INDUCTION OF MICROSONAL AND PEROXISOMAL 
FATTY ACID OXIDATION BY CHLOROPHENOXO ACID HERBICIDES

A thesis presented for the degree of Philosophy

By

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SUMMARY

Induction of the cytochrome P-450 mixed-function oxidase and specifically the cytochrome P-450 IVA1 isoenzyme by seven phenoxy acid herbicides in rat liver and kidney, have been studied. My results using liver microsomes demonstrated that the 12-hydroxylation of lauric acid was significantly induced by all compounds (3 - 8-fold), 4-chlorophenoxyacetic acid (CPA) (300 mg/kg) being the weakest and 2,4,5-trichlorophenoxypropionic acid (2,4,5-TP) (200 mg/kg) the most potent inducers respectively. This increase in lauric acid 12-hydroxylase-activity was accompanied by an increase in the hepatic content of cytochrome P-450 IVA1 as assessed by both a qualitative Western blot procedure and a quantitative ELISA method. Furthermore, there was a parallel increase in cytochrome P-450 IVA1 mRNA and a similar increase in peroxisomal \( \beta \)-oxidation subsequent to exposure to these compounds. In addition, benzphetamine-N-demethylase, a marker of cytochrome P-450 IIB1 and IIB2 activities, was not affected by any of the herbicides, whereas cytochrome P-450 IA1 and IA2, as assayed by ethoxyresorufin-O-deethylase activity, was significantly increased (up to 2.2-fold) by some of the compounds. Kidney microsomal parameters were not affected by any of these compounds.

My in vivo studies using antipyrine, pentobarbital and zoxazolamine indicated that the metabolism of these substrates was marginally affected by only some of the compounds.
In order to highlight the possible involvement of a metabolite of the chlorophenoxy acids in the induction of cytochrome P-450, I investigated four related chlorophenols. There was no significant change in cytochrome P-450 isoenzyme levels in rat liver and kidney microsomes nor was there any increase in peroxisomal β-oxidation.

Taken collectively, the results presented in this thesis indicate that the chlorinated phenoxy acid herbicides studied preferentially induce the cytochrome P-450 IVA1 isoenzyme and peroxisomal β-oxidation in a pattern similar to the typical inducers of this isoenzyme such as clofibrate. A scheme is presented whereby induction of catalytically competent cytochrome P-450 IVA1 is required for the phenomenon of peroxisome proliferation by these chlorophenoxy acid derivatives.
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CHAPTER ONE

INTRODUCTION
Enzyme induction plays an important role in normal development and differentiation of the cell; in the regulation of essential metabolic pathways, in the cellular action of various hormones and in the response of mammals to drugs and other lipophilic xenobiotics (Gelehrter, 1976). It is now many years since Brown et al (1954) noticed the stimulatory effect of foreign compounds on liver microsomal enzymes, in particular the dietary factors that influence the activity of hepatic aminoazo dye-N-demethylase.

Further studies revealed that administration of small doses of polycyclic aromatic hydrocarbons, such as 3-methylcholanthrene, increased several-fold the activities of the liver microsomal enzymes which are able to oxidise and reduce a variety of functional groups in drugs and other xenobiotics (Conney, 1967). Furthermore, it was found that the activity of enzymes which catalyze the covalent binding of aminoazo dyes to proteins, was also enhanced by co-administration of polyaromatic hydrocarbons. Barbiturates, among other drugs, was discovered to stimulate liver microsomal drug metabolism (Lu and West, 1980). These early observations inspired an intense research effort over the ensuing years into the nature of the enzyme(s) induced, the molecular characteristic of enzyme-inducing agents and the molecular mechanisms of enzyme induction.
Drugs are metabolised by a number of enzyme systems which, collectively appear to have the ability to interact with an unlimited number of organic chemicals. Studies in animals, demonstrating sex and strain differences and genetic polymorphism, illustrate the ever-increasing awareness of the complexities of cytochrome P-450 enzymes, and other enzymes involved in reduction, hydrolysis and conjugation of endogenous and exogenous compounds (Lu and West, 1980). Many different forms of cytochrome P-450 have now been isolated and characterised from many animal species and man. Most of these forms have been isolated from animals treated with inducing agents such as phenobarbitone, and 3-methylcholanthrene (Nebert et al., 1987), and each of these agents induces several forms of cytochrome P-450.

1.1 HEPATOMEGALY

Hepatomegaly is liver enlargement measured as an increase in the liver-to-body weight ratio, and it appears to be a characteristic response in laboratory animals exposed to a variety of xenobiotics (Reddy and Lalwani, 1983). This enlargement may be associated with an increase in the number and size of peroxisomes, and in the accumulation of lipid droplets (Hawkins et al., 1987). The administration of hypolipidaemic agents and chlorinated phenoxyacids to rodents is predictably associated with hepatomegaly, the extent of which is dependent upon the agent involved (Lake et al. 1975, Reddy 1980, Kawashima et al. 1983 and 1984a & b, Fournel et al. 1985). The nature of the hepatomegalic effect produced by clofibrate in the rat was initially studied by Hess et al., (1965).
A daily oral dose for a period of 14 days resulted in a significant enlargement of the liver. The relative weight increased rapidly during the first week and fell to permanent levels after withdrawal of clofibrate at 22-30 days. Liver enlargement has been observed in both male and female rats, and this has been attributed, in part, to an increase in phospholipid and total protein levels (Azarnoff et al., 1965). An associated increase in microsomal and cell sap protein concentration has also been noted (Platt and Cockrill, 1969).

It has been established that the liver enlargement induced by clofibrate and nafenopin is the result of hypertrophy and hyperplasia, which may be dependent on the dose and duration of treatment (Beckett et al., 1972). A marked increase in cell size was observed in centrilobular hepatocytes and the increase in cell number correlated with increases in hepatic DNA content in nafenopin-treated animals (Beckett et al., 1972). The nafenopin-induced hepatomegaly may be attributed to hypertrophy associated with a marked proliferation of the smooth endoplasmic reticulum and peroxisomes (Reddy et al., 1973). Chronic administration of nefenopin and clofibrate results in an accumulation of protein, phospholipids and RNA, with the absence of cholesterol or triglycerides, indicating an adaptive hepatomegaly opposed to a pathological condition associated with fat droplets and high cholesterol and/or triglycerides levels (Dalton et al., 1974).
Wy-14,643-induced liver enlargement is attributed to hypertrophy and hyperplasia (Reddy et al., 1979). Short-term administration of this compound stimulates DNA replication and cell division. Administration of bezafibrate for 5 days causes a marked hepatic enlargement associated with an alteration of the mixed-function oxidase system (Facino and Carini, 1981). Chronic administration of fenofibrate, methyl clofenapate, and fibric acid results in a dose-dependent increase in liver size of a comparable magnitude markedly greater than the clofibrate-treated group (Orton and Parker, 1982).

2,4,5-T treatment of rats produced hepatomegaly at lesser magnitude than that produced by clofibrate (Kawashima et al., 1984a) whereas 2,4-D treatment did not cause hepatomegaly, linking the hepatomegaly-induced to the induction of peroxisomal B-oxidation enzymes by clofibrate and 2,4,5-T.

It would appear that administration of hypolipidaemic agents characteristically results in a hepatomegaly of a hypertropic and hyperplastic nature. Hypertrophy may be associated with a combined proliferation of the smooth endoplasmic reticulum and peroxisomes, and in some instances a contributory increase in mitochondrial number (Hess et al., 1965). However, induction of peroxisomal B-oxidation dissociated from hepatomegaly and peroxisome proliferation has been reported with bezafibrate, indicative that hepatomegaly is dose-dependent and may or may not reflect ultrastructural changes (Lazarow et al., 1982).
1.2 Cytochrome P-450 IA1 and 2 induction

As a result of intensive studies on the metabolic fate of xenobiotics in animals, it is now recognised that many chemical carcinogens are not active per se, but require biotransformation to reactive species, which are ultimately recognisable for the initiation of tumorigenic processes. The membrane bound cytochrome P-450 system has been shown to play a central role in the activation of such potent carcinogens as hydrazine derivatives, nitrosamines, aromatic amines, and polycyclic aromatic hydrocarbons (for review see Miller 1978, Guengerich et al., 1985, Parke 1987).

Arylamine activation is postulated to be catalysed most efficiently in rats by hepatic cytochrome P-450 IA1 and IA2 isoenzymes and their orthologues in other tissues and species; Thomas et al., 1982, Masson et al., 1983, Yamazoe et al., 1984.

Several forms of cytochrome P-450 have been purified from rat liver microsomes (for review see Lu and West 1980, Guengerich 1987, Nebert 1987) and can now be quantified individually using specific antibodies. Using this approach it has been shown that a number of xenobiotics each induce several different isoenzymes of cytochrome P-450. For example, treatment of animals with 2,3,7,8-tetrachlorodibenzo-P-dioxin (TCDD), 3-methylcholanthrene, isosafrole or certain polyhalogenated biphenyls increases the level of both cytochromes P-450 IA 1 and 2 (c and d), but to different extents (Thomas et al 1983, Parkinson et al 1983, Dannan et al 1983). Although the molecular mechanism of induction is still far from
clear, the induction of cytochrome P-450 IA1 seems to be mediated by a cytoplasmic receptor with high affinity for TCDD, 3-methylcholanthrene, α-naphthoflavone and some polychlorinated biphenyls, all bind to the same receptor with different affinities (Bandira et al 1982, Poland and Glover 1976). Thus, it is generally accepted today that TCDD is a true inducer of some drug-metabolising enzymes.

It is now well-known that many toxic chemicals are metabolically converted into reactive species by the action of microsomal mixed-function oxidases (Ioannides and Parke, 1980). This enzyme system requires oxygen and reduced NADPH and comprises cytochrome P-450 reductase and the haemoprotein cytochrome P-450 acting as the terminal oxygenase (Cooper et al, 1965; Phillipson et al 1982). Cytochrome P-450 IA is preferentially induced by many carcinogens and is also involved in their activation (Ioannides et al 1981, 1984), as well as in the activation of certain drugs such as paracetamol to reactive metabolites responsible for their toxicity (Ioannides et al 1983, Steel et al 1983). The activity of cytochrome P-450 IA1 may be determined using substrate and reactions which are exclusively catalysed by this form of cytochrome, such as 2-hydroxylation of biphenyl, the O-deethylation of ethoxyresorufin,EROD, (Burke and Mayer 1975) and the activation of benzo[a]pyrene to mutagens (Ioannides et al 1981, Phillipson 1985). An excellent direct correlation has been observed between EROD and metabolic activation of benzo[a]pyrene to mutagens.
The possibility of the presence of trace amounts of TCDD and similar compounds in the chlorinated phenoxy acids cannot be ruled out since this compound can be formed from 2,4,5-TCP or similar phenolic compounds. During the past three decades, halogenated aromatic hydrocarbons such as polychlorinated biphenyls and dioxins have generated intense public and scientific concern because of their widespread occurrence as environmental contaminants, their resistance to degradation, and their biological potency. The prototypical halogenated aromatic hydrocarbon, TCDD, produces a diverse set of responses in experimental animals, inducing a wasting syndrome, immunological alteration, teratogenic effects, tumor promotion, epithelial hyperplasia/metaplasia, and the induction of several drug metabolising enzymes (Poland and Knutson, 1982). In many cell types, TCDD induces the activity of aryl hydrocarbon hydroxylase (AHH) many times greater than that of 3MC and \( \beta \)-naphthoflavone (Poland and Glover, 1976), which is primarily catalysed by an isoenzyme of cytochrome P-450, cytochrome P-450 IA1 in rat (Whitlock, 1986). The AHH system is responsible for the metabolic activities and detoxification of polycyclic aromatic hydrocarbons (Gelboin, 1980) and TCDD is the best inducer of AHH activity. TCDD induces the AHH activity by increasing the rate of transcription of the cytochrome P-450 IA1 gene (Israel and Whitlock 1984), by means of binding to a receptor with properties similar to the steroid receptor (Wilhelmsson et al 1986) and differ in their ligand binding domain, because steroids do not bind TCDD receptors, nor does TCDD bind to steroid receptors (Poellinger et al 1983). The binding of TCDD to the receptor results in the formation of
TCDD-receptor complex (Israel and Whitlock, 1989) which is a DNA-binding protein (Hannah et al 1986., Durrin et al 1987).

1.3 Cytochrome P-450 IVA1 induction

Recent work from this laboratory has shown that pre-treatment of rats with the hypolipidaemic drug clofibrate resulted in a significant increase in the w-and (w-1)-hydroxylation of lauric acid - the former pathway being preferentially induced (Gibson et al 1982). Not only does clofibrate pretreatment increase fatty acid oxidation, but a specific isoenzyme of cytochrome P-450 (P-450 IVA1) is induced by this hypolipidaemic agent. Cytochrome P-450 IVA1 has been subsequently purified to electrophoretic homogeneity from clofibrate-treated rat liver and the protein biochemistry of the enzyme described in detail (Tamburini et al 1984). Comparison of the highly purified cytochrome P-450 IVA1 with other drug-induced cytochrome P-450s (phenobarbital and B-naphthoflavone) has uniquivocally shown that cytochrome P-450 IVA1 has unique properties, one of which is high substrate specificity for the w-oxidation of lauric acid (Tamburini et al 1984, Bains et al 1985). a specificity not usually observed in the cytochrome P-450 group of hepatic enzymes. Furthermore, a specific polyclonal antibody raised against clofibrate-induced purified cytochrome P-450 IVA1 in sheep recognised its homologous antigen but did not cross-react with purified cytochrome P-450 isoenzymes induced by either PB or B-naphthoflavone (Tamburini et al 1984). These antibodies recognised a single protein of the correct molecular weight (51,500) for cytochrome P-450 IVA1 in Western blot procedure (Sharma et al 1988a)
and gave one reactive spot in 2-D electrophoresis, viz. electrofocusing followed by SDS-page (Gibson, G.G. personal communication). It should be noted that the precise mechanism of cytochrome P-450 IVA1 induction still remains speculative in that a cytosolic receptor mediating induction has been proposed (Lalwani et al 1983). A clofibrate-binding protein or receptor of molecular weight of 70000 was purified with very low affinity towards clofibrate (Lalwani et al., 1983, 1987).

Recently Milton et al (1988) demonstrated the lack of detectable specific binding of hypolipidaemic drugs to hepatic homogenates (receptor) and therefore cast considerable doubt on the existence of such a receptor. They indicated that the peroxisome proliferating hypolipidaemic drugs bind to serum albumin and possibly to other cellular proteins not involved in the activation of genes necessary for peroxisome proliferation. These findings support a previous result (Chatterjee et al 1987) that concluded, using a cDNA probe to the peroxisomal bifunctional β-oxidation enzyme, that proliferation is inconsistent with a receptor-mediated mechanism. Whether hypolipidaemic agents, such as clofibrate, act via a cytosolic receptor or directly influence cytochrome P-450 IVA1 gene regulation is still debatable, but it is abundantly clear that this class of compounds regulate the transcriptional activation of the cytochrome P-450 IVA1 gene (Hardwick et al 1987, Gonzalez 1989).

As reviewed by Reddy and Lalwani (1983) and Hawkins et al., (1987) clofibrate and related drugs produce three characteristic
liver changes in rodents; namely, proliferation of the endoplasmic reticulum, peroxisomal proliferation and hepatocellular carcinomas on chronic exposure. All the evidence indicates a coupling between the proliferation of peroxisomes and the endoplasmic reticulum (in particular the induction of cytochrome P-450 IVA1) with high correlation between some of the enzymes involved (Sharma et al 1988 a & b; Gibson and Sharma 1988).

Sharma et al (1988a) presented a scheme (Fig.1,1) whereby the hypolipidaemic agent (such as clofibrate) is taken up by the hepatocytes and induces cytochrome P-450 IVA1 synthesis. This induction of cytochrome P-450 IVA1 results in the increased w-hydroxylation of fatty acids (medium and long-chain) and is further metabolised through the cytosolic oxidation to long-chain dicarboxylic acids. These latter compounds are then taken up by the peroxisome thus presenting this organelle with a substrate overload of one of its preferred substrate (Singh et al 1984), since the mitochondria cannot readily metabolise long-chain fatty acids, (Alexon and Cannon 1984).

Bains et al (1985) demonstrated the presence of cytochrome P-450 IVA1 in uninduced rat liver microsomes at a level which can be elevated several fold as a result of clofibrate treatment. It has also been established that the profile of arachidonic acid metabolites from clofibrate-treated rats is markedly different to that of control animals. Furthermore, Capdevila et al (1985) demonstrated that pre-treatment of rats with the fibric acid type
Figure 1.1

Possible Mechanisms Leading to the Induction of Microsomal and Peroxisomal Enzymes in the Rat

HEPATO CYTE

Hypolipidaemic Agent

Induction

↑ P-452

e.r.

↑ Medium and Long Chain Fatty Acids

Induction

Hepatic Fatty Acids

w-OH

Fatty Acids

Cytosolic Oxidation

↑ (medium and long chain) Dicarboxylic Acids

↑ Lipolysis

Diabetes/Starvation

↑ Medium and Long Chain Fatty Acids

↑ β-Oxidation

Peroxisome

↑ β-Oxidation

Fatty Acids

Further β-Oxidation

Excretion of Short Chain Fatty Acids

reproduced from Sharma et al. (1988).
hypolipidaemic drug, ciprofibrate, results in a 7-fold stimulation of \( \omega \)-and \( (\omega-1) \)-hydroxylation of arachidonic acid. These results suggest that the main function of cytochrome P-450 IVA1 is in the hepatic metabolism of endogenous substrates, namely fatty acids—for example, arachidonic acid. The use of lauric acid in the study of cytochrome P-450 IVA1 is because of its availability, stability and the limited resulting metabolites.

The metabolism of arachidonic acid (AA) by rat hepatic microsomes in the presence of NADPH and oxygen was reported by Capdevila et al. (1981). Studies using various inhibitors indicated that metabolism of arachidonic acid to hydroxy derivatives was by a lipoxygenase-like function of the membrane bound cytochrome P-450. The involvement of cytochrome P-450 in the metabolism of AA to a variety of products including 19- and 20-hydroxy derivatives, has been established (Oliw and Oates, 1981). Further investigation led to the identification of several new metabolites from hepatic microsomal metabolism of AA including four novel epoxy acid derivatives (Chacos et al. 1982), hydroxyicosatetraenoic acids (HETES), (Capdevila et al 1982), four epoxide intermediates (Oliw et al 1982) and epoxyeicosatrienoic acids (Chacos et al 1983a). It has been demonstrated that the hepatic metabolism of docosahexaenoic acid, presumably by the cytochrome-P-450 mono-oxygenases, results in the formation of 19,20-, 16, 17, 13, 14-, 10, 11- and 7,8-dihydroxydocosapentaenoic acids, 22-hydroxydocosahexaenoic acid and 21-hydroxydocosahexanoic acid (Van Rollins et al 1984). In addition 5- and 8-HETE metabolites of arachidonic acid have been isolated.
(Falck et al 1984). The metabolism of leukotriene B4 by rat liver microsomes in the presence of oxygen and NADPH and in a reconstituted system with cytochrome P-450 LM2, has been reported (Bosterling and Trudill 1983). The metabolism of arachidonic acid and the formation of, and biological actions of epoxxygenase-derived eicosanoids has been reviewed recently by Fitzpatrick and Murphy (1989).

The involvement of cytochrome P-450 IVA1 in the omega-hydroxylation of saturated fatty acids has been established and it has been unequivocally demonstrated with the same isoenzyme hydroxylates arachidonic acid (Bains et al., 1985). It is plausible that the isoenzyme cytochrome P-450 IVA1 with omega-hydroxylase activity is capable on the basis of broad substrate specificity of metabolising other endogenous substrates such as saturated and unsaturated medium to long-chain fatty acids. The elucidation of the precise substrate specificity is of fundamental importance in clarifying the physiological role of this induced haemoprotein.
1.4 Peroxisome proliferation and associated enzymes

Two structured classes of hypolipidaemic agents have been identified as inducers of peroxisomal proliferators: clofibrate and its structural analogous (fig.1.2) and compounds structurally unrelated to clofibrate such as the phthalate ester plasticisers. In this section, the chlorinated phenoxyacid herbicides (fig.1.3), structurally related to clofibrate, will be considered with clofibrate and other inducers of peroxisomal proliferation.

There are a large number of lipid classes, each having a unique anabolic and catabolic pathway. Since fatty acids are the structural components of the simple and complex lipids, I will discuss only certain aspects of their catabolism with particular emphasis on the $\beta$-oxidation of medium and long chain fatty acids. Fatty acids are found mostly esterified, e.g. acylglycerols and phosphoacylglycerols. Once released from the ester linkage by the action of lipases, the free fatty acids can be further degraded to provide carbon and/or energy or be re-utilized in the biosynthesis of various lipids.

At the turn of the century, Knoop (1905) established that the aliphatic hydrocarbon chains of fatty acids are degraded by the sequential removal of two carbon units ($CH_3COO^-$), proceeding from the carboxyl end of the molecule. This is termed the process of $\beta$-oxidation, signifying that each round of chemistry involves the oxidation of the $\beta$ carbon prior to bond cleavage between C-\(\beta\) at the C-C-COO terminals.
**Figure 1.2 Hypolipidaemic Agents**

Clofibrate and its Structural Analogues

- **Clofibrate**
  \[
  \text{Cl} - \overset{-\text{CH}_3}{\circlearrowright} - \overset{\text{COOH}}{\text{C}} - \text{COOC}_2\text{H}_5
  \]

- **Bezafibrate**
  \[
  \text{Cl} - \overset{-\text{CH}_3}{\circlearrowright} - \overset{\text{COOH}}{\text{C}} - \text{NHCH}_2\text{CH}_2 - \overset{-\text{CH}_3}{\circlearrowright} - \overset{\text{COOH}}{\text{C}}
  \]

- **Ciprofibrate**
  \[
  \text{Cl} - \overset{-\text{CH}_3}{\circlearrowright} - \overset{\text{COOH}}{\text{C}}
  \]

- **Clobuzarit**
  \[
  \text{Cl} - \overset{-\text{CH}_3}{\circlearrowright} - \overset{\text{COOH}}{\text{C}} - \text{CH}_2\text{O}
  \]

- **Fenofibrate**
  \[
  \text{Cl} - \overset{-\text{CH}_2\text{CH}_2\text{CH}_2}{\circlearrowright} - \overset{\text{COOH}}{\text{C}} - \text{CH}_3
  \]

- **Gemfibrozil**
  \[
  \text{CH}_3 - \overset{-\text{CH}_2\text{CH}_2\text{CH}_2}{\circlearrowright} - \overset{\text{COOH}}{\text{C}} - \text{CH}_3
  \]

- **Methyl Clofenapate**
  \[
  \text{CH}_3 - \overset{-\text{COOH}}{\text{C}} - \text{CH}_2\text{O}
  \]

- **Nafenopin**
  \[
  \text{CH}_3 - \overset{\text{COOH}}{\text{C}}
  \]

- **SaH-42,348**
  \[
  \text{Cl} - \overset{-\text{OH}}{\circlearrowright} - \overset{\text{COOH}}{\text{C}} - \text{CH}_2\text{O}
  \]
Figure 1.3 Chemical structure of phenoxy acids.

