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# THE EFFECT OF SHORT-TERM PRETREATMENT WITH PEROXISOME PROLIFERATORS ON THE ACUTE TOXICITY OF VARIOUS TOXICANTS, INCLUDING PARACETAMOL

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*How to get there*

*Go to the end of the path until you get to the gate*
*Go through the gate*
*and head straight out towards the horizon*
*Keep going towards the horizon*
*Sit down and have a rest every now and again*
*But keep on going*
*Just keep on with it*
*Keep on going as far as you can*

*That's how you get there*

*Michael Leunig*

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## ABSTRACT

Repeated administration of a diverse group of chemicals known as peroxisome proliferators (PxP) causes an increase in the number of peroxisomes in rodent livers. In addition to the increased abundance of these organelles, a range of other biochemical changes also occur in the livers of PxP treated rodents. A distinctive outcome is liver cancer, which involves a hyperproliferative response mediated by a specific receptor, termed the peroxisome proliferator activated receptor. However, hepatocarcinogenesis also seems to involve other mechanisms and thus investigators have suggested that an overproduction of reactive oxygen species contributes to the development of PxP induced hepatic tumours.

Among the other biochemical changes produced by PxPs that are important from a toxicological perspective are various alterations in specific enzymes and cofactors involved in biotransformation and detoxication of xenobiotics. These changes affect enzymes involved in both Phase I and Phase II biotransformations, as well as various ancillary enzymes that protect hepatocytes against chemical injury, including oxidative stress. Due to these biochemical alterations during shortterm treatment with PxPs, the metabolism and toxicity of other xenobiotics may well be altered in PxP-treated rodents. This premise forms the basis of the experiments described in this study which used both *in vivo* and *in vitro* approaches to studying the effect of PxPs on the toxicity of other xenobiotics.

After establishing a course of treatment to induce peroxisome proliferation in mice, a 10 day exposure to the hypolipidemic PxP, clofibrate was shown to profoundly protect against the acute hepatotoxicity of paracetamol (AAP). As this protection was also observed following treatment with other PxPs, namely the plasticiser, DEHP and the herbicide, Silvex, it appears associated with PxP as a class rather than a distinctive effect of clofibrate. Subsequently, an investigation of the time and dose dependency of clofibrate induced hepatoprotection suggested that expression of chemoresistance is correlated with the increase in peroxisome numbers upon commencement of exposure, but not necessarily the decrease in numbers that occurred following cessation of treatment.

The toxicity of AAP involves the formation of the reactive CYP450 generated metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is detoxicated via conjugation with the endogenous cytoprotective tripeptide, glutathione (GSH). This pathway competes with two Phase II biotransformation processes that act directly on the parent drug, namely glucuronidation and sulphation. Urinary metabolite analysis revealed that following clofibrate pretreatment there was no increase in either glucuronide or sulphate metabolites compared to control mice, indicating no change in conjugation prior to bioactivation of AAP. There was also no difference in the ability of microsomal fractions prepared from either clofibrate or

control treated mice to form NAPQI conjugates. However, on the basis of radiolabel studies there was a reduction in the amount of NAPQI being bound within the hepatocyte, which was accompanied by a lack of mitochondrial damage in AAP-intoxicated clofibrate pretreated mice.

The logical explanation for this decrease in NAPQI binding was an increase in the availability of intracellular GSH. However, determination of the absolute levels of GSH and also measurement of the activities of GSH dependent enzymes suggested that there was no alteration in GSH homeostatis that could explain the extent of hepatoprotection observed. Indeed, the hepatoprotective effect of pretreatment with PxP against AAP toxicity was still present in both *in vivo* and *in vitro* models, following almost complete depletion of GSH by prior exposure to GSH conjugating agent, diethylmaleate.

To characterise the hepatoprotective effect further, the toxicity of a variety of diverse-acting toxicants was investigated. The acute *in vivo* hepatotoxicity of the radical generating carbon tetrachloride, and to a lesser extent, the electrophile forming bromobenzene, was reduced in clofibrate pretreated mice. While these results further established that GSH was not the primary protective mechanism, they suggested that the hepatoprotection may well be related to an increase in antioxidant capacity in the livers of PxP treated mice. This conclusion was supported by an *in vitro* study utilising 9 toxicants with diverse mechanisms of action. Thus, hepatocytes isolated from PxP treated mice consistantly resisted prooxidant induced damage compared to control cells.

