REGULATION AND EXPRESSION OF CYTOCHROME 
P450 4A1 AND ITS RELATIONSHIP TO PEROXISOME 
PROLIFERATION.

A thesis submitted in accordance with the requirements of the University of Surrey 
for the degree of Doctor of Philosophy

by

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Dedicated to my Dad
and
in loving memory of my Mum.
Summary

1. Species differences in elements of the peroxisome proliferator domain (PPD), including the cytochrome P450 4A subfamily of fatty acid hydroxylases and peroxisomal fatty acid β-oxidation have been examined.

2. Microsomal preparations from a panel of human livers showed cross-reactivity with a sheep anti-sera to rat cytochrome P450 4A1 and gave a single band on Western blot analysis with a relative $M_r$ of approximately 51kDa (compared to approximately 51.5kDa for the rat). Spectral analysis of ferrous-CO complexes revealed peak maxima between 450-452nm with the total cytochrome P450 specific content and NADPH-cytochrome P450 reductase activity displaying about three and two-fold variations, respectively. Immunoquantification of the cytochrome P450 4A1 human orthologue by an ELISA protocol gave between 4-12% of total cytochrome P450 content. NADPH-fortified human liver microsomal preparations from all the samples used both laurate and arachidonate as substrate and formed the ω- and (ω-1)- metabolites at varying rates. Moreover, anti-rat P450 4A1-enriched IgG fraction inhibited the human ω-lauiate hydroxylase activity to greater than 55% (20% in the rat) at a concentration of 5mg/nmol total cytochrome P450. Taken collectively, the information thus presented is strongly suggestive of the expression of a member(s) of the cytochrome P450 4A subfamily in the panel of human liver tissues examined.

3. To further elucidate the mechanism of cytochrome P450 4A1 induction and peroxisome proliferation by a diversity of peroxisome proliferators, I also examined the stereochemical specificity in the induction of members of the peroxisome proliferation domain by optically active enantiomers of a clofibrate-analogue. In all the parameters examined, the R(-)- enantiomer was identified as the eutomer and its corresponding S(+) -antipode as the distomer, with the racemic mixture intermediate in inducing these enzyme activities.
4. The effect of a relatively newly described peroxisome proliferator, perfluoro-n-decanoic acid (PFDA), on the peroxisome proliferation domain in a responsive (rat) and a non-responsive (guinea pig) species was also investigated. At the dose level investigated (20 mg/ kg), PFDA administration did not appear to impair food intake nor weight gain but resulted in distinct differences in the hepatic and renal response between the two species examined. In the rat, hepatomegaly was observed with the coordinate induction of total cytochrome P450 levels (1.5 fold), NADPH-cytochrome P450 reductase (1.5-fold), palmitoyl CoA oxidase (6-fold), carnitine acetyl transferase (peroxisomal and mitochondrial, 15-fold) and preferential cytochrome P450 4A1-mediated ω-laurate hydroxylase activity (5-fold). In addition, a substantial inhibition in the activity of cytochrome P450 1A1-associated enzyme activity as measured by ethoxyresorufin-O-deethylase (EROD) activity was observed in the rat liver (74%) and kidney (39%). Western blotting analysis of hepatic and renal microsomes for cytochromes P450 4A1 and 1A1 isozymes reflected the above changes. A similar analysis showed significant induction of the trifunctional protein of the rat hepatic peroxisomal β-oxidation spiral. Generally, the guinea pig appeared to be non-responsive at this dose level. However, a significant inhibition of the EROD activity was detected in both the liver (35%) and kidney (31%). A similar pattern of results was obtained for the PROD activity in both tissues. Generally, the rat kidney was less responsive than the liver.

5. The ligand-binding site for peroxisome proliferators in the peroxisome proliferator-activated receptor (PPAR) has been computer-modelled and used as a basis to discuss the structural diversity of peroxisome proliferators.

6. Species differences in response to peroxisome proliferators have been discussed in terms of lipid homoeostasis and/or regulatory elements of the relevant genes.
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Almighty God I acknowledge your works with greatness!

E.C. Chinje

September, 1992
CHAPTER ONE

General Introduction
General Introduction

1.1 Nomenclature and Multiplicity of Cytochrome P450 Gene Superfamily

What is now known as the cytochrome P450 gene superfamily (EC 1.14.14.1) encodes numerous enzymes that are remarkable in the variety of chemical reactions catalysed and the number of substrates handled [Guengerich, 1991]. They comprise a family of b-type haemoproteins with an identical prosthetic group and mechanism of catalysis, but different apoprotein structures which are responsible for the different substrate specificities. Cytochrome P450 evolution is believed to have begun 2-3 billion years ago, with only a few genes coding P450 forms that were engaged in the metabolism of endogenous substrates such as steroids, fatty acids and eicosanoids [Nebert et al., 1991]. Humans and rats are believed to have diverged from common ancestors some 80 million years ago, with one of the consequences of the human cytochrome P450 gene evolution being the polymorphism of drug metabolism, leading to marked differences in the response of individuals to the toxic and carcinogenic effects of drugs and other environmental chemicals [Guengerich, 1989; Nebert et al., 1991].

Indeed no exaggeration is made in stating that cytochrome P450 is the most versatile and abundant biological catalyst known, considering the rapid progress that has been made in recent years in the characterisation of over 150 isoforms. There are presently 154 P450 genes and seven putative pseudogenes that have been described in 23 eukaryotes (including nine mammalian and one plant species) and in six prokaryotes [Nebert et al., 1991]. Of at least 27 gene families described so far, 10 exist in all mammals comprising 18 subfamilies or clusters of genes, of which 16 have been mapped on the human gene.
Most of the cytochrome P450's were discovered primarily through protein purification research, which meant many of them demonstrated broad and overlapping substrate specificities which precluded establishing a nomenclature system based upon distinct catalytic functions. However, within the last decade the isolation and sequencing of P450 proteins, genes and cDNAs have facilitated the development of a P450 classification system based on primary amino acid sequence alignment data [Nebert and Gonzalez, 1987].

Since many of the individual cytochrome P450's catalyse multiple reactions, the incompatibility of individual designation and symbols for identical forms resulted in a joint effort to devise a more systematic nomenclature based on structural homology [Nebert et al., 1991]. This system of nomenclature is constantly evolving as more P450's are isolated and characterised. According to this classification scheme, those cytochrome P450 proteins with 40% or greater sequence homology are included in the same family (designated by an Arabic number), and those with greater than 55% identity are included in the same subfamily (designated by a Capital letter). The individual genes are arbitrarily assigned numbers in order of their discovery. However, even two very similar rat forms CYPs 2B1 and 2B2, with less than 3% difference in sequence homology, have distinct genes. The main advantage of the unified nomenclature is that structurally identical or highly similar P450's are easily recognisable, regardless of the source (species, tissue, or organelle), the inducer, or the catalytic activity examined.

Our knowledge of the scope of cytochrome P450 catalysed reactions is still incomplete as these haemoproteins are widespread in nature and many isoforms have yet to be fully characterised or even identified. Mammalian cytochromes P450 can be roughly divided into two major groups based on their intracellular location and the enzyme from which they receive electrons [Gonzalez, 1990]. On the one hand, the mitochondrial P450s, such as the side-chain cleavage enzyme (cytochrome
P450 scc) and steroid 11 β-hydroxylase (cytochrome P450 11β) synthesised by membrane-free polyribosomes [Nabi et al., 1983] are found in the adrenal cortex and are involved in steroid hormone biosynthesis, whereas the second group found primarily in the endoplasmic reticulum (i.e. membrane bound) are synthesised by membrane-bound polyribosomes [Gonzalez and Kasper, 1980].

The orientation of eukaryotic cytochromes P450 with respect to the membrane of the endoplasmic reticulum has also been investigated [Edwards et al., 1991] and results from such studies suggest that cytochrome P450 is most likely oriented such that the haem is not fixed horizontally to the plane of the membrane. This may be relevant to the intramolecular movements of cytochrome P450 during its catalytic cycle and the partitioning of substrates for cytochrome P450 between the cytosol and membrane.

1.2 Cytochrome P450 4 Family Proteins

An unusual aspect of some forms of cytochrome P450 is their capacity to preferentially oxidise primary rather than secondary or tertiary carbon-hydrogen bonds. This is particularly evident for fatty acid substrates such as lauric acid, arachidonic acid and the eicosanoids where the hydroxylation of the terminal carbon is referred to as ω—hydroxylation [Kupfer, 1980]. The ω—hydroxylated fatty acids are subsequently oxidised further to dicarboxylic acids [Bjökhem, 1976], which are elevated in several species during ketotic states such as starvation or diabetes [Horie et al., 1981]. These lipid substrates are additionally hydroxylated by several members of other cytochrome P450 isoenzymes at various positions in the alkyl chain, and it appears that hydroxylation at the terminal carbon atom is most characteristic of the cytochrome P450 4A subfamily.

The cytochrome P450 enzymes that catalyse ω—hydroxylation reactions appear to comprise a distinct family of haemoproteins belonging to the cytochrome P450 4 family, in the uniform system of nomenclature [Nebert et al., 1991; Gibson and
Lake, 1991]. The complementary DNAs of this family of proteins have been isolated from cDNA libraries from liver, kidney, prostate and lung of various species including the rat, mouse, rabbit and human [Gibson and Lake, 1991]. Some of these P450 gene/ protein designates are shown in Table 1.1. The cytochrome P450 4A subfamily consists of eleven members to date arranged in three subfamilies and all eleven of the corresponding genes have been sequenced and their amino acid sequences predicted. Of these eleven gene designates, nine are cDNAs and only two (P450 4A1 and 4A2) are derived from genomic libraries. As such, these contain introns and 5' upstream flanking regions, the latter containing putative regulatory sites that may interact with its inducer.

A cDNA corresponding to a rat liver lauric acid ω—hydroxylase, P450 4A1, has been cloned and characterised in the livers of clofibrate-treated rats [Hardwick et al., 1987; Earnshaw et al., 1988]. In addition, two genomic clones for P450 4A2 and 4A3 have been isolated [Kimura et al., 1989a; 1989b]. The mRNAs for these three forms were also induced in the kidney by clofibrate. Moreover, cytochrome P450 4A2 was constitutively expressed at a higher level in the kidney and was only slightly induced by clofibrate treatment in this tissue [Kimura et al., 1989b].

A rabbit lung isozyme, which is involved in the ω—hydroxylation of prostaglandins and induced during pregnancy or after progesterone treatment, showed close similarities to the derived amino acid sequence of the rat cytochrome P450 4A1 and was designated cytochrome P450 4A4 [Matsubara et al., 1987; Nebert et al., 1989]. However, preparations of cytochrome P450 4A4 exhibit negligible lauric acid ω—hydroxylase activity [Yamamoto et al., 1984], suggesting that members of the cytochrome P450 4A subfamily, distinct from cytochrome P450 4A4, may be expressed in the rabbit. This led to the isolation of cDNAs for three rabbit kidney lauric acid ω—hydroxylases denoted cytochromes P450 4A5, 4A6 and 4A7 [Johnson et al., 1990]. A similar or possibly identical protein to cytochrome P450 4A4 has been isolated from livers of pregnant rabbits [Kikuta et
<table>
<thead>
<tr>
<th>P450 Genes/ protein designation</th>
<th>Species</th>
<th>Tissue</th>
<th>References a</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP 4A 1</td>
<td>rat</td>
<td>liver, kidney</td>
<td>Hardwick et al., 1987; Earnshaw et al., 1988</td>
</tr>
<tr>
<td>4A 2</td>
<td>rat</td>
<td>liver, kidney</td>
<td>Kimura et al., 1989a</td>
</tr>
<tr>
<td>4A 3</td>
<td>rat</td>
<td>liver, kidney</td>
<td>Kimura et al., 1989b; Aoyama et al., 1990</td>
</tr>
<tr>
<td>4A 4</td>
<td>rabbit</td>
<td>lung, kidney, liver, uterus, placenta</td>
<td>Matsubara et al., 1987; Kikuta et al., 1989</td>
</tr>
<tr>
<td>4A 5</td>
<td>rabbit</td>
<td>liver, kidney, small intestine</td>
<td>Johnson et al., 1990</td>
</tr>
<tr>
<td>4A 6</td>
<td>rabbit</td>
<td>kidney, liver</td>
<td>..</td>
</tr>
<tr>
<td>4A 7</td>
<td>rabbit</td>
<td>kidney</td>
<td>..</td>
</tr>
<tr>
<td>4A 8</td>
<td>rat</td>
<td>prostate, kidney</td>
<td>Stromstedt et al., 1990</td>
</tr>
<tr>
<td>4A 9</td>
<td>human</td>
<td>liver</td>
<td>Hardwick, J.P., 1991 [personal communication]</td>
</tr>
<tr>
<td>4B 1</td>
<td>rat, human, rabbit</td>
<td>lung, kidney, intestine</td>
<td>Nhamburo et al., 1989; Gasser and Philpot, 1989</td>
</tr>
<tr>
<td>4C1</td>
<td>cockroach</td>
<td>fat body</td>
<td>Bradfield et al., 1991</td>
</tr>
</tbody>
</table>

a Reference with complete cDNA and amino acid sequence, regulation of gene expression stated.
Another member of the cytochrome P450 4A subfamily has been very recently tentatively identified in human liver [Hardwick et al., 1991, personal communication] and designated cytochrome P450 4A9.

Based on structural information derived from a nucleotide sequence, a cytochrome P450 designated isozyme 5, remarkably similar to the rabbit cytochrome P450p_2 [Gasser and Philpot, 1989], is also expressed in the rat and placed in a previously unrecognised cytochrome P450 4B gene subfamily. Indeed, the conservation of primary structure seen with the cytochrome P450 4B members was estimated at 5 to 15% greater than between other pairs of cytochrome P450 isozymes from rat and rabbit that have been assigned to the same gene subfamilies [Matsubara et al., 1987]. More recently, a human form was isolated from a lung cDNA library using the rat CYP 4A1 cDNA as a probe [Nhamburo et al., 1989]. The cDNA-deduced amino acid sequence of this P450, designated 4B1, consisted of 511 amino acids and coded for a protein of relative M_r 59.5 kDa with 51%, 53%, and 52% amino acid homologies to CYP 4A1, 4A2, and rabbit P450p_2, respectively. However, the protein was shown only to be constitutively expressed in the lung but could not be identified in the livers of test patients.

The finding that the sequence derived for a human pulmonary cytochrome P450 4B isozyme [Nhamburo et al., 1989; Gonzalez, 1989, personal communication] is 81% identical to the sequence of rabbit isozyme 5, provides additional evidence of extensive conservation of structure within the cytochrome P450 4B subfamily [Vanderslice et al., 1987]. Cytochrome P450 4B subfamily proteins mainly mediate the pulmonary N-oxidation of aromatic amines such as the promutagen 2-aminofluorene. Unlike the rabbit orthologous form, human cytochrome P450 4B1 was unable to metabolise this compound despite the presence of cytochrome P450 4B1 mRNA in three out of four lungs analysed [Nhamburo et al., 1989]. In contrast to its expression in lung, cytochrome P450 4B1 mRNA was undetectable in livers obtained from 14 individuals (including those from which the lungs were
Similarly, cytochrome P450 4B1-related mRNA was also expressed in rat lung but was undetected in untreated rat liver. Expression of cytochrome P450 4B1 in rabbit but not rat or human liver suggests an interesting species difference in tissue-specific expression of this gene. Yet another newly identified member of the cytochrome P450 4 family has been reported in the cockroach (Blaberus discoidalis) and the gene represented by the cloned cDNA has been named CYP4Cl [Bradfield et al., 1991]. The sequence of this cDNA is 32-36% identical to mammalian family 4A and 4B enzymes and contains a 13-residue sequence characteristic of the cytochrome P450 4 family suggesting that substrates for these enzymes might be closely related. It is mainly present in the fat bodies and the physiological role for cytochrome P450 4C1 is highly speculative but the mRNA level is known to be elevated by both the hypertrehalosemic hormone (HTH) and starvation. A principal function of HTH is stimulation of fat body glycogenolysis, thus providing precursors for the formation of trehalose, the main circulating carbohydrate in insects. Bradfield and co-workers concluded from their studies that cytochrome P450 4C1 may be hormonally regulated in association with energy substrate mobilization. However, its protein catalytic activity and regulation of the corresponding gene by HTH still remains to be ascertained.

A model for the positioning of lauric acid in the active site of cytochrome P450 4A1 has been proposed [CaJacob et al., 1988] and is depicted in Figure 1.1. It has been suggested that the active site of lauric acid ω-hydroxylase is highly structured in the vicinity of the activated oxygen and sterically suppresses (ω-1)-hydroxylation in order to deliver oxygen to the thermodynamically disfavoured terminal carbon of the methyl group. This is probably achieved by the active site cleft allowing the terminal methyl group to reach the haem's activated oxygen. Furthermore, tolerance of varying lengths of fatty acids suggests that the terminal methyl specificity is not governed by specific interactions of the protein with the acid's carboxyl group, but by steric constraints within the catalytic site.
1.3 *Cytochrome P450 Catalysed Reactions*

The cytochrome P450-mediated mixed-function monooxygenases of liver microsomal membranes are of central importance in the metabolism of a variety of xenobiotics, including substances that occur biologically but are foreign to animals, such as antibiotics and unusual compounds in plants, vitamins as well as many drugs, carcinogens, synthetic organic chemicals, environmental pollutants and a variety of steroids and physiologically occurring lipids [Cheng and Schenkman, 1983, 1984; Ryan *et al.*, 1982; Ryan and Levin, 1990; Boddupalli *et al.*, 1990; Guengerich, 1991]. These enzyme systems are also present in many other tissues and in a wide range of organisms including animals, plants and microorganisms [Kato, 1979; Nebert *et al.*, 1991].

The principal role of cytochromes P450 in the metabolism of xenobiotics is to introduce a polar hydroxyl group into the xenobiotic molecule, thus providing a "handle" for the phase II or conjugating reactions which introduce additional polar groups, usually of endogenous origin. This then facilitates the elimination of such unwanted metabolites from the body which otherwise would be retained in the body thereby causing deleterious effects. Hence because of the existence of multiple forms of cytochromes P450 in any single organism, they may afford quite a strong physiological and defensive ability to handle a wide range of substrates. Some of the cytochromes P450, such as those involved in steroid and fatty acid biotransformations are fairly selective in their choice of substrates with substantial regio- and stereo- selectivity, whereas others particularly those in liver microsomes, have unusually broad and overlapping substrate specificity.

Most of the cytochrome P450 catalysed reactions begin with the transfer of electrons from NAD(P)H to either NADPH-cytochrome P450 reductase and in some cases cytochrome b5 in the microsomal system (or a ferredoxin reductase and a non-haem iron protein in the mitochondrial and bacterial systems), and then to cytochrome P450, thus leading to the reductive activation of molecular oxygen.
followed by the insertion of one oxygen atom into the substrate. The scheme presented (Figure 1.2) is in accord with the established stoichiometry of the cytochrome P450 catalysed hydroxylation reaction, where RH represents the substrate.

\[
\text{RH} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NADP}^+ 
\]

The scheme begins with the binding of the substrate (RH), which perturbs the spin state equilibrium of the cytochrome P450, thereby facilitating the uptake of the first electron. It is suggested that substrates that undergo reduction rather than oxygenation, such as epoxides, accept two electrons in a stepwise fashion to yield \(\text{RH}(\text{H}_2)\) [Porter and Coon, 1991].

To initiate the oxidative reactions, \(\text{O}_2\) is bound to the ferrous cytochrome P450 to form an intermediate, \(\text{Fe}^{3+}(\text{O}_2^-)\), with the substrate still attached. Transfer of the second electron then occurs, with the possible involvement of cytochrome b5 as an additional electron donor in mammalian microsomal systems [Schenkman et al., 1976]. The next step involves splitting of the oxygen-oxygen bond with the uptake of two protons (\(2\text{H}^+\)) and the formation of an "activated oxygen" species and simultaneous release of water. It has been suggested that oxygen insertion into the substrate involves hydrogen abstraction from the substrate and recombination of the resulting transient hydroxy and carbon radicals to give the product [Groves et al., 1978] but this is still debatable as the identity of the powerful oxidant that is necessary for oxygen insertion into the substrates is still not known. The cycle is completed with the dissociation of the product (ROH), which restores the cytochrome P450 to the starting ferric state ready to handle yet another substrate molecule.
Figure 1.2 Microsomal cytochrome P450-mediating electron transport cycle. Fe$^{3+}$ represents oxidised cytochrome P450; RH, substrate; RH(H)$_2$, a reduction product; ROH, a monooxygenation product, and XOOH, a peroxo compound that can serve as an alternative oxygen donor. (Reproduced from Porter and Coon, 1991).
1.4 Pleiotropic Response to the Administration of Peroxisome Proliferators

Administration of several groups of structurally diverse compounds including hypolipidaemic agents such as clofibrate, do cause different characteristic changes in the morphology and biochemistry of the liver in susceptible species such as the rodents and more particularly the mouse and rat [Reddy et al., 1982a]. These hypolipidaemic agents among other classes of compounds such as the phthalate esters, fatty acid analogues and high fat diets, especially those rich in very-long chain fatty acids (VLCFA), are termed peroxisome proliferators (PPs) as they lead to the proliferation of this organelle. Examples of some peroxisome proliferators are listed in Table 1.2. and generally, the prolonged use of some of these compounds as lipid-lowering drugs has been associated with occasional side-effects of varying magnitude in humans (Table 1.3). Some of the proliferative effects of these compounds are summarised below.

1.4.1 Hepatomegaly

The pleioptropic responses observed following the administration of peroxisome proliferators may either be hypertrophic i.e associated with organelle proliferation or hyperplastic due to increase in cell number and size which may all be dependent on the dose and duration of treatment [Beckett et al., 1972]. Usually the first response visible is hepatomegaly and is reflected in a significant increase in the liver to body weight ratio [Hess et al., 1965]. Also associated with such response, is mid-zonal and periportal accumulation of lipid droplets and an increase in phospholipid and total protein levels in the liver [Azarnoff et al., 1965, Mann et al., 1985]. It would appear that administration of hypolipidaemic agents characteristically results in hepatomegaly of a hypertrophic and hyperplastic nature. Hypertrophy may be associated with a combined proliferation of the smooth
Table 1.2 Uses of some compounds that are peroxisomal proliferators

<table>
<thead>
<tr>
<th>Peroxisome proliferator</th>
<th>Use</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Drugs</strong></td>
<td></td>
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<tr>
<td>Clofibrate</td>
<td></td>
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<tr>
<td>Ciprofibrate</td>
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<tr>
<td>Bezafibrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>Anti-inflammatory</td>
<td>Oesch and Schladt, 1987</td>
</tr>
<tr>
<td>POCA</td>
<td>Hypoglycaemic</td>
<td>Bone et al., 1982</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>Tranquillizer</td>
<td>Vamecq et al., 1987</td>
</tr>
<tr>
<td>1-Benzylimidazole</td>
<td>Anti-fungal</td>
<td></td>
</tr>
<tr>
<td>Valproic acid</td>
<td>Anti-epileptic</td>
<td>Horie and Suga, 1985</td>
</tr>
<tr>
<td><strong>Industrial Chemicals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isooctane</td>
<td>Industrial solvent</td>
<td>Lock et al., 1987</td>
</tr>
<tr>
<td>Prudhoe Bay crude oil</td>
<td>Crude petroleum mix</td>
<td>Khan et al., 1989</td>
</tr>
<tr>
<td>Perfluorinated fatty acids</td>
<td>Surfactants, flame retardants</td>
<td>Ikeda et al., 1986</td>
</tr>
<tr>
<td><strong>Pesticides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tridipane, 2,4-D, 2,4,5-T</td>
<td>Herbicide</td>
<td>Lundgren et al., 1987</td>
</tr>
<tr>
<td>Lactofen</td>
<td>Herbicide</td>
<td></td>
</tr>
<tr>
<td>Dimethrin</td>
<td>Insecticide</td>
<td>Hruban et al., 1974</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEHP, DEHA</td>
<td>Plasticizers</td>
<td>Sharma et al., 1988b</td>
</tr>
<tr>
<td>Citral, Linolol</td>
<td>Food flavouring</td>
<td>Roffey et al., 1990</td>
</tr>
</tbody>
</table>

Abbreviations used: POCA, 2-[5-(4-chlorophenyl) pentyl] oxirane-2-carboxylate; DEHP, di-(2-ethylhexyl) phthalate; DEHA, di-(2-ethylhexyl) adipate.
Table 1.3  Drug management of hyperlipidaemia

<table>
<thead>
<tr>
<th>Hypolipidaemic Agent</th>
<th>Parameter Affected</th>
<th>Side Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clofibrate</td>
<td>Decreases in triglycerides, cholesterol, VLDL, LDL, fibrinogen levels as well as increases in HDL and fibrinolysis</td>
<td>Nausea, diarrhoea, skin rash, cardiac arrhythmias, synergism with oral anti-coagulants</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>Decreases in VLDL and triglycerides, increase in HDL</td>
<td>Diarrhoea, nausea, abdominal pain, synergism with oral anti-coagulants</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>Decreases in triglycerides and cholesterol levels</td>
<td>Abdominal discomfort, nausea, rarely pruritus</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>Decreases in triglycerides, cholesterol, LDL, VLDL and increase in HDL</td>
<td>Dyspepsia, nausea, pruritus, cholestatic jaundice and hyperuricaemia</td>
</tr>
<tr>
<td>Probufol</td>
<td>Decrease in HDL, LDL and cholesterol synthesis</td>
<td>Nausea, vomiting, abdominal pain, diarrhoea</td>
</tr>
<tr>
<td>Dextrothyroxine</td>
<td>Decrease in cholesterol levels</td>
<td>Angina in patients with pre-existing sub-clinical coronary artery disease, tachycardia</td>
</tr>
</tbody>
</table>

Abbreviations used: VLDL, very low density lipoproteins; LDL, low density lipoproteins and HDL, high density lipoproteins.
endoplasmic reticulum and a contributory increase in mitochondrial numbers [Hess et al., 1965].

Liver enlargement occurs rapidly in response to administration of peroxisome proliferators (such as clofibrate, Wy-14643, methylclofenepate and ciprofibrate), reaching a steady state level within two weeks, and is maintained throughout treatment, returning to pretreatment levels within a similar period of time [Reddy and Lalwani, 1983; Rao and Reddy, 1987]. Following clofibrate pretreatment in the rat, the relative liver weight increased rapidly during the first week and fell to pretreatment levels after withdrawal of the drug at three to four weeks [Lake et al., 1975].

1.4.2 Peroxisomal and Mitochondrial Changes

Treatment of rats with peroxisome proliferators results in several observable changes in hepatic peroxisomes and mitochondria, which include an increase in both peroxisome numbers and volume, proliferation of the mitochondria, structural changes, enlargement and induction of certain enzymes [Reddy and Lalwani, 1983; Gibson et al., 1982; Svoboda and Azamoff, 1966]. These proteins, including among others the cytochrome P450 4A subfamily of fatty acid hydroxylases, members of the peroxisomal β-oxidation spiral as well as the cytosolic fatty acid binding protein, have been classified as belonging to a distinct class, termed the peroxisome proliferator domain (PPD) [Watanabe et al., 1985]. The peroxisome may account for up to 25% of the hepatocyte cytoplasmic volume in the livers of rats and mice treated with potent peroxisome proliferators such as ciprofibrate [Rao and Reddy, 1987]. This represents a 10-20 fold increase which is also reflected in the induction of some enzymes of the peroxisomal β-oxidation system [Reddy et al., 1986].
Catalase activity is only slightly induced, often not more than 2-fold and the activity of carnitine acetyl transferase, a peroxisomal enzyme which is also located in the mitochondrial membrane, may be induced to a much greater extent (which could be several hundred fold), than that of peroxisomal β-oxidation under similar experimental conditions [Stott, 1988]. Likewise, carnitine octanoyltransferase, a purely peroxisomal enzyme, can be induced over 40-fold by DEHP [Miyazawa et al., 1983].

Increases in the activity of acyl-CoA synthetase, carnitine acetyltransferase, carnitine palmitoyltransferase and acyl-CoA hydrolase have been observed in hepatic mitochondria isolated from rats treated with peroxisome proliferators [Leighton et al., 1982; Berge et al., 1983]. A slight induction in the mitochondrial β-oxidation of short-chain acyl-carnitines has been demonstrated in clofibrate-treated rats [Christiansen et al., 1978]. Clofibrate administration has also been shown to inhibit branched-chain amino acid metabolism in the mitochondria [Wagenmakers et al., 1985].

Several other hepatic peroxisomal and mitochondrial enzymes are also affected in varying degrees and are well documented [Hess et al., 1965; Cohen and Grasso, 1981; Antonenkov et al., 1983]. Similarly, cytosolic changes have been reported. For instance, inhibition of cytosolic glutathione peroxidase, glutathione transferase and superoxide dismutase have been observed when rats have been treated with a number of peroxisome proliferators for a period of 30 days or more [Lake et al., 1987, 1989]. The administration of peroxisome proliferators to rats also induces certain cytosolic proteins, namely palmitoyl-CoA hydrolase, cytosolic epoxide hydrolase and fatty-acid binding protein [Berge et al., 1987; Moody et al., 1985, 1991]. The proportion of catalase in rat liver cytosol is also increased and may reflect an altered fluidity of the peroxisomal membrane [Crane et al., 1985].
1.5 Perfluorocarboxylic Acid Derivatives and Peroxisome Proliferation.

Interest in the toxicity of the perfluorocarbons and their carboxylic acid derivatives began when a non-ionic form of fluoride consistent with the presence of a fluorocarbon was found in the serum of humans [Taves, 1968]. Subsequent studies led to its isolation and identification as perfluorooctanoic acid (PFOA), [Guy et al., 1976]. Generally, perfluorocarbons are considered metabolically inert and relatively non-toxic compounds [Sargent and Seffl, 1970]. Recently, however, perfluorinated compounds such as perfluorooctanoic acid (PFOA), perfluorodecanoic acid (PFDA), and ammonium perfluorooctanoate have been shown to be peroxisome proliferators [Ikeda et al., 1985; Pastoor et al., 1987; Harrison et al., 1988; Borges et al., 1992]. Hence, these compounds like the other well characterised peroxisome proliferators, such as the hypolipidaemic drugs (clofibrate, fenofibrate and structurally-related analogues) [Hawkins et al., 1987, Milton et al., 1990; Chinje and Gibson, 1991], plasticizers (phthalate derivatives), [Sharma et al., 1988b] and chlorophenoxy acid herbicides [Bacher and Gibson, 1988], do induce hepatomegaly, endoplasmic reticulum, and peroxisomal proliferation as well as peroxisomal and mitochondrial metabolism of fatty acids. Collectively, these chemicals are classified as peroxisome proliferators and concern has been expressed about their safety because they are non-genotoxic carcinogens in susceptible species, particularly rodents [Reddy et al., 1980; Reddy and Lalwani, 1983; Rao and Reddy, 1987], probably as a result of indirect DNA damage caused by oxidative stress mechanisms [Elliot et al., 1986; Takagi et al., 1990].

Studies on the induction of peroxisome proliferation by perfluorinated fatty acids and their analogues (Figure 1.3 ) has also shown the importance of chain length and the presence of a carboxylic acid group in the molecule [Ikeda et al., 1985]. Because of their general metabolic inertness, they are considered as ideal for structure activity relationship (SAR) studies. For instance following a single i.p.
Figure 1.3 Structures of some perfluoro fatty acids and their derivatives.
dose, PFDA, PFOA and 1-H,1-H-pentadecafluoro-n-octanol (PFOL) were shown to be inducers of the peroxisome proliferation-associated polypeptide (PPA-80) and lauric acid ω—hydroxylase, whereas perfluoro-n-butyric acid (PFBA) and the perfluorinated paraffins, perfluorododecane (PFD) and perfluoroctane (PFO) did not induce these enzymes. Similarly, several chain length perfluorinated fatty acids have been examined for their ability to inhibit mitochondrial acyl-CoA synthetase (ACS) [Vanden Heuvel et al., 1991]. ACS is an enzyme essential for both oxidation and esterification of fatty acids. Short-chain perfluorinated fatty acids (perfluoropropionic and perfluorobutyric acids) did not inhibit ACS activity, whereas medium-chain perfluorinated acids, such as perfluoroctanoic and perfluorodecanoic acids, were found to be potent inhibitors of ACS in isolated mitochondria. However, these workers did not establish whether ACS inhibition was causally related to PFDA-induced peroxisome proliferation and altered lipid metabolism seen in vivo.

The biological effects of the perfluoro-fatty acids appear to be of a persistent nature [Van Rafelghen et al., 1987; George and Andersen, 1986] and exposure to rodents have been demonstrated to give rise to a peculiar "wasting syndrome" resulting in severe weight loss and hypothermia [Langley, 1990]. It has been reported that the body weights of rats provided with a diet containing PFDA ceased to increase, even though these animals consumed as much food as their control counterparts [Borges et al., 1990]. More recent studies carried out by Borges et al. [1992], showed that rats treated with cumulative doses of PFDA < 12 mg/kg did not differ from their pair-fed control counterparts, in growth or feed intake, while those which received cumulative doses of > 40 mg/kg lost weight and decreased their feed intake. Similarly, total peroxisomal β-oxidation was decreased in a dose-related manner, whereas the liver to body weight ratio and activities of the individual enzymes comprising the peroxisomal β-oxidation system, namely fatty
acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and thiolase were increased.

It has also been reported that rats treated with doses of PFDA between 40 and 150 mg/kg exhibit delayed lethalities occurring 2-3 weeks after treatment with the xenobiotic [Osln and Andersen, 1983]. The target organ of PFDA-induced toxicity is the liver, although thymic atrophy, fatty changes in the proximal tubular epithelium of the kidney and testicular seminiferous tubular degeneration have also been reported [Van Rafelghem et al., 1982, Ikeda et al., 1985; George and Andersen, 1986; Harrison et al., 1988]

Comparative toxicity of PFOA with its 10-carbon analogue PFDA, revealed that the latter was more toxic having a 30-day LD₅₀ of approximately 41 mg/kg, which was considerably less than that for PFOA (189 mg/kg) [Osln and Andersen, 1983]. Moreover, delayed lethalities occurring 2-3 weeks following PFDA administration at doses between 40 and 150 mg/kg were not seen with PFOA administration. PFDA is poorly metabolized by rats and is excreted at the rate of about 0.5% of the administered dose per day [Vanden Heuvel et al., 1991; Ylinen and Auriola, 1990], the urinary elimination being a minor route of elimination. In an experiment carried out by Vanden Heuvel and co-workers, in which the percentage dose of PFDA-derived ^14C per gram tissue was estimated after a single intraperitoneal dose of 9.4 mmol [1-^14C] PFDA/ kg, it was observed that 24h after administration, the liver (5.9%) contained 10-fold that of the kidney (0.62%). A similar distribution pattern was maintained for up to 4 weeks with the liver still having the highest distribution (3%), followed by the plasma (0.32%), kidney (0.25%), heart (0.2%), epididymal fat pad (0.1%) and testis (0.05%).