- 4-Chlorophenoxyacetic acid (CPA)
- 2,4-Dichlorophenoxyacetic acid (2,4-D)
- 2,4-Dichlorophenoxypropionic acid (2,4-DP)
- 2,4-Dichlorophenoxybutyric acid (2,4-DB)
- 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)
- 2,4,5-Trichlorophenoxypropionic acid (2,4,5-TP)
- 4-Chloro-2-methylyphenoxyacetic acid (MCPA)
The biochemical details of the fatty acid β-oxidation process, involving intermediates, sequence of conversion, enzymes and coenzymes were unravelled about fifty years later.

The conversion of saturated fatty acids to mono-unsaturated fatty acids is catalysed by a microsomal desaturation system, composed of reduced (NADH)-cytochrome b5 reductase, cytochrome b5 and a terminal desaturase enzyme (cyanide-sensitive factor Shimakata et al 1972). For example, the terminal desaturase catalyses the conversion of stearoyl-CoA to oleoyl-CoA. The activity of stearoyl-CoA desaturase in rat liver is increased considerably by clofibric acid, 2-(4-chlorophenoxy)-propionic acid and 2-(4-chlorophenoxy)-2-methyl-propionic acid (Kawashima et al 1984b). Moreover, 2,4,5-T also increased the desaturase activity, although the inducing potency was very weak compared to that of clofibric acid. 2,4-D, CPA, 2-(phenoxy)-propionic acid and 2-chlorophenoxyacetic acid only minimally induced the desaturase activity and had no influence on NADH-cytochrome b5 reductase activity, cytochrome b5 content and terminal desaturase activity. This supports previous results suggesting that peroxisomal proliferators, regardless of their
structure, induce the fatty acid desaturase activity (Kawashima et al 1983).

Clofibric acid increased the activity of stearoyl-CoA desaturation in hepatic microsomes and in vivo (Kawashima and Kozuka 1982). This increase was due to the increase in the activity of terminal desaturase as measured by the rate constant for cytochrome b₅ reoxidation, but not due to changes in cytochrome b₅ content or NADH-cytochrome b₅ reductase activity.

Peroxisomes contain many enzymes including oxidases, dehydrogenases, aminotransferases, reductases, hydrolases, catalase and the enzymes of fatty acid β-oxidation and the glycolate cycle and have been identified in a variety of plant and animal tissues. Since the predominant reactions in peroxisomes are oxidative, the function of peroxisomes is geared toward the catabolic pathway, although some products are used as precursors in cellular anabolic processes.

The β-oxidation of fatty acids and bile acid synthesis also occurs in peroxisomes (Lazarow 1982). The peroxisomal fatty acid β-oxidation system consists of four enzyme activities: acyl-CoA oxidase, 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase, and 3-hydroxyacyl-CoA dehydrogenase (Lazarow 1982). The latter two enzyme activities are associated with a single bifunctional enzyme protein of molecular weight 80,000 daltons (Reddy et al 1980, Osumi and Hashimoto 1979).
The administration of peroxisome proliferators to rats results in an increase in both peroxisome number and volume and may account for up to 25% (10-20 fold increase) of the hepatocyte cytoplasmic volume in the liver of rats and mice treated with peroxisome proliferators such as Wy-14,643, methylclofenapate and ciprofibrate (Rao and Reddy 1987). A similar magnitude of induction is seen in the enzymes of $\beta$-oxidation (Reddy et al 1986) and microsomal lauric acid $\omega$-hydroxylase activity (Sharma et al 1988a).

2,4-D and 2,4,5-T, like clofibrinic acid, has been shown to induce peroxisomal $\beta$-oxidation enzymes (Kawashima et al 1984a). The ability of 2,4,5-T to induce peroxisomal $\beta$-oxidation was less pronounced than that of clofibrinic acid, whereas the effect of 2,4,5-T on peroxisomal enzymes was greater than that of 2,4-D. Most of the peroxisome proliferators are known to cause hepatomegaly and a small induction of catalase as well as of peroxisomal $\beta$-oxidation and carnitine acetyl transferase (Hawkins et al 1987). 2,4,5-T pre-treatment results in hepatomegaly and induction of peroxisomal $\beta$-oxidation and carnitine acetyl transferase activity (Kawashima et al 1984a). However, 2,4-D differed from 2,4,5-T in not causing hepatomegaly. Lazarow et al (1982) pointed out that an increase in the activity of peroxisomal $\beta$-oxidation is not always accompanied by an increase in catalase activity or liver size. The potency of 2,4-D and 2,4,5-T to induce peroxisomal enzymes may be due to their structural similarity to clofibrinic acid. However, it should be noted that phenoxyacetic acid, 2-chlorophenoxyacetic acid and CPA (4-chlorophenoxyacetic acid) hardly caused hepatomegaly or an
increase in the activity of peroxisomal $\beta$-oxidation, catalase and carnitine acetyl transferase (Kawashima et al 1984a). These latter authors also showed that 2,4-D and 2,4,5-T treatment effectively increased the concentration of a polypeptide with a molecular weight of approximately 80,000 daltons in the light mitochondrial fraction. The polypeptide has been reported to be increased markedly in peroxisomes of livers from rats treated with peroxisome proliferators (Reddy and Kumar 1977).

Osumi and Hashimoto (1979) showed that a peroxisomal protein having activities of both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, corresponds to the above peroxisome-specific polypeptide with a molecular weight of 80,000 daltons. Furthermore, 2,4-D and 2,4,5-T treatment increased the activity of cyanide-insensitive palmitoyl-CoA oxidation, although the extent of the increase is less pronounced than that obtained by treatment with clofibrac acid (Kawashima et al, 1984a), and additionally decreased the serum levels of triglycerides and cholesterol.

The hepatotoxicity (carcinogenicity) of chlorophenoxy acids and clofibrate may be due to enhanced accumulation of hydrogen peroxide and other oxygen-derived compounds (Reddy et al 1980, Vainio et al 1983), in which peroxisomal proliferation greatly exceeds the increase in catalase activity after exposure of rats to these chlorinated phenoxy compounds (Lazarow et al 1982). The peroxisomal proliferation may subsequently enhance the production of oxygen-centered reactive agents and this might be one mechanism for
the carcinogenic effects of peroxisomal proliferators such as clofibrate and chlorophenoxy acids (Reddy et al 1980, Hawkins et al 1987). Cytoplasmic glutathione peroxidase activity may also be of importance as a cellular defence mechanism (Neat et al 1980), in that this enzyme catalyses the oxidation of reduced glutathione and results in consumption of hydrogen peroxide and organic hydroperoxides. This enzyme activity has been reported to be increased after MCPA treatment (Hietanen et al 1985).

The effect of peroxisome proliferators on liver biochemistry parameters are rapidly reversible following the termination of dosing. Within a few weeks of the cessation of treatment, peroxisomal numbers, liver weight and enzyme activities return to control values (Reddy et al 1978, Chatterjee et al 1983, Eacho et al 1986). This indicates the requirement of the continued presence of the peroxisome proliferator for the maintenance of elevated numbers of peroxisomes, enhanced activities of peroxisomal enzymes and the generation of their metabolic by-products (Stott, 1988).

The bifunctional protein of peroxisomes from rat liver expresses both the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase enzymatic activities which form part of the fatty acid β-oxidation pathway in peroxisomes as noted above (Osumi and Hasimoto 1979). The protein is inducible, its content in peroxisomes being increased markedly following treatment of rodents with peroxisome proliferators such as clofibrate (Reddy et al, 1980).
Chatterjee et al (1987) reported that the proliferation of rat liver peroxisomes by the hypolipidaemic drug Wy-14,643 is associated with a concomitant induction of peroxisomal enzymes involved in the \( \beta \)-oxidation of fatty acids. They concluded that the induction of the peroxisomal bifunctional enzyme enoyl CoA hydratase, 3-hydroxylacyl-CoA dehydrogenase (ECH) activity by Wy-14,643 is due to an enhancement of the rate of transcription of the bifunctional enzyme gene and hence an increase in the enzyme mRNA, after ten hours of incubating hepatocytes with Wy-14,643 \textit{in vitro}. The relatively slow induction (10-15 hours) of the bifunctional enzyme mRNA \textit{in vitro} (Chatterjee et al, 1989) is in striking contrast to the \textit{in vivo} results of Reddy et al (1986) where a maximal induction of the bifunctional enzyme in rat liver was detected within one hour of intragastric administration of the peroxisomal proliferators. Because of this relatively long lag period of induction, it has been suggested that the induction of bifunctional enzyme mRNA may involve an indirect effect of the drug on the transcription of this gene. This is in agreement with the results of Milton (1989) and Sharma et al (1988a) who suggested that the induction of cytochrome P-450 IVa1 precedes the induction of \( \beta \)-oxidation.

Based on \textit{in vivo} and \textit{in vitro} data, Lalwani et al (1983) and Reddy et al (1986) proposed that hypolipidaemic drugs act through a receptor mechanism similar to that for steroid hormones. However, regulation of the genes for peroxisomal enzymes by structurally diverse inducing agents through a common receptor system seems unlikely and the evidence for the existence of such a receptor has
not been substantiated (Milton et al, 1988). Peroxisome proliferation is more likely to be a multistep cascade process for the transcriptional stimulation of the target genes of peroxisomal enzymes linked to the induction of cytochrome P-450 IVA1 (Sharma et al 1988a, Milton 1989). The cascade mechanism may be mediated through a feedback control and substrate induction. Conditions that cause accumulation of hepatocellular acyl-CoA compounds, such as a high-fat diet and inhibition of mitochondrial $\beta$-oxidation, are also known to induce peroxisome biogenesis and peroxisomal $\beta$-oxidation (Berze and Aarsland, 1985).

Dietary exposure of mice to 2,4-D and 2,4,5-T resulted in a substantial increase in the activities of cytosolic and microsomal epoxide hydrolases in mouse liver and generally less pronounced increases in the cytosolic glutathione transferase activity and microsomal content of cytochrome P-450 (Lundgren et al, 1987a & b). 2,4-D and 2,4,5-T treatment also resulted in extensive proliferation of peroxisomes (as judged by the total levels of carnitine acetyltransferase, cyanide-insensitive palmitoyl-CoA oxidation and catalase) and increase in total cytochrome oxidase activity whereas CPA treatment did not cause extensive proliferation of peroxisomes, but increased the specific activity of cytochrome oxidase in mouse liver (Lundgren et al, 1987a & b).

Recently it was reported that both microsomal and mitochondrial glycerol phosphate acyltransferase activities are also raised in liver by the administration of clofibrate (Pollard and
Brindly, 1982), indicating that clofibrate could induce not only peroxisomal enzymes, but also enzymes which are considered to be involved in lipid synthesis and are located in organelles other than peroxisomes.

Cultured rat hepatocytes have been suggested to be a valuable in vitro tool to study biochemical mechanisms of peroxisome proliferation and hepatotoxicity for this class of agents (Feller et al 1987, Lake et al 1987). Clofibric acid, ciprofibrate (Feller et al, 1987) and phthalate monoesters (Lake et al, 1987) increased the activities of laurate hydroxylase, fatty acyl-CoA oxidase, carnitine acetyltransferase and a polypeptide with a molecular weight of 80,000 daltons in cultured rat hepatocytes, parameters which have been shown to be induced in vivo in the livers of rats pre-treated with ciprofibrate (Hawkins et al, 1987). Furthermore, Lewis et al (1987) reported that like clofibrate, a number of chlorinated phenoxy compounds including clofibric acid, 2,4-D, 2,4-DP, 2,4-DB, 2,4,5-T and 2,4,5-TP induced cyanide-insensitive palmitoyl-CoA oxidation in rat hepatocytes in culture.

It has been observed, in addition to xenobiotics, that several nutritional and physiological states can also cause peroxisome proliferation in rat liver. These include high fat diets, vitamin E deficiency, diabetes, starvation and cold adaptation. For a review of this area, see Hawkins et al (1987).
1.5 In vivo induction of drug metabolism

There are 3 principal methods which can be used for the assessment of enzyme induction and enzyme inhibition.

1. Investigation of the pharmacokinetics of model drug substrates in vivo. The pharmacokinetic disposition and urinary elimination of drug and metabolites are measured and the concentration-time profile related to enzyme activity by pharmacokinetics analysis.

2. Direct measurement of enzyme activity in vitro, using whole tissue and/or a tissue fraction such as microsomes.

3. Non-invasive methods are defined as those which do not involve administration of a test substance. Change of disposition of an endogenous substance, the formation of which is related to the activity of the drug-metabolising enzymes, are monitored before, during and after exposure to the potential inducer or inhibitor.

Ideally, one would like to perform parallel in vitro and in vivo studies in which changes in enzyme activity may be related to an alteration in drug disposition.
1.5.1 Clearance

For a drug to be a useful model substrate for the assessment of drug metabolising enzyme activity, its clearance from the body of the rat with the appearance of a metabolite or metabolites (e.g. in urine), should be directly related to the activity of the enzymes responsible for its metabolism. When a drug is eliminated entirely by metabolism, e.g. if metabolism occurs exclusively in the liver, then its clearance is a measure of hepatocellular enzyme activity.

For a drug that forms more than one metabolite, hepatic clearance will be the sum of the clearance of the individual metabolites. Therefore, an increase in hepatic drug-metabolising enzyme activity mediated by enzyme induction will increase the clearance of the drug. Furthermore, for a drug which undergoes several routes of metabolism, the increase in hepatic clearance will be equivalent to the sum of the increase in clearance to the products of the induced enzymes.

Antipyrine is a good drug to study in vivo because it exhibits first order elimination, entirely dependent on hepatic metabolism. It is absorbed rapidly and completely after oral administration, all routes of metabolism are known and it has no toxicological properties at the dose administered.

The rate of elimination of the metabolites should be rapid compared with its rate of formation so that metabolism is the rate-limiting step in the appearance of the metabolite in urine; the
metabolite excreted by the kidney entirely, the metabolite should not be further metabolised except to conjugates which can be hydrolysed quantitatively, and it should be produced exclusively by the enzymes in which one is interested.

The problems in correlating in vitro and in vivo studies of drug metabolism have been reviewed recently by Gillette (1984).

1.5.2. Antipyrine metabolism

Since antipyrine gained popularity in the last decade as a model drug for studying drug-metabolizing enzymes, the antipyrine test is widely used as a research tool in clinical pharmacology to assess the in vivo effects of numerous factors on drug metabolism in animals and man. Metabolite excretion is of interest for investigations with antipyrine. Effectively, this drug is converted into several Phase I metabolites, of which 4-hydroxyantipyrine, norantipyrine and 3-hydroxymethylantipyrine are the principal metabolites. Detailed investigations into the metabolism of antipyrine have shown that formation of these metabolites depends on different enzymes of the cytochrome P-450 system (Danhof et al 1979, Toverud et al 1981, Teunissen et al 1983, Breimer 1983).

Several studies have indicated that the three major oxidized metabolites of antipyrine are produced by different forms of cytochrome P-450 in rats, pigs and humans. In rats, a selective increase in the rate of OHA formation was obtained following 3-methylcholanthrene treatment (Danhof et al 1979, Inaba et al 1980,
Teunissen et al 1983). In pigs, OHA is the only known metabolite that is formed in appreciable quantities; HMA and NORA are almost totally absent (Van den Broek et al 1981). In humans, induction studies with pentobarbital, rifampicin and antipyrine showed a selective increase in NORA formation (Danhof et al 1982b, Toverud et al 1981, Loft et al 1987).

Inhibition of antipyrine metabolism by propranolol appeared to be most prominent for HMA, although in some subjects clearances for production of NORA and OHA were also decreased (Bax et al 1981). Oral contraceptive steroids inhibited antipyrine metabolism non-selectively (Teunissen et al 1982). Boobis et al (1981) compared the in vivo and in vitro rates of formation of antipyrine metabolites in subjects with normal hepatic function and in patients with suspected liver disease. They found that the rank order of the rate of formation (clearance for production) of the three metabolites of antipyrine (OHA, NORA and HMA) was similar in vivo and in vitro (Vmax/Km). Also, there was no significant correlation between the relative rates of formation of any pair of antipyrine metabolites in vivo and in vitro. Furthermore, these latter authors found a significant correlation between in vivo and in vitro rates of formation of each of the three metabolites in the same group of patients. Although these studies are indicative of the involvement of different forms of cytochrome P-450 in the metabolism of antipyrine in humans, in vitro studies with purified forms are ultimately needed to assess the product selectivity of the different forms for antipyrine. A difficulty in human in vivo studies is the inability to study many different inducers and substrates which have
proven to be very useful in the elucidation of the multiplicity of the cytochrome P-450 system in animals (Lu and West 1980, Guengrich 1987).

Using liver microsomes and purified cytochrome P-450 isoenzymes from phenobarbital and 3-methylcholanthrene pre-treated rats, Buppodom et al (1986) showed that the formation of 4-hydroxyantipyrine was significantly increased in phenobarbital pre-treated rats (5.4-fold) and to a lesser extent by 3-methylcholanthrene pre-treatment (1.7-fold), while HMA formation was not enhanced at all. Recently, Loft and Poulsen (1989) and Chenery et al (1987), using isolated rat hepatocytes, showed that phenobarbital increased the formation of HMA (2.5-fold), NORA (4-fold) and OHA (10-fold). In hepatocytes from 3-methylcholanthrene pre-treated rats the Km and Vmax values for antipyrine metabolite formation were not significantly altered, partly in agreement with studies on rat liver microsomes in which unchanged NORA and OHA, but considerably decreased HMA formation have been observed (Kahn et al 1982). This is in contrast to previously reported results from in vivo studies which reported an increase in antipyrine metabolism and clearance of OHA and NORA(5-10-fold) and a decrease in HMA (0.5-fold) after 3-methylcholanthrene administration (Danhof et al 1979, Teunissen et al 1983).

Aminopyrine, like antipyrine, is metabolised mainly by N-demethylation and hydroxylation by microsomal cytochrome P-450 system in liver (Aust and Stevens 1971, Inoue et al 1983). The N-demethylation activity was attributed to the phenobarbital inducible
cytochrome P-450 (P-450IIB1 and IIB2) (Guengerich et al 1982). Recently, Imaoka et al (1988) showed that purified 3MC-inducible cytochrome P-450 (P-450 IA1 and IA2) is also involved in aminopyrine metabolism. It was also noted that the sex differences apply in that male rats have a higher catalytic activity for aminopyrine than female rats (Kato, 1974).

1.5.3. Barbiturate-induced sleeping time

Measuring barbiturate sleeping time and zoxazolamine paralysis time has been used as a rapid and simple method to obtain a rough estimate of both abundance and ratio of cytochrome P-450b (P-450 IIB1) and C-type (P-450 IA1) isoenzyme activity, respectively (Ioannides and Parke 1973, Wiebel et al 1976, Kim and Carlson 1983). Also, the measurement of sleeping time and/or paralysis time in combination with determination of pharmacokinetic parameters has been reported (Buchel et al 1974 a and b, Yasata et al 1978, Griffeth et al 1984, Parkinson et al 1982).

The correlation in the metabolism of two related compounds - hexobarbital and heptobarbital - in the rat has been studied under conditions of a different status of induction of cytochrome P-450 (Van der Graaff et al 1983b). A correlation with $r = 0.97$ was observed. In a subsequent study, the correlation between the metabolism of hexobarbital and antipyrine was addressed. Hexobarbital clearance is known to be increased selectively by phenobarbital pre-treatment. Antipyrine clearance and the formation of antipyrine metabolites, however, are differently affected: 3-methylcholanthrene increases the percentage of 4-hydroxyantipyrine,
whereas phenobarbital increases the production of 3-hydroxymethylantipyrine (Breimer et al 1984). A weak correlation between hexobarbital and antipyrine clearance was expected ($r=0.08$). However, correlation between the intrinsic clearance of hexobarbital and the partial clearance for production of 3-hydroxymethylantipyrine was excellent, with $r=0.89$ (Van der Graaff et al 1983a and 1984). Clearance data of simultaneously administered hexobarbital and aminopyrine also correlated well ($r=0.92$) (Van der Graaff et al 1986). For all these experiments, it was found that phenobarbital pre-treatment increased the clearances whereas 3-methylcholanthrene pre-treatment slightly reduced the total clearance values. Thus, it was concluded that all the metabolic reactions were preferentially mediated by the major phenobarbital-inducible cytochrome P-450 subspecies.

The *in vitro* metabolism of hexobarbital in the rat was studied using purified cytochrome P-450 IAI and P-450 IIB1 (Miyano and Toki 1980, Miyano et al 1980, Ryan et al 1982). Results showed that phenobarbital-induced cytochrome P-450 (P-450 IIB1) but not 3-methylcholanthrene-inducible (P-450 IAI) metabolise hexobarbital and benzphetamine. The latter cytochrome P-450 isoenzyme selectively metabolised benzo(a)pyrene, zoxazolamine and 7-ethoxycoumarine (Ryan et al 1982).
1.5.4. Zoxazolamine

Zoxazolamine is widely used for a pharmacologic test (paralysis time) that serves as a convenient indicator of changes in \textit{in vivo} cytochrome P-450 activity in rodents (Gram \textit{et al} 1967, Burns \textit{et al} 1958 and Baird \textit{et al} 1975). It is a centrally acting skeletal muscle relaxant of the benzazole type that inhibits reflex pathways within the spinal cord (Geiger \textit{et al} 1958). It is partly metabolised to chlorzoxazone, which is used clinically to relieve localized muscle spasm. Both compounds are centrally acting skeletal muscle relaxants but zoxazolamine is no longer used clinically due to hepatotoxicity (Smith 1965 and Franz 1975). In rodents and in man zoxazolamine is metabolised mainly by 3-methylcholanthrene-inducible cytochrome P-450 (Wiebel \textit{et al} 1976 and Krevsky and Hitchcock 1977) and it is thought to proceed mostly via the 6-hydroxylation in the benzene nucleus leading to 6-hydroxyzoxazolamine and to a lesser extent to chlorzoxazone, which are excreted in a conjugated form in the urine (Conney \textit{et al} 1960, Tomaszewski \textit{et al} 1976, Desiraju \textit{et al} 1983).