Such resistance to oxidative damage was also observed in liver homogenate preparations, where both a resistance to prooxidant induced lipid peroxidation (as measured by malondialdehyde formation) and a decrease in endogenous protein oxidation (as measured by protein carbonyls) was found to accompany clofibrate pretreatment. These observations could not be explained on the basis of alterations in the ratio of unsaturated to saturated fatty acids in the livers of PxP treated mice. Rather, subsequent experiments implied an increase in the antioxidant capacity in the soluble fraction of the liver.

In conclusion, this work has shown that pretreatment with PxP protects mice against the acute hepatotoxicity of AAP, in addition to a number of other toxicants. While the mechanism of PxP-induced hepatoprotection is not conclusively identified, on the basis of results reported in this study, an increase in antioxidant capacity in the soluble fraction of the liver appears the most likely explanation.

## DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when depoisted in the University Library, being available for loan and photocopying.

SIGNED: DATE: 1st June, 1998

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Nicholls-Grzemski FA, Calder IC and Priestly BG (1991) Silvex induced peroxisome proliferation protects against paracetamol- induced hepatotoxicity in mice. Society for Free Radical Research, Lorne, Victoria, Australia

Laslett TJ, Nicholls-Grzemski FA, Calder IC and Priestly BG (1991) A modifying role for glutathione in peroxidative damage in hepatocytes from peroxisome proliferator fed rats? Society for Free Radical Research, Lorne,Victoria, Australia

Priestly BG, Nicholls-Grzemski FA and Calder IC (1992) Clofibrate decreases *in vitro* hepatic lipid peroxidation in mice. International Congress of Toxicology VI, Rome, Italy

Nicholls-Grzemski FA, Burcham PC, Calder IC and Priestly BG (1995)
Pretreatment with peroxisome proliferators protects mice against some but not all hepatotoxins. Peroxisomes: Biology and Role in Toxicology and Disease-International Symposium, Aspen, Colorado, USA

Nicholls-Grzemski FA, Burcham PC, Calder IC and Priestly BG (1995) Effect of pretreatment with clofibrate on acetaminophen toxicity in mouse hepatocytes. International Congress of Toxicology-VII, Seattle, Washington, USA

Nicholls-Grzemski FA, Burcham PC, Belling B, Calder IC and Priestly BG (1995) Hepatoprotection associated with pretreatment of mice with peroxisome proliferators is not due to changes in hepatic fatty acid levels. Australasian Society of Clinical and Experimental Pharmacology and Toxicology Meeting, Adelaide, South Australia, Australia

Nicholls-Grzemski FA and Burcham PC (1995) Hepatocytes from peroxisome proliferated mice are resistant to some but not all hepatotoxicants. Australasian Society of Clinical and Experimental Pharmacology and Toxicology, Adelaide, South Australia, Australia

# ABBREVIATIONS

| | |
|---|---|
| AAP | paracetamol, acetaminophen |
| AAP:GSH | paracetamol-glutathione conjugate |
| AAP-$SO_4$ | paracetamol-sulphate conjugate |
| ALAT | alanine aminotransferase |
| ANOVA | analysis of variance |
| ASAT | aspartate aminotransferase |
| ATP | adenosine triphosphate |
| BCNU | 1,3-*bis*-(2-chloroethyl)-1-nitrosourea |
| BrB | bromobenzene |
| $CCl_3OO_o$ | trichloromethylperoxyl radical |
| $CCl_3o$ | trichloromethyl radical |
| $CCl_4$ | carbon tetrachloride |
| CDNB | 1-chloro 2,4-dinitrobenzene |
| cEH | epoxide hydrolase - cytosolic form |
| CoA | coenzyme A |
| CYP1A1 | cytochrome P450 1A1 isoform |
| CYP2E1 | cytochrome P450 2E1 isoform |
| CYP450 | cytochrome P450 |
| CYP4A1 | cytochrome P450 4A1 isoform |
| DEHP | di(2-ethylhexyl) phthalate |
| DEM | diethyl maleate |
| DFO | desferrioximine |
| DMSO | dimethylsulfoxide |
| DPM | disintegrations per minute |
| DPPD | diphenyl-*p*-phenylene diamine |
| EDTA | ethylene diamine tetraacetic acid |
| $FADH_2$ | flavine adenine dinucleotide (reduced) |
| $FeSO_4$ | ferrous sulphate |
| Gred | glutathione reductase |
| GSH | glutathione |
| GSH:AAP | glutathione conjugate of paracetamol |
| GSHPx | glutathione peroxidase |
| GSHt | glutathione transferase |
| $H_2O_2$ | hydrogen peroxide |
| HSP | heat shock protein |
| i.p. | intraperitoneal |
| %LBW | liver weight as percent body weight |

