296}$		++/+	++	++/+
		anti-d(-c)		++	+/-	-
		Control IgG		+/-	-	-
1	None	3/4/2	++/+	++/-	+/-	-
		C8	++/+	++/+	+/-	
		anti-$d_{290-296}$		++	++/+	-
		anti-d(-c)		+	+	+/-
		Control IgG		+	-	-
	MC	3/4/2	+++	++/+		++
		C8	++	++/+	+/-	-
		anti-$d_{290-296}$		+++	++	+
		anti-d(-c)		+++	++/+	+/-
		Control IgG		+	-	-
5	MC	3/4/2	+++	+++	+++	
		C8	+++	+++	+++/++	++/+
		anti-$d_{290-296}$		++	++/+	+
		anti-d(-c)		++	+	+/-
		Control IgG		+	-	-

Table 4.7 continued:

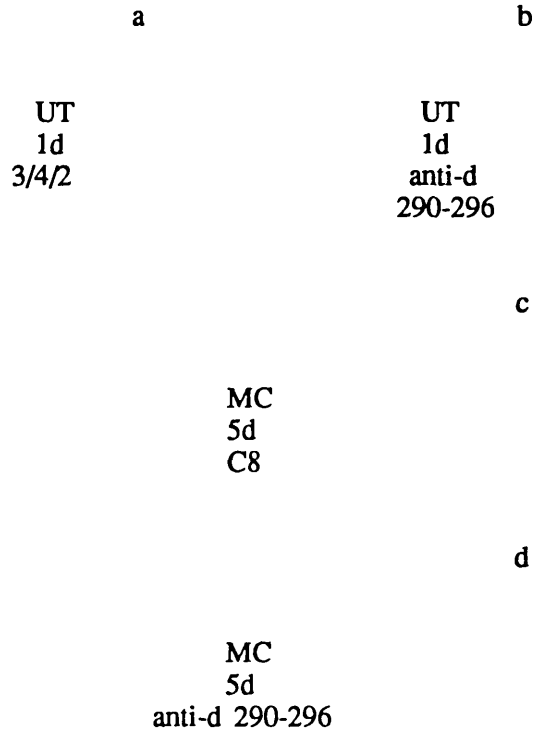
Adult	None	3/4/2	+	-	-	
		C8	+	-	-	
		anti-$d_{290-296}$		++	+	+/-
		anti-d(-c)	+++	++	+	-
		Control IgG		-	-	-
	MC	3/4/2	+++	++	++	+
		C8	+++	+++	++	++/+
		anti-$d_{290-296}$		++/+	+/-	-
		anti-d(-c)	++	+	-	
		Control IgG	+	-	-	

Intensity of staining: +++ intense, ++ moderate, ++/+ some sections moderate others weak, ++/- some sections moderate others none, + weak, +/- some sections weak others none, - none.

*Control IgG= pre-immune rabbit serum

Figure 4.10: Effect of age on the localization of P450IA1 and P450IA2 in rat small intestine

Sections from untreated 1 day old rat (a, b) or MC-treated 5 day old rats (c, d) were incubated with 3/4/2 at a dilution of 1:10 (a), C8, at a dilution of 1:500 (c) or anti-$d_{290\text{-}296}$ at a dilution of 1:50 (b, d). v, villus tip; LP, lamina propria; ➡ intensely stained enterocytes. Magnifications, (a) x60; (b), x405; (c) x440 and (d) x550.



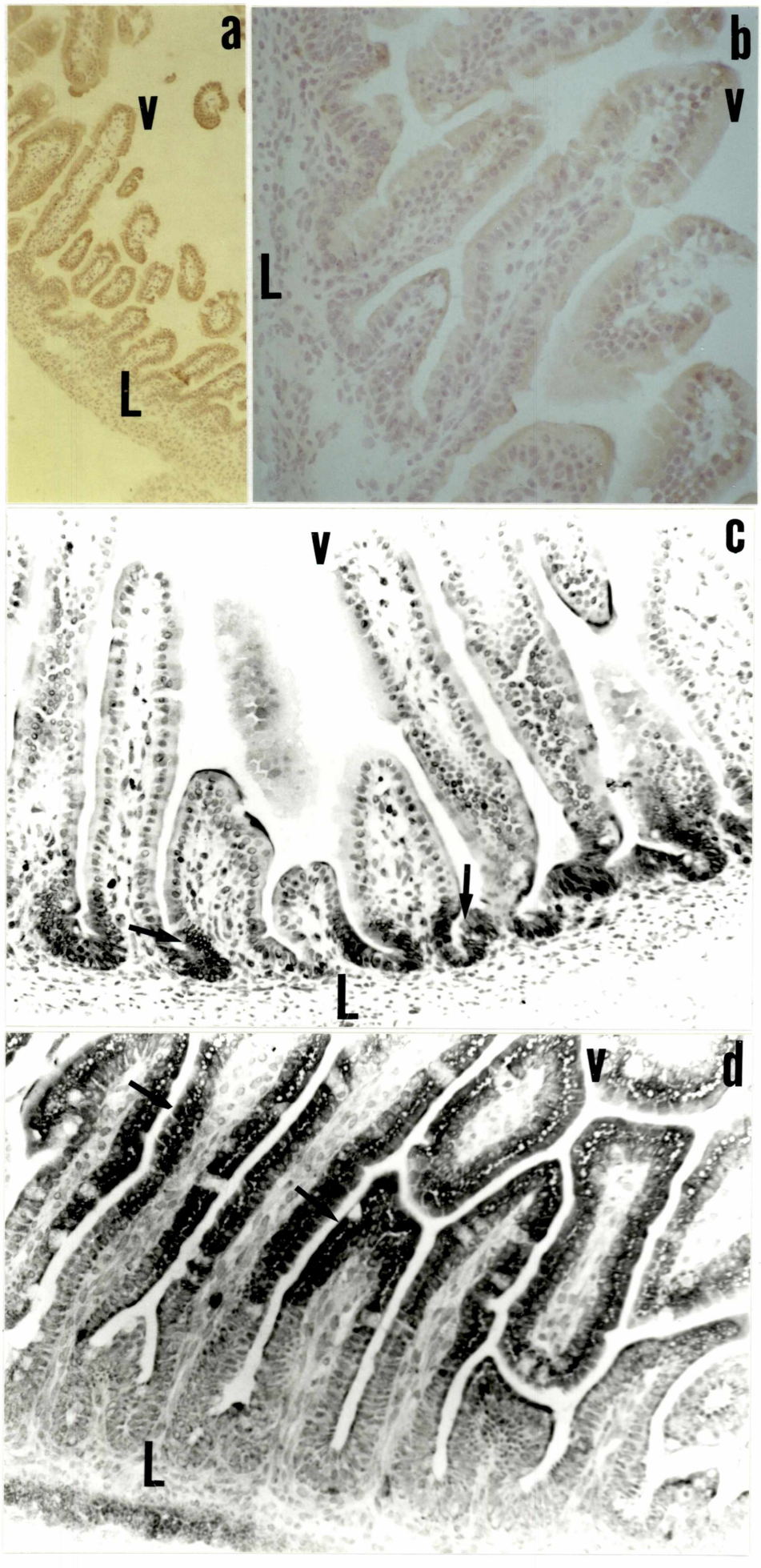
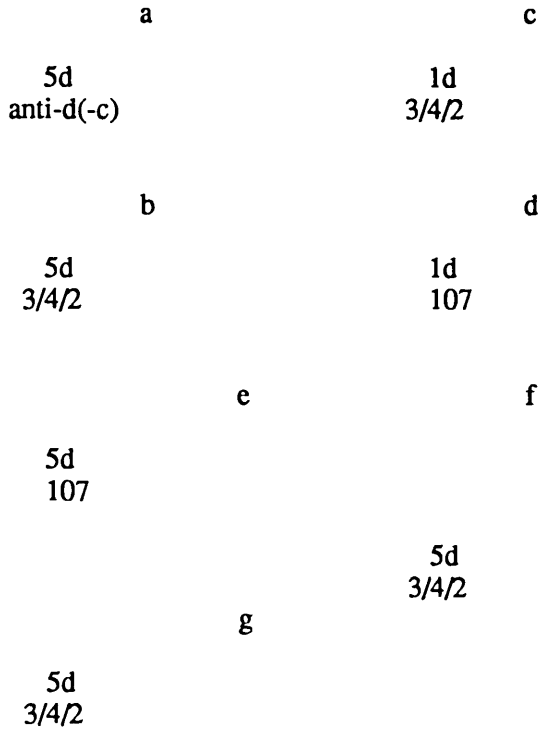
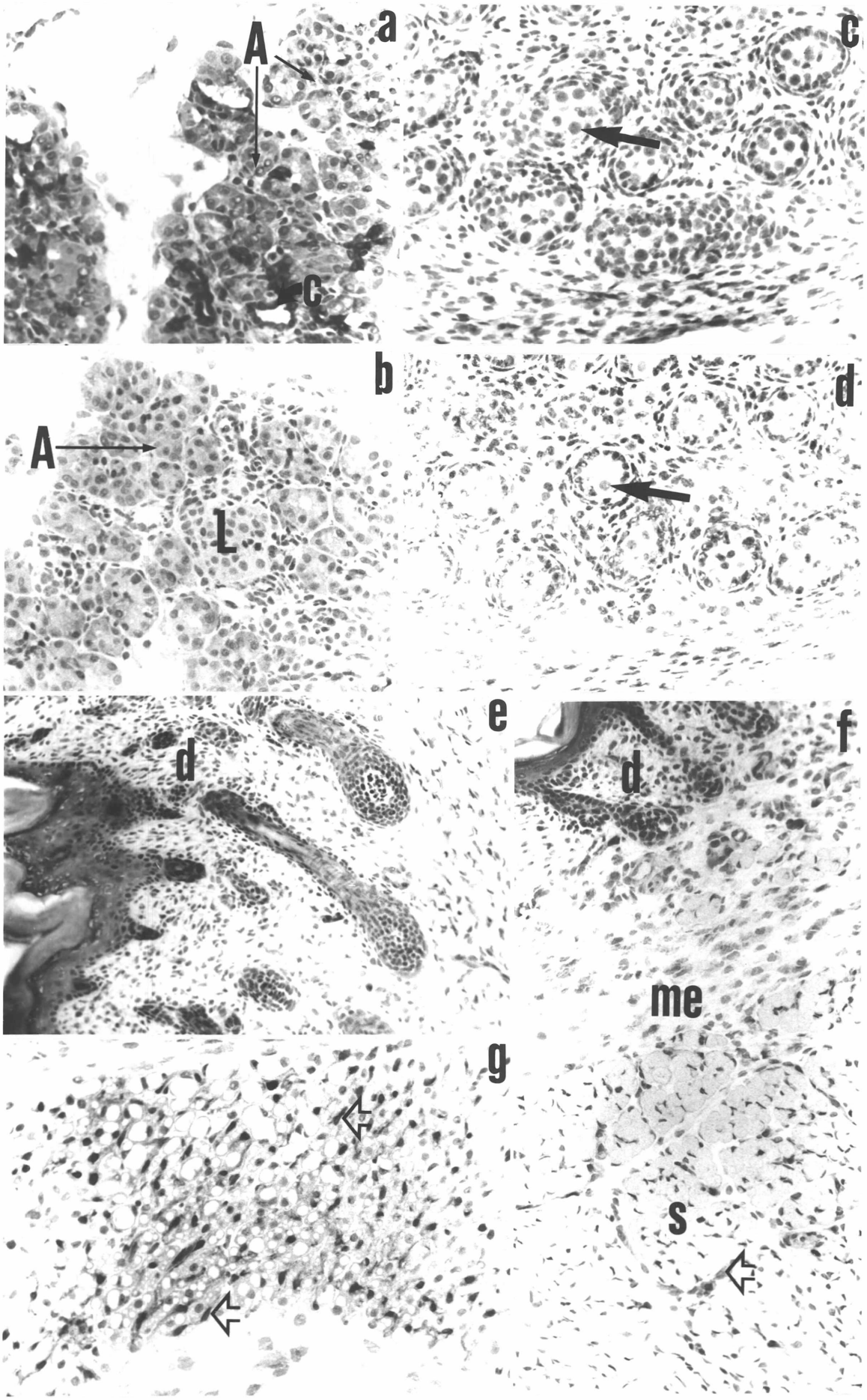

a
v
L
b
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L
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c
L
v
d
L

Figure 4.11: Immunocytochemical localization of P450IA in some extrahepatic tissues of perinatal MC-treated rats

(a, b) Sections of pancreas from 5 day old rat, incubated with anti-d(-c), diluted 1:50 (a) or 3/4/2, diluted 1:10 (b). (c, d) Sections of testis from 1 day old rats incubated with 3/4/2 (c) or 107 (d) each diluted 1:10. (e-g) Sections of mesenchymal and muscle layers beneath the skin in the trunk region from 5 day old rats, incubated with either 107 (e) or 3/4/2 (f, g) at dilutions of 1:10. A, acinar cells; c, centroacinar cells; L, islets of Langerhans; ➡ spermatogonocytes; s, skeletal muscle; me, mesenchymal cells; d, dermis and ⇨ intensely stained interstitial cells. Magnifications (a-d, f) x210; (e) x130 and (g) x250.




a
A
c
c
b
A
L
d
d
e
d
f
d
me
g
s

for P450IA1 was evident only in acinar cells with the centroacinar cells of ducts being negative. No specific staining was apparent in the Islets of Langerhans following incubation with either antibody (Fig. 4.11b).

Very high levels of background staining, presumably due to non-specific absorption of the horseradish peroxidase conjugated detecting antibodies, were observed in the epidermal layers of the skin (Fig. 4.11e). Hence, although in some sections, probed with 3/4/2 or C8, staining of skin was very intense following MC-treatment of mothers or perinates, it was difficult to assess unequivocally whether or not induction of IA1 had occurred in this tissue. A very strong immunoreaction with C8 was also observed in smooth muscle cells of many tissues, but particularly of blood vessels, small intestine and the bladder, in sections from both untreated and MC-treated rats. Such staining in muscle of small intestine probed with 3/4/2 and 1-7-1 was never as strong as that with C8 and more often than not diluted out upon a 5-fold dilution of the antibody (not shown). It is likely that this staining is due to non-specific binding of the primary antibody to this tissue.

Each of the monoclonal antibodies produced staining of interstitial cells of mesenchymous tissue lying between developing organs (Fig. 4.11f and Fig. 4.11g), particularly in limb areas and in other cells of testis, possibly spermatogonocytes (Fig. 4.11c) in sections from perinates, but only at low antibody dilutions (<1:50) and only following MC-treatment of dams. Since these cells did not stain in sections from untreated perinates nor in sections probed with inappropriate antibodies (Fig. 4.11d), such staining likely reflects induction of P450IA1.

4.3 Discussion

The data of the present study confirm the findings of previous reports which indicated that during ontogenesis, PAHs differentially affect the levels of P450IA1 and IA2 in both rats (Guenthner & Nebert, 1978; Cresteil *et al.*, 1986) and rabbits (Atlas *et al.*, 1977; Norman *et al.*, 1978). In rats of all ages, P450IA1 represents less than 4% of the total P450 pool, whilst IA2 represents less than 17% and 24% of total P450 in adult rats and rabbits, respectively. This compares favourably with earlier reports in which the isoenzymes were quantified by Western blotting (Thomas *et al.*, 1983; Goldstein & Linko, 1984; Domin *et al.*, 1984; Cresteil *et al.*, 1986). Induction of IA1 occurs prior to birth whereas that of IA2 is effected a few days later. However, some differences were evident, particularly in the expression of P450IA2.

No immunoreactive band corresponding to IA1 was detected in Western blots of hepatic microsomal samples of untreated rats of any age by MAb 12/2/3/2. This correlates well with the quantitative data. In respective microsomal samples from MC-treated rats, an immunoreactive band co-migrating with IA1 was evident at 24 h pre- and post-partum, indicating transplacental induction of this isoenzyme. Yet, levels remained below the limits of detection in dot blots probed with 3/4/2. That this was due to differences in the sensitivity of the techniques used is unlikely since in both cases the microsomal protein was denatured, that being adsorbed to nitrocellulose sheets for dot blotting, having been pre-treated with 8 M urea. This was a necessary step since the epitope to which 3/4/2 binds is more accessible in the denatured state. Instead, it is likely that differences in the affinities of the two antibodies used, which do bind to different epitopes on IA1 (Sesardic *et al.*, 1986), account for the inconsistency. In addition, it is possible that a foetal isoenzyme variant of the P450I family is induced prior to birth, which contains the epitope to which 12/2/3/2, but not 3/4/2, binds. Interestingly, 3/4/2 does recognise a protein in foetal rabbit livers, which co-migrates with IA1 in Western blots. In fact, the inducibility of this protein by MC appeared to decrease with increasing gestational age further supporting the concept that a foetal isoenzymic variant is expressed.

Levels of immunoreactive IA2 in hepatic microsomes from untreated rats, as determined by dot blotting, were consistently 100 pmoles.mg^{-1} protein, regardless of the age of the animals. Thus, constitutive levels of this isoenzyme relative to total P450 were higher in foetal and neonatal rats than in adults. As a consequence, the fold increase in IA2 following MC-treatment was much smaller in younger animals (1.6-fold cf 3-7-fold). These results are contrary to the ontogenic profile obtained in Western blots for this isoenzyme, both in this study and that of Cresteil *et al.* (1986), where no IA2 could be detected in untreated or MC-treated rats younger than 15 and 5 days of age, respectively. Indeed, a very dramatic increase in the intensity of the immunoreactive band was evident in microsomes from 5 day old MC-treated rat livers, which in the blots of Cresteil *et al.* (1986) was estimated at being 30-fold more intense than that in untreated animals.

The discrepancy in these results could be due to differences in either the sensitivity of the two techniques used, Western blotting vs. dot blotting, the latter of which reduces denaturation of proteins, or in the specificity of the antibodies. The absolute amount of protein transferred from a gel to nitrocellulose for Western blotting is uncertain, and in the system used in these experiments was never one hundred percent even when the transfer period was increased or the protein load was reduced. Hence this technique may underestimate the amount of antigen originally present.

Alternatively, the isoenzyme in foetal and neonatal tissues may actually be a different isoenzyme from that in the adult, and thus a polyclonal antibody raised against the purified protein from the adult may not bind to the foetal form with the same affinity. The amino acid sequence of IA2 to which the anti-peptide antibody used in these studies was raised, could represent a region that is conserved in the foetal isoenzyme but which is denatured by SDS-PAGE treatment. If this is true, it is reasonable to predict that the extent of expression of this protein will decrease following parturition whilst that of the adult form increases. However, a relatively high constitutive expression of this protein is maintained through to adulthood in dot blots, whilst no immunoreactive band co-migrating with IA2 could be detected in untreated liver microsomal samples in Western blots with either the anti-peptide antibody or 12/2/3/2. It is therefore possible that the anti-peptide antibody reacts with some other unrelated protein containing the same or very similar linear sequence of amino acids when undenatured, which adopts a similar 'binding shape' at the protein surface.

As in the rat, hepatic P450IA2 is not detectable in the rabbit until after birth, and levels are consistently very low until the time of weaning. Even following MC-treatment, levels remain below detection in foetuses and do not increase significantly until 6 days after birth. A surge in the levels of this isoenzyme occurs in 2-3 week old animals and is not unlike that observed for IA2 in the rat (Cresteil *et al.*, 1986). The dramatic induction of this isoenzyme coincides with a sharp decrease in AHH activity (Atlas *et al.*, 1977). Benzo[a]pyrene is a specific substrate for P450IA1 in MC-treated animals, but it is unlikely that this isoenzyme becomes refractive to induction at this age, as increases in the intensity of the immunoreactive 57 000 molecular weight band in microsomal fractions from 3 week old animals were evident following treatment with MC. A possible explanation may be that the very high levels of IA2 divert the fluorescent metabolites of benzo[a]pyrene measured in the AHH assay into non-fluorescent species. Indeed, recent work in this department confirms that this does occur. In the presence of MAb 107, an inhibitor of IA2 activity, there was a 2-fold stimulation of AHH activity (Murray *et al.*, 1989). Increases in IA2 in these species corroborate previous reports in which the induction of an electrophoretic band of 54 000 daltons was correlated with PAH-inducible N-acetylarylamine N-hydroxylase and acetanilide 4-hydroxylase activities (Guenthner & Nebert, 1978; Atlas *et al.*, 1977).

Immunocytochemically, both isoenzymes were detectable in hepatocytes of foetal rats and rabbits, although in the latter species, IA2 was found only in a limited number of cells around central veins. This technique is undoubtedly much more sensitive than that of

either dot or Western blotting, thus accounting for differences between the data. For example, constitutively, IA1 is below detection in blots but is readily detected in tissue sections. In fact, the immunocytochemical data correlate well with the results from Northern blotting studies where mRNA to both IA1 and IA2 was detected from as early as two weeks or a few days prior to parturition, respectively (Tuteja *et al.*, 1985; Kimura *et al.*, 1986; Giachelli & Omiecinski, 1987). By using antisense probes from 3'-specific regions of the Cyp1A1 and Cyp1A2 cDNAs, *in situ* hybridization studies have revealed that mRNA to IA1 is localized in all hepatocytes of the developing foetal mouse liver from as early as 12.5 days of gestation, but only after MC-treatment (Dey *et al.*, 1989). No mRNA to IA2, however, was detected in either untreated or MC-treated mice until the neonatal period. In the rat, the constitutive development of these two isoenzymes, with respect to their distribution, appears to be co-ordinately controlled, with each protein eventually being preferentially concentrated in centrilobular cells of adult livers. In contrast, the development of these two proteins in the rabbit is clearly regulated in a different manner. Cytochrome P450IA1 is expressed homogeneously throughout the lobule, its distribution being very similar to that of the rat orthologue. In contrast, expression of cytochrome P450IA2 is markedly defined at each stage of development. It is essentially absent prenatally and begins to occur in a few hepatocytes scattered across the lobule, perinatally. At 6 days, expression is confined to a very narrow band of hepatocytes around central veins. With increasing age there is a gradual recruitment in the number of cells expressing this isoenzyme until, in the adult, all cells are positive.

The different and changing patterns in the distribution of P450IA2 presumably reflect differences in factors such as nutrient supply and levels of hormones, which undoubtedly vary at each stage of the life cycle and which will vary from one mammalian species to another. For instance, plasma corticosteroids and catecholamines are very low in suckling neonates whereas plasma growth and thyroid hormones are elevated during the first week of life (Girard & Ferre, 1982). The susceptibility of this isoenzyme to its environment has been emphasized by recent studies. The inclusion of Nu-serum, a medium supplement with newborn calf serum, various growth factors, insulin, transferrin and other hormones, amino acids and nutrients, to tissue culture medium promoted the expression of P450IA2 in hepatocyte cultures as also did maintenance on Engelbreth-Holm-Swarm mouse tumours (Pasco *et al.*, 1988). Steroid hormones, such as glucocorticoids are known to regulate genes by activation and inhibition of transcription as well as by altering mRNA turnover and processing. More specifically, it has been shown that dexamethasone acts to increase the content of cytochrome P450IA1 mRNA and its rate of transcription, in foetal rat hepatocytes in primary culture, by interacting with specific regions within the first intron

of the gene (Mathis *et al.*, 1989). Whether this can be extrapolated to the *in vivo* situation remains to be determined. In the developing foetus, products of the foetal adrenal may play an important role in the modulation of the effects of xenobiotics by regulating metabolic activation or detoxication reactions. Additionally, a recent study suggests that unidentified maternal factors also affect cytochrome P450 specific content and the activities of acetanilide 4-hydroxylase and AHH (Simpson *et al.*, 1988). Interestingly, these maternal factors are without effect on the development of testosterone 15α- and 16α-hydroxylases and NADPH-cytochrome c reductase activities, the former of which are regulated by sex-dependent factors (Waxman *et al.*, 1985).

In both species, the factors controlling the distribution of these isoenzymes on MC-treatment are subject to differential control. No dramatic changes occur in the distribution of IA1, but induction of IA2 within different hepatocytes differs as a function of age. As discussed in the previous chapter, induction is greatest in those cells which are constitutively deficient. MC treatment of animals leads to developmentally advanced livers which not only have specific contents of total cytochrome P450 approaching those of adults, but which also, immunohistochemically, resemble livers of animals at least 10-20 days older, in their distribution of IA2. This will have important implications for the toxicology of compounds activated by this isoenzyme. For example, Juchau *et al.* (1985) showed that the incidence of foetal rat malformations elicited by exposure to AAF were markedly increased following transplacental induction with MC, but not with PB. Furthermore, certain enzymes associated with phase II detoxication such as UDP-glucuronosyltransferases, do not become functional until after birth (Leakey, 1983). Thus, the developing foetus will be particularly vulnerable to activated metabolites generated by P450IA-mediated biotransformation.

In summary, these data indicate that both isoenzymes are expressed prior to birth, albeit at low levels, and are homogeneously distributed across the lobule. Treatment of animals with MC confirms previous reports (Atlas *et al.*, 1977; Guenthner & Nebert, 1978), that regulatory factors leading to the induction of cytochrome P450 are separated developmentally but only by a few days.

At present, the issue of the tissue-specific expression of P450IA1 and IA2 is unclear. Certainly, data from this and other studies show that IA1 is detectable in many tissues but evidence for extrahepatic IA2 is weak. Cytochrome P450IA1 is induced in the convoluted proximal tubule of the kidney of MC-treated rats and TCDD-treated rabbits, although constitutively expression is low or absent, respectively (Foster *et al.*, 1986; Dees *et al.*,

1982). Expression is also very low in the lung of untreated animals but is induced following treatment with various PAHs in both these species (Foster *et al.*, 1986; Serabjit-Singh *et al.*, 1983; Domin *et al.*, 1984) although IA2 appears refractive to treatment with TCDD (Liem *et al.*, 1980; Domin *et al.*, 1984). Messenger RNA for IA2 has been demonstrated in several extrahepatic tissues, but levels are so low as to be negligible (Tuteja *et al.*, 1985; Pasco *et al.*, 1988). Although the IA2 gene is transcribed, no mature mRNA was found in the kidney, indicating the absence of this protein from this tissue. The lack of an immunoreactive band in Western blots of kidneys and lungs, in this study, supports the view that IA2 is absent extrahepatically.

Immunocytochemistry, however, is a very sensitive means of detecting low levels of protein. The results of its application here are such as to suggest that both IA1 and IA2 are expressed extrahepatically, albeit at low levels. Yet, only IA1 was found to be inducible in the tissues examined. The expression of these isoenzymes and the induction of IA1, varied as a function of tissue and age. Thus, both isoenzymes are found in the proximal tubules of the kidney and in the epithelial cells lining small intestinal villi, not only in adult rats but also in immature animals. Cytochrome P450IA1 is inducible in kidneys and gut of rats as early as 24 hours after parturition, whilst induction in rabbit kidneys is less obvious, indicating species differences. A transient surge in inducible AHH activity (25-fold) in kidney homogenates of two week old rats has been reported (Klinger *et al.*, 1978). Inducibility in animals either side of this peak was low to negligible. A similar surge (47-fold) in the induction of AHH activity by MC was observed in kidneys of rabbits, but in much younger animals, namely, 3 days after parturition. As in rats, inducibility fell sharply to less than a 3-fold increase in activity in adult rabbits (Atlas *et al.*, 1977). Interestingly, no mRNA to either of these isoenzymes could be detected in foetal kidney or gastrointestinal tracts of mice during organogenesis, although inducible IA1 enzyme activity is known to exist in these tissues of the newborn (Dey *et al.*, 1989).

Induction of IA1 in the lung of perinates is confined to unidentified interstitial cells of mesenchymal tissue lining endodermal tubes. Inducible mRNA to this isoenzyme was also detected in foetal lungs of mice (Dey *et al.*, 1989). At this time the foetal circulation is quite well established and MC arriving from the maternal circulation will come into early contact with this tissue as well as the liver. Strong staining in similar cells was noticed in other areas, for example, mesenchymal tissue surrounding limbs and other organs. It is tempting to speculate that these cells have an important part to play in controlling the development of tissues, perhaps by secreting growth factors. The association then, of cytochrome P450 with such cells, which disappear in rats within 5 days of parturition,

suggests a development role for this haemoprotein. The temporal control of genes encoding these proteins is much more complex than previously thought, since not only is the ontogeny of expression and induction subject to differential regulation but also the cellular distribution in both hepatic and extrahepatic tissues.

A wide range of compounds are metabolized, not only by rodents foetal livers but also by livers from human foetuses. Several antibodies have been used in an attempt to correlate inhibition of ethoxycoumarin *O*-deethylase activity with a particular isoenzyme but none was found using antibodies directed against the IA, IIB or III families of cytochrome P450 (Cresteil *et al.*, 1986; Ladona *et al.*, 1988). In contrast, the abundance of another human isoenzyme, identified by cross-reacting antibodies raised against human liver P450 P_5, correlated with epoxidation of aldrin, N-demethylation of benzphetamine and 16α-hydroxylation of dehydroepiandrosterone (Cresteil *et al.*, 1985). Although proteins of the P450IA, IIB and IIE families are low to absent in rat and human foetal livers, there are a considerable number of reports which demonstrate the presence of members of the P450III family (Kitada *et al.*, 1985; Barnes *et al.*, 1987; Kitada *et al.*, 1988; Wrighton *et al.*, 1988; Ladona *et al.*, 1988; Barnes *et al.*, 1989; Simmons & Kasper, 1989; Wrighton & Vandenbranden, 1989; Komori *et al.*, 1989). Moreover, immunocytochemical studies with a monoclonal antibody, MAb-HL3, which recognises a major isoenzyme of human liver P450, have shown that the immunoreactive foetal form is evenly distributed across the lobule, whilst in the adult expression is confined to centrilobular hepatocytes (Barnes *et al.*, 1987). The foetal environment, with high levels of steroid hormones, would certainly favour a glucocorticoid type of induction early in development for this isoenzyme. Considering the spectrum of monooxygenase activities identified during development in both the rat and human, it seems likely that as yet unidentified foetal isoenzymes of cytochrome P450 are present, which appear during development but disappear following parturition.

Chapter Five

Immunohistochemical localization of the major phenobarbital-inducible isoenzymes of P450 in hepatic and extrahepatic tissues of the rat

5.1 Introduction

The major subfamily of cytochrome P450 inducible by phenobarbitone (PB) has been designated P450IIB (Nebert *et al.*, 1989). The two major members of this subfamily, namely P450IIB1 and P450IIB2, are coordinately induced in the liver by PB and several other unrelated compounds (Thomas *et al.*, 1983; Kelley *et al.*, 1987). These two proteins have also been referred to as P450b (Ryan *et al.*, 1982), PB_{3a} (Wolf *et al.*, 1987), PB-B (Guengerich *et al.*, 1982) or PB-4 (Waxman & Walsh, 1982) and P450e (Ryan *et al.*, 1982), PB_{3b} (Wolf *et al.*, 1987), PB-D (Guengerich *et al.*, 1982) or PB-5 (Waxman & Walsh, 1982) respectively. They will be referred to here as IIB1 and IIB2.