It has been demonstrated that treatment of rats with polycyclic aromatic hydrocarbons such as 5,6-benzoflavone and 3-methylcholanthrene displays increased rates of microsomal zoxazolamine 6-hydroxylation (Tomaszewski \textit{et al} 1976). Ethanol pretreatment was also shown to increase zoxazolamine metabolism in the rat as exemplified by decreased paralysis time (Ioannides and Parke 1973). When hamsters were treated with ethanol or isoniazid, the metabolism of zoxazolamine was increased but, in contrast to
other rodent species, this drug metabolising activity was decreased in hamster liver microsomes after treatment with either phenobarbital or 5,6-benzoflavone (Ardies et al 1987). Recently, Van der Graaff et al (1986) studied the dose-dependent pharmacokinetics of zoxazolamine in the rat. It was concluded that pharmacokinetic parameters of zoxazolamine can be used to reflect changes in the activity of hepatic cytochrome P-450 in the rat only when doses lower than 25 mg/kg are administered. Similar studies suggested that zoxazolamine is a more suitable agent than chlorzoxazone for subsequent pharmacodynamic studies of disease effects in rats (Yasuhsara and Levy 1988). This is in support of previous studies which showed that model traumatic injury to rats caused prolonged zoxazolamine paralysis time as well as increasing the sleeping time caused by hexobarbital (Griffeth et al 1984). Such increases may be expected since that trauma was shown to decrease hepatic microsomal cytochrome P-450 content and activity, as measured by in vivo studies (Ciaccio and Francillo 1979). Furthermore, a more detailed study demonstrated that the observed post-traumatic decrease in hepatic cytochrome P-450 content was parallelled by a depression of antipyrine metabolism in vivo (Griffith et al 1983). Moreover, this effect was seen after a model injury which could be classified as only mild to moderate in severity and was not accompanied by histopathological changes in the liver or by marked serum transaminase elevation. Similar effects of interferons on cytochrome P-450 content and the in vitro rate of metabolism of benzphetamine and zoxazolamine in mice have been demonstrated (Parkinson et al 1982).
1.6 Phenol metabolism

As phenols are potential metabolites of the chlorophenoxy acids, it is informative to discuss their metabolism. It has been reported that the oxidation of benzene to phenol and phenol to hydroquinone is catalysed by the cytochrome P-450-dependent mono-oxygenase system (Gonasun et al 1973, Gollmer et al 1986). The haematotoxicity of benzene is not manifested by the parent compound (Dean 1985, Kalf 1987). The identity of the proximate toxin and/or leukemogen is not firmly established although it is clear that the primary metabolite, phenol, is not haematoxic when administered alone (Bruce et al 1987). It was suggested that hydroquinone and catechol (or the oxidized forms semiquinone and benzaquinone) or the reactive α, β-unsaturated dialdehyde muconaldehyde may be involved in haematotoxicity (Pellack-Walker et al 1985, Latriano et al 1986): similar metabolites were formed after 4-chlorophenol metabolism in rat liver (Phornchirasilp et al 1989). Furthermore, Eastmond et al (1987) reported that the mylotoxicity observed after benzene exposure could be reproduced by the coadministration of hydroquinone and phenol and the administration of the compounds individually did not elicit the toxic effects. Recently (Koop et al 1989) identified ethanol-inducible cytochrome P-450 (P-450 IIE1) from rabbit liver as being the isoenzyme responsible for catalysing benzene and phenol biotransformation. In this context, it should be noted that chlorinated benzenes induce several forms of cytochrome P-450 in particular, cytochrome P-450IA1, IA2 and P-450IIB1 and IIB2 which were induced significantly by hexachlorobenzene (Goldstein et al 1986).
Hesse et al (1978) and Lau et al (1984) have suggested that chemically reactive quinone species of catechol-derived metabolites of chlorobiphenols and 4-bromophenol are bound to tissue protein in vitro. In this regard, catechol compounds are presumed to bind to protein by forming highly reactive semiquinones and quinones. This reaction may be mediated in part by the presence of superoxide anions with the involvement of cytochrome P-450 (Nelson et al 1976). Furthermore, Phomchirasilp et al (1989) proposed that 4-chlorophenol may be oxidized by hepatic mixed-function oxidase system to a 4-chlorocatechol by a nonenzymatic rearrangement via an epoxide intermediate, and that 4-chlorocatechol might be further oxidized and converted to the corresponding semiquinone metabolites. This is similar to the recent finding that showed during the in vitro metabolism of 2,4,5-trichlorophenol by rat liver S-9 fractions, monomeric and dimeric products were identified using gas chromatography-mass spectrometry (Butte et al 1988). The monomeric compounds were 3,4,5-trichlorocatechol and a dihydroxy-dichlorobenzene. A dimerization process resulted in the formation of a dihydroxy-hexachlorobiphenyl, a dihydroxy-pentachlorobiphenyl ether, two hydroxy-pentachlorobiphenyl ethers, a hydroxy-hexachlorobiphenyl ether and a hydroxy-hexachlorodioxin or hydroxy-hexachlorodiphenoquinone.

In toxicity testing, in Chinese hamster cell line V79, neither 2,4-dichlorophenol nor MCPA interfered with cell growth or mutagenicity, whereas 2,4-dichlorophenol only showed a dose-
dependent toxicity as well as a weak mutagenicity in a cell-mediated test (Fiskesjo 1988).

1.7 Conjugation

In view of the fact that the carboxyl groups of the chlorophenoxy acid herbicides studied are readily conjugated, this pathway will now be considered. Conjugation with glucuronic acid and sulphuric acid are most important biotransformations for foreign and endogenous compounds, and conjugation with glutathione provides a major defence mechanism against the toxicities produced by chemically reactive metabolites formed in phase I oxidation reactions. In rat liver, glucuronidation is catalysed by at least five independent forms of glucuronyl transferase: nitrophenol, morphine, bilirubin, testosterone and esterone being representative substrates of different forms (Bock et al 1984) with overlapping substrate specificities.

Glutathione is available in liver in a high concentration, and any electrophilic metabolites formed in liver will undergo interaction with liver thiol, forming adducts resulting in a lowering of liver thiol content (Gillette et al 1974).

Glutathione S-transferases, which exist in multiple forms (Jakoby and Habig 1980) catalyse the reaction in which the electrophilic center of many xenobiotics is conjugated to the -SH group of glutathione (Chasseaud 1979, Smith and Litwack 1980). They are particularly abundant in liver where they can constitute up to
10% of the total soluble fraction protein (Jakoby 1978, Boyer et al 1983). Of these many forms of glutathione S-transferase, Vessey and Boyer (1984) showed that 2,4-D and 2,4,5-T inhibited forms A and C whereas form AA was activated by the two compounds. The microsomal membrane-bound form of glutathione S-transferase (form B) was also inhibited by 2,4-D and 2,4,5-T. This is similar to other results (Dierickx 1983) who showed the mixed induction and inhibition of GST isoenzymes by 2,4-D in rat liver. While GST AA isoenzyme was slightly stimulated by 2,4-D and MCPA, each of the other GST isoenzymes (A, B, C, E and M) were inhibited by 2,4-D, MCPA, 2,4-DP and MCPP (2-(2-methyl-4-chlorophenoxy) - propionic acid) (Dierickx 1983). The effect on GST by these compounds was by means of direct binding to these proteins.

Dierickx (1988) showed that 1,4-benzoquinone and 2,4-D inhibited cytosolic and microsomal glutathione S-transferase from rat liver and may therefore contribute to their toxicity, since glutathione S-transferase plays an important part in the detoxification process. In the mouse, cytosolic glutathione S-transferase activity was shown to be increased by 2,4-D and 2,4,5-T treatment (Lundgren et al 1987a & b).

Hietanen et al (1983) showed that MCPA treatment increased the hepatic ethoxycoumarin O-deethylase activity by 2-fold. Both 2,4-D and MCPA increased the hepatic epoxide hydrolase activity and decreased the hepatic glutathione S-transferase activity. UDP
glucuronosyl transferase activity was also decreased by 2,4-D and clofibrate.

The UDP glucuronosyl transferases are a family of membrane-bound enzymes which catalyse the conjugation of various endogenous compounds such as bilirubin and steroid hormones as well as exogenous chemicals and potential carcinogens, with UDP glucuronic acid (Dutton 1980). At least two distinctive proteins presenting partially overlapping substrate specificities have been described on the basis of their separate purification (Falany and Tephly 1983, Bock et al 1988), immunological properties, tissue distribution, and chemical structure of the conjugated aglycone (Mackenzie et al 1984, Boutin et al 1984). Differential regulation by inducers was described by Lilienblum et al (1982) and Watkins et al (1982). One form (G\text{I}) which metabolises planner phenols such as 4-nitrophenol (group I substrate), is preferentially induced by 3-methylcholanthrene, whereas the other form (G\text{II}) enhanced by phenobarbital type inducers, catalyses the conjugation of bulkier structures such as morphine (group II substrate) (Wishart 1978). Recently, Fournel et al (1985) showed a structure-dependent increase in glucuronidation of bibirubin after treatment of rats with CPA, CPP and clofibric acid – the latter being the highest inducer. Furthermore, a close linkage was suggested between bilirubin UDP glucuronosyl transferase induction and that of cytochrome P-450 IVA1, as shown by enhanced \( \omega \)-oxidation of lauric acid.
1.8 Renal responses to administration of peroxisome proliferators

Unlike the liver, no kidney enlargement or renal proliferation of the endoplasmic reticulum has been observed in experimental animals treated with hypolipidaemic agents. The major cellular response of the kidney to treatment is a small peroxisome proliferation, with a corresponding increase in peroxisome B-oxidation enzymes and induction of the polypeptide PPA 80,000 (Hawkins et al., 1987).

Whereas cytochrome P-450-dependent mono-oxygenases in liver microsomes have been extensively studied, only a limited amount of information is available from studies on kidney microsomes. The observation that saturated fatty acids are ω-hydroxylated by kidney microsomal preparations and that this reaction is inhibited by carbon monoxide (Wada et al., 1968; Ichihara et al., 1969) prompted Jakobsson and co-workers (1970) to examine further this hydroxylation system. These latter investigators demonstrated that rat kidney cortex microsomes catalyse the ω-oxidation of laurate at a similar rate to that obtained with liver microsomes. By contrast the oxidative metabolism of aminopyrine and testosterone was negligible by rat kidney cortex compared to liver microsomes. Furthermore, Jakobsson et al., (1970) observed that laurate stimulated the rate of reduction of NADPH-linked cytochrome P-450 in kidney cortex. However, by contrast, neither aminopyrine nor testosterone, which are poor substrates for the kidney mono-oxygenase, exhibited this stimulatory activity. Of particular interest is the observation of feeding rats with about 10% lauric acid in the diet stimulated in the kidney the formation
of more cytochrome P-454, increased by 4-fold the laurate generated type I spectral change and increased 1.5-fold the w-oxidation of laurate. By contrast, phenobarbital had no observable effect on either kidney cortex cytochrome P-454 or on the w-oxidation of laurate. These findings suggest that kidney and liver mono-oxygenases are markedly different. Subsequent studies from the same laboratory demonstrated that starvation of rats increased kidney cortex cytochrome P-454 and laurate hydroxylation (Ellin and Orrenius, 1971). Further investigations showed that the hydroxylation of laurate by rat kidney cortex microsomes occurs at the w- and (w-1)-positions, yielding 12-hydroxy- and 11-hydroxy-laurate, respectively (Ellin et al., 1972). The ratio of w-/(w-1)-hydroxylation of laurate remained 2:1 in a series of experiments with microsomes from control and from starved rats, though in the latter case the total hydroxylation activity was enhanced about 2-fold (Ellin et al., 1973). These findings may suggest that the hydroxylation of laurate at w- and (w-1)-positions in the kidney cortex is catalysed by a single enzyme.

Parker and Orton (1980) demonstrated the induction of a kidney microsomal cytochrome P-450 with specificity towards the hydroxylation of fatty acids following treatment with the hypolipidaemic drug, clobuzarit. Later, Imaoka and Funae (1986) reported the isolation of the major renal cytochrome P-450 from untreated rats. This protein was shown to catalyse the w-and (w-1) hydroxylation of lauric acid, but was inefficient in the N-demethylation of benzphetamine and the O-dealkylation of 7-ethoxycoumarin.
Recently Sharma (1988) and Makowska (1988) showed that treatment of rats with clofibrate, ciprofibrate, clobuzarit, bezafibrate, nafenopin, DEHP, MEHP and Wy-14,643 significantly increased the cytochrome P-450IVAl isoenzyme, accompanied by an increase in the ω-hydroxylation of lauric acid and to a lesser extent the (ω-1)-hydroxylation, but that the ethoxyresorufin-O-deethylase activity was significantly reduced.

Measurement of specific cytochrome P-450IVAl by an ELISA technique indicated that approximately 30% of the total cytochrome P-450 in control kidney microsomes is immunochemically related to hepatic microsomal cytochrome P-450IVAl and the level of this enzyme was seen to increase 2-3-fold in rat kidney after treatment of rats with hypolipidaemic drugs (Sharma, 1988). Furthermore, this increase was correlated with an increase in total lauric acid metabolism (3-5-fold).

The renal metabolism of fatty acids occurs either by β-oxidation through the mitochondrial pathway or by ω-and (ω-1)-oxidation in the microsomal subcellular fraction. In addition, an extra-mitochondrial β-oxidation pathway has also been demonstrated with this being located in peroxisomes in the medulla and medullary rays (Le Hir and Dubach, 1982).

Previous workers have reported the major cellular response of the kidney to treatment with hypolipidaemic agents is peroxisome proliferation with a corresponding increase in peroxisomal β-oxidation and induction of the polypeptide PPA 80,000 molecular weight protein (Lalwani et al., 1981; Small et al., 1982). Other workers have reported the induction of
mitochondrial carnitine acetyl CoA transferase in the kidney as a result of clofibrate treatment (Mittal and Kurup, 1981).

Peroxisome proliferation has been demonstrated in clofibrate treated kidney cells (Svobada et al., 1969). This proliferation was sex-related with male rats displaying more extensive peroxisome proliferation than females at the same dose levels (Henry and De Morrow, 1985). Induction of peroxisomal \( \beta \)-oxidation, enoyl-CoA hydratase and PPA 80,000 with an associated peroxisome proliferation was reported in mouse kidney following methyl clofenapate, BR-931 and Wy-14,643 treatment (Lalwani et al., 1981).

The effect of a structurally diverse range of peroxisome proliferators on renal mitochondrial and peroxisomal enzymes has been studied in rats and other animals (Sharma, 1988; Makowska, 1988). Their results showed a 1.5-3-fold induction in peroxisomal \( \beta \)-oxidation following clofibrate, ciprofibrate, nafenopin, bezafibrate and DEHP treatment. A maximal 2-fold induction in carnitine acetyltransferase, carnitine palmitoyltransferase, total enoyl-CoA hydrates (mitochondrial and peroxisomal) and peroxisomal enoyl-CoA hydratase activities resulted from treatment (Sharma, 1988). The induction of renal enzymes was considerably lower than in the liver of many animals (Makowska, 1988).
1.9 Aims of the present investigation:

The present study is designed to characterise the effect of chlorinated phenoxy acids pretreatment on the cytochrome P-450 system and peroxisomal β-oxidation, in vivo and in vitro, and in particular cytochrome P-450IVAm and to see if a structure induction relationship can be drawn and compared to existing data on related chemicals such as clofibrate.
CHAPTER TWO

MATERIALS AND METHODS
CHAPTER 2

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2.1 MATERIALS

Acetylacetone, acrylamide, Amberlite MBI resin, ammonium acetate, ammonium persulphate, bromophenol blue, citric acid, copper sulphate, EDTA, diethylpyrocarbonate, Folin phenol reagent, gelatine, glycine, hydrogen peroxide, magnesium chloride, potassium chloride, potassium cyanide, potassium phosphate, sodium bicarbonate, sodium carbonate, sodium chloride, sodium dithionite, sodium-potassium tartrate, sodium persulphite, sucrose, sodium N-lauryl sarcosine and Triton-X-100 were purchased from BDH Limited (Poole, Dorset).

Benzphetamine, acetyl CoA, bovine serum albumin, carnitine, Coenzyme A, 5,5'-dithiobis (2-nitrobenzoic acid), dithiothreitol, dianobenzidine chloride, L-alpha-dilauryl phosphatidylcholine, dextran sulphate, antifoam A emulsion, ethidium bromide, FAD⁺, 4-hydroquinoline, kynuramine, lauric acid, orthophenyl-diamine, NAD⁺, NADH, NADPH, nicotinamide, MOPS (3(N-morpholino)propanesulphonic acid), palmitoyl CoA, pentobarbital, salmon sperm DNA (Sigma type III, sodium salt), sodium cholate, sodium alpha-glycerophosphate, sodium malonate, sodium succinate, Tris (Trizma-base), Tween 20 were purchased from Sigma Chemicals Company Limited (Poole, Dorset). The
herbicides 4-chlorophenol, 4-chlorophenoxy-, 2,4-dichlorophenoxy-, 2,4,5-trichlorophenoxy-, and 4-chloro-2-methylphenoxyacetic acid, 2,4-dichlorophenoxy butyric acid, 2,4-dichlorophenoxypropionic acid, 2,4,5-trichlorophenol, 2,4,5-trichlorophenoxypropionic acid and uric acid were also purchased from Sigma Chemicals Company Limited (Poole, Dorset).

Antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one, phenazine), 4-hydroxyantipyrine, 4-dimethylaminoantipyrine, p-acetophenetidide (phenacetin), zoxazolamine (2-amino-5-chlorobenzoxazole) and the phenolics 4-chloro-2-methylphenol and 2,4-dichlorophenol were purchased from Aldrich Chemicals Company Limited (Dorset, UK).

Radioactive labelled (α-P32) deoxycytidine triphosphate (specific activity 2000Ci/mmol) and C14-lauric acid was obtained from Amersham Radiochemical Centre (Amersham, Buckinghamshire); glycerol from Fision Scientific Equipment Company (Loughborough, Leicestershire); ethoxyresorufin and resorufin from Molecular Probes Inc. (Junction City, Oregon, USA) and the nitrocellulose filters (0.45 m) were purchased from Anderman and Company Limited (Kingston-Upon-Thames, Surrey) or from Sigma Chemical Company Limited. Emulgen 911 was obtained from KAO Atlas Company (Tokyo, Japan) and control donkey anti-sheep IgG enzyme label was kindly supplied by Guildhay Antisera Limited (Guildford, Surrey).
The ultrapure enzyme grade agarose, caesium chloride, guanidine hydrochloride, guanidium isothiocyanate, NACS Prepac columns and nick translation kits were obtained from Bethesda Research Laboratories (Maryland, USA).

All other chemicals were obtained as Analar grade 3 wherever possible, or the highest purity grade available.

2.2 METHODS

Animals: Male Wister rats (150-200g body weight) (University of Surrey Breeders) were used throughout the study. The rats were divided into groups (3 animals in each group) and were allowed free access to water ad libitum and laboratory food (Spratts animal diet No.1) and were housed in a room with 12h light/12h dark cycles and controlled humidity (50±5%) and temperature (21±1%). Animals were given daily intragastric doses of the test compounds suspended in peanut oil at the dose levels of 50, 100 or 200 mg/kg (MCPA and CPA were given as the free acid as doses of 100, 200 or 300 mg/kg) for three days. Control animals received corresponding quantities (5ml/kg) of the vehicle (peanut oil) or saline, and no significant differences were noted between these two control values for all parameters studied.

All animals were killed 24h after the last dose. The livers and kidneys were removed and the livers were then perfused with 0.9%(w/v) saline prior to homogenisation. Livers were blot dried on
tissue paper and weighed. The kidneys were decapsulated before homogenisation. The liver and kidneys were scissor minced and homogenised in 0.25M sucrose (25-30% (w/v) and 15% (w/v) respectively) using a Potter Elvehem glass teflon homogeniser.

Aliquots of the homogenates were frozen at -70°C for use in the analysis of palmitoyl CoA β-oxidation and for RNA analysis.

2.3 PREPARATION OF MICROSOMAL FRACTIONS

Microsomes were prepared either by the calcium precipitation method, as described by Cinti et al., (1972) or by a modification of the ultracentrifugation method of Omura and Sato (1964).

In the calcium precipitation method liver and kidney homogenates in 0.25M sucrose were centrifuged at 12,600g at 4°C for 30 minutes in 14 x 50ml rotor in a Beckman J2-21 centrifuge. After discarding the pellets, calcium chloride stock (80mM) was added to the supernatants to give a final concentration of 8mM and this was then re-centrifuged at 27,400g at 4°C for 15 minutes. The resulting pellets were resuspended in 15ml of 0.15M potassium chloride to remove the CaCl and re-centrifuged at 27,400g for 15 minutes. The final pellets were resuspended in ice-cold 50mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol and were stored at -70°C until used.
In the ultracentrifugation method, tissue homogenates in sucrose, after being centrifuged at 12,600g for 30 minutes at 4°C, the supernatants were centrifuged at 105,000g for 60 minutes at 4°C in a 60Ti rotor, Beckman L5-6S ultracentrifuge. The resulting microsomal pellets were resuspended in phosphate buffer 50mM (pH 7.25) and stored at -70°C until used.

Microsomal protein concentration was determined by the modified method of Lowry et al., (1951) using bovine serum albumin as standard.

2.4 ENZYME ASSAYS

2.4.1 DETERMINATION OF TOTAL CYTOCHROME P-450 CONTENT

Total cytochrome P-450 was determined according to the spectral method of Omura and Sato (1964) using a difference absorption coefficient (450 to 490nm) of 91mM^{-1} cm^{-1} for the sodium dithionite-reduced carbon monoxide adduct. Liver microsomes were diluted to 1mg/ml with buffer and the baseline was recorded in DW-2UV/VIS Varian spectrophotometer. A few grains of sodium dithionite were added to both cuvettes to reduce cytochrome P-450. Carbon monoxide was bubbled through the sample cuvette (1 bubble/second for 30 seconds) and the reduced difference spectrum was recorded between 500-400nm.
For kidney microsomal cytochrome P-450 determinations, sodium succinate (10mM) and NADH (25 1, 2% (w/v) solution) was included in the dilution buffer to reduce the cortex mitochondrial electron transport enzyme contamination.