| | |
|---|---|
| LDH | lactate dehydrogenase |
| MDA | malondialdehyde |
| mEH | epoxide hydralase - microsomal form |
| NAD+ | nicotine adenine dinucleotide (oxidised) |
| NADH | nicotine adenine dinucleotide (reduced) |
| NADP+ | nicotine adenine dinucleotide phosphate (oxidised) |
| NADPH | nicotine adenine dinucleotide phosphate (reduced) |
| NAPQI | N-acetyl-p-benzoquinoneimine |
| 8-OHG | 8-hydroxyguanosine |
| PAPs | 3'-phospho-adenosine 5'phosphosulpate |
| PGHS | prostaglandin H synthetase |
| PPAR | peroxisome proliferator activated receptor |
| PPRE | peroxisome proliferator response element |
| PxP | peroxisome proliferator |
| RCR | respiratory control ratio |
| ROS | reactive oxygen species |
| RXR | retinoid X receptor |
| SOD | superoxide dismutase |
| SOt | sulphotransferase |
| TBARs | thiobarbituric acid reactive substances |
| tBHP | *tert*-butylated hydroperoxide |
| TCDD | tetra chloro dibenzo-*p*- dioxin |
| TMPD | N,N,N',N'-tetramethyl-p-phenylenediamine |
| UDPGt | uridine diphosphate glucuronide transferase |

## ERRATUM

Page 17 *paragraph 3* "physiochemical" *should read* "physicochemical"
"the the both" *should read* "the both"

Page 27 *paragraph 2* "((Dahlin" *should read* "(Dahlin"

Page 50 *legend 3.5A to D* "Samples was" *should read* "Samples were"

Page 58 *legend for Figure 4.1* "0, 20 50 or 500mg/kg/day"
*should read* "0, 20, 50, 200 or 500mg/kg/day"

Page 78 *paragraph 1* "protein assay" *should read* "protein assay kit"

Page 86
*y* axis on both graphs should read "treatment". *x* axis on lower graph should read "RCR"
Lower graph should be labelled B: in accordance with upper graph, graph A:

Page 113 *paragraph 2* "(Chapters 3,4 and 5))" *should read* "(Chapters 3,4 and 5)"

Page 127 *paragraph 2* "activates $CCl_4$" *should read* "reductively activates $CCl_4$"

Page 147 *paragraph 1* "controls GSH" *should read* "control GSH"

Page 151 *paragraph 2* "treatment group control"
*should read* "treatment control group"

Page 164 *paragraph 2* "precious" *should read* "previous"

Page 166 *paragraph 2* "there were" *should read* "there was"

Page 170 *legend* # = $P<0.05$ should be included in text

Page 183 *paragraph 1* "were not" *should read* "was not"

Page 184 *paragraph 1* "upon treatment of clofibrate hepatocytes with proxidants"
*should read* "upon exposure of clofibrate pretreated hepatocytes "

Appendix 4 *legend* "circles" *should read* "triangles"

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# CHAPTER 1

# INTRODUCTION

## 1.1 Discovery of peroxisome proliferators

It has long been known that an increase in the number of the intracellular organelle, the peroxisome, can occur following exposure to certain chemicals. Best and Duncan (1964) observed that treatment with the hypolipidemic drug, clofibrate, caused hepatomegaly in rats. While investigating this phenomenon, a concurrent increase in the number of cytoplasmic "dense bodies" in rat hepatocytes was also detected (Paget, 1965). As these bodies lacked a crystalloid core, they were initially identified as lysosomes, however subsequent studies revealed that these organelles were in fact peroxisomes (Hess *et al*, 1965, Svoboda and Azarnoff, 1966). In 1975, the term "peroxisome proliferator" (PxP) was coined to describe "any drug or xenobiotic which induces the proliferation of peroxisomes in liver cells" (Reddy *et al*, 1975). PxPs have attracted considerable attention from toxicologists, largely due to the demonstration of hepatocarcinogenic potential following repeated long-term exposure in rats and mice (Reddy *et al*, 1979; Reddy and Lalwani, 1983).