Results from a number of independent groups suggest that there are at least four distinct PB-inducible forms of cytochrome P450 in the liver, the contents of which show remarkable strain and/or colony differences in the rat (Vlasuk *et al.*, 1982; Sakai *et al.*, 1983; Reik *et al.*, 1985). Three of these isoenzymes have been purified and characterized and have been referred to as P450b, from Long Evans rats, cytochrome P450bH, a strain variant of P450b from Holtzmann rats and P450e, common to both strains (Reik *et al.*, 1985).

Studies of genetic crosses and analysis of cloned cDNA have led to the identification of at least four genes/pseudogenes related to IIB1 and two, or possibly three genes/pseudogenes to IIB2 (Rampersaud & Walz, 1983; Atchison & Adesnik, 1983). How many of these are true genes is not yet known and only those for IIB1 and IIB2 have been completely sequenced and characterized (Fujii-Kuriyama *et al.*, 1982; Yuan *et al.*, 1983). A third gene, designated P450b*, indicative of the fact that it is more similar to P450b than to P450e, has also been sequenced but its relationship to previously identified genes in this subfamily is unclear (Shepherd *et al.*, 1987). These data confirm that there are at least three isoenzymes encoded by distinct genes. However, the fact that Vlasuk *et al.* (1982) recognised four proteins suggests that at least one other gene is active.

Cytochromes P450 IIB1 and IIB2 differ in only 13 of 491 amino acids (Yuan *et al.*, 1983)

with most of these substitutions restricted to a hypervariable region spanning about 50 amino acids in exon 7 (Suwa *et al.*, 1985). It is not surprising then that they show remarkable similarities in their minimum molecular weights (Black & Coon, 1986), spectral characteristics (Sakai *et al.*, 1983), substrate specificities (Waxman & Walsh, 1983; Swinney *et al.*, 1987) and peptide maps following proteolytic digestion (Vlasuk *et al.*, 1982) and are immunochemically indistinguishable with polyclonal antibodies against either isoenzyme (Thomas *et al.*, 1987b) or indeed even with monoclonal antibodies (Reik *et al.*, 1985; Christou *et al.*, 1987). They are sufficiently different, however, that they can be resolved by two-dimensional isoelectric focusing-SDS-PAGE (Vlasuk *et al.*, 1982; Pickett *et al.*, 1983) and by one-dimensional SDS-PAGE performed under optimal conditions (Reik *et al.*, 1985; Wilson *et al.*, 1987) and exhibit tissue specific expression and large differences in their relative activities toward some substrates (Ryan *et al.*, 1982; Wolf *et al.*, 1988), particularly 7,12-dimethylbenz[a]anthracene (Wilson *et al.*, 1987; Christou *et al.*, 1989).

The hepatic distribution and inducibility of the major PB-inducible isoenzymes of cytochrome P450, assessed immunohistochemically using polyclonal antibodies against apparently homogeneous preparations of P450IIB1, are well established for some rat strains (Baron *et al.*, 1981; Wolf *et al.*, 1984). However, due to the immunochemical relatedness of isoenzymes within the P450II family, these results are likely to reflect not only the combined distribution of IIB1 with IIB2 but also with a number of constitutive forms, such as P450IIC6 and P450IIC7 (Ryan *et al.*, 1984), which share 50% homology with IIB2 in both nucleotide and amino acid sequence (Kumar *et al.*, 1983; Gonzalez *et al.*, 1986).

The tissue-specific regulation of the expression of IIB1 and IIB2 appears quite complex. Recently, Wilson *et al.* (1987) showed that in the liver constitutive levels of P450IIB2 exceed those of P450IIB1. Following PB-treatment of rats, levels of IIB1 are induced to twice those of IIB2. Moreover, the use of specific, short (18-19 mer) oligonucleotide probes has revealed that levels of hepatic mRNA for both isoenzymes is increased by PB administration to rats, with a 4- to 5-fold greater accumulation of P450IIB1 mRNA than of P450IIB2 mRNA (Omiecinski *et al.*, 1985; Shepherd *et al.*, 1987). Transcriptional activation to/ produce these mRNAs is responsible for elevated levels of protein (Hardwick *et al.*, 1983). Omiecinski (1986) has also shown that IIB1, but not IIB2, mRNA is present in lung and testes but unlike that in the liver is refractive to PB-treatment. In contrast, IIB1 mRNA is inducible in small intestinal mucosa (Traber *et al.*, 1988). These isoenzymes have been quantified within these and other extrahepatic tissues by Western blotting of subcellular fractions (Wilson *et al.*, 1987). Together these studies provide evidence for the presence of cytochrome P450 IIB1 and/or IIB2 within the kidney, lung, small intestine and testis, albeit

at very low levels. However, there are some discrepancies between the findings of these groups. For example, it is suggested that IIB1 is expressed in the kidney and induced in the lung (Wilson *et al.*, 1987) but no mRNA for this isoenzyme was detected in the kidney and that present in the lung was not increased following PB treatment (Omiecinski, 1986).

The question of in which of these tissues induction does occur, and to which sub-populations of cells this is restricted, requires clarification since this may increase our understanding of the susceptibility of certain cell populations to toxic compounds, such as 4-ipomeanol. Further, other cell types may be identified which could represent potential cytotoxic targets. The ultimate aim of this study was to further investigate the constitutive expression and inducibility of the major PB-inducible form(s) of P450 (P450IIB1/2) in rat tissues, using immunocytochemical techniques. An absolute pre-requisite to this was characterization of the two monoclonal antibodies to be used in these studies, 10/1 and 1/4, previously prepared in our department (Sesardic *et al.*, 1986).

5.2 Results

5.2.1 Specificity of monoclonal antibodies 10/1 and 1/4

5.2.1.1 Characterization of specificity by the e.l.i.s.a technique

Both antibodies reacted strongly with apparently homogeneous preparations of cytochromes P450IIB1 (10.5 nmoles.mg^{-1} protein) and P450IIB2 (20.2 nmoles.mg^{-1} protein) and with the hepatic microsomal fraction from PB-treated rats (Table 5.1). Neither antibody reacted with P450IA1. Immunotitration revealed that 10/1 had a higher affinity (by approximately 10-fold) than 1/4 for their antigen (Fig. 5.1a). Unlabelled competitive e.l.i.s.a. was used in an attempt to determine whether MAbs 10/1 and 1/4 recognize spatially distinct epitopes. Plates were coated with microsomes from livers of PB-treated rats and incubated with either MAb alone or in the presence of competing antibody at a dilution which alone, produced a 50% response in e.l.i.s.a. ie. 1:10 000 dilution for 1/4 and a 1:50 000 dilution for 10/1. If the antibodies recognise the same epitope there should be no change in the absorbance. However, if spatially distinct epitopes are recognised then a shift of 2-2.5 log units to the right in the dose-response curves would be expected (Curve P, Fig. 5.1b and 5.1c). There was little change in the curves, with less than a 0.5 log unit shift to the right. The data suggest that 10/1 and 1/4 bind to different epitopes that overlap to such an extent that binding of one antibody reduces only partially binding of the other antibody. However, the possibility that binding of either 10/1 or 1/4 alters the conformation of cytochrome

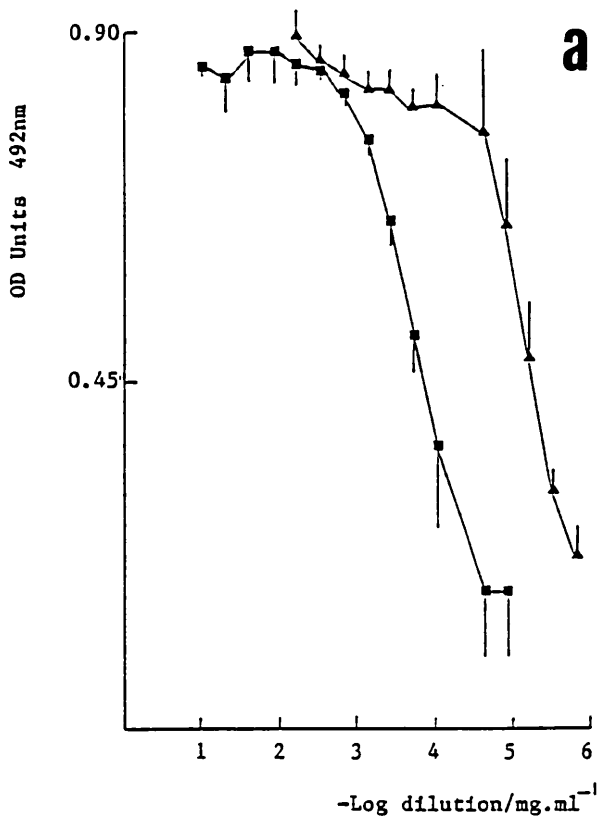
MAb	Antigen	A_{492nm}
1/4	IIB1	0.851 ± 0.039
	IIB2	0.752 ± 0.028
	Microsomes from PB-treated rat	0.707 ± 0.018
	IA1	0.032 ± 0.025
10/1	IIB1	0.980 ± 0.135
	IIB2	0.758 ± 0.030
	Microsomes from PB-treated rat	0.775 ± 0.075
	IA1	0.055 ± 0.005
	PBS alone	0.027 ± 0.011

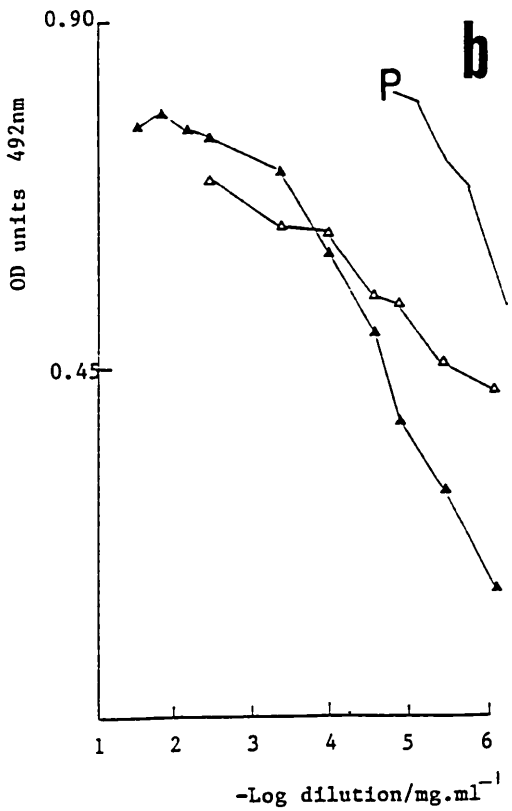
Table 5.1: Reactivity of monoclonal antibodies determined by e.l.i.s.a. assay

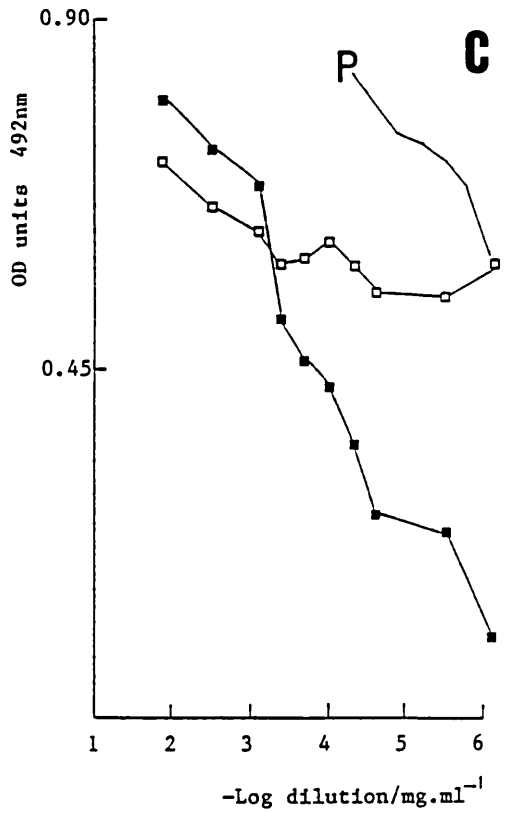
Results are mean ± standard deviation of at least 3 determinations, at antibody dilutions of 2 $\mu g.ml^{-1}$.

Figure 5.1: Titration of 10/1 and 1/4 binding to microsomes from PB-treated rat liver in e.l.i.s.a.

(a-c) Microtitre plates were coated with 100 μg of microsomal protein and exposed to varying dilutions of 10/1 (▲) or 1/4 (■) (a) alone, or in the presence of (b) a 1:10 000 dilution of 1/4 (△) or (c) a 1:50 000 dilution of 10/1 (□), respectively. (a) Results are mean ± standard deviation of 3 separate experiments. (b and c) Results are means of two separate experiments. P = predicted titration curve for additive binding, assuming interaction of the antibodies with spatially distinct epitopes.


a
0.90
0.45
OD Units 492nm
1
2
3
4
5
6
-Log dilution/mg.ml^{-1}


b
P
0.90
0.45
OD units 492nm
1
2
3
4
5
6
-Log dilution/mg.ml^{-1}


c
P
0.90
0.45
OD units 492nm
1
2
3
4
5
6
-Log dilution/mg.ml^{-1}

P450IIB1/2, such that the binding site for the other antibody is modified cannot be excluded from these experiments.

5.2.1.2 Characterization of specificity by the method of dot blotting

The reactivities of MAbs 1/4 and 10/1 and the polyclonal antibody, anti-PB-P450, were also determined by the method of dot blotting, where unseparated proteins are applied directly to nitrocellulose sheets. Apparently homogeneous preparations of IIB1 and IIB2 were applied in the absence or presence of 8 M urea with or without a post-incubation period in 25% (v/v) cold acetone for 15 minutes. The rationale for this approach was to establish i) the effect of denaturation by linearization of the protein, as occurs in SDS-PAGE and ii) the effect of acetone treatment, which was used as a fixative in the immunocytochemical studies, upon the reactivities of these antibodies.

The rank order of reactivity of these antibodies with IIB1 or IIB2 was anti-PB-P450 >>> 10/1 > 1/4 (Fig. 5.2). For both isoenzymes, the response to anti-PB-P450, tested in a single experiment, was linear over the range of 0.125-1 pmole. The response to 10/1 was linear over the range 0.25-2 pmoles, although immunoreactivity varied with each experiment (not shown). In contrast, immunoreactivity to 1/4 was negligible even at 8 pmoles IIB1 or IIB2 (Fig. 5.2c). Following treatment with 8 M urea, the interaction of 10/1 and 1/4 with either isoenzyme was dramatically reduced (Fig. 5.2b and Fig. 5.2c). This treatment also slightly reduced the intensity of the immunoreaction with anti-PB-P450, reactivity being lost at 0.5-1 pmoles (Fig. 5.2a). Post-incubation with 25% acetone, however, increased the intensity of the immunoreaction of both isoenzymes with both MAbs, with the limit of detection for IIB1 (500 fmoles) some 5-10-fold greater than for IIB2 (2 pmoles) (Fig. 5.2b and Fig. 5.2c). The effect of higher concentrations of acetone upon immunoreactivity could not be tested in this system, as concentrations of acetone exceeding 30% (v/v) caused severe deterioration of the nitrocellulose sheets.

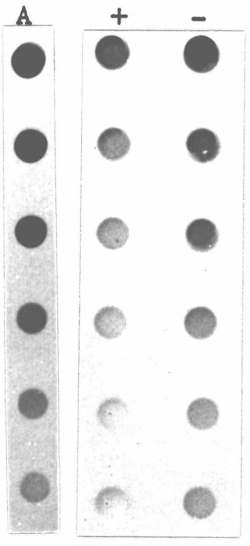
5.2.1.3 Characterization of specificity by the method of Western blotting

Hepatic microsomal fractions from PB-treated rats and apparently homogeneous preparations of IIB1 and IIB2 were electrophoresed in SDS-9% polyacrylamide gels as described in Section 2.2.13, such that these two closely related isoenzymes were separated, then electrotransferred to nitrocellulose sheets and probed with either anti-PB-P450 or 10/1 antibodies.

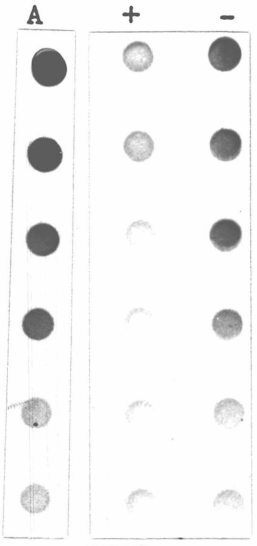
Figure 5.2: Reactivity of antibodies with their homologous antigen determined by dot blotting

Unseparated proteins diluted serially 2-fold, starting at 8 pmoles.well^{-1}, were applied directly to nitrocellulose sheets, in the absence (-) or presence (+) of 8 M urea. Proteins applied in the absence of 8 M urea were also subsequently treated with 25% (v/v) cold acetone for 15 minutes (A). Blots were immunostained with (a) anti-PB-P450 diluted 1:1000; (b) 10/1, 1 μg.ml^{-1} or (c) 1/4, 1 μg.ml^{-1}, as described in Section 2.2.16.

a

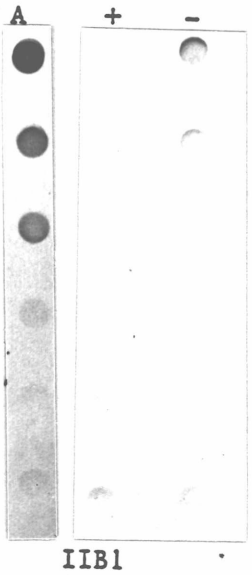


IIB1

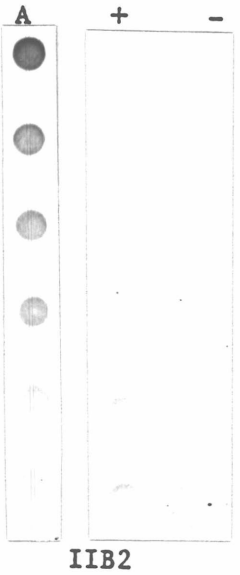


IIB2

b

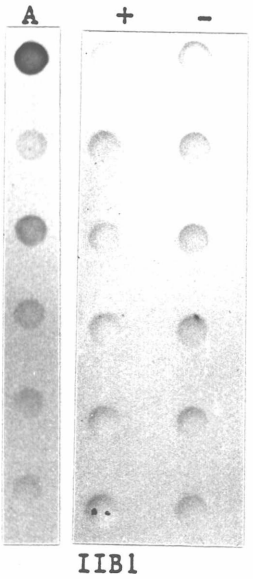


IIB1

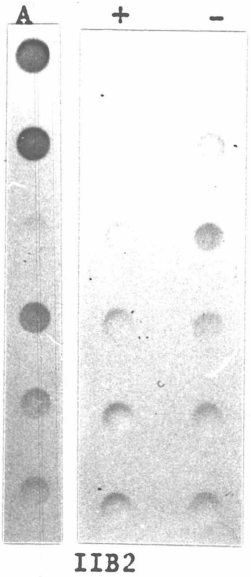


IIB2

c



IIB1



IIB2

The anti-PB-P450 antibody reacted strongly with two polypeptides in the IIB1 preparation with apparent M_r of 51 000 and 52 500, respectively (Fig. 5.3a). Two polypeptides were also recognised in the apparently homogeneous preparation of IIB2, with apparent M_r of 51 500 and 53 000, respectively. In immunoblots of an identical preparation, MAb 10/1 recognised both polypeptides in the IIB1 preparation but reacted only weakly with the lower molecular weight polypeptide in the IIB2 preparation (Fig. 5.3b).

The reactivity of these two antibodies with PB-inducible isoenzymes was also determined in liver microsomal samples from untreated and PB-treated rats of different strains. No immunoreactive bands were detected in immunoblots of microsomes from untreated Wistar, Sprague-Dawley, Lister-Hooded or F344 rats by anti-PB-P450 or 10/1 antibodies (not shown). Not surprisingly, two major bands, co-migrating with cytochromes IIB1 and IIB2 were detected in microsomes from PB-treated Wistar, Sprague-Dawley, Lister-Hooded and F344 rats by the polyclonal antibody (Fig. 5.3a). In contrast, only a single polypeptide band, co-migrating with cytochrome IIB1 was detected in immunoblots of an identical preparation probed with MAb 10/1 (Fig. 5.3b).

In a separate experiment, immunoblots of an identical preparation containing liver microsomal fractions from PB-treated Wistar rats, probed with 10/1 and 1/4, showed that 1/4 also had a weaker affinity for IIB1 in this system, agreeing closely with the results of e.l.i.s.a. (Fig. 5.4a and 5.4b). MAb 10/1 recognised a polypeptide of identical molecular weight to cytochrome IIB1 in microsomes from acetone and isosafrole-treated rats. No bands were detected in microsomes from rats pre-treated with other inducing agents or in hepatic microsomes from other mammalian species (Fig. 5.4c).

These experiments show that MAbs 10/1 and 1/4 recognise IIB1 and IIB2, although interaction with IIB2 is much weaker and following denaturing treatments abolished.

5.2.1.4 The effect of tissue fixation on the specificity as determined by immunocytochemistry

An optimal fixation protocol was established in preliminary experiments in which the reactivities of 1/4 and 10/1 in unfixed frozen liver sections were compared to those in sections which had been fixed with methanol (5 min), acetone (15 min), methanol-acetone, 1:1 (v/v) (5 min) or 0.5% (w/v) *p*-benzoquinone (5 min). The reactivities of these antibodies in paraffin-embedded liver sections were also tested.

Figure 5.3: Reactivity of antibodies determined by Western blotting

Hepatic microsomal proteins from PB-treated rats and purified preparations of IIB1 and IIB2 were separated by SDS-PAGE and then subjected to Western blotting as described in Sections 2.2.13 and 2.2.14. Immunoblots were stained with (a) anti-PB-P450 diluted 1:1000 or (b) 10/1, 1 $\mu g.ml^{-1}$. Protein preparations, 20 µg, were as follows: lanes 1-3, Wistar rats,; lanes 4 and 5, Sprague-Dawley rats; lanes 6-8, IIB1, 1, 2 and 4 pmole respectively; lanes 9-11, IIB2, 1, 2 and 4 pmole respectively; lanes 12-14, Lister-Hooded rats and lanes 15-17, F344 rats.

The apparently homogeneous preparations of P450IIB1 and IIB2 were, infact, under the conditions used here, shown each to be contaminated with other proteins, these possibly representing isozymic variants of IIB1 and/or IIB2.

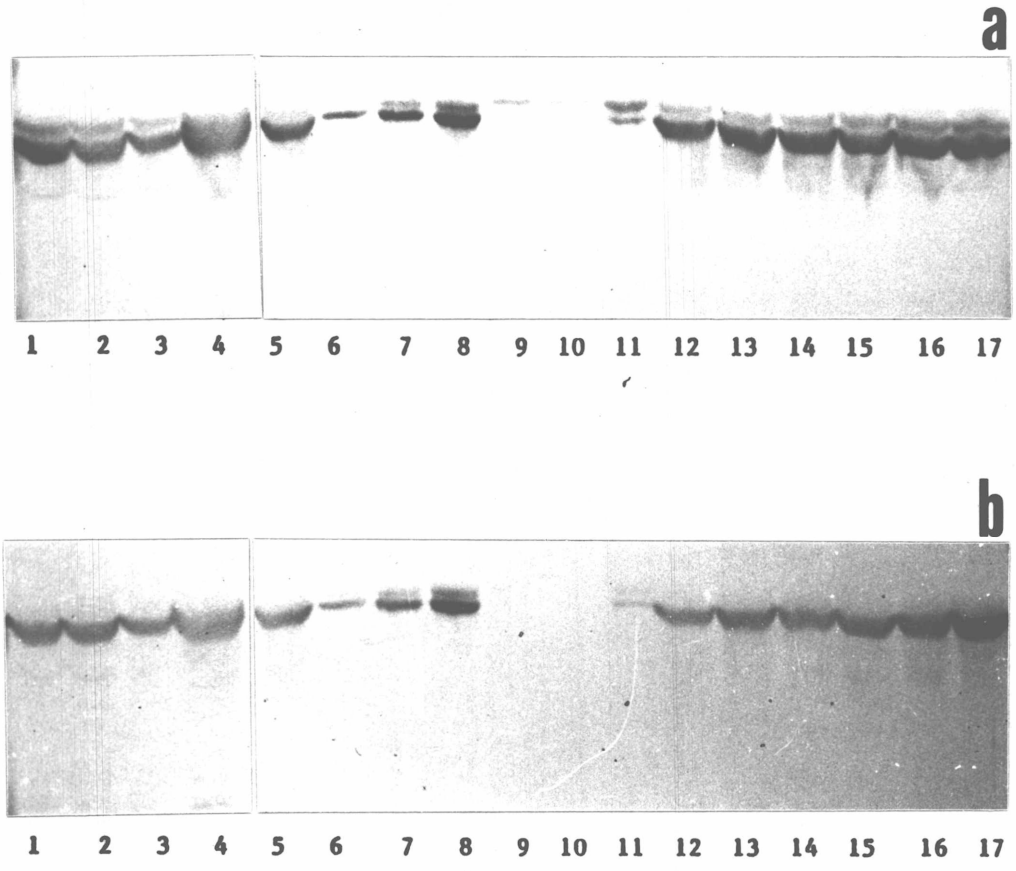
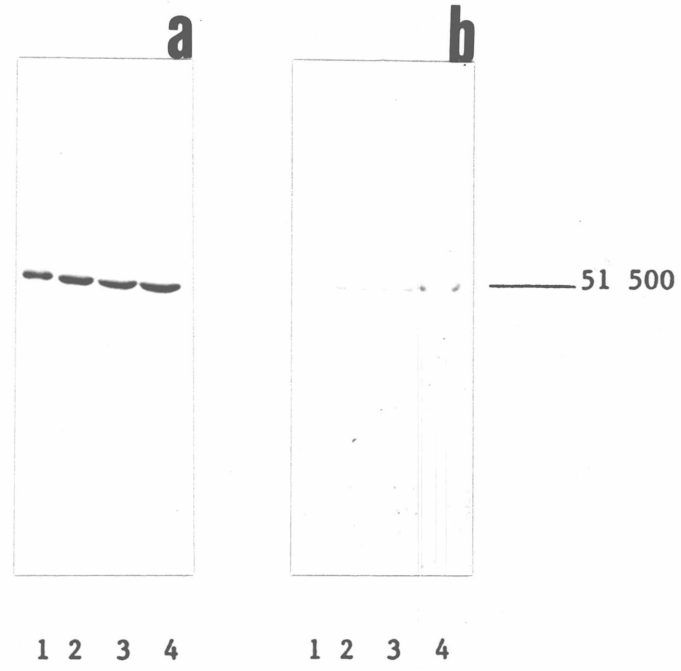

a
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
b
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 5.4: Western blotting of hepatic microsomal fractions from different mammalian species

Microsomal proteins were separated by SDS-PAGE and subjected to Western blotting as described in Sections 2.2.13 and 2.2.14. Immunoblots containing samples from PB-treated rat liver (lanes 1-4: 5, 10, 20 and 30 μg protein, respectively) were immunostained with 1 $\mu g.ml^{-1}$ of (a) 10/1 or (b) 1/4. (c) Immunoblots containing microsomal samples from rats treated with various xenobiotics and from other mammalian species were immunostained with 10/1, 1 $\mu g.ml^{-1}$. Hepatic microsomal fractions (20 μg) were as follows: lane 1, untreated rat; lane 2 and 3, PB-treated rat; lane 4, MC-treated rat; lane 5, acetone-treated rat; lane 6, PCN-treated rat; lane 7, INH-treated rat; lane 8, ISF-treated rat; lane 9, CL-treated rat; lane 10, human; lane 11, purified P450IIB1, 10 pmoles; lane 12, untreated Balb/c mouse; lane 13, untreated New Zealand White rabbit; lane 14, untreated Dunkin Hartley guinea-pig and lane 15, untreated Syrian hamster.


a
b
51 500
1 2 3 4
1 2 3 4

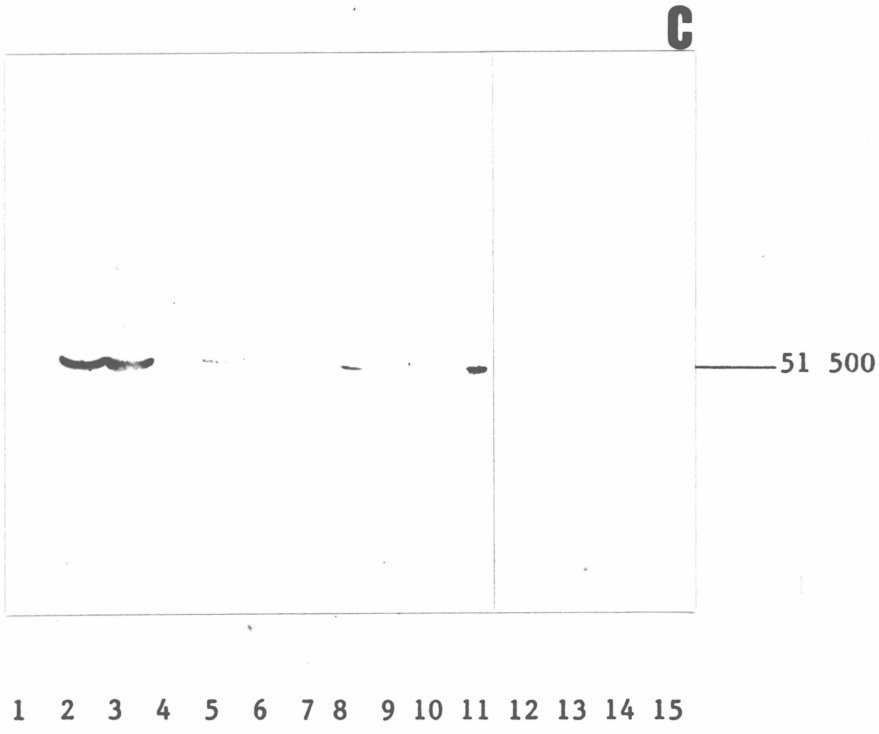
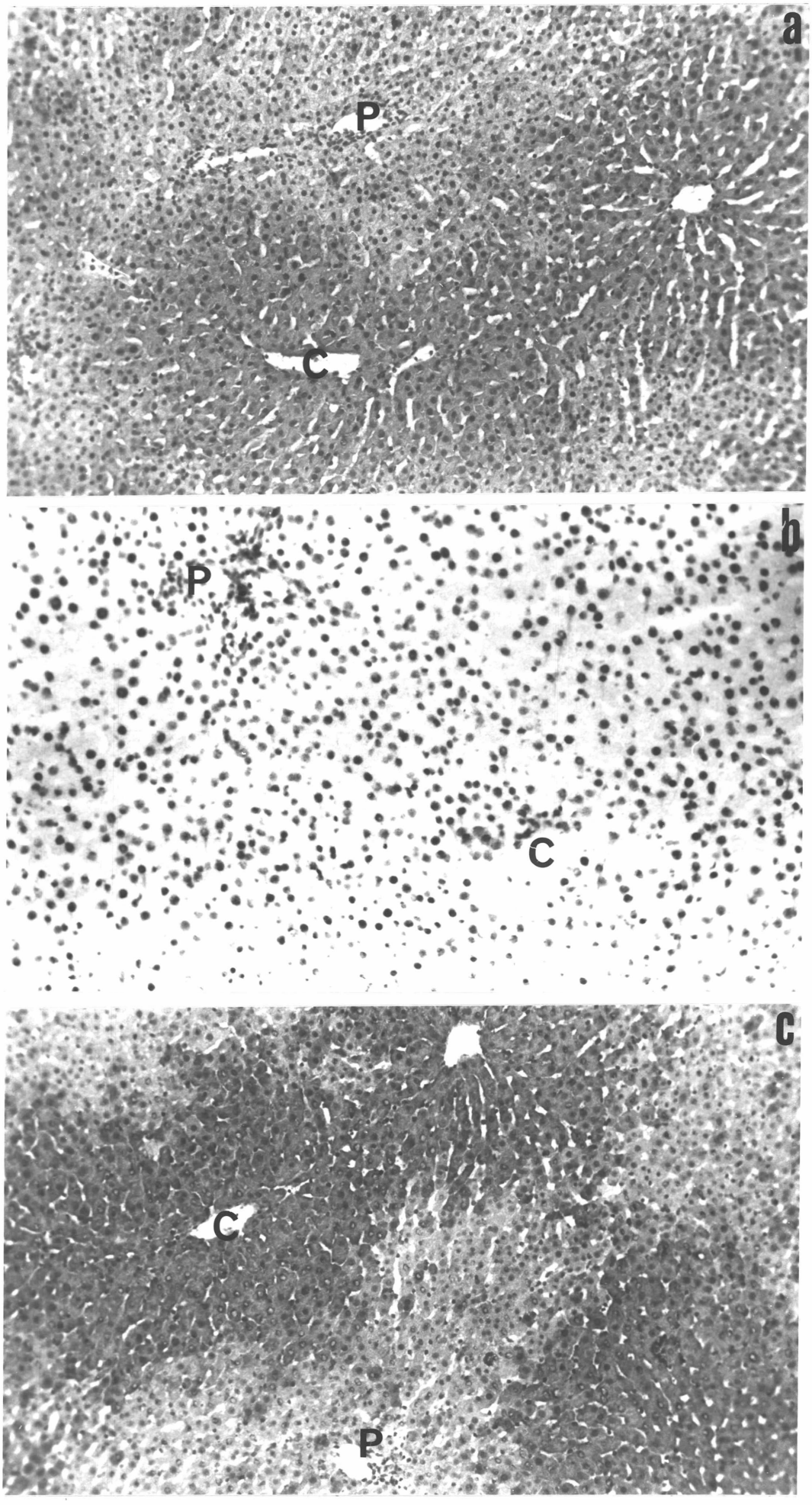

c
51 500
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 5.5: Effect of method of tissue fixation on the specificity of binding of 1/4

Frozen liver sections from PB-treated Wistar rats were (a) unfixed or treated with (b) absolute methanol for 5 min or (c) absolute, cold acetone for 15 min. Sections were immunostained with 1/4 diluted 1:100 as described in Section 2.2.18. C, central vein; P, portal tract. Magnification x154.


a
P
C
b
P
C
c
C
P

The best retention of antigenicity was obtained with frozen sections fixed in acetone, the distribution of staining comparing favourably with that seen in unfixed tissue. In fact, the intensity of the immunoreaction was slightly stronger in the acetone-fixed sections (Fig. 5.5a cf Fig. 5.5c). Methanol abolished all antigenicity (Fig. 5.5b), whilst *p*-benzoquinone markedly reduced the intensity of staining (data not shown). No immunoreactive protein was detectable in tissue subjected to paraffin embedding. Acetone fixation, which appeared not to decrease antigenicity in tissue sections, was therefore used in all subsequent studies with these antibodies.