2.4.2 Determination of Benzphetamine-N-Demethylase Activity

The rate of benzphetamine-N-demethylation was assessed from the rates of formaldehyde formation which was detected according to the method of Nash (1953). The incubation mixture contained 50 g/ml sodium deoxycholate, 15mM magnesium chloride, 50mM potassium phosphate buffer (pH7.25), 0.1 ml microsomal suspension (approximately 1-2 mg protein) and 0.1 ml of 15mM benzphetamine to give a total volume of 1 ml. Following 2-3 minutes of incubation at 37°C the reaction was started by the addition of 40 l of 50mM NADPH.

After 5-10 minutes incubation at 37°C the reaction was stopped by the addition of 0.5ml of ice-cold 12.5% (w/v) trichloroacetic acid (TCA). Samples were mixed and then centrifuged at 12,000g for 10 minutes at 4°C in a Beckman J6B centrifuge. The supernatant (1ml) was then removed for determination of formaldehyde using the Nash assay.
2.4.3 Nash Assay

**Reagent:** Ammonium acetate (150g) was dissolved in 900ml of distilled water. Acetylacetone (2ml) was added and completely dissolved. The pH was adjusted to pH6.0 with glacial acetic acid, and the volume adjusted to 1 litre. The reagent was stored at 4°C.

**Method:** The supernatant from the TCA precipitation (1ml, Section 2.4.2) was added to the Nash reagent (1 ml) mixed and heated in a water bath at 58°C for 10 minutes in the dark. After 5 minutes cooling, the absorbance at 412nm was recorded. A standard curve was plotted using 1ml volumes containing 0, 10, 20, 40, 60, 100 and 140nmol formaldehyde and treated in an identical manner to the test samples. Under these conditions 1.0 A412 = 1000 nmoles formaldehyde.

2.4.4 Determination of Ethoxyresorufin-O-Deethylase Activity

The O-deethylation of ethoxyresorufin was routinely determined by the method of Burke et al., (1977), using the difference in fluorescent properties of ethoxyresorufin (excitation wavelength = 456nm, emission wavelength = 586 nm) and the product resorufin (excitation wavelength = 510nm, emission wavelength = 586nm).

The assay system contained 30-80 ul of microsomal suspension in 2 ml of 0.1M Tris-HCl buffer (pH7.8) at 37°C. Ethoxyresorufin
(stock solution in methanol) was added to give a final concentration of 50μM. The cuvette was placed in a thermostatically controlled cuvette housing of a Perkin Elmer LS-5 luminescence fluorimeter, set at an excitation wavelength of 510nm and emission wavelength of 586nm. After pre-incubation at 37°C for 2 minutes, a steady baseline with time was recorded. The reaction was initiated with a final concentration of 1mM NADPH and the production of resorufin with time monitored as the increase in fluorescence at 586nm. The fluorimeter was calibrated with multiples of 5 and 10μl of resorufin standard (10μM in methanol).

2.4.5 Determination of Lauric Acid Hydroxylation

The metabolism of 14C-lauric acid to 12- and 11-hydroxylated products was evaluated by HPLC analysis as modified from the method described by Parker and Orton (1980). The incubation mixture contained 0.5mg or 1mg protein/ml, 0.55mM 12C-lauric acid and 0.1μCi-14C-lauric acid (in methanol) in a total volume of 1.96ml. The tubes were incubated for 5 minutes at 37°C prior to the addition of 40μl NADPH (0.8mM final concentration) to initiate the reaction. After 5-10 minutes incubation the reaction was terminated by the addition of 0.2 ml of 3M HCl.

10ml of diethyl ether was then added to the incubation mixture, followed by mixing on a rotary shaker for 10 minutes. The
tubes were then left to stand for at least 5 minutes or centrifuged at 2000 rpm for 5 minutes to clarify 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 -70°C prior to analysis.

**HPLC analysis of 11- and 12-hydroxylauric acid:** The dried ether extracts were reconstituted in 150 ml of eluting solvent, water: methanol: acetic acid (45:55:0.1 by volume), 50-10ul aliquots were then injected into the HPLC system. The metabolites of 14C-lauric acid were separated using a linear gradient of water:methanol:acid (45:55:0.1 by volume) to 100% methanol over a 35 minute period passing through a reverse phase ultrasphere (4.6 x 15cm) at a flow rate of 1ml/min. The elution profile of the radioactivity products was monitored using a Berthold LB 503 radiodetector linked to a Commodore PET (Series 4000) allowing separate quantitation of 12- and 11-hydroxylaurate.
2.4.6

Determination of Cyanide-Insensitive Palmitoyl-CoAβ-Oxidation

The assay was performed as described by Bronfman et al., (1979), as follows. A reaction mixture containing CoA (75uM), FAD (180uM), NAD⁺ (555uM), nicotinamide (141mM), DTT (4.2mM), KCN (3mM) and BSA (0.225mg/ml) in 60mM Tris-HCl buffer (pH8.3) was freshly prepared and stored on ice. A 2ml aliquot of the reaction cocktail and 0.96ml of 60mM Tris-HCl buffer (pH8.3) were equilibrated at 37°C for 5 minutes. The liver homogenate was diluted 1.1 with 60mM Tris-HCl buffer (pH8.3) containing 1% (w/v) Triton-X-100 and equilibrated at 37°C for 2 minutes and 40ul of sample was then added to the cocktail buffer, transferred to a cuvette and placed in a Varian 219 spectrophotometer. The reaction was initiated with 20ul of 75uM palmitoyl-CoA and the change in absorbance at 340nm was recorded for 10-20 minutes at 37°C. The assay measures the reduction rate of NAD⁺ to produce NADH and so the results were expressed as nmol product formed/min/mg protein assuming the molar absorbance coefficient of NADH at 340nm is 6220.
2.5.1

QUANTITATIVE AND QUALITATIVE DETERMINATION OF CYTOCHROME P-450IVAI

Enzyme-Linked Immunosorbent Assay (ELISA)

The quantitative studies of microsomal cytochrome P-450 IVAI were carried out by the ELISA technique (enzyme-linked immunosorbent assay) as described by Voller et al., (1978).

Antibodies raised in sheep against clofibrate-induced cytochrome P-450 IVAI (rat derived) were used throughout this study. Microsomes were solubilised in 1mg cholate and 0.2mg Emulgen 911 per mg of protein and stirred on ice for 20 minutes, diluted as appropriate (0.005-0.1pmoles of cytochrome P-450 per assay) with 0.1M sodium carbonate/bicarbonate buffer (pH 9.6) and coated to the walls of a microtitre plate at 4°C overnight in a humid environment. The plates were washed with phosphate-buffered saline (pH 7.4) containing 0.1% (w/v) gelatin and 0.05% (v/v) Tween 20 (PBS-GT). The antisera (cytochrome P-450 IVAI antibody or the pre-immune sera were diluted in PBS-GT buffer as required and applied to the plates in 200ul volumes. The binding of the pre-immune serum to the cytochrome P-450 IVAI antigen was taken as the level of non-specific binding observed in this assay and was subtracted from the test immune value.

Following a 2h incubation at 37°C and washing with PBS-GT, donkey anti-sheep enzyme label (200ul/well) was added and incubated
for a further 2 hours at 37°C and subsequently washed with PBS-GT. The substrate (100ml of 0.025M citric acid, 0.05M sodium hydrogen phosphate buffer, pH5.6, containing 40mg orthophenyldiamine and 40ul hydrogen peroxide) was added to the plates (150ul/well) and incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 2.5M sulphuric acid (50ul/well). The plates were then read at 490nm using a Dynatech plate reader. A calibration curve using purified cytochrome P-450 IVAI was constructed and shown to be linear up to 0.02pmols of cytochrome P-450 IVAI per assay.

2.5.2 Western Blot Analysis

The method used in Western blot analysis was a modification of the procedures of Burnett (1981) which involves the electrophoretic transfer of separated proteins on polyacrylamide gel to nitrocellulose sheets which are then subjected to immunological analysis.

SDS polyacrylamide gel electrophoresis was performed on samples to be analysed according to the method of Laemmli (1970). Prior to transfer, the gels were immersed in transfer buffer (20mM Tris base, 150mM glycine) for 20-30 minutes. This pre-transfer stage was included to allow for any swelling or shrinking in gel size before the transfer.

The transfer was achieved by the use of a plastic sandwich apparatus. A sheet of nitrocellulose paper (0.45um in pore size)
was cut to the appropriate size and laid on two sheets of Whatman 3MM blotting paper immersed in transfer buffer. The tel to be blotted was then laid onto the nitrocellulose sheet with two other sheets of blotting paper being placed on top. The whole assembly was then placed between two Scotch Brite scouring pads supported on a stiff plastic grid. It is important to ensure that no air bubbles are caught between the filters and the gel because of the possibility of low efficiency transfer and band distortion occurring in these areas. The immobilised assembly was then inserted into the electrophoretic transfer apparatus with the nitrocellulose paper facing the anode (+). The tank was filled with transfer buffer and electrophoretic transfer was achieved overnight at 5V/cm.

Following electrophoretic transfer the nitrocellulose sheet was washed in phosphate buffer saline (PBS) containing 1% (w/v) bovine serum albumin and 0.2% (v/v) Triton X-100. Washing was carried out for 15 minutes on a shaking water bath. After three washes the nitrocellulose sheet was washed for 1 hour in buffer containing 60ul anti-cytochrome P-450 IVA1 serum.

Three further 15 minute washes with PBS wash buffer were followed by a second 1 hour wash with the washing buffer containing donkey anti-sheep (500 ul) as the second antibody. Again, 3 further washes in PBS wash buffer of 15 mins duration were required followed by a 1 hour wash with PBS wash buffer containing horseradish peroxidase labelled antibody (100 ul). This was washed 3 times with PBS wash buffer followed by one final wash with PBS alone for 15
mins. The nitrocellulose sheet was then developed in 0.1M Tris-HCl buffer (pH 7.5) containing 0.5 mg/ml diaminobenzidine chloride and 1:5000 H₂O₂. Once the desired image intensity was observed, the sheet was then rinsed in distilled water which effectively stopped the reaction.
2.6

**ISOLATION OF TOTAL CELLULAR RNA**

Total cellular RNA was prepared from liver homogenates of control and treated animals according to the method of Chomczynski and Sacchi (1987).

**Reagents**

Denaturing solution (Solution D)

- 4M guanidium thiocyanate
- 2.5mM sodium citrate, pH7.0
- 0.5% (w/v) sarcosyl
- 0.1, 2-mercaptoethanol
- Phenol (nucleic acid grade) saturated with water

**2.6.1 Experimental Procedure**

One gram of liver, fresh or frozen, was homogenised in a glass teflon homogeniser and subsequently transferred to a 4ml polypropylene tube. 0.1ml of 2M sodium acetate (pH-14), 1ml of phenol (water saturated), and 0.2ml of chloroform isoamyl alcohol mixture (49:1) were added to the homogenate with thorough mixing after the addition of each reagent. The final suspension was shaken vigorously for 10 seconds and cooled on ice for 15 minutes. Samples were centrifuged at 10,000g for 20 minutes at 4°C. After centrifugation, the aqueous phase, which contains the RNA, was transferred to a fresh tube, mixed with 1ml isopropanol and then placed at -20°C for 1 hour to precipitate RNA. The tubes were
centrifuged at 10,000g for 20 minutes at 4°C to sediment the RNA, and the resulting pellet was dissolved in 1.3ml of solution D, and precipitated with one volume of isopropanol at -20°C for 1 hour. After centrifugation at 10,000g for 10 minutes at 4°C the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried and dissolved in 200ul water (sterile) at 65°C for 10 minutes and stored at -70°C until used.

To determine the purity of the RNA preparations, 10ul aliquots were diluted 1:100 with sterile water and transferred to 1ml quartz cuvettes. The samples were scanned between 200 and 300nm and the absorption at 260 and 280nm was determined. A 260-280nm ratio of 2.0 was taken as indicative of pure RNA. The RNA yield was calculated assuming:

\[ \text{A}_{1cm}/1\% \text{ at } 260 = 40 \text{ug/ml} \]

2.6.2 RNA Dot Blots with Nitrocellulose

Dot blotting was carried out routinely using an adaptation to the method of White and Bancroft (1982). RNA samples were diluted in sterile water as required to give a final RNA concentration of 1, 2, 5 and 10ul and then 150ul of 6.15M formaldehyde and 150ul of 20 x SSC (3M sodium chloride and 0.3M sodium citrate, pH8) were added, mixed and the samples were incubated at 65°C for 15 minutes.

The nitrocellulose filter was soaked in 10 x SSC and sterile water for 5 minutes each time. The dot blot apparatus (BRL) was cleaned with distilled water and acetone and assembled with the
filter according to the manufacturer's instructions. The RNA samples were applied to the wells, under a slow vacuum and then washed with 400ul 10 x SSC; the filter was removed and dried between Whatman 3MM paper prior to baking at 80°C for 2-3 hours. The baked filters were stored at room temperature between sheets of Whatman 3MM paper prior to hybridisation.

2.6.3 Nick Translation

The nick translation kit was obtained from BRL and the accompanying instructions followed:

**Reagents**

- 200ng rat cytochrome P-450 DNA
- 5ul dA, dG, dT
- 100ug dC
- and sterile water to give a total volume of 40ul.

This was vortexed briefly and 5ul of DNA polymerase added. The solution was mixed and centrifuged rapidly prior to incubation for 2 hours at 15°C. The reaction was stopped by the addition of 5ul of 0.3M EDTA, mixed and stored on ice. A further 200ul of TNE buffer (10mM Tris, 100mM NaCl and 1mM EDTA, pH8) and 5ul of salmon sperm DNA (2mg/ml) were added.
2.6.4 Removal of Unincorporated Alpha $^{32}$ P dCTP

NACS prepac cartridges (1cm x 0.5cm) contain NACS 52, a derivative of hydroxylapatite, were used to remove unincorporated alpha $^{32}$ nucleotides (Maniatis et al., 1982). Under low salt concentration DNA is bound to the column by the phosphates whereas nucleotides do not bind. In conditions of high salt concentration, DNA is eluted. The column was attached to a 2ml syringe and hydrated with 5 x 1ml volumes of 2M NaCl in 10m Tris, 1mM EDTA 9pH8) (TE) and equilibrated with 10 x 1ml washes of 0.5M NaCl in TE buffer. The column was secured in a retort and the nick translation volume was loaded onto the column via the syringe barrel. The column was washed with 10 x 1ml volumes of 0.5M NaCl/TE buffer collected into Eppendorfs and eluted with 5 x 0.1ml aliquots of 2M NaCl in TE buffer.

2.6.5 Filter Hybridisation with Radioactive Probes

The application of nucleic acid hybridisation has been studied extensively (Hames and Higgins, 1985) allowing for modification to optimise conditions. The backed filters were soaked in 5 x SSC before being transferred to 250ml pre-hybridisation buffer, warmed to 42°C in a plastic container. Pre-hybridisation was carried out at 42°C in a shaking water bath for 7 hours. The buffer was removed and replaced with hybridisation buffer containing the radioactive probe which had previously been boiled for 5 minutes to denature the double stranded DNA. The filters were hydridised for a minimum of 15 hours at 42°C in a shaking water bath.
### Prehybridisation Buffer

<table>
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<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
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<tr>
<td>Deionised formamide</td>
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</tr>
<tr>
<td>50 x Demhardet solution</td>
<td>50ml</td>
</tr>
<tr>
<td>(2% w/v Ficoll, 2% w/v BSA and 2% w/v polyvinyl-pyrrolidine)</td>
<td></td>
</tr>
<tr>
<td>20 x SSC</td>
<td>125ml</td>
</tr>
<tr>
<td>Boiled salmon sperm DNA (10mg/ml)</td>
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<tr>
<td>Sterile water</td>
<td>62.5ml</td>
</tr>
<tr>
<td>Poly A (20mg/ml stock solution)</td>
<td>0.25ml</td>
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<tr>
<td>Poly C (20mg/ml stock solution)</td>
<td>0.25ml</td>
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### Hybridisation Buffer

<table>
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<tr>
<th>Component</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>Deionised formamide</td>
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<tr>
<td>50 x Demhardt's solution</td>
<td>10ml</td>
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<tr>
<td>20 x SSC</td>
<td>125ml</td>
</tr>
<tr>
<td>50% Dextran sulphate</td>
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<tr>
<td>Boiled salmon sperm DNA (10mg/ml)</td>
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</tr>
<tr>
<td>Poly A (20mg/ml stock solution)</td>
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</tr>
<tr>
<td>Poly C (20mg/ml stock solution)</td>
<td>0.25ml</td>
</tr>
</tbody>
</table>
2.6.6 Washing of Filters Following Hybridisation

The hybridisation buffer was removed and replaced with 2 x SSC, 0.1% (w/v) SDS for three consecutive washes at room temperature for 30 minutes followed by one wash in 0.5 x SSC, 0.1% (w/v) SDS for 45 minutes at 55°C. The final wash was completed at room temperature in 5 x SSC for 30 minutes. The filters were dried between sheets of Whatman 3MM paper at 37°C for 20 minutes and then placed in an X-ray cassette with an intensifying screen with X-ray film (Kodak GB X2) for four days at -70°C. The X-ray films were developed and qualitative and quantitative assessment of RNA dot blots made by the use of densitometry (ACD-18 Automatic Computing Densitometer, Gelman Inst. Co., Ann Arbor, MI, U.S.A.).
2.7 IN VIVO DETERMINATION OF DRUG METABOLISING ACTIVITY

2.7.1 Determination of Antipyrine Metabolism

Antipyrine (AP) and its main metabolites in rat urine were determined according to the method of Teunissen et al., (1983) with some modification.

Animals were treated with the appropriate compound as previously described, and were housed individually in metabolic cages to allow for separate urine collection. Urine was collected 24 hours prior to AP dosing (70mg/kg by gavage 24 hours after the last treatment with the appropriate compounds) and for a further 24 hours from the AP dosing. Urine volume (24 hours collection) was measured and sodium pyrosulphite ($Na_2S_2O_5$) was added to the urine to prevent the oxidation or breaking down of AP and metabolites to give a final concentration of 2g/l. The urine samples were stored at -20°C until used.

Analytical Procedure

To 0.5ml of urine, 0.5ml of 0.5M acetate buffer (pH 4.5), 40mg of $Na_2S_2O_5$ and 10mg of limpet acetone powder were added. The mixture was incubated for 2-3 hours at 37°C in a shaking water bath. After the incubation 10ug of phenacetin (an internal standard in 50ul of ethanol) was added to the samples and the mixture was saturated with 200mg NaCl and extracted with 5ml of chloroform-ethanol (9:1 v/v). Organic layer was collected and evaporated under reduced pressure at room temperature. The residue was dissolved in 0.2ml of methanol.
and diluted with 0.5ml of 10mM acetate buffer containing 12.5mg of Na₂S₂O₅ (freshly prepared). Of this solution, 15ul was injected into the HPLC system.

**Calibration Graphs and Standard Curves**

Calibration graphs were prepared by spiking blank samples with AP or the metabolites to be measured ranging from 1.5-10ug/ml (from stock solutions of 1mg/ml) and were subsequently treated as the urine samples.

**2.7.2 Metabolite Separation and Chromatographic Conditions**

The liquid chromatographic system (Kipp Analytica, Holland) consists of a 9208 pumping device and a 9203 spectrophotometer detector set at 245nm connected to a chart recorder. The separation of the AP and its metabolites was carried out by the use of a column (I.D., 4mm x 11cm) packed with Hypersil 3 DS, 5μm (Technical Assoc., Canaga Park, CA, U.S.A.). The eluent for the separation consisted of a mixture of 20mM phosphate buffer (pH7.2) and acetonitrile (100:10) containing Na₂S₂O₅(2g/l). The flow rate was set at 1.5ml/min.

Metabolite quantitation was calculated from the peak height of the internal standard, the drug and metabolites as well as reference to a standard curve.
2.7.3 Pentobarbital Sleeping Time

Wistar-albino rats weighing 80-100g (10 rats per group) were dosed with the appropriate compounds, as described previously. Pentobarbital (30mg/kg in volumes of 0.09-0.13ml) was given intraperitoneally to the rats 24 hours after the last dose of the herbicides. Sleeping time was measured by means of the rats gaining the right of reflex after they were put on their backs.

2.7.4 Zoxazolamine Paralysis Time

Rats of Wistar-albino strain (100-120)(10 rats per group, 15 rats for control) were dosed with the appropriate compound for three days as previously described. Zoxazolamine was given at 70mg/kg dose intraperitoneally and the zoxazolamine paralysis time was recorded.

2.8 STATISTICAL ANALYSIS

Statistical evaluation was carried out by the Student's t-test. Multi-dimensional regression analysis was performed using r (multiple correlation coefficient) and s (standard error of the estimate) to test the quality of the regression equations.
CHAPTER THREE

EFFECT OF CHLOROPHENOXYACID HERBICIDES ON MICROSONAL CYTOCHROME P-450 IVA1 IN RAT LIVER AND KIDNEY
3.1. INTRODUCTION

In rodents, the administration of several hypolipidaemic agents is associated with hepatomegaly and with marked proliferation of both peroxisomes and the endoplasmic reticulum (Hess et al., 1965; Reddy and Lalwani, 1983; Hawkins et al., 1987). The early hepatic changes observed in rats on administration of hypolipidaemic agents such as clofibrate include hepatomegaly, proliferation of peroxisomes and more recently, the induction of a microsomal isoenzyme of cytochrome P-450 (termed cytochrome P-450 IVA1), the latter specifically catalysing the hydroxylation of fatty acids (Tamburini et al., 1984). The relationship between the induction of microsomal and peroxisomal enzymes following hypolipidaemic challenge is thought to be closely linked (Lake et al., 1986; Sharma et al., 1988a and b).

Structurally related to the oxyisobutyrate class of hypolipidaemics, the phenoxy acid herbicides have been shown to induce hepatic β-oxidation and peroxisomal proliferation in rodents (Vainio et al., 1983; Kawashima et al., 1984a; Hietanin et al., 1985). In addition, these herbicides induce several other enzyme activities in rodent liver including catalase (Vainio et al., 1983), glutathione reductase and glutathione peroxidase (Hietanin et al.,
In view of the above, the present study was undertaken to investigate the effect of seven phenoxy herbicides on the cytochrome P-450 mixed-function oxidase system in rat liver and kidney microsomes, and in particular, the previously described, hypolipidaemic-induced cytochrome P-450 IVAl isoenzyme.