A single i.p dose of PFDA (50 mg/kg) administered to Sprague-Dawley rats caused disruption of the endoplasmic reticulum, mitochondrial swelling and increases in intracellular lipid droplets in hepatocytes [Harrison et al., 1988]. These
effects were quite similar to those reported for 2,3,7,7-tetrachlorodibenzo-p-dioxin, (TCDD), however, dioxin does not cause peroxisome proliferation. PFDA administration to rats has also been shown to rather increase the plasma triglyceride levels while causing a decrease in plasma cholesterol levels. This finding was in contrast to that observed for other classical peroxisome proliferators, such as the hypolipidaemic agent, clofibrate and has led to the classification of PFDA as a "non-hypotriglyceridaemic" peroxisome proliferator [Borges et al., 1990].

One of the current hypothesis concerning the signal for peroxisome proliferation is based on the formation of a thioester between the proliferator and coenzyme A [Lock et al., 1989]. However, it remains to be demonstrated if PFDA and other analogues, such as perfluorooctanoic acid (PFOA), are also linked to this mechanism. Perfluorooctane and perfluorodecane do not appear to cause peroxisome proliferation in rat liver [Ikeda et al., 1985], suggesting that the carboxylate group may be essential in triggering peroxisome proliferation. PFDA and PFOA may both act in a similar manner, as an uncoupler of oxidative phosphorylation and as an inhibitor of the electron transport chain, as observed in rats [Langley, 1990].

1.6 Role of Peroxisomes and Mitochondria in β-Oxidation of Fatty Acids

Peroxisomes often occur in close association with elements of the endoplasmic reticulum [Zaar et al., 1987], highlighting the earlier belief that peroxisomes were formed by budding from the endoplasmic reticulum [De Duve and Baudhuin, 1966]. However, the polypeptide composition of the peroxisomal membrane and the endoplasmic reticulum have been shown to be markedly dissimilar [Fujiki et al., 1982]. The current model of peroxisome biogenesis suggests that peroxisomes are formed by fission from pre-existing organelles [Yamamoto and Fahimi, 1987;
Fujiki et al., 1984]. Unlike the enzymatic complement of many other organelles, the enzymology of the peroxisome may vary considerably from one tissue to another and between species.

Generally peroxisomes provide a compartment for alternative pathways in the metabolism of fatty acids [Lock et al., 1989]. In several tissues examined so far, three main functional cellular compartments that are responsible for fatty acid oxidation, have been well distinguished. These include (i) the extra-mitochondrial compartment comprising the endoplasmic reticulum, the cytosol and the outer mitochondrial membrane, (ii) the peroxisomes, and (iii) the mitochondrial matrix. The metabolic function of these different compartments varies depending on the fatty acid chain length as depicted in Figure 1.4. Peroxisomes participate in a number of specific metabolic pathways that lead to energy production and oxidative metabolism [De Duve and Baudhuin, 1966].

Although the reactions involved during peroxisomal β-oxidation are identical to those of mitochondrial β-oxidation, the enzymes are quite distinct from their mitochondrial counterparts. One outstanding difference is that fatty acids are incompletely oxidised by peroxisomes and long chain acyl-CoA has been found to undergo 2-5 cycles of β-oxidation [Lazarow and De Duve, 1976; Osmundsen, 1982]. Since C_{22} fatty acids are poor substrates for mitochondria, chain shortening of these fatty acids leads to fatty acids which are more easily oxidised by the mitochondria. Peroxisomal β-oxidation of these fatty acids therefore becomes a mechanism by which poorly oxidisable fatty acids are converted to metabolically more degradable products. As this is achieved by chain shortening, rather than complete oxidation, a minimum of metabolic energy is wasted. It has also been suggested that β-oxidation in peroxisomes may be a chain-shortening mechanism for long-chain dicarboxylic acids formed during fatty acid ω—oxidation [Hemmelgarn et al., 1977].
Figure 1.4 Compartmentation of fatty acid metabolism in the liver.

Abbreviations used: FFA, free fatty acids; Glyc.P, glycero-3-phosphate; P-lip., phospholipids; Ac-CoA, acetyl-CoA; AcAc, acetooacetate; β-ox., β-oxidation. The circles across the mitochondrial membrane represent the carnitine-dependent transport of acetyl and long-chain acyl groups from extramitochondrial to intramitochondrial CoA. (Derived from Bremer and Osmundsen, 1984).
In terms of capacity to generate acetyl groups, the capacity of the peroxisomes is relatively low, being around 10% of the mitochondrial capacity in rats treated with clofibrate [Osmundsen, 1982; Neat et al., 1981]. However, if chain-shortening of fatty acids is considered a major function of peroxisomal β-oxidation, then it is the peroxisomal capacity to chain shorten e.g. a C22:1 fatty acid which is significant. The peroxisomal capacity to chain shorten a C22:1 fatty acid may be as much as 100-300% of the mitochondrial capacity to oxidise this fatty acid [Bremer and Osmundsen, 1984]. Hence, there appears to be a peroxisomal capacity which would be of significant physiological value. It has been reported that patients suffering from the inheritable disease adrenoleukodystrophy show accumulation of very long-chain fatty acids (C25 and C26) in adrenals as well as in the central nervous system, probably due to a defect in peroxisomal β-oxidation [Singh et al., 1981].

Subsequent identification of additional peroxisomal enzymes led to further speculation on the metabolic role of these organelles. A number of enzymes such as oxidases, dehydrogenases, catalase, acyl-transferases and those of the fatty acid oxidation cycle have been identified in peroxisomes isolated from a variety of plants and animals. Beta-oxidation of both mono- and di-carboxylic fatty acids [Lazarow, 1982] are due to the presence of four main enzymes namely: acyl CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase (Figure 1.5). The last three are found on the so-called trifunctional protein [Goel et al., 1986] or the peroxisome proliferator-associated protein 80,000 daltons (PPA-80) [Osumi and Hashimoto, 1979].

The enzymes of peroxisomal β-oxidation differ from mitochondrial enzymes with respect to their catalytic and molecular properties. For instance, it has been shown that, unlike the carnitine-dependent cyanide-sensitive mitochondrial system, peroxisomal β-oxidation enzymes are cyanide-insensitive in vitro and do not require carnitine [Lazarow, 1982]. The discovery of carnitine acetyltransferase in
Figure 1.5 β-Oxidation sequence in peroxisomes.
peroxisomes was of special interest because this enzyme was considered to be primarily located in the mitochondria. Various chain lengths of the acyl transferase have been described ranging from short- through medium- to long- chain acyltransferases. These acyltransferases and in particular, the short- and medium-chain lengths have seen their barely detectable levels of activity in livers of untreated rats and mice, increase between 30 - 160 fold in animals following treatment with peroxisome proliferators [Moody and Reddy, 1974; 1978]. It has been suggested that these transferases may play a role in the removal of end products of metabolism in the peroxisomes [Lazarow, 1982].

Peroxisome proliferation appears to be a tissue and species specific phenomenon. The liver is the most susceptible organ, though the kidney, heart, intestinal mucosa and skeletal muscle all respond depending on the dose and duration of exposure to peroxisome proliferators [Stott, 1988]. The induction of mitochondrial and peroxisomal enzymes involved in the \(\beta\)-oxidation of fatty acids, in conjunction with other known cellular responses, points to a common mechanism involved in their hypolipidaemic effect.

1.7 **Induction of Cytochrome P450 4A Subfamily Proteins and Their Role in the Metabolism of Endogenous Substrates.**

The cytochromes P450 associated with the microsomal fraction of the liver and other organs such as the kidney and lung, catalyse the NADPH- and oxygen-dependent metabolism of a variety of lipophilic foreign and endogenous compounds. Many of these cytochromes P450 are present at very low but measurable constitutive levels in untreated rats and may be under hormonal regulation [Schenkman et al., 1989]. They may also be induced several fold by a variety of structurally diverse foreign compounds [Guengerich, 1991; Boobis and Davies, 1984].
Associated with smooth endoplasmic reticulum proliferation is the induction of one of the most active cytochrome P450 fatty acid-metabolising haemoproteins, designated cytochrome P450 4A1 [Hardwick et al., 1987; Tamburini et al., 1984; Gibson et al., 1982]. The metabolism of lauric acid, as substrate, to its $\omega$— and $(\omega-1)$-hydroxyl metabolites has been attributed to the activity of this isozyme [Gibson, 1989; Sharma et al., 1989]. Consideration of the fact that the concentration of both free and esterified laurate is low in the hepatic endoplasmic reticulum membrane was indicative of the presence of other endogenous substrates. The involvement of cytochromes P450 in the metabolism of arachidonic acid to a wide variety of oxygenated products, has been well documented [Sharma et al., 1989; Oliw and Oates, 1981; Capdevila et al., 1985; Chacos et al., 1983].

Arachidonic acid is an important constituent of biological membranes and is almost invariably found esterified to the 2-position of various cellular glycerophospholipids. Phospholipases of the A$_2$-type are responsible for hydrolysing this fatty acid thereby releasing it into the cell medium. The actions of lipoxygenases, cyclooxygenases, lead to the production of intermediates that serve as precursors of a variety of compounds with potent physiological and pharmacological properties. Such by-products include the prostaglandins, thromboxanes, prostacyclins, hydroxyeicosatetraenoates and leukotrienes.

Arachidonic acid also serves as an excellent substrate for the clofibrate-induced cytochrome P450 4A1 hydroxylase system in the rat [Bains et al., 1985; Sharma et al., 1989]. Pretreatment of rats with ciprofibrate resulted in an 8-fold stimulation of $\omega$— and $(\omega-1)$-oxidation of arachidonic acid with a simultaneous net decrease in the formation of the other oxygenated metabolites [Capdevila et al., 1985]. Recently, it was reported that $\omega$— and $(\omega-1)$-hydroxyarachidonates had potent biological activities [Schwartzman et al., 1989]. Whereas $\omega$—hydroxyarachidonic acid shows properties of a vasoconstrictor, the $(\omega-1)$-product, has been demonstrated as a stimulator of Na$^+$/ K$^+$- ATPase.
It would seem plausible that members of the cytochrome P450 4A subfamily proteins with \( \omega-1 \) (\( \omega-1 \))-hydroxylase activities, are capable on the basis of broad substrate specificity to metabolise other substrates containing \( \omega-1 \) and \( \omega-1 \)-carbon atoms. Interestingly, this is not the case with the leukotrienes (e.g. leukotriene B\(_4\), found in polymorphonuclear leukocytes), which serve as important mediators of the inflammatory process [Samuelsson, 1983]. Studies on the metabolism of leukotriene B\(_4\) (LTB\(_4\)) suggest that this is a relatively poor substrate for both the renal and hepatic cytochrome P450 4A1-dependent enzyme system as it was demonstrated that both the \( \omega-1 \) and \( \omega-1 \)-LTB\(_4\) hydroxylase activities were decreased in both tissues following pretreatment of rats with clofibrate [Sharma et al., 1989]. Such findings prompted these workers to suggest that the induction or specific gene "switch on" of laurate hydroxylase by clofibrate appeared to result in a specific gene "switch off" of LTB\(_4\) hydroxylase.

LTB\(_4\) hydroxylases are present in rat liver and kidney as constitutive isozymes of cytochrome P450 but are distinct from those which mediate the hydroxylation of laurate and possibly, prostaglandins [Romano et al., 1987]. Similarly, it has been suggested that prostaglandin hydroxylases are distinct from laurate hydroxylases and possibly LTB\(_4\) hydroxylases [Matsubara et al., 1987]. The isolation and sequencing of a prostaglandin \( \omega-1 \)-hydroxylase from rabbit lung (cytochrome P450 p-2) 4A4, showing about 74% amino acid sequence homology with that of laurate \( \omega-1 \)-hydroxylase (cytochrome P450 4A1), prompted its classification into a new cytochrome P450 gene subfamily, CYP 4B1 [Nhamburo et al., 1989].

As one would expect because of the multiplicity of cytochrome P450, there is considerable diversity in the mechanisms of regulation of these enzymes. Transcriptional regulation is the most common means of gene regulation and has been reported for a number of mammalian cytochrome P450 isozymes, the most extensively characterised being cytochrome P450 1A1 [Nebert and Jones, 1989]. This is the only cytochrome P450 for which a receptor-mediated mechanism of
induction has been clearly demonstrated via an Ah or TCDD receptor [Poland et al., 1976; Fujisawa-Sehara et al., 1987]. Another form of regulation may be post-translational. The relative proportion of a particular form of P450 and its associated enzyme activities are determined by multiple steps ranging from gene transcription, through mRNA stabilisation to enzyme stabilisation via translation. In contrast to P450 1A1, which is primarily regulated by transcriptional control, 1A2, a closely related isoform is regulated for the most part [Kimura et al., 1986], apparently through enhanced stability and intranuclear processing of the 1A2 mRNA precursor [Silver and Krauter, 1990].

There is considerable documentation of mRNA stabilisation as a mechanism of induction for several other cytochromes P450, as exemplified by the ethanol-inducible cytochrome P450 2E1. This isozyme is induced by other small molecules such as acetol, acetone, pyrazole and is thought to be via specific protein stabilisation effects of these molecules [Eliasson et al., 1990; Song et al., 1989]. Other factors such as diabetes and fasting have each been reported to produce up to a 10-fold elevation in cytochrome P450 2E1 mRNA, the increase with diabetes being as a result of mRNA stabilisation [Song et al., 1987] and that by fasting, due to gene transcription [Johansson et al., 1990].

1.8 Peroxisome Proliferators and Hepatocarcinogenesis

A good correlation exists between the incidence of liver cancer and the effectiveness of the administered dose in inducing peroxisomes [Reddy and Lalwani, 1983]. These workers were able to show that the genesis of tumours in rats and mice by peroxisome proliferators requires continuous exposure for 60 to greater than 500 days depending upon the potency of the carcinogen and that once tumour formation has begun, its progression is not inhibited by cessation of exposure to the carcinogen. Numerous studies indicate that these agents are not mutagenic and do
not interact directly with nor damage DNA [Butterworth et al., 1984; Glauert et al., 1984; Warren et al., 1980]. More recently, Schiestl, [1989], has developed a system for chromosomal recombination in yeast which is inducible by a number of non-genotoxic carcinogens and a number of hypolipidaemic peroxisome proliferators tested in this system, were without effect [Schiestl and Reddy, 1990], thus supporting the notion that peroxisome proliferators are truly non-mutagenic carcinogens. Subchronic treatment of rodents with a variety of structurally diverse compounds leads to hepatic peroxisome proliferation, whilst life-time feeding with such compounds is associated with an elevated incidence of hepatocellular carcinomas [Reddy et al., 1980], a finding which prompted these latter workers to suggest that peroxisome proliferators form a novel class of carcinogens.

In view of the heterogeneous nature of agents which induce peroxisome proliferation and their widespread and varied use, human exposure to such compounds is inevitable. In general, peroxisome proliferators are not considered genotoxic i.e they are not thought to interact directly with cellular DNA. This is borne out by the fact that several such agents did not induce DNA-repair in cultured human lymphocytes [Warren et al., 1980] nor could covalent binding to hepatocellular DNA be demonstrated [Goel et al., 1985]. It was due to such considerations that led to the postulation that peroxisome proliferators may induce DNA damage by indirect mechanisms [Reddy et al., 1980]. Furthermore, no carcinogenic peroxisome proliferators, including the hypolipidaemic drugs and phthalate plasticizers displayed any capacity to covalently modify or damage cellular DNA either in vivo or in vitro [Glauert et al., 1984; Linnainman, 1984]. The carcinogenicity of these non-mutagenic and non-DNA adduct-forming peroxisome proliferators appears to be related to biologically active products of the proliferated peroxisomes rather than a direct chemical effect.
1.8.1 The Cellular Oxidative Stress Hypothesis

The oxidative stress hypothesis was advanced to explain how peroxisome proliferation may result in hepatocellular carcinomas. Long-term administration of peroxisome proliferators to rats and mice is often associated with increased incidences of hepatocellular carcinomas [Reddy et al., 1980; Rao and Reddy, 1987]. To date, all peroxisome proliferators that have been tested have been shown to produce hepatic tumours in responsive species such as the rat and mouse. In the proposed model (Figure 1.6), the enhanced production of hydrogen peroxide (via the peroxisomal fatty acid β-oxidation system, see earlier) overcomes the ability of peroxisomal catalase, or other hydrogen peroxide degrading enzymes and results in a slow accumulation of oxidative damage to the genome. Evidence for this model has gained support from a number of laboratories which have demonstrated in vitro damage to DNA associated with proliferated peroxisomes [Fahl et al., 1984], increased production of hydroxy radical by proliferated peroxisomes [Elliot et al., 1986] and a close association between lipofuscin accumulation and carcinogenesis of peroxisome proliferators [Conway et al., 1989; Reddy et al., 1982a].

Persistent increase in the formation of 8-hydroxydeoxyguanosine was observed in rats continuously fed a diet containing the peroxisome proliferator, ciprofibrate, for 16 to 20 weeks [Kasai et al., 1989]. Strikingly this could not be found within 3 to 4 hours after a single dose of the same peroxisome proliferator, suggesting that persistent peroxisome proliferation is required for the formation of oxidative damage to DNA. Similar response in the formation of 8-hydroxydeoxyguanosine was observed in rat liver and not the kidney when treated with both DEHP and DEHA for 1 and 2 weeks, respectively [Takagi et al., 1990].

The oxidative stress is related to the tissue/ cell specific increase in mRNAs of the β-oxidation system and this cell specificity is attributed to specific recognition factors in responsive cells. This provides compelling evidence that hepatocellular carcinoma formation in laboratory animals treated with peroxisome proliferators is
Figure 1.6 The oxidative stress hypothesis for peroxisome proliferator-induced hepatocarcinogenicity. (Derived from Reddy and Lalwani, 1983; Kasai et al., 1989).
directly linked to the persistent increase in peroxisomes. At the present time, there is no conclusive evidence of a causal link between peroxisome proliferation associated oxidative stress and hepatocarcinogenicity. There is, however, good evidence of a strong association between these processes. The findings by some workers using hepatocytes, have indicated that induction of peroxisomal $\beta$-oxidation and stimulation of DNA synthesis do not always go hand-in-hand [Bieri et al., 1988]. This observation is supported by further findings that cast doubt on the correlation between peroxisome proliferation and neoplasia [Marsman et al., 1988]. The studies carried out by these researchers was based on a previous report that rats fed with DEHP at 1.2% in the diet produced a 10% incidence of hepatocellular carcinoma after two years, whereas Wy-14,643 at 0.1% in the diet resulted in a 100% incidence after 15 months [Kluwe et al., 1982; Lalwani et al., 1981]. The lack of a quantitative correlation between the degree of peroxisome proliferation to hepatocarcinogenicity in such study, despite little differences in the magnitude of peroxisomal enzyme induction between the two compounds, suggests that measurements of peroxisome proliferation by such class of xenobiotics are not reliable predictors of the carcinogenic potential of these chemicals. Consequently, in any study of the species differences on the effect of peroxisome proliferators, the induction of hepatocellular DNA synthesis should also be taken into account.

### 1.8.2 Peroxisome Proliferators as Tumour Promoters

The requirement for long-term continuous feeding of peroxisome proliferators to produce tumours and their non-genotoxic nature suggest that non-traditional mechanisms may be involved in the mechanism of carcinogenesis. Peroxisome proliferators have been tested for their promotion effects in a number of studies [Conway et al., 1989; Cattley and Popp, 1989; Kraup-Grasl et al., 1990, 1991]. In general, they act as positive promoters when studies were carried out to sufficient length for tumour formation to be used as the end-point, but gave variable results in
short-term studies which only measured altered cell foci formation. The eventual neoplasia is a result of selection and clonal expansion of these pre-initiated focii in older rodents which are generally more susceptible to hepatocarcinogenesis by peroxisome proliferators (Kraup-Grasl et al., 1991). However, such reports have to be rationalised with work indicating that some of these tumour promoters can act as inhibitors [Staubli et al., 1984; Numoto et al., 1984; Popp et al., 1985]. Increased levels of oncogene products have been observed within 28 days of rat treatment with nafenopin and have been implicated in the process of tumour formation [Bentley et al., 1988], although the precise details and molecular mechanisms of regulation remains to be established.

Peroxisome proliferators as tumour promoters provide an alternative proposal to explain how peroxisome proliferation may result in hepatocellular carcinomas. In this model, it is proposed that peroxisome proliferators may act through promotion of spontaneously initiated foci in the liver [Cattley and Popp, 1989; Glauert et al., 1986; Schulte-Hermann et al., 1983; Ward et al., 1986]. Quite recently, the promotional activities of the peroxisome proliferators have been associated with their ability to stimulate foci or tumour growth through localised increases in cell replication [Marsman et al., 1988]. This proposal merits further study and may be valid, offering a unique form of initiation-promotion.

The use of initiation-promotion protocols is providing greater insight into the carcinogenic action of peroxisome proliferators [Garvey et al., 1987; Ward et al., 1986; Cattley et al., 1989, 1991; Glauert and Clark, 1989]. To date, the results of initiation-promotion and hepatocyte replication studies suggest that peroxisomal proliferating chemicals may be causing promotion of spontaneously initiated sites rather than initiating the hepatocarcinogenic response in rodents. Therefore, there is need for further clarification as to the involvement of peroxisome proliferators in the rodent model before clear health assessment issues for humans can be established.
The importance of cell signalling in peroxisome proliferation is only just beginning to emerge [Bronfman et al., 1986, 1989; Itoga et al., 1990; Watanabe et al., 1991]. Protein kinase C (Ca$^{2+}$-activated, phospholipid-dependent protein kinase; pkc) has been implicated in tumorigenesis by the observation that this protein represents the major cellular receptor for tumour-promoting phorbol esters [Nishizuka, 1984]. It has been suggested that the carcinogenic effects of many peroxisome proliferators are activated to their corresponding CoA thioesters and that the latter (but not the free acids) were able to stimulate protein kinase C, which may result in the phosphorylation of regulatory proteins [Bronfman et al., 1986, 1989]. However, the significance of this process is not known.

It is well established that protein kinase C activity depends on the presence of Ca$^{2+}$ and it has recently been shown that the Ca$^{2+}$ antagonist, nicardipine, suppresses peroxisome proliferation and cytochrome P450 4A1 induction [Itoga et al., 1990, Watanabe et al., 1991]. It is highly likely that this suppression may be due to a decreased activity of protein kinase C. Furthermore, rats treated with clofibrate, and other hypolipidaemic drugs, show an increased liver concentration of natural long chain acyl-CoAs [Berge and Aarsland, 1985], which would also help to stimulate protein kinase C activity.

1.8.3 Proposed Mechanisms for the Induction of Peroxisome Proliferation

The exact underlying cellular mechanism responsible for the induction of peroxisome proliferation and associated enzyme activities, remains to be fully ascertained. A number of theories have been put forward which remain to be fully explored.
i) Receptor-mediated hypothesis for carcinogenesis

The mechanism by which structurally diverse peroxisome proliferators produce a similar pleiotropic response is not known but the tissue specific biological response and rapid rate of transcription of peroxisomal fatty acid β-oxidation enzyme genes support the hypothesis that these agents act by binding to a specific recognition molecule. The diverse nature of the chemical structures of the agents known to produce essentially the same pleiotropic response makes it difficult to explain how they interact with a single type of binding protein having a single recognition site. If more than one type of receptor molecule exists, then these must converge on a common final pathway to induce similar biological and biochemical effects in hepatocytes. The demonstration of a reversible, specific, albeit weak binding of nafenopin, a peroxisome proliferator, to a cytosolic protein in rat liver [Lalwani et al., 1983] led to the proposal that peroxisome proliferators evoke their action possibly by a receptor-mediated mechanism [Reddy and Lalwani, 1983; Rao and Reddy, 1987].

Circumstantial evidence supporting the existence of a specific receptor for the xenobiotic peroxisome proliferator have been previously reported [Fournel et al., 1985; Lundgren et al., 1987, 1988]. It was also suggested that the peroxisome proliferator binding protein (PPbp), purified from rat liver cytosol by affinity and ion-exchange chromatography [Lalwani et al., 1987], acts in a similar manner to that proposed for the Ah receptor [Nebert, 1979]. In such a scheme, it is postulated that the peroxisome proliferators enter the cell by passive diffusion through the plasma membrane and then bind to the receptor inside the cell. The binding of the ligand activates the receptor and this ligand-receptor complex then binds with increased affinity to selective site(s) in cellular DNA, thereby possibly triggering the transcription of the genes of the peroxisome proliferator domain. However, extensive studies by other research groups in exploiting the above observation about a peroxisome proliferator receptor were unsuccessful and the identity and nature of
this postulated receptor remain unknown with a number of questions being raised about the existence of such a receptor [Milton et al., 1988; Siest et al., 1988].

More research has since been devoted to further characterising the postulated cytosolic receptor [Alvares et al., 1990]. These workers were able to show that this protein is homologous with the 'Heat shock' protein HS P70 family by analysis of amino acid sequences of isolated peptides from trypsin-treated clofibrate acid binding protein and by cross-reactivity with a monoclonal antibody raised against the conserved region of the 70 kDa heat shock protein. Furthermore, the clofibrate acid-sepharose column could bind HS P70 protein isolated from various species, which could then be eluted with either clofibrate acid or ATP, suggesting that clofibrate acid preferentially interacts with P72 (a constitutively expressed member of the HS P70 family) at or near the ATP binding site. It remains to be shown whether these interactions are directly linked to the events that lead to peroxisome proliferator-induced liver pathophysiology. Nevertheless, the isolation of a single protein with high affinity for three fibric acid analogues favourably argues for a specific binding protein. Additional work is required to ascertain if binding occurs in vivo and if this plays a role in transcriptional activation.

The presence of a peroxisome proliferator binding protein and the ability of peroxisome proliferators to activate gene transcription suggested that the mechanism by which these chemicals act could be similar to that of steroid hormone action [Reddy and Rao, 1986]. Quite recently, there have been more convincing reports on the existence of a receptor which could be activated by the presence of some of the well known peroxisome proliferators, which should by and large explain the mechanism by which peroxisome proliferators regulate gene expression and induce liver tumours in rodents [Green, 1992]. The identification of such a putative transcription factor specifically activated by peroxisome proliferators suggest that this receptor directly mediates the effects of this class of chemical [Issemann and Green, 1990, 1991]. These workers, by screening a mouse liver cDNA library
using a consensus probe derived from the combined nucleotide sequences of several nuclear hormone receptors, were able to identify four new members of the hormone receptor family. The nuclear hormone receptor superfamily comprises at least 25 mammalian genes that encode receptors for the classical steroid and thyroid hormones [Carson-Jurica, 1990; Evans, 1988]. It further demonstrated that one of the four newly identified members of the hormone receptor family could be activated by the addition of a variety of peroxisome proliferators and therefore termed Peroxisome Proliferator-Activated Receptor (PPAR) [Issemann and Green, 1991]. A possible role in the regulation of responsive genes by this receptor subsequent to the administration of a peroxisome proliferator is depicted in Figure 1.7. According to this scheme, the receptor-ligand complex is thought to switch-on acyl-CoA oxidase gene expression by the interaction with putative regulatory elements in the 5' flanking region of the gene. The precise molecular details of this gene regulation are only now beginning to emerge and it is theoretically possible that the peroxisome proliferator-PPAR complex or a lipid-PPAR complex regulates the corresponding responsive genes.

There is tissue specific expression of PPAR and the highest reported levels of expression are observed in the liver and to a lesser extent in brown adipose tissue, kidney, heart, brain and testis. It will be important to determine whether all of the effects of peroxisome proliferators are receptor-mediated. If they are, then learning more about the role and function of PPAR presents an exciting and unique opportunity to understand more about the role of peroxisome proliferators in hypolipidaemia, peroxisome proliferation and etiology of cancer. Furthermore, examination of the expression and function of PPAR in humans could have important implications in assessing the hazard that peroxisome proliferators may represent to the human population.
Figure 1.7 A possible mechanism of action of the peroxisome proliferator-activated receptor (PPAR) in hepatic pleiotropic responses to peroxisome proliferators.
ii) The "Cascade" or Substrate Overload Theory

Early suggestions indicated that an influx of fatty acids into the liver as a result of treatment leads to a lipid excess, therefore triggering peroxisomal \( \beta \)-oxidation [Reddy and Lalwani, 1983]. Fatty acids serve as substrates for the microsomal cytochrome P450 hydroxylase system as well as for peroxisomal \( \beta \)-oxidation, and both activities undergo substantial induction in hypolipidaemic-treated animals.

The close relationship between the induction of cytochrome P450 4A1 and the endoplasmic reticulum on the one hand, and peroxisomal and mitochondrial enzyme induction on the other, led to an alternative proposed mechanism for the co-induction of hepatic microsomal cytochrome P450 4A1 and peroxisomal \( \beta \)-oxidation enzymes by hypolipidaemic agents [Sharma et al., 1988b; Lock et al., 1989]. In this scheme (Figure 1.8), the hypolipidaemic agent or peroxisome proliferator is taken up by the hepatocyte followed by initially inhibition of fatty acid oxidation by the dual mechanism of inhibition of carnitine acyl transferase in the mitochondrion [Lock et al., 1989] or sequestration of essential CoA by the peroxisome proliferator itself [Bronfman et al., 1989]. Induction of cytochrome P450 4A1 synthesis then occurs as an early event, possibly via a receptor as discussed above. This is supported by the observation that there is an increase in the rate of cytochrome P450 4A1 gene transcription as early as 1hr after the administration of clofibrate [Hardwick et al., 1987]. This increase was then followed by an elevation of cytochrome P450 4A1 mRNA immunochemically detectable protein, and its associated catalytic activity [Hardwick et al., 1987; Earnshaw et al., 1988; Milton et al., 1990]. This specific sequence of events has also been demonstrated in vitro in rat hepatocyte primary culture [Bieri et al., 1991].

Subsequent to the induction of cytochrome P450 4A1 by these hypolipidaemic agents, there is an enhanced \( \omega-1 \)-hydroxylation of medium- and long-chain monocarboxylic fatty acids, such as lauric acid and arachidonic acid. Further
Figure 1.8 Possible inter-relationship between cytochrome P450 4A1 induction and peroxisome proliferation. (Derived from Sharma et al., 1988a and Lock et al., 1989).
metabolism of these hydroxy monocarboxylic acids into their keto-derivatives and dicarboxylic acids, is catalysed by cytosolic alcohol and aldehyde dehydrogenases [Mitz and Heinrikson, 1961; Robbins, 1968]. It is then proposed that these dicarboxylic acids are then taken up by the peroxisome, thus presenting the organelle with a substrate overload of one of its preferred substrates [Singh et al., 1984], because the mitochondrion cannot readily metabolise long chain fatty acids [Alexson and Cannon, 1984]

The hypolipidaemic drug induction of peroxisomal β-oxidation enzymes may be considered as a secondary adaptive cellular response by the hepatocyte in an attempt to clear long chain dicarboxylic fatty acids for subsequent chain shortening by the mitochondrion [Alexson and Cannon, 1984], thereby maintaining lipid homoeostasis. This theory is supported by the observation that dicarboxylic acids are substrates for peroxisomal β-oxidation enzymes [Mortensen, 1986; Vamecq and Draye, 1987]. In addition, an elevated level of dicarboxylic acids has been suggested as a possible mechanism of stimulation of peroxisomal β-oxidation in hepatocyte cultures [Mitchell and Elcombe, 1986]. Evidence for the dicarboxylic acid-mediated induction of peroxisomes may well explain a number of other physiological factors that have been found to induce peroxisome proliferation, such as high fat diets [Osmundsen, 1982; Nilsson et al., 1984], cold adaptation, starvation and diabetes [Ishii et al., 1980; Horie et al., 1984].

It may well be possible that the above sequential mechanism put forward by Sharma and co-workers for peroxisomal β-oxidation, may not be dependent on cytochrome P450 4A1 induction, thus suggesting a casual relationship between the two phenomena. An alternative mechanism is possible whereby the commonality is related to structurally similar regulatory elements in the 5' upstream flanking regions of the cytochrome P450 4A1 and acyl-CoA oxidase genes. This would imply the peroxisome proliferator may directly interact with the common regulatory sequences or indirectly modulate gene expression by lipids derived as a direct result
of inhibition of mitochondrial fatty acid β-oxidation described above or from lipids displaced from fatty acid binding proteins [Cannon and Eacho, 1991]. If this alternative mechanism is plausible, then this commonality of gene expression may also rationalise why many proteins are induced by peroxisome proliferators and the so-called peroxisome proliferator domain may simply reflect the existence of common regulatory elements in what would appear to be functionally unconnected proteins. This alternative mechanism may also provide an explanation as to why several structurally diverse inducers of cytochrome P450 4A1 and acyl-CoA oxidase exist, in that the peroxisome proliferators may not all directly interact with the inducible genes but indirectly act by influencing lipid disposition or a common perturbation of lipid biotransformation.

1.9 Species Differences in Response to Peroxisome Proliferators

There exists pronounced species difference in xenobiotic-induced peroxisome proliferation and this has rendered the extrapolation of data from other species to human, in assessing risk involvement rather difficult. Extrapolation of rodent toxicity data to assess potential human risk, following exposure to peroxisome proliferators requires certain pre-conditions. Amongst such conditions, is the need to consider whether such changes occurring in rodents represent a rodent specific phenomenon or a species common response.

Some of the species that have been examined for their response after challenge by various peroxisome proliferators, include various rat strains, hamster, dog, rhesus monkey, guinea pig, mice, ferret, rabbit, marmoset and humans [De La Iglesia et al., 1982; Gray and De La Iglesia, 1984; Orton et al., 1984; Reddy et al., 1984; Gariot et al., 1983; Makowska et al., 1991]. The results obtained have so far revealed that higher mammalian species are considerably less sensitive than rats, or totally insensitive to the effects of known peroxisome proliferative agents at
tolerated or therapeutic doses. Thus, compounds such as cloburazit, gemfibrozil, fenofibrate, which cause as much as a 10-20 fold increase in rats, cause only slight increases, or no change in dogs and non human primates at much higher dose levels. Similar differences between rats, mice and higher mammalian species have been reported for DEHP and trichloroacetic acid [Elcombe et al., 1985; Elcombe and Mitchell, 1986] and this was also reflected in both laurate ω-hydroxylase and peroxisomal β-oxidation activities [Lhuguenot, 1988]. Caution must be shown when categorising rodents as sensitive species to peroxisome proliferators [Stott, 1988] since this may be misleading because, guinea pigs which are non-responsive, hamsters which are weakly responsive, and rats and mice which are highly responsive species, all belong to the order rodentia.