5.2.2 Distribution of cytochromes P450IIB1/2 in liver

5.2.2.1 Localization of P450IIB1/2 in Wistar rat liver

In liver from untreated animals, specific immunostaining was detected with both 1/4 and 10/1, but only at dilutions of 1:100 or less (Table 5.2). Staining was most intense in a few, single randomly distributed hepatocytes radiating out from the central vein over a short distance of not more than 3-4 cell rows (Fig. 5.6a). The intensity of the immunoreaction varied amongst cells. Strongly reacting hepatocytes were interspersed with moderate to weakly stained hepatocytes. A very weak immunoreaction was detected in hepatocytes throughout the rest of the lobule. No staining of the bile duct epithelium was apparent. The immunoreactive product was diffuse, appearing homogeneous and uniformly dispersed throughout the cytoplasm. When sections were incubated with anti-PB-P450 antibody (at dilutions of 1:500-1:2000), a relatively strong immunoreaction was observed, which was distributed more evenly throughout the lobule (Table 5.2). There was, however, a slightly more intense immunoreaction in randomly distributed hepatocytes radiating out from the central vein. Sections stained with MAb 107 or normal rat serum as a negative control, showed no specific staining (Fig. 5.6b).

Following pre-treatment of rats with PB immunohistochemical staining for cytochrome P450IIB1/2 was apparent at monoclonal antibody dilutions as high as 1:1000 using indirect immunoperoxidase detection (Table 5.2) and greater than 1:10 000 with IGSS detection (Fig. 5.6c), thus confirming previous results (Section 3.2.2) that silver enhancement increases the limit of detection by greater than 10-fold. All cells within the centrilobular and most in the midzonal regions were intensely stained. Some scattered single hepatocytes in the periportal region were also positively stained (Fig. 5.6d). In comparison, the distribution of immunoreactivity following incubation with anti-PB-P450 antibody did not change, except for a slight increase in intensity in a few centrilobular cells (Table 5.2).

Table 5.2: Immunotitration of 1/4, 10/1 and anti-PB-P450 antibody binding to rat liver in immunocytochemistry

Rat Strain	Treatment	Antibody	Antibody dilution 1:10	1:100	1:500	1:1000	Distribution
Wistar	None	1/4	++	+	-		Few scattered CL cells
		10/1	++	+	-		Few scattered CL cells
		anti-PB-P450		+++	+++	+++	WL and+++ few CL cells
	PB	1/4	+++	+++	++		CL
		10/1	+++	+++	+++	+++	CL
		anti-PB-P450		+++	++	++	WL, very intense CL
Lister-Hooded	None	1/4	++	-	-		PP
		10/1	++	++/+	+/-	-	PP
		anti-PB-P450	+++	++	++	+	WL
	PB	1/4		+++	+	+/-	CL and MZ
		10/1	+++	+++	++	++	CL and MZ
		anti-PB-P450		+++	+++	++	CL and MZ
Sprague Dawley	None	1/4	++	++/+			WL
		10/1	++	++	++	+	WL
		anti-PB-P450	+++		++	++	WL, +++ CL
	PB	1/4		+++	+	+/-	WL
		10/1	+++	+++	++	++	WL
		anti-PB-P450		+++	+++	++	WL

Table 5.2 continued:

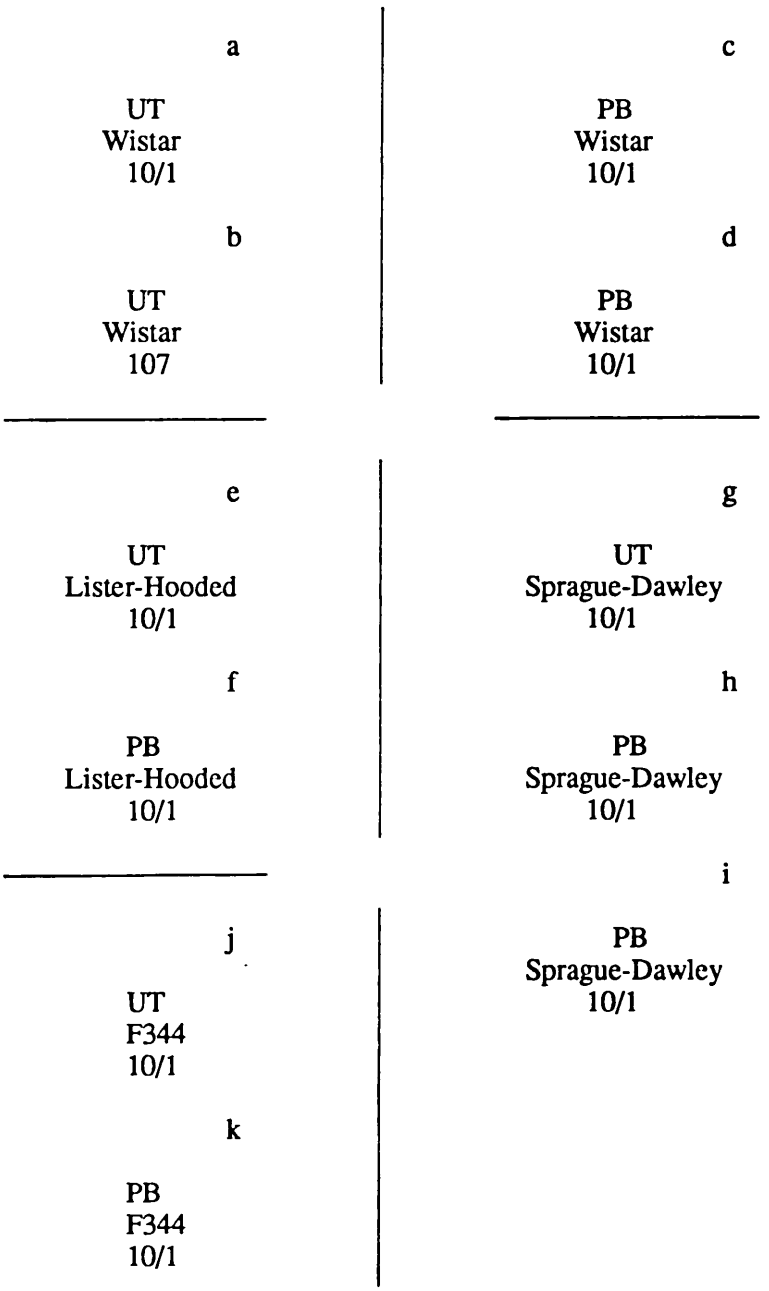
F344	None	1/4	++	+/-	-		CL and PP
		10/1	++	++/+	+	+/-	CL and PP
		anti-PB-P450	+++	+++	++	++	WL, +++ CL
	PB	1/4		+++	++	+/-	WL
		10/1	+++	+++	+++	+++	WL
		anti-PB-P450		+++	+++	++	WL

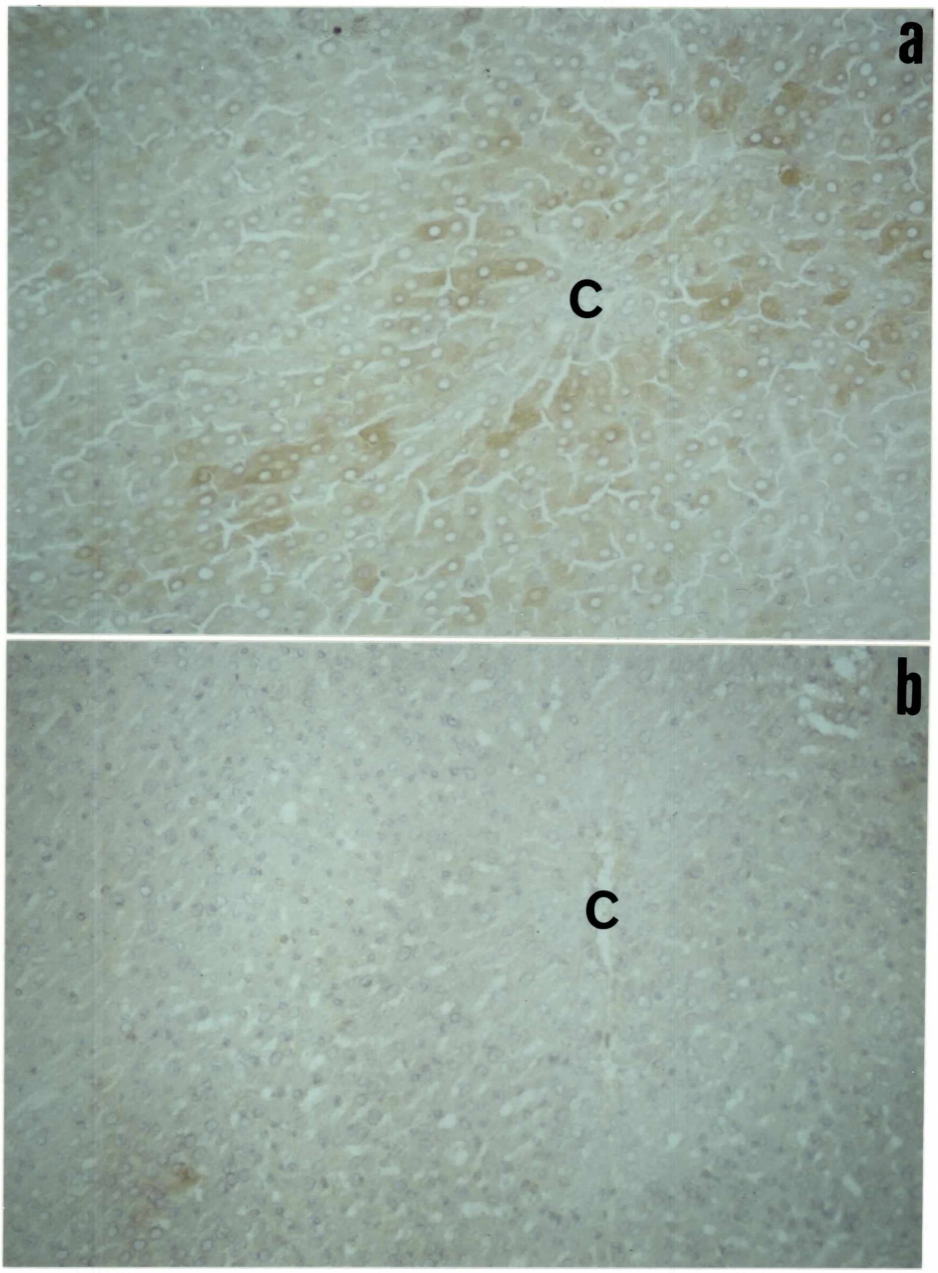
Intensity of staining: +++ intense, ++ moderate, ++/+ some sections moderate others weak, + weak, +/- some sections weak others negative, - negative.

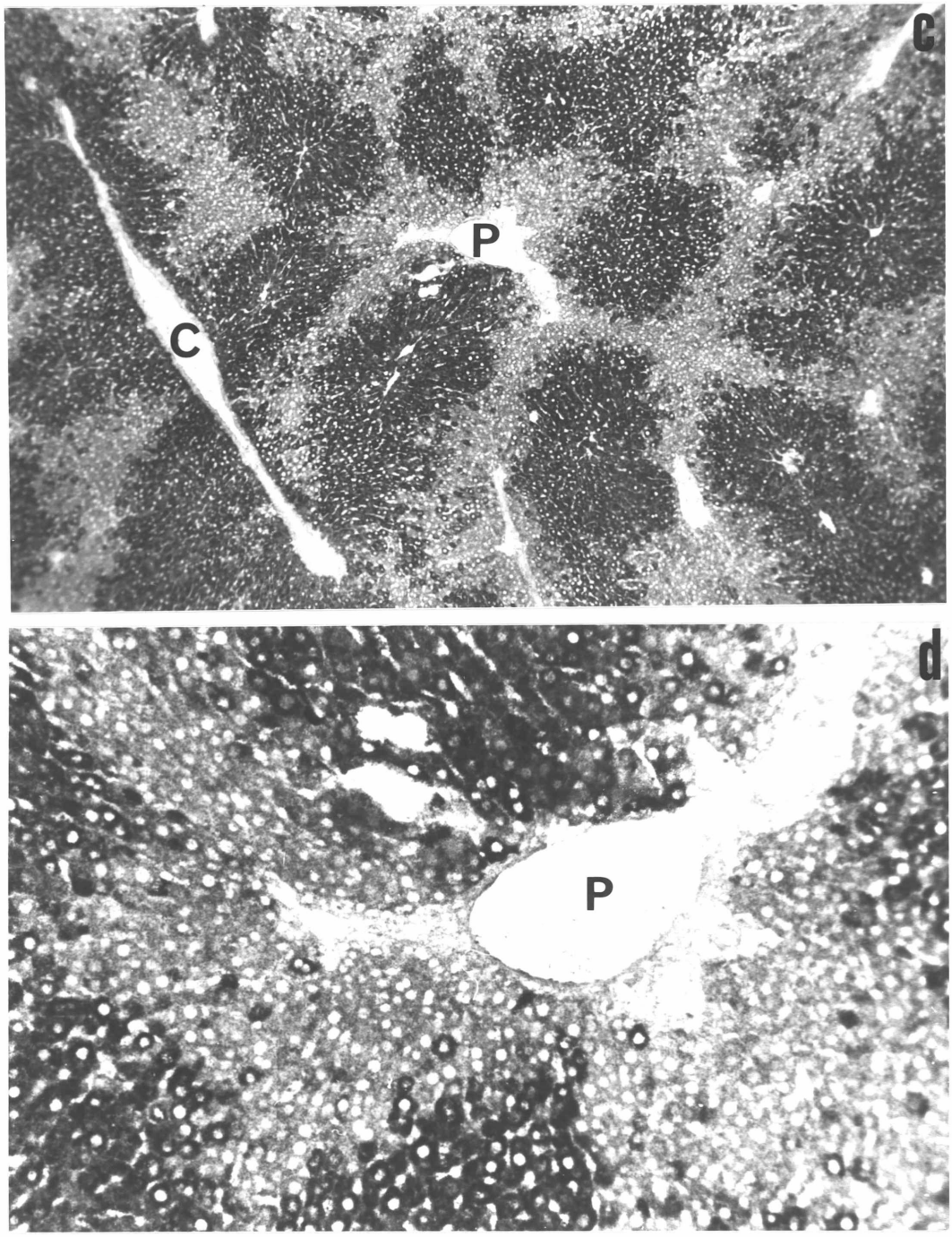
WL, whole lobule; CL, centrilobular hepatocytes; MZ, midzonal hepatocytes; PP, periportal hepatocytes.

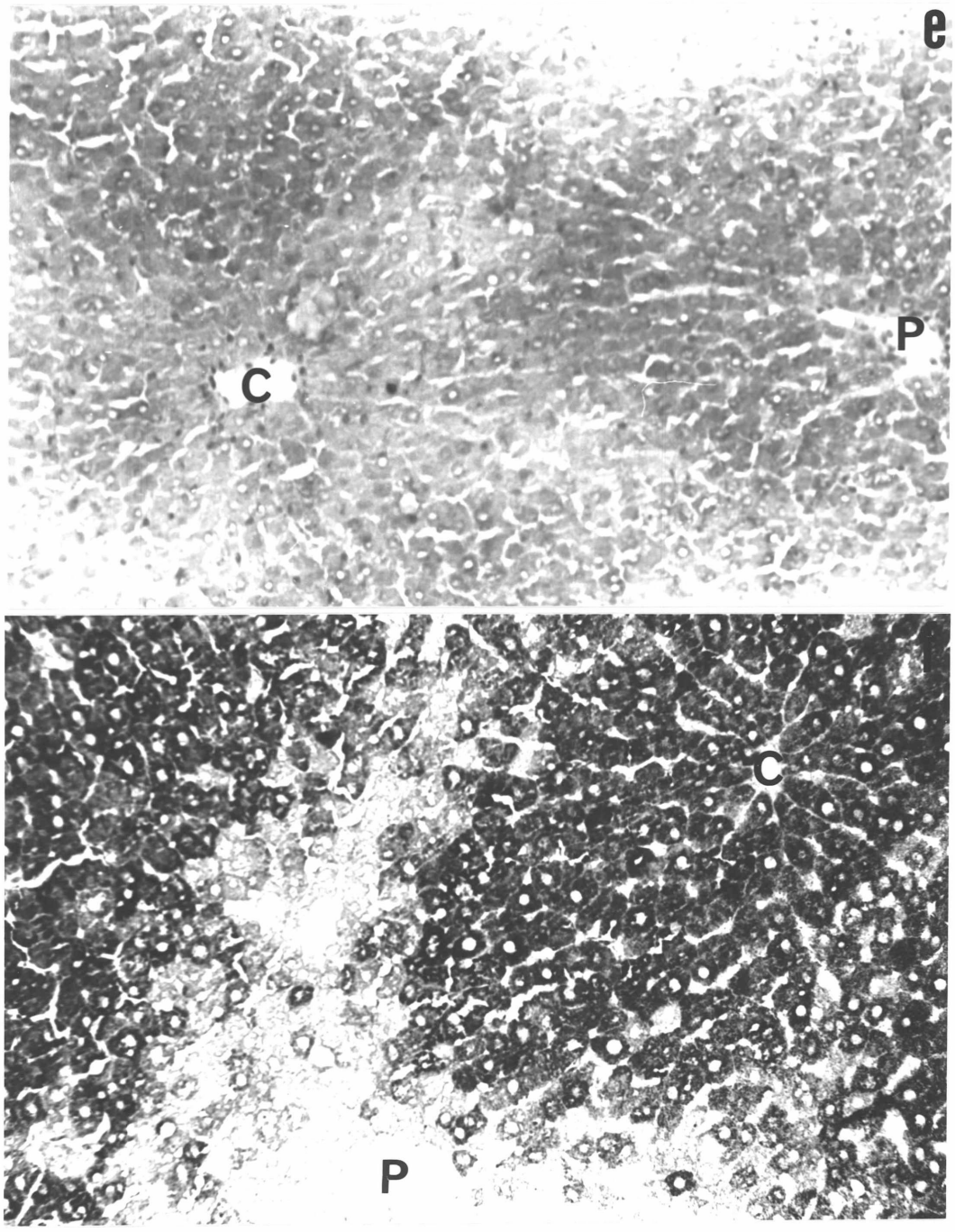
Figure 5.6: Immunocytochemical localization of P450IIB1/2 in livers of rats

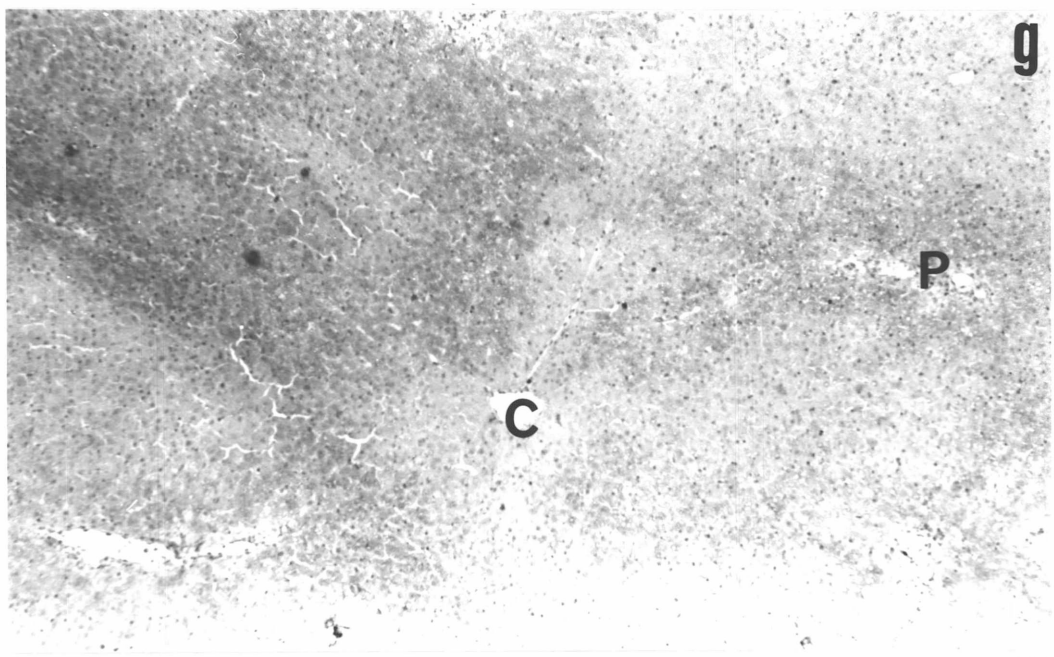
Sections were incubated with either 10/1 (a, c-k) or 107 (b) at the dilutions indicated on the micrographs. Sections were from untreated (a-b, e, g, j) and PB-treated (c-d, f, h-i, k) Wistar, Lister-Hooded, Sprague-Dawley or F344 rats respectively. (c, d, f) specific immunoreaction was detected using GAM conjugated to 5 nm colloidal gold and the signal enhanced with silver enhancement solution. C, central vein; P, portal tract; ➡ inflammatory cells. Magnification ranged from x50 to x500.

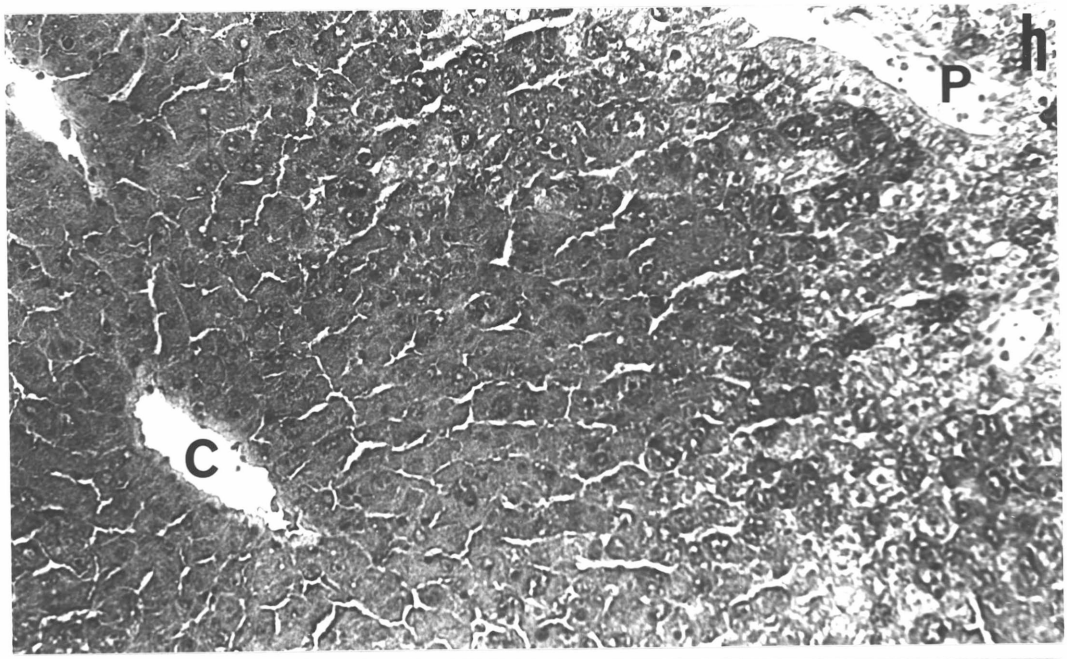


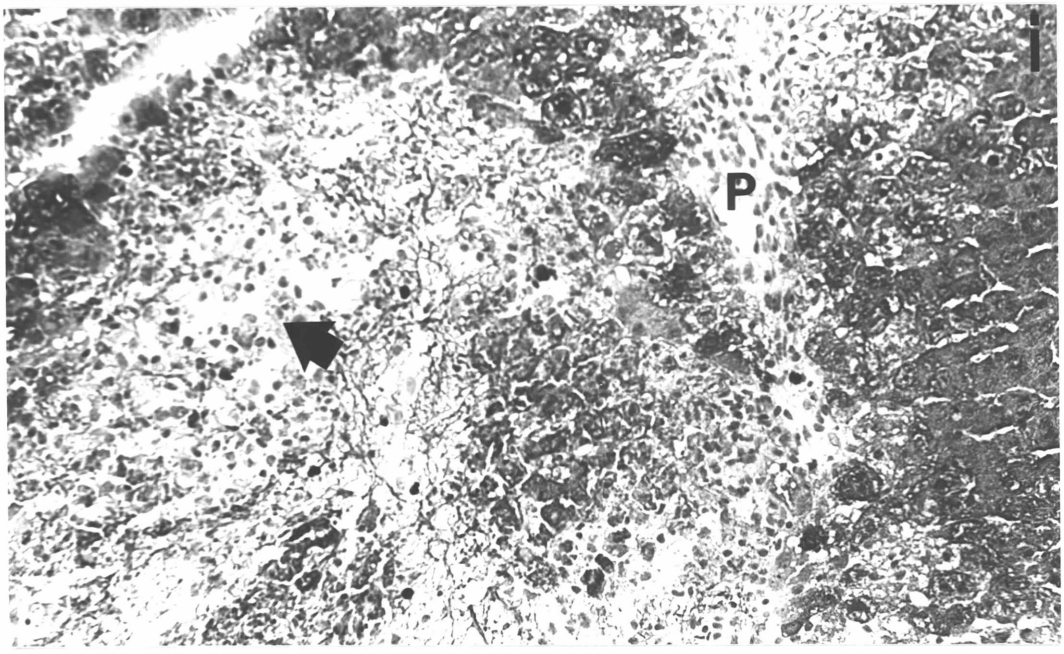

a
C
b
C

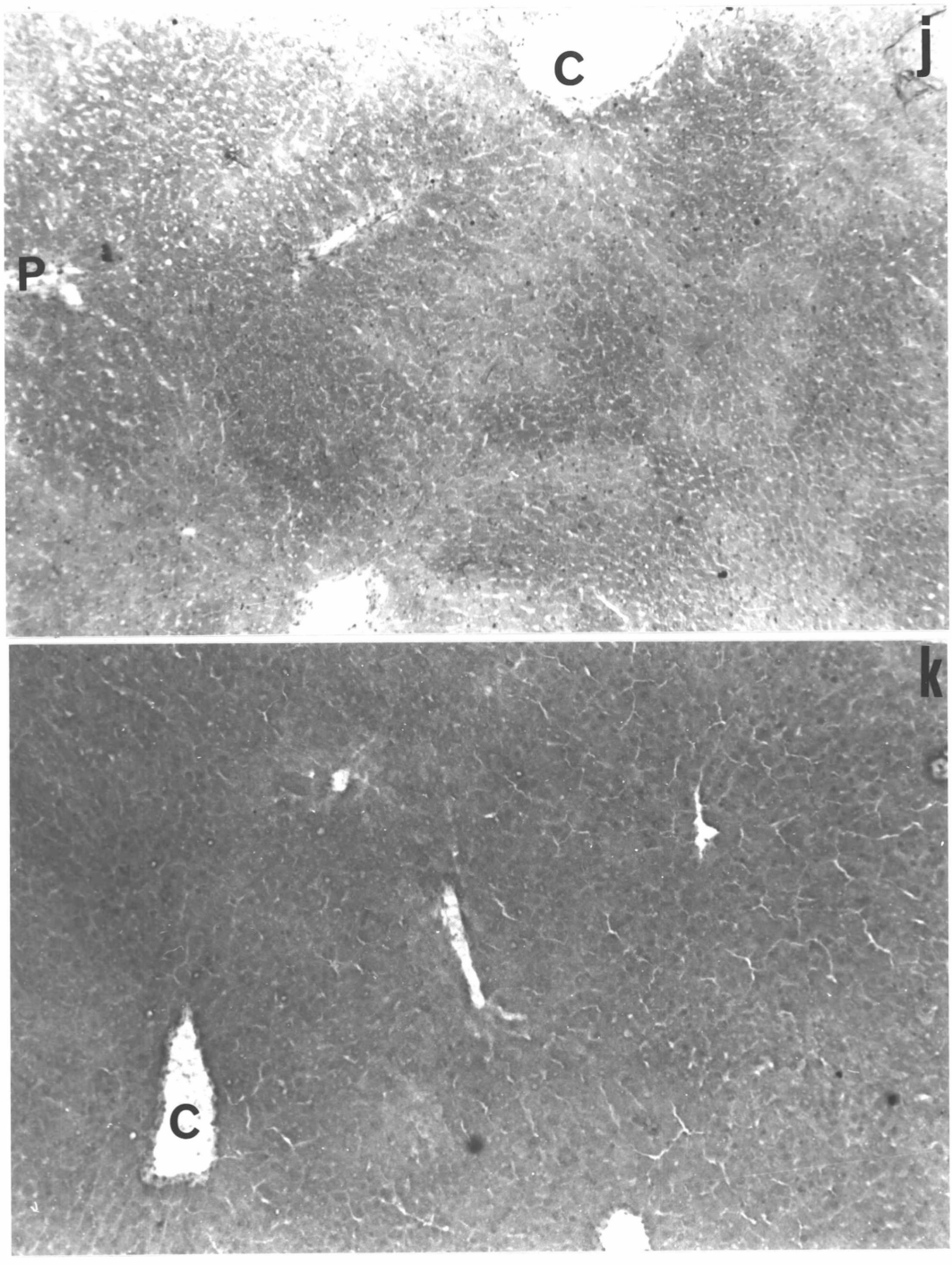

c
P
C
d
P


e
C
P
C
P


g
P
C


h
P
C


i
P


C
j
P
k
C

5.2.2.2 Localization of P450IIB1/2 in Lister-Hooded rat liver

Immunohistochemical staining for this isoenzyme(s) in liver sections from untreated rat, in contrast to results with Wistar rat, was detected in a zone of cells radiating out from portal tracts (> 8 cell rows) following incubation with either 10/1 or 1/4 (Fig. 5.6e). The intensity of the immunoreaction did not vary amongst these hepatocytes. In contrast, a moderate immunoreaction, evenly distributed across the lobule, was observed following incubation with anti-PB-P450 antibody.