PxPs are a diverse group of chemicals, both in structure and commercial use (Table 1.1). In addition to being united in their common characteristic of increasing the level of cellular peroxisomes, PxP also alter enzymes and cellular factors involved in the metabolism and protection against xenobiotic toxicity. Investigations into the toxicology of PxPs has primarily focused on the chemicals themselves and not on the implications of PxP pretreatment on the toxicity other xenobiotics. Due to the number of cellular alterations induced by PxP, it seems likely that exposure to PxP would effect the toxicity of other xenobiotics and this postulate forms the basis of the studies described in this thesis.

## 1.2. The Peroxisome

### 1.2.1 Discovery of the Peroxisome

Peroxisomes were first detected as organelles containing a fine granular matrix surrounded by a single membrane in the proximal convoluted tubule of the mouse kidney (Rhodin, 1954) and were later identified in rat parenchymal cells (Roullier and Bernard, 1956). As these organelles contained high concentrations of oxidase enzymes which produce hydrogen peroxide ($H_2O_2$) as a byproduct of their enzymatic activity, for example, urate oxidase and D-amino oxidase, and also catalase which metabolises $H_2O_2$ to water and oxygen, they were named "peroxisomes" due to their inherent ability to both produce and degrade $H_2O_2$ (de Duve, 1965).

| | |
|---|---|
| **phenoxyacetic acids and analogues** | **alkyl-aryl carboxylic acids and precursors** |
| Beclobric acid | Clobuzarit |
| Bezafibrate | Gemfibrozil |
| Ciprofibrate | LK-903 |
| Clofibrate | RMI-14,514 |
| Clofibric Acid | DL-040 |
| 2,4-dichlorophenoxyacetic acid | Ethyl 4-(4-chlorophenoxy)butanoate |
| Fenofibrate | OKY-1581 |
| Halofenate | DG-5685 |
| Methyl chlorophenoxyacetic acid | DH-6463 |
| Methyl clofenapate | |
| Nafenopin | **o-substituted benzoic acids and precursors** |
| S-8527 | Acetylsalicylic acid |
| SaH-442348 | Butyl benzyl phthalate |
| Silvex | Di(2-ethylhexyl)phthalate |
| Simfibrate | Dibutyl phthalate |
| 2,4,5-trichlorophenoxyacetic acid | Diisodecyl phthalate |
| Wy-14,643 | Diisononyl phthalate |
| | Diundecyl phthalate |
| **n-alkyl carboxylic acids and their precursors** | Lactofen |
| Dimethrin | Mono-(2-ethylhexyl)phthalate |
| Hexanoic acid (plus methyl and ethyl derivatives) | Mono-n-octyl phthalate |
| 1-mono(carboxyethylthio) tetradecone | Tibric acid |
| 1-mono(carboxymethylthio) tetradecone | |
| Perfluorobutyric acid | **Non-carboxylic acids** |
| Perfluorodecanoic acid | Perfluoroocytyl sulphuric acid |
| Perfluorooctanoic acid | LY-171883 (and certain other substituted tetrazoles) |
| Trichloroacetic acid | |
| Valproic acid | |
| **n-alkyl carboxylic acids precursors** | **Other compounds** |
| Chlorinated paraffins (12-14) | Cinnamyl anthranilate |
| Citral | LS-2265 |
| Di(2-ethylhexyl)adipate | Tridiphane |
| Di(2-ethylhexyl)phosphate | |
| Di(2-ethylhexyl)sebacate | |
| 2-ethylhexanol | |
| 2-ethylhexylaldehyde | |
| Perchloroethylene | |
| Trichloroethylene | |
| 2,2,4-trimethylpentane | |
| **dicarboxylic acids and their precursors** | **Pathological:Physiological conditions** |
| bis(carboxymethylthio)-1,10-decane | High fat diet |
| Gemcadiol | Diet containing increased levels of n-3 fatty acids |
| 2,2,4,4,6,8,8-heptylmethylnonane | Starvation |
| Hexadecanedioic acid | Cold stress |
| Medica 16 | Diabetes |
| Niadenate | |
| Tiadenol | |

**Table 1.1:** Chemicals that are reported to induce peroxisome proliferation in the liver (adapted from Ashby *et al,* 1994)

### 1.2.2 Morphology of the Peroxisome

In rat liver parenchymal cells, peroxisomes are either spherical or oval in shape with an average diameter of 0.5 μm (range 0.1-1.5 μm). They comprise about 1.5% of total parenchymal cell volume (Staübli, 1969) and 2.5 % of total cellular protein (Leighton *et al*, 1968). Within the cell, peroxisomes exist in close association with the endoplasmic reticulum (de Duve, 1973). They are derived from the division of preexisting peroxisomes and are

interconnected to each other via a vast membranous network throughout the cell (Lazarow and Fujiki, 1985).