3.2 RESULTS

3.2.1 Liver Microsomes

As can be seen from Fig. 3.1 intragastric exposure to some phenoxy acid herbicides had a significant effect on rat liver/body weight ratio. Body weight gain was normal in rats treated with 2,4-DB, CPA and MCPA compared to control groups, whereas treatment of rats with 2,4-DP and 2,4,5-Tp resulted in loss of about 6-7% of body weight at the highest dose treatment. At the lower doses there was no clear influence of drug treatment on body weight gain when compared to control. The liver/body weight ratio, as an indication of change in liver size, was normal in rats treated with 2,4-DB, MCPA and CPA over the three dose levels (Fig.3.1), whereas 2,4-DP (25%), 2,4,5-T (25%) and 2,4,5-Tp (50%) increased the liver/body weight ratio significantly. The increase in liver/body weight ratio
Figure 3.1 Effect of phenoxy acid pretreatment on liver:body weight ratio.

Compounds were administered at 50, 100 and 200 mg/kg/day for 3 days (CPA and MCPA were given at 100, 200 and 300 mg/kg/day) and were killed 24 hrs later. * statistically significant (student's t-test) when $p < 0.05$. 
did not correlate well, in general, with the increase in total cytochrome P-450 content (Figs. 3.1 and 3.2).

Only 2,4-D treatment at 200mg/kg showed a decrease (11%) in liver/body weight ratio as compared to control (Fig. 3.1). The decrease in body weight and liver/body weight ratio after treatment with 2,4-D(200mg/kg) may be explained by the effect of the compound on the ability of the rats to move and feed. At the above dose level, 2,4-D treatment increased the total cytochrome P-450 content by 100% over the control levels (p < 0.05) (Fig. 3.2).

Total cytochrome P-450 content was increased significantly by all seven compounds in a dose-dependent manner (Fig. 3.2). The increase of total cytochrome P-450 content in liver microsomes at the highest dose level ranging from 148% (p > 0.05) in the case of CPA (300mg/kg) to 228% (p > 0.01) after treatment with 2,4,5-T (200mg/kg). The correlation between liver/body weight ratio and cytochrome P-450 content showed significance in the case of 2,4-DP, 2,4,5-T, 2,4,5-TP (Fig. 3.1 and 3.2): it should be noted that as in liver/body weight ratio, 2,4,5-T and 2,4,5-TP produced the highest increase in total cytochrome P-450 content (128%, 89% respectively) over the control level and the better correlation over the three dose levels.

Among the seven compounds tested, the greatest induction of ethoxyresorufin-O-deethylase activity (a marker substrate for cytochrome P-450 IA1) was obtained after MCPA treatment (Fig. 3.3).
Figure 3.2  
Influence of phenoxy acid on the total cytochrome P-450 content in liver microsomes.

Compounds were administered and animals killed as described in methods. CPA AND MCPA were given at doses of 100, 200 and 300 mg/kg. * p<0.05.
Figure 3.3  Influence of phenoxy acid on ethoxyresorufin-O-deethylase activity in liver microsomes.

Ethoxyresorufin-O-deethylase activity (nmol/min/mg protein)

Dose (mg/kg)

CPA and MCPA were given at doses of 100, 200 and 300 mg/kg and animals killed 24 hrs. later.  * p<0.05, ** p<0.01, *** p<0.001.
MCPA treatment (200mg/kg and 300mg/kg) increased ethoxyresorufin-O-deethylase activity significantly (2.2 - 2.4-fold) with statistical significance of p < 0.01. 2,4-D and 2,4,5-T at a dose of 200mg/kg also produced significant (1.8 and 1.6-fold respectively with p < 0.05) increase of the enzyme activity over the control levels (Fig. 3.3). Other compounds produced no significant changes in the enzyme activity over the different dose levels given. By contrast, none of the compounds tested had any significant influence on benzphetamine-N-demethylase activity (a marker substrate for cytochrome P-450 IIB1) (Fig. 3.4).

The structural similarity of these phenoxy acids to clofibrate suggested that they may have an influence on fatty acid metabolism. Since lauric acid was shown to be a suitable substrate for clofibrate-induced cytochrome P-450 IVA1 (Tamburini et al., 1984). I then investigated the ability of the above seven phenoxy acid compounds to induce lauric acid hydroxylase activity (namely, cytochrome P-450 IVA1 which is responsible for the 12-hydroxylation of lauric acid).

Upon the administration of the phenoxy acids to the rats, the liver microsomal laurate 12-hydroxylase activity was elevated by all seven compounds significantly at the highest dose level (Fig. 3.5). At the lowest dose of 50mg/kg, 2,4,5-TP produced the greatest induction (8.2-fold), reaching an apparent saturation level, whereas 2,4-DP showed about a 5-fold increase in the 12-hydroxylation of lauric acid. With the exception of the above two compounds, 2,4-D,
Figure 3.4 Effect of phenoxy acid on benzphetamine-\textsuperscript{14}C-\textsuperscript{N}-deethylase activity in liver microsomes.

- 2,4-D
- 2,4-DP
- 2,4-DB
- 2,4,5-T
- 2,4,5-TP
- MCPA
- CPA

CPA and MCPA were given at doses of 100, 200 and 300 mg/kg, and animals killed 24 hrs. later.
Figure 3.5 Induction of laurate 12-hydroxylase activity in liver microsomes by phenoxo acid.

CPA and MCPA were given at 100, 200 and 300 mg/kg, and animals killed 24 hrs. later. * p<0.05, ** p<0.01, *** p<0.001.
2,4-DB and 2,4,5-T showed a steady increase in the laurate 12-hydroxylation over the three dose levels (Fig. 3.5). The other two compounds, MCPA and CPA, given at dose levels of 100, 200 and 300mg/kg, as indicated in Methods, produced the highest induction (8.3-fold in the case of MCPA at 300mg/kg) and the lowest (3-fold) as in the case of CPA (Fig.3.5). At the highest dose levels, the 12-hydroxylation of lauric acid induction was statistically significant at $p < 0.01$ (2,4-D and CPA) and $p < 0.001$ (2,4-DP, 2,4,5-T, 2,4,5-TP and MCPA) as compared to the control value.

The 11-hydroxylation of lauric acid was also induced by several of the phenoxy acid tested (Fig. 3.6). Although the induction of this activity was substantially less marked than for the corresponding 12-hydroxylase activity, it was significantly induced by 2,4-DP (167%) ($p < 0.05$), 2,4,5-TP (264%) ($p < 0.001$), MCPA (237%) ($p < 0.01$) and CPA (251%) ($p < 0.01$) and statistically significant when compared to control. 2,4-D, 2,4-DB and 2,4,5-T showed no significant induction of 11-hydroxylation of lauric acid over the dose levels administered. From Figs. 3.5 and 3.6 it is clear that, with the exception of CPA, most inducers of 12-hydroxylase activity are also inducers of 11-hydroxylase activity with good correlation (Table 3.2 and 3.3).

In view of the fact that all seven compounds induced the microsomal 11- and 12-lauric acid hydroxylase activity (Figs. 3.5 and 3.6), the influence of these compounds on the amount of immunochemically-determined cytochrome P-450 IVA1 was investigated.
Figure 3.6 Influence of phenoxy acid pretreatment on the 11-hydroxylation of lauric acid in liver microsomes.

CPA and MCPA were given at 100, 200 and 300 mg/kg, and animals killed 24 hrs. later.  
* p<0.05, ** p<0.01, *** p<0.001.
As shown in Fig. 3.7, with the exception of CPA, all phenoxy acids substantially induced (3-5-fold) the level of this isoenzyme in rat liver microsomes. It is clear from the graph that, with the exception of 2,4,5-TP, the induction of cytochrome P-450 IVA1 was dose-dependent with a good linearity over the three dose levels. It is also clear that 2,4,5-TP produced the greatest increase of this isoenzyme as in the case of 12-hydroxylation of lauric acid (Fig. 3.5) at 200mg/kg dose level. As in Table 3.2 and 3.3 it is not surprising to see a good correlation between lauric acid 12-hydroxylation and the specific content of cytochrome P-450 IVA1 and also to note the statistically significant induction (p < 0.01 or p < 0.001) of this isoenzyme at the highest dose levels given (Table 3.1).

Using antibody raised against clofibrate-induced cytochrome P-450 IVA1, I blotted the control and test microsomal preparations against this antibody (Fig. 3.8). As can be seen from Fig. 3.8, a low constitutive level of the isoenzyme was identified in control liver, the magnitude of which was substantially increased in phenoxy acid-pretreated liver microsomal fractions at the top dose levels given. It is clear that the antibody used only reacts with a major protein band of a molecular weight corresponding to authentic cytochrome P-450 IVA1, thus lending further credence to the ELISA-derived values for cytochrome P-450 IVA1 given in Table 3.1 and Fig. 3.7. Taking this into account, I could not see any real increase in protein in the range of 51,500 molecular weight on SDS-PAGE 9Fig. 3.9).
Figure 3.7  
Specific cytochrome P-450IVa1 content in liver microsomes:  
Influence of phenoxy acid pretreatment.

ELISA analysis was performed as described in methods. CPA and MCPA were given at doses of 100, 200 and 300 mg/kg, and animals killed 24 hrs. later.

* p<0.05, ** p<0.01, *** p<0.001.
Figure 3.8 Western blot analysis for cytochrome P-450IVA1 of liver microsomal samples from rats treated with phenoxy acid herbicides.

Western blot analysis was carried out as described in methods. The samples were loaded as follows: track 1) and 7) 2pmol of pure rat cytochrome P-450IVA1; track 2) and 8), control; 3) 2,4,5-T; 4) 2,4,5-TP; 5) MCPA; 6) CPA; 9) 2,4-D; 10) 2,4-DFP and 11) 2,4-DB loaded at 5 pmol of total cytochrome P-450 content. Animals were pretreated with the top dose of each compound and killed as described in Materials and Methods. Control animals received peanut oil as described in Materials and Methods.
Samples were loaded as follows: tracks (1) and (10) standard molecular weight markers as described in Materials and Methods, track (2) control, (3) 2,4-D, (4) 2,4-DP, (5) 2,4-DB, (6) 2,4,5-T, (7) 2,4,5-TP, (8) MCPA and track (9) CPA loaded at 15 mg of microsomal protein. Test doses were as described in the legend to Figure 3.8.
Western blot analysis using antibody raised against 3-methylcholanthrene-induced cytochrome P-450 IA1 (Fig. 3.10) showed no difference between microsomes from control and treated animals which may indicate that the increase in ethoxyresorufin-deethylase activity (Fig. 3.3) with some of the phenoxy acids is only the result of direct activation of the enzyme protein and not the result of any increase in the amount of enzyme protein.

3.2.2 Kidney Microsomes

Different effects on the renal cytochrome P-450 system were observed after the treatment of rats with the individual phenoxy acid compounds (Table 3.4) at the highest dose level.

Kidney microsomal total cytochrome P-450 content was not induced by any of the compounds tested (Fig. 3.11) whereas 2,4-DB, 2,4,5-TP and MCPA treatment showed about 100% increase in ethoxyresorufin-O-deethylase activity over the control level (Fig. 3.12). The picture is similar to that of the ethoxyresorufin-O-deethylase activity in liver microsomes (Fig. 3.3) in showing that MCPA being the highest inducer (120% over control level). Similar compound-dependent effects can be seen with benzphetamine-N-demethylase activity in a manner comparable to that of liver microsomes (Fig. 3.13). Again MCPA showed 80% induction over the control whereas 2,4,5-T showed the highest induction with 100% over
Western blot analysis was carried out as described in methods. Track (1) contains 0.05 pmol of pure 3-MC inducible cytochrome P-450, tracks (2) control, (3) CPA, (4) MCPA, (5) 2,4-D, (6) 2,4-DP, (7) 2,4-DB, (8) 2,4,5-T and (9) 2,4,5-TP loaded at 5 pmol of total cytochrome P-450.
Figure 3.11  Total cytochrome P-450 content in kidney microsomes: Influence of phenoxy acid pretreatment.

Values are the mean of duplicate determinations on pooled samples derived from three or more animals pretreated with the weight dose of the compound (200 mg/kg) (CPA and MCPA at 300 mg/kg).
**Figure 3.12** Ethoxyresorufin-O-deethylase activity in kidney microsomes: influence of phenoxy acid pretreatment.

Dose levels are given in the legend to Fig. 3.11.
Figure 3.13. Benzphetamine-N-demethylase activity in kidney microsomes: Influence of phenoxy acid pretreatment.

Dose levels are given in the legend to Fig. 3.11.
control. Decrease in this enzyme activity was seen with 2,4,5-TP (65% of control) and to a lesser extent 2,4-DB.

Renal lauric acid 11- and 12-hydroxylation was also minimally affected by the phenoxy acetic acids. In contrast to the liver it is surprising to see a decrease in the laurate 12-hydroxylase activity with some of the compounds (CPA = 60% and 2,4,5-T = 68% of control) (Fig.3.14). None of the compounds increased the 11- or 12-hydroxylation of lauric acid (Fig. 3.14 and 3.15). There was a decrease in the 11-hydroxylation of lauric acid by some of the compounds, specifically 2,4-D, 2,4,5-T and CPA (53%, 73% and 67% of control respectively) (Fig. 3.15). This was made clearer by the ELISA analysis for the specific cytochrome P-450 IVA1 (Fig.3.16). It is clear that no significant change can be seen in the level of this isoenzyme in kidney microsomes. From these results with kidney microsomes, it was concluded that, unlike liver microsomes, the chlorophenoxy acids studied have no profound effect on the cytochrome P-450 mixed-function oxidase in the kidney microsomes (Table 3.4).

3.3. DISCUSSION

Although chlorophenoxy acid compounds have been documented to induce several hepatic enzyme activities (Vainio et al., 1983; Kawashima et al., 1984a; Vessey and Boyer, 1984; Hietanin et al., 1985; Lundgren et al., 1987a & b), their ability to influence the cytochrome P-450 dependent mixed function oxidase system is not so
Figure 3.14 Laurate 12-hydroxylase activity in kidney microsomes: Influence of phenoxy acid pretreatment.

Dose levels are given in the legend to Fig. 3.11.
Figure 3.15  Laurate 11-hydroxylase activity in kidney microsomes: Influence of phenoxy acid pretreatment.

Legend as in figure 3.11.
Figure 3.16 Influence of phenoxy acid on cytochrome P-450IVA1 content in kidney microsomes.

Legend as in figure 3.11.
well defined. In this study my results have shown that structurally-related chlorophenoxy acids are relatively specific inducers of the previously described, hypolipidaemic-induced cytochrome P-450 IVAl isoenzyme (Gibson et al., 1982; Tamburini et al., 1984; Bains et al., 1985). Looking at Table 3.1 it is clear that all seven compounds significantly increased the specific cytochrome P-450 IVAl isoenzyme at the top dose levels. This induction of specific cytochrome P-450 IVAl isoenzyme is coupled with a significant increase in laurate 12-hydroxylase activity (Figs. 3.5 and 3.7). For CPA, the significant increase in laurate 12-hydroxylase activity was not coupled with significant cytochrome P-450 IVAl induction. Thus, by using these chlorinated acids as an experimental tool, I have shown the close correlation that exists between lauric acid 12-hydroxylase activity and the amount of the cytochrome P-450 IVAl (Tables 3.2 and 3.3).

Although I have not attempted to define precisely a structure-activity relationship for the chlorophenoxy acids, it is apparent that a greater degree of chlorination in the aromatic ring results in a greater induction of laurate 12-hydroxylase activity (compare 2,4,5-T > 2,4-D > CPA and 2,4,5-TP > 2,4-DP) (Fig. 3.5) when considered on a mg/kg dose basis. These extensively chlorinated, potent inducers are even more potent still, when compared on a moles/kg basis (data not shown). Whether this apparent relative potency is a function of lipophilicity or pKa and therefore the 'true bio-availability' of the inducer or a preferred steric requirement of the inducer, is not clear from my studies, and
### Table 3.1

**Effects of Phenoxyacid Herbicides on Rat Liver Weight and Microsomal Cytochrome P-450**

The compounds (defined in Fig.1.3) were administered by gastric intubation once a day for 3 days at a dose level of 200 mg/kg. Each value represents mean ± S.D. Statistical significance in comparison with controls: *P<0.05; **P<0.01; P<0.001.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Liver/body wt. (%)</th>
<th>Total cytochrome P-450 (nmol/mg)</th>
<th>Specific P-450 IVAl nmol/nmol P-450 (%) of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>5.5 ± 0.3</td>
<td>0.80 ± 0.14</td>
<td>0.05 (6.8)</td>
</tr>
<tr>
<td>2,4-D</td>
<td>9</td>
<td>4.9 ± 1.0</td>
<td>1.60 ± 0.28</td>
<td>0.32 (20.2)**</td>
</tr>
<tr>
<td>2,4-DP</td>
<td>5</td>
<td>6.9 ± 0.2**</td>
<td>1.32 ± 0.02***</td>
<td>0.30 (22.5)**</td>
</tr>
<tr>
<td>2,4-DB</td>
<td>3</td>
<td>6.0 ± 0.2</td>
<td>1.44 ± 0.11**</td>
<td>0.22 (15.3)**</td>
</tr>
<tr>
<td>2,4,5-T</td>
<td>9</td>
<td>6.8 ± 0.3**</td>
<td>1.83 ± 0.3**</td>
<td>0.37 (20.3)**</td>
</tr>
<tr>
<td>2,4,5-TP</td>
<td>3</td>
<td>8.2 ± 0.5***</td>
<td>1.51 ± 0.23**</td>
<td>0.53 (34.6)**</td>
</tr>
<tr>
<td>MCPA</td>
<td>6</td>
<td>5.8 ± 0.5</td>
<td>1.27 ± 0.3*</td>
<td>0.23 (21.5)**</td>
</tr>
<tr>
<td>CPA</td>
<td>6</td>
<td>6.0 ± 0.8</td>
<td>1.18 ± 0.09*</td>
<td>0.07 (7.3)</td>
</tr>
</tbody>
</table>
remains a topic for further exploration.

The inter-relationship between the cytochrome P-450 dependent parameters induced by this class of compounds is clearly seen in the correlation matrices of Tables 3.2 and 3.3. For example, the correlation between 12-hydroxylase activity and specific cytochrome P-450 IVA1 induced by 2,4,5-TP is high (0.968), whereas the correlation between both benzphetamine N-demethylase and ethoxyresorufin-O-deethylase and cytochrome P-450 IVA1 is very low (0.181 and 0.315 respectively). Similarly, the lack of correlation between 12-hydroxylase and both ethoxyresorufin-O-deethylase and benzphetamine N-demethylase (0.086 and 0.058 respectively) indicates that these three substrates are preferentially metabolised by different isoenzymes of cytochrome P-450. This latter conclusion is consistent with previously published data on the substrate specificity of purified cytochrome P-450 IVA1, P-450 IIB1 and P-450 IAI in reconstituted enzyme systems (Tamburini et al., 1984). It is worth noting that an excellent correlation (0.975) was observed between cytochrome P-450 IVA1 content and lauric acid 11-hydroxylase activity after 2,4,5-TP treatment (Table 3.3). This observation may be rationalised by the fact that highly purified, electrophoretically homogeneous cytochrome P-450 IVA1 retains residual 11-hydroxylase activity (Gibson et al., 1982; Tamburini et al., 1984) and would thus feature strongly in the correlation matrix. However, it must be emphasised that cytochrome P-450 IVA1 preferentially hydroxylates lauric acid in the 12-position, as reflected in the 12:11 product ratio of approximately 10.1 (Gibson et al., 1982; Tamburini, 1984). It should be noted that all other
TABLE 3.2
CORRELATION MATRIX BETWEEN VARIOUS ISOENZYMES ACTIVITIES OF CYTOCHROME P-450 INDUCED BY PHENOXY ACIDS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(Laurate 12-hydroxylation/total cytochrome P-450)</th>
<th>Laurate 11-hydroxylation/total P-450</th>
<th>(Laurate 12-hydroxylation/cytochrome P-450 IVAL)</th>
<th>(Ethoxyreborufin-O-deethylase/total P-450)</th>
<th>(Total P-450/cytochrome P-450 IVAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>0.926</td>
<td>0.889</td>
<td>0.935</td>
<td>0.921</td>
<td>0.898</td>
</tr>
<tr>
<td>2,4-DP</td>
<td>0.968</td>
<td>0.958</td>
<td>0.811</td>
<td>-0.249</td>
<td>0.765</td>
</tr>
<tr>
<td>2,4-DB</td>
<td>0.644</td>
<td>0.159</td>
<td>0.761</td>
<td>0.633</td>
<td>0.504</td>
</tr>
<tr>
<td>2,4,5-T</td>
<td>0.988</td>
<td>0.271</td>
<td>0.960</td>
<td>0.660</td>
<td>0.919</td>
</tr>
<tr>
<td>2,4,5-TP</td>
<td>0.821</td>
<td>0.985</td>
<td>0.968</td>
<td>0.376</td>
<td>0.923</td>
</tr>
<tr>
<td>MCPA</td>
<td>0.974</td>
<td>0.979</td>
<td>0.940</td>
<td>0.848</td>
<td>0.947</td>
</tr>
<tr>
<td>CPA</td>
<td>0.505</td>
<td>0.319</td>
<td>0.702</td>
<td>0.232</td>
<td>0.404</td>
</tr>
</tbody>
</table>

Regression analysis was performed using a programmable calculator to obtain the multiple correlation co-efficient (r) between the mean of each group over the three dose levels (+ control).

Parameters are significant at 95% probability if the r value given in table is \( \geq 0.666 \).
TABLE 3.3
CORRELATION MATRIX FOR THE INTER-RELATIONSHIP BETWEEN MIXED FUNCTION OXIDASE PARAMETERS INDUCED BY 2,4,5-TP

The correlation coefficient relating any two parameters in a linear regression analysis is significant at 95% probability if the r value given in the Table is $\geq 0.666$. Abbreviations used (units) are: total P-450, total carbon monoxide discernible cytochrome P-450 (nmol/mg protein); EROD, ethoxyresorufin-O-deethylase activity (pmol product/min/mg protein); BZP, benzphetamine N-demethylase (nmol product/min/mg protein; 12-OHlase, lauric acid 12-hydroxylase activity (nmol product/min/mg protein); 11-OHlase, lauric acid 11-hydroxylase activity (nmol product/min/mg protein; specific P-450 IVAl (nmol cytochrome P-450 IVAl/nmol total cytochrome P-450).