Treatment of rats with PB resulted in intense staining of all hepatocytes in centrilobular and midzonal regions following incubation with any one of the three antibodies (Table 5.2; Fig. 5.6f). Those hepatocytes immediately surrounding central veins were less intensely stained and at high monoclonal antibody titres were unstained. In sections from the three rats examined, a narrow band of hepatocytes surrounding portal tracts was also unstained and appeared to show signs of damage. There was some infiltration of inflammatory cells into these areas.

5.2.2.3 Localization of P450IIB1/2 in Sprague-Dawley rat liver

Exposure of liver sections from untreated rats to 10/1, at dilutions of less than 1:100, resulted in moderate to weak staining of most hepatocytes across the lobule. A more intense immunoreaction was apparent in hepatocytes around either central veins or portal tracts in sections from some rats (Fig. 5.6g). A more intense immunoreaction was observed in all hepatocytes following exposure to anti-PB-P450 antibody, although cells around central veins were most intensely stained. Upon titration of this antibody to 1:1000, only staining of centrilobular cells remained (Table 5.2).

Following PB-treatment of rats an intense immunoreaction was observed in most hepatocytes across the lobule, following exposure to either monoclonal antibody (Fig. 5.6h). A similar phenomenon to that seen in livers from Lister-Hooded rats was also apparent here, namely a narrow band of hepatocytes surrounding portal tracts were negative. However, the occasional periportal hepatocyte was intensely stained. Once again, there were signs of damage to cells in these regions. Liver sections from one of the three rats showed signs of necrosis around a large periportal vein and there was infiltration of inflammatory cells, not only in periportal regions but also in centrilobular areas (Fig. 5.6i). The anti-PB-P450 antibody produced even staining throughout the lobule, although a more intense immunoreaction was apparent in some single, scattered cells.

5.2.2.4 Localization of P450IIB1/2 in F344 rat liver

A moderate to weak, specific immunoreaction was evident in sections from untreated rats following exposure to either MAb, at dilutions of 1:100 or less, (Table 5.2). In contrast to the pattern of staining observed in the three outbred strains, immunoreactive protein was apparent in a narrow band of hepatocytes surrounding both central and periportal tracts (5-8 cell rows) (Fig. 5.6j). The intensity of staining amongst hepatocytes within any one area also varied, although this is not apparent in the figure. Following exposure to the polyclonal antibody, a more even immunoreaction was observed, with centrilobular cells slightly more intensely stained (Table 5.2).

Treatment of rats with PB resulted in a marked increase in the intensity of the immunoreaction in all hepatocytes across the lobule following exposure to either monoclonal antibody (Fig. 5.6k). No variation in the intensity of the immunoreaction amongst hepatocytes was apparent. A strong, even immunoreaction was also observed following exposure to the polyclonal antibody (Table 5.2).

5.2.3 Distribution of P450IIB1/2 in rat kidney

Kidney sections from untreated and PB-treated Wistar rats exposed to either 10/1 or 1/4 showed no immunostaining at antibody dilutions as low as 1:10 (Table 5.3). This also proved to be the case in 75% of kidney sections examined from either untreated or PB-treated Sprague-Dawley, Lister-Hooded and F344 strains. In the other 25%, a very weak immunoreaction was observed in the proximal tubule cells.

The possibility that the lack of detectable immunoreactivity was due to insensitivity of the second antibody was excluded by using immunogold-silver staining, which increased the limits of detection of the indirect two-stage technique by 10-20-fold in the liver. It was still not possible to detect immunoreactive product above background levels, even after PB treatment of the animals (Fig. 5.7a). Only with MAb 3/4/2, used as a positive control in these experiments, was staining apparent, which was identical to that previously described (Foster *et al.*, 1986). Cytochrome P450IA1 was localized in cells of the S_3 portion of the proximal convoluted tubule (Fig. 5.7b).

In contrast to results with MAb 1/4 and 10/1, exposure of kidney sections from all strains to anti-PB-P450 antibody, produced specific immunostaining in most structures, other than the innermost medulla region. However, cells of the proximal tubule were most intensely

Table 5.3: A comparison of the immunocytochemical titration of 10/1, 1/4 and the polyclonal antibody, anti-PB-P450, in kidney from different strains of rat

Strain	Treatment	Antibody	Antibody dilution			
			1:10	1:100	1:200	1:500
Wistar	None	1/4 or 10/1	-	-	-	-
		Anti-PB-P450		++	+	-
	PB	1/4 or 10/1	-	-	-	-
		Anti-PB-P450		++	+	+/-
Lister-Hooded	None	1/4 or 10/1	+/-	-	-	
		Anti-PB-P450		+++	++	+/-
	PB	1/4 or 10/1	+/-	-	-	
		Anti-PB-P450		+++	++	+/-
Sprague-Dawley	None	1/4 or 10/1	+/-	-	-	
		Anti-PB-P450		+++	++/+	+/-
	PB	1/4 or 10/1	+/-	-	-	
		Anti-PB-P450		+++	++	+
Fischer	None	1/4 or 10/1	+/-	-	-	
		Anti-PB-P450		+++	++	+/-
	PB	1/4 or 10/1	+	-	-	
		Anti-PB-P450		+++	++	+

Intensity of staining: +++ intense, ++ moderate, ++/+ some moderately stained areas with weak areas, + weak, +/- some sections showed very weak staining, others negative, - negative.

Figure 5.7: Immunocytochemical localization of P450IIB1/2 in rat kidney

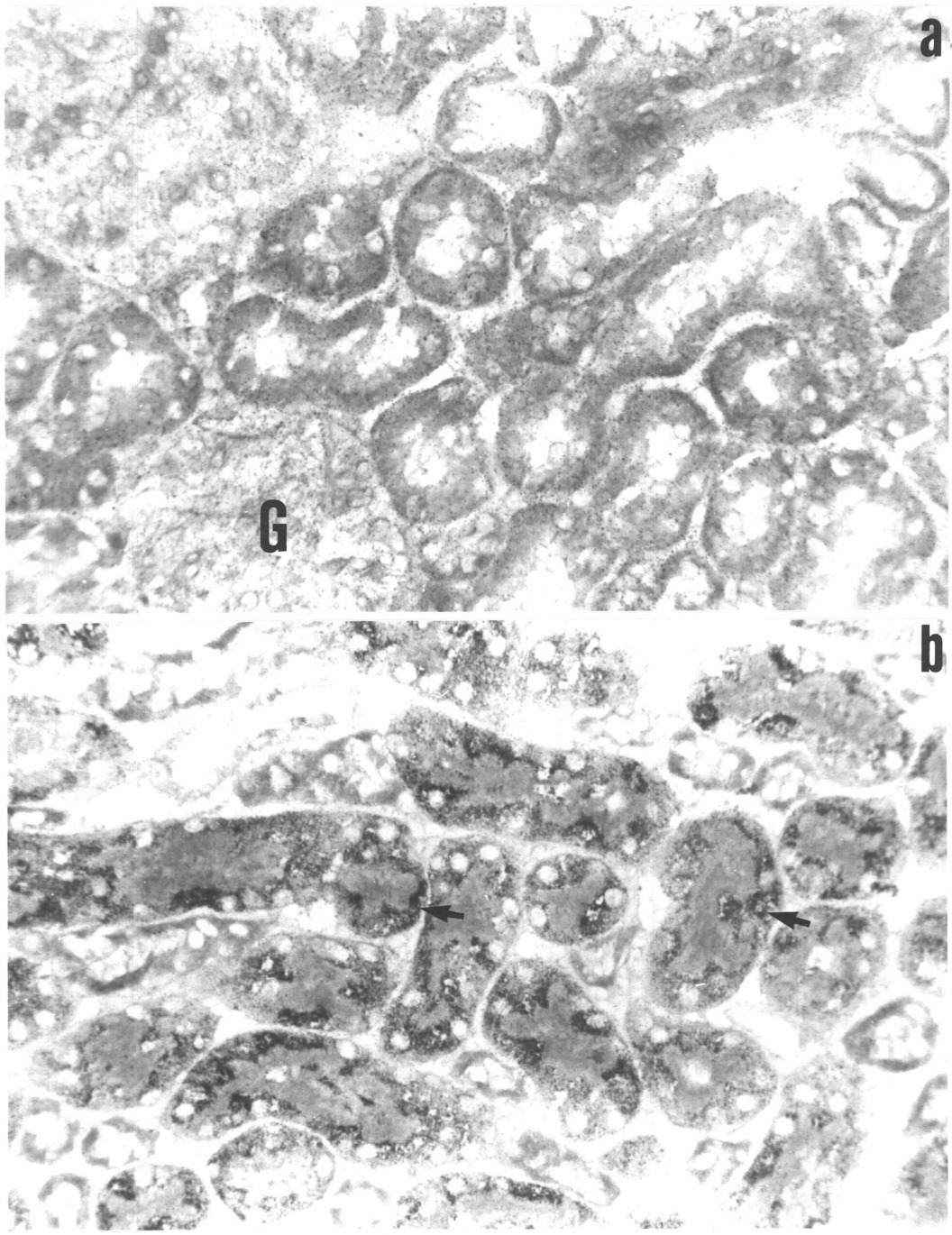
(a) Sections of kidney from a rat treated with PB, incubated with MAb 10/1 at low dilution (1:10). No immunoreactive P450IIB1/2 is apparent. (b) A section from the same kidney incubated with MAb 3/4/2 at a dilution of 1:10 showing specific staining for P450IA1 in proximal tubule cells (➡). Specific immunoreaction was detected using GAM conjugated to 5 nm colloidal gold and the signal enhanced with silver enhancement solution. G, glomerulus. Magnification x320.

a

PB
10/1

b

PB
3/4/2


a
G
b

stained, particularly those in the immediate vicinity of glomeruli (the S_1 portion of the proximal tubule). Weak staining was apparent in the glomeruli and connective tissue around blood vessels. Immunotitration of the antibody resulted in a reduction in the intensity of staining in all positively reacting structures (Table 5.3). The intensity and distribution of staining was unchanged following PB-treatment of rats (Table 5.3).

5.2.4 Distribution of P450IIB1/2 in rat lung

In sections of lung from untreated Wistar rats incubated with 1/4 immunoreactive cytochrome P450 was clearly evident in the bronchial epithelial cells and in the non-ciliated bronchiolar epithelial, or Clara, cells (Fig. 5.8a and 5.8c). Clara cells were readily identifiable by their bulbous, domed shape. Although the intensity of the immunoreaction product varied amongst these cells it was always greater in the apical than in the basal cytoplasm (Fig. 5.8c). No staining was apparent in adjacent ciliated cells, even at low antibody titres. The distribution of cytochrome P450IIB1/2 in the lung was not changed by treatment of the animals with PB, nor was there any apparent induction, the reaction product in sections from both untreated and treated animals, being clearly visible at antibody dilutions greater than 1:10 000 (Table 5.4). Interestingly, this isoenzyme(s) was strongly expressed in the Clara cells of lungs from untreated rats from as early as one day of age.

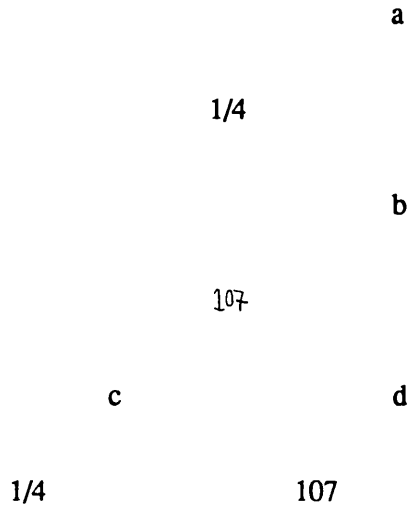
Due to the limitations of the techniques employed, resolution of other specific pulmonary cell types, particularly in alveolar cell walls, was difficult. Furthermore, to preserve the integrity of the sections, endogenous peroxidase was not blocked by pre-incubation in H_2O_2. Consequently, there was relatively high background staining, particularly in the connective tissues of blood vessels and alveolar walls. Hence, it was not possible in these experiments to determine the extent to which the antibodies may have preferentially bound to cell types within these regions. In contrast, MAb 3/4/2, again used as a positive control in these experiments, produced only a moderate immunoreaction in the same cell types at antibody dilutions < 1:50 (Table 5.4). No specific staining was apparent in Clara or other cells when the antibodies were replaced with MAb 107 as a negative control (Fig. 5.8b and 5.8d).

5.2.5 Distribution of P450IIB1/2 in rat small intestine

A very weak, specific immunoreaction with 10/1 was observed in enterocytes in sections cut from the duodenal area of the small intestine of untreated Wistar rats. There was a gradient of staining from the crypts to the tips of the villi (Fig. 5.9). This pattern of staining was maintained but with a considerable increase in intensity, when the rats were pre-treated with

Figure 5.8: Immunocytochemical localization of P450IIB1/2 in rat lung

Sections of lung from PB-treated Wistar rats were incubated with 1/4 (a, c) or 107 (b,d) at dilutions of 1:200. (a, c) Staining is apparent in bronchiolar epithelium (→) and Clara cells (➡). Magnification (a, b) x132 and (c, d) x320.



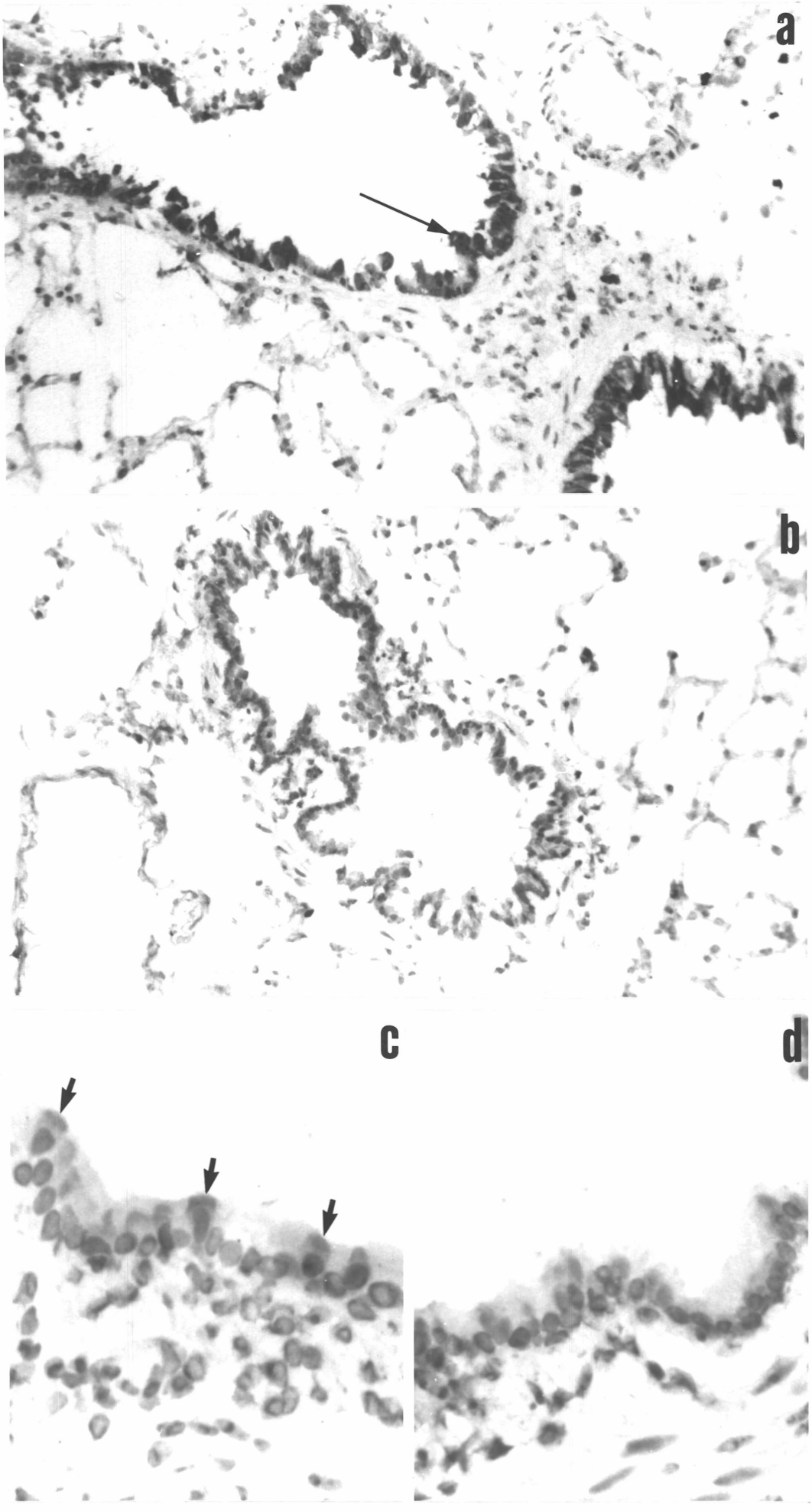

a
b
c
d

Table 5.4: Immunotitration of the binding of MAbs 1/4, 10/1 and 3/4/2, to lung from rat

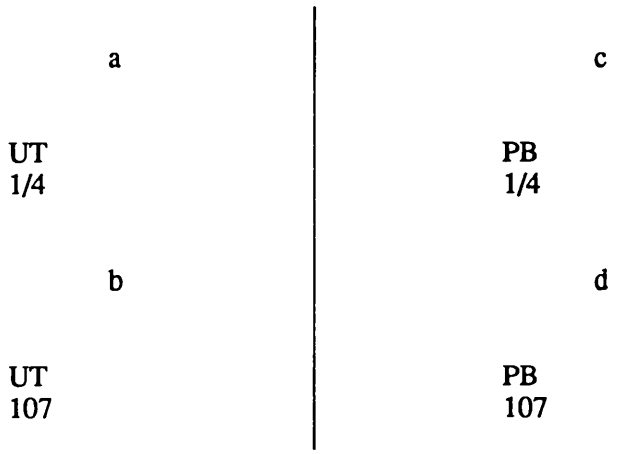
Treatment	MAb	MAb Dilution					
		1:10	1:50	1:100	1:1000	1:10 000	1:50 000
None	1/4	+++	+++	+++	+++	++	++
	10/1	+++	+++	+++	+++	++	++
	3/4/2	++	++/+	+/-	-	nd	nd
PB	1/4	+++	+++	+++	+++/++	++	++
	10/1	+++	+++	+++	+++	++	++
	3/4/2	++	++/+	+/-	-	nd	nd

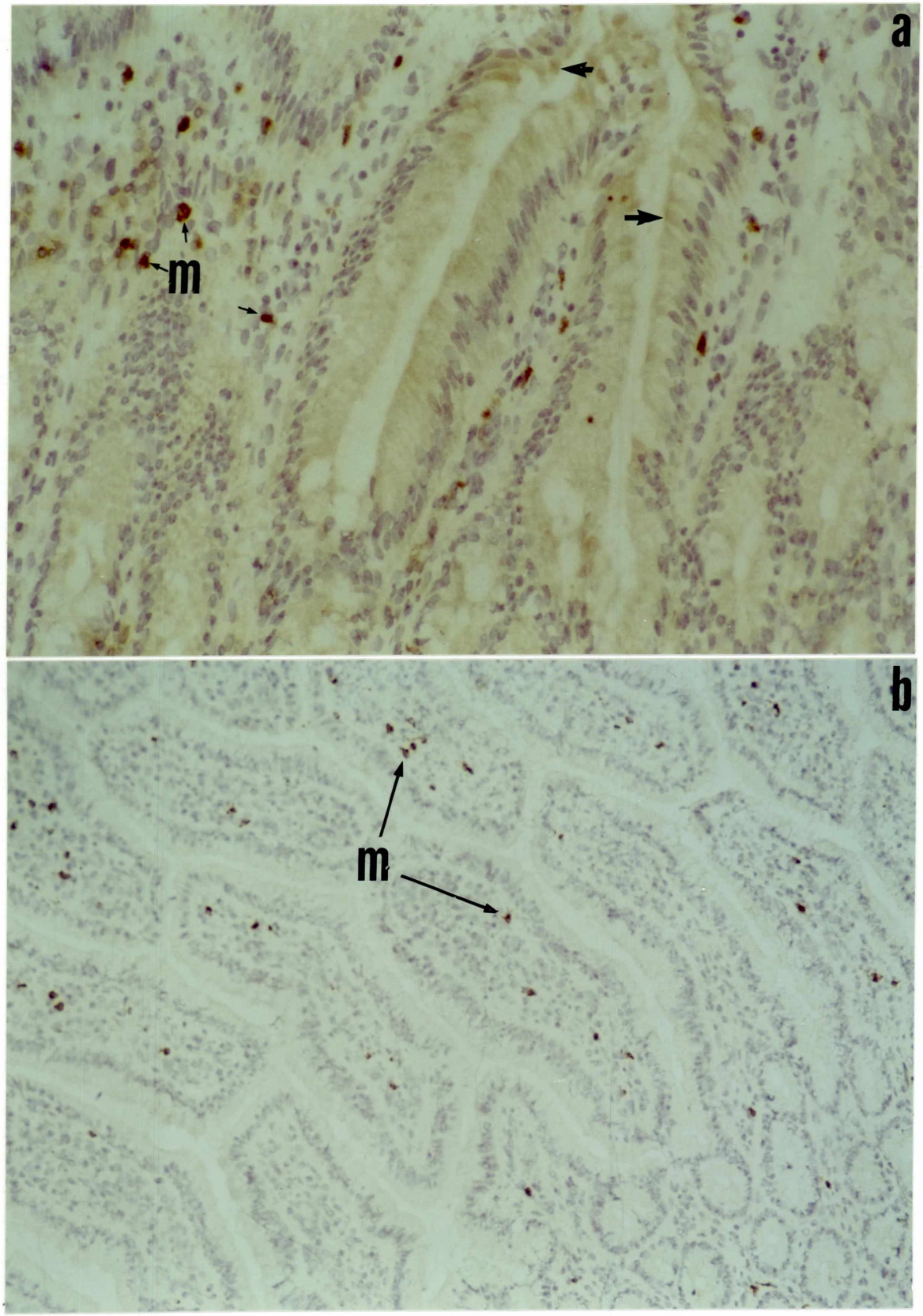
Intensity of staining: +++ intense; ++ moderate; + weak; +/- some weakly positive areas; - none.

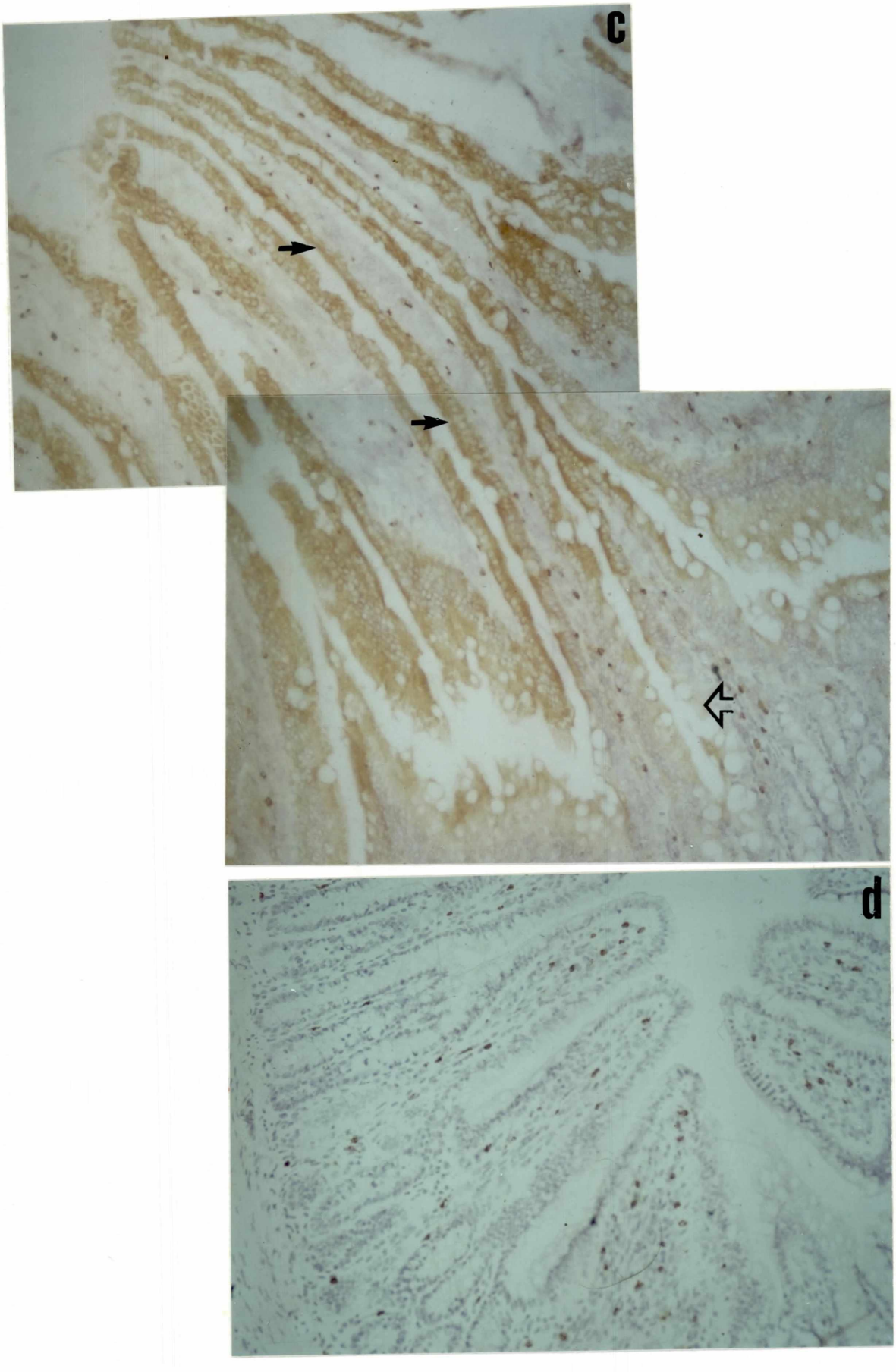
nd not determined.

Figure 5.9: Immunocytochemical localization of P450IIB1/2 in the small intestine of untreated and PB-treated rats

Sections were incubated with either MAb 1/4 (a, c) or with control MAb 107 (b, d) at a dilution of 1:10. (a, b) Sections from untreated rats. (a) Specific staining is stronger at the tips of the villi and appears to be more intense at the apical side of epithelial cells (➡). (c, d) Sections from PB-treated rats. (c) An intense immunoreaction is apparent along the length of the villi (➡), but this is slightly weaker in cells of the crypts of Lieberkuhn (⇨). MAb 107 produced negligible staining of sections from either untreated (b) or PB-treated (d) rats. Non-specific staining of macrophages (m) is evident in sections incubated with either MAb 1/4 or MAb 107. Magnification: (a) x360 (b) x280, (c, d) x200.




a
m
b
m


c
d

PB. Both the cells lining the length of the villi and those within the crypts of Lieberkuhn were positively stained, albeit very weakly in the latter case only. Whereas staining was apparent in sections from untreated animals with antibody dilutions only of 1:10, following PB treatment immunoreactive protein was detectable at MAb dilutions as high as 1:200, indicating considerable induction (Table 5.5). Paneth cells were not stained in sections from either untreated or PB-treated animals, with either of the specific antibodies used. Appropriate control sections treated with MAb 107 showed no staining of any cells (Fig. 5.9). There was intense staining of macrophages in sections of intestine from both control and PB-treated animals. However, this was presumably due to high residual levels of peroxidase, as similar staining was observed with the control antibody, MAb 107, and when only the second antibody was used.