The induction of peroxisome proliferation in humans resulting from therapy with several hypolipidaemic agents appear to be questionable and it may be speculated that peroxisome proliferation is either a species-dependent or a dose-dependent phenomenon. With respect to non-human primate toxicity studies, dubious, if not misleading information may be generated when using excessively high doses in comparison to the relatively low levels employed in human therapy. It should be noted that peroxisome populations in human liver vary considerably in number and volume densities, hence complicating the evaluation of peroxisome numbers.

The only substantiated report of a chemically induced increase in the number of peroxisomes in humans was made by Hanefeld et al. [1983]. A morphological analysis of liver biopsy tissue obtained from hyperlipidaemic patients receiving a therapeutic dose level of clofibrate (20-40 mg/kg/day) revealed a 1.5-fold increase in the peroxisome number. Likewise, a 23% increase in the number of hepatocellular peroxisomes in hypolipidaemic patients receiving therapeutic dose levels of gemfibrozil or fenofibrate have also been reported [Gariot et al., 1983, 1987]. Liver peroxisomal proliferation ascribed to chronic cumulative leakage of phthalate ester plasticizers from dialysis tubing and haemodialysis transfusion
containers was also reported in patients treated by haemodialysis for more than one year [Ganing et al., 1984]. Similarly, prolonged use of clofibrate in the treatment of patients with myocardial infarction was investigated in a 5-year US Coronary Drug Project in 1975, but the results were inconclusive and have since restricted the use of clofibrate reflecting doubts about the effectiveness of its therapeutic action and the possibility of latent side-effects.

Interspecies differences in the response to peroxisome proliferators demonstrated in vivo, have likewise been reproducible in vitro, using cultured hepatocytes [Mitchell et al., 1985; Allen et al., 1987; Hertz et al., 1987]. This was in contrast to earlier suggestion that phylogenetic differences in the responses could be attributed simply to interspecies differences in the pharmacokinetics in handling these compounds [Berge et al., 1984]. Thus rat and mouse hepatocyte cultures were found to respond to peroxisome proliferators, whereas guinea pig, marmoset monkey, cynomologus monkey and human hepatocyte cultures were not responsive.

In general, the sensitivity of species to peroxisome proliferators followed the trends: rats, mice >> hamster >> guinea pig >> rabbit >> dog, primates including man. On the basis of limited studies in humans, it has been argued by some researchers that humans are at limited risk from the carcinogenic effects of peroxisome proliferators. This may be a reasonable argument, but to be convincing peroxisome proliferation must be proven as the cause of the carcinogenic response.
1.10 Objectives of Thesis

In recent years, growing concern has arisen with respect to the long term exposure of man to hypolipidaemic agents and many industrial chemicals that have been shown to induce peroxisome proliferation. Such concern is largely based on the ever increasing evidence that these compounds constitute a class of hepatic carcinogens, at least in rodents. The phenomenon of peroxisome proliferation has invariably been associated with induction of member(s) of the cytochrome P450 4A subfamily proteins, a relationship which at present is still not well defined. Accordingly, the main objectives of this thesis are three-fold:

1- To further substantiate the mechanistic interrelationship between peroxisome proliferation and co-induction of cytochrome P450 4A1 by employing stereoisomers of a clofibrate analogue.

2- To characterise the expression of the human orthologous form(s) of cytochrome P450 4A subfamily in human liver with structural comparison to the well-characterised rat form. This, I hope, will serve as a preliminary step to better understanding of the implications of extrapolating animal data to humans.

3- A recently proposed alternative mechanism of co-regulation of cytochrome P450 4A1 and acyl-CoA genes of the peroxisome proliferator domain suggests it may be mediated through the recently described peroxisome proliferator activated receptor (PPAR). It may well be that fatty acids are natural ligands to this receptor. Accordingly, I have therefore explored the use of perfluoro-fatty acids which are also peroxisome proliferators and may mimic the natural ligands in vivo, a combination of characteristics which make them highly useful in studies designed to reveal the mechanism of peroxisome proliferation.
CHAPTER TWO

Isolation, Purification and Characterisation of Clofibrate-
inducible Cytochrome P450 4A1 (452) in the Male Rat and
Characterisation of a Corresponding Antibody
Isolation, Purification and Characterisation of Clofibrate-inducible Cytochrome P450 4A1 (P452) in the Male Rat and Characterisation of a Corresponding Antibody

2.1 Introduction

The cytochrome P450-mediated mixed-function monooxygenases of mammalian liver microsomal membranes are of central importance in the metabolism of a wide range of xenobiotics and steroid hormones, including many drugs, environmental pollutants and carcinogens [Hietanen et al., 1982; Murray and Reidy, 1990]. These enzyme systems are also present in many other tissues and in a wide variety of organisms [Lu and West, 1980; Nebert and Negishi, 1982]. Much progress has been made in recent years in understanding the importance of such enzymes with the advent of methodologies for purification and resolution of these isozymes from hepatic and extra-hepatic tissues of a number of species.

One of these isozymes, cytochrome P450 4A1 (formerly termed P452), is induced following pretreatment of animals with the hypolipidaemic agent, clofibrate, (ethyl 2-(p-chloro-phenoxy)isobutyrate [Gibson et al., 1982]. Induction resulted in significant and selected increases in cytochrome P450 levels with the cytochrome P450 4A1 having a high activity towards the \( \omega \) and \( (\omega - 1) \)-hydroxylation of fatty acids such as lauric acid [Tamburini et al., 1984; Bains et al., 1985; Lake et al., 1984b; Sharma et al., 1989].

The initial methodology employed to purify this isozyme was described by Tamburini et al., [1984] and subsequently by Hardwick et al., [1987]. The nomenclature of cytochrome P452 was originally based on the wavelength absorption maxima of the ferrous-carbon monoxide adduct of the haemoprotein and was alternatively designated as P450 LA\(_{\omega} \) by Hardwick and co-workers, to reflect the catalytic activity of the isozyme. More recently, a nomenclature based on
Divergent evolution has been proposed [Nebert et al., 1987; 1991] and the above enzyme redesignated cytochrome P450 4A1. Highly purified cytochrome P450 4A1 isolated from the hepatic microsomes of clofibrate-pretreated rats has been compared to the major isozymes isolated from hepatic microsomes derived from phenobarbital- and β-naphthoflavone-treated rats [Tamburini et al., 1984]. This comparison indicated marked structural, immunological, catalytical and spectrophotometric differences between the three forms, indicating the uniqueness of the cytochrome P450 4A1 haemoprotein.

Several of the cytochrome P450 isozymes are present in detectable constitutive levels which are readily modulated by xenobiotics. To study the basal and induced levels of the cytochrome P450 4A1 in tissues of various species such as those from the rat (Chapters 3 and 4), guinea pig (Chapter 4) and human (Chapter 5), a sensitive and quantitative assay system was required. Accordingly, this study was initiated with a view to isolating and purifying to electrophoretic homogeneity the major clofibrate-inducible cytochrome P450 isozyme. The procedure described in this chapter is the most up-to-date methodology applied in our laboratories to obtain cytochrome P450 4A1 in a highly electrophoretic homogeneous form and of greatly improved yield. As such the purified protein was then used to reboost a previously immunised sheep and the polyclonal antibody obtained further characterised for use in immunological studies. It is worth mentioning here that such an antibody has been previously used to isolate a full length cDNA coding for cytochrome P450 4A1 [Hardwick et al., 1987; Earnshaw et al., 1988].
2.2 Materials and Methods

2.2.1 Chemicals

The chemicals and materials used in these studies are listed below under the various suppliers. Analytical grade solvents were either supplied by the British Drug Houses Ltd (Poole, Dorset, UK) or Fisons (Loughborough, Leicestershire, UK).

Amersham Radiochemicals (Buckinghamshire, UK): [1-14C] Lauric acid.

British Drug Houses Ltd (Poole, Dorset, UK):
Ammonium persulphate, ammonium sulphate, citric acid, cyanogen bromide, EDTA, glycine, sodium dodecyl sulphate, sodium cholate, potassium chloride, potassium cyanide, potassium phosphate, sodium carbonate, sodium dithionite sodium potassium tartrate, sodium hydrogen carbonate, sodium succinate, sodium azide, sucrose, sodium sulphate, magnesium chloride.

Fisons (Loughborough, Leicestershire, UK):
Glacial acetic acid, acetone, diethylether, absolute ethanol, glycerol, hydrochloric acid, hexane, methanol, trichloroacetic acid, acetonitrile, ethylacetate, sodium hydroxide.

Pharmacia Fine Chemicals Ltd. (Milton Keynes, UK):
DEAE-sephacel, hydroxylapatite, sepharose 4B.

Sigma Chemical Co. Ltd (Poole, Dorset, UK):
2-mercaptoethanol, bromophenol blue, coomassie brilliant blue R-250, N,N-tetramethylethylene diamine (TEMED), Triton X-100, diaminobenzidine chloride, Tween 20, bovine cytochrome oxidase, bovine serum albumin, L-α-dilauroyl phosphatidylcholine (DPLC), clofibric acid, cytochrome c, dithiothreitol (DTT), lauric acid, NADH, NADPH, trizma base, 30% (v/v) hydrogen peroxide solution, cyanogen bromide-activated sepharose 4B, phenobarbital, 3-methylcholanthrene.
Other suppliers:
Nitrocellulose filters (Anderman and Co Ltd. Kingston-upon-Thames, UK); Dialysis tubing (FSA Supplies, Loughborough, Leicestershire, UK); acrylagel and bisacrylagel (National Diagnostics, Aylesbury, UK); emulgen 911 (KAO Atlas Company, Tokyo, Japan); 3MM blotting paper and DEAE cellulose (Whatman, Maidstone, Kent, UK); slab gel apparatus (Hoefer Scientific Instruments, Newcastle, Staffs. UK); power packs (BioRad, Watford, Herts. UK); amicon ultrafiltration cell, model 8400 (Amicon Corp. Danvers, USA); flat well supreme series microplates (Alpha Laboratories, Eastleigh, Hampshire, UK).

2.2.2 Animals and drug pretreatment

Male wistar albino rats (150-200g body weight, University of Surrey Breeders) were housed in cages with sawdust bedding and allowed food (Spratts Animal Diet No. 1) and water ad libitum. A twelve-hour light/ dark cycle was maintained at 22°C and 50% relative humidity. The animals were dosed with sodium clofibrate (250 mg/kg i.p in 0.9% (w/v) saline) once daily for three days and sacrificed on the fourth day, i.e one day after the last injection.

2.2.3 Isolation of hepatic microsomes

Rats were sacrificed by cervical dislocation and the livers were quickly removed perfused with 0.9% (w/v) ice-cold saline, blotted dry and weighed. Livers from thirty rats were pooled together, scissor-minced and homogenised in ice-cold 0.25M sucrose using a Potter-Elvehjem glass-teflon homogeniser and adjusted to 25-33% (w/v) with the same sucrose solution. At this stage, aliquots of whole liver homogenate were frozen at -80°C for subsequent analyses. All subsequent stages were carried out at 4°C.
The homogenate was centrifuged in a JA-17 rotor in a Beckman J2-21 centrifuge at 12,600g for 30 min to remove cell debris and mitochondrial fractions. The resulting supernatant was subjected to a further ultra-spin at 105,000g for 1h in a Beckman preparative ultracentrifuge, model L7-65 (Beckman Co., USA), using a 60-Ti rotor. Aliquots of the cytosol were also frozen at -80°C in case it was required. The microsomal pellet was resuspended in ice-cold 50mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol and aliquoted out in 0.5-1ml volumes in eppendorfs and stored at -80°C until required. Under these storage conditions, enzyme activities remained stable for several months.

2.2.4 Solubilisation of hepatic microsomes

Although the solubilisation of functional membrane proteins is a central task in modern membrane biochemistry, systematic experimentation directed at achieving this goal has not been successful. However in the present work, sodium cholate was employed because of its physico-chemical properties (Table 2.1).

In the initial solubilisation reaction medium, 21% (w/v) sodium cholate stock was used to achieve a final concentration of 1.8% (w/v). Solubilisation was carried out under an atmosphere of nitrogen at 4°C and at a microsomal protein concentration of 10 mgml⁻¹. The appropriate volume of the 21% (w/v) sodium cholate stock solution was added under these conditions dropwise with stirring over 20 min and then left for a further period of 40 min. The mixture was then centrifuged at 35,000 rpm for 1h in a 50 Ti-rotor head in the Beckman centrifuge. The pale pellet was discarded and the reddish supernatant diluted with the appropriate buffer to give a suspension having a final cholate concentration of 0.6% (w/v). An aliquot from this pool was taken out for total cytochrome P450 and protein determinations. The remaining material was processed immediately on a previously prepared 8- aminoxyt sepharose 4B affinity chromatography column.
Table 2.1 Properties of sodium cholate as detergent

<table>
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<tr>
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<th>Data</th>
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<td>Critical micelle concentration [% (w/v)]</td>
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<tr>
<td>Binds divalent cations</td>
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</tr>
<tr>
<td>Significant absorbance at 280 nm</td>
<td>No</td>
</tr>
<tr>
<td>Interference with protein assays</td>
<td>No</td>
</tr>
</tbody>
</table>
2.2.5 General column chromatography procedures

a) Column pouring and equilibration

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

After allowing the medium to settle for 12h in the column, adequate packing was achieved under a constant pressure head at the flow rate required during chromatography. All chromatography columns were equilibrated with at least 3 column volumes of equilibration buffer, during which time the pH of the eluting buffer was regularly monitored.

b) Sample loading and elution

About 5000-6000 nmol of cytochrome P450 were loaded per packed column (2.6x38 cm). While loading, the columns and reservoirs were covered in aluminium foil to minimise light-dependent haem degradation. The flow rate was adjusted to 20-30ml h⁻¹ and the loading eluate was retained to check if the cytochrome P450 was indeed retained by the column.

c) Ultrafiltration of pooled fractions

An Amicon PM-30 membrane of molecular weight exclusion of 30 kDa, was routinely used and was placed in an Amicon ultrafiltration cell of 350ml capacity (Amicon Corp., Danvers, U.S.A.). Prior to ultrafiltration, the assembled chamber was filled with distilled water and attached via a high pressure tubing to a nitrogen cylinder. A constant pressure of 20psi was then applied to force water through the amicon membrane. This procedure was repeated twice before introducing the protein-containing pooled fractions. Ultrafiltration was accomplished using 30psi
pressure. The eluate was checked to ensure the membrane wasn't flawed or the chamber incorrectly assembled. The chamber was maintained throughout at 4°C by placing it on a tray of ice on a magnetic stirrer to prevent the membrane pores from clogging up.

The resulting concentrate was poured into prepared dialysis tubings of appropriate lengths as described below and dialysis carried out at 4°C. A minimum of three changes of the dialysis buffer (3L each) was found adequate, with 12h period between changes. On each occasion the dialysis container was wrapped up in tin foil to minimise degradation of the cytochrome P450.

d) **Preparation of dialysis tubing**

The dialysis tubing was cut into pieces of convenient length (usually 10-20 cm) and boiled for 10 min in a large volume of 2% (w/v) sodium bicarbonate and 1mM EDTA, pH 8. The tubing was then rinsed thoroughly in distilled water and boiled for another 10 min in 1mM EDTA, pH 8. After allowing the tubing to cool to room temperature, it was then stored at 4°C until required. From this point onward it was important not to handle the tubing without gloves and each time before use, both the inside and outside of the tubing was washed with distilled water.

### 2.2.6 8-Aminooctyl Sepharose 4B Affinity Chromatography

a) **Method of synthesis**

Diaminooctane was coupled to sepharose 4B utilising cyanogen bromide activation as described by Cuatrecasas [1970]. Finely divided cyanogen bromide (100g) was added with stirring to 400 ml (packed volume) of sepharose 4B present as a 1:1 suspension in distilled water. The pH and temperature were maintained at 11 and 20°C respectively by either using a 10N sodium hydroxide solution or by adding ice. When the pH of the solution no longer showed a tendency to drop, large amounts of ice were added, followed by rapid washing under suction with 3L
of 50mM sodium carbonate/bicarbonate buffer, pH 10, on a sintered glass funnel. The activated sepharose 4B was then transferred into a beaker containing 400ml of 25% (w/v) diaminooctane adjusted to pH 10, and gently stirred at 4°C for 20h. The derivatised sepharose 4B was washed on a sintered glass funnel consecutively with 3L of the above buffer, followed by distilled water and 100mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 0.7% (w/v) sodium cholate, 1mM dithiothreitol, 1mM EDTA, 2μM FMN, and was stored in the last buffer containing 0.02% (w/v) sodium azide at 4°C.

b) Method of column regeneration

To achieve a successful regeneration, it was important to elute NADPH cytochrome P450 reductase and cytochrome b5 whilst packed in a column by using the appropriate buffers. Following column chromatography, the media was transferred to a sintered glass funnel and washed consecutively under suction (with occasional stirring) by utilising 2L of each of the following: 100mM potassium phosphate buffer, pH 7.25, containing 0.4% (v/v) emulgen 911; the same buffer containing 0.4% sodium deoxcholate; distilled water; 10% (v/v) aqueous dioxane; distilled water again and finally 500mM potassium phosphate buffer, pH 7.25, containing 0.7% (w/v) sodium cholate, 1mM dithiothreitol, 1mM EDTA and 2μM FMN.

c) Column Loading and Elution

The solubilised microsomal supernatant derived above, was applied to two columns of 8-aminooctyl sepharose 4B (2.6x38 cm) previously equilibrated with 3 column volumes of buffer A (100mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 1mM EDTA, 1mM dithiothreitol, 2μM FMN, 0.6% (w/v) sodium cholate). Upon loading, a dark red band (cytochrome P450) was seen to form at the top of the column. Loading was terminated when the band had increased to occupy a third to half the column. The columns were then washed with at least 3 column volumes of buffer A in which the sodium cholate concentration
had been decreased to 0.42% (w/v) to remove unadsorbed protein. Cytochrome P420 and membrane lipids were also eluted at this stage.

Elution of cytochrome P450 was achieved by washing the columns with buffer B (100 mM potassium phosphate buffer, pH 7.25, containing 20% (w/v) glycerol, 0.33% (w/v) sodium cholate, 1 mM dithiothreitol, 1 mM EDTA, 2 μM FMN and 0.08% (v/v) emulgen 911), whereupon the dark red band was seen to migrate slowly and sharpen upon descending the column. The cytochrome P450 content of eluted fractions was routinely monitored spectrophotometrically at 417 nm. Fractions displaying an absorbance greater than a third of the elution peak value were pooled and dialysed against two successive 3.5 L volumes of buffer C (20 mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 0.2% (v/v) emulgen 911), for 12-15 h period. The high absorbance of emulgen 911 at 280 nm present in subsequent buffers prevented the rapid estimation of the purity of cytochrome P450 by utilising the 417/280 nm absorbance ratio.

2.2.7 DEAE Sephacel Anionic Exchange Chromatography

a) Preparation of media

The required amount of the medium was transferred to a sintered glass funnel and washed under gravity with distilled water to remove the storage solution. The chloride counter ion was exchanged with phosphate and the medium set to the correct pH by transferring an excess volume of 400 mM potassium phosphate, pH 7.25. This was left standing for 24 h before replacing the buffer with the equilibration buffer and packing the column.

b) Regeneration of column material

DEAE sephacel was regenerated by standing the media in an excess volume of 400 mM potassium phosphate, pH 7.25, overnight, followed by extensive washing with 500 mM sodium chloride and distilled water. The medium was stored in
c) **Loading and Elution**

Pooled and dialysed cytochrome P450 concentrate from 8-aminooctyl sepharose 4B column chromatography was applied onto a DEAE sephacel column (30x2.6 cm) previously equilibrated with the last dialysis buffer and the flow rate adjusted to 30-40 ml/h. Upon loading, a tight red band developed at the top of the column. However, some of the cytochrome P450 did not stick to the column but passed straight through. When loading was completed, the column was washed with at least four column volumes of 5mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 0.2% (v/v) emulgen 911. This process eliminates some hydrophobic molecules that are still present on the column. Elution of cytochrome P450 was then achieved by washing with the same buffer in which the ionic strength had been increased stepwise from 5mM to 50mM in increments of 5mM. Cytochrome P450 in the eluate was detected and pooled as judged by their purity on calibrated 10% (w/v) SDS-PAGE gels. Aliquots from each pool was assayed for total cytochrome P450 and protein contents.

2.2.8 **Hydroxylapatite (Biogel HT)**

a) **Preparation of media**

The required amount of hydroxylapatite was transferred into a beaker containing an excess of the equilibration buffer and gently swirled into suspension. The gel was allowed to settle, leaving a cloudy buffer volume above, containing slowly sedimenting 'fines', which were decanted. Fresh buffer was added, and the procedure repeated 4 times. The medium was discarded after use.

b) **Loading and Elution**

The pooled and dialysed cytochrome P450 concentrate obtained from the DEAE
sephacel column was applied onto a prepared hydroxylapatite column (2.6x15 cm). The column was washed with 2 volumes of buffer D (5mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol and 0.2% (v/v) emulgen 911). Cytochrome P450 was eluted with a linear gradient of the same buffer ranging from 5-150 mM concentrations. Fractions eluted were monitored at 417nm and an aliquot from every second tube was used to check the purity on calibrated 10% (w/v) SDS-PAGE gels and pools were made based on the information obtained. Pooled fractions were dialysed against two successive 3L volumes of buffer D for 12h period.

2.2.9 Total CO-Discernible Cytochrome P450 Determination

Cytochrome P450 was routinely determined as the reduced carbon monoxide adduct [Omura and Sato, 1964]. Microsomes were diluted with 50mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol to give a protein concentration of 1-2 mg/ml. Reduction to the ferrous form of the haemoprotein was achieved by the addition of a few milligrams of sodium dithionite. The sample was then split between two quartz cuvettes and a baseline recorded using a Kontron-Uvikon 860 spectrophotometer (Kontron Instruments Ltd, Switzerland). Carbon monoxide (30 bubbles at approximately one per second) was gassed through the sample cuvette only and the contents of the cuvettes were rescanned between 400-500nm. The concentration of cytochrome P450 was calculated using the difference extinction coefficient (450-490 nm) of 91 mM⁻¹cm⁻¹.

2.2.10 Determination of Cytochrome b₅

Cytochrome b₅ was determined from the difference spectrum between NADH-reduced and air-saturated microsomes (under aerobic conditions), essentially according to the method of Omura and Sato [1964]. In NADH-reduced microsomes cytochrome P450 was mostly in the oxidised form whereas cytochrome b₅ was in
the reduced form. The increment of the molar extinction between 424 and 409nm in the difference spectrum was assumed to be 185 mM\(^{-1}\) cm\(^{-1}\).

The microsomal suspension contained approximately 2mg of protein per ml in 50mM phosphate buffer, pH 7.25, containing 20% (v/v) glycerol. The mixture was saturated with air and split between two cuvettes for baseline recording between 400 and 500nm, prior to the addition of 25\(\mu\)l of 2% (w/v) NADH in 1% (w/v) sodium bicarbonate to the sample cuvette. The sample was rescanned until there was no further spectral development at 424nm.

**2.2.11 Determination of NADPH-Cytochrome P450 Reductase Activity**

The activity of the flavoprotein cytochrome P450 reductase was determined by the method of French and Coon [1979], according to its ability to reduce cytochrome c.

The microsomal sample was diluted with 100mM Tris-Cl buffer, pH 7.5, to 2 mg/ml and 200\(\mu\)l of this together with 1.0ml of cytochrome c (0.46 mg/ml) were added to each cuvette. The final volume was 3.0ml after addition of all the other components. The reaction was followed at 550nm until a baseline was established and then 100\(\mu\)l of 40mM NADPH in 1% (w/v) sodium bicarbonate solution (1mM, final concentration) was added to the sample cuvette and the initial reaction rate was measured. The activity was calculated using the extinction coefficient for reduced cytochrome c of 18.5 mM\(^{-1}\) cm\(^{-1}\).

**2.2.12 Protein Determination**

Protein concentration was determined by the method of Lowry et al. [1951] using bovine serum albumin (Fraction V, Sigma Co.) as standard.
2.2.13 Reconstitution Methodology for Assaying Cytochrome P450 4A1

The metabolism of a model substrate by purified preparation of cytochrome P450 was routinely determined using the standard reconstituted system of Haugen et al., [1975].

The components of the reconstitution system for lauric acid metabolism were added sequentially to achieve the following final concentration or amounts: L-α-dilauroyl phosphatidylcholine (30μg), cytochrome P450 (0.1-0.3μM), purified NADPH-cytochrome c reductase (0.5-0.9 U/ml), sodium deoxycholate (50μg/ml), 50mM potassium phosphate buffer, pH 7.25, magnesium chloride (15mM), saturating amounts of lauric acid as substrate (200nmol), and distilled water added to the required volume of 1ml.

The system was pre-incubated for 5 min, on a shaking water bath set at 37°C, prior to the initiation of the reaction by the addition of NADPH to a final concentration of 1mM. The reaction was then allowed to proceed for 10 min after which it was terminated by the addition of 300μl of 3M HCl.

2.2.14 Analysis of Lauric Acid Metabolism by h.p.l.c.

The metabolism of [1-14C] lauric acid to ω- and (ω-1)-hydroxylated products was evaluated essentially as described by Parker and Orton [1980].

2 ml incubation volume contained [1-14C] lauric acid (0.1μCi in 10μl methanol) and 0.1mM [1-12C] lauric acid (200mM stock solution in methanol diluted to 1mM in 0.5M Tris-HCl buffer, pH 7.4). 1mg of protein was used per assay. The tubes were pre-incubated for 5 min at 37°C prior to the addition of 40μl of 40mM NADPH to initiate the reaction. Microsomal blanks contained distilled water instead of NADPH. Control and treated samples were incubated at 37°C for 10
The reaction was terminated by the addition of 300μl of 3M HCl. Incubations were carried out in polypropylene stoppered tubes.

10ml of diethylether was then added to the incubation mixture followed by mixing on a rotary shaker for 20 min. The tubes were then spun at 1000g for 2-3 min to separate the phases after which the upper (ether) layer was transferred to a test tube and evaporated to dryness under a stream of nitrogen. The test tubes were sealed with parafilm and stored at -80 °C until analysed.

The dried ether extracts were reconstituted in 150μl of the eluting solvent (water : methanol : glacial acetic acid. 45: 55: 0.05 by volume) of which 100μl was then injected onto a reverse phase Microbondapak MCH-10 column (30x4.4 cm, Varian Associates Ltd, Walton-on-Thames, UK.). The metabolites were resolved using a linear gradient of water : methanol (45: 55 containing 0.1% acetic acid) to 100% methanol over a 45 min period at a flow rate of 1 ml/min.

The eluate was passed through a Berthold LB 506 C-1 Radioactivity Monitor (Lab-Impex, Twickenham, UK), containing a 200μl flow cell and interfaced with a microprocessor (Motorola 68B09 CPU). This was linked to a PC IV 286 AT-Compact computer (Opus Technology plc, Redhill, UK), enabling quantitative analysis of lauric acid metabolism. The (ω—1)- and ω—hydroxylated products were successively eluted followed by the unchanged lauric acid. The rates of hydroxylation were calculated from the fractional conversion of substrate to ω— and (ω—1)-hydroxyl products obviating the need for the estimation of recovery of radioactive products. The fractional conversions of the substrate were obtained on data print-outs which gave relative percentages after correction for background counts. The rate of formation (R) of individual metabolites was quantified using the following formula:

\[ R = \frac{\text{fractional conversion of substrate to metabolite}}{\text{time elapsed}} \]
\[
R = \frac{S \times m}{t \times p}
\]

- **S** (nmol) = amount of original substrate in incubation mixture (nmol)
- **m** = percentage of substrate converted to metabolite
- **t** = incubation time in minutes
- **p** = amount of protein per incubate (mg)

**R** is then expressed as: nmol product formed/ min/ mg protein.

Alternatively, the rate of product formation may be expressed per nmol cytochrome P450 and given by the formula:

\[
E = \frac{R}{Q}
\]

where, **Q** = cytochrome P450 content (nmol/ mg protein);

R as defined above and **E** = nmol product formed/ min/ nmol P450.

### 2.2.15 Antiserum Production, Immunopurification and Characterisation

#### a) Cytochrome P450 4A1 antibody production

Production of a sheep polyclonal antiserum was achieved with a single hepatic protein purified as above (cytochrome P450 4A1). The sheep used in this study had been previously primed and boosted with this same antigen which resulted in the production of a high titre antibody with specificity and avidity [Sharma, 1988; Ph.D Thesis, University of Surrey].

In the second boosting experiment (after the monitored antibody level had returned to basal levels, approximately 24 months later), 100μg of the highly homogeneous antigen (cytochrome P450 4A1) was added to an equal volume of non-ulcerative Freunds complete adjuvant containing heat-killed BCG mycobacteria. The use of Freund's complete adjuvant results in a far greater antibody response than is stimulated by antigen in a simple water-in-oil emulsion because of the greater persistence of the antigenic complex within the tissues of the test animal,
thus delaying absorption. Similarly the presence of the heat-killed mycobacteria serve to stimulate the entire reticuloendothelial system of the animal with the droplets slowly removed by the macrophages of this system.

The antigen-adjuvant emulsion is obtained by the double-hubbed needle method. This involves repeated aspiration and expulsion from an all-glass syringe fitted with an all-metal needle which makes the possibility of attacks by components of the adjuvant very remote. The stability of the emulsion was always ascertained by placing a single drop in a beaker of cold water and this was not expected to break up. Once satisfied as to the above, immunisation was achieved and the levels of antibody to cytochrome P450 4A1 were monitored every two weeks from serum prepared from large bleeds (approximately 300ml) by the ELISA technique. Serum is often preferred to plasma because it is less likely to form cryoprecipitates and was stored with 0.1% (w/v) sodium azide as preservative at 2-4°C. Under these conditions the serum was stable for at least six months.

b) **Titre and avidity determinations**

An antiserum displacement curve was constructed as an indirect means of determining the avidity of the anti-cytochrome P450 4A1 antiserum to its specific antigen, in this case cytochrome P450 4A1. Essentially a microtitre plate was coated with a fixed amount of the antigen (0.02pmol in 200μl volumes) and incubated overnight at 4°C in a humid environment. Following incubation, the wells were washed with Phosphate Buffered Saline, pH 7.4, containing 0.1% (w/v) gelatin and 0.05% Tween 20, (PBSGT). Various dilutions of the antiserum were then prepared in PBSGT ranging from 1:500 - 1:256000 and 200μl of each was then added in triplicates to a section of the wells either alone or with added free antigen (0.02pmol), the latter ensuring there was competition between the fixed and free antigens for the antibody binding sites.
After further incubation of the plate at room temperature for 2h, it was re-washed with PBS/GT and the donkey anti-sheep enzyme-labelled conjugate, horseradish peroxidase (200μl/well) was applied at a dilution of 1:5000. After a further 2h incubation at 37°C and a PBS/GT wash, the substrate (75ml of 0.025M citric acid, 0.05M disodium hydrogen phosphate buffer, pH 5.6, containing 30mg orthophenylenediamine (OPD) tablet and 30ml of 30% (v/v) hydrogen peroxide was added to the plate (150μl/well). Following incubation for 10 min at 37°C, the reaction was terminated by the addition of 2.5M sulphuric acid (50μl/well).

The absorbance of the wells was read at 492nm using a Titertek plate reader (Flow Laboratories, UK) and were plotted (after adjustments of the absorbances for non-specific binding i.e pre-immune sera values) against the serial dilutions of the antiserum on a logarithmic scale yielding two sigmoid curves. The extent of displacement of the antiserum dilution curve to the left was used as an indication of the avidity of the antiserum to its specific antigen. Likewise, the reciprocal of the antiserum dilution producing 50% of the maximum absorbance reading was described as the titre.

c) **Immunopurification of cytochrome P450 4A1 polyclonal antibody**

Approximately 100μg of purified cytochrome P450 4A1 was immobilised on 100μg (300μl) of activated porous silica. It was then reacted with 1ml of antiserum diluted with 1ml 100mM citrate/phosphate buffer, pH 7.0, in a 7ml disposable chromatography column, No D823 (Lab M, Bury, Lancs. England). This was left on a roller-mixer for 15 min after which the column was washed off with 2.5ml of the same citrate/phosphate buffer, pH 7.0. The adsorbed antibody was eluted by reducing the pH in a step-wise manner with 4.5ml of 100mM citrate/phosphate buffer pH’s of 5.5, 4.0 and finally 2.5. Pooled antibody fractions from the various pH elutions were equilibrated to pH 5.5 by the addition of 0.5ml of 2.5M acetate buffer, pH 5.5.
Protein concentration of the samples were estimated by measuring the optical density at 280nm. Calculations were made based on the information that serum contains about 40mg albumin per ml and that at 280nm, 1mg IgG/ml solution has an absorbance of approximately 0.6 units whereas, immune serum contains between 10-40 mg IgG/ml and at 280nm, a solution of 1mg IgG/ml has an absorbance of 1.4 units.

d) Preparation of IgG-enriched fractions

Immunoglobulin fractions from normal sheep and immunised sera were isolated by a modification of the method of Walker and Mayer [1976].

Essentially, 25ml of saturated solution of ammonium sulphate was added dropwise from a burette into 25ml of serum in a conical flask, while stirring on ice, to achieve a 50% saturation solution. Following this step, the precipitate formed was pelleted down at 12,600g for 20 min in a J2-21 Beckman centrifuge. The precipitate was then washed three times with ice-cold 1.75M ammonium sulphate to achieve a clean whitish pellet. This ensured albumin, transferrin and α-proteins, including haptoglobin and haemoglobin which may be present, were eliminated. The final white pellet was dissolved in 5ml of 10mM potassium phosphate buffer, pH 7.0, and dialysed overnight against 5L of water. Dialysis was then repeated against 2 x 3L of 10mM potassium phosphate buffer, pH 7.4, over a 16h period. The preparation was then loaded onto a DEAE cellulose Whatman DE 52 column (5x12 cm), previously equilibrated with the same buffer. The column was washed with the same buffer until all the IgG was eluted. Eluted fractions were monitored spectrophotometrically at an absorbance wavelength of 280nm. The fractions were then aliquoted and freeze-dried in quantities of 50-100 mg/vial for each of the pre-immune and immune IgG preparations.
2.2.16 Enzyme-linked immunosorbent assay (ELISA)

The technique of ELISA as described by Voller et al. [1978] and modified for the quantification of cytochrome P4504A1 by Sharma et al. [1988a] was utilised to quantify this protein in microsomal samples.

Microsomes were solubilised by the addition of 1mg cholate and 0.2mg emulgen 911 per mg microsomal protein with stirring for 20 min on ice. The solubilised microsomes were suitably diluted with 0.1M sodium bicarbonate/ carbonate buffer, pH 9.6, and 200µl aliquots loaded onto microtitre plates on protein basis. The plates were left overnight at 4°C in a humid box. Subsequently, the plates were washed with phosphate buffered saline, pH 7.4, containing 0.1% (w/v) gelatin and 0.05% Tween 20 (PBSGT). The antisera to cytochrome P450 4A1 or the pre-immune sera were diluted in PBSGT as required and applied to the microtitre plate at 200µl/ well.