<table>
<thead>
<tr>
<th></th>
<th>Liver/body wt.</th>
<th>Total P-450</th>
<th>EROD</th>
<th>BZP</th>
<th>12-OHlase</th>
<th>11-OHlase</th>
<th>Specific P-450 IVAl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver/body wt.</td>
<td>1</td>
<td>0.827</td>
<td>0.201</td>
<td>0.052</td>
<td>0.931</td>
<td>0.898</td>
<td>0.896</td>
</tr>
<tr>
<td>Total P-450</td>
<td>1</td>
<td>0.376</td>
<td>0.522</td>
<td>0.821</td>
<td>0.985</td>
<td>0.923</td>
<td></td>
</tr>
<tr>
<td>EROD</td>
<td>1</td>
<td>0.989</td>
<td>-0.058</td>
<td>0.086</td>
<td>0.498</td>
<td>0.367</td>
<td>0.181</td>
</tr>
<tr>
<td>BZP</td>
<td>1</td>
<td>1</td>
<td>0.907</td>
<td>0.367</td>
<td>1</td>
<td>0.975</td>
<td></td>
</tr>
<tr>
<td>12-OHlase</td>
<td>1</td>
<td></td>
<td>0.907</td>
<td>0.367</td>
<td>1</td>
<td>0.975</td>
<td></td>
</tr>
<tr>
<td>11-OHlase</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific P-450 IVAl</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
compounds used produced a significant correlation (higher than 0.666) between laurate 12-hydroxylase and cytochrome P-450 IVA1 (Table 3.2) with the exception of 2,4-DB and CPA, all other compounds produced good correlation between total cytochrome P-450 content and laurate 12-hydroxylase and also between total cytochrome content and specific cytochrome P-450 IVA1 as shown in Table 3.2.

My data indicates that some of the chlorinated phenoxy acids (particularly MCPA, 2,4-D and 2,4,5-T) may function as 'mixed inducers' of cytochrome P-450, in that ethoxyresorufin-O-deethylase activity (cytochrome P-450 IAl-dependent) is additionally induced. However, this is not true for all the compounds studied (Fig. 3.3). For the above active inducers, the extent of ethoxyresorufin-O-deethylase induction is clearly less (approximately 2-fold) than the 12-hydroxylase induction (approximately 8-fold) after 2,4,5-TP treatment. It is only 2,4,-D and MCPA that produced good correlation between total cytochrome P-450 content and ethoxyresorufin-O-deethylase (Table 3.2). By contrast to the above, cytochrome P-450 IIB1-dependent benzphetamine N-demethylase activity is not induced by any of the seven compounds tested even at the highest dose administered. Taken collectively, this information indicates that the cytochrome P-450 IVA1, P-450 IIB1 isoenzymes are differentially regulated by their corresponding inducers, a conclusion that is consistent with the available literature (Hardwick et al., 1987; Nebert et al., 1987).
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>2,4-D</th>
<th>2,4-DB</th>
<th>2,4-DB</th>
<th>2,4,5-T</th>
<th>2,4,5-TP</th>
<th>HCPA</th>
<th>CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cytochrome P-450 content (nmol/mg)</td>
<td>0.12±0.02</td>
<td>0.13±0.01</td>
<td>0.15±0.04</td>
<td>0.15±0.02</td>
<td>0.13±0.03</td>
<td>0.13±0.04</td>
<td>0.11±0.03</td>
<td></td>
</tr>
<tr>
<td>Ethoxyresorufin-O-deethylase (Pmoles/mg/min)</td>
<td>11.1±4</td>
<td>15.1±5</td>
<td>15.8±3</td>
<td>22.0±7</td>
<td>15.1±2</td>
<td>19.9±4</td>
<td>24.1±4</td>
<td>11.7±3</td>
</tr>
<tr>
<td>Benzphetamine-N-demethylase (nmole/mg/min)</td>
<td>1.85±0.7</td>
<td>2.73±0.8</td>
<td>1.81±0.5</td>
<td>1.66±0.5</td>
<td>3.68±0.7</td>
<td>1.21±0.4</td>
<td>3.35±0.8</td>
<td>1.85±0.5</td>
</tr>
<tr>
<td>Lauric acid hydroxylation (nmole/mg/min)</td>
<td>3.54±1.5</td>
<td>3.46±0.9</td>
<td>3.66±1</td>
<td>4.21±1.5</td>
<td>2.43±1</td>
<td>3.35±1</td>
<td>3.91±0.9</td>
<td>2.12±1.1</td>
</tr>
<tr>
<td>12-hydroxy</td>
<td>2.02±1</td>
<td>1.06±0.4</td>
<td>2.37±0.7</td>
<td>2.31±0.5</td>
<td>1.48±0.4</td>
<td>2.33±0.2</td>
<td>2.51±0.8</td>
<td>1.36±0.9</td>
</tr>
<tr>
<td>Specific cytochrome P-450 IVaL: nmol/nmol P-450 (% of total)</td>
<td>0.027</td>
<td>0.020</td>
<td>0.030</td>
<td>0.036</td>
<td>0.029</td>
<td>0.026</td>
<td>0.029</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Each value represents the mean of 2-3 determination of 1-3 pools of kidney microsomes from rats given the highest dose levels (200 mg/kg/day except for MCPA and CPA which were given at 300 mg/kg).
For kidney microsomes, no statistical analysis could be completed on the data due to an insufficient number of kidney samples (one microsomal pool per dose level). From Table 3.4 there was no clear pattern of increase or decrease in the enzyme activity tested. It is only with benzphetamine-N-demethylase and ethoxyresorufin-O-deethylase activity that some of the parameter was increased to a maximum of 2-fold. The only significant result that can be noted is that the kidney microsomal content of cytochrome P-450 IVA1 (22.5% of total P-450) is much higher than that of liver microsomes (6.8% of P-450) at the control level. From this I conclude that the chlorophenoxy acids studied, after their metabolism in the liver (or the parent compound) have no apparent effect on the kidney parameters tested, which may be explained, in part, by the lack of either uptake or renal cellular bioavailability of the inducer.

In conclusion, I have shown that chlorophenoxy acids are relatively specific inducers of the cytochrome P-450 IVA1-dependent, lauric acid 12-hydroxylase enzyme. In view of the structural similarity of these chlorinated phenoxy acids to clofibrate and their ability to produce peroxisomal proliferation in rodent liver (Vainio et al., 1983; Kawashima et al., 1984; Heitanin et al., 1985), they may share a common mode of induction with the oxyisobutyrate class of peroxisome proliferators. This also suggests that my results and previous results using these phenoxy acid class of compounds can fit into the scheme proposal by Sharma et al., 1988a, in which an hypothesis is presented whereby an early
biological response in the hypolipidaemic induction of microsomal cytochrome P-450 IVA1 resulting in ω-hydroxy fatty acids and their subsequent further oxidation to dicarboxylic acids, the latter providing the peroxisomal stimulus for peroxisomal proliferation.
CHAPTER FOUR

EFFECT OF PRETREATMENT WITH CHLOROPHENOLS ON RAT HEPATIC AND KIDNEY CYTOCHROME P-450 MIXED FUNCTION OXIDASE ACTIVITIES
4.1 INTRODUCTION

In view of the fact that chlorinated phenoxy acids preferentially induced fatty acid hydroxylation and cytochrome P-450 IVAI in rat liver microsomes (Chapter 3). It was decided to investigate the possibility of post-metabolism involvement of derivatives of these phenoxy acids in the induction process, namely the corresponding phenols, and four phenols, 4-chlorophenol (CP), 2,4-dichlorophenol (DCP), 2,4,5-trichlorophenol (TCP) and 4-chloro-2-methylphenol (MCP) Fig.4.1 were chosen for study. These were used in this study to determine their possible inductive effect on the cytochrome P-450 system in rat liver and kidney microsomes, as they may possibly be formed from the corresponding chlorophenoxy acids. Although alkyl carboxylic acids may also be theoretically produced from the parent compounds, these carboxylic acids were not investigated further.

Although phenolic compounds are well known to be excreted in urine by man and many other species (Williams 1969), the effect of phenols, and in particular chlorinated phenols on the cytochrome P-450 system is not very well documented. The metabolism of phenols has been studied quantitatively and qualitatively by Capel et al (1972 a,b) and Deichmann and Keplinger (1981). The metabolism of phenolic compounds and their excretion as phenyl glucuronic acid and
Figure 4.1 Chemical structure of phenols studied.

4-Chlorophenol (CP)

2,4-Dichlorophenol (DCP)

2,4,5-Trichlorophenol (TCP)

4-Chloro-2-methylphenol (MCP)
phenyl sulphate metabolites have been described by Kao et al (1979), with phenol sulphotransferase being the enzyme catalysing the sulphate conjugation of phenols (Weinshilboum, 1986).

Chlorophenols (the subject of this study) are used primarily as pesticides and as intermediates in the production of pesticides (Freiter, 1979). 4-chlorophenol and 2,4-dichlorophenol are used principally as an intermediate in the production of herbicides such as CPA, 2,4-D, 2,4-DB, 2,4-DB and related compounds, whereas 4-chloro-2-methylphenol is used in the manufacture of MCPA.

The major use of 2,4,5-trichlorophenol (TCP) has been as an intermediate in the manufacture of industrial and agricultural chemicals, including 2,4,5-T and 2,4,5-TP. 2,4,5-trichlorophenol and its salts have been used as preservatives in the textile industry, in the adhesives industry for polyvinyl acetate emulsions, in the leather industry and in the automotive industry for preservation of rubber gaskets. The water soluble sodium salts have been used to preserve casein-derived adhesives, to preserve and stabilize metal cutting fluids and as an antimicrobial agent in cooling tower water and in pulp and paper mills (Freiter 1979).

To establish whether the induction of cytochrome P-450 system as described in chapter 3 is the result of the phenoxyacids themselves or a result of a possible phenol formation, I investigated the effect of four phenol compounds (corresponding to the phenoxyacids) on the
cytochrome P-450 system in liver and kidney microsomes of Wistar Albino rats.

4.2 RESULTS

4.2.1. Liver Microsomes

As described in methods, the phenols: (4-chlorophenol, 2,4-dichlorophenol, 2,4,5-trichlorophenol and 4-chloro-2-methylphenol (Fig. 4.1),) were given to rats at doses of 50, 100 and 200 mg/kg/day for three days. None of the phenols produced any effect on body weight gain and on liver/body weight ratio (Fig. 4.2) as compared to control. Signs of liver enlargement were therefore not observed, an indication of the absence of hepatomegaly produced by some of the chlorinated phenoxyacids previously studied (Chapter 3).

Total content of cytochrome P-450 in liver microsomes was not induced by any of the phenols studied. In contrast, there was a slight but significant decrease after DCP and TCP treatment (90% of control) at 200 mg/kg/day for three days (Fig. 4.3). At the lower dose of 50 mg/kg, CP, TCP and MCP, treatment produced a 10-15% decrease in total cytochrome P-450 content. None of the phenol treatments showed a consistency in decreasing the total cytochrome P-450 content.
Figure 4. Effect of phenol pretreatment on liver/body weight ratio.

Phenols were administered orally at 50, 100 and 200 mg/kg/day for three days. Each data point is the mean of at least three animals.
Figure 4.3  Effect of Phenol pretreatment on cytochrome P-450 total content in liver microsomes.

Each point represents the mean of at least three individual animals.

Cytochrome P-450 total content (nmol/mg protein)
Figure 4.4 Influence of phenol pretreatment on benzphetamine-N-demethylase activity in liver microsomes.

Benzphetamine-N-demethylase activity (nmol/min/mg protein)

Dose (mg/kg)

*p < 0.05, **p < 0.01.
The activity of cytochrome P-450 IIB1 was measured by means of using benzphetamine as the substrate. Benzphetamine-N-demethylase was significantly reduced by some of the phenols treatment (Fig.4.4). The most effective phenol in reducing the enzyme activity was CP in a dose-dependent manner. At the highest dose level (200 mg/kg), CP, DCP and TCP (P<0.01 and P<0.001) reduced significantly the enzyme activity (32%, 25% and 77% of control respectively). Although MCP significantly reduced benzphetamine-N-demethylase activity at 50 mg/kg and 100 mg/kg (80% and 76% of control respectively), 200 mg/kg treatment resulted in no effect on the enzyme activity. For ethoxyresorufin-O-deethylase activity, as a marker for cytochrome P-450 IA1 and P-450 IA2, the effect is quite the opposite (Fig.4.5). All of the four phenol compounds significantly induced this enzyme activity. The most potent inducer was MCP over the three dose levels. At 200 mg/kg dose level MCP induced the ethoxyresorufin-O-deethylase activity significantly (262% over the control, P<0.001) whereas CP was the least active inducer (145% over the control, P<0.001). Overall, the induction of this latter enzyme over the three dose levels gave a good correlation in regard to induction/dose relationship (Fig.4.5).

Unlike their chlorephenoxy acid analogues the immunochemically determined specific cytochrome P-450 IVA1 concentrations as determined by ELISA showed no significant change from that of control after the phenol pre-treatment (Fig.4.6). This conclusion was supported by Western blot analyses of the microsomal fractions derived from the livers of phenol-pre-treated animals. As shown in
Figure 4.5 Influence of phenol pretreatment on ethoxyresorufin-o-deethylase activity in liver microsomes.

- CP
- DCP
- TCP
- MCP

Ethoxyresorufin-O-deethylase activity (pmol/min/mg protein)

Dose (mg/kg)

*p < 0.05, **p < 0.01.
Figure 4.6 Effect of phenol pretreatment on cytochrome P-450IVA1 specific content in liver microsomes.

Cytochrome P-450IVA1 specific content (% of total P-450) vs Dose (mg/kg)

Control=0.04 nmols P-450IVA1/nmol P-450.
Western blot analysis and dosing was carried out as described in methods. The samples were loaded as follows: track (1) 2 pmol of electrophoretically pure rat P-450Iva1, (2) control, (3) CP, (4) DCP, (5) TCP and (6) MCP, loaded at 5 pmol of total cytochrome P-450. Control animals received peanut oil.
Fig. 4.7, when the microsomal preparations were blotted against the cytochrome P-450 IVAI antibody, the magnitude of the isoenzyme level identifiable in the control was quite similar to that of phenol-pretreated liver microsomal fractions. By contrast, the associated cytochrome P-450 IVAI-driven lauric acid hydroxylase is slightly induced by some of the compounds. Laurate 12-hydroxylase activity was only marginally induced by pretreatment with CP and MCP (Fig. 4.8). There was an irregular increase and decrease in the enzyme activity over the different dose levels after the pretreatment with CP, DCP and TCP. Similarly laurate 11-hydroxylase activity was induced by some of the compounds (Fig. 4.9). MCP was shown to be the highest inducer (165% of control, P<0.01), whereas DCP showed a slight but not significant decrease (88% of control) of enzyme activity. None of the enzyme activities could be correlated with each other.

4.2.2 Kidney microsomes

The effect of phenols administration on total cytochrome P-450 content is shown in Fig. 4.10. A small decrease (80% of control) in the total content of the enzyme was seen after MCP treatment and less (85%) with TCP treatment. CP and DCP showed no apparent change in the total cytochrome P-450 content. The catalytic activities of phenol-treated kidney microsomes showed differing effects on the dealkylation of both benzphetamine and ethoxyresorufin, good markers for the cytochrome P-450 IIBI and P-450 IAI isoenzymes respectively. Benzphetamine-N-demethylase activity in kidney microsomes was
Figure 4.8
Influence of Phenol Pretreatment.

nmol/min/mg protein

Lauaret 12-hydroxylyase activity
Figure 4.9 Laurate 11-hydroxylase activity in liver microsomes: Influence of phenol pretreatment.

Dose (mg/kg)

* p < 0.05, ** p < 0.01.

Laurate 11-hydroxylase activity (nmol/min/mg protein)
Each value represents the mean of duplicate determination on pooled samples derived from three individual animals of the highest dose level (200 mg/kg).
Figure 4.11 Benzphetamine-N-deethylation activity in kidney microsomes: Influence of phenol pretreatment.

Benzphetamine-N-demethylase activity (nmol/min/mg protein)

---

Legend as in figure 4.10.
slightly reduced (78% of control) by TCP and MCP at the highest dose level (200 mg/kg) (Fig.4.11), whereas only CP showed some increase in the enzyme activity (149% of control at a dose of 200 mg/kg). With respect to ethoxyresorufin-O-deethylase activity only MCP produced a noticeable increase in the enzyme activity (204% of control) (Fig.4.12). TCP reduced this latter enzyme activity to 67% of control, whereas CP and DCP showed a slight increase at the top dose level.

Specific cytochrome P-450 IVA1 isoenzyme levels in kidney microsomes from control animals were shown to be 25% of the total cytochrome P-450 population (Fig.4.13) and none of the phenols studied showed any real effect on these isoenzyme levels. In addition to the above, with the exception of MCP, none of the phenols studied had any noticeable effect on the laurate 12-hydroxylase activity (Fig.4.14,15). MCP showed a slight decrease in the laurate-12-hydroxylase activity (80% of control). This is also the case with laurate 11-hydroxylase activity (Fig.4.16) where only DCP showed some increase (190% of control) in the enzyme activity whereas TCD and MCP reduced the enzyme activity (75% of control) at the top dose level of 200 mg/kg.
Figure 4.12 Ethoxyresorufin-O-deethylase activity in kidney microsomes: influence of phenol pretreatment.

Legend as in Fig. 4.10. No statistical comparison shown because pooled kidney samples were used.

( pmol/min/mg protein)
Figure 4.13 Influence of phenol pretreatment on cytochrome P-450IVA1 specific content in kidney microsomes.

Control = 0.03 nmol P-450IVA1/nmol P-450.
Western blot analysis was performed as described in methods. Tracks represent: (1) standard (2 pmol pure cytochrome P-450IVA1), (2) control; (3) CP; (4) DCP; (5) TCP; and (6) MCP loaded at 5 pmol of total cytochrome P-450.
Figure 4.15 Laurate 12-hydroxylase activity in kidney microsomes: Influence of phenol pretreatment.

Legend as in figure 10.
Figure 4.16 Laurate 11-hydroxylase activity in kidney microsomes: Influence of phenol pretreatment.

Laurate 11-hydroxylase activity

nmol/min/mg protein

Dose (mg/kg)
TABLE 4.1
EFFECT OF CHLORINATED PHENOLS ON LIVER SIZE, CYTOCHROME P-450 CONTENT, BENZPHETAMINE-N-DEMETHYLASE, ETHOXYRESORUFIN-O-DEETHYLASE AND CYTOCHROME P-450 IVAl. COMPOUNDS IN PEANUT OIL WERE ADMINISTERED BY GAVAGE FOR 3 DAYS AT 200 mg/kg/DAY. CONTROLS RECEIVED 5 ml/kg PEANUT OIL. VALUES ARE MEANS ± S.D. (3 INDIVIDUAL ANIMALS). SIGNIFICANT DIFFERENCES (STUDENT'S T TEST) VERSUS CONTROL.

* = P<0.05  ** = P<0.01  *** = P<0.001.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body wt.</th>
<th>Liver wt. (n mol/mg)</th>
<th>Total P-450 (nmol/min/mg)</th>
<th>Bnz.</th>
<th>EROD (Pmol/min/mg)</th>
<th>Specific cyt. P-450 IVAl (nmol/mg)</th>
<th>P-450 IVAl (% of total P-450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut oil (control)</td>
<td>6.0 ± 0.30</td>
<td>0.81 ± 0.07</td>
<td>2.7 ± 0.2</td>
<td>23.5 ± 4</td>
<td>0.040 ± 0.01</td>
<td>5.01</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>4.5 ± 0.4 *</td>
<td>0.88 ± 0.12</td>
<td>0.43 ± **</td>
<td>33.9 ± 9</td>
<td>0.044 ± 0.02</td>
<td>5.22</td>
<td></td>
</tr>
<tr>
<td>DCP</td>
<td>6.3 ± 0.8</td>
<td>0.73 ± 0.03</td>
<td>2.29 ± 0.4</td>
<td>49.8 ± 8</td>
<td>0.041 ± 0.02</td>
<td>5.01</td>
<td></td>
</tr>
<tr>
<td>TCP</td>
<td>6.5 ± 0.4</td>
<td>0.72 ± 0.07 *</td>
<td>2.07 ± **</td>
<td>38.7 ± 7</td>
<td>0.035 ± 0.02</td>
<td>5.25</td>
<td></td>
</tr>
<tr>
<td>MCP</td>
<td>5.2 ± 0.3</td>
<td>0.79 ± 0.08</td>
<td>2.76 ± 0.3</td>
<td>61.6 ± 8.5</td>
<td>0.041 ± 0.01</td>
<td>5.15</td>
<td></td>
</tr>
</tbody>
</table>
4.3. DISCUSSION

The data presented in this chapter demonstrate the lack of real effect of chlorinated pre-treatment on the cytochrome-P-450 mixed-function oxidase system in rat liver and kidney microsomes. In the previous study (Chapter 3) I showed that some of the chlorinated phenoxyacids preferentially induced the cytochrome P-450 IVAI isoenzyme in the rat liver microsomes in a magnitude which could not be achieved by pre-treating the rats with the corresponding chlorinated phenols. This may suggest that either chlorophenoxyacids directly induce the activity of cytochrome P-450 IVAI in liver microsomes or that a different phenol metabolite is involved.

As can be seen in table 4.1 and 4.2, total cytochrome P-450 content, cytochrome P-450 IIB1 and cytochrome P-450 IA1 was not induced significantly in rat liver and kidney microsomes as a result of chlorophenol pre-treatment. An important finding is the lack of induction of cytochrome P-450 IVAI in the rat liver microsomes (Fig. 4.6-9) which suggests that chlorinated phenols are not involved in the induction of this particular isoenzyme (Chapter 3). This may be expected since earlier reports on the metabolism of chlorinated phenoxyacids in animals and man suggest that a very high percentage of the compounds are excreted in the urine unchanged (Piper et al 1973, Gehring et al 1973, Elo 1976).