5.2.6 Distribution of P450IIB1/2 in other extrahepatic tissues of the rat

Comparison of sections stained with MAb 10/1 and 107, showed that no specific staining of IIB1/2 was apparent in pancreas or testis from Wistar rats either before or after treatment with PB (Fig. 5.10).

5.2.7 Immunoquantification of P450IIB1/2 in liver and extrahepatic tissues of the rat

Levels of cytochrome P450IIB1/2 in liver and extrahepatic tissues of untreated Wistar rats are very low or absent. Indeed, no band corresponding to this isoenzyme(s) could be detected in Western blots of microsomes from kidney, lung or gut with either 10/1 or 1/4 (data not shown). Therefore, in an attempt to quantify the immunocytochemical observations, the method of dot blotting, where unseparated proteins are applied directly to nitrocellulose membranes, was used. There was a linear relationship between the amount of protein applied to the nitrocellulose sheets and the density of the dot for the P450IIB1 preparation and for all of the microsomal preparations studied, over the range of concentrations used ($r \geq 0.94$). The gradients of the concentration-optical density lines were the same within a tissue before and after treatment of the animals with PB. However, there was a slight difference in the gradients amongst tissues, leading to a maximum discrepancy in calculated IIB1/2 content between tissues of approximately 2-fold. Thus, within tissue comparisons of IIB1/2 content should be quantitative and between-tissue comparisons semi-quantitative.

In agreement with the immunocytochemical data, constitutive levels of IIB1/2 in the liver were low and represented only a minor fraction (<6%) of total, spectrally-determined

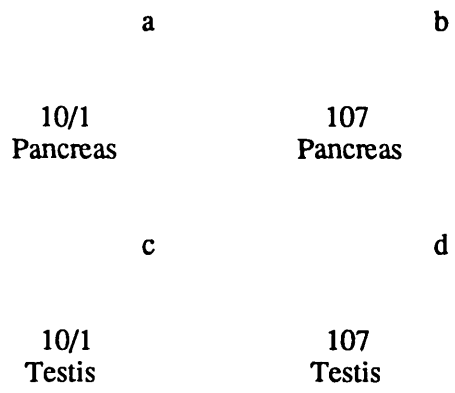
Table 5.5: Immunotitration of the binding of MAbs, 1/4 and 10/1, to small intestine from rat

Treatment	MAb	MAb Dilution		
		1:10	1:50	1:200
None	1/4	+/-	-	-
	10/1	+	-	-
PB	1/4	+++	++	++
	10/1	+++	+++	++

Intensity of staining: +++ intense; ++ moderate; + weak; +/- some weakly positive areas; - none.

Figure 5.10: Immunocytochemical staining for P450IIB1/2 in other extrahepatic organs

Sections of (a, b) pancreas and (c, d) testis of rat treated with PB exposed to MAb (a, c) 10/1 or (b, d) 107, each at a dilution of 1:10. No specific immunoreaction was apparent in either tissue. A, acinar cells; L, Islets of Langerhans; S, seminiferous tubules; I, interstitial areas. Magnification (a) x128 and (b-d) x205.



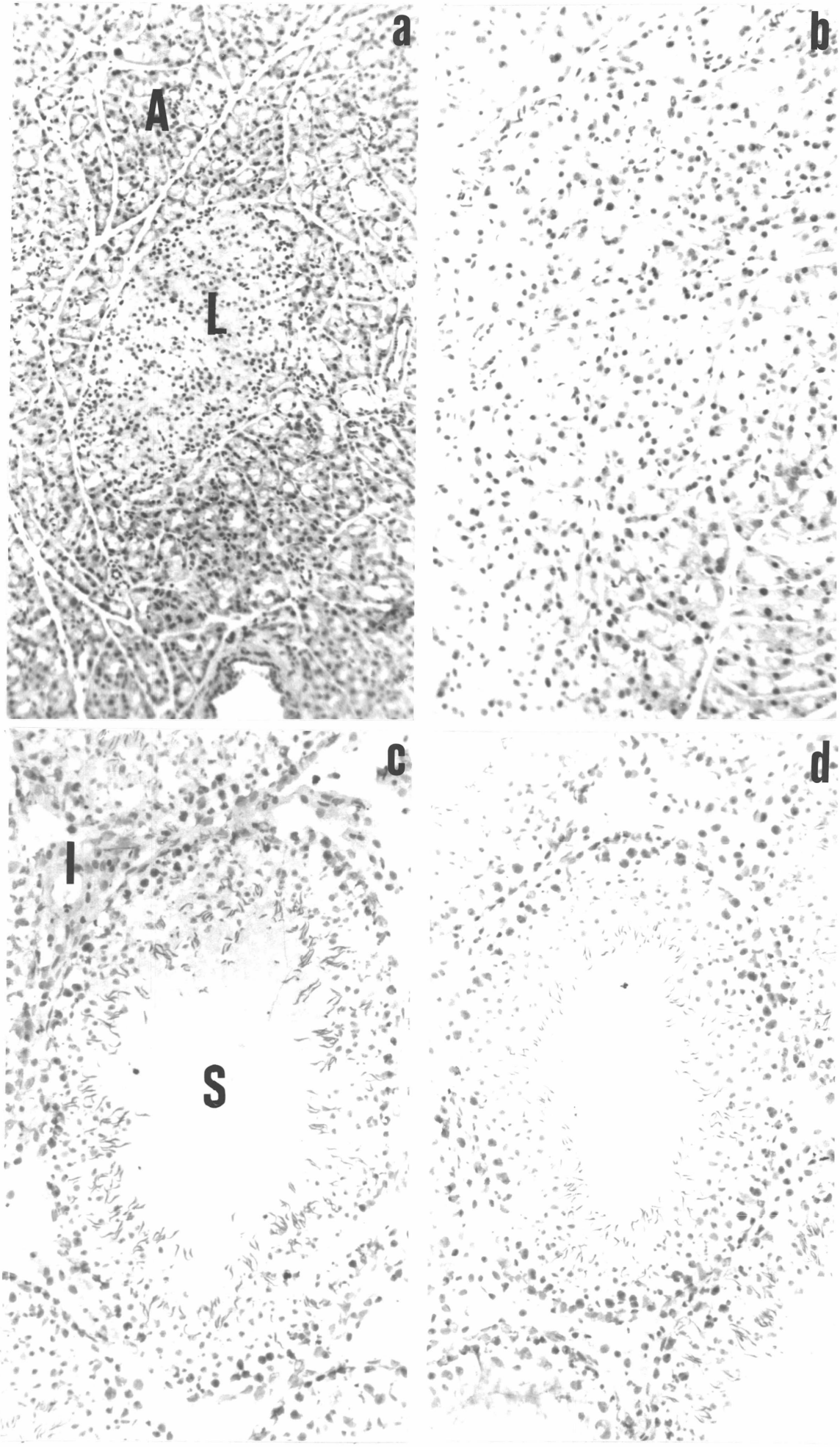

a
A
L
b
c
I
S
d

Table 5.6: Immunoquantification of P450IIB1/2 in liver and extrahepatic tissues of the rat by dot blotting

Tissue	Treatment	Total P450	Immunoreactive P450
		pmoles.mg^{-1} protein	
Liver	None	755 ± 115 (6)	44 ± 6 (3)
	PB	1290 ± 258 (6)	1830* ± 316 (9)
Kidney	None	119 ± 5 (3)	bd (4)
	PB	102 ± 42 (3)	bd (4)
Lung	None	38 ± 7 (3)	24
	PB	40 ± 3 (3)	21
Gut	None	46 ± 14 (3)	8 ± 2 (3)
	PB	84 ± 17 (4)	42* ± 10 (8)

Results are means ± standard deviation, where shown, from at least two separate microsomal preparations. Numbers in parentheses indicate the number of determinations.

bd: below detection.

* Significantly different from untreated animals at $P < 0.001$.

Immunoblotting was performed with MAb 10/1, 1μg.ml^{-1}.

cytochrome P450. These levels increased >40-fold upon PB-treatment (Table 5.6). No cytochrome P450IIB1/2 could be detected in microsomes from kidney of either untreated rats or rats pre-treated with PB (Table 5.6). Constitutive levels of this isoenzyme in lung microsomes were very low, although they did represent approximately 50% of total cytochrome P450. They were refractive to PB-treatment. In contrast, the barely detectable levels of immunoreactive protein in microsomes from small intestine of untreated rats were increased 6-fold by PB treatment (Table 5.6). This was reflected in only a doubling of the total cytochrome P450 pool.

5.3 Discussion

The tissue distribution of cytochrome P450IIB1/2 has been determined by immunocytochemistry utilizing two monoclonal antibodies raised against the major PB-inducible isoenzyme(s) of cytochrome P450. The specificity (affinity) of these antibodies for the closely related isoenzymes IIB1 and IIB2 was shown to vary depending on the immunochemical procedure used. However, 10/1 always bound with greater affinity to IIB1/2 than 1/4, suggesting that the epitopes to which these antibodies bind are not identical. Unlabelled competitive e.l.i.s.a. further showed that the epitopes are not spatially distinct. Despite specific interaction with IIB2 in e.l.i.s.a. and dot blots, these antibodies react with only a single protein band in Western blots of hepatic microsomes from rats treated with PB, with the same molecular weight as that of purified cytochrome P450IIB1 (under conditions such that IIB1 was resolved from IIB2). A low affinity interaction might not be apparent in Western blots, but might be detected under conditions, such as those used here in immunocytochemistry, where the holoprotein is subject to less severely denaturing treatment. Indeed, in screening various fixatives for their effect on the reactivities of 1/4 and 10/1 in immunocytochemistry, it was apparent that the epitope to which these MAbs bind is particularly susceptible, not only to harsh tissue treatments such as paraffin-embedding, but also to relatively mild denaturing treatments. The procedures used in Western blotting lead to unfolding of the protein so resulting in perturbation of epitopes that might be accessible in the native proteins such as in tissue sections, and this may well reduce antigenicity. Indeed, the reaction of IIB2 with 10/1 and 1/4 proved to be more sensitive than that of IIB1 to denaturing treatments, such as 8 M urea and SDS-PAGE. This suggested that the epitope on IIB2 to which these antibodies bind is slightly different to that with which they react on IIB1. Hence, the possibility that these antibodies react with IIB2 in acetone-fixed tissue sections, although any such binding would be with much lower affinity than to IIB1, cannot be excluded.

It is of interest that, to date, all antibodies (polyclonal or monoclonal) raised to individual PB-inducible holoenzymes, either IIB1 or IIB2, cross-react with both forms and in some instances, also with variant forms of IIB1, if not in Western blots, then in reconstituted systems to inhibit catalytic activity (Ryan *et al.*, 1982; Letawe-Goujon *et al.*, 1984; Reik *et al.*, 1985; Wilson *et al.*, 1987). This has been attributed to the very high homology (>97%) in amino acid sequence between these two isoenzymes. However, a monoclonal antibody against IIB1 has been described that recognises IIB2 only under certain conditions, and even then with much reduced affinity (Reik *et al.*, 1985). It is possible that 10/1 and 1/4 similarly react with a modified epitope on IIB2 in tissue sections. For this reason, in the present study specific immunocytochemical staining has not been assumed to reflect interaction of MAbs with only IIB1 but possibly also with its variant forms and with IIB2. Such binding has been referred to as with P450IIB1/2. In support of this assumption, recent studies have shown that in the liver of untreated rats the content of IIB2 greatly exceeds that of IIB1, whereas in the lung IIB1 exceeds IIB2 (Christou *et al.*, 1987).

The expression of IIB1/2 in livers from untreated animals is low and its distribution as determined with monoclonal antibodies 10/1 and 1/4 varies with the strain of rat. These differences presumably reflect differences in the expression of IIB1 and IIB2 in the liver. In liver of F344 rats equal amounts of IIB1 and IIB2 are expressed, whereas in the Sprague-Dawley and Wistar strains IIB2 is preferentially expressed, with smaller amounts of a variant form of IIB1 (Wilson *et al.*, 1987). Thus, it appears that IIB1 and/or its variant forms is/are expressed periportally and/or diffusely throughout the lobule, whilst IIB2 is preferentially expressed in centrilobular cells. Such a well-defined localization of a P450 isoenzyme has been reported previously for PB_1 and PB_2 (isoenzymes which are probably analogous to either f, g, h or i referred to as IIC7, IIC13, IIC11 and IIC12 according to Nebert's nomenclature (Nebert *et al.*, 1989)) and/or UTA and UT1 (Wolf *et al.*, 1987)) where expression was confined to a narrow band of cells around central veins (Wolf *et al.*, 1984). Clearly, further studies, such as *in situ* hybridization with highly specific oligonucleotide probes or anti-peptide antibodies to each isoenzyme, are required to resolve this question.

The cellular localization of IIB1/2 in tissues of untreated rats found in the present study contrasts with that described in previous reports in which polyclonal antibodies were employed (Baron *et al.*, 1986; Schwartz *et al.*, 1987). However, the pattern of relatively moderate and evenly distributed immunostaining throughout the lobule, with some indication of a preferential centrilobular localization, described by these groups is very similar to that found here with a polyclonal anti-PB-P450 antibody. Thus, the major cause of the

difference in staining pattern between this and other studies appears to be the nature of the antibodies used.

Generally, the localization of P450IIB1/2 in virtually all hepatocytes as found here, following treatment of rats with phenobarbitone, was similar to that described by others (Wolf *et al.*, 1984; Baron *et al.*, 1986; Kolyada, 1986; Schwartz *et al.*, 1987; Moody *et al.*, 1988). Hence, the degree and localization of inducibility depended to some extent on the level to which the isoenzyme(s) was constitutively expressed. Thus, in Wistar and Sprague-Dawley or Lister-Hooded rats, induction of IIB1/2 is maximal in centrilobular areas as constitutive expression is either diffuse throughout the lobule or predominantly in periportal cells, respectively. In the inbred F344 strain, induction is maximal in midzonal hepatocytes, as constitutive expression is both centrilobular and periportal. In contrast to the rat, no strain-related differences in the immunolocalization of PB-inducible isoenzymes of cytochrome P450 were found in the mouse (Forkert *et al.*, 1988).

These studies show that not only is the expression of P450IIB1/2 non-homogeneous between zones, but also amongst individual hepatocytes within a zone, as exemplified by the staining pattern of sections from untreated Wistar rats. Furthermore, although not all cells within a zone constitutively express cytochrome P450IIB1/2 at detectable levels, they all exhibit increased levels of isoenzyme on PB-treatment of the rats. Thus, the extent to which individual hepatocytes respond to induction varies. This suggests that the mechanisms regulating both the constitutive expression and the inducibility of P450IIB1/2 differ from cell to cell. Whether this is due to variation in the regulation of different isoenzyme variants or in the amount of a single isoenzyme has yet to be determined. The intracellular distribution of staining of hepatocytes is consistent with localization of P450IIB1/2 in the endoplasmic reticulum.

Initially it was suggested that induction of P450 isoenzymes by PB is restricted to liver (Guengerich & Mason, 1979). However, more recent studies (Omiecinski, 1986; Christou *et al.*, 1987; Traber *et al.*, 1988), together with the present work, have shown that P450IIB1/2 is inducible in extrahepatic tissues by PB. However, such induction is tissue-specific.

Constitutive levels of P450IIB1/2 in the small intestine are very low but detectable, increasing from the crypts to the tips of the villi. Following PB-treatment of rats, staining of P450IIB1/2 in the small intestine is considerably increased. These findings, which at the time of writing represent the first description for the cellular localization of P450IIB1/2 in the small intestine, confirm previous work by Bonkovsky and co-workers, who quantified

levels of this isoenzyme in microsomes prepared from cells isolated from upper villus, lower villus and crypts, and found that levels increase in the cells as they mature (Bonkovsky *et al.*, 1985). Interestingly, there have been reports of cytochrome P450-dependent monooxygenases in microsomes from small intestinal mucosa which are inducible by PB treatment and which, after PB induction, are inhibited (>70%) by an anti-P450IIB1/2 monoclonal antibody, 2-66-3 (Christou *et al.*, 1987; Hietanen *et al.*, 1987). Further, in a recent study it was shown that although there is constitutive expression of both IIB1 and IIB2 mRNA in the small intestinal mucosa of the rat, only IIB1 mRNA is increased by treatment with PB, polyhalogenated biphenyls or organochlorine pesticides (Traber *et al.*, 1988). Moreover, *in situ* hybridization studies revealed that induction of this mRNA occurred only in epithelial cells of villi with no induction in crypts. Western blot analysis (Bonkovsky *et al.*, 1985; Christou *et al.*, 1987), and levels of 12-methoxy hydroxylation of 7, 12-dimethylbenz[a]anthracene (reflecting IIB2 activity) relative to 7-methoxy hydroxylation (reflecting IIB1 activity) suggest that only IIB1 is expressed in this tissue (Christou *et al.*, 1989). Hence, one interpretation of these findings is that the P450IIB2 mRNA identified in untreated small intestine (Traber *et al.*, 1988) is translated very poorly or not at all. However, as pointed out by this group, caution must be exercised in concluding that the mRNAs found in the small intestine are identical to those in the liver or indeed in other tissues. The oligonucleotide probes used in this and other studies (Omiecinski, 1986) have identical complementary sequences to three genomic clones in addition to IIB1 (Adesnik & Atchison, 1986).

The gradient of P450IIB1/2 staining in the intestinal mucosa is maintained following PB-treatment of the rats, and is not related to the route of administration of the inducer, as no differences were found in the pattern or intensity of staining when PB was administered either intraperitoneally or orally as a 0.1% solution in the drinking water for 7 days. It has also been shown that both oral and intraperitoneal injection of PB produce the same pattern and extent of induction of IIB1/2 mRNA (Traber *et al.*, 1988).

Levels of cytochrome P450IIB1/2 in the Clara cells of the lung of untreated Wistar rats are very high. This contrasts with the low expression of IIB1/2 in the hepatocytes of such animals, in which specific staining is more than 100-fold less than in Clara cells, as shown by antibody titration. Christou *et al.* (1987) have shown that where it is IIB2 that is mainly expressed in the liver, it is IIB1 that is expressed in the lung. The specific content (per mg microsomal protein) of constitutive PB-inducible cytochromes P450 is approximately the same in the liver and the lung (Christou *et al.*, 1987). However, whilst in liver this reflects expression in many hepatocytes, in lung it reflects expression in only a very limited number

of specific cells. Hence, expression of IIB1/2 appears to be much greater in the Clara cell than in the average hepatocyte.

The specific localization of IIB1/2 in the Clara cells found in the present study is in agreement with that reported by other groups (Baron *et al.*, 1986; Serabjit-Singh *et al.*, 1988; Keith *et al.*, 1987). Nevertheless, the presence of this isoenzyme in additional pulmonary cells, such as type II pneumocytes, as reported for both rat and rabbit (Dees *et al.*, 1982; Serabjit-Singh *et al.*, 1988; Keith *et al.*, 1987), cannot be excluded as it is notoriously difficult to differentiate between alveolar cell types at the light microscope level. This is particularly true in the present study since the preservation of immunoreactivity depended upon the use of mild fixation (acetone), which was achieved at a reduction in resolution of cell types. Dees *et al.* (1982) have suggested that IIB1 is also present in rabbit alveolar type I pneumocytes. However, this conclusion depended entirely upon indirect immunofluorescence, a technique with less resolution and precision than immunoperoxidase staining.

The inducibility of IIB1 in lung is equivocal. In the present study it was shown that IIB1/2 in the lung is refractive to PB-treatment and this is consistent with the very high level of this isoenzyme constitutively expressed in Clara cells. The levels of mRNA present after PB-treatment support this view (Omiecinski, 1986). However, in studies using Western blot analysis very small increases in the levels of IIB1 after PB treatment have been reported (Christou *et al.*, 1987), but these appear to be strain-specific (Christou *et al.*, 1989). For example, levels of IIB1 were increased 1.5-fold in the F344 strain but not at all in the Marshall strain. Similarly, PB-treatment had no effect on the rates of formation of any of the metabolites of 7,12-dimethylbenz[a]anthracene produced by microsomes from Marshall rats and failed to increase the extent of inhibition of this metabolism by anti-P450IIB1/2 IgG (Christou *et al.*, 1989). The lack of response to PB treatment, may be due to the fact that the lung of untreated Marshall rats already expresses comparatively high levels of IIB1 (180 pmoles.mg^{-1} protein cf 50 pmoles. mg^{-1} protein in the F344 strain). Hence, it appears that where levels of this isoenzyme are already high (perhaps due to maximal expression), induction does not occur.

No immunoreactive cytochrome P450IIB1/2 could be detected in kidney from Wistar or other strains of rat, even after PB-treatment, a finding supported by recent data showing that renal monooxygenase activity of PB-treated rats could not be inhibited by a monoclonal antibody which recognises all of the PB-inducible isoenzymes (Hietenan *et al.*, 1987). In addition, no expression of mRNAs coding for IIB1 and IIB2 could be demonstrated in

kidney, even after PB-treatment, using highly specific 18mer synthetic oligonucleotide hybridization probes (Omiecinski, 1986). In contradiction, however, Shepherd and co-workers have reported the presence of mRNA to cytochrome IIB1 in kidney (Shepherd *et al.*, 1987). Further, one laboratory has reported the presence of very low, but detectable levels of immunoreactive cytochrome P450IIB1/2 in kidney, but only 3 of 6 groups of Sprague-Dawley rats treated with PB were positive (Christou *et al.*, 1987). The relative proportion of PB-inducible isoenzymes in untreated liver differs amongst rat strains (Wilson *et al.*, 1987), and the same would appear to be true for extrahepatic organs (Christou *et al.*, 1989). Although this may account for some discrepancies an additional factor that will tend to confound comparison between laboratories is the reactivity profile of the antibody used. A comparison of the reactivity profile produced by the polyclonal antibody, anti-PB-P450, with the monoclonal antibodies 10/1 and 1/4, gives credence to this supposition. Anti-PB-P450 produced indiscriminate staining of most structures, regardless of the rat strain whereas the monoclonal antibodies produced weak to negligible staining. Although antibody affinity may also contribute, in part, to these differences, it seems likely that the anti-PB-P450 antibody used here and polyclonal antibodies used by other groups recognise possibly one or more isoenzyme(s) of similar amino acid sequence, in the P450II family.

Comparatively little is known about the expression or inducibility of cytochrome P450IIB1/2 in pancreas or testis, although immunoreactive P450IIB1/2 has been demonstrated in acinar cells of the exocrine pancreas of hamsters but not of rats (Kawabata *et al.*, 1984). Solution hybridization experiments using 18 mer oligonucleotide probes showed the presence of IIB1 in the testis. This was refractive to PB-treatment (Omiecinski, 1986). In contrast, using highly purified monoclonal antibodies, no IIB1/2 could be detected in either of these organs, either before or following PB-treatment of the animals. Hence, at least in the testis, protein levels must be very low or the mRNA is not translated. Alternatively, the mRNA detected was not that of IIB1, but of a closely-related variant.

Chapter Six

Ultrastructural localization of hepatic cytochrome P450

6.1 Introduction

Electron microscopy, combined with immunocytochemistry allows the visualization of specific proteins at the ultrastructural level. The most commonly applied "on-grid" labelling technique is that of ultra-thin, resin-embedded tissue sections (Newman & Jasani, 1984). An alternative method, increasing in popularity, involves labelling of ultrathin frozen sections (Tokuyasu, 1980). In the former method, tissues are fixed, dehydrated and embedded in resin. In contrast, in the second method tissues are fixed and then subsequently cryoprotected by infusion with 2.3 M sucrose prior to freezing. In the majority of immunoelectron microscopical studies, the colloidal gold particle has been the marker of choice, probably due to the fact that it is commercially available in a number of sizes, allows easy identification of labelled structures and has the potential for quantitative evaluation of the intensity of labelling.

The necessity of establishing conditions for tissue processing that optimize labelling in electron microscopic studies is just as important as that at the light microscope level. The problem of obtaining good ultrastructural preservation which allows clear identification of labelled organelles together with retention of antigenicity of the protein of interest is confounded by the need to use crosslinking fixatives such as glutaraldehyde and formaldehyde and resins which ensure stability of sections under the electron beam. Such constraints have more often than not precluded the utilization of monoclonal antibodies in such studies. Polyclonal antibodies have therefore remained important reagents for immunoelectron microscopy. Nevertheless, preservation of antigenicity, even with these antibodies, has imposed certain criteria on the choice of resin used for imparting rigidity to the tissue for sectioning.

In general, the best results and greatest flexibility of technique have been obtained with two broad groups of resins, (i) the cross-linking epoxy resins and (ii) the hydrophilic acrylics (Causton, 1984). Of the epoxy resins available, Araldite is the most popular, the major disadvantage of the others (Epon and Spurr type resins) being their toxicity. Araldite (75% diglycidyl ether of bisphenol A, and 25% dibutyl phthalate) is a hydrophobic resin, the curing system of which is of the very thermally stable, diphatic anhydride/amine type, usually dodecyl succinic anhydride. The resultant cross-linking endows sections with good

beam stability, but can prevent subsequent antibody penetration. Hence, prior to immunostaining, sections require treatment (etching) with an oxidising agent, usually H_2O_2, which thus increases the hydrophilicity of the resin by introducing hydroxyl or carboxyl groups into the hydrophobic alkane side chains.

The acrylate embedding media arose out of a need to maintain low temperatures during the processing of well-frozen specimens (Carlemalm *et al.*, 1982). Five of these have been developed and two are commercially available, Lowicryl K4M, a hydrophilic resin usable down to -35°C, and HM20, a hydrophobic resin usable down to -70°C (Carlemalm *et al.*, 1986). Both resins can be polymerized by ultraviolet light (360 nm) at low temperatures. K4M is particularly suited to immunocytochemistry, not only because of its hydrophilic nature, but also because it can be polymerized with up to 5% (v/v) water in the block, allowing the specimen to be kept in a partially hydrated state. Tissue specimens may be embedded via normal, ambient temperature fixation with subsequent gradual lowering of the temperature during the dehydration stages or by freeze-substitution so that the frozen specimen is never warmed up until it has been fully infiltrated with resin and polymerized.

This chapter describes results obtained from preliminary experiments designed to determine the optimum conditions for using polyclonal antibodies, in particular, anti-peptide antibodies, directed against cytochrome P450 at the ultrastructural level. The effects of fixation and embedding on the retention of antigenicity of cytochromes P450IA2 and P450IIB1/2 was assessed.

6.2 Results

6.2.1 The effect of tissue fixation and embedding on ultrastructural morphology

A comparison of liver sections fixed with 2% *p*-formaldehyde containing 0.2% glutaraldehyde with those that were unfixed but rapidly frozen in liquid nitrogen slush, embedded in either Araldite or K4M, revealed that hepatocyte ultrastructure was best preserved in pre-fixed liver subsequently embedded in Araldite (data not shown). Morphology compared well with that obtained by conventional processing of the tissue ie. fixation in 4% glutaraldehyde at ambient temperature for 2-3 h followed by post-fixation in osmium tetroxide and embedding in Araldite (Fig. 6.1a cf 6.1b and Fig. 6.2a cf 6.2b). Hepatocytes were easily identified by their polyhedral shape. Intercellular contacts were linear and the sinusoidal faces were studded with numerous microvilli. Intracellular organelles, such as mitochondria, lysosomes, glycogen granules, nuclei and their envelopes

Figure 6.1: Typical ultrastructure of hepatocytes in Araldite-embedded livers of untreated rats

Livers were fixed with (a) 4% glutaraldehyde and post-fixed in OsO_4 or (b) 2% *p*-formaldehyde containing 0.2% glutaraldehyde, then embedded in Araldite. (b) Sections were immunostained with anti-$d_{290\text{-}296}$ at a dilution of 1:100 and specific antigen-antibody reactions were revealed by incubation with GAR IgG conjugated to 30 nm gold. N, nucleus; mt, mitochondria; RER, rough endoplasmic reticulum; B, bile canaliculus; Si, sinusoidal cell. Magnification, (a) x5 850 and (b) x4 425.