The plates were incubated at 37°C for 2h, re-washed with PBSGT, and the donkey anti-sheep enzyme labelled conjugate, horseradish peroxidase (200µl/well) was applied and the remaining steps processed as outlined in Section 2.2.15(b). A calibration graph using electrophoretically homogeneous cytochrome P450 4A1 was constructed between 0 and 0.04 pmol of pure protein. The amount of cytochrome P450 4A1 in the microsomal samples was determined from the calibration curve after adjustment of the absorbances for non-specific binding (pre-immune sera values).

2.2.17 Western blot analysis

Western blotting involves the electrophoretic transfer of separated proteins from polyacrylamide gels to nitrocellulose sheets which are then subjected to immunological analysis. The methods employed were modifications of the basic procedures of Towbin et al., [1979], Burnette [1981] and reviewed by Bers and
a) Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The method of Laemmli [1970] was routinely used to resolve the microsomal proteins. Electrophoresis was performed at room temperature using a vertical slab gel assembly (Hoefer Scientific Ltd) in which the gel was contained in a glass and perppex cassette.

All samples were diluted in sample solubilisation buffer, boiled for 2 min and subjected to a 10% (w/v) SDS-polyacrylamide gel electrophoresis. The following lyophilised proteins of known molecular weights in a supplied kit (SDS-7B, Sigma, Poole Dorset) were routinely employed:

- α2- Macroglobulin: 180,000
- β- Galactosidase: 116,000
- Fructose-6-phosphate kinase: 84,000
- Pyruvate kinase: 58,000
- Fumarase: 48,500
- Lactate dehydrogenase: 36,500
- Triosephosphate isomerase: 26,600

For cytochrome P450 4A1 Western blots, 1.0pmol of purified cytochrome P450 4A1 was loaded per tract as an authentic marker. Samples were in general loaded on equiprotein basis and a constant current of 40mA was maintained throughout electrophoresis. When electrophoresis was complete, the gels were either stained with coomassie R-250 dye for protein visualisation or subjected to immunoblotting analysis.

b) Electrophoretic transfer and immunochemical staining

The lower separating gel, having been removed from the gel cassette, was immersed in transfer buffer (20mM Tris-HCl, 150mM glycine, 20% (v/v) methanol) for 45 min prior to transfer to nitrocellulose sheet. This was to allow the gel to shrink, thus avoiding distortion of the bands during transfer. The transfer
process was achieved using an electrophoretic transfer apparatus (BIORAD) at room temperature and at a constant voltage of 30V overnight.

Following electrophoretic transfer the nitrocellulose sheet was washed in phosphate buffered saline containing 1% (w/v) bovine serum albumin and 0.2% (v/v) Triton X-100 (PBSBT) for 2h at room temperature. Subsequent washes were carried out at this temperature on a shaker. The PBSBT was then removed and replaced with fresh PBSBT containing anti-cytochrome P450 4A1 polyclonal antibody diluted 1:2000, in 50ml of PBSBT and incubation was left on for 1h. The primary antibody was then removed and the nitrocellulose sheet subjected to two 20 min washes with PBSBT. Donkey anti-sheep enzyme label (horseradish peroxidase) was then applied to the filter in PBSBT while shaking for 1h. After further 2x20 min washes, the nitrocellulose sheet was then developed in 0.1M Tris-HCl, pH 7.5, containing 0.6 mg/ml 4-chloronaphthol and 1:5000 hydrogen peroxide (30vols). The reaction was terminated when judged appropriate by rinsing the filter several times with distilled water. This was to ensure that no substrate was left on the filter prior to storage. The developed blots were photographed for permanent record and quantified by using a CS-9000 Dual Wavelength Flying-spot Scanner (Shimadzu Corp, Japan).
2.3 Results and Discussion

The purification protocol used is represented schematically in Figure 2.1 and a typical purification result presented in Table 2.2.

2.3.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis:

Following a step-wise gradient elution from DEAE-sephacel column, with potassium phosphate buffer, pH 7.7, five pools were obtained based on purity of individual fractions eluted. These pools were derived from elutions with 5, 10, 15, 20 to 50, and 150mM concentrations of the buffer. Figure 2.2 shows the purity of some of the pools on a 10% SDS-PAGE gel and included two pools of the 15mM buffer elution fractions. Based on this information, further purification steps utilised the 15mM pool that showed more homogeneity (track 6). This was loaded onto a hydroxylapatite chromatography column and eluted with a linear gradient system of the potassium phosphate buffer, pH 7.7, ranging from 5-100 mM. Three main fractions were obtained designated fractions 1, 2, and 3 and their purities were checked on a calibrated 10% (w/v) SDS-PAGE gel (data not shown). Fraction 2 was further processed on another hydroxylapatite column and eluted with 320mM phosphate buffer, pH 7.7. This last stage was carried out to minimise the emulgen content, a process referred to as desoaping. This final preparation and samples from the previous purification steps were separated on a calibrated 10% (w/v) SDS-PAGE gel as shown in Figure 2.3.

The purified rat hepatic cytochrome P450 was judged to have a molecular weight of approximately 51.5kDa, based on migration on a 10%(w/v) calibrated SDS PAGE gel. The homogeneity and extent of purity of this haemoprotein was indicated by the lack of protein staining bands elsewhere on the gel. The haemoprotein also exhibited electrophoretic mobility in a similar SDS-polyacrylamide gel, identical to cytochrome P450 4A1 previously isolated in our laboratory.
Figure 2.1 Flow chart for the purification of cytochrome P450 4A1 isolated from the endoplasmic reticulum of clofibrate-induced rats.
<table>
<thead>
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<th>Purification step</th>
<th>Volume (ml)</th>
<th>Protein conc. (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Cyt. P450 (nmol/ml)</th>
<th>Total cyt. P450 (nmol)</th>
<th>Cyt. P450 specific cont. (nmol/mg protein)</th>
<th>Overall yield (percentage)</th>
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<td>Microsomes</td>
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<td>DEAE sephacel stepwise gradient (15 mM pool)</td>
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<tr>
<td>Hydroxylapatite linear gradient (5 - 150 mM)</td>
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<td>25.1</td>
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</table>

* n.d = not determined.
Figure 2.2 SDS-PAGE analysis on a 10% (w/v) gel of some pools obtained following DEAE sephacel chromatography of cytochrome P450 was performed as described in Materials and Methods. Track 1, prestained molecular weight markers (0.2μg); track 2, 5mM pool (1.5pmol); track 3, 10mM pool (1.5pmol); track 4, authentic cytochrome P450 4A1 (5μg); track 5, 15mM pool A (1.5pmol); and track 6, 15mM pool B (1.5pmol).
Figure 2.3 Various purification stages of clofibrate-induced cytochrome P450 4A1 analysed on a 10% (w/v) SDS-PAGE gel. The procedure was carried out as described under Materials and Methods Section. An aliquot from each purification step was loaded as follows: track 1, clofibrate-induced microsomes (30µg); track 2, solubilised microsomes (25µg); track 3, fraction following sepharose 4B chromatography (50µg); track 4, fraction following DEAE sephacel chromatography (15mM pool, 20µg); track 5, authentic cytochrome P450 4A1 (5µg); track 6, fraction following hydroxylapatite chromatography (5µg).
2.3.2 Spectral characteristics and catalytic activity.

Several spectral parameters have been used to delineate differences among purified isozymes of rat liver cytochrome P450s. The results from the present purification show that the absorbance maxima of the ferrous-CO complex of the haemoprotein occurred between 451 and 452 nm, for three separate determinations (data not shown) and was quite similar to that at 451.5 nm, previously reported in our laboratory. This preparation also had a specific content of 9.95 nmol/mg protein which was almost a ten-fold increase over crude microsomal preparation.

Cytochrome P450 4A1 (P452) has previously been demonstrated by several workers (including our laboratory) to be induced following pretreatment of animals with the hypolipidaemic agent, clofibrate [Gibson et al., 1982, Tamburini et al., 1984; Bains et al., 1985; Lake et al., 1984a, Sharma et al., 1989]. These workers also showed that induction resulted in significant and selective increases in one of the cytochrome P450 isozyme levels (P450 4A1) having a high activity towards hydroxylation of lauric (dodecanoic) acid at both the ω— and (ω—1)-positions, the former emerging as the predominant metabolite.

The catalytic activity of purified cytochrome P450 4A1 derived from clofibrate-induced rats was assessed by way of a reconstituted system. The reconstitution media usually contained saturating amounts of purified NADPH-cytochrome c reductase (previously prepared in our laboratories from phenobarbitone-induced microsomes) and optimal concentrations of dilaurylphosphatidylcholine. Results obtained from the reconstitution studies show that the cytochrome P450 4A1 haemoprotein catalyses both ω— and (ω—1)-hydroxylation of lauric acid, a marker substrate for this isozyme. The ω—hydroxylated product was preferentially formed at a rate of 11.2 nmol/min/nmol P450 4A1 compared to 17.32 nmol/min/nmol P450 4A1, previously reported in our laboratory for similar purification procedures. The reason for such discrepancy was not very obvious, however, previous workers
included cytochrome b5 which has been demonstrated to stimulate lauric acid hydroxylation mediated by cytochrome P450 4A1. Moreover, the preparation of NADPH cytochrome c reductase like that of cytochrome P450 4A1 contained trace amounts of emulgen 911 despite attempts of its removal on a hydroxylapatite column and this is well known to interfere with catalytic activity assays.

2.3.3 Polyclonal antibody production and immunochemical reactivity

The sheep received a second boosting with 100μg of purified cytochrome P450 4A1 almost 24 months after it was last boosted, by which time the anti-cytochrome P450 4A1 concentration in the serum had returned to basal levels. The effect of a second boosting on the levels of anti-cytochrome P450 4A1 in sheep sera collected at two-week intervals is shown in Figure 2.4 and screened by an earlier established ELISA protocol [Sharma, 1988; Ph.D Thesis University of Surrey].

Generally, an immunisation programme will yield a number of antisera from which one or more must be selected for use in an assay. The criteria for this selection are specificity, affinity and titre all of which depend on the requirements of such an antiserum. The titre is defined as the reciprocal of the antiserum dilution giving half the maximum absorbance value when absorbance was plotted against the serial dilutions of the antiserum on a logarithmic scale. A typical antiserum curve obtained with bleed number 3 (two weeks after immunisation) is shown on Figure 2.5A. From such information, the titre was estimated by extrapolation and corresponded to a titre value of approximately 1:25,000. This titre value was used in subsequent analyses.

An indication of the avidity of the antiserum to its specific antigen (in this instance, cytochrome P450 4A1 apoprotein), was demonstrated by constructing an antiserum displacement curve as shown in Figure 2.5B. This is a measure of the ability of the free antigen to compete with the same antigen bound to a microtitre
Figure 2.4 Antibody levels in sheep bleeds following reboosting with a homogeneous preparation of cytochrome P450 4A1. The point of reboosting is indicated by an arrow and bleeds were collected every two-week interval and processed as described in Materials and Methods. The antiserum was used in a dilution of 1:25k and the enzyme label, 1:5k. The wells were coated with 0.02pmol cytochrome P450 4A1 each and values were corrected for non-specific binding with pre-immune sera.
Figure 2.5 Titre and avidity assessments of cytochrome P450 4A1 polyclonal antibody levels in the reboosted sheep. The procedures used in obtaining these curves have been detailed in Section 2.2.15 of Materials and Methods.
plate (possibly resulting in a slightly altered conformation), for binding sites in the IgG molecules present in the antiserum. The data presented clearly illustrates this point as the antiserum dilution curve is displaced to the left, thus indicating a good affinity of our prepared antiserum to its corresponding antigen, cytochrome P450 4A1.

Moreover, the specificity must be the primary criterion; unless an antiserum has the appropriate specificity it will be of no value in the assay regardless of its affinity and titre. The specificity of the raised antibody was further investigated by Western blot analysis and quantification by densitometry (Figure 2.6). The data presented was derived from hepatic microsomes of, clofibrate-, phenobarbital-, isoniazid-, and 3-methylcholanthrene- induced rat livers, representing members of the 4A, 2B, 2E and 1A subfamily haemoproteins, respectively. The data demonstrates the binding of cytochrome P450 4A1 by reacting the antibody with various cytochrome P450 isozymes as antigen. As would be expected, treatment with clofibrate led to several-fold increase in the level of immunodetectable cytochrome P450 4A1, whereas 3-methylcholanthrene and isoniazid treatments had the opposite effect in suppressing the P450 4A1 level, thus highlighting important changes occurring at the molecular level. Phenobarbital had a slight inductive effect but this was not significantly different from that of untreated rat.

The low levels of cross-reactivity seen with untreated, phenobarbital-, isoniazid- and 3-methylcholanthrene- induced microsomes may be as a result of a combination of contributions. Firstly it is highly likely that these microsomes possess low but immunochemically quantifiable constitutive levels of cytochrome P450 4A1 or secondly that the antibody recognises very similar epitopes to this haemoprotein. However, in a similar preparation, no cross-reactivity was noted between cytochrome P450 4A1 (P452) antibody and electrophoretically homogeneous preparations of P450b and P450c by immunodiffusion analysis [Tamburini et al., 1984].
Figure 2.6 Quantitation of cytochrome P450 4A1 in xenobiotic-induced liver microsomes by densitometry. A polyclonal sheep antibody (1:2000 dilution) to electrophoretically homogeneous rat liver cytochrome P450 4A1 was used in a Western blot procedure as described in Materials and Methods. Equivalent amounts of pooled microsomal protein (10μg) derived from control- (CONT.), clofibrate- (CLOFIB.); phenobarbital- (PB); isoniazid- (INZ) and 3-methylcholanthrene- (3-MC) pretreated rat livers (n=3), were separated on a 7.5% SDS-PAGE gel electrophoresis. Using authentic cytochrome P450 4A1 as reference (1.0pmol), results are expressed as pmol P450 4A1/ mg protein. Data represents the average of 2 determinations.
A typical calibration curve using the purified cytochrome P450 4A1 and raised polyclonal antibody, under optimised ELISA conditions, was constructed as depicted in Figure 2.7. It could be seen that there was a linear response up to about 0.04 pmol of the purified protein, indicating that very low amounts of this isozyme in various biological samples could be detected, as demonstrated in the subsequent chapters.

2.3.4 Immunopurification of cytochrome P450 4A1 polyclonal antibody

When rat hepatic-derived microsomes was separated on a 7.5% SDS-PAGE gel, blotted with our polyclonal antibody to highly homogeneous cytochrome P450 4A1, two closely related bands that co-migrated were routinely obtained (Figure 2.8). The lower of the two bands, with relative molecular mass of approximately 51.5 kDa, corresponded to cytochrome P450 4A1 and the other band is believed to be a closely-related member of the same P450 4A subfamily.

By using immunoaffinity columns, an attempt was made to improve the specificity of this antibody reacting with each of these bands. The experiment utilizes IgG-enriched fractions eluted from an immunoaffinity column with pH's 2.5, 4.0 and 5.5 used on Western blotting analysis. The results obtained (data not shown) do not appear to improve the specificity of the antibody to the P450 4A1 apoprotein and therefore suggests that the other co-migrating band may be intimately related to this isozyme which presents similar epitopes to the anti-cytochrome P450 4A1 polyclonal antibody.

In summary, the results presented for the above studies describe the purification and partial characterisation of highly homogeneous isoenzyme of the cytochrome P450 superfamily from hepatic microsomes of clofibrate-pretreated rats. Based on its molecular weight, spectral property (absorption maxima of the ferrous-CO
Figure 2.7 Typical calibration graph with purified cytochrome P450 4A1 was constructed as described in Materials and Methods (section 2.2.16). Each data point represent triplicate determinations.
Figure 2.8 Western blotting analysis on hepatic microsomes derived from untreated and clofibrate-induced rat liver was carried out as outlined in Materials and Methods. Track 1, authentic cytochrome P450 4A1 (0.5pmol); tracks 2, clofibrate-induced liver microsomes (20μg) and track 3, untreated rat liver microsomes (20μg).
complex) and catalytic activity towards lauric acid, I believe this isoenzyme is cytochrome P450 4A1 previously isolated in our laboratory. Other approaches used to confirm the identity of cytochrome P450 4A1 have been described elsewhere. This includes amongst those described above, uv - visible spectral properties for the spin state [Gibson et al., 1982], peptide fragmentation pattern [Tamburini et al., 1984] and molecular properties such as cDNA nucleotide and amino acid sequences [Hardwick et al., 1987; Earnshaw et al., 1988].
CHAPTER THREE

Stereochemical Specificity in the Induction of Cytochrome P450 4A1–Dependent Fatty Acid Hydroxylation and Peroxisome Proliferation in the Rat
Stereochemical Specificity in the Induction of Cytochrome P450 4A1-Dependent Fatty Acid Hydroxylation and Peroxisome Proliferation in the Rat

3.1 Introduction

The administration of clofibrate and various other hypolipidaemic agents including non-therapeutic compounds such as the phthalate ester plasticizers, results in several characteristic hepatic changes in rodents. These induced hepatic responses include hepatomegaly, proliferation of smooth endoplasmic reticulum and induction of cytochrome P450 4A1-dependent fatty acid ω-hydroxylase activity, peroxisome proliferation with associated changes in enzyme composition and alteration in mitochondrial number and structure with concomitant increases in certain enzyme levels [Orton and Higgins, 1979; Gibson et al. 1982; Reddy et al. 1982b; Sharma et al. 1988a]. These subcellular changes are toxicologically important as sustained proliferation of peroxisomes in rodents is frequently associated with the development of hepatocellular carcinomas and this has led to the suggestion that peroxisome proliferators constitute a novel class of non-genotoxic hepatocarcinogens [Reddy et al. 1980; Reddy and Lalwani, 1983; Lalwani et al. 1981].

In particular, the relationship between the xenobiotic-mediated induction of cytochrome P450 4A1 and peroxisome proliferation is at present still not clear. Various suggestions have been proposed including the presence of a common or related organelle biogenesis [Zaar et al. 1987] or a cytosolic "receptor protein" [Lalwani et al. 1983]. The involvement of such a receptor in the induction phenomenon would, by its presence or absence, readily explain the well-documented tissue, sex and species differences in response to these agents, but the existence of this putative receptor still remains a matter of debate [Milton et al. 1988].
More recently, screening a mouse liver cDNA library using a probe derived from the combined nucleotide sequences of several nuclear hormone receptors, has led to the identification of four new members of the hormone receptor family [Issemann and Green, 1990, 1991]. Importantly, one of these receptors was shown to be activated by the addition of a variety of peroxisome proliferators including a number of the hypolipidaemic drugs investigated [Issemann and Green, 1990] and was termed the Peroxisome Proliferator Activated Receptor (PPAR). Data obtained by these workers suggested that the PPAR could mediate the biological effects of peroxisome proliferators as well as explaining the tissue-specific response to these compounds.

Other attempts have been made to explore any correlation between the induction of several enzymes related to both drug and lipid metabolism following treatment with structurally diverse peroxisome proliferators [Sharma et al. 1988a]. Results from such studies have led to the proposal that catalytically-competent cytochrome P450 4A1 is a necessary prerequisite for peroxisome proliferation by these xenobiotics, as verified by more recent studies [Milton et al., 1990]. The proposed initial liver response is induction of microsomal cytochrome P450 4A1, which will generate increased cellular concentrations of long chain dicarboxylic acids. As these latter metabolites are preferentially degraded by β-oxidation in the peroxisome (as opposed to the mitochondrion), it has been proposed that dicarboxylic acids then form the proximal stimulus for peroxisome proliferation in an attempt by the cell to maintain lipid homoeostasis [Sharma et al. 1988a].

Based on preliminary studies at ICI Pharmaceuticals (U.K) by Dr Brian Holloway, this chapter further examines the above hypothesis proposed by Sharma and co-workers, by comparing the relative abilities of optically-active enantiomers and the racemate of a clofibrate structural analogue to induce microsomal cytochrome P450 4A1 and/or peroxisome proliferation.
3.2 Materials and Methods

3.2.1 Chemicals.

The optically pure R(-)-isomer, S(+)-isomer and the racemic mixture of a clofibrate analogue [2-[4-(4-chlorophenyl)benzyloxy]-2-phenylacetic acid], Figure 3.1a, were kindly provided by ICI Pharmaceuticals (Macclesfield, U.K) and synthesised by Dr M.E. Edge. Lauric acid, NADPH and prestained molecular weight markers (stock No. SDS-7B) were purchased from the Sigma Chemical Co. (Poole, U.K.). [1-14C]Lauric acid was supplied by the Radiochemical Centre (Amersham, U.K.)

Electrophoretically homogeneous cytochrome P450 4A1 and the corresponding anti-serum were prepared in our laboratories as outlined in Chapter Two. Antitrifunctional peroxisomal enzyme serum was a kind gift from Dr D. Cinti (University of Connecticut Health Centre, CT, U.S.A.). Donkey anti-sheep enzyme label, donkey anti-rabbit enzyme label, pre-immune sheep and pre-immune rabbit sera were kindly supplied by Guildhay Antisera Ltd (Guildford, U.K.).

3.2.2 Animals and Drug Pretreatment

Male Long Evans hooded rats (125-150 grams body weight, University of Surrey Breeders) were pretreated by gavage once daily for three consecutive days with each enantiomer or the racemic mixture at a dose level of 80mg/kg. Compounds were administered in gum tragacanth as vehicle and the control group received the same volume of vehicle only. All animals were sacrificed at the start of the fourth day, i.e. 24h after the last dose.

The livers, following excision, were rinsed and perfused with 0.9% (w/v) sodium chloride to remove contaminating blood, blotted dry and weighed. All subsequent steps were performed at 4°C. The livers were scissor-minced and
Figure 3.1 Structures of the clofibrate analogue, 2-[4-(4-chlorophenyl)benzyloxy]-2-phenylacetic acid (a), showing the chiral centre (*) and clofibrin acid (b).
homogenized in 0.25M sucrose using a Potter-Elvehjem glass-Teflon homogenizer. The liver homogenate was then adjusted to 25-33% (w/v) by the addition of 0.25M sucrose. Aliquots of homogenate were frozen at -80°C for subsequent analysis.

The homogenates were then processed by the standard centrifugation procedures for preparing microsomal pellet as previously described in the Materials and Methods section of Chapter Two. The resuspended microsomal pellet was stored as 1ml aliquots at -80°C and under these conditions the cytochrome P450 content and related enzyme activities remained stable for periods of up to several months.

3.2.3 Spectrophotometric Enzyme Assays

Total homogenate and microsomal protein contents, as well as total carbon monoxide-discernible cytochrome P450 were routinely determined by methods of Lowry et al., [1951] and Omura and Sato, [1964] respectively, as outlined in Materials and Methods, Chapter Two.

3.2.4 Determination of Lauric Acid Hydroxylase Activity

The metabolism of lauric acid to (ω-1)- and ω-hydroxylated products was evaluated by reverse phase h.p.l.c analysis essentially as described by Parker and Orton [1980] and as detailed in Materials and Methods, Chapter Two.

3.2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

The technique of ELISA as described by Voller et al., [1978] and modified for cytochrome P450 4A1 was utilised to quantify this haemoprotein in microsomal samples, as outlined in Materials and Methods, Chapter Two.
3.2.6 Western Blot Analysis

The methods employed were modifications of the basic procedures of Towbin et al. [1979], Burnette [1981] and reviewed by Bers and Garfin [1985] which are detailed under Materials and Methods, Chapter Two.

3.2.7 Estimation of Cyanide-Insensitive Palmitoyl CoA β-Oxidation

The method utilised was that of Bronfman et al., [1979]. The oxidation of palmitoyl CoA results in the reduction of FAD and NAD⁺. In mitochondria, these are re-oxidised by the cytochrome chain which can be blocked by the introduction of cyanide into the assay media. However, in peroxisomes, FADH is re-oxidised by molecular oxygen but the NADH accumulates and therefore an assay of NADH production in the presence of cyanide serves as a measure of the peroxisomal fatty acid oxidase system, as the palmitoyl CoA oxidase is the first and rate limiting enzyme in the system.

The assay medium containing, at a final concentration coenzyme A (75μM), FAD (180μM), NAD⁺ (555μM), nicotinamide (141mM), DTT (4.2μM), KCN (3μM) and BSA (255μg/ml) in 60mM Tris-HCl buffer (pH 8.3) was freshly prepared and stored on ice. Both sample and reference cuvettes each contained 25μl aliquots of rat liver homogenate solubilised with an equal volume of 60mM Tris-HCl buffer (pH 8.3) containing 1% (w/v) Triton X-100 at 37°C for 2 minutes. To the solubilized homogenate was added 2ml of reaction mixture and 0.9ml of the above Tris-HCl buffer. The resultant incubation mix was allowed to equilibrate for a further period of 5 minutes at 37°C. The reaction was initiated by the addition of 20μl palmitoyl CoA (75μM) and the change in absorbance at 340nm at 37°C was recorded using a Cary 219 spectrophotometer. Results were expressed as nmol NADH produced per minute per mg of protein, using the molar extinction coefficient of 6220 M⁻¹ cm⁻¹ for NADH at 340nm.
3.2.8 Measurement of Carnitine Acetyl Transferase Activity

Total (peroxisomal and mitochondrial) carnitine acetyl transferase activity was measured by the method of Bieber et al., [1972] and Bock et al., [1980]. The assay is based on the measurement of free CoA formed during the acyl transfer from acyl-CoA to added carnitine; the free CoA being determined by the sulphhydryl reagent, 5,5'-dithio-bis-2-nitrobenzoic acid (DNB).

The 2ml incubation medium contained 200μl of diluted homogenate (1:5 in 116mM Tris-HCl, pH 8.0 containing 2.5mM EDTA, 0.2% (w/v) Triton X-100); 100μl of acetyl-CoA (2mM in distilled water) and 1.7ml of DNB (0.5mM in the above Tris-HCl buffer). The solution was mixed and centrifuged briefly at 13,000rpm (MSE minicentrifuge). The incubation mixture was then divided between two 1ml quartz cuvettes and the reaction initiated by the addition of 10μl of carnitine (1.0M in Tris-HCl buffer). The change in absorbance was recorded at 412nm, 25°C, in a Cary 219 spectrophotometer. The rate of reaction was calculated using the molar extinction coefficient of the product at 412nm of 13,600 M⁻¹cm⁻¹.

3.2.9 Immunological Detection of Peroxisomal Trifunctional Protein

a) Enzyme-Linked Immunosorbent Assay (ELISA)

The method previously described for the quantification of cytochrome P450 4A1 was adapted for the quantification of peroxisomal trifunctional protein.

Freeze-dried sera from rabbits immunised with trifunctional protein was reconstituted in phosphate buffered saline (PBS) prior to use. Homogenate samples were solubilised with an equal volume of 60mM Tris-HCl buffer (pH 8.3) containing 1% (w/v) triton X-100 at room temperature for 1h. The solubilised samples were then diluted as appropriate in sodium bicarbonate/ carbonate buffer (0.1M, pH 9.6) and loaded on a nanogram-protein basis in a total volume of 200μl.
per well. A standard curve of solubilised homogenate from control rat liver was constructed from 0 to 2.0μg protein (Figure 3.2).

b) Western Blot Analysis

The SDS-PAGE gel was cast as previously described except that the acrylamide and bis-acrylamide concentrations of the upper gel were 3% and 0.13% respectively. Samples were solubilised as before and loaded on an equiprotein basis (usually 30μg in 10μl total volume). The other difference with the protocol previously described, was the use of donkey anti-rabbit enzyme label (horseradish peroxidase) as the primary antibody was raised in the rabbit.
Figure 3.2 Immunodetection of hepatic trifunctional protein of the β-oxidation spiral in total homogenate of untreated rat. A rabbit polyclonal antibody to this protein was used in the ELISA procedure as described in Materials and Methods at a dilution of 1:20,000. Linearity in the quantitation of the trifunctional protein was established. Each data point is derived from triplicate determinations.
3.3 Results

The effect of administration of the pure enantiomers and racemic mixture of the clofibrate analogue on the liver/body weight ratio (a measure of hepatomegaly), total carbon monoxide-discernible cytochrome P450 and specific cytochrome P450 4A1 isozyme levels are presented in Table 3.1.

Induction of specific cytochrome P450 4A1 was quantified by employing the enzyme-linked immunosorbent assay (Table 3.1). The cytochrome P450 4A1 isozyme level in the control group was approximately 5% of the total cytochrome P450 population, and induction by the isomers and racemate led to 1.5-4-fold induction over this constitutive level. The highest fold increase in cytochrome P450 4A1 was observed after treatment with the R(-)-enantiomer and corresponded to an eudismic ratio of approximately 3. Furthermore, the influence of treatment on cytochrome P450 4A1-dependent (ω-1)- and ω— hydroxylation of lauric acid, as determined by reverse phase h.p.l.c. analysis is shown in Figure 3.3. The data shows preferential induction of the ω—hydroxylase activity with the highest fold increase of 4 produced by the R(-)-enantiomer and a 1.3-fold induction with the corresponding S(+)-antipode. Such a preferential induction of ω—hydroxylase activity has been previously demonstrated with other peroxisome proliferators [Sharma et. al., 1989], and reflects the regioselectivity of the cytochrome P450 4A1 isozyme for lauric acid ω— hydroxylation [Gibson et al., 1982].

Western blotting analysis of hepatic microsomes derived from the various treatment groups was performed using a sheep polyclonal antibody raised against purified rat hepatic cytochrome P450 4A1 (Figure 3.4). In particular, the data presented demonstrates that the anti-cytochrome P450 4A1 serum recognises two proteins from both control and treatment groups. It appears that the lower of these two bands, which is preferentially induced, co-migrates with authentic hepatic cytochrome P450 4A1. Furthermore, following treatment (particularly with the R(-)
Table 3.1  Stereoselective Induction of Hepatomegaly and Cytochrome P450/ P450 4A1 in the Male Long Evans Hooded Rat.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver/body weight ratio (%)</th>
<th>Total cytochrome P450 specific content (nmol/mg protein)</th>
<th>Cytochrome P450 4A1 Quantitation (nmol/mg protein)</th>
<th>% Total P450</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.64 ± 0.31</td>
<td>0.48 ± 0.06</td>
<td>0.028 ± 0.003</td>
<td>5.95 ± 0.88</td>
</tr>
<tr>
<td>R(-)-isomer</td>
<td>6.67 ± 0.41*</td>
<td>0.57 ± 0.06*</td>
<td>0.107 ± 0.012**</td>
<td>19.40 ± 3.270**</td>
</tr>
<tr>
<td>S(+) - isomer</td>
<td>5.83 ± 0.19</td>
<td>0.49 ± 0.07</td>
<td>0.037 ± 0.007**</td>
<td>7.44 ± 0.41**</td>
</tr>
<tr>
<td>Racemate</td>
<td>5.97 ± 0.26</td>
<td>0.55 ± 0.09*</td>
<td>0.059 ± 0.006**</td>
<td>10.88 ± 0.750**</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of six animals (control) or three animals in the test groups (80 mg/kg as detailed in Materials and Methods). *P < 0.01, **P < 0.005.
Figure 3.3 Stereochemical induction of lauric acid hydroxylation by rat liver microsomes. Rats (six animals in control and three in the test groups) were pretreated with the clofibrate structural analogues and both ω- and (ω-1)- laurate hydroxylase activities were determined by reverse phase h.p.l.c. as detailed in Materials and Methods. Each set of data points is generated from triplicate analyses.

*P< 0.01, **P< 0.005
Figure 3.4 Western blot analysis of cytochrome P450 4A1 induction in the rat. Rats were pretreated with the R(-)- isomer, the S(+)- isomer or the racemate of the clofibrate analogue and Western blot analysis carried out as described in Materials and Methods. 10µg of microsomal protein (from pooled microsomal fractions) were loaded on each track as follows: track 1, prestained molecular weight markers; track 2, control microsomes; track 3, racemate pretreatment; track 4, S(+) enantiomer pretreatment; track 5, R(−) enantiomer pretreatment; track 6, authentic cytochrome P450 4A1 (0.5pmol). The antibody was used at a dilution of 1:2000.
-enantiomer, the levels of hepatic cytochrome P450 4A1 as determined by ELISA, in addition to the induction of the cytochrome P450-dependent ω-hydroxylation of lauric acid noted above, approximated more closely to the induction of the lower of the two bands. This data therefore lends further support to the conclusion that in the liver, the cytochrome P450 4A1 isozyme is represented by the lower molecular mass species, as previously observed [Sharma et al., 1989]. From the relative intensities of the protein bands judged by visual examination, the R(-)-isomer was again the more potent inducer of cytochrome P450 4A1 haemoprotein.

The effect of treatment on peroxisomal and mitochondrial lipid-metabolising enzymes is presented in Table 3.2. The data presented are for peroxisomal cyanide-insensitive palmitoyl-CoA oxidation (PCOA) and total (peroxisomal plus mitochondrial) carnitine acetyl transferase activity (CAT). The R(-)-enantiomer was more potent than the S(+) isomer in inducing peroxisomal β-oxidation activity, the induction corresponding to an eudismic ratio of approximately 2.2. Similarly, carnitine acetyl transferase activity was also demonstrated to show stereoselectivity in the induction process. Although this latter enzyme, unlike palmitoyl CoA oxidase, is not a specific peroxisomal marker, much of the observed increase in enzyme activity may be ascribed to induction of peroxisomal rather than mitochondrial enzyme activity [Zaar et al., 1987]. An eudismic ratio of approximately the same magnitude as that for KCN-insensitive palmitoyl CoA oxidation was observed (Table 3.2).

The high sensitivity of an adapted ELISA method for the quantification of the trifunctional protein would be expected to highlight some differences which may otherwise escape detection during visual comparison of the Western blot analysis. A preliminary screening on the response of this protein, in the total homogenate from various treatments, to the antibody was carried out. The results obtained demonstrate a linear interaction was apparent up to approximately 500 nanograms for both the racemate and R(-)-isomer-treated groups (Figure 3.5) and about 1000
Figure 3.5 Immunodetection of hepatic trifunctional protein of the β-oxidation spiral in total homogenate of rats treated with either the R(−)-isomer or the racemic mixture of the clofibrate analogue. A rabbit polyclonal antibody to this protein was used in the ELISA procedure as described in Materials and Methods at a dilution of 1:20,000. Linearity in the quantitation of the trifunctional protein was established. Each data point is derived from triplicate determinations.
Table 3.2  Differential induction of palmitoyl CoA oxidase and carnitine acetyl-CoA transferase activities by stereoisomers of a clofibrate structural analogue.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Palmitoyl CoA oxidation (nmol NADH/min/mg protein)</th>
<th>Carnitine acetyl transferase activity (nmol CoA/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.42 ± 0.91</td>
<td>1.42 ± 0.44</td>
</tr>
<tr>
<td>R(-)-isomer</td>
<td>11.67 ± 2.66**</td>
<td>14.68 ± 4.90*</td>
</tr>
<tr>
<td>S(+) -isomer</td>
<td>5.20 ± 0.62</td>
<td>2.71 ± 0.79*</td>
</tr>
<tr>
<td>Racemate</td>
<td>8.71 ± 1.42**</td>
<td>6.01 ± 0.61**</td>
</tr>
</tbody>
</table>

R/S activity ratio 2.24 5.42

Values are for mean ± SEM of either six animals (control) or three animals in the test groups and treated as detailed in Materials and Methods.