Peroxisomes are present in all animal cells with the exception of erythrocytes (Novikoff *et al,* 1973). Hepatic peroxisomes from many species typically contain an electron dense crystalloid core comprising of urate oxidase (Hruban and Swift, 1964) however, this core is not seen in either extrahepatic peroxisomes or in human hepatic peroxisomes (Afzelius, 1965). In addition, the morphology of peroxisomes varies according to their stage of development, with newly synthesised "microperoxisomes" being readily distinguishable from their parent organelles (Novikoff *et al* 1973).

### 1.2.3. Biochemical and cellular properties of the peroxisome

A number of enzymes are found in the peroxisome (Table 1.2), the majority of which are involved in lipid metabolism. Peroxisomes are also involved in the synthesis of plasmalogens, cholesterol, bile salts and dolichol and have been implicated in other important cellular functions, including respiration, gluconeogenesis, thermogenesis and purine catabolism (Reddy and Lalwani, 1983).

| Enzyme Group | Enzyme |
|---|---|
| Oxidases | urate oxidase (not in primates)<br>D-amino acid oxidase<br>hydroxyacid oxidase |
| Acyl transferases | carnitine acetyl-CoA transferase<br>carnitine transferase (short and medium chain)<br>acyl-CoA:dihydroxy-acetone phosphate transferase |
| ß-oxidation | fatty acid acyl CoA oxidase<br>enoyl-CoA hydratase<br>thiolase<br>fatty acid CoA synthetase<br>2,4-dienoyl CoA reductase<br>3-hydroxyacyl-CoA dehydrogenase |
| NAD(P)-linked Dehydrogenases | glucose 6-phosphate dehydrogenase<br>NAD+-α /ß-glycerol phosphate dehydrogenase<br>NADH-glycolate reductase<br>isocitrate dehydrogenase<br>xanthine dehydrogenase |
| Others | catalase<br>superoxide dismutase<br>NADH-cytochrome c reductase (antimycin insensitive)<br>Acyl-CoA synthetase |

**Table 1.2:** Enzymes found in the peroxisome. Table adapted from Bentley *et al* ,1993.

Peroxisomal fatty acid ß-oxidation differs from its mitochondrial counterpart in that it neither requires carnitine nor is inhibited by potassium cyanide (Hawkins *et al*, 1987). Unlike mitochondria which tend to metabolise short chain acyl CoA derivatives, peroxisomes metabolise long and very long chain fatty acids (Lazarow and de Duve 1976). Despite these

differences, mitochondrial and peroxisomal ß-oxidation follow a similar 4 step procedure as illustrated in Figure 1.3.

Peroxisomal ß-oxidation commences with a fatty acid containing 8 or more carbon units being metabolised to its acyl CoA derivative in a step that is catalysed by acyl CoA synthetase, an ATP dependent enzyme found in the peroxisomal membrane (Lazarow, 1978). The subsequent steps occur within the peroxisomal matrix. The fatty acyl CoA is metabolised stepwise to ultimately produce acetyl CoA and a saturated fatty acyl CoA comprising of two fewer carbon atoms than the original molecule. Depending on its carbon unit length, this shorter fatty acyl CoA is able to reenter the peroxisomal ß-oxidation pathway or leave the peroxisome to undergo metabolism by ß-oxidation in the mitochondria.