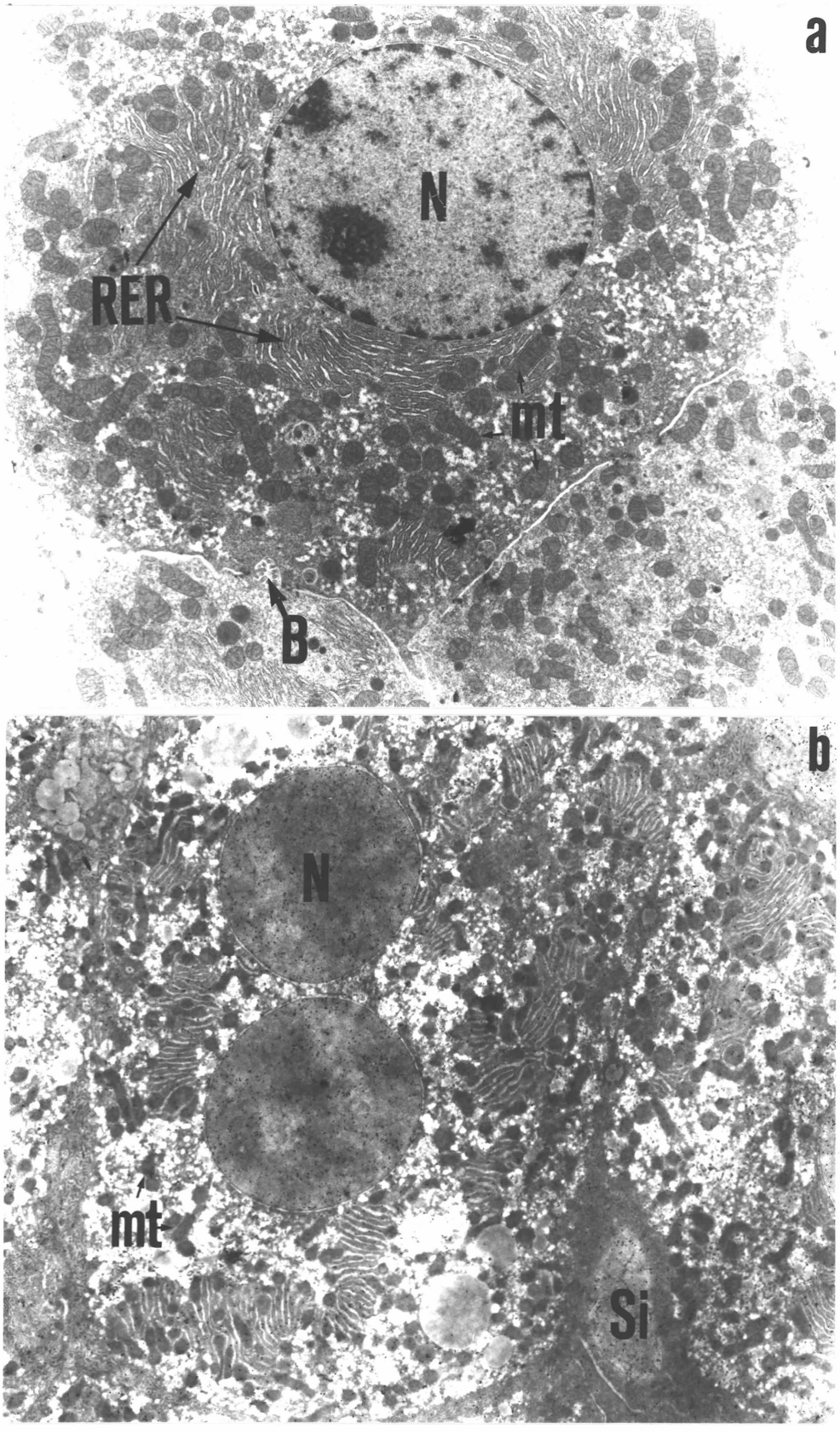
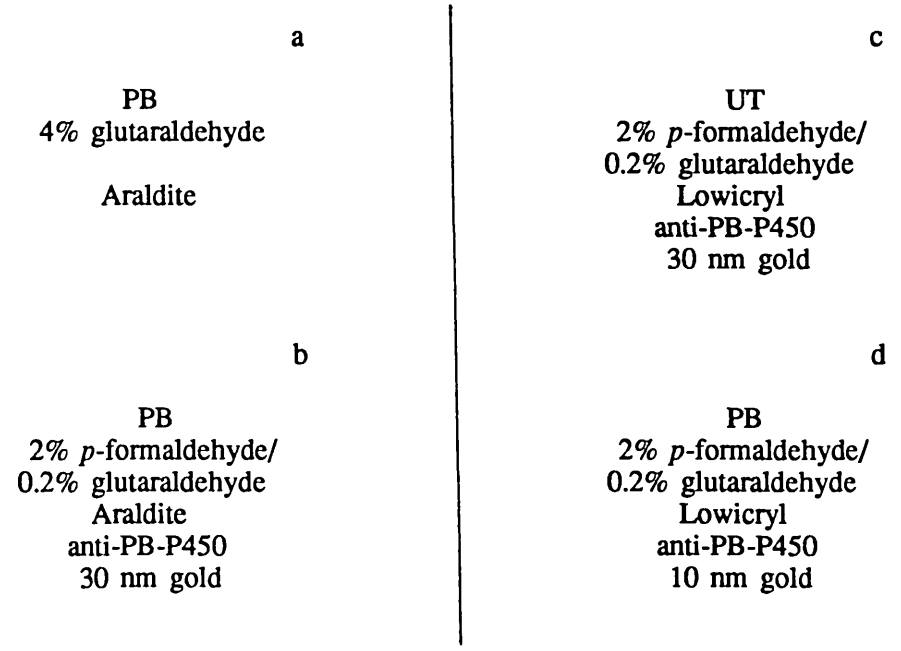
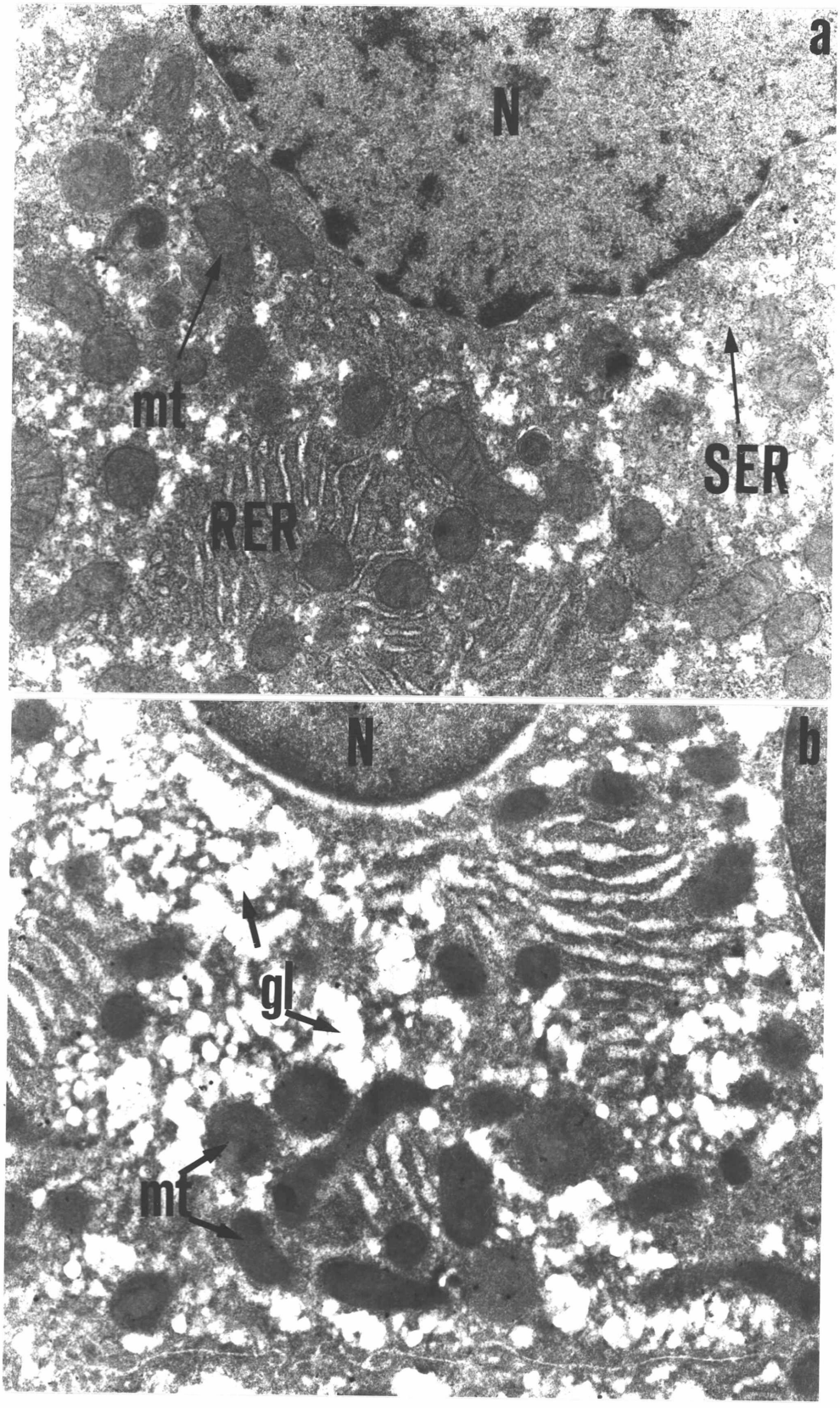
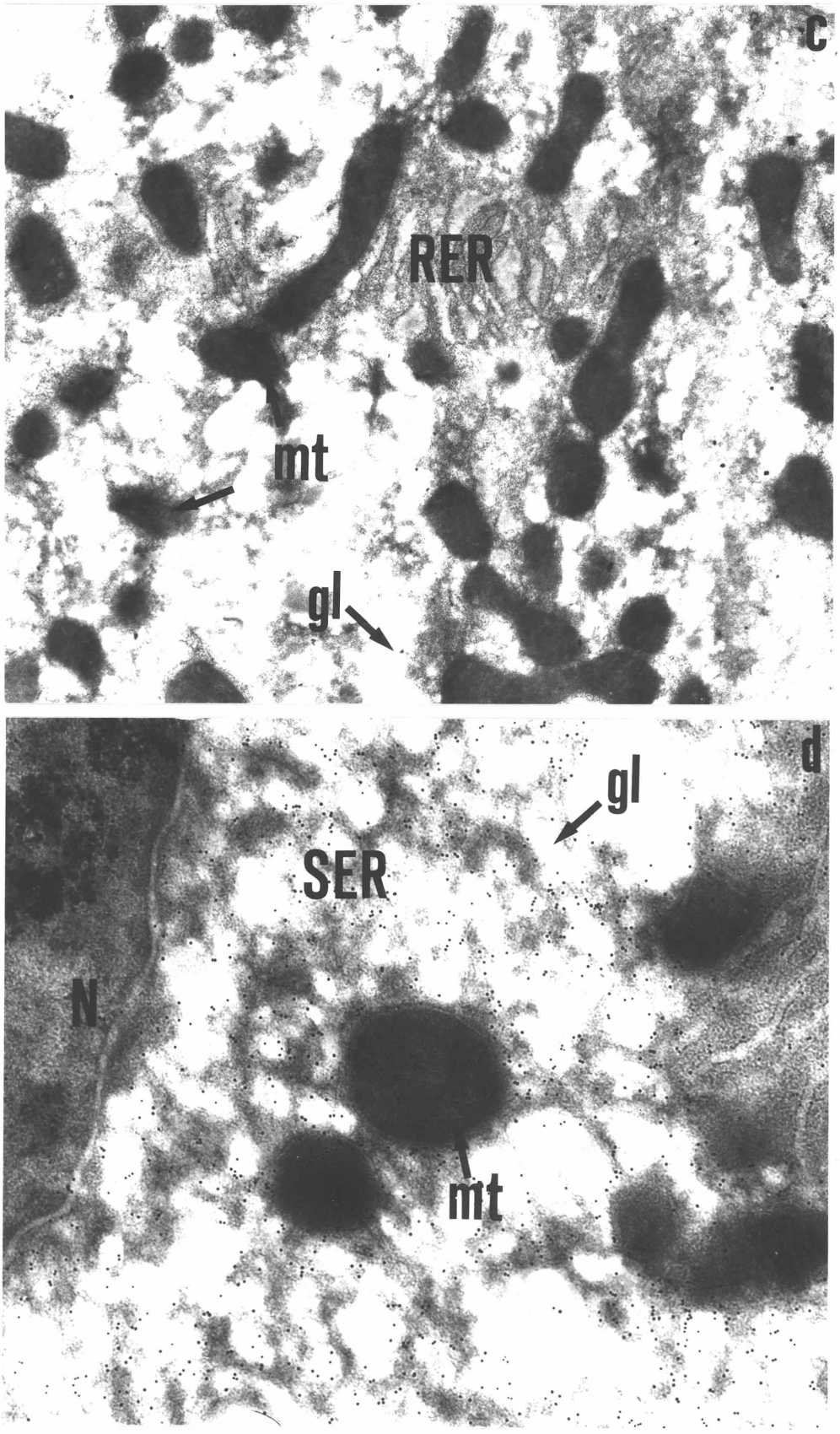

a
N
RER
mt
B
b
N
mt
Si

Figure 6.2: Ultrastructural immunolocalization of P450IIB1/2 in hepatocytes

Liver from PB-treated (a, b, d) and untreated (c) rats was fixed in (a) 4% glutaraldehyde or (b-d) 2% *p*-formaldehyde containing 0.2% glutaraldehyde and embedded in either Araldite (a, b) or Lowicryl K4M (c, d). Sections were immunostained with anti-PB-P450 antibody at a dilution of 1:50 and specific antigen-antibody reactions revealed by incubation with GAR IgG conjugated to 30 nm (b, c) or 10 nm (d) colloidal gold. N, nucleus; mt, mitochondria; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; gl, glycogen granules. Magnification (a-c) x17 250 and (d) x33 600.




a
N
mt
RER
SER
N
b
gl
mt


c
RER
mt
gl
d
gl
SER
N
mt

and reticular membranes, were readily identifiable. However, the finer details of some of these structures were less well preserved eg. cristae of mitochondria.

Nuclei and their envelopes, mitochondria and endoplasmic reticular membranes were also readily identified in pre-fixed liver embedded in K4M, but the cytoplasmic matrix was fragmented and sections were extremely vulnerable to the electron beam (Fig. 6.2c, 6.2d and 6.3a). Moreover, the quality of sections was not consistent, varying considerably between tissue blocks.

Sections of unfixed liver from untreated, PB- and MC-treated rats were devoid of all morphological detail, and only the sinusoids and plasma membranes of cells could be distinguished (not shown). Consequently tissue prepared in this manner was excluded from further study.

6.2.2 The effect of tissue fixation and embedding on immunoreactivity of antigens

In previous experiments (Sections 5.2.1.4) it was shown that the antigenicity of P450IIB1/2 towards monoclonal antibodies 1/4 and 10/1 was lost following fixation of tissue with methanol or 95% ethanol containing 1% acetic acid and subsequent paraffin-embedding. Similarly, fixation of liver from MC-treated rats with 4% glutaraldehyde or 95% ethanol containing 1% acetic acid and subsequent paraffin-embedding, resulted in a total lack of immunostaining by the monoclonal antibody 3/4/2 (not shown). Not surprisingly, none of these antibodies produced specific immunostaining in ultrathin sections of liver embedded in either Araldite or K4M (not shown).

The anti-peptide antibody, anti-$d_{290\text{-}296}$, did produce immunostaining of cytochrome P450 in tissue fixed with 95% ethanol containing 1% acetic acid and subsequently embedded in paraffin, although the intensity of the immunoreaction was substantially reduced when compared to frozen tissue sections (not shown). Nevertheless, this antibody appeared to be a suitable reagent, together with another polyclonal antibody, anti-PB-P450, for immunostaining of ultrathin sections for electron microscopy.

Immunogold particles are electron opaque and stood out clearly against tissue lightly counterstained with uranyl acetate and lead citrate (Fig. 6.1b, 6.2d and 6.3b). The retention of antigenicity of cytochrome P450IA2 at the ultrastructural level varied with the resin employed, being detectable with anti-$d_{290\text{-}296}$, at dilutions of 1:500 or less, in liver from either untreated or MC-treated rats, only in sections embedded in Araldite (Fig. 6.1b and Fig. 6.3b

Figure 6.3: Ultrastructural immunolocalization of P450IA2 in hepatocytes

Liver from untreated (a) or MC-treated (b, c) rats was fixed with 2% *p*-formaldehyde containing 0.2% glutaraldehyde and embedded in either Lowicryl K4M (a) or Araldite (b, c). Sections were incubated with anti-$d_{290\text{-}296}$ (a, b) or pre-immune rabbit serum (c), at a dilution of 1:100, and specific antigen-antibody interactions were revealed by incubation with GAR IgG conjugated to 10 nm colloidal gold. N, nucleus; mt, mitochondria; RER, rough endoplasmic reticulum; B, bile canaliculus; ➡ sites of immunogold particles. Magnification (a) x10 800 and (b) x21 000.

a

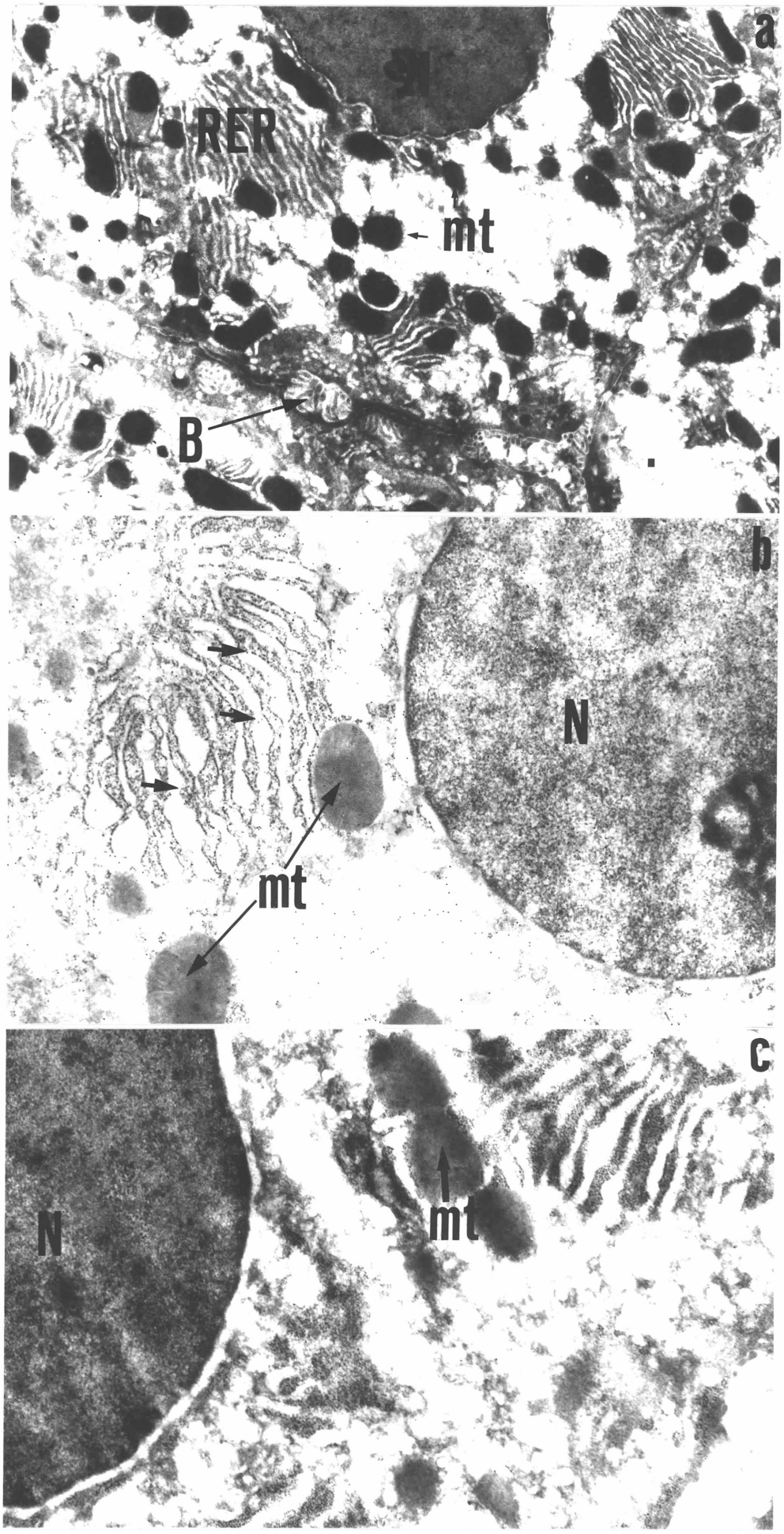
UT
Lowicryl
anti-d
290-296

b

MC
Araldite
anti-d
290-296

c

MC
Araldite
Pre-immune rabbit serum


a
RER
mt
B
b
N
mt
c
N
mt

cf Fig. 6.3a). This staining was specific, as after incubation of similar sections with pre-immune rabbit serum, at the same protein concentrations, the density of immunogold labelling was negligible (Fig. 6.3c and Fig. 6.4d).

In contrast, with the polyclonal anti-PB-P450 antibody, no immunoreactivity was seen in liver embedded in Araldite, from either untreated or PB-treated rats (Fig. 6.2b). However, very strong labelling was apparent in sections of K4M-embedded livers from PB-treated rats following incubation with anti-PB-P450, at dilutions of 1:50 to 1:500 (6.2d). Immunogold labelling in liver sections from untreated rats was negligible (Fig. 6.2c), as was that in liver sections from MC-treated rats.

6.2.3 The effect of immunogold particle size on the density of labelling

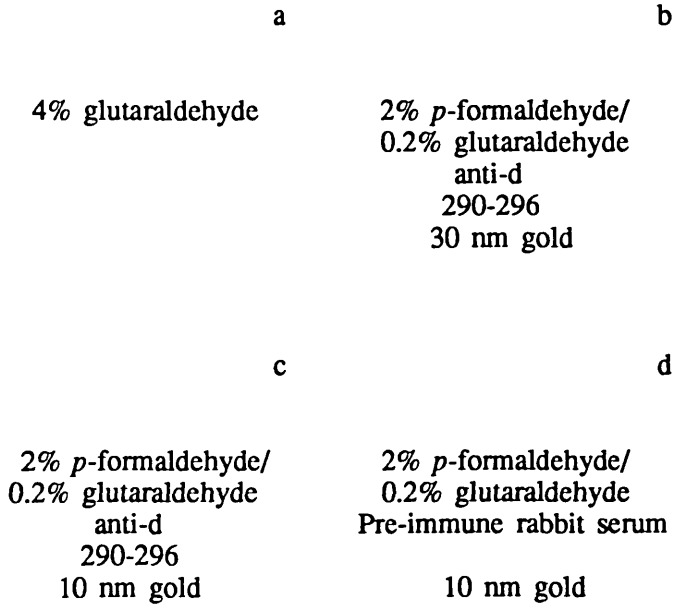
Immunogold particles, conjugated to immunoglobulin molecules, are available in a number of sizes, namely 5, 10, 15 and 30 nm. The degree of penetration of the probes into resin-embedded tissue will obviously depend upon the size of the gold particle. Two sizes of gold particle were used in this study, one of 10 nm and the other of 30 nm diameter. A comparison of the immunolabelling produced by these antibodies following pre-incubation of liver sections from MC-treated rats with anti-$d_{290\text{-}296}$ is shown in Fig. 6.4. Clearly, the signal obtained with the 30 nm gold particle is readily discernable, whilst that of the 10 nm gold particle requires closer scrutiny for visualization. Moreover, the smaller gold particle, although more electron dense, has a similar size to the ribosomes on endoplasmic reticular membranes (Fig. 6.4a and 6.4c). However, with the smaller particle, the intensity of specific labelling was greater, with 5-10-fold more particles per unit surface area of the rough endoplasmic reticulum than with the larger particle (Fig. 6.4b and 6.4c).

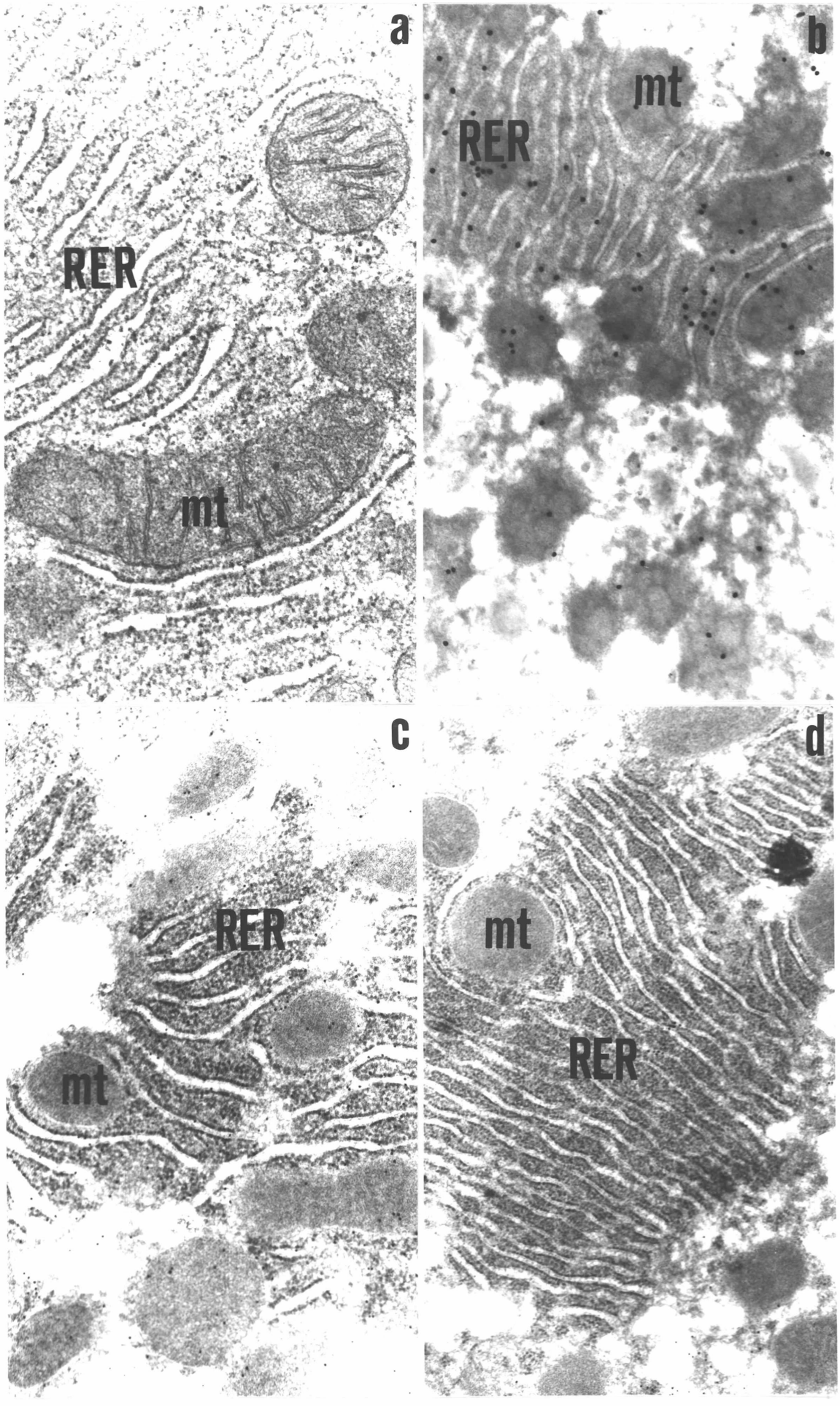
6.2.4 Ultrastructural localization of cytochrome P450

Electron microscopy is largely a qualitative technique. However, the extent of immunogold staining in post-embedded sections can be semi-quantified with some reliability, as most of the factors are controlled (Bendayan, 1985; Griffiths & Hoppeler, 1986). In sections from post-embedded material, only those sites exposed at the surface of the section are accessible to antibody binding. Furthermore, since the size of the gold particles used for labelling is uniform, the binding capacity of each particle can be assumed to be the same. However, the exact number of gold particles per P450 molecule is not known and therefore data reflects only absolute numbers of gold particles and not levels of protein. Nevertheless, the relative labelling of organelles, and hence of antigen present, within a section can be

Figure 6.4: The effect of colloidal gold size on the density of labelling; sites of P450IA2 expression on endoplasmic reticulum

Livers from MC-treated rats were fixed with 4% glutaraldehyde (a) or 2% *p*-formaldehyde containing 0.2% glutaraldehyde (b-d) and embedded in Araldite. Sections were immunostained with anti-$d_{290\text{-}296}$ (b, c) or pre-immune rabbit serum (d), diluted 1:100. Immunogold particles were 30 nm (b) or 10 nm (c, d) in diameter. RER, rough endoplasmic reticulum; mt, mitochondria. Magnification (a) x42 750, (b) x24 800 (c) x39 000 (c) and (d) x24 000.




a
RER
mt
b
mt
RER
c
RER
mt
d
mt
RER

evaluated.

The density of labelling is related to the surface area occupied by the different cellular compartments. For quantification, the surface area of each compartment can be evaluated morphometrically according to the principles of Weibel (1979). The density of labelling can then be calculated from the number of gold particles present over the same compartment. This technique was applied here to compare the labelling intensity of various intracellular compartments. Random liver sections, from at least two separate preparations, which were of good preservation of morphology, were selected and photographed at a primary magnification of 10 000 and 16 000. Negatives were enlarged approximately three-fold. A simple lattice, of known dimensions, was superimposed over the micrographs and the number of gold particles over a unit area of organelle counted. No account of the cell volume, total volume of organelles per cell or surface-to-volume ratio of organelles or of the total cell, hence section thickness, was taken. The results of this estimation are shown in Table 6.1.

Immunogold labelling of cytochrome P450IA2 was evident in both the rough and smooth endoplasmic reticulum in liver sections from both untreated and MC-treated rats. Considerable staining of the nucleus was observed, with a similar density to that of the rough endoplasmic reticulum (Fig. 6.1b and Fig. 6.3b; Table 6.1). Immunogold label appeared to be associated with the densely stained, condensed chromatin at the periphery of the nucleus and with dispersed chromatin throughout the nucleus. The nucleolus was unstained. The nuclei of sinusoidal cells were also stained (Fig. 6.1b). Other organelles, such as the mitochondria, peroxisomes and lysosomes of hepatocytes were labelled to a lesser extent or not at all (Table 6.1).

Cytochrome P450IIB1/2 was located predominately in the smooth endoplasmic reticular membranes, and to a lesser extent in the rough endoplasmic reticulum of sections from livers of PB-treated rats (Fig. 6.2d; Table 6.1). There were a few gold particles associated with the outer membrane of the nuclear envelope. Mitochondria, lysosomes, peroxisomes, glycogen granules and nuclear contents were unstained.

6.3 Discussion

The aim of these experiments was to establish an optimum protocol for the ultrastructural localization of cytochrome P450 in hepatocytes, with a view to extending this to other tissues which contain many different cell types not resolvable at the light microscope level,

Table 6.1: Semi-quantification of immunogold labelling on ultrathin sections of rat liver

Treatment	Antibody	Resin	Number of gold particles.μm^{-1} Mitochondria	Nucleus	RER	SER
MC	Anti-$d_{290-296}$	Araldite	5.9 ± 2.4	24.0 ± 8.5	20.4 ± 5.7	11.0 ± 2.6
MC	Control IgG[a]	Araldite	2.4 ± 2.6	8, 9	2.6 ± 2.8	bd
PB	Anti-PB-P450	Lowicryl	7.7 ± 4.4	3.1 ± 3.3	14.5 ± 7.6	25.6 ± 6.6

Results are the mean ± standard deviation of counts taken from a minimum of 4 different micrographs.
bd: below detection.

[a] Control IgG= pre-immune rabbit serum

such as the lung. A suitable protocol was achieved for the liver by modification of fixation and embedding regimens and optimization of the immunogold staining procedures.

The successful immunolocalization of cytochromes P450IA2 and IIB1/2 depended on the resin used. The polyclonal anti-PB-P450 antibody undoubtedly recognises a number of different epitopes. It was expected that at least some of these epitopes would retain their antigenicity, regardless of the fixation and embedding protocol employed. This was also thought to be the case for the anti-peptide antibody. The lack of staining with anti-PB-P450 in Araldite-embedded liver may be a consequence of the excessive heating required for chemical curing of the resin, which might have denatured all of the epitopes. It was therefore surprising that successful immunostaining was obtained with anti-$d_{290\text{-}296}$, in liver subject to extensive heating (60°C for 18h), and even more surprising that no staining was apparent with this antibody in liver subject to low temperature embedding. The reason for the differential effects of fixation on the binding of these antibodies must lie, not only with differences in the nature of the epitopes with which they react, but also in the nature of the chemical reaction of the resins with tissue components. In this respect, epoxy resins covalently cross-link with biological components ie. proteins, whereas Lowicryls do not (Kellenberger *et al.* 1987). This group showed that microtomy of resin-embedded tissue produces sections which are not smooth or flat, but which show a pattern of relief characteristic for the resin employed. During sectioning of Lowicryl-embedded tissue, the surface of cleavage was found to follow areas of least resistance eg. the interfaces between resin and proteins, and as a consequence epitopes on the surface of proteins were revealed. In contrast, the strong covalent bonds formed in Epon-embedded tissue, resulted in a smoother relief (1-2 nm cf 2-6 nm) following sectioning and consequent shielding of epitopes with resin. It is not yet clear whether it is such shielding of epitopes with resin that causes loss of antigenicity or whether it is the transient use of temperatures between 40°C and 60°C, as required for chemical curing of Epon. Certainly, etching of Epon sections with H_2O_2 does reveal previously "hidden" antigenic sites (Baskin *et al.* 1979), although this was not found for P450IIB1/2. Obviously, there are currently insufficient data upon which to base general rules for the preservation of antigenic sites. Whilst loss of antigenicity of cytochrome P450IIB1/2 may be explained in accordance with the above, that of IA2 in Lowicryl-embedded liver remains inexplicable.