*P < 0.01, **P < 0.005.
nanograms for the S(+) -isomer (data not shown). Based on this preliminary screening procedure, appropriate dilutions were then subjected to the ELISA protocol. Results for ELISA-based quantification of the trifunctional protein of the peroxisomal beta-oxidation spiral are presented in Figure 3.6, and demonstrates that the R(-) -isomer was more potent in inducing this protein (R/S activity ratio of 5.4). Further evidence lending support to this latter conclusion was obtained from Western blotting analysis using a polyclonal antibody to the trifunctional protein raised in the rabbit (Figure 3.7). Because of non-availability of the authentic trifunctional protein as a standard, this protein was identified by its co-migration with prestained molecular weight markers. The observed molecular weight of approximately 80kDa for this peroxisome associated polypeptide agreed with that of previous reports [Reddy et. al. 1981]. Two lower molecular weight proteins were also recognized by the antibody to the trifunctional protein, and are believed to be previously described proteolysis products [Nemali et. al. 1989].
Figure 3.6 Induction and immunochemical quantitation of peroxisomal trifunctional protein. ELISA determinations were carried out as described in Materials and Methods from pooled homogenates (six in control group, three in test groups). 100ng of protein were loaded per well from each of the R(-)-isomer and racemate-treated groups and 500ng from the S(+) -treatment group. Freeze-dried anti-trifunctional enzyme immune serum was reconstituted and diluted 1:20,000. Data are expressed as (units) mg control animal protein equivalents/ mg protein and represents mean ± SEM of 3 to 6 animals in each group. *P < 0.01, **P < 0.001.
Figure 3.7  Induction and Western blot analysis of the peroxisomal trifunctional protein. Rats were pretreated with the R(-)- isomer, the S(+) - isomer or the racemate of the clofibrate analogue and Western blot analysis carried out as described in Materials and Methods. 30μg of total liver homogenate protein was loaded on each track (pooled from 3 to 6 animals) as follows. Track no.: 1 and 6, prestained molecular weight standards; 2, control homogenate; 3, R(-)-enantiomer pretreatment; 4, S(+) -enantiomer pretreatment; 5, racemate pretreatment.
3.4 Discussion

It is well established that \textit{in vivo} treatment with a number of structurally diverse hypolipidaemics to susceptible species such as the rat and mouse results in changes in liver biochemistry and morphology (Reddy \textit{et al.}, 1980; Bains \textit{et al.}, 1985; Sharma \textit{et al.}, 1988a). The results of the present study clearly demonstrates that there is a high degree of enantiomeric induction selectivity in lipid-metabolising enzymes and that the R(-)-isomer of a clofibrate analogue appears to be the more potent of the enantiomeric forms administered. My results also demonstrate the close relationship that exists between induction of the microsomal and peroxisomal lipid-metabolising enzymes following challenge with these xenobiotics. The following induction potency was obvious in all the parameters investigated in the present study: R(-)-isomer > racemate > S(+)-isomer > control. Moreover, the R(-)-enantiomer caused approximately double the inductive effects of the racemate suggesting that, at the dose levels administered and the duration of study, it is this enantiomeric form that mainly contributes to the observed changes in hepatic enzymes.

The induction of cytochrome P450 4A1 is mirrored by the concomitant parallel increase in lauric acid hydroxylase activity. Cytochrome P450 4A1 has previously been documented as the major hepatic isozyme of cytochrome P450 that exhibits a preferential regioselectivity for the $\omega-$hydroxylation of lauric acid in the rat [Gibson \textit{et al.}, 1982; Sharma \textit{et al.}, 1989], although more recent results have indicated that other members of the cytochrome P450 4A subfamily exhibit this substrate specificity [Nebert \textit{et al.}, 1991; Gibson and Lake, 1991]. Results obtained by reverse phase h.p.l.c. analysis (Figure 3.3), further substantiated the phenomenon of enantioselectivity in the induction process and again the R(-)-isomer was much more potent in inducing lauric acid $\omega-$hydroxylase activity. Very clearly, an eudismic ratio of approximately 3 is further evidence for enantioselectivity in this process. Hence, although the administration of these
clofibrate analogues caused rather small increases in total microsomal cytochrome P450 content, the large increase in ω-hydroxylase activity for lauric acid as exemplified with the R(-)-isomer, demonstrates that there are significant changes occurring among some constitutive forms present in the rat liver microsomes.

Further support for enantioselective induction is provided by Western blot data which demonstrates that both control and induced hepatic microsomes contained two cytochrome P450 4A1-related polypeptides that are recognized by the antibody (Figure 3.4). These two polypeptides are of unequal intensity with the lower molecular mass band predominating in all the various treatment groups. The molecular mass of the lower band is consistent with that of authentic rat hepatic cytochrome P450 4A1 (51.5 kDa) and the higher band estimated to be 52 kDa. At present, the precise nature of the 52 kDa polypeptide is unknown, but is believed to be a closely related member of the cytochrome P450 4A subfamily possibly cytochrome P450 4A3 [Kimura et al., 1989a]. This observation is based on the isolation of a novel P450 cDNA, designated Cyp4A3, isolated from a λgt11 cDNA library from clofibrate-treated rat liver by screening with the lauric acid ω-hydroxylase, 4A1, cDNA probe. Cytochromes P450 4A1 and 4A3 display 72% amino acid sequence similarity and are expressed in livers of rats treated with the hypolipidaemic drug, clofibrate [Kimura et al., 1989a]. These findings were further substantiated by the work carried out by Aoyama et al., [1990], who were able to demonstrate laurate hydroxylase catalytic activities for cytochromes P450 4A1 and 4A3 by cDNA-directed expression using vaccinia virus.

Many critical features of the biological action of xenobiotics arise from their interaction with endogenous chiral molecules such as receptors or enzymes and it is therefore not surprising that the biological actions of chiral, pharmacologically active molecules reside predominantly in one enantiomer [Testa, 1988; Jenner and Testa, 1973; Simonyi et al., 1986; Ariens, 1983]. For example, the 2-arylpropionic acids, an important group of non-steroidal anti-inflammatory drugs
(the profen series), contain a chiral centre and in vitro studies on inhibition of prostaglandin synthesis demonstrates that activity resides almost exclusively in the S(+)-isomer with a eudismic ratio of 160 [Adams et al., 1976]. However, in vitro, this activity ratio is dramatically reduced to 1.3, due to the unidirectional metabolic inversion of the chiral centre from the inactive R(-)-isomer to the S(+)-antipode [Caldwell et al., 1988]. Although the metabolic disposition and potential metabolic inversion of the enantiomeric clofibrate analogues described herein have not been investigated, it would appear unlikely that metabolic chiral inversion occurs in this case as may be deduced from the induction studies reported herein.

The stereospecificity of liver enzyme induction noted in this study may be explained by differences in xenobiotic absorption, distribution or tissue uptake. Alternatively, enantiospecific selectivity may reside in the specific interaction of the inducer with a chiral intracellular macromolecule, which then initiates the induction process. This macromolecule is probably not a classical cytosolic protein receptor as was suggested by Lalwani et al., [1983] and subsequently disputed [Milton et al., 1988], but may be associated with the 5'-flanking regulatory segments of the cytochrome P450 4A1 and/or peroxisomal fatty acid beta-oxidation genes or the associated nuclear binding proteins, based on the fact that clofibrate induction of cytochrome P450 4A1 is by transcriptional activation of the corresponding gene [Hardwick et al., 1987], a concept that will be developed further in Chapter Six (Final discussion).

In conclusion, I have been able to demonstrate that there is stereochemical specificity in the induction of microsomal cytochrome P450 4A1-dependent fatty acid hydroxylase activity. These results identify the R(-)-enantiomer as the eutomer and its corresponding S(+)-antipode as the distomer, with the racemic mixture intermediary in inducing this enzyme activity. Also an identical enantiomeric selectivity was observed for the phenomenon of peroxisome proliferation by these compounds. Thus, taken collectively, the above findings are not inconsistent with
the previously stated hypothesis [Sharma et al., 1988a] that cytochrome P450 4A1 induction and peroxisome proliferation are intimately linked. Whether the observed stereochemical selectivity resides in xenobiotic recognition or disposition still remains to be determined.
CHAPTER FOUR

Co-induction of Cytochrome P450 4A Subfamily Proteins and Peroxisome Proliferation in the Rat and Guinea Pig by Perfluoro-n-Decanoic Acid [PFDA]
Co-induction of Cytochrome P450 4A Subfamily Proteins and Peroxisome Proliferation in the Rat and Guinea Pig by Perfluoro-n-decanoic Acid [PFDA]

4.1 Introduction

The perfluoro-fatty acids are widely used in chemical industries as lubricants, surfactants, wetting agents and corrosion inhibitors [Olson and Andersen, 1983; Bryce, 1964; Shinoda and Nomura, 1980]. The choice of compound to be used for a specific purpose is largely dictated by economic factors, performance indices and their toxicity. The latter is an important consideration because of the potential occupational exposure to workers involved in the production and use of such products. Perfluorinated compounds have also been proposed for use as vascular replacement fluids and as contrast media in computer-assisted tomography, and as such, patients undergoing treatment designed to prevent heart attack are also at risk [Takagi et al., 1991].

Perfluorinated compounds are generally considered to be metabolically inert [Clark et al., 1973]. However, recent studies involving the use of polychlorotrifluoroethylene (PCTFE), a perhalogenated chlorofluorocarbon, has shown that such compounds could be metabolized to carboxylic acid derivatives in rats which may be implicated in their toxicity [Brashear et al., 1992; Greene et al., 1992] The relatively recent discovery that perfluoro-fatty acids also cause a number of hepatotoxic effects, raises some questions on their safety [Ikeda et al., 1983; Pastoor et al., 1987; Harrison et al., 1988; Just et al., 1989; Abdellatif et al., 1991; Vanden Heuvel et al., 1991]. In particular, these halogenated hydrocarbons produce hepatomegaly (hyperplasia and hypertrophy), peroxisome proliferation and induction of smooth endoplasmic reticulum, the latter being accompanied by induction of a spectrum of enzymes including the cytochrome P450 superfamily of
isoenzymes [Nebert et al., 1991]. This spectrum of biological responses is very similar to that extensively studied in our laboratory [Gibson et al., 1982; Bacher and Gibson, 1988; Sharma et al., 1988a,b; Milton et al., 1990; Chinje and Gibson, 1991] in the safety assessment of phthalate ester plasticisers, chlorophenoxy acid herbicides and lipid-lowering drugs of the oxy-isobutyrate class, such as clofibrate and its analogues. Collectively, these chemicals are classified as peroxisome proliferators and concern has been expressed about their safety because they are non-genotoxic carcinogens in susceptible species, particularly rodents [Reddy and Lalwani, 1983], probably as a result of indirect DNA damage caused by oxidative stress mechanisms [Elliot et al., 1986; Takagi et al., 1990].

A number of bioassays have been used to assess the genotoxic potential of PFDA amongst other similar compounds [Godin et al., 1992] By utilising the chromosomal aberration assay, PFDA indicated positive only when conducted in the presence of S9 fraction and in an in vivo/in vitro unscheduled DNA synthesis (UDS) assay, it induced S-phase synthesis 48h after dosing [Godin et al., 1992]. Although there are no available data on nephrotoxicity of the perfluoro-fatty acids, it has been suggested that PFOA has some effects on the kidney [Goldsworthy and Popp, 1987]. Generally, the biological effects appear to be of a persistent nature [Van Rafelghen et al., 1987] and exposure of rodents has been demonstrated to give rise to a peculiar "wasting syndrome" resulting in severe weight loss and hypothermia [Langley, 1990]. It has been reported that the body weights of rats provided with a diet containing PFDA ceased to increase, even though these animals consumed as much food as their control counterparts [Borges et al., 1990].

Much information on the hepatotoxicity of oxy-isobutyrate drugs is available, such as species differences in species response [Makowska et al., 1991], dose-response relationships [Sharma et al., 1988b] and molecular mechanisms of toxicity [Lock et al., 1989; Milton et al., 1990; Gibson, 1989], however, very little equivalent information is available for the perfluoro-fatty acids. Accordingly, this
chapter is aimed at further investigating the biochemical liver and kidney changes elicited in response to a representative member of the perfluoro-fatty acids, PFDA, and by comparing the information obtained, with better characterised peroxisome proliferators, will provide the valuable biological information for their safety assessment and ultimately provide a valuable input to choice of product development and human exposure.
4.2 Materials and Methods

4.2.1 Chemicals

Perfluoro-n-decanoic acid (PFDA recrystallised, approximately 99.5% pure), was kindly provided by Dr B. Jamot, of the Biochemistry Branch, Toxic Hazards Division, Wright-Patterson Air Force Base, Dayton, Ohio, U.S.A.

All other reagents and chemicals used were as previously described in Materials and Methods Section (Chapter Two).

4.2.2 Animals and Drug Pretreatment

Male Wistar albino rats (150-200g body weight, University of Surrey Breeders) and male Dunkin Hartley guinea pigs (350-450g body weight, David Hall, Burton-On-Trent, Staffs.) were housed separately on wood chip bedding in a temperature controlled room with an 11-hour/13-hour light/dark cycle. The rats were fed a standard rat chow (LAD1 breeder) and the guinea pig, a diet supplemented with vitamin C (FD1 breeder + stock). All diets used for feeding were supplied by Special Diets Services (Witham, Essex, England). All animals were provided water ad libitum throughout the experiment.

In all experiments, PFDA treatment consisted of a single i.p administration of 20 mg/kg body weight dissolved in a propylene glycol-water (1:1(v/v)) vehicle delivered in a volume of 1ml/kg body weight. Weight-matched pair-fed controls were given the same vehicle in a volume of 1ml/kg body weight and then pair-fed to the daily feed consumption record of the appropriate PFDA-treated animals. Ad libitum-fed control animals received the vehicle only. All the animals were killed at the start of the eighth day after commencement of treatment and the livers and kidneys were quickly removed. Microsomes were prepared from 33% (w/v) tissue homogenate in 0.25M sucrose, as previously described (Chapter Two, Section 2.2.3).
4.2.3 Total Protein Determination

Protein content in all instances, was determined according to the method of Lowry et al., [1951] and as outlined in Chapter 2, using crystalline bovine serum albumin as the standard.

4.2.4 Spectrophotometric Enzyme Assays

Total CO-discernible cytochrome P450 content, cytochrome b5 specific content and NADPH-cytochrome c reductase activity were determined as outlined in Sections 2.2.9; 2.2.10 and 2.2.11, respectively of Chapter Two.

4.2.5 Determination of Lauric Acid Hydroxylation

The metabolism of [1-14C] lauric acid to (ω−1)- and ω−hydroxylated products was evaluated using h.p.l.c by employing the method described by Parker and Orton [1980] and as outlined in Section 2.2.14, Chapter Two.

4.2.6 Determination of Alkoxyresorufin-O-Deethylation

The O-deethylation of ethoxyresorufin and pentoxyresorufin were determined spectrophotometrically, according to the method of Burke and Mayer [1974] and as outlined below.

The assay system composed of 2ml of 0.1M Tris-HCl buffer (pH 7.8), either 10μl ethoxyresorufin (0.1mM stock solution made in DMSO) or 5μl pentoxyresorufin (1.0mM stock in DMSO), and an appropriate volume of microsomes (equivalent to 0.5-2mg protein). The assay was performed in a fluorimeter cuvette placed in a thermostatically controlled cuvette housing of a Perkin-Elmer MPF-3 fluorimeter, set at an excitation wavelength of 510nm and an emission wavelength of 586nm with slit widths of 10 and 2.5nm respectively. After
preincubation at 37°C for 2 minutes, a steady baseline with time was recorded. The reaction was initiated with 10µl of 50mM NADPH (1mM final concentration) and the production of resorufin with time monitored as the increase in fluorescence at 586nm. The fluorimeter response was calibrated with multiples of 2µl of resorufin standard (10mM stock solution in DMSO).

4.2.7 Measurement of Peroxisomal and Mitochondrial Enzyme Activities

Whole liver and kidney homogenates were assayed for the activities of KCN-insensitive palmitoyl-CoA oxidation and carnitine acetyl-CoA transferase as outlined in sections 3.2.7 and 3.2.8, respectively, of Chapter Three.

4.2.8 Western Blotting Analysis

Western blotting procedures employing polyclonal antisera to cytochromes P450 4A1 and 1A1 as well as the trifunctional protein of the peroxisomal β-oxidation spiral, were conducted as previously described (Section 2.2.17, Chapter Two). Western blots were also quantified by densitometry using a CS-9000 Dual Wavelength Flying-spot scanner (Shimadzu, Tokyo, Japan).

4.2.9 Statistical Analysis

The main effects of treatment (PFDA versus pair-fed) were analysed by two-way analysis of variance (ANOVA). Linear regression analysis was used to describe the relationship between cumulative feed intake and change in body weight at 7 days following treatment and statistical difference between PFDA-treated rats and those receiving vehicle was evaluated by simple Students' t-test for small sample size (two-tailed). In all cases, significance levels were set at either 5% (P < 0.05) or 1% (P < 0.01).
4.3 Results

4.3.1 General Toxicity of PFDA

At the dose level investigated (20 mg/kg body weight), PFDA did not show any significant variability in body weight change and feed intake over the 7-day period following dosing for any of the species studied. Rats receiving this dose of PFDA had a cumulative feed intake that was similar to the vehicle-treated group with unlimited access to feed (ad libitum-fed control). Moreover, these PFDA-treated rats did gain slightly more weight than either the ad libitum-fed or the pair-fed animals (Table 4.1), although this was not judged to be statistically significant (P > 0.05). In contrast, male guinea pigs receiving the same dose of PFDA, showed a slight decrease in cumulative feed intake which was statistically significant when compared to the ad libitum-fed control group. However, these treated guinea pigs, like their pair-fed counterparts, did not gain as much weight as those receiving vehicle with unlimited feed intake (Table 4.1).

The relationship of cumulative feed intake (grams/7 days) to changes in body weight, with respect to day zero for the rat was further examined using linear regression analysis (Figure 4.1). Similar information was also obtained with the guinea pig treatment groups (Figure 4.2). As shown on the plots, the relationship was linear within all the experimental groups.

4.3.2 Some Pathological Changes Associated with PFDA Dosing

The only striking pathological feature of PFDA toxicity in the rat but which was not seen in the guinea pig, was enlargement of the liver over the corresponding pair-fed group eight days post-dosing. The increase in relative liver weight (expressed as percentage of animal body weight) in this species, was about
Table 4.1 Effects of single dose of PFDA on body weight and cumulative feed intake in the male rat and guinea pig

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment Group</th>
<th>Initial Body Weight (g)</th>
<th>Final Body Weight (g)</th>
<th>Change in Body Weight (g)</th>
<th>Cumulative Feed Intake (g/7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>AL</td>
<td>216.6 ± 19</td>
<td>272.9 ± 19</td>
<td>56.3 ± 1.8</td>
<td>163.1 ± 1.2</td>
</tr>
<tr>
<td>RAT PFDA</td>
<td>215.9 ± 14</td>
<td>277.6 ± 14</td>
<td>61.7 ± 1.8</td>
<td>163.8 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>PF</td>
<td>225.3 ± 11</td>
<td>278.7 ± 11</td>
<td>53.5 ± 2.8</td>
<td>162.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>AL PFDA</td>
<td>433.4 ± 26</td>
<td>532.7 ± 28</td>
<td>99.3 ± 4.6</td>
<td>299.2 ± 9.8</td>
<td></td>
</tr>
<tr>
<td>GUINEA PFDA</td>
<td>450.1 ± 6</td>
<td>517.2 ± 11</td>
<td>67.2 ± 8.4**</td>
<td>271.2 ± 12*</td>
<td></td>
</tr>
<tr>
<td>PIG PF</td>
<td>453.7 ± 10</td>
<td>519.0 ± 13</td>
<td>65.3 ± 7.9**</td>
<td>267.1 ± 28*</td>
<td></td>
</tr>
</tbody>
</table>

Values are reported as the mean ± SEM for six animals per group. Final body weight was determined 7 days after treatment as outlined in Materials and Methods (i.e at the start of the 8th day). AL = Ad libitum-fed; PFDA = perfluoro-n-decanoic acid-treated; PF = Pair-fed. *P < 0.05; **P < 0.01
Figure 4.1 Relationship of cumulative feed intake in the rat to change in body weight in different treatment groups.
Figure 4.2 Relationship of cumulative feed intake in the guinea pig to change in body weight in different treatment groups.
1.4-fold over that of its pair-fed control partners (Figure 4.3). Concomitant with this effect in the rat, was a decrease in microsomal protein content expressed as mg/gram wet liver tissue. Similarly, the data obtained for hepatomegaly in the guinea pig was also included in Figure 4.3 and showed a slight liver enlargement which was not statistically significant (P>0.05). No significant changes were observed in the relative kidney to body weight ratio expressed as a percentage in either of the species examined (Figure 4.4). In the present study, no macroscopical hepatocellular observations in these species were investigated, however, some swellings were noted in at least two of the PFDA-dosed rat livers and their precise nature was not determined.

4.3.3 Changes in Microsomal Enzymes

Table 4.2 summarises the induction of the mixed function monooxygenase system in liver-derived microsomes from the rat and guinea pig. A similar information obtained in kidney-derived microsomes, is presented in Table 4.3. In rat liver, PFDA treatment resulted in statistically significant induction (P< 0.01) of total cytochrome P450 specific content as well as NADPH-cytochrome c reductase activity when compared to the pair-fed controls. No spectral shifts were observed for CO-cytochrome P450 adduct in any of the experimental group (data not shown) In the guinea pig liver, PFDA-induced change in total cytochrome P450 content was not statistically significant over the pair-fed group. In addition there was a slight decrease in NADPH-cytochrome c reductase activity (P< 0.05). Determinations of cytochrome b5 in all the treatment groups of each species revealed no significant differences (Table 4.2). In general, pair-feeding appeared to have minimal effects on the liver monooxygenase system when compared to ad libitum-fed controls. With the exception of NADPH-cytochrome P450 reductase activity in the guinea
Figure 4.3 The effect of PFDA on guinea pig and rat liver sizes. Animals were treated as outlined in Materials and Methods and the values presented are the mean ± SEM from 6 animals per group. *P values for results significantly differed between PFDA-dosed versus pair-fed and ad libitum-fed versus pair-fed. *P < 0.05.
Figure 4.4 The effect of PFDA on guinea pig and rat kidney sizes. Animals were treated as outlined in Materials and Methods and the values presented are the mean ± SEM from 6 animals per group.
Table 4.2  Modulation of Hepatic Microsomal Mixed Function Monooxygenase System in the Rat and Guinea Pig

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Protein Content (mg/gram liver)</th>
<th>Total Cytochrome P450 Specific Content (nmol/mg)</th>
<th>NADPH-Cytochrome P450 Reductase Activity (nmol/min/mg)</th>
<th>Cytochrome b₅ Specific Content (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>13.4 ± 1.6</td>
<td>1.01 ± 0.06</td>
<td>74.6 ± 6.3</td>
<td>0.41 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>RAT</td>
<td>13.7 ± 0.7</td>
<td>1.30 ± 0.10**</td>
<td>95.6 ± 6.0**</td>
<td>0.37 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>PF</td>
<td>16.1 ± 1.5*</td>
<td>0.91 ± 0.04</td>
<td>64.8 ± 4.3</td>
<td>0.35 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>AL</td>
<td>29.3 ± 1.2</td>
<td>0.84 ± 0.03</td>
<td>52.0 ± 1.5</td>
<td>0.50 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>GUINEA</td>
<td>PFDA</td>
<td>32.6 ± 3.1*</td>
<td>0.96 ± 0.04</td>
<td>56.3 ± 2.5**</td>
<td>0.50 ± 0.03</td>
</tr>
<tr>
<td>PIG</td>
<td>PF</td>
<td>29.2 ± 2.8</td>
<td>0.90 ± 0.03</td>
<td>63.7 ± 3.0**</td>
<td>0.55 ± 0.02</td>
</tr>
</tbody>
</table>

Values quoted are the mean ± SEM for triplicate determinations and six animals per group. Animals were treated as outlined in Materials and Methods. Abbreviations are as described in Table 4.1. Main effects of treatment were evaluated by comparing PFDA versus PF and AL versus PF. *P < 0.05; **P < 0.01
<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Protein Content (mg/gram liver)</th>
<th>Total Cytochrome P450 Specific Content (nmol/mg)</th>
<th>NADPH-Cytochrome P450 Reductase Activity (nmol/min/mg)</th>
<th>Cytochrome b5 Specific Content (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>PFDA</td>
<td>13.7 ± 1.6</td>
<td>0.11 ± 0.01</td>
<td>18.09 ± 2.63</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>RAT</td>
<td>PFDA</td>
<td>11.5 ± 0.3**</td>
<td>0.14 ± 0.01</td>
<td>18.10 ± 2.07**</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>AL</td>
<td>PF</td>
<td>19.4 ± 1.3*</td>
<td>0.12 ± 0.01</td>
<td>11.51 ± 0.02</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>GUINEA</td>
<td>PFDA</td>
<td>21.8 ± 2.7</td>
<td>0.23 ± 0.01</td>
<td>16.76 ± 1.51</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>PIG</td>
<td>PF</td>
<td>17.2 ± 0.3*</td>
<td>0.22 ± 0.01*</td>
<td>14.61 ± 1.39**</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>PIG</td>
<td>PF</td>
<td>20.1 ± 1.9</td>
<td>0.18 ± 0.01</td>
<td>22.75 ± 2.81**</td>
<td>0.25 ± 0.01</td>
</tr>
</tbody>
</table>

Values quoted are the mean ± SEM for triplicate determinations from pooled samples and six animals per group. Animals were treated as outlined in Materials and Methods. Abbreviations are as described in Table 4.1. Main effects of treatment were evaluated by comparing PFDA versus PF and AL versus PF. *P < 0.05; **P < 0.01
pig which was significantly inhibited (P<0.01), following PFDA treatment, the kidney was generally refractory to the above microsomal changes (Table 4.3).

Concomitant with PFDA-induced hepatomegaly in the rat, reverse phase h.p.l.c. analysis demonstrated that ω—laurate hydroxylation was preferentially induced over (ω—1)-hydroxylation (Figure 4.5). This change is reflected by a relatively small increase (about 1.5-fold) in (ω—1)-hydroxylase activity when compared to a 5 to 6-fold increase in the corresponding ω—hydroxylase activity. Again, the effects of pair-feeding in the rats on laurate hydroxylation were minimal since no statistically significant changes were seen when compared to the ad libitum-fed group. Unlike in rats, the guinea pig liver was refractory to induction of lauric acid metabolism (marker substrate for cytochrome P450 4A1-mediated metabolism and other members of the cytochrome P450 4A subfamily), (Figure 4.5).

Similarly, reverse phase h.p.l.c. analysis of renal microsomal laurate hydroxylation in response to PFDA treatment exhibited differential response in both species (Figure 4.6). In rats, the level of total (i.e (ω—1)- plus ω—hydroxylation) lauric acid metabolism in control kidney microsomes, expressed as nmol/min/nmol P450, was 10-fold higher than the corresponding control value for liver microsomes, and about 2-fold higher in the PFDA treatment group. In the guinea pig, total laurate metabolism in untreated kidney was approximately twice that of the liver and the same ratio was maintained eight days after PFDA treatment suggestive of the refractory nature of this species.

The present study also showed that in the rat, marked increase in liver laurate hydroxylation activity (probably mediated by cytochrome P450 4A1 hydroxylase activity), was associated with a decrease in the dealkylation of ethoxyresorufin, marker substrate for cytochrome P450 1A1 isoenzyme (EROD activity) (Table 4.4). This decrease was about 74% that of the corresponding pair-fed group. In contrast, pentoxyresorufin-O-deethylation (PROD) activity (marker enzyme for
Figure 4.5 Modulation of hepatic 11- and 12-laurate hydroxylase activities in the rat and guinea pig by single dose of PFDA. Animals were treated as outlined in Materials and Methods and lauric acid 11- (ω-1) and 12- (ω)-hydroxylase activities were determined by reverse phase h.p.l.c. analysis. P values for results significantly differed between PFDA-dosed versus pair-fed and ad libitum-fed versus pair-fed. *P < 0.05, **P < 0.01.
Figure 4.6 Modulation of renal 11- and 12- laurate hydroxylase activities in the rat and guinea pig by single dose of PFDA. Animals were treated as outlined in Materials and Methods and lauric acid 11 (ω-1)- and 12 (ω)-hydroxylase activities were determined by reverse phase h.p.l.c. analysis. P values for results significantly differed between PFDA-dosed versus pair-fed and ad libitum-fed versus pair-fed. *P < 0.05.
Table 4.4 PFDA Induction of Hepatic Alkoxynresorufin-O-Deethylase Activities in the Rat and Guinea Pig

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Ethoxyresorufin-O-Deethylase Activity (EROD)</th>
<th>Pentoxynresorufin-O-Deethylase Activity (PROD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group</td>
<td>(pmol/ min/ mg protein) (pmol/ min/ nmol P450)</td>
<td>(pmol/ min/ mg protein) (pmol/ min/ nmol P450)</td>
</tr>
<tr>
<td>AL</td>
<td>PFDA</td>
<td>96.62 ± 8.3</td>
<td>28.32 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>PF</td>
<td>95.3 ± 4.4</td>
<td>22.08 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>AL</td>
<td>76.46 ± 5.0</td>
<td>64.97 ± 5.9</td>
</tr>
<tr>
<td>RAT</td>
<td>PFDA</td>
<td>7.70 ± 0.87</td>
<td>10.30 ± 0.70</td>
</tr>
<tr>
<td></td>
<td>PF</td>
<td>5.78 ± 0.60</td>
<td>8.15 ± 0.90</td>
</tr>
<tr>
<td></td>
<td>AL</td>
<td>37.74 ± 1.44</td>
<td>26.50 ± 2.8</td>
</tr>
<tr>
<td>GUINEA PIG</td>
<td>PFDA</td>
<td>31.30 ± 1.50</td>
<td>25.15 ± 2.20</td>
</tr>
<tr>
<td></td>
<td>PF</td>
<td>41.92 ± 1.0</td>
<td>26.50 ± 2.8</td>
</tr>
</tbody>
</table>

Values are reported as the mean ± SEM for six animals per group. Animals were pretreated as outlined in Materials and Methods. Abbreviations were as defined in Table 4.1. Main effects of treatment were evaluated by comparing PFDA versus PF and AL versus PF. *P < 0.05; **P < 0.01
cytochrome 2B1 isoenzyme) was slightly induced by approximately 1.3-fold over pair-fed animals. In the guinea pig liver microsomes, enzymatic activities were significantly reduced by 35 and 37% for EROD and PROD, respectively, over the pair-fed animals. Similar information obtained with the kidneys of both species is presented on Table 4.5. The results show both EROD and PROD activities were significantly lowered and more pronounced with PFDA-dosed guinea pigs.

Western blotting analysis of rat and guinea pig hepatic microsomes, by comparing the effects of various treatments on cytochrome P450 4A1 apoprotein levels, is shown in Figure 4.7. Immunoblotting involved the use of a polyclonal antibody raised against homogeneous rat cytochrome P450 4A1 (Chapter Two). The enhanced interaction between microsomes from PFDA-treated rat liver microsomes and the P450 4A1 polyclonal antibody was readily visualised and showed two intimately associated bands that co-migrate, the lower and more intense band being cytochrome P450 4A1. This induction was confirmed by scanning on a densitometer (data not shown) and showed an approximate 3-fold increase over its pair-fed counterparts. The difference between pair-fed and ad libitum-fed controls was not significant. Similar results with guinea pig-derived liver microsomes is included in Figure 4.7. The data also shows the rat polyclonal antibody to cytochrome P450 4A1 cross-reacts with, most likely, guinea pig orthologues. Two very distinct and well-resolved bands were obtained, with the lower band most likely representing the guinea pig cytochrome P450 orthologous form. Neither of the two bands was induced as assessed by densitometric analysis (data not shown). When the kidney-derived microsomes were investigated for interaction with the same rat liver polyclonal antibody to cytochrome P450 4A1, only the rat renal apoprotein indicated an enhanced interaction after eight days post-exposure to the single dose of PFDA (Figure 4.8). The response in the guinea pig kidney was very different and apart from showing only one major band, there was significant suppression of the cytochrome P450 4A1 orthologue apoprotein level.
Table 4.5  PFDA Induction of Renal Alkoxyresorufin-O-Deethylase Activities in the Rat and Guinea Pig

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Ethoxyresorufin-O-Deethylase Activity (EROD)</th>
<th>Pentoxyresorufin-O-Deethylase Activity (PROD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group</td>
<td>(pmol/ min/ mg protein) (pmol/ min/ nmol P450)</td>
<td>(pmol/ min/ mg protein) (pmol/ min/ nmol P450)</td>
</tr>
<tr>
<td>RAT</td>
<td>AL</td>
<td>13.62 ± 0.01</td>
<td>118.1 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>PF</td>
<td>7.02 ± 0.80*</td>
<td>51.0 ± 0.70**</td>
</tr>
<tr>
<td></td>
<td>PFDA</td>
<td>9.50 ± 5.0*</td>
<td>83.0 ± 4.5**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GUINEA PIG</td>
<td>AL</td>
<td>38.07 ± 2.0</td>
<td>169.0 ± 9.0</td>
</tr>
<tr>
<td></td>
<td>PF</td>
<td>23.01 ± 1.0*</td>
<td>64.97 ± 5.9**</td>
</tr>
<tr>
<td></td>
<td>PFDA</td>
<td>27.50 ± 0.5*</td>
<td>153.01 ± 2.5</td>
</tr>
</tbody>
</table>

Values are reported as the mean ± SEM for triplicate determinations of pooled samples (six animals per group). Animals were pretreated as outlined in Materials and Methods. Abbreviations were as defined in Table 4.1. Main effects of treatment were evaluated by comparing PFDA versus PF and AL versus PF. *P < 0.05; **P < 0.01
Figure 4.7 Western blot analysis of rat and guinea pig hepatic microsomes with anti-cytochrome P450 4A1 polyclonal antibody. Animals were pretreated as outlined in Materials and Methods. Pooled microsomes were loaded on protein basis (20μg) as follows: track 1, ad libitum-fed rat; track 2, pair-fed rat; track 3, PFDA-induced rat; track 4, ad libitum-fed guinea pig; track 5, pair-fed guinea pig; track 6, PFDA-induced guinea pig and track 7, authentic cytochrome P450 4A1 (0.5pmol). Ratios of PFDA/pair-fed and pair-fed/ad libitum-fed band intensities for the rat was evaluated by densitometry and this corresponded to 3 and 1.1 respectively.
Figure 4.8 Western blot analysis of rat and guinea pig renal microsomes with anti-cytochrome P450 4A1 polyclonal antibody. Animals were pretreated as outlined in Materials and Methods. Pooled microsomes were loaded on protein basis (20µg) as follows: tracks 1 and 2, *ad libitum*-fed rat; tracks 3 and 4, PFDA-induced rat; tracks 5 and 6, pair-fed rat; tracks 7 and 14, authentic cytochrome P450 4A1 (0.5pmol); tracks 8 and 9, *ad libitum*-fed guinea pig; tracks 10 and 11, FFDA-induced guinea pig and tracks 12 and 13, pair-fed guinea pig.
Significant inhibition of microsomal activity associated with the deethylation of ethoxyresorufin (marker substrate for cytochrome P450 1A subfamily) was reported earlier (see Table 4.4). Attempts to quantify this response to PFDA exposure, was demonstrated by Western immunoblotting analysis employing a polyclonal antibody against the rat P450 1A subfamily (Figure 4.9). Microsomes derived from 3-methylcholanthrene-induced rats was also included as a positive control. Firstly, the results indicate a difference in migration of recognised apoproteins on a 10% SDS-PAGE gel, the guinea pig bands migrating slightly lower than the rat bands. Secondly, in the rat only one band (as opposed to two in the guinea pig) cross-reacted with the antibody. Thirdly, in the guinea pig the upper of the two recognised bands showed less immunoreactivity and corresponded to the more intense rat bands, which in turn corresponded to the lower of the two (more or less equally intense) 3-MC-induced bands believed to be cytochrome P450 1A2. However, both the PFDA-exposed rat and guinea pig liver microsomes showed substantial decrease in the cytochrome P450 1A level over their pair-fed partners when quantified by densitometry, even though the changes seen with the latter species were very marginal. A similar trend of results was obtained with the kidneys but the low degree of cross-reactivity made quantification rather difficult (data not shown).