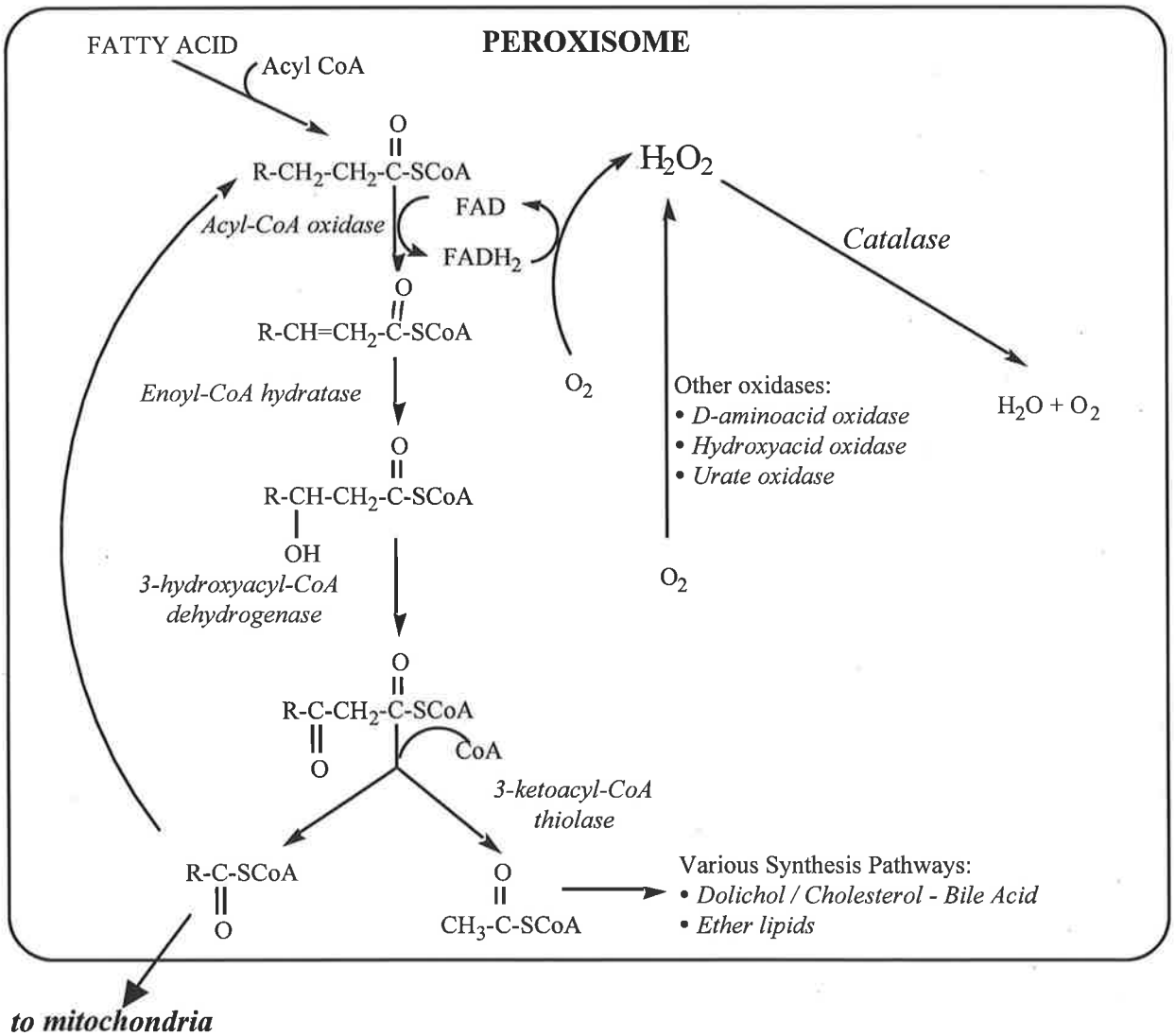



**Figure 1.3:** Biochemical pathways in the generalised peroxisome.

A key difference between mitochondrial and peroxisomal ß-oxidation is the fate of $FADH_2$ at the step when the fatty acyl-CoA ester is dehydrogenated to the trans-enoyl CoA (Figure 1.3).

In the mitochondria, $FADH_2$ formed by acyl CoA dehydrogenase is oxidised by the electron transport chain, ultimately generating 2 ATP molecules. In the peroxisome however, the production of $FADH_2$ by acyl CoA oxidase is directly coupled to the reduction of molecular oxygen to $H_2O_2$ (Lenter *et al,* 1986). In addition, other oxidases present in the peroxisome also generate $H_2O_2$ as a byproduct of their enzymatic action. The $H_2O_2$ produced during these reactions are metabolised to oxygen and water by the enzyme catalase, which is present within the peroxisomal matrix (Lenter *et al,* 1986).

### 1.3. What initiates a proliferation of hepatic peroxisomes?

Peroxisomes are proliferated following exposure to various endogenous and also exogenous compounds (Table 1.1). PxPs exhibit varying potencies in their ability to induce peroxisome proliferation, as determined by an increase in activity of the peroxisomal marker enzyme, palmitoyl CoA oxidase (Bentley *et al*, 1993).

In addition, a number of physiological and pathological conditions can also increase the levels of peroxisomes. Since the major function of the peroxisome is the metabolism of fatty acids, it is not surprising that increases in cellular fatty acid levels initiate an increase in peroxisome number. A high dietary fat increases peroxisome numbers, as do conditions involving utilisation of fatty acids as an energy source, including starvation, diabetes and cold stress adaptation (Neat *et al*, 1980; Ishii *et al*, 1980a; Ishii *et al,* 1980b; Horie *et al*, 1981; Osmundsen, 1982; Pollera *et al,* 1983). Collectively, these observations indicate that the cellular level of peroxisomes is under homeostatic control and is increased on demand when required by the hepatocyte.