The results obtained in the present study emphasize the importance of characterizing the immunoreactivity of each antibody under a variety of experimental conditions. A number of additional methods, not tested in this investigation, are available in which the retention of antigenicity of heat-labile antigens might be improved. For example, it has been found that

reduction of the temperature of polymerization of an Araldite block containing tissue from 60°C to 37°C is successful, although the process then takes much longer (in excess of a week) and the tissue structure is less well preserved (Probert *et al.*, 1981). Samples can instead be embedded in polyethylene glycol prior to ultrathin sectioning (Bosman & Go, 1981). Alternatively, embedding may be avoided altogether by cryoultramicrotomy of prefixed samples (Tokuyasu, 1980). These latter methods, although still involving fixation with aldehydes, are applicable to studies with monoclonal antibodies, as demonstrated by Brands *et al.* (1985). The widespread use of cryoultramicrotomy, however, has been hampered by the considerable expense, both of acquiring the equipment and its use, due to the very large amounts of liquid nitrogen required by current instruments.

The pattern of staining of P450IA2 is consistent with and explains the punctate nature of the immunoreaction observed at the light microscope level following incubation of sections with the anti-peptide antibody. In a similar study, but using frozen cryosections with the monoclonal antibody 1-7-1, Brands *et al.* (1985) demonstrated that P450IA1/2 is ultrastructurally localized in both the rough and smooth endoplasmic reticular membranes of the liver of MC-treated rats. In their study, no P450IA1/2 was found in the nucleus, although, there was some staining of the outer nuclear envelope. This contrasts with the distribution of P450IA2, obtained in this study, which, although apparent in the nuclear envelope, predominates in the nuclear interior. Whether this represents the localization of P450IA2, or that of another protein which shares the same epitope(s), remains unclear. Certainly, similar sections immunostained with pre-immune rabbit serum did not show staining in the nucleus. Although no specific immunostaining in nuclei of cells was apparent at the light microscope level, this may have been because the amount of protein present was too low to enable visualization of product. There are reports supporting the presence of P450 in the nucleus. Nuclei of cultured AC2F cells, an hepatic cell line, stained positively for P450IA2 but not IA1 following incubation with highly specific monoclonal antibodies (Hishinuma *et al.*, 1987). This cell line, which lacks microsomal P450, activates a number of carcinogenic amines (Trp P-1 and Glu P-2), suggesting a catalytic role for nuclear cytochrome P450. Interestingly, cytochrome P450IA2, but not P450IA1, has been immunodetected, by Western blotting, in preparations of nuclei from untreated rats, the levels of which were elevated by 0.1% AAF in the diet for one week (Freidman *et al.*, 1989).

The ultrastructural localization of cytochrome P450IIB1/2 in hepatocytes from PB-treated rats was predominantly over the smooth endoplasmic reticulum and to a lesser extent the rough endoplasmic membranes. This observation is consistent with previous reports in which the

localization of PB-inducible cytochrome P450 isoenzymes on various membrane fractions of rat liver cells was studied (Matsuura *et al.*, 1978; De Lemos-Chiarandini *et al.*, 1987). Matsuura and co-workers (1981) also reported that this isoenzyme is present in the outer nuclear envelope, supporting the findings of the present study.

The protocols devised for the successful ultrastructural localization of cytochromes P450IA2 and IIB1/2 in the liver have been applied to studies of the lung. Preliminary results, not detailed above, confirm light microscopical observations, that P450IA2 is not expressed to any significant extent in cells of the lung of untreated or MC-treated rats. However, immunogold particles, representing specific staining, were found over the nuclei of most cells, although the level of staining was not as dense as that seen in nuclei of hepatocytes. No P450IIB1/2 was apparent in Araldite-embedded lung tissue, not surprising in view of the results obtained in liver. Future experiments using Lowicryl-embedded tissue are required to answer the question of whether or not P450IIB1/2 is expressed in alveolar cells of the rat lung.

In a recent study, Serabjit-Singh *et al.*, (1988) compared the cellular localization of isoenzymes IIB4 and IVB1 (forms 2 and 5, respectively) and NADPH-cytochrome P450 reductase in rabbit lung at the ultrastructural level. The preservation of epitopes in this study was also found to depend on mild fixation and low temperature methods. Furthermore, osmication of tissue, an effect not tested here, was detrimental to immunoreactivity. All three proteins were found in regions of agranular endoplasmic reticulum in the cytoplasm of Clara cells, whilst only IIB4 and IVB1 were found in type II alveolar cells. Type I alveolar cells were unstained. Considerable staining of other structures, not previously observed in light microscopical studies, such as microvilli tips of ciliated cells, secretory granules of goblet cells and plasma membranes of endothelial cells, was evident.

Electron microscopy of cytochromes P450 using suitable antibodies and procedures for tissue preparation will enable the intracellular localization of specific isoenzymes of P450 in a variety of tissues and cell types to be determined by immunogold labelling. Such studies should help in understanding the specific toxicity and the biochemical and toxicological changes manifest within individual cells by P450.

Chapter Seven

Conclusion

The sensitivity of monoclonal and anti-peptide antibodies, together with secondary marker immunoglobulins (gold- or enzyme-conjugated), has allowed the visualization of isoenzymes of the cytochrome P450IA and IIB families, not only at the tissue and cellular level but also ultrastructurally.

Under optimal conditions of fixation and preparation of tissue for immunostaining, low levels of constitutively expressed cytochromes P450 of the IA and IIB families were detected in liver and extrahepatic tissues. Previously described hepatic distributions for these isoenzymes have differed between laboratories as a result of the specificity of the, more often than not, polyclonal antibodies employed. The use of highly, specific monoclonal and anti-peptide antibodies in this study has not only confirmed, but clarified some previous findings. Thus, P450IA1 was homogeneously expressed both in untreated and MC-treated animals, whilst P450IA2 exhibited a non-homogeneous distribution, residing in a narrow band of centrilobular hepatocytes in untreated animals and in a wider band of cells following treatment of animals with MC. Cytochrome P450IIB1/2, previously described as a centrilobular isoenzyme(s), was shown to exhibit a more precisely defined distribution in untreated animals, which varied with the strain examined. Thus, in Wistar rats P450IIB1/2 was most predominant in a discrete number of hepatocytes, sometimes even single cells, of this region, whilst in the Lister-Hooded strain, expression was confined to periportal hepatocytes and in the F344 strain was found in both regions. Nevertheless, following treatment of animals with PB, induction was greatest in the centrilobular cells, regardless of the strain, with the exception of the F344 rat, where expression was increased throughout the lobule.

Similarly, this study has shown that the effects of xenobiotics are sometimes more complex than apparent from simple measurement of enzyme activities or immunoblots, in that not only do levels of isoenzymes increase but also the extent to which any one cell responds may vary not only with the strain, but also with the age of the animal. For example, the expression of IA2 in livers of foetuses and neonates is limited to a discrete, but increasing number of hepatocytes, as a function of age. This characteristic appears ultimately to determine and/or control the distribution pattern for a particular isoenzyme and was inherent in hepatocytes from as early as a few days prior to birth. Hormonal or dietary factors may

also have some role to play in stimulating the production of certain isoenzymes within these individually pre-programmed cells. Such data emphasises the importance of using immunocytochemistry as an adjunct to other biochemical methods from which this type of information would not be forthcoming, thus complimenting or reinforcing present concepts of the cytochrome P450 monooxygenase system.

The expression of these isoenzymes was also shown to vary considerably among different extrahepatic organs and cell types of the rat. Moreover, each isoenzyme was subject to differential induction in either a tissue- and/or age-specific manner. For example, whereas IIB1/2 was absent from the kidney, even following pre-treatment of rats with PB, IA1 and IA2 were constitutively expressed, with IA1 residing in the S_3 cells and IA2 in the S_2 cells of the proximal tubule. Isoenzymes of both these families were expressed in the epithelial cells of the small intestinal villi, and in the lung. In the latter, IIB1/2 was focally concentrated in the Clara cells, whilst IA1 was distributed more widely and IA2 not at all. Although these findings confirm previous reports, they also strongly indicated that IIB1/2 was not induced in this tissue, perhaps as a result of being so concentrated within one cell. No P450IIB1/2 was found in the testis or pancreas, but, in contrast, IA1 was expressed weakly in all acinar cells and to a lesser extent in the epithelial cells lining the inter- and intra-lobular ducts whilst IA2 was strongly expressed in the latter only. Extrahepatically, IA2 appeared totally refractive to MC treatment of rats whilst induction of IA1 occurred in most tissues following parturition. In contrast, P450IIB1/2 could be induced by PB-treatment of rats, only in the small intestine. Clearly, these data beg the question as to how isoenzyme expression is controlled at a cellular level and one to which only a molecular biological approach is likely to find an answer.

Immunocytochemical findings, nevertheless, must be interpreted with caution since specific binding may not always reflect the presence of catalytically active isoenzymes. However, additional data, such as the demonstration of mRNAs to various isoenzymes of P450 and the presence of other [illegible] enzymes such as NADPH-cytochrome P450 reductase and phase II conjugating enzymes in the same tissues or cells, or metabolism of specific substrates can be supportive of this. In this respect, NADPH-cytochrome P450 reductase, epoxide hydrolase, glutathione-S-transferases and UDP-glucuronylsyltransferases have been shown to share the same distribution with cytochrome P450 in the liver and in some extrahepatic tissues (Wolf *et al.*, 1984; Baron *et al.*, 1986; McManus *et al.*, 1987; Moody *et al.*, 1988; Knapp *et al.*, 1988; Hall *et al.*, 1989). Similarly, metabolism of substrates specifically catalysed by isoenzymes of the P450IA and IIB families, has been demonstrated in many extrahepatic tissues as discussed in previous chapters.

Extrapolation of immunocytochemical data obtained from different mammalian species describing the precise intra- and inter- tissue distributions of cytochromes P450, particularly of the IA family, to man, may help in explaining the toxicological effects of xenobiotics activated by these isoenzymes. Certainly, this study has shown similarities in the distribution of isoenzymes of the P450IA family amongst several species and in several tissues including some from human. A particularly good example which stresses this point is seen for acetaminophen (paracetamol) which causes hepatotoxicity, manifest as centrilobular necrosis, when it is ingested in large quantities in both animals and man (Moldeus *et al.*, 1978). The involvement of cytochrome P450IA2, an isoenzyme shown here to be predominant within centrilobular hepatocytes, in activating acetaminophen to a reactive metabolite, N-acetyl-*p*-benzoquinoneimine in human liver was demonstrated by inhibition of catalysis with monoclonal antibodies directed against this isoenzyme (Raucy *et al.*, 1989). Normally, this metabolite is conjugated to glutathione, but in large doses this protective system becomes saturated resulting in initiation of damage.

The development of more mono-specific anti-peptide antibodies directed against the human PAH-inducible isoenzymes should allow a more extensive study than was possible here, with the monoclonal antibody 3/4/2, of the distribution patterns of constitutive and inducible isoenzymes in human tissues. For example, a comparison of tissues from smokers and non-smokers and from alcoholics and non-alcoholics would be particularly revealing.

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Selective destruction of cytochrome P-450d and associated monooxygenase activity by carbon tetrachloride in the rat

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1. The effects of acute treatment of 3-methylcholanthrene (MC)-induced rats with carbon tetrachloride (CCl_4) on the content and activity of the polycyclic aromatic hydrocarbon-inducible forms of cytochrome P-450 (P-450c and P-450d) in liver and kidney have been determined.

2. Post-treatment of MC-induced rats with CCl_4 *in vivo* decreased the specific content of total, spectrally determined, P-450 in both hepatic and renal microsomes, by 60% and 40%, respectively. CCl_4 treatment destroyed almost all of the hepatic P-450d (specific content after 6 h, $<2\%$ of control), but had no effect on P-450c, which increased slightly over the 6 h, to 30% above control values.

3. Immunocytochemical measurements demonstrated greater loss of P-450d from the centrilobular and midzonal than from periportal regions of the liver.

4. Hepatic phenacetin *O*-deethylase, an activity catalysed specifically by P-450d in this tissue, was dramatically decreased following administration of CCl_4 to MC-induced rats. Loss of monooxygenase activity was highly correlated with the decrease in P-450d content ($r=0{\cdot}947$, $P<0{\cdot}001$). Aryl hydrocarbon hydroxylase activity of liver, catalysed almost entirely by P-450c, was unchanged and neither activity was affected in kidney.

5. Treatment of MC-induced rats with CCl_4 causes a selective loss of hepatic P-450d and associated monooxygenase activities. Phenacetin *O*-deethylation is catalysed specifically by P-450d in liver, but not in kidney. The mechanism for this destruction of P-450d may be suicide activation of CCl_4, but the rate of such activation appears to be much lower than with P-450b. Alternatively, P-450d may be particularly sensitive, and P-450c particularly resistant, to the active metabolite of CCl_4 diffusing from a distant site of formation.

Introduction

Polycyclic aromatic hydrocarbon (PAH)-inducible isoenzymes of cytochrome P-450 (P-450), the products of the P-450IA gene sub-family (Nebert *et al.* 1987), activate a large number of xenobiotic compounds to carcinogenic or mutagenic intermediates. In the rat, cytochrome P-450d is generally the most specific isoenzyme catalysing the oxidation of aromatic amines, whereas P-450c most specifically oxidizes polycyclic aromatic hydrocarbons. Both isoenzymes are capable of activating pro-carcinogens. Thus, differences in the expression and induction of these isoenzymes can influence the site of, and individual susceptibility to, toxic or carcinogenic effects. P-450d is constitutively expressed in excess of P-450c in the liver of untreated animals (Chiang *et al.* 1982, Kelley *et al.* 1987, Sesardic *et al.* 1988). Following treatment of rats with 3-methylcholanthrene (MC), both isoenzymes are induced, but there is a preferential induction of P-450c (Thomas *et al.* 1983, Yeowell *et al.* 1985, Kelley *et al.* 1987). However, in extrahepatic tissues, PAHs induce only P-450c (Goldstein and Linko 1984, Boobis *et al.* 1987). Some

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substrates specific to P-450d in the liver are metabolized only by P-450c in extrahepatic tissues following treatment of the animals with PAHs, as only P-450c is present in such circumstances (Davies *et al.* 1988). Thus, it is of importance to determine the substrate specificity of these isoenzymes within the tissues of interest, and not only when they are isolated and reconstituted. Specificity has been assessed in a number of ways, including the use of specific inhibitory antibodies, competitive inhibitors and selective reversible or irreversible chemical inhibitors. In this last instance, work by Degawa *et al.* (1987) with carbon tetrachloride (CCl_4) has shown considerable promise.

Carbon tetrachloride is both hepatotoxic and nephrotoxic, although there are marked sex and species differences in these effects. Its toxicity depends upon metabolism by the P-450-dependent monooxygenase system to a highly reactive radical intermediate. In addition to initiating cytotoxicity, this radical interacts with the haem of P-450, thereby destroying the isoenzyme(s) responsible for its formation. Thus, CCl_4 acts as a suicide inhibitor of P-450. This is reflected in the decrease in total hepatic P-450 content which is apparent following treatment of animals with the compound. Loss of P-450 can also be demonstrated during the incubation of CCl_4 with hepatic microsomal fractions.

There is evidence that the activation of CCl_4 is catalysed by specific forms of P-450. Treatment of animals with a low dose of the compound protects them against a subsequent dose that would otherwise be lethal (Glende 1972). In addition, a number of inducing agents, including phenobarbitone (PB) and ethanol, have been shown to increase the toxicity of CCl_4. In parallel with this there was an increased loss of P-450. A number of groups have attempted to identify the specific isoenzymes of cytochrome P-450 involved. Evidence has been obtained for the involvement of P-450b/e, the major PB-inducible forms (Noguchi *et al.* 1982a, 1982b, Davies *et al.* 1985, 1986), and of P-450j, the ethanol-inducible form (Gadeholt 1984, English and Anders 1985). Of considerable interest was the suggestion that an MC-inducible form might also be selectively destroyed by treatment of rats with CCl_4 (Enosawa and Nakazawa 1986, Degawa *et al.* 1987). Curiously, MC treatment of rats decreases the hepatoxicity of CCl_4 (Suarez *et al.* 1972).

Degawa *et al.* (1987) have suggested that treatment of MC-induced rats with CCl_4 might provide a convenient means of investigating the specificity of PAH-inducible forms of P-450 in the liver. We have investigated this possibility, using specific antibodies to P-450c and P-450d, together with assays of monooxygenase activity specific to each isoenzyme, aryl hydrocarbon hydroxylase (AHH) and phenacetin *O*-deethylase (POD), respectively. In view of the fact that CCl_4 can cause nephrotoxicity, as well as hepatotoxicity, its effects on PAH-inducible forms of P-450 in the kidney were determined.

Materials and methods

Chemicals

NADPH, *n*-undecane, 3-methylcholanthrene, benzo[*a*]pyrene and prestained molecular weight markers were from Sigma Chemical Co. Ltd (Poole, Dorset, UK). Carbon tetrachloride, hydrogen peroxide, phenacetin, paracetamol, dichlorodifluoromethane (Arcton), synthetic mounting medium containing resin in xylene (Pertex) and TissueTek II embedding medium were from BDH Chemicals Ltd (Poole, Dorset, UK). Deuterated [$^2H_3$]paracetamol was synthesized as previously described (Murray and Boobis 1986). 3,5-Bistrifluoromethylbenzoyl chloride was supplied by Fluorochem Ltd (Glossop, UK). All gel filtration and ion-exchange resins were from Pharmacia (Milton Keynes, UK).

Both benzo[*a*]pyrene and phenacetin were recrystallized before use. All reagents for sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis and for Western blotting were from BioRad Laboratories (Watford, Herts, UK) or National Diagnostics (Aylesbury, Bucks, UK). Nitrocellulose membrane (Hybond C) was obtained from Amersham International plc (Amersham, Bucks, UK) and anti-mouse IgG conjugated to horseradish peroxidase from ICN Biomedicals (High Wycombe, Bucks, UK). All other chemicals were obtained from either Sigma Chemical Co. or BDH Chemicals and were of Analar grade or the best equivalent.

Treatment of animals and preparation of tissues

Male Wistar rats (Olac, Bicester, Oxon, UK), weighing between 180 and 250 g, were maintained under constant heating and lighting cycles prior to induction. Animals were treated with 80 mg/kg of MC in corn oil by i.p. injection, or with PB (80 mg/kg) in saline for 4 days, also by i.p. injection. Sixty-two hours later the rats treated with MC received CCl_4, as a 5% solution in corn oil (800 mg/kg), given by i.p. injection. Groups of animals were killed, by stunning and exsanguination, at 0, 1, 3 and 6 h after CCl_4 treatment. Rats were permitted free access to food (PRD diet, Labsure Animal Products, Poole, Dorset, UK), until 18 h before CCl_4 treatment and to tap water *ad libitum*. Livers and kidneys were removed and microsomal fractions prepared from individual livers and the pooled kidneys from three animals, by differential ultracentrifugation as previously described (Boobis *et al.* 1985). Microsomal fractions were suspended in 0·25 M potassium phosphate buffer, pH 7·25, containing 30% (v/v) glycerol, to a protein concentration of >15 mg/ml, and stored at −80°C until required.

For immunocytochemistry, tissue blocks of approx $0{\cdot}5 \times 1\ cm^2$ were prepared from the medial lobe of liver and from kidney, snap frozen in melting Arcton (Freon, −156°C, precooled in liquid nitrogen) and stored at −80°C until required for staining.

Preparation of antibodies

Cytochromes P-450c and P450d were purified from the livers of rats treated with MC as previously described (Sesardic *et al.* 1986, Edwards *et al.* 1988). A monoclonal antibody (MAb) specific to rat cytochromes P-450c and P-450d (12/2/3/2), that has been described previously (Sesardic *et al.* 1986) was used. In addition, MAbs specific to rat cytochrome P-450c: C8, C9 (Thomas *et al.* 1984) and 3/4/2 (Boobis *et al.* 1985) and a monospecific polyclonal antibody to rat P-450d, D1, previously called αP-450d(-c) (Ryan *et al.* 1980), were used. All antibodies were purified by fast protein liquid chromatography using a Mono-Q column (Pharmacia, Milton Keynes, UK), as previously described (Edwards *et al.* 1988).

Immunological assays

Immunoquantification of P-450c and P-450d was performed by Western blotting, essentially as described previously (Sesardic *et al.* 1986). The stained bands obtained on Western blotting were quantified by reflectance densitometry using a GS 300 Transmittance/Reflectance Scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA, USA) and analysed using a Waters Model 840 Data and Chromatography control station (Millipore (UK) Ltd, Harrow, UK). Standard curves were constructed using purified rat cytochrome P-450c. The limit of detection for P-450c with MAb 3/4/2 was 30 fmol, giving a limit of sensitivity of 0·5 pmol P-450c/mg microsomal protein. With MAb 12/2/3/2, the limit of the assay was 2 pmol P-450 (either P-450c or P-450d)/mg microsomal protein.

Immunoinhibition of metabolic activity was assessed by incubating purified antibody with microsomal fraction at room temperature for up to 30 min in a total volume of 200 μl phosphate buffered saline (PBS: 10 mM sodium–potassium phosphate buffer pH 7·4, containing 137 mM sodium chloride and 2·6 mM potassium chloride) prior to addition of cofactors. With MAbs the ratio of antibody to P-450 was between 0·1 and 1·0 mg Ig/nmol P-450 and with the polyclonal antibody it was between 5·0 and 20·0 mg Ig/nmol P-450.

P-450c and P-450d were localized immunocytochemically in sections of frozen tissue by the indirect two-stage immunoperoxidase method. Sections of approximately 7 μm thickness were cut on a cryostatic microtome (Slee, London, UK) at −20°C, mounted onto poly-L-lysine coated glass slides (Huang *et al.* 1983), air dried overnight at room temperature and stored, with desiccant, at −80°C until required, at which time the sections were warmed to room temperature, fixed with cold acetone and rehydrated in PBS, pH 7·4. Non-specific binding sites were blocked by incubating the sections with 1% bovine serum albumin in PBS for at least 10 min. Specific antibody was applied to the tissue sections, which were then incubated overnight at 4°C in a humidified chamber. After washing with PBS, the sections were incubated with the appropriate second antibody for 90 min at room temperature, usually at a dilution of 1:200, in PBS containing 5% normal rat serum. Detection of bound antibody was accomplished by incubating the sections with 0·025% 3,3-diaminobenzidine tetrachloride in PBS containing 0·03% hydrogen peroxide for 20 min. Sections were counterstained with Cole's haematoxylin, dehydrated and mounted in Pertex.

Sections were stained for fatty acids by the method of Drury and Wallington (1980), which involved staining the unfixed sections with oil red *O*-triethyl phosphate, prepared as a 1% solution in 60% aqueous triethyl phosphate, for 10–15 min. Sections were counterstained as described above.

Enzyme assays

Phenacetin *O*-deethylase (POD) activity of microsomal fractions was determined by the method of Murray and Boobis (1986). The reaction mixture contained Tris-HCl buffer, pH 7·4 (75 mM), $MgCl_2$ (3 mM), NADPH (1 mM), crystalline bovine serum albumin, fraction V (0·7 mg) and microsomal protein (0·02–2·0 mg), in a final volume of 1 ml. The reaction was started by the addition of recrystallized phenacetin, in 20 μl methanol, to give a final concentration of 4 μM for liver and 20 μM for kidney. Samples were incubated for 10 min at 37°C in a shaking water bath. Deuterated paracetamol was used as internal standard. The product, paracetamol, together with its internal standard, were extracted, derivatized with 3,5-bistrifluoromethylbenzoyl chloride, and finally dissolved in *n*-undecane. Analysis was by combined gas chromatography/negative ion chemical ionization mass spectrometry (Model 4500, Finnigan-MAT, San José, CA, USA) using a fused silica capillary column (SE 54, 30 m × 0·27 mm, J&W Scientific Inc., Folson, CA, USA). The limit of detection for the assay was 0·2 ng of paracetamol per incubation.

Aryl hydrocarbon hydroxylase (AHH) activity was assayed by a modification of the method of Atlas *et al.* (1976). The reaction mixture (1 ml) contained Tris-HCl buffer, pH 7·25 (50 mM), $MgCl_2$ (3 mM), crystalline bovine serum albumin, fraction V (0·7 mg), NADPH (1 mM) and microsomal protein (5–50 μg). Benzo[*a*]pyrene, in methanol, was added to give a final concentration of 5 μM. Samples were incubated for 5 min at 37°C in a shaking water bath. Fluorescent metabolites were extracted and analysed by spectrophotofluorometry (Aminco-Bowman, American Instrument Co., Silver Spring, Maryland, USA) with an excitation wavelength of 396 nm and an emission wavelength of 522 nm. The assay was standardized with authentic 3-hydroxybenzo[*a*]pyrene, with quinine sulphate as a secondary standard (Nebert 1978).

Effect of CCl_4 in vitro

Hepatic microsomal fraction, 4 mg protein, from MC-treated rats was incubated in 75 mM Tris-HCl buffer, pH 7·4, containing $MgCl_2$ (3 mM), NADPH (1 mM) and CCl_4 (1–4 mM in 10 μl methanol). Samples were incubated in a shaking water bath at 37°C for 20 min. The reaction was terminated by the addition of 5 ml of ice cold 0·25 M potassium phosphate buffer, pH 7·4. The microsomal fraction was re-isolated by ultracentrifugation for 60 min at 105,000*g* and then resuspended in 1 ml of 0·25 M potassium phosphate buffer, pH 7·25, containing 30% (v/v) glycerol by gentle homogenization using a hand-operated Teflon/glass homogenizer. Portions of the resuspended microsomal fraction were used for the assay of protein, P-450 content and monooxygenase activities as described elsewhere in the Methods section.

Other assays

Protein concentration was determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin, fraction V, as standard. Total hepatic P-450 content was measured by the method of Omura and Sato (1964), assuming an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ between 450 and 480 nm. Total P-450 content of renal microsomes was determined by the method of Johannesen and DePierre (1979), in which the difference between oxidized CO-bound and reduced CO-bound P-450 was determined, and an extinction coefficient of 105 $mM^{-1}\,cm^{-1}$ between 475 and 450 nm was assumed. All P-450 spectra were recorded on a model 555 split-beam scanning spectrophotometer with microprocessor-controlled background correction (Perkin-Elmer Ltd, Beaconsfield, Bucks, UK).

Results

Microsomal protein yield was 3-fold greater from liver than from kidney of MC-treated rats. The specific content of P-450 in hepatic microsomes from these animals was 6–7-fold that in renal microsomes (table 1).

Treatment of MC-induced rats with CCl_4, at a relatively high dose, caused a significant time-dependent decrease in hepatic microsomal protein yield, such that 6 h after the dose of CCl_4, protein yield was reduced to 61% of control values (table 1). Renal microsomal protein yield was not affected by CCl_4 treatment of the animals. The specific content of total P-450 of both hepatic and renal microsomes was decreased in CCl_4-treated rats, hepatic P-450 content being decreased by 60% over 6 h and renal P-450 content being decreased by 40%. Thus, the loss of P-450 per g wet weight of tissue from the liver, 6 h after treatment with CCl_4, was 75%, whereas from the kidney it was only 35%.

In a previous study it was shown, by immunoquantification, that in the liver of untreated rats P-450d content is almost 10 pmol/mg, whereas the level of P-450c is

Table 1. Effect of post-treatment of MC-induced rats with carbon tetrachloride *in vivo* on protein and spectrally determined content of cytochrome P-450 in hepatic and renal microsomes.

Time after CCl_4 (h)	Tissue	Protein		P-450 content	
		mg/g	% Control	nmol/mg	% Control
0	Liver	24·8±4·0	100±16·1	1·71±0·2	100±11·7
1	Liver	21·5±1·5	86·7±6·0	1·02±0·1	59·7±5·9
3	Liver	20·2±0·5	81·5±2·0	0·795±0·2	46·6±11·7
6	Liver	15·3±1·1	61·2±4·4	0·681±0·03	39·9±1·8
0	Kidney	7·71	100·0	0·257	100·0
1	Kidney	9·75	126·5	0·284	110·5
3	Kidney	6·75	87·6	0·178	69·3
6	Kidney	9·60	124·5	0·158	61·5

All rats were treated with MC, at a dose of 80 mg/kg, i.p. in corn oil, 62 h prior to treatment with carbon tetrachloride (800 mg/kg). The results for liver are means for three different animals±SD. For renal samples, results are average of duplicate determinations on the pooled tissues from three animals.

below the limit of detection (0·5 pmol/mg) (Sesardic *et al.* 1989). Treatment of rats with MC results in a considerable increase in the content of both of these isoenzymes in the liver, such that together they account for the majority of the P-450 present. As treatment of MC-induced rats with CCl_4 resulted in a loss of total P-450 of 60% after 6 h, the effect of this treatment on the specific content of P-450c and P-450d was investigated. The isoenzymes were quantified following Western blotting with a monoclonal antibody (MAb 12/2/3/2) which recognizes only these two forms of P-450 in the rat. Immunoblotting of hepatic microsomes from MC-treated rats revealed two bands, at M_r 54,000 and M_r 57,000, corresponding to P-450d and P-450c, respectively (figure 1, 0 h). Following treatment of MC-induced rats with CCl_4 there was a time-dependent decrease in the intensity of the P-450d band, with some loss of staining apparent within 1 h of administering the hepatotoxin. At 6 h there was only a faint trace of a band at M_r 54,000. The intensity of the P-450c band appeared unchanged after CCl_4 administration, even at 6 h. This is perhaps more apparent from densitometric scans of the blots (figure 2), which revealed an almost complete loss of P-450d, in the presence of levels of P-450c comparable to those in the control animals.