Sharma et al (1988a) presented a scheme whereby a relationship between the induction of cytochrome P-450 IVAI and peroxisomal P-oxidation has been proposed. In this scheme the induction of
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total P-450 (nmol/mg)</th>
<th>Bnz (nmol/min/mg)</th>
<th>EROD (pmol/min/mg)</th>
<th>Laurate hydroxylation (11-OH) (pmol/min/mg)</th>
<th>Laurate hydroxylation (12-OH) (pmol/min/mg)</th>
<th>Specific cyt. P-450 (nmol/min/mg)</th>
<th>P-450 IVA1 (% of total P-450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut oil (control)</td>
<td>0.11</td>
<td>1.11</td>
<td>12.1</td>
<td>1.86</td>
<td>5.7</td>
<td>0.028</td>
<td>25</td>
</tr>
<tr>
<td>CP</td>
<td>0.12</td>
<td>1.64</td>
<td>13.4</td>
<td>2.1</td>
<td>7.5</td>
<td>0.028</td>
<td>23.3</td>
</tr>
<tr>
<td>DCP</td>
<td>0.11</td>
<td>1.17</td>
<td>13.1</td>
<td>3.5</td>
<td>5.6</td>
<td>0.030</td>
<td>27.2</td>
</tr>
<tr>
<td>TCP</td>
<td>0.1</td>
<td>0.86</td>
<td>8.1</td>
<td>1.4</td>
<td>4.9</td>
<td>0.025</td>
<td>25</td>
</tr>
<tr>
<td>MCP</td>
<td>0.09</td>
<td>0.86</td>
<td>24.7</td>
<td>1.3</td>
<td>4.0</td>
<td>0.020</td>
<td>22.2</td>
</tr>
</tbody>
</table>
cytochrome P-450 IVA1 by hypolipidaemic drugs is proceeding, followed by an increase in the medium and long chain fatty acids and this provides a substrate for the increase in peroxisomal and mitochondrial β-oxidation. The chlorinated phenoxyacids studied in the previous chapter can be fitted in this scheme since they significantly induced cytochrome P-450 IVA1 and were previously shown to induce peroxisomal β-oxidation (Vaino et al 1983, Kawashima et al 1984a and b, Hietanen et al 1985). In regard to chlorinated phenols, there is no significant induction of this isoenzyme, with the lack of reports of peroxisomal β-oxidation induction which rule out the possibility of fitting into this scheme.

It is surprising to see a real decrease in liver microsomal benzphetamine-N-demethylase activity after CP treatment. This isoenzyme activity was reduced in a dose-dependent manner reaching 32% of control at the highest dose level (Fig.4.4). None of the other compounds had any significant effect on this isoenzyme in liver or kidney microsomes. It is not clear why such a drop in the catalytic activity of cytochrome P-450 IIB1 occurs after CP treatment. Cytochrome P-450 IA1 was significantly induced at the highest dose levels by MCP and DCP and to a lesser extent by TCP and CP. This picture is similar to that seen with chlorophenoxyacids treatment. It is possible that chlorophenol and chlorophenoxyacid induction of cytochrome P-450 IA1 is the result of further metabolism of these compounds to monomeric and dimeric compounds, including catechol and quinone metabolites similar to that seen with TCP metabolism by rat liver S-9 fractions (Butte et al 1988).
In conclusion, the pre-treatment of rats with chlorophenols showed no real effect on liver and kidney microsomal cytochrome P-450 IIB1 and P-450 IVA1. The small induction of cytochrome P-450 IA1 seen after chlorophenol and phenoxy acid treatment is not compatible with the large induction seen with typical inducers of this isoenzyme such as 3-methylcholanthrene and this may be attributed to a common contaminant or metabolite in some of these compounds or indeed be simply reflective of a low potency of the parent compound itself.
CHAPTER FIVE

EFFECT OF CHLOROPHENOXYACID PRE-TREATMENT ON IN VIVO DRUG METABOLISM IN THE RAT
CHAPTER 5

EFFECT OF CHLOROPHENOXYACID PRE-TREATMENT ON IN VIVO DRUG METABOLISM IN THE RAT

5.1 INTRODUCTION

In rat as well as in man, antipyrine is almost completely metabolised by hepatic cytochrome P-450, with only a few percent of the dose being excreted unchanged in the urine (Brodie and Axelrod, 1950). The excretion into urine of unchanged antipyrine and the three major metabolites, 4-hydroxyantipyrine (OHA), 3-hydroxy-methylantipyrine (HMA) and norantipyrine (NORA) (Fig. 5.1), accounts for 65-70% of the dose (Danhof et al, 1979a and 1982), and has therefore been proposed as a probe for the drug oxidation enzymes in animals and man. Several groups have investigated the effects of induction of the cytochrome P-450 system with inducers such as phenobarbital and 2-naphtoflavone on antipyrine metabolism patterns in rat urine (Danhof et al, 1979a and b, 1982, Bottcher et al, 1982a and b, Rhodes and Houston 1982, Teunissen et al, 1983) and in cultured rat hepatocytes (Chenery et al, 1987) and in rat liver microsomes (Dupont et al, 1987) in the presence of monoamine oxidase inhibitors. All of these authors found selective changes in the metabolite profile as a consequence of induction, suggesting that several isoenzymes of cytochrome P-450 metabolise antipyrine. For example, the formation of HMA is associated with the phenobarbital-inducible type of cytochrome P-450 (P-450 11A1), that of OHA with the 3-methylcholanthrene-
inducible type (P-450 1A1), while NORA is probably also formed by a 3-methylcholanthrene-inducible type of cytochrome P-450. Hence the assessment of rates of formation of antipyrine metabolites enables the quantitation of different drug-oxidizing enzymes in one test. This topic was reviewed recently by Poulsen and Loft (1988), Parke and Kitteringham (1988) and Breimer et al (1984).

Measurement of both barbiturate sleeping time and zoxazolamine paralysis time have frequently been used to distinguish between the activities of phenobarbital and 3-methylcholanthrene-inducible cytochrome P-450 activity (cytochrome P-450 IIIB1 and P-450 IAl respectively) (Ioannides and Parke 1973, Wiebel et al 1976, Tomazewski et al 1976, Krevsky and Hitchcock 1977, Smith et al 1973, Graham et al 1981, Hatanaka et al 1988a and Van der Graaff et al 1988). Therefore, like antipyrine, pentobarbital and zoxazolamine (Fig. 5.1) have been used as model substrate to study the in vivo drug metabolism and the influence of cytochrome P-450 induction on its metabolism.

The aim of the study reported in this chapter was to assess the influence of chlorophenoxy acids treatment on the in vivo metabolism of antipyrine, pentobarbital and zoxazolamine in the rat and to correlate the results with the induction data reported in chapter three.
Figure 5.1 The chemical structure of antipyrine, zoxazolamine and pentobarbital and major metabolites

Antipyrine

Norantipyrine (NORA)

3-Hydroxymethylantipyrine (HMA)

4-Hydroxyantipyrine (OHA)

Zoxazolamine

6-Hydroxyzoxazolamine

Pentobarbital

Pentobarbital Alcohol
5.2 Results
5.2.1 Antipyrine metabolism

Initially I selected four compounds to study their effects on antipyrine metabolism: CPA, 2,4-DP, 2,4,5-T and 2,4,5-TP, given at 200 mg/kg/day to rats for 3 days. Treatment of animals with 2,4,5-T and 2,4,5-TP affected their mobility in the cages so that their food and water intake diminished and a low urine excretion was noted over 24 hours and a great decrease in body weight, necessitating that the animals were killed before the end of the experiment. The same effect was not observed with the control group or other pre-treatments. Urine collection was started 24 hours before antipyrine dosing and this was taken as a background reading to see the presence of peaks that may coincide with the metabolite peaks which can be detected by the HPLC system. Antipyrine (70 mg/kg) was given to the animals 24 hours after the last treatment and urine collection started immediately for 24 hours. Sodium pyrosulphite as an anti-oxidant was added to the urine samples and stored at -20°C until used for analysis.

The metabolites of antipyrine are excreted in urine mainly as conjugates (Bottcher et al 1982a). Therefore enzymatic hydrolysis with glucuronidase-sulphatase was performed prior to the metabolite assay, since it resulted in complete cleavage of the conjugates in the presence of sodium pyro-sulphite (16
mg/ml) as an antioxidant to prevent decomposition of metabolites.

The extraction and separation procedures were performed as described in methods. Evaluation of the chromatograms were based on peak height as calculated by the internal standard method (phenacetin). The identification of the peaks was based on the method described by Teunissen et al (1983) and the comparison of the chromatographic charts of urine samples before and after antipyrine dose (Fig. 5.2a,b). As shown in Fig.5.2a,b the extra peak in CPA-treated rats made it difficult to identify a peak corresponding to HMA.

Both compounds used (CPA and 2,4-DP-200 mg/kg/day for 3 days) significantly increased urine volume excreted over the 24 hours (8.5±3 and 16-7 ml compared to control 6.8 ml per 24 hours). The effect of CPA and 2,4-DP treatment on antipyrine metabolism were compared to control. From Fig. 5.3, a general decrease in the antipyrine metabolites detected in urine of CPA and 2,4-DP pre-treated rats were observed. It is clear that 2,4-DP had the greater effect on OHA, NORA and HMA formation when 24 hours urine volume was taken into consideration (Fig. 5.4). As for OHA (Fig.5.5), the availability of the compound enabled us to draw a standard curve and to evaluate its metabolism in mg excretion in urine. It is quite clear that there was a small decrease in OHA excreted per ml. urine when compared to control (91% and 80% of control after CPA and 2,4-
Figure  Influence of CPA and 2,4-DP pretreatment on antipyrine metabolism.

Chromatograms represent the HPLC separation chart of urine samples collected from rats (over 24 hrs period) pretreated with CPA and 2,4-DP (200mg/kg) and from control rats (received 5ml/kg peanut oil). (A) represents urine samples taken prior to antipyrine dosing and (B) represent urine samples collected after antipyrine dose (70mg/kg). 0.5ml of urine was used in the HPLC separation and the metabolites; HMA (1), NORA (2), OHA (3), antipyrine (4) and the internal standard (5) was identified essentially as described by Teunissen et al. (1983).
Figure 5.3 Influence of chlorophenoxy acid pretreatment on the in vivo metabolism of antipyrine.

Legend:
- [ ] CONTROL
- [ ] CPA
- [ ] 2,4-OP

AMOUNT OF METABOLITE

Bars represent the mean of 3 animals.

(a) no data can be estimated due to the presence of a secondary peak at the same distance (see figure 5.2). *p<0.05, **p<0.01.
Figure 5.4  Influence of chlorophenoxy acid pretreatment on the in vivo metabolism of antipyrine.

Bars represent the mean of 3 animals (peak height ratio).
(a) no data can be estimated due to the presence of a secondary peak at the same distance (see Figure 5.2). *p<0.05.
Bars are the mean±S.D of three individual animals. Values were read from the standard curve. **p<0.01.
DP treatment respectively), and showed a slight increase when adjusted to the total urine volume of 24 hours collection (114% and 188% of control). This is because of the increase in urine volume excreted in CPA and 2,4-DP treated rats.

Fig. 5.4 shows the 24 hours elimination of OHA, NORA and HMA. It is clear that the 24 hours elimination levels differ from the units/ml (peak height ratio) (Fig. 5.3). Only 2,4-DP showed a significant increase in OHA elimination in 24 hours urine collection, (188% of control, p 0.01) (28.6 ug/ml urine). Although NORA and HMA showed a slight decrease from the control levels, it was not found to be statistically significant due to the high difference in individual elimination.

5.2.2 Pentobarbital Sleeping time

A pilot experiment was done to compare the effect of 2,4-D, 2,4,5-T (200 mg/kg) and clofibrate (250 mg/kg) on pentobarbital sleeping time (Fig. 5.6). Rats were divided into groups (10 animals per group) and were treated with the appropriate compound for three days as described in Methods. Control rats were given peanut oil (5 ml/kg), pentobarbital 30 mg/kg was administered 24 hours after the last treatment. Sleeping time was recorded as described in Methods. As can be seen in Fig. 5.6, pentobarbital treatment showed a sleeping time of 38+9 minutes in the control group whereas 2,4-D and
Fig. 5.6 Influence of chlorophenoxy acid pretreatment on pentobarbital sleeping time.

![Graph showing sleeping time for different treatments](image)

**TREATMENT (200mg/kg)**

Ears are the mean ±S.D. of three individual animals given 200 mg/kg/day for 3 days of the test compound. Control received peanut oil. Pentobarbital (30 mg/kg) was administered 24 hrs. after the last dose. p<0.05 (*)
clofibrate pre-treatment significantly prolonged the sleeping time (142% and 129% respectively of control). It was noted that the 2,4-D pretreated group was not as healthy as the other groups and this may be a significant factor in prolonging the sleeping time. 2,4,5-T treatment showed no significant change in pentobarbital sleeping time. These results may be taken in comparison with the lack of induction of cytochrome P-450 IIBI seen in chapter 3.

5.2.3 Zoxazolamine paralysis time

Treatment of rats with zoxazolamine (70 mg/kg) (Fig. 5.7), a muscle relaxant which causes temporary paralysis, resulted in 92 minutes of paralysis time in the control group (average of 10 animals). When compared to the control group 2,4-DP and 2,4,5-TP, pre-treatment significantly reduced the sleeping time to 67% and 48% of control respectively. It is only 2,4-D pre-treatment which prolonged markedly the paralysis time - 44% over the control level.

This may suggest that 2,4-DP and 2,4,5-TP induced zoxazolamine metabolising enzyme (cytochrome P-450 IAI). This suggestion is not in line with our previous results: chapter 3 which suggested that these compounds have no clear effect on the ethoxyresorufin-O-deethylase activity. One might expect that MCPA pre-treatment may induce zoxazolamine metabolism and
Fig. 5.7  Influence of chlorophenoxy acid on zoaxazolamine paralysis time in the rat.

Compounds were given at 100, 200, and 300 mg/kg/day for 3 days. Zoaxazolamine was given at 70 mg/kg 24 hrs. after the last dose. Bars represent the mean +S.D. of 8-10 individual animals  *p<0.05,  ** p<0.01,  *** p<0.001. Treatment was: (A) control, (B) 2,4-D, (C) 2,4-DB, (D) 2,4-DP, (E) 2,4,5-T, (F) 2,4,5-TP, (G) MCPA, and (H) CPA.
hence shorter paralysis time, based on our previous results, but that was not shown. CPA, 2,4-DB and 2,4,5-T pre-treatment did not show any significant effect on the zoxazolamine paralysis time.

5.3. Discussion

To compare the induction of in vitro drug metabolism, (seen in chapter 3), and in vivo drug metabolism, three substrates commonly used to investigate drug metabolism in vivo, namely, antipyrine, pentobarbital, and zoxazolamine, were used in the present study.

Antipyrine was chosen as a convenient "prototype" drug which has become popular for the estimation of in vivo cytochrome P-450 - mediated drug oxidations. Changes in the hepatic cytochrome P-450 system have been correlated directly with changes in in vivo antipyrine metabolism and the "antipyrine test" has thus been used extensively in both man and laboratory animals for the assessment of cytochrome P-450 mixed function oxidase activity (Stevenson 1977, Vesell 1979, Danhof et al 1979a and 1982). I assessed the metabolism of antipyrine and the excretion of the three main metabolites of antipyrine in urine.

My results demonstrate the lack of real effect on antipyrine metabolism by the treatment of rats with CPA and
2,4-DP (200 mg/kg), consistent with the lack of induction of cytochromes P-450 IA1 and P-450 IIB1 by CPA and 2,4-DP treatment reported previously (Figs. 3.3 & 3.4). These two isoenzymes of cytochrome P-450 have previously been shown to be involved in antipyrine metabolism leading to different metabolite formation (namely OHA, NORA and HMA respectively) (Danhof et al. 1982, Rhodes and Houston 1982, Bottcher et al. 1982 a & b).

Fig.5.5 showed a slight increase in OHA excretion in rat urine after 2,4-DP treatment (188% of control), coincident with a small decrease in paralysis time caused by zoxazolamine (Fig. 5.7). This was not supported by a positive increase in microsomal ethoxyresorufin-O-deethylase activity (Fig.3.3). This may suggest that the small increase in cytochrome P-450 IA1 isoenzyme activity seen in the in vivo experiments is not significant enough and could not be detected in the liver or kidney microsomes. Neither NORA nor HMA was affected by CPA or 2,4-DP pre-treatment (Fig. 5.4). It is only OHA which we were able to quantitate in ug (Fig.5.5) since I had the pure compound and we were able to construct a standard curve.

It is known that zoxazolamine is metabolised mainly to 6-hydroxyzoxazolamine, and chlorzoxazone as a minor metabolite, by 3-methylcholanthrene induced P-450 IA1) (Tomaszewski et al. 1976, Conney et al. 1960, Van der Graaff et al. 1986, Yasuhara and Levy 1988).
My earlier results indicated a small induction of microsomal ethoxyresorufin-O-deethylase activity by some of the compounds studied in chapter 3. Therefore, I attempted to correlate that of microsomal enzyme activity with the in vivo enzyme activity by means of using zoxazolamine paralysis time. One may expect to see a real decrease in paralysis time mainly by MCPA since it was the highest inducer of ethoxyresorufin-O-deethylase activity (140% over control levels). On the contrary, MCPA showed no effect on paralysis time (zoxazolamine metabolism)(Fig 5.7). In contrast 2,4,5-TP showed the highest effect on zoxazolamine paralysis time. It reduced significantly the paralysis time to 40% of control (P<0.001), whereas 2,4-D was the only compound that increased the paralysis time to 145% of control. This is because of the effects of 2,4-D on the mobility of the animals. Other compounds that decreased paralysis time were 2,4-DP, 2,4,5-T and CPA (67%, 89% amd 87% of control respectively), when only 2,4,5-T slightly increased microsomal ethoxyresorufin-O-deethylase activity to 160% of control.

In summary, my results demonstrate lack of real effect of the chlorinated phenoxyacids studied on the in vivo metabolism of the substrates used, namely, antipyrine, zoxazolamine and pentobarbital. Since these substrates are metabolised mainly by cytochrome P-450 IA1 and/or P-450 IIB1, the results presented here support my earlier results, suggesting that the compounds studied have little effect, if any, on these two
isoenzymes both in vivo and in vitro, supporting the earlier conclusion that chlorophenoxyacids are relatively specific inducers of cytochrome P-450 IVA1 isoenzyme. Thus it may be concluded that cytochrome P 450 IVA1 plays no role in the metabolism of the compound tested; a conclusion not inconsistent with the narrow substrate specificity (i.e. for fatty acids) of this particular isoenzyme (Tamburini et al., 1984).
CHAPTER SIX

EFFECTS OF CHLOROPHENOXACYCIDS AND CHLOROPHENOLS
ON CYTOCHROME P-450 IVA1 mRNA
6.1 INTRODUCTION

In order to understand the molecular basis of cytochrome P-450 induction and peroxisome proliferation much of the research in this field is concentrated on the molecular biology and the events that occur at the molecular level following the administration of peroxisome proliferators to animals and isolated cell lines.

The cytochrome P-450 IVA1 gene has been cloned from rat liver (Hardwick et al., 1987, Earnshaw et al., 1988), as well as the genes for several of the enzymes of the peroxisomal β-oxidation spiral including 3-hydroxyacyl-CoA dehydrogenase bifunctional protein (Osumi et al., 1965, Chatterjee et al., 1987). Several groups have used cDNA probes to study the changes in mRNA levels following the administration of peroxisome proliferators to rats. These studies have shown that observed increases in the mRNA for cytochrome P-450 IVA1 and the bifunctional protein are due to transcriptional activation of these genes (Osumi et al., 1985, Chatterjee et al., 1987, Hardwick et al., 1987, Milton, 1989). These increases in the rate of transcription have been shown to occur in vivo as early as one hour after the administration of inducers such as clofibrate (Milton, 1989, Osumi et al., 1985, Hardwick et al., 1987).
In this chapter the influence of pre-treatment of rats with four chlorophenoxy acids and related phenols on the mRNA coding for cytochrome P-450 IVA1 and the bifunctional protein has been studied in an attempt to rationalise on a molecular level the previously described changes in both enzyme activity and apoprotein induction.

6.2 Results

Total hepatic RNA was isolated from rats treated with CPA, MCPA, 2,4-DP, 2,4,5-TP and the related chlorophenols CP, MCP, DCP and TCP at 200 mg/kg/day for 3 days and from control rats (Peanut oil treated) by the method of Chomczynski and Sacchi (1987) as outlined in Chapter 2. The samples were hybridised with actin cDNA (as a control), the cytochrome P-450 IVA1 cDNA and the bifunctional enzyme cDNA probes. The cDNA probes used in this study were either provided as insert DNA (cytochrome P-450 IVA1 and actin) or as insert DNA in plasmid (bifunctional protein). A marked increase in the mRNA hybridising to the cytochrome P-450 IVA1 cDNA probe was observed in the 2,4,5-TP, 2,4-DP and MCPA treated rat (fig.6.1). By using densitometric analysis, I was able to compare the density of the RNA dots as a percentage of the control value (fig. 6.2). There was a 2.7-fold increase in the level of mRNA after 2,4,5-TP pretreatment and 2,4-DP and MCPA pretreatment resulted in 2.4- and 1.8-fold increases in P-450 IVA1 mRNA, respectively. All other treatments showed no significant increase in the cytochrome P-450 IVA1 in mRNA. The bifunctional protein mRNA level showed a similar pattern of increase as that of cytochrome P-450 IVA1 mRNA level.
Rats were pretreated with phenoxy acids (CPA, 2,4,5-TP, 2,4-DP and MCPA) and phenols (CP, TCP, DCP, and MCP) at a dose of 200 mg/kg/day for 7 days and RNA was extracted from the liver as described in methods. A DNA dot blot was performed as described in methods. A plasmid of 2.1 kbp was used to hybridise the specific cytochrome P-450IVA1 mRNA.
Figure 6.2  Densitometric analysis of RNA dot blot with a cytochrome P-450IVAl cDNA probe (Fig. 6.1).

% OF CONTROL

TREATMENT (200mg/kg)

Each value represents the mean ± S.D. of three dots with different concentration (1,2 and 5 ug), details as in Fig. 6.1. *** p<0.001.
after 2,4-DP, 2,4,5-TP and MCPA treatment (data not shown). There was no change in the level of actin mRNA throughout the different samples (data not shown). The level of actin mRNA serves as a positive control for the hybridization analysis, as its expression is not affected and can be used to show that there has not been a general, non-specific, increase in mRNA production and that equivalent amounts of RNA have been loaded per dot. The increase in cytochrome P-450 IVA1 mRNA is in accordance with the increases seen in cytochrome P-450 IVA1 apoprotein and lauric acid w-hydroxylase activity (reproduced in fig. 6.3 and 6.4) and the detailed results reported in Chapters 3 and 4.