### 1.4. Are all species responsive to PxPs?

#### 1.4.1 Non-primate Species

Despite the occurrence of peroxisomes in the majority of cell types in all eukaryotic species, there are considerable interspecies variations in response to PxPs. Most studies investigating peroxisome proliferation have used rats and mice due to their exquisite sensitivity to these compounds, with morphological and biochemical evidence of an increase in peroxisomal number evident within 3 days of commencement of exposure (Moody and Reddy, 1976). Other rodent species have been found to be less sensitive. Syrian hamsters administered clofibrate at doses attaining greater plasma concentrations than those that produce peroxisome proliferation in rats and mice, found no increase in palmitoyl CoA oxidase activity, with other studies also indicating little or no effect in this species (Lake *et al*, 1984a; Orton *et al,* 1984; Pourbaix *et al*, 1984; Lake *et al*, 1989a). Guinea pigs have been found to be quite non-responsive or producing weak responses reportedly 10-20 fold smaller than those seen with equivalent doses in the rat and mouse (Osumi and Hashimoto, 1978; Oesch *et al*, 1988; Lake

*et al*, 1989a). Similarly, the Jerboa is also only weakly responsive to PxP compared to the rat (el Kebbaj *et al*, 1996).

Although a number of non-rodent species have been investigated, there is limited data on individual species which makes rigorous comparisons difficult. Non-rodent species tend to be either non-responsive, for example, dogs or having a small but measurable response, for example; cats, chicken, and pigeons, amphibians and various species of fish (Reddy *et al,* 1984; Eacho *et al*, 1986; Ciolek and Dauca, 1991; Scarano *et al,* 1994) .

### 1.4.2 Primate species

Primates are considered non-responsive to the peroxisome proliferative effects of PxPs. Studies using a variety of different PxPs typically revealed no induction of palmitoyl CoA oxidase activity in the livers of rhesus monkeys or the marmoset (Eacho *et al*, 1986; Orton *et al,* 1984) during chronic exposure to PxP. However, when a dose of ciprofibrate 5 times higher than that inducing a 35 fold increase in palmitoyl CoA oxidase activity in rats was administered to marmosets for 22 weeks, a weak but measurable 3.5 fold increase in the activity of this marker enzyme was found (Makowska *et al,* 1990). Similar experiments have estimated that monkeys are 40 to 70 times less responsive than rats (Reddy *et al*, 1984). This suggests that when PxP are administered at very high doses, monkeys are capable of responding, albeit less dramatically than rats or mice.

### 1.4.3 Humans

*In vivo* studies involving patients on hypolipidemic drug therapy have concluded that humans are non-responsive to the effects of PxP. While some studies have suggested small alterations of up to 50% increase in peroxisomal number, interpretation of such studies is hampered by the large individual variation observed, possible diurnal variations between sampling times, small sample sizes and lack of suitable controls (de la Iglesia *et al,* 1982; Hanefield *et al,* 1983; Gariot *et al,* 1987).

Another data source for human PxP exposure is patients undergoing renal dialysis who are exposed to phthalate esters present in the polyvinyl chloride pump tubing. An increased number of peroxisomes in the cytoplasm of liver cells in patients with renal failure was reported following 12 months of dialysis, however, this study was not biochemically quantitated (Ganning *et al,* 1984). Collectively, the alterations in peroxisomal number observed in human studies were marginal compared to the many fold induction observed in other species. Consequently, it is difficult to definitively conclude as to whether any peroxisome proliferation occurs in humans.

The use of isolated hepatocytes allows an ease of comparison across species which is not possible *in vivo.* Isolated hepatocyte studies have paralleled *in vivo* findings with rat and mouse cells being highly responsive, compared to limited response of both guinea pig and hamster with dog, monkey and human cells essentially non-responsive (Lake *et al,* 1986, Foxworthy *et al,* 1990; Mennes *et al,* 1994; Cornu-Chagnon *et al,* 1995; Richert *et al,* 1996). Investigations with various doses of PxP reveal that hepatocytes isolated from guinea pig, rabbit or monkey livers are at least 30 times less sensitive to the peroxisome proliferative effects of PxPs than rat hepatocytes (Dirven *et al,* 1993). Also, as peroxisome proliferation was observed in cell culture, species differences cannot be simply explained by variations in the *in vivo* disposition or metabolism of PxP. The intrahepatic nature of peroxisome proliferation indicates that intrinsic biochemical difference(s) probably account for the different responsiveness between species.