The specific content of P-450c and P-450d was quantified by scanning of the blots, followed by integration of the peaks as described in Materials and Methods. The specific content of P-450d decreased from 285±18 pmol/mg to only 4·4±0·9 pmol/mg over the 6 h from administration of CCl_4, whilst the specific content of P-450c increased slightly, from 252±60 pmol/mg to 311±43 pmol/mg, over the same period. The data have been expressed as a percentage of control, with no exposure to CCl_4 (0 time) (figure 3a). Within 1 h of treatment with CCl_4 the content of P-450d had declined to 30%, and by 6 h to <2%.

It has been shown previously (Goldstein and Linko 1984, Boobis *et al.* 1987) that treatment of rats with PAHs induces only cytochrome P-450c in extrahepatic tissues. The content of P-450c in the kidney of MC-induced rats treated with CCl_4 was determined by Western blotting with a monoclonal antibody (MAb 3/4/2) specific to this isoenzyme (figure 4). Hepatic microsomes were similarly blotted, for comparison. P-450c was not detectable in the kidney of untreated rats (data not shown). MC treatment resulted in the appearance of a densely staining band at

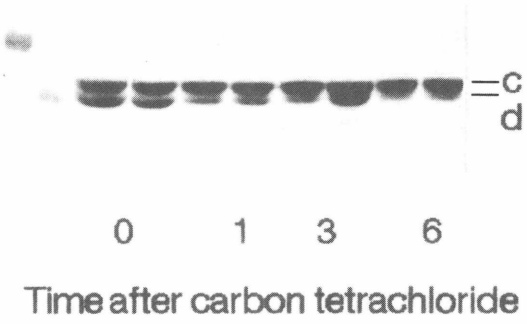


Figure 1. Western blot of hepatic microsomal fractions from rats treated with CCl_4 at various times after administration of MC with a monoclonal antibody specific to P-450c and P-450d.

Hepatic microsomal proteins (25 μg) were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membrane and blotted with MAb 12/2/3/2 as described in the text and elsewhere (Sesardic *et al.* 1986). Blots of samples, from two rats taken at random in each group, are shown. **c** and **d** indicate the relative positions of P-450c and P-450d, at M_r 57,000 and M_r 54,000, respectively. The first two lanes contained prestained molecular weight markers. Animals were treated with MC (80 mg/kg) and then exposed to CCl_4 (800 mg/kg) for the times indicated.

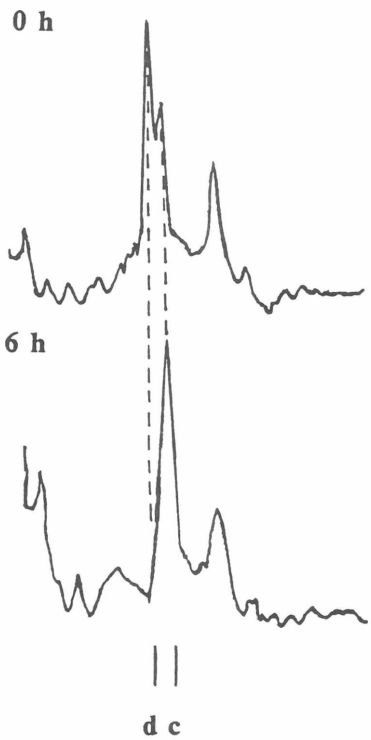
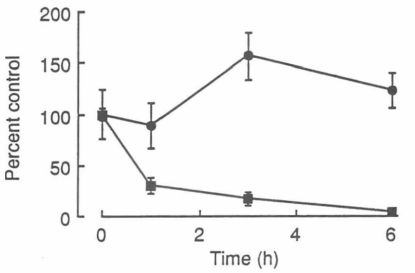
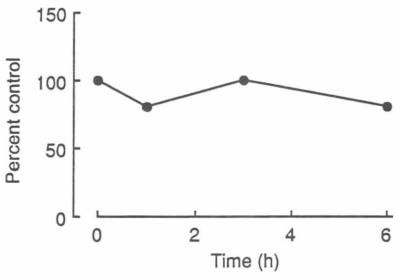


Figure 2. Densitometric scans of Western blots obtained as described in the legend to figure 1.

The *upper trace* is from a representative sample from a rat not exposed to CCl_4 and the *lower trace* is from a typical sample from a rat treated 6 h earlier with CCl_4. Proteins migrated from left to right. **c** and **d**, together with the corresponding broken vertical lines, indicate the relative positions of P-450c and P-450d, respectively. The blots were scanned using reflectance densitometry, as described in Methods.



(*a*)



(*b*)

Figure 3. Effect of treating MC-induced rats with CCl_4 on (*a*) hepatic content of P-450c and P-450d and (*b*) renal content of P-450c.

Microsomal proteins were subjected to Western blotting and P-450c (●) and P-450d (■) were immunoquantified as described in Methods. Values for liver are means ± SD, for three different animals. Where an error bar is not visible it falls within the symbol. Values for kidney were obtained from pooled samples from three rats at each time point. P-450 contents have been expressed as a percentage of control values, from animals not exposed to CCl_4 (0h). In control animals the specific content of P-450c and P-450d in liver was 252 ± 60 pmol/mg and 285 ± 18 pmol/mg, respectively, and of P-450c in kidney it was 40–50 pmol/mg.

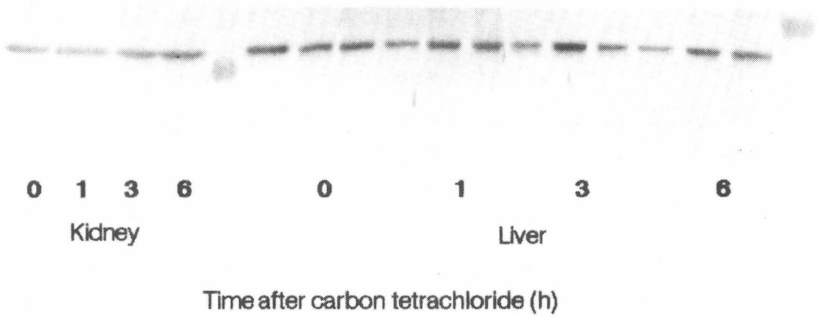


Figure 4. Western blot of hepatic and renal microsomal fractions from rats treated with CCl_4 at various times after administration of MC with a monoclonal antibody specific to P-450c.

Hepatic microsomal proteins (2·5 μg) from individual animals, three per group, and renal microsomal proteins (50 μg) from the pooled tissues of three animals at each time point, were separated on 10% SDS-polyacrylamide gels and subjected to Western blotting with MAb 3/4/2 as described in Methods. Animals were treated with MC (80 mg/kg) and then exposed to CCl_4 (800 mg/kg) for the times indicated.

M_r 57,000, P-450c, with an identical relative mobility to that of the hepatic form. CCl_4 treatment of the rats appeared to have very little effect on the renal content of P-450c. Similarly, hepatic P-450c assessed with MAb 3/4/2, as with 12/2/3/2, showed no significant decrease following CCl_4 exposure. The specific content of P-450c in the kidney of MC-treated rats was 40–50 pmol/mg, determined on the pooled tissue from three animals. Over the 6 h from the time of administration of CCl_4, renal P-450c content decreased by less than 20% (figure 3b). The level of cytochrome P-450d in renal microsomes from MC-treated rats, determined with an antibody specific to this isoenzyme, was $<2{\cdot}0$ pmol/mg, even before the administration of CCl_4.

The hepatotoxicity of CCl_4 is characterized by centrilobular necrosis. There is also evidence that many of the isoenzymes of P-450 show zonal distribution in the liver. Thus, the effect of CCl_4 treatment of MC-induced rats on the distribution of P-450c and P-450d was determined by immunocytochemistry, to assess the localization and specificity of action of this hepatotoxin on P-450. Liver sections from MC-induced rats before (figure 5a) and up to 6 h after (figure 5b) exposure to CCl_4 were stained with a monoclonal antibody (MAb 3/4/2) specific to rat P-450c. There was even staining across the liver lobule, which was unaffected by treatment of the rats with CCl_4. A gradual increase in the number of cytoplasmic vacuoles in hepatocytes with increasing time of exposure to CCl_4 was noted. Staining of unfixed sections with oil red *O*-triethyl phosphate, a lipid-soluble dye, showed that this was due to an increase in fatty acid deposits, which were lost upon acetone fixation of the sections, leaving characteristic vacuoles (data not shown). These vacuoles had no effect on immunostaining by the antibodies used in this study.

Liver sections from MC-induced rats before (figure 6a) and up to 6 h after (figure 6b) treatment with CCl_4 were stained with a monospecific polyclonal antibody to P-450d. In sections from the liver of rats treated with MC but not exposed to CCl_4 strong immunostaining was apparent, with an even distribution, indicating the presence of P-450d in all hepatocytes. In sections from MC-induced rats treated with CCl_4 there was a considerable reduction in the intensity of specific staining of P-450d in the hepatocytes of the midzonal and centrilobular regions, most evident 6 h after CCl_4 (figure 6b). In addition, the normally homogeneous staining of the hepatocyte cytoplasm by the product of the diaminobenzidine/hydrogen peroxide reaction was replaced by a more punctate, or stippled staining, particularly in the cells of the centrilobular region. Thus, CCl_4 treatment of the animals changed both the distribution and amount of P-450d within the liver.

We have previously reported the cellular localization of P-450c in rat kidney following treatment of the animals with MC (Foster *et al.* 1986). The effect of exposure of MC-induced rats to CCl_4 on this distribution was determined in the present study. In MC-treated rats, specific staining for P-450c was detected along the whole length of the proximal tubule, with no staining in the glomerulus or in the collecting duct. There was no change in either the distribution or intensity of this staining in sections of kidney from animals further treated with CCl_4 up to 6 h earlier (data not shown).

The effect of CCl_4 treatment on the catalytic activity of PAH-inducible isoenzymes was assessed using specific substrates for each form of P-450. In previous studies we have shown that the high affinity component of POD activity in rat liver is catalysed exclusively by P-450d (Davies *et al.* 1988), and a number of

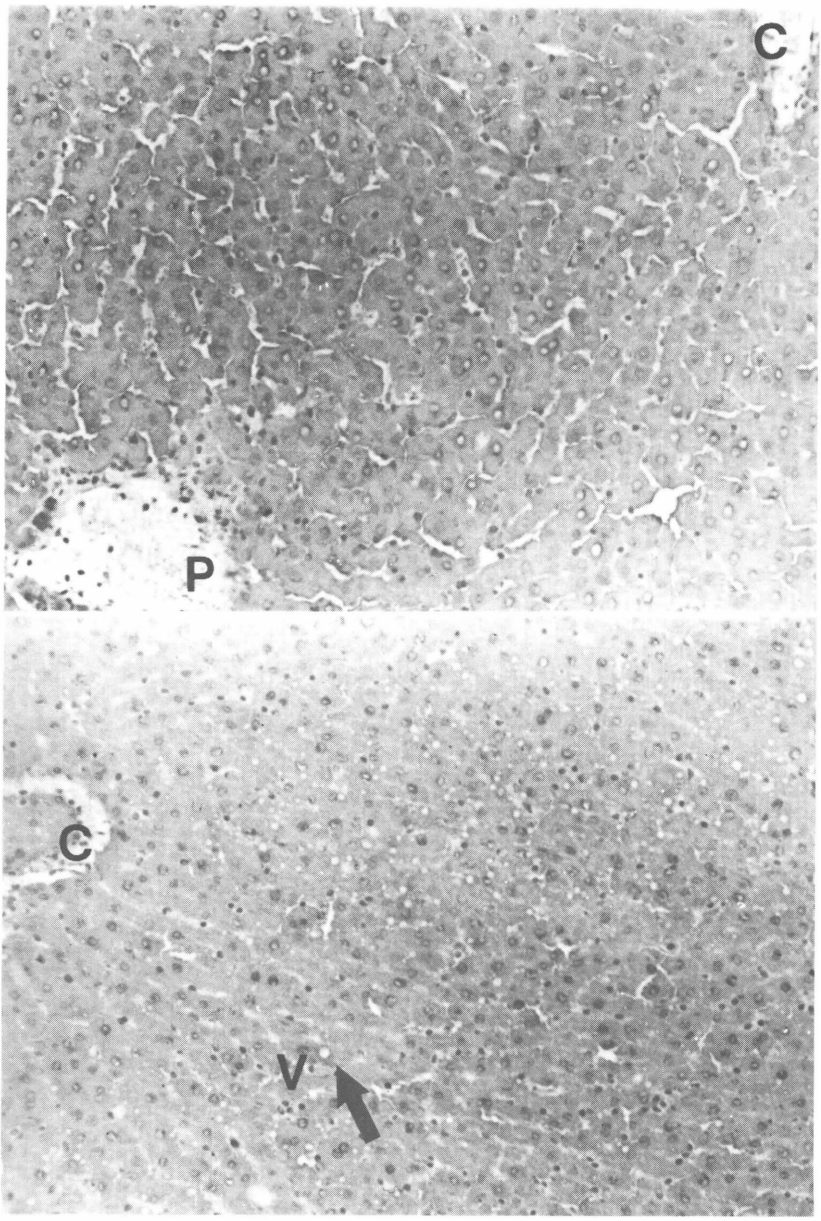


Figure 5. Immunocytochemical localisation of P-450c in liver of MC-induced rats (*a*) before and (*b*) 6 h after exposure to CCl_4.

P-450c was visualized using the indirect two-stage immunoperoxidase method, with MAb 3/4/2, specific to P-450c, as the first antibody. Results are typical of those observed on examination of five sections from each of three animals. C: Central vein; P: portal vein; V: fatty acid vacuole. (Magnification × 212.)

groups have reported that, following MC treatment of rats, AHH activity is catalysed almost entirely by P-450c (Ryan *et al.* 1979, Guengerich *et al.* 1982). The specificity of the activities used, AHH and POD, was confirmed in the present study, using specific inhibitory antibodies to P-450c and P-450d, respectively. In

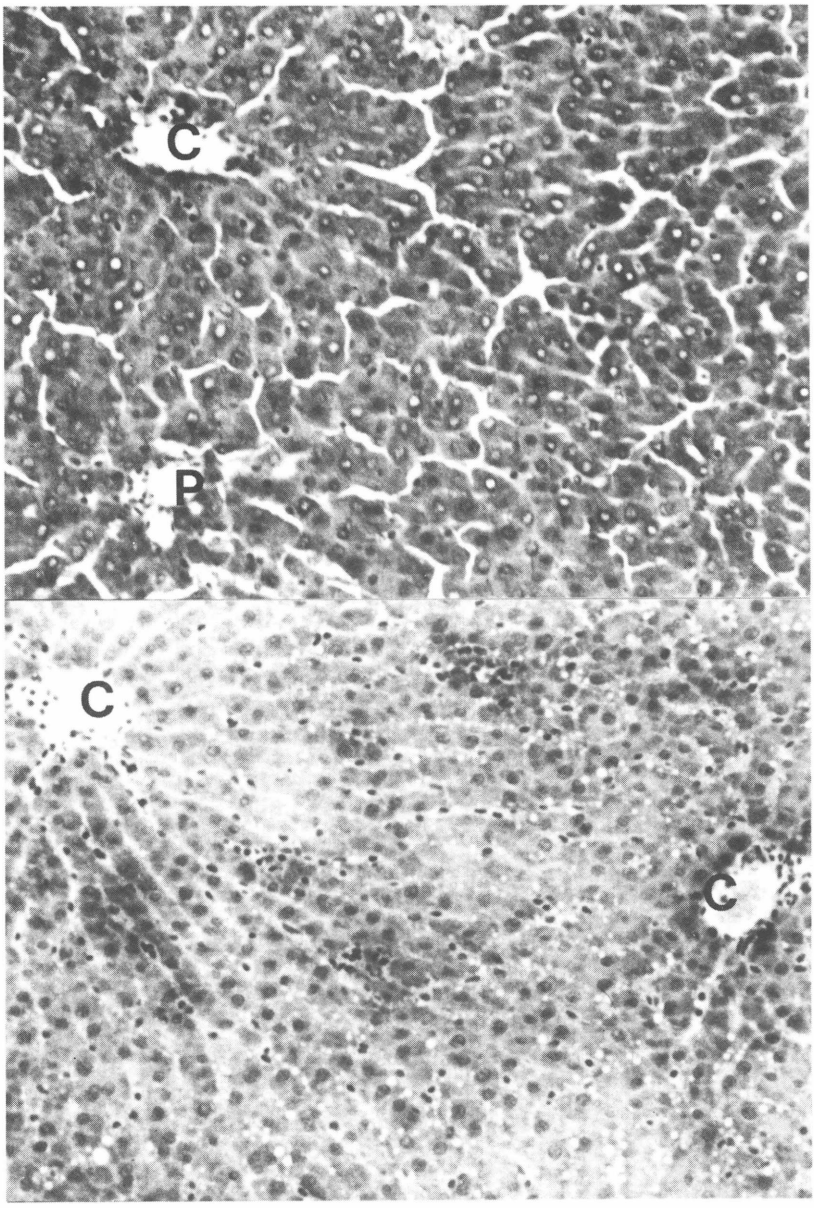


Figure 6. Immunocytochemical localization of P-450d in liver of MC-induced rats (*a*) before and (*b*) 6 h after exposure to CCl_4.

P-450d was visualized using the indirect two-stage immunoperoxidase method, with polyclonal antibody D1, specific to P-450d, as the first antibody. Results are typical of those observed on examination of five sections from each of three animals. C: Central vein; P: portal vein. (Magnification ×148.)

the liver of rats pretreated with MC, the monoclonal antibody to P-450c, C8, inhibited 85% of AHH activity, but did not affect POD activity (table 2). However, the antibody specific to P-450d, D1, inhibited 85% of hepatic POD activity, but had no effect on AHH activity. In contrast to the results obtained in liver, both renal AHH and POD activities of MC-treated rats were inhibited by >85% only

Table 2. Effect of antibodies on hepatic and renal aryl hydrocarbon hydroxylase (AHH) and phenacetin *O*-deethylase (POD) activities mediated by microsomal fractions from rats pretreated with MC.

		AHH activity		POD activity	
Tissue	Antibody	pmol/mg per min	% Control*	pmol/mg per min	% Control*
Liver	None	9,530	100	1,700	100
Liver	C9	10,000	105	1,860	110
Liver	C8	1,570	16·6	2,000	118
Liver	D1	10,490	110	254	15·0
Kidney	None	1,030	100	24·8	100
Kidney	C9	1,100	107	28·1	114
Kidney	C8	100	9·7	3·7	14·9
Kidney	D1	1,230	120	23·5	94·7

The results are means of duplicate determinations on pools containing tissues from three animals and are typical of five different experiments. Monoclonal antibodies C9 and C8, to rat P-450c, were used at a concentration of 1 mg Ig/nmol P-450 and a polyclonal antibody to rat P-450d, D1, was used at 15 mg Ig/nmol P-450.

*Activity has been expressed as a percentage of that of the control in the absence of antibody.

by the antibody specific to P-450c (C8). The antibody to P-450d had no effect on either of the renal activities.

Treatment of MC-induced rats with CCl_4 had very little effect on hepatic AHH activity, which decreased from $9{\cdot}5 \pm 0{\cdot}2$ nmol/mg per min to a minimum of $8{\cdot}0 \pm 0{\cdot}3$ nmol/mg per min 6 h after administration of CCl_4, a reduction of only 15% (figure 7a). In marked contrast, hepatic POD activity decreased from 1700 ± 357 pmol/mg per min to only 73 ± 10 pmol/mg per min, a reduction of 95%, over the same interval. The time-course for the loss of hepatic POD activity was parallel to that for the loss of immunologically determined content of P-450d. Indeed there was a highly significant correlation between POD activity and P-450d content ($r = 0{\cdot}947$, $P < 0{\cdot}001$). Renal activity was, on average, 10-fold lower than that of liver. AHH activity of microsomal fractions from kidney of MC-induced rats before treatment with CCl_4 was 1030 pmol/mg per min, and 6 h after exposure it was 858 pmol/mg per min, representing a 17% reduction, at most. Renal POD activity was 80-fold lower than that of the liver. Prior to CCl_4 treatment, activity was 24·8 pmol/mg per min and 6 h after exposure it was 28·1 pmol/mg per min. Thus, AHH activity was reduced by a maximum of only 17% in either hepatic or renal microsomes from MC-induced rats following their treatment with CCl_4, whereas POD activity was specifically reduced, by more than 95%, in hepatic microsomes (figure 6a) with no reduction in renal POD activity (figure 7b).

The effects of CCl_4 on monooxygenase activity and P-450 content were also determined *in vitro*, with hepatic microsomal fractions from rats pretreated with MC (table 3). Incubation with CCl_4 at 1 mM had no effect on total P-450 content, AHH or POD activities, but at 4 mM the total, spectrally determined P-450 content and the activity of POD were decreased by approximately 30%, in the absence of any decrease in AHH activity. Indeed, specific AHH activity increased somewhat following incubation with this concentration of CCl_4. When CCl_4 at 4 mM was incubated with microsomal fraction from rats treated with PB as a positive control, total P-450 content was decreased by 60% (data not shown), in agreement with a previous report of Davies *et al.* (1986).

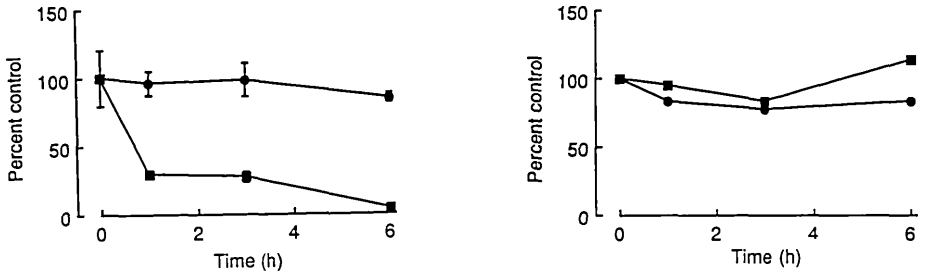


Figure 7. Effect of treatment of MC-induced rats with CCl_4 *in vivo* on (*a*) hepatic and (*b*) renal microsomal aryl hydrocarbon hydroxylase (●) and phenacetin *O*-deethylase (■) activities.

Values for liver are means ± SD of duplicate determinations of samples from three animals. Where an error bar is not visible it falls within the symbol. Values for kidney are means of duplicate determinations on the pooled tissue from three animals. Activities have been expressed as a percentage of control values, from animals that were not treated with CCl_4. Control activities were: hepatic AHH and POD, 9·5 ± 0·2 nmol/mg per min and 1700 ± 357 pmol/mg per min, respectively; renal AHH and POD activities, 1·03 nmol/mg per min and 24·8 pmol/mg per min, respectively. Animals were treated with MC (80 mg/kg) and then exposed to CCl_4 (800 mg/kg) for the times indicated.

Table 3. Effect of carbon tetrachloride *in vitro* on the total cytochrome P-450 content, and aryl hydrocarbon hydroxylase (AHH) and phenacetin *O*-deethylase (POD) activities of hepatic microsomal fractions from rats pretreated with MC.

CCl_4 (mM)	Cytochrome P-450 content	AHH activity	POD activity
0·0	100	100	100
0·5	93·9	111	119
1·0	84·4	104	113
2·0	76·4	110	63·7
3·0	67·0	107	67·6
4·0	70·3	129	74·2

The results were normalized for protein content and have been expressed as percentages of the control values, obtained by incubating samples with methanol only, in place of CCl_4. P-450 content, AHH and POD activities were determined as described in Methods. Control values (0·0 mM CCl_4) were as follows: P-450 content, 2·12 nmol/mg; AHH activity, 10,700 pmol/mg per min; POD activity, 1650 pmol/mg per min.

Discussion

It has been known for many years that CCl_4 is a suicide substrate for hepatic P-450, with a loss of both spectrally determined P-450 content and enzyme activity. The inactivation of P-450 is through the formation of a reactive intermediate, probably the trichloromethyl radical, by the reductive metabolism of the halocarbon (Poyer *et al.* 1980). There is now good evidence that haem is both the site of and the target for the suicidal activation of CCl_4 by P-450 (Manno *et al.* 1988). The alkylated haem products bind covalently to the P-450 apoprotein. This, possibly in combination with direct alkylation of the protein by a reactive metabolite of CCl_4 and/or peroxidative damage to the microsomal membrane, results in a specific loss of the holoenzyme of P-450.

A number of studies have suggested that a specific isoenzyme of P-450 is inactivated by CCl_4. Some of the early data were obtained in studies of the hepatotoxicity of CCl_4. Thus, it was shown that PB treatment of rats increases the toxicity of CCl_4, whereas MC treatment has little effect (Shah and Carlson 1975) or even slightly reduces both the toxicity of the compound (Suarez *et al.* 1972) and the loss in monooxygenase activity (Stripp *et al.* 1972). The involvement of PB-inducible forms of P-450 was shown more directly by Noguchi *et al.* (1982 a, 1982 b). More recently, it has been shown that the ethanol-inducible form of P-450, P-450j, is very effective in catalysing the activation of CCl_4 (Gadeholt 1984, English and Anders 1985, Johansson *et al.* 1988).

Evidence for a selective effect of CCl_4 on P-450d in PAH-induced rats came from a study by Enosawa and Nakazawa (1986), who showed that the lighter of the two PAH-inducible proteins in rat liver was selectively lost following treatment of the animals with CCl_4, yet AHH activity was unaffected. These observations were confirmed and extended by Degawa *et al.* (1987), who showed that P-450d is, indeed, preferentially decreased by treatment of MC-treated rats with CCl_4.

In the present study we confirmed the dramatic and selective loss of P-450d from the liver of MC-induced rats following treatment with CCl_4 *in vivo*, in the absence of any marked change in the level of P-450c. The time course for the loss of P-450d was broadly in agreement with that found by Degawa *et al.* (1987). However, these authors found some discrepancy between the results obtained with two different antibodies to P-450d, which was attributed to differential lability of the epitopes. We have made a similar observation, in that the loss of P-450d determined immunologically by Western blotting with a polyclonal antibody was not as great as that found with a monoclonal antibody (unpublished data). Despite this, the M_r of P-450d was identical when determined with the two antibodies. Thus, the P-450d remaining within the microsomal membrane does not undergo fragmentation due to exposure to CCl_4. It should also be noted that the loss of putative P-450d reported by Enosawa and Nakazawa (1986) was determined by chromatography of the solubilized proteins. Thus, there does appear to be substantial loss of P-450d from the microsomal membrane following CCl_4 treatment.

It is of interest that catalytic activity specific to P-450d, the *O*-deethylation of phenacetin, in hepatic microsomes from CCl_4-treated MC-induced rats declines in parallel with the loss of P-450d assessed with an inhibitory monoclonal antibody. It is thus possible that the epitope on P-450d with which MAb 12/2/3/2 interacts is the site, or one of the sites, inactivated by CCl_4.

CCl_4 treatment of MC-induced rats decreases hepatic total P-450 content by 60%. This is more than the total amount of P-450d present in these microsomes. Thus, it seems likely that forms of P-450, other than P-450d, are also inactivated in such animals. The other major form present is P-450c. However, this isoenzyme showed no decrease in amount or activity. Indeed, the slight increase in specific content of P-450c over the 6 h following administration of CCl_4 may be due to the relative enrichment of the microsomal membranes with this protein, as other proteins are lost. Over the 6 h there was a 30% decrease in specific microsomal protein content. Evidence for the loss of other forms of P-450 from hepatic microsomes was provided earlier by Enosawa and Nakazawa (1986), who showed a decrease in both ethylmorphine demethylase and aniline hydroxylase activities.

Immunocytochemical studies showed that both P-450c and P-450d are distributed evenly throughout the liver lobule of rats treated with MC. The pattern of

staining for P-450c was not affected by exposure of the animals to CCl_4. Thus, there is no sub-population of cells in the liver in which P-450c is more susceptible to the effects of CCl_4 than in others. However, this is not so for P-450d. Staining for this isoenzyme shows greater loss from centrilobular and midzonal regions than from periportal regions. Although these regions are also those most sensitive to the cytotoxic effects of CCl_4, it is unlikely that the two are causally related. However, there may be a common link, such as zonal oxygen tension. Similarly, Moody *et al.* (1986) have presented immunohistochemical evidence for the specific loss, following CCl_4 intoxication, of cytochromes P-450b from PB-treated rats, and P-450a and $P\text{-}450_{UN}$ from untreated rats from hepatocytes primarily in the centrilobular region. Immunocytochemistry of the kidney confirmed the lack of effect of CCl_4 treatment on P-450c content of this tissue.

POD activity of liver microsomes from MC-treated rats is selectively inhibited by an antibody against P-450d. The involvement of P-450d in catalysing POD activity is confirmed by the almost complete loss of this activity following CCl_4 treatment. However, POD activity of kidney microsomes is not inhibited by an antibody to P-450d, whereas an antibody to P-450c almost completely inhibits this activity, in addition to AHH activity. CCl_4 treatment of MC-induced rats causes a modest reduction in renal P-450 content but has very little effect on the amount of P-450c present. This is echoed by the lack of effect that CCl_4 treatment has on either AHH or POD activity of the kidney. Thus, POD activity in kidney is catalysed by P-450c. It is also apparent that CCl_4 treatment causes the loss of forms of P-450, other than P-450c, from the kidney.

The reason for the selective destruction of P-450d and not P-450c is not known. It has been shown that CCl_4 is not metabolized by P450c (Masuda and Yasoshima 1987), which could explain why this isoenzyme is spared. However, it is not known whether CCl_4 is a substrate for P-450d. This does seem the most likely explanation, the halomethane then acting as a suicide substrate.

MC treatment of rats does not increase the hepatotoxicity of CCl_4. Thus, if CCl_4 is indeed activated by P-450d then either the turnover number for this reaction is much lower than that for P-450j and P-450b, or the fate of the reactive metabolite generated by P-450d is different from that produced by other isoenzymes of P-450. It is possible that the radical produced interacts with P-450d more readily than with other isoenzymes, thus preventing its escape from its site of formation to cause toxicity. From the studies *in vitro* it appears that P-450d does indeed have a lower turnover rate for CCl_4 than P-450b. Thus, *in vivo* P-450d may activate CCl_4 at a relatively low rate, but once formed the radical would then interact very readily with the isoenzyme, inactivating it. An alternative possibility is that the radical is formed by another form of P-450, neither P-450c nor P-450d, and that this, or a further product such as a reactive oxygen species, then diffuses from its site of formation to selectively destroy P-450d, whilst not affecting P-450c, which appears to be relatively stable to oxygen radical damage.

The present study has demonstrated the value of the CCl_4-treated MC-induced rat in determining substrate and isoenzyme specificity of the two members of the P-450IA gene subfamily. Using this model we have confirmed the lack of involvement of P-450c in catalysing high-affinity POD activity in the liver, and have obtained further evidence for the sole involvement of P-450d in this reaction.

Acknowledgements

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