4.3.4 Peroxisomal and mitochondrial changes in response to PFDA treatment

PFDA resulted in differential induction profiles in liver and kidney whole homogenates of the two species. In rat liver, PFDA treatment resulted in significant increase in cyanide-insensitive palmitoyl-CoA oxidation (PCOA) activity of the peroxisomal β-oxidation spiral, with concomitant induction of total (mitochondrial and peroxisomal) carnitine acetyl-CoA transferase (CAT) activity (Figure 4.10). The latter enzyme is involved in the transfer of fatty acyl-CoA
Figure 4.9 Interaction between rat and guinea pig liver-derived microsomes and anti-cytochrome P450 1A polyclonal antibody. Western blotting technique was applied on pooled microsomal samples from various treatment groups as outlined in Materials and Methods. Samples were loaded on protein basis (20μg/track) as follows: track 1, ad libitum-fed rat; track 2, PFDA-induced rat; track 3, pair-fed rat; track 4, ad libitum-fed guinea pig; track 5, PFDA-induced guinea pig; track 6, pair-fed guinea pig and track 7, 3-MC-induced rat microsomes (10μg).
A- KCN-Insensitive Palmitoyl-CoA Oxidation Activity (PCOA)

![Bar chart showing liver PCOA activity in rats and guinea pigs under different treatments.](chart)

B- Carnitine Acetyl-CoA Transferase Activity (CAT)

![Bar chart showing liver CAT activity in rats and guinea pigs under different treatments.](chart)

Figure 4.10 Influence of PFDA on the liver β-oxidation system in the rat and guinea pig. KCN-insensitive PCOA and total CAT (mitochondrial and peroxisomal) were determined in whole liver homogenates derived from both species following different treatments as detailed in Materials and Methods. Data represents mean ± SEM of duplicate determinations from 6 animals per group. The effect of treatment was evaluated by comparing PFDA-treated versus pair-fed and likewise ad libitum-fed versus pair-fed. *P < 0.05; **P < 0.01.
derivatives through the membranes of the mitochondria and in the peroxisomes it is mainly required for the outward movement. Guinea pig liver from PFDA-dosed animals did not show any significant difference from pair-fed and *ad libitum-fed* control animals (Figure 4.10). However, the CAT activity in untreated guinea pig liver was at least four times that of untreated rat, whereas the PCOA activity was about the same.

The effect of PFDA treatment, eight days after administration, on renal PCOA and CAT activities in both species is presented in Figure 4.11. The results presented show a significant increase in rat PCOA activity over its corresponding pair-fed partners (about 1.5-fold, *P*<0.05), whereas the guinea pig again was refractory to the rat kidney changes. CAT activity did not reveal any significant changes in either species. Further evidence lending support to the peroxisome proliferation potency of PFDA treatment in rat liver was obtained from Western blotting analysis using a polyclonal antibody to the trifunctional protein of the peroxisomal $\alpha$-oxidation spiral (Figure 4.12). The observed molecular weight of approximately 80kDa for this peroxisome-associated polypeptide (PPA-80), agreed with that of previous reports [Reddy *et al.*, 1981; Osumi and Hoshimoto, 1979]. The two lower molecular weight proteins recognised by the antibody and which were not detectable in guinea pig hepatic microsomes are believed to represent proteolysis products as previously reported [Nemali *et al.*, 1989]. In accordance with the enzyme data previously presented in this Chapter, the guinea pig showed little, if any response to the induction of this protein. The refractory nature of this protein in PFDA-treated guinea pig kidney was also reflected on Western blotting analysis (data not shown). Meanwhile, results from the different rat kidney groups demonstrated an increase in the PPA-80 level in PFDA-dosed group over its pair-fed controls (data not shown).
Figure 4.11 Modulation of renal β-oxidation system in the rat and guinea pig by a single dose of PFDA. KCN-insensitive PCOA and total CAT (mitochondrial and peroxisomal) were determined in whole liver homogenates derived from both species following different treatments as detailed in Materials and Methods. Data represents mean ± SEM of triplicate determinations from 6 animals per group. The effect of treatment was evaluated by comparing PFDA-treated versus pair-fed and likewise ad libitum-fed versus pair-fed. *P < 0.05.
Figure 4.12 Western blotting analysis of rat and guinea pig hepatic microsomes with antibody to the trifunctional protein. With the exception of track 7 (prestained molecular weight markers, see section 2.2.17 of Chapter Two), all tracks contained equal amounts of protein (30μg) as follows: 1, ad libitum-fed rat; 2, PFDA-induced rat; 3, pair-fed rat; 4, ad libitum-fed guinea pig; 5, PFDA-induced guinea pig and 6, pair-fed guinea pig.
4.3 Discussion

The results of this study have demonstrated various renal and hepatic enzyme changes (microsomal and peroxisomal), following administration of the perfluoro-fatty acid, PFDA to male rats and guinea pigs. In general there were quantitative and qualitative differences in liver and kidney responses within the two species. Unlike the rat, the guinea pig was refractory to most of the parameters investigated.

Administration of single intraperitoneal dose of PFDA (20mg/kg body weight) resulted in differential feed intake and body weight gain in both species. In the guinea pig, PFDA treatment resulted in an initial decrease in feed intake and body weight when compared to ad libitum-fed animals. This was followed by a slow and gradual increase from the day 2, the result being an overall decrease in body weight change and cumulative feed intake (Table 4.1 and Figure 4.2). A similar initial decrease seen in the pair-fed group may be accounted for as due to the restricted amount of feed provided. This may well suggest a more profound initial impact of this compound in the guinea pig as opposed to the rat which showed a steady increase in feed intake and body weight gain in all the groups (Table 4.1). When compared to its pair-fed counterparts receiving an equivalent amount of feed, PFDA-dosed rats did not show any statistically significant difference in cumulative feed intake, suggesting the dose level used in this study did not cause any observable alterations in energy balance. The influence of PFDA on energy metabolism, evaluated through the calculation of the feed intake needed for maintenance at zero change of body weight [Fischer and Canolty, 1983], was not addressed in the present investigation. The main areas of energy utilisation have been defined as: energy storage, cost of energy storage and cost of maintenance [Hill et al., 1984]. In the rat, measured feed intake throughout the duration of the study, was not different for PFDA (163.8 gram feed/ 7days) compared to the pair-fed (163.1 gram feed/ 7 days) or ad libitum-fed (163.1 gram feed/ 7days) treatment.
Studies involving PFDA treatment at higher doses (>50 mg/kg body weight as compared to the 20 mg/kg dose level used in my study) have often resulted in a number of toxicity symptoms, the liver being the major target organ. This includes progressive reduction in body weight and feed intake, delayed lethality, with deaths occurring in the second and third week post-treatment. However, in the present study, no such drastic changes were apparent 8 days after animals were exposed to PFDA even though there were significant changes occurring at the level of enzymatic activities, especially within the rat. Because of these reasons, we found it necessary to use this lower dose level to avoid complications of PFDA and metabolic changes, the latter can also change cytochrome P450 4A subfamily as in fatty acid diet studies.

A striking response to PFDA treatment in rats but which was lacking in guinea pigs, was hepatomegaly with concomitant increases in total cytochrome P450 specific content as well as NADPH-cytochrome P450 reductase activity (Table 4.2). The fact that liver enlargement was not very pronounced, if at all present, in the guinea pig may be related to the pharmacokinetics of PFDA in this species. It is known that the rate of elimination from the body is important in relation to the capacity of many substances to induce liver enlargement [Schulte-Hermann, 1974]. It may well be possible that a difference in the persistence of this compound in the two species studied may account partially for the difference in the degree of liver enlargement and associated enzyme changes.

In general, there is good indication that the fatty acid-metabolising enzyme complement in the kidney and liver are similar even although substantial quantitative tissue differences exist between control and induced states. Increased \( \omega \)-hydroxylation of lauric acid in rat liver and kidney following PFDA exposure, reflected changes occurring within the cytochrome P450 4A subfamily enzymes, the latter enzymes mediating the \( \omega \)-hydroxylation of this substrate. Rat kidney cortex has previously been shown to contain a cytochrome P450 monooxygenase with a
preference for fatty acid hydroxylation [Parker and Orton, 1980] and medium chain saturated fatty acids, such as lauric acid, display a high affinity for kidney cytochrome P450. Subsequent studies by Sharma et al., [1989] confirmed that cytochrome P450 4A1 (or a very closely related isoenzyme in the same gene family) is a major constitutive haemoprotein in rat kidney microsomes and actively supported ω—hydroxylation of lauric acid as well as arachidonic acid. Similarly, the cloning and expression of three rabbit kidney cDNAs encoding lauric acid ω-hydroxylases has been reported [Johnson et al., 1990]. This clearly indicates that the kidney cytochrome P450 4 family genes play a major role across various species.

By employing a polyclonal antibody against rat liver cytochrome P450 4A1 on Western blotting analysis (Figures 4.7 and 4.8), further evidence on the differential modulation of the cytochrome P450 apoprotein levels in the liver and kidney by PFDA was provided. Similar to previous reports by other workers [Sharma et al., 1989, Hardwick et al., 1987], our polyclonal antibody recognised two bands in rat hepatic and renal microsomes, a major band characterised by a monomeric molecular mass of approximately 51.5kDa (similar to authentic homogeneous hepatic cytochrome P450 4A1) and a minor one at 52 kDa, most likely representing cytochrome P450 4A3 [Aoyama et al., 1990]. Levels of cytochromes P450 4A1, 4A2 and 4A3 mRNA in untreated rat have been analysed in liver and kidney and were present at very low levels in the liver. These are coordinately induced in rats treated with clofibrate. In the kidney, cytochromes P450 4A1 and 4A3 mRNAs were present in low levels and were induced by clofibrate in a manner similar to that in liver. In contrast, the level of cytochrome P450 4A2 mRNA expression in the kidney of untreated rats was similar to that of the maximally induced cytochrome P450 4A2 mRNA in liver. A major cytochrome P450 from the kidney of untreated rats which was shown to catalyse the ω— and (ω—1)- hydroxylation of lauric acid had earlier been isolated, purified and N-terminal sequenced by two
groups [Imaoka et al., 1990; Yoshimoto et al., 1986] and based on limited amino acid similarities appears to correspond to cytochrome P450 4A2.

The effect of PFDA on guinea pig renal and hepatic cytochrome P450 mediating the hydroxylation of lauric acid (marker substrate for cytochrome P450 4A1 and related isoforms) was also investigated. Because of the high refractory nature of this species to most peroxisome proliferators examined to date, it is often classified as a 'non-responsive' rodent. My results showed liver and kidney microsomes were refractory to cytochrome P450 4A1-mediated ω-hydroxylation of lauric acid when the PFDA-dosed group was compared to its corresponding pair-fed partners (Figures 4.5 and 4.6). However, when the constitutive levels in untreated hepatic microsomes derived from both species was compared, they were found to be approximately the same. This similar catalytic activity in liver-derived microsomes was not reflected in Western immunoblotting for the cytochrome P450 4A1 apoprotein (Figure 4.8). Ad libitum-fed rat liver microsomes produced a greater interaction with the P450 4A1 polyclonal antibody than with similar guinea pig liver-derived microsomes. Moreover, resolution of the two bands recognised by the antibody was much better with the lower major band most likely representing the guinea pig liver orthologue of the rat cytochrome P450 4A1. The upper minor band had a monomeric molecular mass slightly higher than that seen with the rat but may well still represent a member of the cytochrome P450 4A subfamily. None of the bands seen in the guinea pig liver was affected significantly by PFDA treatment and the precise nature of these (presumably) cytochrome P450 isoforms and their relationship to the rat enzymes awaits further characterisation and clarification.

Differential interaction between rat and guinea pig microsomes and the cytochrome P450 4A1 antibody was further highlighted with the response of kidney-derived microsomes. Whereas the induction of laurate hydroxylase activity seen in PFDA-treated rat kidney was reflected by a similar response on Western immunoblotting, the refractory response seen in the guinea pig kidney laurate
hydroxylase activity was transformed to a very significant inhibition of the orthologous apoprotein (Figure 4.8). These results suggest that the kidney cytochrome P450 4A1 orthologue in both species is distinctively different from the well characterised liver form. This observation is supported by the studies carried out by Sharma and co-workers, [1989]. These workers were able to demonstrate that, although the kidney supported fatty acid ω-hydroxylase activity, it was refractory to inhibition by a polyclonal antibody to liver cytochrome P450 4A1.

If the gene coding for guinea pig kidney cytochrome P450 4A1 orthologue is different from that in the rat, then modulation of its activity may also be different and it is plausible that PFDA administration could result in a gene switch-off phenomenon resulting in the expression of a different member of the cytochrome P450 4A subfamily hitherto unidentified. Measurement of specific isoenzyme changes using changes in the in vitro assessment of catalytic activities of cytochromes P450 2B1 and P450 1A1 mediated O-deethylation of pentoxyresorufin (PROD) and ethoxyresorufin (EROD), respectively indicated in microsomes derived from the liver and kidney of both species, these activities were decreased to varying extents with similar trends in the liver and kidney (Tables 4.4 and 4.5). The decrease in ethoxyresorufin-related activity (mainly contributed by cytochrome P450 1A2) was further confirmed by Western immunoblotting analysis on hepatic-derived microsomes (Figure 4.9). These results appear to indicate there is a specific gene "switch-on" for cytochrome P450 4A1 with concomitant gene "switch-off" for the 1A subfamily.

Peroxisome proliferation and induction of hepatic peroxisomal fatty acyl-CoA oxidation are characteristic effects of several xenobiotics [Moody and Reddy, 1978; Cohen and Grasso, 1981; Moody et al., 1991]. The present study also examined any possible relationship between induction of hepatic and renal enzymes and the peroxisome proliferative potentials of PFDA. PFDA has previously been shown to affect hepatic lipid metabolism in rats by altering fatty acid profiles with increases
in long-chain fatty acids such as palmitic and oleic acids [George and Andersen, 1986], and the capacity of this class of compounds to induce peroxisomes in rats has recently been confirmed [Ikeda et al., 1985; Borges et al., 1992]. These effects on lipid metabolism are consistent with the substrate overload hypothesis of peroxisome proliferative response in the rat [Sharma et al., 1988a]. In rat liver, treatment with PFDA resulted in an obvious positive correlation between induction of cytochrome P450 4A subfamily protein(s) and peroxisome proliferation as evidenced by the enhanced laurate hydroxylase activity and the peroxisomal parameters investigated such as cyanide-insensitive palmitoyl CoA oxidation (Table 4.6) and Western immunoblotting of the "trifunctional protein of the β-oxidation spiral (Figure 4.10).

The rat kidney produced a similar trend of results but with less dramatic changes (Table 4.7 and Figure 4.10). In contrast PFDA administration to the guinea pig did not lead to an induction in peroxisomal β-oxidation parameters (an indirect assessment of peroxisome proliferation) in either tissue (Tables 4.4 and 4.5). Neither was there induction in total carnitine acetyl-CoA transferase (CAT) activities (localised in peroxisomal and mitochondrial subcellular fractions) in this species. This same activity was induced by PFDA treatment in the rat liver by approximately 15-fold over pair-fed controls. The mitochondrial fatty acid β-oxidation system is dependent on this latter enzyme, as it functions as an intermediate carrier of acyl substrates into the mitochondrial matrix. The peroxisomal β-oxidation system is not dependent on carnitine, but this transferase may play a role in the removal of end products of metabolism [Lazarow, 1982]. In untreated animals, the constitutive level of CAT was 3-4 fold higher in the guinea pig liver when compared to similar activity in the rat, whereas the levels in the kidney were about the same. It is possible that selective enzyme induction and tissue toxicity by peroxisome proliferators may be masked by the fact that specific cell types in the kidney are indeed very susceptible to peroxisome proliferation but
are obscured by whole-tissue-homogenate activity analyses, a hypothesis that may be addressed by *in situ* hybridization studies with appropriate probes.

The above relatively refractory nature of rat kidney and guinea pig enzymatic parameters to modulation by PFDA treatment coupled with the low basal activities recorded, may have profound implications for the species/organ susceptibility to toxicity by this class of compounds and to the extrapolation of the information so obtained to humans. This problem is exacerbated by reports that peroxisome proliferation and induction of palmitoyl-CoA oxidation activity in the liver results in a dramatic increase in peroxisomal production of hydrogen peroxide, which may be responsible for the hepatocarcinogenicity of these xenobiotics in rodents [Reddy and Lalwani, 1983]. The lack of reported incidences of renal carcinomas, in direct contrast to the high susceptibility to toxicity of mouse and rat liver, may be related to the poor respondent nature of this tissue or may be due to low levels of the recently identified peroxisome proliferator-activated receptor [Green, 1992].

In conclusion, I have examined the effect of a single i.p dose of PFDA (7 days post-administration) in male rats and guinea pigs. The findings of the present study indicate there are differential pleiotropic responses in both species. In all the parameters examined, the rat proved very responsive and the guinea pig either weakly responsive or non-responsive. As exemplified by the rat, there is very good agreement between PFDA-induction reflected in PCOA, peroxisome proliferation and cytochrome P450 4A1 activities, suggesting both processes may be activated through a common mechanism [Sharma *et al.*, 1988a]. Association of PFDA exposure and hepatocarcinogenesis must be proven as a preliminary step to extrapolating the risk implications of this class of compounds to the human population. Also a deeper understanding of PPAR in a non-responsive species such as the guinea pig may help in better understanding its modulation in humans, if it is expressed at all.
CHAPTER FIVE

Characterisation of Human Liver Cytochrome P450 4A Subfamily Proteins.
5.1 Introduction.

The cytochrome P450-mediated mixed-function monooxygenases of liver microsomal membranes are of central importance in the metabolism of steroid hormones and xenobiotics, including many drugs, environmental pollutants as well as many carcinogens [Coon et al. 1980]. Marked species differences in xenobiotic metabolism in the liver seriously limit the extrapolations from animals to man and therefore necessitates metabolic studies with human liver. Such studies are sparse in the literature and in general, apart from limited access to human tissues, the material used in such studies is often not completely characterized with respect to the morphology, homogenisation, fractionation, and storage procedures. Tissue from diseased patients or from livers probably having marked post-mortem changes has also been used making it difficult to satisfactorily preserve a range of drug metabolism activities in man. In one such study cytochrome P450 in human liver decreased to undetectable levels within 5h of death [Schenkman et al. 1969] and other qualitative modifications have been reported [Barker, 1974].

Of about twenty-seven cytochrome P450 gene families so far described, ten exist in all mammals, which comprise eighteen subfamilies of which sixteen have been mapped in the human genome [Nebert et al. 1991]. Individual human variations are well known in the metabolism of some foreign compounds such as therapeutic drugs [Conney, 1982]. This metabolic variability may be due, in part, to genetically determined factors relating to the structure and expression of cytochrome P450 isozyms. The existence of multiple forms of cytochrome P450 in humans has been directly demonstrated by the isolation, from tissues, of several cytochrome P450 isozyms that vary in their catalytic activity towards drugs and carcinogens [Wang...
et al., 1983; Nhamburo et al., 1989; Johnson et al., 1990; Stromstedt et al., 1990; Yokotani et al., 1991.

The role and function of $\omega$- and $(\omega-1)$-oxidation of fatty acid metabolism has received considerable attention [Kupfer, 1982; Sharma et al., 1989]. It is well documented that prostaglandins and leukotrienes can serve as substrates for such a reaction, suggesting that this may represent a pathway for the further metabolism of these physiologically important compounds [Okita et al., 1981; Feinmark et al., 1981; Tanaka et al., 1990]. Similarly, the finding that lauric acid and more importantly yet another endogenous substrate, arachidonic acid are $\omega$- and $(\omega-1)$-oxidised by liver microsomal cytochrome P450 4A subfamily proteins, raises the question of whether therapy with agents of the fibric acid type, such as clofibrate (an inducer of some of the cytochrome P450 4A subfamily isozymes) can perturb the in vivo steady-state levels of these eicosanoids. Evidently, any such changes may have pronounced effects on cellular homoeostasis and may contribute to the hypolipidaemic functions of these types of drugs. Arachidonic acid is a starting material in the in vivo synthesis of potent bioactive substances such as prostaglandins and leukotrienes, and their metabolism in the renal tissues may contribute to kidney function.

Recently, it was reported that some of the metabolites of arachidonic acid are biologically active with several roles, including those of a vasodilator [Carroll et al., 1987], an inhibitor of ion transport in the collecting tubules of rabbit kidney and a $\text{Na}^+$/ $\text{K}^+$-ATPase inhibitor [Jacobson et al., 1984; Schwartzman et al., 1985]. Furthermore, the $\omega$-hydroxyeicosatetraenoic acid (20-HETE) metabolite produced by the kidney is a potent vasoconstrictor, which may affect renal vascular resistance and contribute to the pathogenesis of hypertension, and the corresponding $(\omega-1)$-hydroxyeicosatetraenoic acid (19-HETE) metabolite, a stimulator of $\text{Na}^+$/ $\text{K}^+$-ATPase [Escalante et al., 1989; Schwartzman et al., 1989, 1990]. Thus by manipulating $\text{Na}^+$/ $\text{K}^+$-ATPase activity, dilating and/or constricting vascular
smooth muscles, these metabolites may influence renal function and contribute to the development of a pathological state such as hypertension.

In the present chapter, I have examined the existence/ similarity of cytochrome P450 4A1 reported in other animal models to that which may exist in human liver tissue. It is hoped the results obtained would serve as a prelude in the assessment of human risks following exposure to hypolipidaemics.
5.2 Materials and Methods

5.2.1 Chemicals

The chemicals used in the present study were essentially as listed in Materials Section (Chapter 2), but with a few additions: \([1-^{14}C]\)-arachidonic acid and \([1-^{12}C]\)-sodium arachidonate were purchased from the Radiochemical Centre (Amersham, U.K.) and Sigma Chemical Co. (Poole, Dorset, U.K.) respectively.

5.2.2 Human Liver samples

Liver tissue samples were kindly provided by Drs Mike Tarbit and Alan Boobis of Glaxo Group Research and the Royal Postgraduate Medical School, London, respectively, and ethical permission for cytochrome P450 characterisation was previously obtained by these workers. The samples were transported in dry ice and once received, they were immediately stored at \(-80^\circ\text{C}\) until processed. For most of the samples, extensive case histories were not available. The limited information available is presented in Table 5.1.

5.2.3 Animals and Drug Pretreatment

Isozymes of cytochrome P450 corresponding to the 1A, 2B and 4A subfamilies were also assessed in the panel of human livers and were compared with similar forms in the rat. Rats were dosed intraperitoneally as follows: 80mg/kg of phenobarbitone followed by 0.1% in drinking water for 3 days; 20mg/kg 3-methylcholanthrene for three consecutive days; clofibrate at 250mg/kg body weight also for three consecutive days and the control group received 0.9% (w/v) saline for three consecutive days. The rats were all killed by cervical dislocation 24h after the last injection and microsomes were prepared by standard centrifugation procedures as previously described in Materials and Methods, Chapter Two.
**Table 5.1** Available Clinical Data on Human Liver Samples

<table>
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<tr>
<th>Code</th>
<th>Sex</th>
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<th>Date obtained</th>
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<th>Drug history</th>
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<td>45</td>
<td>22-12-88</td>
<td>moderate smoker obstructive jaundice</td>
<td>Temazepam Thiopentone</td>
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<tr>
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<td>10-11-88</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>08-03-89</td>
<td>liver resection</td>
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</tr>
<tr>
<td>HLM-4</td>
<td>F</td>
<td>33</td>
<td>29-03-89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HLM-5</td>
<td>M</td>
<td>-</td>
<td>16-08-89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HLM-6</td>
<td>-</td>
<td>-</td>
<td>04-11-89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HLM-7</td>
<td>-</td>
<td>-</td>
<td>01-11-89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HLM-8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\) Information not available

\(^{\text{b}}\) M = male, F = female
5.2.4 Preparation of Human Liver Microsomes

Human liver, especially that of older patients, contains connective tissue, which makes homogenisation difficult. Microsomes were therefore prepared by a slight modification of the procedure used for the rat. All manipulations were performed at 4°C. Frozen liver pieces were partially thawed out in 0.9% (w/v) sodium chloride, diced with scissors, washed 0.01M Tris, pH 7.4, containing 1.15% (w/v) KCl and a 25-33% (w/v) homogenate prepared in a buffer containing 0.25M sucrose, 0.1M Tris HCl and 1mM EDTA, pH 7.4 using initially an ultra-Turrax for 10-20s and further homogenisation with a motor-driven Potter-Elvehjem device. The homogenate was successively centrifuged at 12,500g for 30 min after which the supernatant was further centrifuged at 105,000g for 1h to isolate the pelleted microsomal fraction. The microsomal pellet was washed with 100mM sodium pyrophosphate, pH 7.5, to eliminate as much of the contaminating haemoglobin as possible. The microsomes were then resuspended in 50mM phosphate buffer, pH 7.4, containing 20% (v/v) glycerol, and stored at -80°C until required. The cytochrome P450-dependent enzymatic activities were verified not to decrease under these storage conditions for at least a few months.

5.2.5 Spectrophotometric Assays

Total microsomal protein content, total carbon monoxide-discernible cytochrome P450, cytochrome b5 and NADPH-cytochrome P450 (cytochrome c) reductase were determined as previously described in Materials and Methods (Chapter Two).

5.2.6 Determination of Alkoxyresorufin O-dealkylase Activities in Human Liver Microsomes

The O-dealkylation of ethoxy- and pentoxy- resorufins, was determined spectrophotometrically according to the method of Burke and Mayer, [1974] by using the difference in fluorescent properties of ethoxyresorufin and
pentoxyresorufin (excitation wavelength at 456nm, emission wavelength at 560nm) and the product resorufin (excitation wavelength at 510nm, emission wavelength at 586nm). Details of the procedures is outlined in Materials and Methods (Chapter Four).

5.2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

Human hepatic microsomal cytochrome P450 4A1-related material was quantified by the ELISA protocol as described in Materials and Methods (Chapter Two).

5.2.8 Determination of Lauric Acid Metabolism

This was carried out as described under Section 2.2.14 (Chapter Two). 2mg of human microsomal protein was used per incubation for 20 minutes. Reaction rates were initially verified to be linear as a function of incubation time.

5.2.9 Arachidonic Acid Metabolism

The method used in the determination of arachidonic acid metabolism was essentially that described by Capdevila et al. [1985]. The products of arachidonic acid metabolism were isolated from a constantly stirred incubation mixture containing either human liver microsomes (2mg/ml), control rat microsomes (1mg/ml) or clofibrate-treated microsomes (1mg/ml), 10mM magnesium chloride, and 50mM Tris-HCl buffer, pH 7.5.

After temperature equilibration at 25°C for 5 min with constant stirring, [1-14C] sodium arachidonate (0.6mCi/ mmol) was added to a final concentration of 0.1mM. NADPH (1mM final concentration) was then added to initiate the reaction. The reaction mixture was then transferred to a 37°C shaking water bath. The reaction was allowed to proceed for 20 min and then terminated by the addition
of 2ml ethyl acetate containing 0.05ml of 1M HCl and 0.1% (w/v) butylated hydroxytoluene (BHT) as anti-oxidant. Following two further extractions with ethyl acetate (2ml), the combined extracts were dried over anhydrous sodium sulphate, filtered and evaporated to dryness under a stream of nitrogen. About 95% of the radioactivity added was reported to be recovered in the organic extracts under these procedures [Capdevila et al., 1985], and therefore no correction for recoveries was applied to my data.

The dried ethyl acetate extracts were reconstituted in 150μl of starting solvent (water : acetonitrile, 70:30 containing 0.1% (v/v) acetic acid). The metabolites were separated by reverse phase h.p.l.c. on a C\textsubscript{18} Ultrasphere ODS 5mm column (4.6mm x 25cm, Beckman Instruments, Inc., CA, U.S.A.), using a shallow elution gradient ranging from the starting solvent to 100% acetonitrile (containing 0.1% (v/v) acetic acid), at a flow rate of 1ml/min for 50 min. The elution profile of the radioactive products was monitored using a Berthold LB 506 C-1 Radioactivity monitor (Lab Impex, Twickenham, UK) containing a 150μl flow cell and interfaced with a microprocessor. This was linked to a PC-IV 286 AT-compact computer (Opus Technology Plc, Redhill, UK) enabling quantitative analysis of the arachidonic acid metabolites, essentially as described for lauric acid metabolites quantification (Section 2.2.14, Chapter Two).

### 5.2.10 Antibody Inhibition of Microsomal Laurate Metabolism

Inhibition of ω— and (ω—1)- laurate and arachidonate hydroxylase activities in human liver microsomes was studied using cytochrome P450 4A1 and pre-immune IgG antibody-enriched fractions, prepared as previously described (Section 2.2.15, Chapter Two). Human liver microsomes (2mg/ml) and control rat microsomes (1mg/ml) were each separately preincubated with 5 and 10 mg/nmol P450 of the pre-immune and immune IgG fractions at 25°C for 10 min. The reaction mixture
was then maintained at 4°C and the remaining components for lauric acid metabolism assay added. The ω- and (ω-1)-hydroxylated products of lauric acid were quantified on reverse phase h.p.l.c. chromatography, as previously described under Materials and Methods (Chapter Two).

5.2.11 Western Blotting Analysis

Human hepatic cytochromes P450 1A1-, 2B1- and 4A1- related materials were analysed using specific rat antibodies to these isoforms, essentially as was previously described in Materials and Methods (Chapter Two).
5.3 Results

Only a single microsomal preparation was made from each human liver sample, of which the results are discussed in this chapter. Liver tissue wedges from eight individuals with little available clinical history were investigated. A summary of the results obtained for the monooxygenase components in these samples is presented in Table 5.2. The values obtained for total CO-discernible cytochrome P450 and cytochrome b5 are quite similar to those published by other workers [Darby et al. 1970, Nelson et al. 1971, Beaune et al. 1986; Distelrath and Guengerich, 1988]. Mean specific contents of $0.36 \pm 0.19$ and $0.32 \pm 0.06$ nmol/mg microsomal protein were obtained for total cytochrome P450 and cytochrome b5 respectively. The average value for cytochrome P450 was less than that obtained with control rat microsomes ($0.56 \pm 0.06$ nmol/mg). The absorption maxima of the cytochrome P450 CO-adduct ranged between 450-452nm. Some of the microsomal preparations also showed a peak at 420nm which was due to haemoglobin contamination even though this was minimised by washing the microsomes during preparation with sodium pyrophosphate.

The NADPH-cytochrome P450 reductase activity recorded for all eight human samples is also included in Table 5.2. The values ranged between 13.6 and 75.2nmol product formed/min/mg of microsomal protein with a mean activity of $51.34 \pm 16.9$nmol product formed/min/mg microsomal protein. The corresponding rat activity was $85.4 \pm 2.9$ nmol product formed/min/mg protein. The lowest activity was obtained with HLM-1 and the highest with HLM-4. In general, these values were slightly lower than those cited in literature for human autopsy samples that ranged between 70-193nmol reductase/min/mg protein [von Bahr et al. 1980, Beaune et al. 1986].