Chlorinated phenoxy acid pre-treatment also showed a significant increase in cyanide-insensitive palmitoyl-CoA oxidation of the peroxisomal β-oxidation pathway (reproduced in fig. 6.5) whereas, the related chlorophenols, showed no change in the enzyme activity, a pattern similar to that of cytochrome P-450 IVA1 and laurate 12-hydroxylase induction (fig. 6.3 and 6.4). Furthermore, 2,4,5-TP was the most potent inducer of the different enzyme activities studied as well as for the mRNA coding for cytochrome P-450 IVA1 and the bifunctional enzyme.
Figure 6.3  Cytochrome P-450IVAl specific content in liver microsomes.  
Influence of phenoxy acid and phenol pretreatment

Bars represent the mean ± S.D. of at least three animals pretreated with the phenoxy compounds: CPA, (B); MCPA, (C); 2,4-DP, (D); 2,4,5-TP, (E); and the phenols: CP, (F); MCP, (G); DCP, (H); TCP, (I) at 200 mg/kg/day for 3 days as described in methods. Reproduced from Chapter 3 and 4.

*** P 0.001. Control (A) received 5 ml/kg peanut oil.
Figure 6.4 Induction of laurate 12-hydroxylase activity in liver microsomes by phenoxy acid and phenol pretreatment.

Bars represent the mean ± S.D of three individual animals treated with the test compound (200 mg/kg) for 3 days * p<0.05, ** p<0.01, *** p<0.001.
Figure 6.5  
Induction of peroxisomal β-oxidation by phenoxy acid and phenol pretreatment.

Palmitoyl-CoA oxidation activity (nmol/min/mg protein)

Palmitoyl-CoA oxidation was determined as described in methods.

\*p<0.05, \**p<0.01, ***p<0.001.
6.3 Discussion

The data presented in this chapter demonstrates a good relation between the induction of cytochrome P-450 IVA1 and palmitoyl-CoA oxidase in regard to the different compounds studied. It is clear from fig. 6.3 - 6.5 that the most potent inducer of the specific content of cytochrome P-450 IVA1 and laurate 12-hydroxylase (2,4,5-TP) (4-fold and 7-fold respectively) is also the most potent inducer of peroxisomal \(^\beta\)-oxidation (3-fold) as indicated by the induction of palmitoyl-CoA oxidation. This increase in the enzyme activity is accompanied by an increase in cytochrome P-450 IVA1 mRNA (fig. 6.1) and the bifunctional protein mRNA (data not shown, due to technical difficulties). These findings and the previous results reported in Chapter 3 for the chlorophenoxy acid herbicides are comparable to results reported by Sharma (1988) and Milton (1989) using clofibrate and related hypolipidaemic drugs.

The bifunctional protein of peroxisomes from rat liver expresses both the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase enzyme activities which form part of the fatty acid \(^\beta\)-oxidation pathway in peroxisomes (Osumi and Hasimoto, 1979). In peroxisomes, the activities of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase have been shown to copurify on one single polypeptide of molecular weight 80,000 daltons whereas in mitochondria the enzyme activities reside on separate protein molecules (Boemer and Osumundsen, 1984). The protein was markedly induced in peroxisomes following treatment of rodents with
peroxisome proliferators such as clofibrate (Reddy et al, 1980, Makoswka, 1988).

Recently, Chatterjee et al, (1987) demonstrated that the treatment of hepatocytes in culture with Wy-14,643 resulted in an increase in the peroxisomal bifunctional enzyme. It was concluded that the induction of the peroxisomal bifunctional enzyme by Wy-14,643 in hepatocytes is due to an increase in the rate of transcription of the enzyme gene and so increase in the enzyme mRNA (Chatterjee et al, 1987). This induction of the bifunctional enzyme mRNA in hepatocytes was shown to take between 10-15 hr after Wy-14,643 treatment. In the intact rat, it was shown that this compound increased the hepatic bifunctional enzyme mRNA within 1 hr of treatment (Reddy et al, 1986). This is similar to a recent result in this laboratory where the induction of the bifunctional enzyme mRNA by clofibrate was shown to be detectable in rat liver shortly (approximately one hour) after a single clofibrate dose (Milton, 1989). Furthermore, this increase was preceded by an increase in the cytochrome P-450 IVA1 mRNA in a time dependent manner. Milton (1989) showed that there was a biphasic response in the cytochrome P-450 IVA1 mRNA levels in rat liver after a single i.p. dose of sodium clofibrate and that the bifunctional protein mRNA appeared later than the earlier burst of cytochrome P-450 IVA1 mRNA increase. Similarly, there was a dose and time dependent increase in the enzymes of $\beta$-oxidation and cytochrome P-450 IVA1, and the increase in the latter enzyme preceded the enzymes of $\beta$-oxidation in a time dependent manner (Sharma, 1988, Milton, 1989).
This lead to a proposed scheme suggesting that the induction of cytochrome P-450 IVA1 precedes the induction of $\beta$-oxidation at the enzymatic and molecular level (Sharma et al., 1988 a). This scheme has additionally been verified on in vitro experiments in cell culture, using both mono- and dicarboxylic fatty acids, and on the relationship of the enzyme activities involved (C.R. Elcombe, Personal Communication). The data presented in this thesis for the chlorehpenoxy acids are not inconsistent with this scheme, which is presented in Chapter 1.

In conclusion, my results have demonstrated that there is a close association between the induction of cytochrome P-450 IVA1 and its mRNA, its associated laurate 12-hydroxylase activity and the peroxisomal fatty acid $\beta$-oxidation pathway. Although no attempt was made to identify structure-induction relationships, my results have clearly shown that there is a potency order of induction since 2,4,5-TP > 2,4-DP > MCPA > CPA in inducing the various parameters studied.
CHAPTER 7

DISCUSSION
DISCUSSION

For drug design it is important to understand structure activity relationships and toxicology studies. Many workers have studied the structure activity relation of peroxisome proliferators in rodents (Katoh et al., 1984; Fournel et al., 1985; Lundgren et al., 1987a, b, 1988; Sharma et al., 1988a). It appears that the blocking of the carbon atom such as clofibrlic acid, 2,4,DP and 2,4,5-TP results in more potent induction of peroxisomal \( \beta \)-oxidation. My results support such a proposal in regard to cytochrome P-450 IVA1 induction since 2,4,5-TP (among the seven compounds studied) showed the highest induction level of this enzyme at the protein and molecular level. CPA, being the smaller of the compounds, showed the least induction capacity. The positioning of the chlorine atoms on the phenyl ring is also of importance since 2,4,5-TP \( > \) 2,4-DP and 2,4,5-T \( > \) 2,4-D \( > \) CPA induces cytochrome P-450 IVA1 or \( \beta \)-oxidation enzyme activities. However, the effect of the blocked carbon atom, and position of and extent of chlorination of the phenyl ring, is not as simple as it first seems as 2,4-DB appears to be less potent than 2,4-DP or 2,4-D. This is in contrast to the effect of the substitution of the carbon atom on the induction of peroxisomal \( \beta \)-oxidation suggested by Katoh et al. (1984), and serves to highlight the interactive nature of the structural elements of the peroxisome proliferators including chlorophenoxyacids. This is inconsistent with the proposal that all these compounds appear to consist of a carboxylic function carried on a hydrophobic backbone to yield an amphipathic carboxylate (Hertz
et al., 1988). The carboxylic function may either be present initially, as in the case of clofibric acid and related drugs and chlorophenoxyacids, or may be derived by metabolic oxidation via the respective alcohols. The free carboxylic acid function, or a derivative thereof, is presumably directly involved in the induction process, whilst the nature of the hydrophobic backbone may determine the potency of the compound. To shed some light on the importance of the carboxylic acid group, my results using chlorinated phenols (related to chlorophenoxy acids) showed no significant potency in inducing cytochrome P-450 IVA1 or peroxisomal \( \beta \)-oxidation. It is possible that chlorphenols, like phenol, are actively metabolised by Phase II conjugation enzymes (Dodgson, 1977; Bruce et al., 1987), thus rapidly removing the inductive stimulus. Further structure-activity relationship analysis may help us define which parameters are important for peroxisome proliferation and/or hypolipidaemic activity.

My results have shown that all of the chlorophenoxy acids studied increased the activity of lauric acid 12-hydroxylation (Fig.3.5) accompanied by increase in the cytochrome P-450 IVA1 protein (Table 3.1). Furthermore, 2,4,5-TP, 2,4-DP and MCPA significantly increased the levels of cytochrome P-450 IVA1 mRNA. This, with the other results which showed that the enzyme activities of benzphetamine-N-demethylase and ethoxyresorufin-O-deethylase (substrates for cytochrome P-450 IIB1 and P-450 IAI respectively) were not significantly changed by some of the compounds after treatment, suggest that the cytochrome P-450 IVA1, P-450 IIB1 and P-
450 IA1 isoenzymes are differentially regulated by corresponding inducers, a conclusion that is consistent with the available literature (Hardwick et al., 1987, Nebert et al., 1987). This observation is also in support of the suggestion that the genes coding for the cytochrome P-450 isoenzymes can be regulated in several different ways (Nebert and Gonzalez, 1985; Adesnik and Atchison, 1985; Whitlock, 1986; Gonzalez, 1989). The most studied isoenzyme of cytochromes P-450 is cytochrome P-450 IA1 and IA2 which have been shown to be regulated by a soluble intracellular protein receptor which binds specific aromatic hydrocarbons such as 3-methylcholanthrene and dioxines such as TCDD (Okey and Vella, 1982; Poellinger et al., 1983; Welchamsson et al., 1986; Durrin et al., 1987), and is termed the Ah receptor. There is evidence that these latter isoenzymes may be regulated by increasing the rate of transcription (and decreasing cytochrome P-450 IA mRNA degradation) following the binding of the inducing agent, such as TCDD (Israel and Whitlock, 1984; Gonzalez and Nebert, 1985). It is also possible that the regulation of these isoenzymes may involve a distinct 4S trans-regulatory protein (Houser et al., 1985; Bresnick et al., 1988) and/or post-transcriptional regulation (Pasco et al., 1988; Silver and Krauter, 1988). Recently Foldes and Bresnick (1989) have demonstrated, (by using 3-MC and cycloheximide to study the inducibility of cytochrome P-450 IA1 mRNA in rat liver), the existence of a specific labile repressor of cytochrome P-450 IA1 gene expression in vivo. However, the precise details of this trans-regulatory mechanism remain to be fully elucidated. The mechanism(s) of induction of cytochromes P-450 IIB1 and P-450 IIB2
and P-450 IVA1 is still far from clear. Evidence for cytosolic receptor involved in the induction of these isoenzymes is still debatable (Fonne and Mayer, 1987; Milton et al., 1988). Furthermore, Milton (1989) showed that there was no protein dependency for the induction of cytochrome P-450 IVA1 mRNA, thereby excluding the intermediacy of a labile, regulatory protein in the induction process.

A scheme was proposed by Sharma et al., (1988a) (Fig. 1.1), whereby xenobiotics can perturb hepatic lipid homeostasis by interacting with the enzymes of fatty acid metabolism, thereby causing a lipid overload. It has been suggested that the stimulus for the induction of peroxisomal \( \beta \)-oxidation is long-chain dicarboxylic fatty acids formed initially by the \( \omega \)-hydroxylation of the corresponding monocarboxylic fatty acids by cytochrome P-450 IVA1 and subsequent oxidation of the proximal peroxisomal proliferator, namely the decarboxylic acid metabolite. The induction of cytochrome P-450 IVA1 is postulated to occur before the induction of peroxisomal \( \beta \)-oxidation. Support for this scheme came from the results of Milton (1989) which showed that cytochrome P-450 IVA1 was induced before peroxisomal \( \beta \)-oxidation although the events do appear to be closely related temporally. It may be possible that the signal for the induction of peroxisomal \( \beta \)-oxidation may be generated in part by constitutive cytochrome P-450 IVA1. This may mean that the induction of cytochrome P-450 IVA1 and peroxisomal \( \beta \)-oxidation, although distinct events, may be closely related temporally. The co-induction of the microsomal fatty acid
hydroxylase and the peroxisomal \( \beta \)-oxidation system (the latter being indicated by the induction of palmitoyl-CoA oxidation) may be the result of disturbances in the metabolism of lipids (Cappuzi et al., 1983; Mitchell and Hinton, 1988). Following clofibrate treatment there is an accumulation of lipid in the liver in a similar fashion to that observed in animals receiving high fat diets (Neat et al., 1981). The way in which this lipid overload causes the observed increased rates of gene transcription is unknown although it is possible that cellular second messenger systems are involved, a possibility being actively pursued in this laboratory.

Much of the current research on the molecular biology of the cytochrome P-450 system is concerned with the elucidation of the genetic and molecular mechanisms responsible for the existence of a multiplicity of forms of cytochrome P-450 and also for the induction of distinct forms by specific inducing agents (Adesnik and Atchison, 1986). Using an antibody to clofibrate inducible cytochrome P-450 IVA1, the isoenzyme was immunochemically detected in non-induced rat liver and kidney microsomes (Tamburini et al., 1984; Bains et al., 1985). The level of this isoenzyme was markedly elevated after clofibrate treatment due to an activation of cytochrome P-450 IVA1 mRNA synthesis as determined using the corresponding cDNA probe (Hardwick et al., 1987). This rapid transcriptional increase is similar to the transcriptional activation of fatty acyl-CoA oxidase and enoyl-CoA hydratase/3-hydroxy acyl-CoA dehydrogenase induced by clofibrate in rat liver (Reddy et al., 1986; Milton, 1989). It appears that cytochrome P-450 IVA1 is regulated by the same mechanism that regulates these peroxisomal enzymes, and may form a
battery of genes that are co-regulated by clofibrate and its structural analogues. Recently in our laboratory, a 1Kbp cDNA probe to cytochrome P-450 IVA1 was isolated from a clofibrate-induced rat liver cDNA library in the bacteriophage expression vector Lambda gt11 using anti-cytochrome P-450 IVA1 sera and a biotin-streptavidin linked immunoassay (Earnshaw et al., 1988). This cDNA probe as well as a 2.1Kbp cDNA probe to cytochrome P-450 IVA1 are being used in our laboratory to examine the corresponding mRNA levels in untreated and treated animals utilising hybridisation methodology.

Induction of cytochrome P-450 IVA1 by peroxisome proliferators exhibited a negative correlation when compared to benzphetamine-N-demethylase and ethoxyresorufin-O-deethylase activities indicating a specific gene switch-off for these two activities (Gibson and Sharma, 1987). My results are in support of these suggestions since a poor correlation was seen between the enzymes indicated above following 2,4,5-TP pretreatment (Table 3.3). Furthermore, there was a positive correlation between cytochrome P-450 IVA1 and laurate 12-hydroxylation and total cytochrome P-450 with all of the chlorophenoxy acids with the exception of 2,4-DB and CPA (Table 3.2).

The lack of real induction in benzphetamine-N-demethylase and ethoxyresorufin-O-deethylase activities is exemplified also by the in vivo results in chapter 5 where there was no real induction in the metabolism of pentobarbital or zoxazolamine except for marginal increases by some of the compounds studied. Furthermore, the
increase in antipyrine metabolism to 4-hydroxyantipyrine after 2,4-DP pre-treatment needs to be further investigated using other compounds since formation of this metabolite can be influenced by phenobarbital and 3-methylcholanthrene (Danhof et al., 1979; Teunissen et al., 1983; Buppodom et al., 1986; Chenery et al., 1987; Loft and Poulsen, 1989).

Imaoka and Funae (1986) have reported the isolation of the major cytochrome P-450 isoenzyme from kidneys of untreated rats. This protein was shown to catalyse the ω- and (ω-1)-hydroxylation of lauric acid and its monomeric molecular weight was determined by SDS-PAGE to be 52,000 Da. This would correspond to the upper band in clofibrate-treated kidney microsomes reported by (Sharma 1988) as visualised by Western blotting. This upper band may represent the active laurate hydroxylation in kidney microsomes with which although recognised by the polyclonal anti-cytochrome P-450 IVA in an inhibition study (Sharma, 1988), is sufficiently distinct so that its catalytic activity is uninhibited.

The high basal level of cytochrome P-450 IVA1 in rat kidney suggests that this enzyme represents a major form of cytochrome P-450 in rat kidney. This is supported by measurement of benzphetamine and ethoxyresorufin dealkylation activities (Table 3.4), the latter two enzymes are generally being accepted as good markers for the cytochrome P-450 IIIB1 and P-450 IA1 isoenzyme respectively. These two isoenzymes activity were 10-fold lower than that seen in hepatic microsomes. These results are supported by the
results of Hardwick et al., (1987) who reported that in non-treated animals cytochrome P-450 IVA1 was readily detectable in rat kidney, whereas cytochrome P-450 IAI and 2, and P-450 IIB1 and 2 were undetectable.

There is much debate at present on whether the carcinogenicity and/or peroxisomal proliferative activity induced in rats by realistic exposure doses of certain widely used compounds is, in fact, likely to constitute a problem for man when exposed to these substances. Acute toxicity in rats following exposure to a variety of xenobiotics may result in carcinogenicity. With the administration of peroxisome proliferators, enzyme induction occurs but this is not related to gross toxicity. However, hepatocarcinogenesis develops in treated rats between 1 and 2 years (Reddy and Lalwani, 1983). These compounds, including chlorophenoxyacids, are uniformly negative in a wide range of mutagenicity tests indicating a class of non-genotoxic carcinogens. No binding of clofibrate nor its metabolites and related drugs to hepatic nuclear DNA has been detected (Von Dankin et al., 1981) and nafenopin and Wy-14,643 exhibited no significant binding to DNA in vitro or in vivo (Goel et al., 1985) and no peroxisome proliferator DNA adducts were detected in hepatocytes under in vivo and in vitro conditions with clofibrate and other peroxisome proliferators (Gupta et al., 1985).

In order to explain the carcinogenicity of peroxisome proliferators in rodents, it has been suggested that the marked
increase in peroxisomal $\beta$-oxidation leads to an excessive production of hydrogen peroxide by fatty acyl-CoA oxidase (Reddy and Lalwani, 1983; Elliot et al., 1986). It has also been suggested that stimulated peroxisomes may be leaky and that excessive hydrogen peroxide may diffuse into the cytoplasm of the cell. The hydrogen peroxide may interact with a non-DNA target producing active oxygen species. The active oxygen species may cause lipid peroxidation, DNA attack and hepatocarcinogenesis. Consistent with this lipid peroxidation proposal of neoplastic transformation is the observed accumulation of lipofuscin in the livers of treated rats (Reddy et al., 1982). This is supported by the results of Makowska (1988) and Sharma (1988) which showed that the specific activity of catalase was unchanged by treatment with hypolipidaemic drugs (total activity was increased in parallel with hepatomegaly) and this may be insufficient to remove excessive hydrogen peroxide produced in stimulated peroxisomes. In long-term studies (26 weeks) with bezafibrate and ciprofibrate, the specific activity of catalase was significantly decreased (Makowska 1988). Furthermore, there was a fifty percent decrease in hepatic glutathione peroxidase activity observed with ciprofibrate treatment which may potentiate oxidative stress by inadequate removal of cytosolic hydrogen peroxide (Makowska, 1988). A similar result was shown earlier using chlorophenoxacy acids, such as CPA 2,4-D, 2,4,5-T and MCPA, indicating that these compounds have a similar induction pattern as that of clofibrlic acid in rat and mouse (with less magnitude) (Kawashima et al., 1984a,b; Vainio et al., 1983; Hietanin et al., 1985; Vessey and Boyer, 1984; Lundgren et al., 1987a,b). These results showed that
the chlorophenoxy acid compounds induce several enzymes including the enzymes of hepatic $\beta$-oxidation and peroxisomal proliferation as with catalase, glutathione reductase, glutathione peroxidase, glutathione S-transferase and epoxide hydrolase. These studies suggest that the cytotoxicity and hepatocarcinogenicity (if any) of these compounds may be of similar mechanism to that of the more studied group of compounds such as the hypolipidaemic drugs including clofibrate. Under physiological conditions, the hydrogen peroxide produced in peroxisomes is largely disposed of by catalase whereas hydrogen peroxide arising from mitochondria, endoplasmic reticulum or cytosolic enzymes such as superoxide dismutase, is acted upon by glutathione peroxase (Halliwell and Gutteridge, 1985).

Hydrogen peroxide may also be produced in the endoplasmic reticulum by the uncoupling of cytochrome P-450 mixed function oxidase activity. Metabolic pathways involving cytochrome P-450 enzymes may initiate or modulate oxidative damage due to oxygen radicals (Gonder et al., 1988). In mice oxygen toxicity has been shown to parallel the genetic control of cytochrome P-450 enzyme induction and toxicity developed at a time when the cytochrome P-450 enzyme system was induced by oxygen. With clofibrate administration microsomal and cytosolic epoxide hydrolases were induced and this may be a protective mechanism (Moody et al., 1985).

The research presented in this thesis has shown that the chlorophenoxy acids studied have a similar pattern of enzyme induction in Wistar albino rats as that of clofibrate and related
drugs. Furthermore, the in vivo and in vitro studies reported indicate that this class of compounds are selective in inducing cytochrome P-450 IVA1 and not cytochrome P-450 IA(1 and 2) or cytochrome P-450 IIB(1 and 2); a conclusion supported by research in this laboratory using a large number of hypolipidaemic drugs.

OUTLOOK

The evaluation of the safety of chemicals in man is based largely upon the extrapolation of animal data to predict the likelihood of toxicity. Detailed knowledge of the pharmacodynamic and pharmacokinetic profiles of the compound both in man and animals is required.

The expression of fatty acid metabolising enzymes in rat liver is dependent upon the inductive and suppressive effects of various endogenous and exogenous substances such as clofibrate, the related chlorophenoxy acid and constitutive fatty acids. It is of importance to study and to understand the nature and the mechanism by which these compounds exert their effect. In this context our laboratory, using cDNA probes to cytochrome P-450 IVA1, fatty acyl-CoA oxidase, peroxisomal bifunctional protein and glutathione peroxidase, is studying the molecular expression and regulation of these enzymes. The role of cytochrome P-450 IVA1 in vivo in normal physiological processes and the existence of high levels of this
enzyme in kidney, need to be further studied. More studies are also required to further understand the nature and relationship between induced peroxisome proliferation and hepatocarcinoma.
During the course of this work, two refereed papers have been published.

   Chlorophenoxy acid herbicides induce microsomal cytochrome P-450 IVA1 (p-452) in rat liver.

   Decreased expression of cytochrome P-452 in the resistance phenotype characteristic of putative preneoplastic hepatocyte nodules during hepatocarcinogenesis.
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