Figure 5.1 shows the SDS-PAGE analysis of human and rat liver microsomes. By examining this data very closely, it was obvious that some significant differences
### Table 5.2  Mixed Function Oxidase System in Human Hepatic Microsomes

<table>
<thead>
<tr>
<th>Code</th>
<th>Protein content (mg/gram liver)</th>
<th>P450 $\lambda_{max}$ (nm)</th>
<th>Cyt. P450 specific content (nmol/mg protein)</th>
<th>Cyt. b5 specific content (nmol/mg protein)</th>
<th>NADPH cyt. c reductase (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM-1</td>
<td>4.12 ± 0.20</td>
<td>452</td>
<td>0.07 ± 0.01</td>
<td>0.23 ± 0.03</td>
<td>13.6 ± 2.3</td>
</tr>
<tr>
<td>HLM-2</td>
<td>5.28 ± 0.12</td>
<td>451</td>
<td>0.37 ± 0.02</td>
<td>0.39 ± 0.01</td>
<td>55.6 ± 2.1</td>
</tr>
<tr>
<td>HLM-3</td>
<td>9.58 ± 0.25</td>
<td>451</td>
<td>0.55 ± 0.01</td>
<td>0.37 ± 0.03</td>
<td>50.0 ± 3.5</td>
</tr>
<tr>
<td>HLM-4</td>
<td>7.22 ± 0.10</td>
<td>450</td>
<td>0.64 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>75.2 ± 2.8</td>
</tr>
<tr>
<td>HLM-5</td>
<td>7.35 ± 0.20</td>
<td>451</td>
<td>0.58 ± 0.01</td>
<td>0.36 ± 0.02</td>
<td>63.0 ± 1.3</td>
</tr>
<tr>
<td>HLM-6</td>
<td>10.40 ± 0.34</td>
<td>452</td>
<td>0.17 ± 0.01</td>
<td>0.35 ± 0.02</td>
<td>37.4 ± 1.3</td>
</tr>
<tr>
<td>HLM-7</td>
<td>12.60 ± 0.21</td>
<td>451</td>
<td>0.21 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>42.0 ± 1.5</td>
</tr>
<tr>
<td>HLM-8</td>
<td>13.23 ± 0.35</td>
<td>451</td>
<td>0.25 ± 0.02</td>
<td>0.37 ± 0.01</td>
<td>36.8 ± 3.7</td>
</tr>
<tr>
<td>Mean</td>
<td>8.72 ± 3.10*</td>
<td>0.36 ± 0.20*</td>
<td>0.32 ± 0.06</td>
<td>51.3 ± 16.9*</td>
<td></td>
</tr>
<tr>
<td>Untreated Rat</td>
<td>12.22 ± 0.18</td>
<td>450</td>
<td>0.56 ± 0.06</td>
<td>0.36 ± 0.03</td>
<td>85.4 ± 2.9</td>
</tr>
</tbody>
</table>

Values indicated are the mean ± SEM of triplicate determinations per human liver (n=8) and rat liver (n=3). Mean values of human samples were significantly different from those of untreated rats at *$P < 0.05$. 
Figure 5.1 SDS-PAGE patterns of human liver microsomes (HLM).
Electrophoresis was carried out by using a 1mm thick 10% (w/v) gel as described in Materials and Methods. All tracks contained 20µg of protein loaded as follows: 1 and 12, clofibrate-induced rat; 2 and 13, untreated rat; 3, HLM-1; 4, HLM-2; 5, HLM-3; 6, HLM-4; 7, HLM-5; 8, prestained molecular weight markers (see Section 2.2.17, Chapter 2); 9, HLM-6; 10, HLM-7; 11, HLM-8. Rat cytochrome P450 4A1 is indicated by an arrow.
appeared between the panel of human liver microsomes and that of the rat, as evidenced by the intensities of some of the protein bands. A few of the bands were peculiar only in rat microsomes tracks (1 and 2). Similarly, some of the protein bands were very prominent only in human microsomes. It is probable that some of these bands may correspond to species specific cytochrome P450 types, thus providing a possible explanation for some of the differences in enzyme activities observed with the various monooxygenase components. Of major significance in each human liver-derived microsomal preparation, was the presence of a detectable protein band that co-migrated with that of cytochrome P450 4A1 in clofibrate-induced microsomes (Figure 5.1). Except for HLM-1 (track 3), these bands showed similar intensities to that of untreated rat microsomes and may well represent the orthologous human form of the cytochrome P450 4A1 or other closely-related member(s) of the cytochrome P450 4A subfamily proteins. Figure 5.1 also demonstrates that microsomes prepared from one of the human samples (HLM-1, track 3) showed significant difference in band intensities and this is reflected in its much lower enzymatic activities (see later).

In order to estimate the amount of orthologous human liver microsomal form of cytochrome P450 4A1 that cross reacts with the rat polyclonal antibody by an ELISA technique, it was necessary to establish an antigen-antibody response with increasing amounts of the antigen. A typical protein calibration graph obtained with one of the human liver samples (HLM-3) is depicted in Figure 5.2 and this demonstrates that there is linearity with such a technique within the range of protein concentrations examined (r=0.925). Similar response was shown for all the other human liver samples with the maximum concentration for linearity ranging between 0.4µg and 0.6µg of solubilised microsomal protein /well (data not shown). Based on these findings 0.2µg and 0.4µg protein of each sample were then used together with a rat P450 4A1 standard for ELISA quantification. The results are presented in Table 5.3 and shows that the orthologous human cytochrome P450 4A1 isozyme
Figure 5.2 Linearity of orthologous cytochrome P450 4A1 determination in human liver microsomes. A polyclonal sheep antibody (1:20k dilution) to electrophoretically homogeneous rat liver cytochrome P450 4A1 was used in an ELISA procedure as described in Materials and Methods. Each data point was derived from triplicate determinations with HLM-3 microsomes.
Table 5.3 Immunoquantitation of Orthologous Cytochrome P450 4A1 Apoprotein in Human Liver Microsomes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Orthologous cytochrome P450 4A1 specific content (pmol/ mg protein)</th>
<th>Percentage of total cytochrome P450</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM-1</td>
<td>3.50 ± 0.71</td>
<td>2.01 ± 0.33</td>
</tr>
<tr>
<td>HLM-2</td>
<td>17.75 ± 0.50</td>
<td>4.79 ± 0.13</td>
</tr>
<tr>
<td>HLM-3</td>
<td>23.01 ± 1.56</td>
<td>4.18 ± 0.27</td>
</tr>
<tr>
<td>HLM-4</td>
<td>36.70 ± 2.60</td>
<td>5.78 ± 0.40</td>
</tr>
<tr>
<td>HLM-5</td>
<td>23.90 ± 3.20</td>
<td>4.15 ± 0.54</td>
</tr>
<tr>
<td>HLM-6</td>
<td>20.50 ± 3.54</td>
<td>7.54 ± 1.40</td>
</tr>
<tr>
<td>HLM-7</td>
<td>25.10 ± 1.06</td>
<td>11.63 ± 0.52</td>
</tr>
<tr>
<td>HLM-8</td>
<td>24.20 ± 2.76</td>
<td>9.66 ± 1.09</td>
</tr>
<tr>
<td>Mean</td>
<td>21.80 ± 8.6*</td>
<td>6.22 ± 2.98</td>
</tr>
<tr>
<td>Untreated rat</td>
<td>35.20 ± 1.7</td>
<td>6.41 ± 0.67</td>
</tr>
</tbody>
</table>

Results are the mean ± SEM of triplicate determinations for each sample. Difference in mean cytochrome P450 4A1 specific content in human livers (n=8) and untreated rats (n=3) was significant at *P< 0.05.
levels estimated ranged between 2-12% of the total cytochrome P450 population. The data obtained was of the same order of magnitude as that determined for untreated rat (about 6% of the total cytochrome P450 population).

Lauric acid has been used previously as a specific substrate for the cytochrome P450 4A subfamily proteins in the rat [Gibson et al. 1982]. It was therefore relevant to investigate the metabolic capabilities of the different human liver microsomes towards lauric acid as a substrate. Catalytic activity in human liver samples was determined under conditions in which metabolism was proportional to time of incubation (Figure 5.3) and the results obtained for the human samples presented in Figure 5.4. The information presented shows that the liver microsomes derived from different human subjects display significant activity toward $\omega-$ and ($\omega-1$)-hydroxylation of lauric acid, except for HLM-1 whose activity was barely detected and was close to the limits of sensitivity of our assay procedures (approximately 0.02nmol product/min/nmol P450). The mean activities for human $\omega-$ and ($\omega-1$)-hydroxylation of laurate were $3.23 \pm 1.51$ and $1.59 \pm 0.99$nmol product/min/nmol total cytochrome P450, respectively, corresponding to a ratio of $\omega-$ to ($\omega-1$)-hydroxylase activities of about 2.0. These values are very comparable to that demonstrated with rat liver microsomes for $\omega-$ and ($\omega-1$)-laurate hydroxylases at 4.92 and 2.96nmol product/min/nmol P450 respectively. However, unlike the rat liver cytochrome P450 4A1 isozyme which is well characterised and shown to exhibit regioselectivity for $\omega-$laurate hydroxylation [Gibson et al. 1982], the equivalent detailed information on the cytochrome P450 4A subfamily-related enzymes is only now beginning to emerge. Of relevance to this, were the findings of separate experiments, in which lauric acid hydroxylation by human hepatic microsomes was carried out under the following conditions (i) in the absence of NADPH (ii) using carbon monoxide as an inhibitor to cytochrome P450-mediated metabolism and (iii) in an atmosphere of nitrogen (data not shown). Collectively, these simple experiments were indicative that in human liver
Figure 5.3  Typical lauric acid metabolism time-course study with a human liver microsomal sample. Determination of the rate of formation ω- and (ω-1)-hydroxylated products by HLM-3 microsomes was assessed by reverse phase h.p.l.c. analysis and as detailed in Materials and Methods. Each data point represents the mean of triplicate determinations.
Mean Activities For Human Liver Samples
(nmol product/ min /nmol total P450)

11-OH = 3.23 ± 1.51
12-OH = 1.59 ± 0.99

Figure 5.4 Lauric acid metabolism by human hepatic microsomes.
Reverse phase h.p.l.c. analysis of laurate metabolites following incubations of human (2mg/ml) and rat (1mg/ml) microsomal protein, was conducted as described in Materials and Methods. Triplicate analysis was performed for each sample. Control rat values were 4.92 ± 0.51 and 2.96 ± 0.10 nmol product/min/nmol P450 for ω— and (ω-1)-hydroxylase activities, respectively.
microsomes, lauric acid hydroxylation is most probably cytochrome P450-mediated since no metabolites were detected under these conditions (data not shown).

The results of immune complex inhibition (using an antibody to rat P450 4A1) of lauric acid metabolism by one of the human liver microsomes that showed highest activity towards this substrate is presented in Figure 5.5. The objective of such an experiment was to investigate the influence of the rat antibody to cytochrome P450 4A1 as a potential inhibitor of laurate biotransformation by human liver microsomes and therefore indicative of which cytochrome P450 species might be involved in laurate hydroxylation. The higher concentration of the immune IgG fraction used in this study was such that it caused approximately 50% inhibition of the rat ω—hydroxylase activity. The data presented was also normalized by taking into account any non-specific inhibition by non-immune IgG fraction. From figure 5.5, it could clearly be seen that 10mg immune IgG/nmol P450, that caused approximately 50% inhibition of ω—hydroxylase activity in the rat, was also very potent towards similar activity in the human liver causing up to 70% inhibition. By lowering the concentration of immune IgG used to 5 mg/nmol P450, the human catalytic activity also reduced to about 55% inhibition whereas that for the rat was just below 20%. The picture for (ω—1)-hydroxylation was different since by doubling the IgG concentration from 5 to 10mg, the inhibition percentage of the human activity did not change at about 80% whereas that for the rat decreased from about 40% to about 20%, suggesting more of stimulation. Attempts to use 20mg immune IgG/nmol P450 totally inhibited the human activity beyond detection (data not shown). The effect of pre-immune IgG at 20 mg/nmol P450 was not investigated.

When [1-14C]-arachidonic acid is incubated with rat and human liver microsomes under conditions that support metabolism, i.e. in the presence of NADPH and molecular oxygen, and the organic soluble products isolated and analysed by reverse-phase high performance liquid chromatography analysis, a
Figure 5.5  Inhibition of 11-(ω-1) and 12-(ω) hydroxylation of lauric acid biotransformation in human liver microsomes by an antibody against rat P450 4A1. Human liver microsomes (HLM-3, 2mg/ml) and control rat microsomes (1mg/ml) were each separately preincubated with the pre-immune and immune IgG fractions (5 and 10mg IgG/nmol cytochrome P450) at 37°C for 10 minutes. The reaction mixture was then placed on ice and lauric acid hydroxylase activity determined as described under Materials and Methods. Values for the rate of formation of (ω-1)- hydroxylated products by human and rat in the presence of pre-immune sera were 1.12 and 1.45 nmol/min/nmol P450 respectively and for their corresponding ω-hydroxylated products, 2.12 and 4.28 nmol/min/nmol P450, for human and rat, respectively.
A complex pattern of metabolites is formed. Figure 5.6 shows HPLC chromatograms obtained for such studies with liver microsomal fractions derived from livers of either untreated male rats, clofibrate-induced rats, or human. The formation of these metabolites would appear to be cytochrome P450-dependent, as separate incubations where NADPH was omitted resulted in no apparent metabolism (Figure 5.7). All the human liver microsomal preparations examined (n=5) showed the same qualitative metabolic pattern as that depicted in figure 5.6, except that the rate of formation of the individual peaks was different. Extensive characterisation and identification of these peaks was not possible due to the lack of authentic standards and was not addressed in the current study. However based on previous experiments carried out in our laboratories and reports from studies reported by other workers [Capdevila et al., 1985], some tentative qualitative and quantitative analyses were made, even though the metabolic profiles were not exactly the same. Five major peaks stood out and were common in all the chromatograms. These were designated as peaks I, II, III, IV and V with retention times of approximately 25.2, 26.4, 27.3, 28.1, 29.2 and 30.1 minutes respectively.

Figure 5.8 represents chromatograms derived from combined incubation mixture extracts derived from either human plus untreated rat liver microsomes (A) or human plus clofibrate-induced rat liver microsomes (B). This data lends support to the observation that similar peaks were present in all the microsomal preparations and the metabolites are very similar to each other. Table 5.4 shows the rate of formation of these peaks expressed as pmol. product formed/min/nmol P450 for each hepatic microsomal preparation. The unmetabolised arachidonic acid substrate was eluted at approximately 45.4 min in all the chromatograms. Notably, the overall rate of arachidonic acid metabolism was nearly the same for all the human samples and averaged 2.10 ± 0.22 nmol/min/nmol P450 (n=5). The values obtained for untreated and clofibrate-induced male rats were 2.39 ± 0.02 and 3.89 ± 0.02 nmol/min/nmol P450 respectively. Comparison of the rates of formation of
Figure 5.6 Metabolite profiles generated by NADPH-dependent oxygenation of \([\text{[1-}^{14}\text{C]}\text{-arachidonate by different liver microsomal preparations. Incubation and metabolite analyses were as described under Materials and Methods. The chromatograms shown were obtained using microsomes from untreated rat (A, 1mg/ml), clofibrate-induced rat (B, 1mg/ml), and human (HLM-3) (C, 2mg/ml) livers. Incubation times were 10 and 20 minutes for rat and human microsomes, respectively. The specific contents of microsomal cytochrome P450 were 0.57, 0.87 and 0.53 nmol/ mg protein for untreated, clofibrate-induced and human microsomes, respectively.} \)
Figure 5.7 Lack of arachidonic acid metabolites formation in rat and human liver microsomes in the absence of NADPH. Microsomes derived from untreated rat (A), clofibrate-induced (B), and human (C) livers were subjected to the same procedures as in Figure 5.6 and as detailed under Materials and Methods except for the omission of NADPH. AA = arachidonic acid
Figure 5.8 Reverse-phase chromatograms of the metabolites formed during the NADPH-dependent metabolism of arachidonic acid catalysed by combinations of microsomal preparations: Untreated rat (U) (1mg protein/ml) + Human (H) (2mg protein/ml) liver microsomes (A) and Clofibrate-treated rat (CF) (0.5mg protein/ml) + Human (2mg protein/ml) liver microsomes (B). All incubations were carried out for 10 minutes. The experimental conditions were essentially as described under Materials and Methods. The specific contents of microsomal cytochrome P450 determined for these combinations were 0.51 (U+H) and 0.63 (CF+H) nmol/mg protein for chromatograms A and B, respectively.
Table 5.4  Comparison of the Rate of Formation of Some Arachidonate Metabolite Peaks by Rat and Human Hepatic Microsomes

<table>
<thead>
<tr>
<th>Peak name</th>
<th>Retention time (minutes)</th>
<th>pmol product formed/ min/ nmol P450</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control rat</td>
</tr>
<tr>
<td>I</td>
<td>24 - 25</td>
<td>266.1 ± 8.8</td>
</tr>
<tr>
<td>II</td>
<td>25 - 26</td>
<td>451.8 ± 16.1</td>
</tr>
<tr>
<td>III</td>
<td>26 - 27</td>
<td>191.5 ± 17.3</td>
</tr>
<tr>
<td>IV</td>
<td>29 - 30</td>
<td>178.4 ± 15.9</td>
</tr>
<tr>
<td>V</td>
<td>30 - 31</td>
<td>106.7 ± 14.4</td>
</tr>
<tr>
<td><strong>Total metabolism</strong></td>
<td></td>
<td>2.4 ± 0.02</td>
</tr>
</tbody>
</table>

* This represents the overall rate of formation of all the metabolites after NADPH-dependent metabolism of [1-14C]-Arachidonic acid by microsomes derived from untreated and clofibrate-treated rats and human livers. Data presented for humans is the mean obtained with all the microsomal samples. Units are: nmol product formed/ min/ nmol P450.
individual metabolites revealed major differences. Whereas the predominant metabolite, when untreated rat microsomes was used, eluted at about 26.4 min (Peak II), those for all the individual human microsomal preparations eluted at approximately 25.2 min (Peak I).

Clofibrate is a hypolipidaemic agent which has been unequivocally demonstrated as a potent inducer of the cytochrome P450 isozyme mediating \( \omega \) and \((\omega-1)\)-hydroxylation of arachidonic acid [Gibson et al., 1982; Sharma et al., 1989]. In my studies, pretreatment of rats with this drug resulted in a significant increase in the rate of formation of a peak with retention time of approximately 29.2 min (Peak IV) (Figure 5.6). This peak together with that eluted at approximately 30.1 min (Peak V) were reduced with concomitant increase in the other peaks (data not shown). Based on these findings and those of other workers [Capdevila et al., 1985; Sharma et al., 1989], peaks IV and V were tentatively identified as the \( \omega \) and \((\omega-1)\)-hydroxyarachidonic acid metabolites. According to our current knowledge, the \( \omega \)-hydroxylation of arachidonic acid represents a reaction pathway catalysed by cytochrome P450 4A1 [Sharma et al., 1989]. The results presented here show that the rate of formation of peak IV in clofibrate-induced rat liver microsomes was approximately 8-fold over that of untreated rat microsomes and between 6 to 50-fold over those of human liver microsomes. The rate of formation of individual metabolite peaks by individual human liver microsomes is presented in Table 5.5. Similarly, a summary of the rate of formation of the tentatively identified \((\omega-1)\) and \( \omega \)-hydroxyeicosatetraenoic acid metabolites, by these human liver microsomes compared to that generated with rat liver microsomes, is presented in Table 5.6.

Western blot analysis of hepatic microsomes derived from each human liver sample was performed using a sheep polyclonal antibody raised against the electrophoretically homogeneous rat hepatic cytochrome P450 4A1 (Figure 5.9). Of major significance, was the observation that the anti-cytochrome P450 4A1 serum recognised only one major band in all the human liver samples examined, as
Table 5.5 Rate of formation of some arachidonic acid metabolites by individual human liver microsomes

<table>
<thead>
<tr>
<th>Sample</th>
<th>pmol product formed /min/nmol cytochrome P450</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak I (24-25min.)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>HLM-1</td>
<td>404.5 ± 15.7</td>
</tr>
<tr>
<td>HLM-2</td>
<td>429.0 ± 58.1</td>
</tr>
<tr>
<td>HLM-3</td>
<td>454.0 ± 18.8</td>
</tr>
<tr>
<td>HLM-4</td>
<td>414.1 ± 12.2</td>
</tr>
<tr>
<td>HLM-5</td>
<td>403.3 ± 41.0</td>
</tr>
</tbody>
</table>

Note: The time indicated in brackets for each peak is the retention time in minutes.
n.d = not detectable.
Table 5.6 Rates of formation of tentatively identified arachidonic acid metabolites by rat and human liver microsomal samples

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Rate of metabolite formation (pmol/min/nmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>untreated rat microsomes</td>
</tr>
<tr>
<td>20-HETE</td>
<td>178.4 ± 16</td>
</tr>
<tr>
<td>19-HETE</td>
<td>106.7 ± 15</td>
</tr>
<tr>
<td>Ratio (ω/ω-1)</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Abbreviations: 20-HETE, 20(ω)-hydroxyeicosatetraenoic acid; 19-HETE, 19(ω-1)-hydroxyeicosatetraenoic acid.
Figure 5.9 Immunodetection of hepatic cytochrome P450 4A1 in human and rat hepatic microsomes by Western blotting. Microsomal fractions were subjected to 7.5% SDS-polyacrylamide gel electrophoresis and immunoblotted with an antibody to highly purified rat hepatic cytochrome P450 4A1 as described in Materials and Methods. The samples were loaded as follows: track 1, HLM-1; 2, HLM-2; 3, HLM-3; 4, HLM-4; 5, HLM-6; 6, 0.5pmol of electrophoretically pure rat cytochrome P450 4A1; 7, untreated rat liver microsomes (20μg); 8, HLM-6; 9, HLM-7; 10, HLM-8. All human hepatic microsomes were loaded on equal protein basis (all 30μg/track).
opposed to two bands seen with rat liver microsomes. Moreover, this human liver microsomal protein did not co-migrate to the same position as seen with either the authentic P450 4A1 apoprotein or the lower of the two rat bands representing the rat P450 4A1 isoform [Tamburini et al., 1984; Sharma et al., 1988a; Chinje and Gibson, 1991]. The molecular mass of the human liver orthologous form was estimated on a 7.5% SDS-PAGE gel at about 51kDa, approximately 500 daltons lower than the rat P450 4A1 isoform. Linear regression analysis was used to describe the relationship between Western blotting data (band intensities) and ELISA-determined levels of human liver cytochrome P450 4A1 orthologue. A correlation coefficient (R) of 0.694 was found not to be statistically significant at 5% level of significance. The difference in molecular weights between the rat and human cytochrome P450 4A1 orthologues became more apparent when rat authentic cytochrome P450 4A1 was spiked with human microsomes (Figure 5.10). As shown in this figure, the human species has a slightly lower molecular mass compared to that of the rat.

In order to further characterise the panel of human liver samples, alkoxyresorufin-O-dealkylase activities associated with the cytochromes P450 1A1 and 2B1 isozymes were determined and the results are presented in Figure 5.11. The average ethoxyresorufin-O-deethylase (EROD) activity in the human samples examined (n=8) was 54 ± 34 pmol product formed/min/nmol P450. The rather large standard deviation suggested interindividual variation within the samples investigated and were comparable in magnitude to that of the male rat (n=3), estimated at 56.9 ± 2.0 pmol product formed/min/nmol P450. HLM-1 showed least ethoxyresorufin-related activity when compared to the other samples. The data for pentoxyresorufin-related activity (PROD), representing cytochrome P450 2B1 is also shown in Figure 5.11. The mean rate of production of resorufin from the substrate pentoxyresorufin in human liver was 2.58 ± 1.24 pmol/min/nmol P450 (n=8) and was not very different when compared to that of the rat (n=3) measured
Figure 5.10 Difference in molecular weights between rat and human cytochrome P450 4A1 species. Western blotting analysis was carried out using a rat cytochrome P450 4A1 polyclonal antibody at a dilution of 1:20k, as described in Materials and Methods. Tracks were loaded as follows: track 1, HLM-3 (30µg); track 2, HLM-6 (30µg); track 3, homogeneous cytochrome P450 4A1 (0.5pmol spiked with 20µg HLM-6); track 4, authentic cytochrome P450 4A1 standard (0.5pmol); track 5, untreated rat microsomes (20µg).
Figure 5.11 Alkoxyresorufin-O-dealkylase Activities in Human and Rat Liver Microsomes. Each bar represents the mean ± SEM of triplicate determinations for individually processed microsomal preparations. Human liver samples are numbered from 1 to 8 for HLM-1 to HLM-8 and possess a mean activities of 54.5 and 114.2 pmol product formed/min/nmol P450 for EROD (A) and PROD (B), respectively. Mean values for untreated rat (n=3) were 56.9 ± 1.3 and 3.6 ± 0.2 pmol/min/nmol P450, for EROD and PROD activities, respectively.
at 3.55 ± 0.98 pmol/min/nmol P450. Attempts to immunochemically substantiate the variation recorded in EROD catalytic data by Western blotting analysis, which included positive control microsomes from 3-MC-induced rat liver (data not shown), was not quite successful as very little cross reactivity was detected between the rat cytochrome P450 1A1 antisera and the orthologous human protein.

A relationship was established between the tentatively identified P450 4A-mediated $\omega$- and $(\omega-1)$-hydroxylation products of arachidonic acid and lauric acid based on regression analysis ($r = 0.740$, $P > 0.05$) (Figure 5.12). Likewise, an attempt was made to identify any significant correlations within the rest of the data presented in this chapter and a correlation matrix table was constructed as depicted in Table 5.7.
Figure 5.12 Relationship between cytochrome P450 4A-mediated laurate and arachidonate hydroxylase activities in human liver microsomes (n= 4). Metabolites were quantified by reverse phase h.p.l.c. analysis as outlined under Materials and Methods. Each data point was from duplicate determinations.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Total P450</th>
<th>NADPH-cyt.c</th>
<th>P450A1 (%) Total P450</th>
<th>11-LAOH</th>
<th>12-LAOH</th>
<th>EROD</th>
<th>PROD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total P450</td>
<td>1.000</td>
<td>0.811</td>
<td>0.037</td>
<td>0.820</td>
<td>0.780</td>
<td>1.000</td>
<td>0.000</td>
</tr>
<tr>
<td>NADPH-cyt.c</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>P450A1 (%) Total P450</td>
<td>0.000</td>
<td>0.025</td>
<td>0.137</td>
<td>0.237</td>
<td>0.093</td>
<td>0.037</td>
<td>0.000</td>
</tr>
<tr>
<td>11-LAOH</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>12-LAOH</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>EROD</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>PROD</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 5.7: Correlation Between Different Monooxygenase Activities (levels) in Human Hepatic Microsomes.
5.4 Discussion

The variations often seen in drug-metabolising activities of the livers of patients probably reflects variability of a normal population clinically observed as variation in plasma concentrations of drugs [Sjöqvist and von Bahr, 1973]. In general, the values I have obtained for total cytochrome P450 specific content (0.07-0.64 nmol/mg protein) are quite similar to those published by other workers, ranging between 0.13-0.49 nmol/mg protein [Darby et al. 1970, Nelson et al. 1971, Beaune et al. 1986], and are somewhat lower than that in the rat. One of the samples examined (HLM-1) reflected a very low cytochrome P450 specific content, believed to be due to the presence of cytochrome P420, which may have arisen due to post-mortem changes, possibly arising as a result of poor handling of obtained tissue prior to proper storage for subsequent analyses. The difficulty in perfusing such tissues also renders haemoglobin a source of contamination in microsomal preparations thereby interfering with spectrophotometric determination of cytochrome P450. No information about the immediate processing of the tissues after death of the patients in my study was readily available. It is well established that storage of tissues under the appropriate conditions at -80°C for several months does not alter monooxygenase activity.

To have an insight into the probable causes of the variations between rat and man liver monooxygenase activities, it was important to consider the relative cytochrome b5/P450 and NADPH-cytochrome c reductase/P450 ratios in both species. The reason for such comparison was made obvious because of the importance of NADPH-cytochrome c reductase in metabolism and the role of cytochrome b5 in stimulating cytochrome P450 4A1-mediated hydroxylation of fatty acids. The ratio cytochrome b5/ P450 in untreated rats was 0.64, whereas a similar ratio in man ranged between 0.47-3.29, the highest recording being for HLM-1 which incidentally showed the least enzymatic activity. However, this ratio did not correlate with any of the catalytic activities measured (data not shown). For
instance, HLM-6 showed the second highest ratio of 2.1, but resulted in the highest laurate hydroxylase activity which was similar to that obtained with untreated rat liver microsomes. Likewise, NADPH-cytochrome c reductase/P450 ratios determined did not correlate with any of the enzymatic activities examined (data not shown).

Sex difference, in the tissues used, was not reflected in the total cytochrome P450 specific contents and may not have been obvious because of the relatively small sample size used in my study. This pattern of result was repeated with the determination of cytochrome b5 specific contents and NADPH-cytochrome P450 (c) reductase activities. The measured NADPH-cytochrome c reductase activities were slightly lower than that reported for biopsy samples (between 70-193nmol reductase/min/mg protein), which are often considered to be fresh and more accurate in reflecting in vivo activities [von Bahr et al. 1980]. NADPH-cytochrome P450 reductase activity has been reported to be fairly labile and therefore decreases relatively rapidly after death [Marzella and Glaumann, 1976; Wang et al. 1980]. The results I have obtained with these samples may either be a true reflection of the in vivo situation or an alternative explanation for apparent lower values recorded being as a result of post-mortem changes.

ELISA-based quantification of the human orthologous form of cytochrome P450 4A1, using the rat polyclonal antibody, show that this represents about 7% (n=8) of total cytochrome P450 determined spectrally. It is important to bear in mind when interpreting such data that, by immunological methods, it is the apoprotein that is measured whereas spectrally, only the haem-linked cytochrome P450 is measured. However, until the corresponding human protein to rat cytochrome P450 4A1 is purified to homogeneity and its antibody produced, the rat antibody described and used herein serves as a useful tool in the study of the expression of the cytochrome P450 4A subfamily proteins in human liver. My results suggest that a human
orthologous form(s) to the rat cytochrome P450 4A1 protein is/are expressed in the human liver samples examined.

Further evidence lending support to the existence of the P450 4A subfamily proteins in humans came from the results obtained by Western blotting analysis. My results show our polyclonal antibody recognised only one major band on analysis and this had a slightly lower molecular mass when compared to that of rat liver microsomes and the corresponding authentic P450 4A1. A similar experiment employing human liver microsomal preparations has been reported elsewhere [Dirven et. al., 1991]. However, in addition to the strong reacting band observed in my studies (M_r of approximately 51kDa), these workers also detected a weaker-reacting band with a higher relative molecular weight, estimated at 56.5kDa, in most of the samples. The absence of such an additional reacting band, in the studies reported herein, goes to show some of the interindividual variations that might exist when dealing with human samples and more specifically with the liver.

The difference in molecular weights between the rat and human enzymes became more apparent when authentic rat P450 4A1 was spiked with human microsomes (Figure 5.10). It was possible with such an experiment to establish with some degree of certainty that a slight difference in molecular mass exists between the rat and human liver microsomal specimens examined. The observation that the rat antibody cross-reacted with a single protein band in the human liver microsomes is further evidence of the existence of cytochrome P450 4A1 or a close relative. The varying intensities of the bands after scanning by densitometry did not correlate well with the ELISA quantification data (r=0.69, Figure 5.10). This inconsistency may well be attributed to the difficulties in establishing linearity with Western blot analysis, a technique which is best suited for qualitative studies.

The absence in human hepatic microsomes of an upper protein band of about 52kDa as seen in the rat, raises some questions regarding the measured catalytic
activity with lauric acid as substrate. In rats, apart from the clofibrate-inducible P450 4A1, cytochrome P450 4A3 has also been demonstrated to catalyse the \( \omega \)- and \( (\omega-1) \)-hydroxylation of lauric acid, the latter possibly a 52kDa protein [Aoyama et al. 1990]. The implications of these observations may be interpreted as suggesting that fewer human P450 isozymes of the P450 4A subfamily are involved in generating fatty acid metabolites as compared to rat liver. The exact number of P450 isoforms involved in human liver fatty acid hydroxylation still remains to be determined but it is clear that in all the human samples examined, there is a preferential hydroxylation of lauric acid at the \( \omega \)-position. It is also worthwhile mentioning that quite recently, a cytochrome P450 has been purified to homogeneity from human kidney microsomes (termed P450\(_{\text{HKo}}\)) which catalysed the \( \omega \)- and \( (\omega-1) \)-hydroxylation of fatty acids with no activity for prostaglandins or xenobiotics such as benzphetamine and 7-ethoxycoumarin [Kawashima et al., 1992]. It was therefore suggested by these workers that cytochrome P450\(_{\text{HKo}}\) is a member of the P450 4A gene subfamily. The physiological significance of this fatty acid \( \omega \)-hydroxylase is not known but predominates over other forms of cytochrome P450 in human kidney cortex microsomes [Jakobsson et al., 1973; Okita et al., 1979; Imaoka et al., 1990b].

Unlike the rat, where regioselectivity has been firmly established, such conclusion could not be drawn with certainty in the case of human hepatic microsomes. For instance, there were a few anomalies encountered in this investigation as was seen in the case of HLM-1 which had barely detectable catalytic activity towards laurate metabolism even although quite an appreciable amount of P450 4A1 apoprotein equivalent was immunodetectable by Western blotting and quantified by ELISA. As earlier mentioned, there is a possibility that this may be as a result of post-mortem changes occurring in the tissues after death. Moreover HLM-1 was reported as a moderate smoker, suffering from obstructive jaundice and died of hepatocellular carcinoma. This subject was also taking...
temazepam and thiopentone, and taken collectively probably had an important impact on the cytochrome P450 isozyme complement.

I have also investigated the role of the cytochrome P450 4A subfamily proteins in the metabolism of arachidonic acid by using liver microsomes derived from untreated and clofibrate-induced rats as well as human liver. Liver microsomal cytochrome P450 haemoproteins catalyse essentially three main reactions during arachidonic acid metabolism, namely: olefin epoxidation reactions or formation of epoxyeicosatrienoic acids (EETs), allylic oxidation or formation of hydroxyeicosatetraenoic acids (HETEs) and oxidation of an unactivated $sp^3$ carbon leading to the formation of $20(\omega)$- and $19(\omega-1)$-hydroxyeicosatetraenoic acids. The peaks observed in all the h.p.l.c chromatograms represent metabolites resulting from an interplay of these pathways, however, their identification and full characterisation was not addressed in the present study.

Studies carried out elsewhere, involving the pretreatment of rats with the hypolipidaemic drug, ciprofibrate, resulted in a dramatic change in oxygenation regiospecificity catalysed by the microsomal fraction [Capdevila et al., 1985]. These workers observed that metabolites, with retention times corresponding to those of authentic $\omega-$ and $(\omega-1)$-alcohol standards, were the predominant feature of the reaction catalysed by ciprofibrate-induced microsomal fractions, concomitant with a marked decrease in the ability of the liver microsomal suspension to catalyse both olefin epoxidation and allylic oxidations. In a similar experiment where I used clofibrate to induce rat liver microsomes, there was a significant enhancement of the tentatively identified $\omega-$ and $(\omega-1)$-oxidation products, however, contrary to the results reported by Capdevila and co-workers, other unidentified metabolites were still detected although with a net decrease in their formation rates, possibly the HETEs and EETs. The observed differences may be explained by considering some of the factors that affect drug disposition. First of all, different rat strains were used in each of the above studies even though it is an unlikely reason. Secondly,
the inducer employed in my study was different to that used by these workers, and
thirdly, the use of 1,2, epoxy-3,3,3,-trichloropropane as an epoxide hydrolase
inhibitor, was omitted in my studies. The absence of this inhibitor would result in
the formation of secondary metabolites which may also be enhanced by the slightly
longer incubation periods used in my studies (20 min) compared to 5 min used by
these workers. The enhanced formation of secondary metabolites (mainly polar) as
a result of longer incubation periods of up to 60 min, has been investigated by using
12(R)-hydroxyeicosatetraenoic acid in the presence of NADPH [Jajoo et al., 1992].
These workers used rat liver microsomes and established linearity in product
formation up to 40 min, the two major metabolites being dihydroxyeicosatetraenoic
acids (DHETEs) generated by \(\omega/(\omega-1)\) hydroxylation.

Controversy surrounds the uniqueness of a single cytochrome P450 capable of
catalysing both the \(\omega-\) and \((\omega-1)\)-hydroxylation reactions [Tamburini et al., 1984;
Björkhem and Danielsson, 1970]. However, the results of the findings described
above and those reported elsewhere strongly lends support to the idea of a unique
species of haemoprotein for the catalysis of this reaction [Sharma et al., 1989]. I
have tentatively identified two peaks present in the h.p.l.c. chromatograms
generated after perturbation by clofibrate, which is a classical inducer of the
cytochrome P450 (P450 4A1) mediating such reactions and labelled them as 19(\(\omega-1\))
and 20(\(\omega\))-hydroxylated products of arachidonic acid. These peaks were amongst
others not positively identified, but were all very likely cytochrome P450-mediated
and required the presence of NADPH. The positive correlation between the overall
rate of formation of \(\omega-\) and \((\omega-1)\)-metabolites (Peaks IV and V) of arachidonic
acid and those with lauric acid as substrate \((r=0.74, n=5)\) further supports the
hypothesis that these metabolites are derived from a closely related member(s) of
the cytochrome P450 4A subfamily.

Specific antibodies can be used to inhibit P450-mediated reactions in
microsomes. In principle, the degree of immunoinhibition indicates what species
are involved in the metabolic pathway under investigation [Thomas et al. 1977]. Obviously the interpretation of immunoinhibition experiments depends upon the specificity of the antibodies, especially as in some cases cytochrome P450s within a family are likely to have considerable similarity such that distinguishing individual forms may be quite a difficult task [Guengerich and Shimada, 1991]. The situation can be rendered more complicated as in humans where a complete account of the multiplicity within the gene superfamily and/ or even the CYP4 gene family is not available.

The influence of rat anti-P450 4A1 antibody as a potential inhibitor to laurate biotransformation was investigated and the results obtained were not very conclusive as to which species is/ are involved. The observation that even the immunopurified IgG fractions from normal sheep serum showed a certain extent of inhibitory action at concentrations that had little or no effect on the rat activity, cast some doubts as to the interpretation of these results. However, some valuable information could be obtained when control experiments were normalised using pre-immune IgG fractions from the same animal. At a concentration of 5mg immune IgG/nmol P450 used, the rat ω—laurate hydroxylase activity was inhibited by almost 50% whereas in the humans about 80% of similar activity was lost. No immunoselectivity in inhibition pattern of ω— and (ω—1)-hydroxylation products formation was apparent. The degree of potency exerted by the rat cytochrome P450 4A1 antibody on human liver microsomal laurate hydroxylation meant there was adequate access of the antibodies to the membrane-bound enzyme(s) involved in this metabolic pathway. Since the human liver isozyme(s) responsible for fatty acid hydroxylation is yet to be purified to homogeneity, it is impracticable to carry out direct inhibitory experiments with antibodies directed against the human isoform of interest.

As discussed above, I can only postulate within the limitations of the present findings that there are some indications as to the existence and close similarity of a member(s) of the cytochrome P450 4A subfamily proteins in the human hepatic
microsomes examined to those well documented in the rat and it is still premature at this stage to pin-point cytochrome P450 4A1 as the sole isoform involved.
CHAPTER SIX

General Discussion
General Discussion

6.1 Structural and functional comparison between human and rat liver cytochrome P450 4A subfamily proteins

One of the questions that was not clear prior to the initiation of the studies described in this thesis, was whether there was similar multiplicity in the cytochrome P450 4 gene family in human liver as previously observed for the well characterised rat isoform(s). The extraordinary broad substrate specificity of the cytochrome P450 system results from the multiplicity of distinct molecular forms which have different, but overlapping substrate specificities [Lu and West, 1980; Nebert and Negishi, 1982]. Many different cytochrome P450 isoenzymes have been purified from human liver [Wang et al., 1983; Guengerich, 1988a], their corresponding cDNAs cloned and classified into families and subfamilies based on their DNA and amino acid sequence similarity [Nebert et al., 1991]. These families are mainly involved in steroid biosynthetic processes as well as catalysing the oxidation of fatty acids, drugs and carcinogens [Guengerich, 1991]. Specifically with families 1,2,3 and 4, it appears that a tremendous variability exists between species in the number of cytochrome P450 genes and the substrate specificities of individual isoenzymes [Nebert et al., 1991; Souček and Gut, 1992]. On the basis of these findings, it is difficult, in many instances, to extrapolate toxicological and carcinogenicity studies from rodents to man. Thus, species differences underscore the necessity to directly study human cytochrome P450s. Only limited data are available on the cytochrome P450 4 family in human liver as opposed to the relatively well-documented data on the corresponding rat proteins.

In view of the diverse function of cytochrome P450s, individuality in isoenzyme expression can have severe implications in the risk assessment of xenobiotic exposure. Much of the structural comparison data described herein was based on enzyme-linked immunosorbent assay (ELISA) and Western blotting employing anti-
rat cytochrome P450 4A1 polyclonal antibody (Chapter 5). My results revealed that in the panel of eight human liver samples, hepatic microsomes apparently expressed only one protein that cross-reacted with our rat polyclonal antibody. This protein band was slightly lower in molecular weight (approximately 51kDa) when compared to rat authentic cytochrome P450 4A1 (estimated at 51.5kDa). This result was consistent with those of other workers who observed a similar protein band that immunoreacted with rat anti-cytochrome P450 4A1 antibody [Dirven et al. 1991; Forrester et al., 1992]. Moreover, in addition to the 51kDa protein expressed in human liver microsomes, Forrester and co-workers found two additional prominent cross-reacting protein at about $M_f$ 51.5 and 60kDa in their panel of 12 human livers, which were subject to 30-fold and 15-fold induction variations, respectively.

An intriguing correlation in the data presented by Forrester and co-workers was in the level of expression of the $M_f$ 50 and 51.5kDa proteins. Such co-ordinate regulation would be consistent with the findings in the rat with regard to cytochromes P450 4A1 and 4A3 [Kimura et al., 1989b]. It is worth mentioning here that our polyclonal antibody recognised similar proteins in untreated and clofibrate-induced rat liver-derived microsomes even after attempts to immunopurify the antibody (Chapter 2). Similar co-ordinate regulation was seen in the rat after treatment with stereoisomers of a clofibrate analogue (Chapter 3). There are many examples in rodents where the levels of cytochrome P450 isoenzymes from distinct gene families or subfamilies are regulated by the same foreign compound inducing agents. An example of this phenomenon was seen in Chapter 4, where two proteins, cytochromes P450 1A1 and 1A2, were shown to be regulated by the same inducer (3-methylcholanthrene in this instance) in the rat. On Western blot analysis only one cytochrome 1A protein could be identified, and the evidence indicated that this was cytochrome 1A2. This phenomenon, which to a degree represents co-ordinate regulation, appears to be subject to some species and strain variation, and it
is therefore of central importance to identify which enzymes are co-ordinately regulated in man.

One basis of variation in humans is genetic polymorphism, classically defined as affecting less than 1% of the population. First of all it is plausible that there may be some genetic polymorphism in the expression of the cytochrome P450 4A subfamily proteins in human liver. This assumption is based on the tremendous variation in Western blotting studies carried out in three laboratories (including the work described herein, Chapter 5). Alternatively it is plausible that the antibody may be recognising similar epitopes in proteins that may be structurally unrelated to cytochrome P450 4A1. The second possibility is highly unlikely since the fatty acid hydroxylase activity associated with these proteins could be inhibited in human liver microsomes by the rat antibody.

My studies did not address Northern blotting analysis for mRNA levels in our panel of human livers but results by other workers showed no correlation between the mRNA levels and the level of protein detected by Western blot analysis [Forrester et al., 1992]. This finding suggests that the rate of translation and protein turnover may be important in determining human hepatic cytochrome P450 levels. However, it should be noted, that when analysing human cytochrome P450 4A enzymes by using an antibody raised against rat hepatic cytochrome P450 4A1, the human cytochrome P450 4A levels may be incorrectly estimated due to lack of specificity of the antibody for the human orthologue. Another aspect that may be responsible for the inter-individual variation seen on Western blot and ELISA data may be variation due to enzyme induction. However, it is not trivial to establish that a particular compound is inducing a specific cytochrome P450 isoenzyme in humans. Studies with animal models indicate that a pleiotropic response is often seen with a single chemical and that altered protein levels may have their basis in any of several modes of regulation, including transcriptional activation, mRNA or
protein stabilisation, or even translational enhancement [Porter and Coon, 1991; Nebert et al., 1991].

Preliminary investigation into the possible number of CYP 4A genes in human genomic DNA by Southern blotting analysis, appears to suggest that there is only one member of this gene family [Hood et al., 1992]. These workers screened a human genomic DNA library (after digestion with the restriction endonuclease, BamH1) with a 2.1kb rat CYP 4A1 cDNA probe [Earnshaw et al., 1988] and obtained a single positive clone. This 1.4kb clone was in the region of exons 8-10 which showed strong sequence homology to several cytochrome P450 4 family genes. Similarly, genomic DNAs from a panel of human livers were digested with the same restriction endonuclease as used in the digestion of the human genomic library. These were then subjected to Southern blotting analyses using the above human 1.4kb clone as a probe. The result was the recognition of a single band of the same size as the probe which suggested strong similarity to the rat CYP 4A1. This result was confirmed by employing another restriction endonuclease (EcoR1) in digesting the genomic DNA and the single gene obtained when probed was of the predicted size [Hood, personal communication]. It should be borne in mind that studies using either oligonucleotide or partial cDNA probes are capable of determining only the presence of mRNA encoding a protein and not whether the protein itself is being expressed. Nonetheless, this information lends support to the data presented in this thesis (Chapter 5) in which, a single protein in our panel of human liver-derived microsomes, immunoreacted with the rat anti-cytochrome P450 4A1 polyclonal antibody.

Comparisons of the relative levels of cytochrome P450 isoenzymes in our panel of human livers with activity towards model substrates in vitro is essential in order to define the toxicological consequences of the inter-individual variation in the expression of these proteins in the liver. A few aspects of catalytic specificity are often worth considering with human cytochrome P450 enzymes. With some
reactions, there can be considerable overlap of the different cytochrome P450 enzymes in their efficiency for catalysis. In other circumstances, a single enzyme may be responsible for most of the activity, either because of its intrinsic rate of catalysis or because other isoenzymes showing similar rates are not very well expressed. My studies employed arachidonate and laurate (marker substrates for members of the cytochrome P450 4A subfamily, including P450 4A1) in the characterisation of the human liver orthologous cytochrome P450 4A1. Both substrates were ω—hydroxylated by all the human liver microsomes at rates comparable in magnitude to the rat isoform even though my data showed indications of small interindividual variations (about 2.5-fold). My initial observations were investigated further by antibody inhibition studies with lauric acid as substrate and the results suggested the involvement of member(s) of the cytochrome P450 4A subfamily. The single protein band obtained on Western blot analysis of our panel of human liver suggests that this protein may be responsible for most of the measurable functional activities in mediating (ω-1) and ω-hydroxylase metabolism of the above fatty acids. Correlation between the arachidonate and laurate hydroxylase activities was positive but not statistically significant at 95% confidence interval (r=0.74), thus highlighting the debate on whether both activities are mediated by the same enzyme in human liver. However, it is difficult to infer from such results because of the constraints imposed by limited sample size. We must also await the isolation and purification of the human cytochrome P450 4A orthologue which could be useful in reconstitution studies.

My observation on the lack of correlation between Western blot and lauric acid hydroxylation data is in agreement with those of other workers [Dirven et al., 1991], who were unable to find any observable relation between the staining intensities of the 51kDa protein band and the rate of lauric acid ω-hydroxylation. Other researchers have identified a clofibrate-inducible cytochrome P450 that is not a lauric acid hydroxylase and does not appear to be a member of the cytochrome

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P450 4A subfamily [Swinney et al., 1991]. This highlights possible problems that may be encountered when interpreting catalytic activities due to specific cytochrome P450 inducers. The effects of age, sex or drug history of patients on the cytochrome P450 4A subfamily is not known.

6.2 Rationalisation of structural diversity of cytochrome P450 4A inducers and peroxisome proliferators

Many structurally diverse chemicals are capable of inducing hepatomegaly which is characterised by pronounced proliferation of cellular organelles, including peroxisomes and the smooth endoplasmic reticulum [Hawkins et al., 1987], and some of these compounds, termed peroxisome proliferators, have been associated with an increased incidence of hepatocellular carcinomas in susceptible rodent species [Reddy and Lalwani, 1983]. Concomitant with these xenobiotic-dependent subcellular changes, is an induction of several proteins and associated enzyme activities including the enzymes of the peroxisomal and mitochondrial β-oxidation system and an isozyme of cytochrome P450, termed cytochrome P450 4A1 [Sharma et al., 1988]. Similarly, there is elevation in the expression of the hepatic fatty-acid-binding protein (FABP) [Appelkvist and Dallner, 1980; Kawashima et al., 1983; Bass et al., 1985]. Results from my studies on the relative cytochrome P450 4A1 and peroxisome proliferating induction potencies of the S(+) and R(−)-isomers of a structural analogue of clofibrate, (2-[4-(4-chlorophenyl) benzylxyloxy]-2-phenylacetic acid (Chapter 3), in the rat, as well as a fatty acid analogue, perfluoro-n-decanoic acid (Chapter 4) in a susceptible species (the rat) and a non-responsive species (the guinea pig), further suggested that both peroxisome proliferation and cytochrome P450 4A1 induction are intimately related phenomena. Taken collectively, the above information tends to indicate that the microsomal-peroxisomal-mitochondrial axis plays a prominent and major role in the onset of peroxisome proliferation.

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Although these peroxisome proliferator inducers possess clearly different physico-chemical properties, most of them contain a carboxyl group in their structures (or may be metabolized to one) which may play an important role in induction. This observation is related to the findings of other workers [Bronfman et al., 1986, 1989; Aarsland et al., 1990] who suggested that the acyl-CoA thioesters of compounds like the hypolipidaemic drug, ciprofibrate, which are formed in vitro and in vivo might be the pharmacologically active species of the drugs. The formation of these acyl-CoA derivatives may be responsible for CoA sequestration and subsequent perturbation of lipid metabolism and homoeostasis triggering peroxisome proliferation [Sharma et al., 1988a]. In agreement with this view, Bronfman et al. [1989] showed that the acyl-CoA thioesters of several of these compounds potentiated the activity of protein kinase C (Ca\(^{2+}\)-activated phospholipid dependent protein kinase; pkC), while the free drugs were inactive. PkC is a key enzyme involved in a number of cellular processes, including receptor function, cellular differentiation and carcinogenesis [Nishizuka, 1986; Berridge, 1987].

The presence of the carboxylate moiety in peroxisome proliferators does not appear to be the only requirement in the induction of cytochrome P450 4A1 and peroxisome proliferation and this should argue against the CoA-thioester-formation theory put forward by Bronfmann and co-workers. This observation is drawn from the fact that there exists a number of such compounds which do not have a carboxyl group in their structure nor can they be metabolized to one. Typical examples of such compounds include the N-methylsulphonyl-2-nitrobenzamide derivative, Fomesafen [Elcombe, 1986] and many tetrazole-substituted acetophenone derivatives such as that of the leukotriene D\(_4\) antagonist (LY171883), 2-hydroxy-3-propyl-4-[6-(tetrazol-5-yl)-hexyloxy]acetophenone (4-THA) and other structural analogues [Cannon and Eacho, 1991; Eacho et al., 1986, 1989; Foxworthy and Eacho, 1988]. The structures of some of the latter compounds are presented in
The ability of this class of compounds to elicit peroxisome proliferation with concomitant elevation of the expression of the hepatic fatty-acid-binding protein (FABP) was also reported by these workers. FABP, a 14kDa cytosolic protein binds endogenous fatty acids [Appelkvist and Dallner, 1980], including arachidonic acid and some of its metabolites. This protein is also thought to be involved in the uptake and intracellular transport of fatty acids [Peeters et al., 1989]. It is therefore plausible that the potencies of these compounds, like other peroxisome proliferators, may be related to their ability to bind to FABP, thereby displacing an endogenous ligand from its binding site (which may sequester CoA as observed by Bronfmann and other workers) thus leading to perturbation of lipid homoeostasis and hence development of the pleiotropic response of peroxisome proliferation.

The potency of some of the tetrazole-substituted acetophenone compounds (as well as other known hypolipidaemics, including clofibric acid) in eliciting peroxisome proliferation was assessed by their ability to displace a known ligand (in this case oleic acid), which bounds specifically and saturably to purified FABP [Cannon and Eacho, 1991]. Based on this information, the order of potency was: clofibric acid > LY171883 > LY189585 >> LY213768 and LY163443 (both inactive). LY163443 differs from LY189585 only in the position of the methylene tetrazole on the phenyl ring (para and meta substitutions, respectively, Figure 6.1) and this slight structural difference has a major effect on the in vivo and in vitro peroxisome-proliferating activity of these compounds. Whereas the former has strong peroxisome proliferation activity, the latter is devoid of any such activity [Eacho et al., 1989] and this difference may be attributed to the tertiary conformations of the molecules [Foxworthy et al., 1990a; Eacho et al., 1989]. However, it should be noted that members of this class of compounds eliciting peroxisome proliferation do have a non-carboxylate acidic functional group and it may be that this general structural feature (together with the energetically favourable
Figure 6.1 Chemical structures of some tetrazole-substituted acetophenone derivatives.
tertiary conformations of the molecules) is more essential for ligand binding and receptor activation. LY213768, an analogue of LY171883 in which the acidic nitrogen of the tetrazole is blocked with a methyl group, does not elicit peroxisome proliferation *in vivo and in vitro* [Eacho *et al*., 1989]. Molecular graphic plots of clofibrlic acid and LY171883 using the COSMIC package [Lake *et al*., 1988] is shown in Figure 6.2 and illustrates the structural similarities of these compounds which may well rationalise why both compounds elicit similar pleiotropic response of peroxisome proliferation.

Any consideration of the biological effects of peroxisome proliferators must take into account the structural diversity of the chemicals. The fact that many functionally diverse genes are regulated by peroxisome proliferators would indicate the existence of a receptor or receptor-related events that would explain most of the pleiotrophic responses following the administration of such compounds. The report of a peroxisome proliferator binding protein of $M_t$ 70 kDa in rat liver [Lalwani *et al*., 1983, 1987] together with the known ability of peroxisome proliferators to modulate specific gene transcription rapidly [Hardwick *et al*., 1987; Earnshaw *et al*., 1988], led Issemann and Green [1990] to suggest that peroxisome proliferators could act by a mechanism similar to that of steroid hormones. Indeed a peroxisome proliferator-activated receptor (PPAR) was recently isolated from the mouse, cloned and sequenced [Issemann and Green, 1990, 1991; Green, 1992]. This receptor constitutes a novel member of the steroid hormone receptor superfamily activated by peroxisome proliferators and contains both DNA-binding and ligand-binding domains. These workers argued that PPAR could mediate the action of peroxisome proliferators and therefore proposed that it should recognise peroxisome proliferation response element(s) (PPRE) in the 5' flanking sequence of those genes that are regulated by peroxisome proliferators. This observation prompted Tugwood and co-workers [1992] to demonstrate that PPAR does indeed recognise such PPRE located upstream of the rat acyl-CoA oxidase gene and that the presence
Figure 6.2 Molecular graphic plots of LY171883 (LY) and Clofibric acid (CL) either apart (A) or overlaid (B) to illustrate their structural similarities.
of the receptor is essential for mediating the effects of the peroxisome proliferator, Wy-14,643. The pattern of expression of this latter receptor mirrors the tissue-specific responses to peroxisome proliferators earlier reported [Nemali et al., 1988]. Coincidentally, there is increased transcription of genes required for the peroxisomal β-oxidation of long chain fatty acids [Reddy et al., 1986] as well as genes of the cytochrome P450 4A subfamily [Hardwick et al., 1987; Earnshaw et al., 1988; Kimura et al., 1989a,b; Sharma et al., 1989]. Based on the above observation on the co-induction of cytochrome P450 4A1 and peroxisome proliferation together with the ample evidence presented in this thesis (chapters 3 and 4), it may well be that cytochrome P450 4A1 induction is also mediated via this receptor in a similar manner to that suggested for rabbit kidney CYP 4A6 gene [Muerhoff et al., 1992], a concept that is not inconsistent with molecular modelling studies involving the PPAR [Lewis and Lake, 1992,]. Such modelling studies would be useful in discerning some of the more general structural features of peroxisome proliferators and should provide an insight into aspects of peroxisome proliferator binding and activation of the receptor.

The mouse PPAR consists of two domains, a DNA-binding domain of approximately 70 amino acids that interacts with responsive genes and a ligand-binding domain of approximately 200-250 amino acid residues that binds the inducer and may additionally be involved in transcriptional gene activation. It has been possible to generate computer-derived models of the ligand binding domain based on a prediction of secondary and tertiary configuration by using standard protein folding packages such as the Sybyl molecular modelling package (Tripos Associates, St Louis, MO) [Lewis and Lake, 1992]. This model identifies tyrosine residues in the helical structure which possibly ring-overlap with the ring systems in inducers such as clofibrate. It would appear that the more potent cytochrome P450 4A1 inducers and peroxisome proliferators such as methylclofenepate [Lock et al., 1989] have two phenyl rings in their structure and this apparently provides for better
binding via the aromatic rings. More crucially, the ε-amino group of a lysine residue in the ligand binding domain may play a significant role in binding the carboxyl function of peroxisome proliferators containing this moiety (or may be metabolized to one or contain a chemical grouping which is bioisosteric to this functional group). This feature was present in all the peroxisome proliferators investigated in my studies (see Figures 1.3 and 3.1 earlier). A general inspection of the computer-modelled PPAR ligand-binding domain reveals a likely binding site containing amino acid residues in the appropriate orientation and complementary with key groups of known peroxisome proliferators [Lewis and Lake, 1992]. Therefore, it would appear as if the carboxyl group requirement for both cytochrome P450 4A1 induction (and co-induction of peroxisomal fatty acid β-oxidation) may well be rationalised by the above interactions with basic amino acid residues in the PPAR.

My results from studies on the inductive potencies of the S(+) and R(-) isomers of a structural analogue of clofibrate on cytochrome P450 4A1 and peroxisome proliferation (Chapter 3) demonstrated that the R(-)-isomer was a more potent inducer of both cytochrome P450 4A1 and peroxisome proliferation than the corresponding S(+) -isomer at the dose level administered. The fact that there was a notable stereoselectivity in induction implies that it is reasonably likely that the induction processes are probably receptor-mediated. More support to this hypothesis is derived from the fact that the more potent R(-)-isomer interacts with the PPAR much better than its antipode (Figure 6.3), as reflected in the ΔG values of -16.9 kcal mol⁻¹ and -12.0 kcal mol⁻¹, respectively. Additionally, difference in potency elicited by these isomers may be linked with the rate of formation of the corresponding acyl-CoA thioesters which in turn may be linked to the phenomenon of signal transduction via interaction with pkC [Bronfman et al., 1989]. It is conceivable that the most favoured configuration adopted by the R(-)-isomer would have a higher rate of formation of the acyl-CoA thioester than its corresponding
Figure 6.3 Possible interaction of the S(+)-{A,purple} and R(-)-{B,green} isomers of (2-[4-(4-chlorophenyl)benzyloxy]-2-phenylacetic acid with the peroxisome proliferator-activated receptor (critical residues in binding site are shown in yellow).
antipode thus, underscoring the pivotal role of the acyl-CoA synthetase(s) in mediating this process. Differences in peroxisome proliferation potency by stereoisomers of 2-arylpropionic acid and derivatives have often been associated with chiral inversion of one of the enantiomers and usually mediated via formation of the thioester intermediate [Porubek et al., 1991]. Similarly, the degree to which a particular 2-arylpropionate inhibits the β-oxidation of fatty acids may be governed by the efficiency with which it is transformed to its CoA thioester [Freneaux et al., 1990]. Work would have to be carried out with authentic specimens of the CoA conjugates of these drugs to substantiate the implication of thioesters as key intermediates in this process.

In chapter 4 we also demonstrated that the phenomenon of cytochrome P450 4A1 induction by the perfluorocarboxylic acid, PFDA, in susceptible species such as the rat was invariably associated with peroxisome proliferation. The interaction of this compound with the modelled binding site of PPAR is depicted in Figure 6.4. Its theoretically calculated free energy of interaction with the PPAR (ΔG) was evaluated at -15.3kcal mol⁻¹ (compared to -28.4kcal mol⁻¹ for clofibrate). It is generally accepted that there is a complementarity between a ligand and its receptor site and this comprises a mutual molding of the ligand and receptor macromolecule to take maximum advantage of steric and electronic effects. Under optimal conditions, the energies liberated in binding (ΔG) may be indicative of the affinity of the ligand to the receptor which may also be related to the biological potency of the peroxisome proliferator in question.

It was intriguing, however, that PFDA (20mg/kg, single i.p. dose for 7 days) and clofibrate (250mg/kg, daily for three days) showed about the same biological potency (in inducing cytochrome P450 4A1-mediated fatty acid hydroxylase activity and other enzymes of the peroxisome proliferation domain) and yet their calculated ΔG values were significantly different. This discrepancy may be explained as a result of some of the assumptions made in their calculations. First of all hydrogen
Figure 6.4 Postulated interaction of perfluoro-n-decanoic acid (green) and the peroxisome proliferator-activated receptor (critical residues in the binding site are shown in yellow).
atoms were excluded from the modelled binding site of the PPAR molecule as well as the ligand to simplify the calculations and because of their relatively negligible mass effect. However, in the case of PFDA, fluorine atoms were present (surrogate atoms for hydrogen) and these were considered in ΔG calculations. Moreover, none of the calculated ΔG values fully took into account the solvation energies due to entropy changes as a result of receptor-ligand interaction. This aspect may be well appreciated when one considers the likely high solvation energy of the fluorinated compound due to the presence of extensive hydrogen bonding, which would probably give rise to large entropy changes during binding.

A scheme proposed to explain the relationship between cytochrome P450 4A1 and peroxisome proliferation requires that cytochrome P450 4A1 induction precedes that of peroxisosmal β-oxidation enzymes [Sharma et al., 1988a; Lock et al., 1989]. This scheme proposes that peroxisome proliferators are taken up by the dual mechanism of inhibition of carnitine acyl-transferase in the mitochondrion [Lock et al., 1989] or sequestration of essential CoA by the peroxisome proliferator itself, the result possibly being the accumulation, within the liver cell, of medium and long-chain fatty acids. These preceding events are then judged to be responsible for triggering the induction of cytochrome P450 4A1-fatty acid hydroxylase, in an attempt to maintain cellular lipid homeostasis. The latter enzyme then enhances the formation of long-chain dicarboxylic acids [Sharma et al., 1988a]. Because the resulting long-chain dicarboxylic acids are the preferred substrate for peroxisosmal β-oxidation system, it is proposed that this leads to induction of other elements of the peroxisome proliferator domain, in an attempt to cope with the substrate overload and hence the observed pleiotropic response in peroxisome proliferation.

By contrast, the above sequential mechanism may not be dependent on cytochrome P450 4A1 induction of peroxisosmal β-oxidation. As depicted in Figure 6.5, an alternative regulatory scheme is possible whereby the commonality is related to structurally similar regulatory elements in the 5’ upstream flanking regions of the
cytochrome P450 4A1 and acyl-CoA oxidase genes. Thus the peroxisomal proliferator may directly interact with the common regulatory sequences or indirectly modulate gene expression by lipids derived as a direct result of inhibition of mitochondrial fatty acid β-oxidation [Elcombe et al., 1986; Eacho and Foxworthy, 1988] or derived from lipids displaced from the fatty acid binding proteins [Cannon and Eacho, 1991]. This commonality of gene expression may also rationalise why so many proteins are induced by peroxisome proliferators and the so-called peroxisome proliferator domain may simple reflect the existence of common regulatory elements in what would appear to be functionally unconnected proteins. Although the regulatory elements of the CYP 4A1 gene have yet to be characterised, the corresponding regions of the acyl-CoA oxidase gene are beginning to be elucidated [Osumi et al., 1991] and the preliminary indications are of a complex pattern of regulation.

Figure 6.5  A scheme postulating a common regulatory mechanism of the cytochrome P450 4A1 and the peroxisomal β-oxidation spiral acyl-CoA oxidase genes.
Thus, the scheme shown in Figure 6.5 may serve as a useful paradigm for further experimentation in both the acyl-CoA oxidase and CYP P450 4A1 genes. In addition the above scheme may rationalise why there are so many structurally diverse inducers of cytochrome P450 4A1 and acyl-CoA oxidase, in that the peroxisome proliferators may not all directly interact with the inducible genes but indirectly act by influencing lipid disposition or a common perturbation of lipid biotransformation. However, it remains uncertain if peroxisomal β-oxidation crucially depends on cytochrome P450 4A1 induction as described previously [Sharma et al., 1988a] or if the above commonality of regulation of gene expression plays a more important role. It is hoped that the isolation and characterisation of the 5' flanking regulatory sequences in the CYP 4A1 gene will identify the putative regulatory elements which could then be compared to the equivalent regions in the better characterised acyl-CoA oxidase gene. It is worth mentioning a recent report which indicated sequence similarities between the 5' flanking regulatory sequences in rabbit CYP 4A6 and the corresponding region in the acyl-CoA oxidase gene [Muerhoff, 1992, personal communication].

It is plausible that accumulation of metabolites resulting from lipid perturbation could be the natural constitutive ligand(s) for cytochrome P450 4A1 and the acyl-CoA oxidase genes. In general, natural ligands or physiologically occurring activators have been identified for only a few of the steroid hormone receptors [Evans, 1988]. It is also possible that the species differences in response to PFDA seen in the rat and guinea pig (Chapter 4) may be related to either differences in lipid disposition and biotransformation or differential expression of PPAR or even a combination of these factors. Transactivation studies using chimeric proteins consisting of a cDNA encoding the putative ligand-binding and the amino-terminal and DNA-binding domains of known steroid hormone receptors such as the PPARs have been used to demonstrate their activation by peroxisome proliferators and some intermediates in lipid metabolism [Issemann and Green, 1990; Göttlicher et al.,
Screening compounds related to lipid metabolism revealed that fatty acids such as linoleic (C\textsubscript{18:2}), arachidonic (C\textsubscript{20:4}), or lauric acid (C\textsubscript{12:0}), but not dehydroepiandrosterone (DHEA), cholesterol or its 25-hydroxy-metabolite, activated the reporter gene expressing the chimeric receptor [Göttlicher et al., 1992]. The findings of these workers, in principle, demonstrates that fatty acids can regulate gene expression by activating a member of the nuclear receptor superfamily.

6.3 Multiplicity of PPAR expression and its biological implications

If peroxisome proliferation is mediated by PPAR, then such species differences, as reported in this thesis and by many other researchers, could reflect either variation in PPAR or in the gene networks that are regulated by PPAR. Support for such possibilities comes from the cloning of PPARs from species such as the mouse [Issemann and Green, 1990; Green, 1992], rat [Göttlicher et al., 1992] and Xenopus laevis [Dreyer et al., 1992] which may account for differences in response to a peroxisome proliferator across species. Differential response within the same species by structurally diverse peroxisome proliferators may also suggest multiplicity in PPAR expression as shown by the cloning of three PPARs from Xenopus laevis [Dreyer et al., 1992]. This discovery raises a number of pertinent questions such as: does each of these PPARs exert a specific function, and are there different PPAR signalling pathways within the cell? If these receptors have the same DNA recognition specificity, then they would be expected to recognise similar elements in the acyl-CoA promoter gene and this appears to be so. It would also be interesting to see if there is any differences in ligand specificity of these PPARs when assessed by their response to well known peroxisome proliferators with differing potencies.
Taken collectively, the information presented in this thesis supports a model where PPAR(s) plays a key role in mediating the action of peroxisome proliferators and it is tempting to speculate that this receptor(s), the peroxisome proliferator-inducible and fatty acid-metabolizing cytochrome P450 4A subfamily together with the peroxisomal β-oxidation pathway might constitute an autoregulatory loop in lipid or fatty acid homoeostasis [Elcombe and Mitchel, 1986]. Given both the key role of acyl-CoA oxidase in the β-oxidation spiral and its peroxisome proliferator-dependent stimulation, activated PPARs may represent an essential and obligatory step in the action of diverse peroxisome proliferators. Furthermore, examination of the expression and function of PPAR in humans could have important implications in assessing the hazard that peroxisome proliferators may pose to humans. To this end, it is worth mentioning a transfection experiment carried out to identify putative regulatory DNA elements within the isolated human 4A gene [Hood et al., 1992] in the absence/presence of mouse PPAR. Preliminary results suggested that there is probably no peroxisome proliferator responsive element to PPAR in the first 6kb of 5′ flanking sequence of the human CYP 4A gene [S. Hood, personal communication].
6.4 Suggestions for future work

1) It has previously been suggested that the acyl-CoA thioesters of most peroxisome proliferators, which are formed both *in vivo* and *in vitro*, might be the pharmacologically active species of these compounds in eliciting the observed pleiotropic responses. This is apparently because several of these compounds potentiate the activity of protein kinase C, a key enzyme involved in cellular differentiation and carcinogenesis. I demonstrated in Chapter 3 that there was differential inducing potencies of two enantiomers of a clofibrate structural analogue which may be related to the rate of formation of their corresponding thioester conjugates. It would therefore be interesting to investigate the effect of administering authentic specimens of the CoA conjugates of these drugs on signal transduction mechanisms.

2) My results in Chapter 4 suggested PFDA exhibits a similar potency to clofibrate induced rats. Clofibrate has also been shown to activate a chimera of the rat and mouse PPARs. It would therefore be interesting to see if a similar pattern of result is obtained when repeated with PFDA. The information so-derived would help in the overall classification of PFDA and other structurally-related analogues in relation to other well-characterised peroxisome proliferators. Obviously this information is necessary for evaluation of the ultimate risks posed by using such chemicals which have a valuable input in the aerospace and many other chemical industries.

3) It would also be of interest if the peroxisome proliferator-activated receptor could be isolated, purified and used in binding studies with various ligands or peroxisome proliferators.

4) Finally, the results presented in Chapter 5 suggest a close similarity between the rat cytochrome P450 4A1 and the corresponding human orthologue. This issue
could be better addressed by isolation and sequence analysis of cDNA clones coding for human cytochrome P450 4A1 and comparing it to the well characterised rat form. This should provide a vital contribution to the understanding of the structure, diversity and risk assessment of peroxisome proliferators on humans. Similarly catalytic activities associated with the human orthologous enzyme could be better assessed by the ultimate isolation and purification of the human liver cytochrome P450 4A1 which will be useful in reconstitution studies.
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