## 1.5 Mechanism of Peroxisome Proliferation

The mechanism of peroxisome proliferation has been subject to extensive investigation since this class of chemicals was first identified. Although considerable physiological, morphological and biochemical data suggested that an increase in cellular fatty acids (or peroxisome substrate overload) initiates an increase in peroxisomes, this theory does not explain the rapid gene transcription that occurs after exposure to certain chemical PxPs.

### 1.5.1 The Peroxisome Proliferator Activated Receptor

Early studies indicated that the proliferation of peroxisomes could involve the activation of an intracellular receptor. The first indication of a potential PxP binding site was the discovery of a protein in rat liver cytosol that was retained on a nafenopin bound affinity column (Lalwani *et al*, 1983). When other PxP were used as the affinity ligand, the same protein was isolated suggestive of a common PxP binding domain (Lalwani *et al,* 1987). This protein was identified as a 70 kD protein with close structural homology to a heat shock protein (HSP). However, the existence of a specific receptor for PxPs was finally established when Isseman and Green cloned a nuclear hormone receptor to which they assigned the name "peroxisome proliferator activated receptor" (PPAR) (Isseman and Green, 1990).

PPARs belong to the nuclear receptor family of steroid hormone receptors which act as transcription factors and thus exert their regulatory functions at the gene level. Although their mechanism of action is within the nucleus, the PPAR could exist in the cytosol complexed to and stabilised by HSP in a manner similar to other steroid hormone receptors, including dioxin receptors (Poellinger *et al,* 1992). In support of this premise, the PPAR has been shown to interact directly with HSP72 (Huang *et al,* 1994). Upon ligand binding the HSP dissociates and the cytoplasmic PPAR is translocated to the nucleus where it dimerises with a receptor cofactor, the retinoid X receptor (RXR) for which *cis* 9-retinoic acid is the activating ligand.

This dimer complex is then translocated to the nucleus where it binds to the "peroxisome proliferator response element" (PPRE) and increases the transcription of PxP-responsive genes (Ashby *et al,* 1994). The PPAR has been shown to regulate genes involved in the peroxisomal ß-oxidation and microsomal ω-hydroxylation of fatty acids and also ketogenesis pathways and it is therefore not surprising that the PPRE has been found in the promotor region of several genes including those coding for enzymes involved in fatty acid ß-oxidation, acyl CoA oxidase and peroxisomal bifunctional enzyme and proteins involved in cellular fatty acid storage such as the fatty acid binding protein (Isseman *et al,* 1992; Tugwood *et al,* 1992; Zhang *et al,* 1993). For a recent review on the different genes regulated by PPAR and their roles in intra- and extracellular lipid metabolism, refer to Schoonjans *et al,* 1996.

*1.5.1.1 Subtypes of the PPAR*

Following the discovery of the PPAR, at least three different subtypes of PPAR have been identified (Dreyer *et al,* 1992) including PPARα, PPARβ and PPARγ, the latter having two different subtypes (Mukherjee *et al,* 1997). The subtypes are the products of separate genes located on different chromosomes and differ in their tissue distribution. PPAR-α and PPARβ are mainly expressed in liver, brown adipose tissue and kidney with the PPARα as the dominant form in both quantity and distribution. However, the PPARα also occurs in a number of other tissues including the gut, brain, gonads, lung, spleen (Jones *et al*, 1995). The PPARγ is the major PPAR in brown adipose tissue and other than a small amount in liver, is not present in high quantities elsewhere (Jones *et al*, 1995). Definitive evidence for the involvement of the PPARα in modulating many, if not all, the short term effects of PxP exposure was obtained using transgenic mice without a functional PPARα. These mice showed no biochemical or physiological responses to PxPs, as determined by hepatomegaly, changes in marker enzymes or increase in peroxisome number when compared to wild type mice (Lee *et al,* 1995).

*1.5.1.2 Do PxP directly interact with the PPAR?*

With the knowledge that the PPAR indeed mediates peroxisome proliferation, the question can be asked if the PxP are ligands for these receptors. Although most PxP contain certain general characteristics, namely amphipathic molecules with a hydrophobic backbone and acidic function. The majority of PxP have very diverse structures (Bentley *et al,* 1993). It thus appears that the PPAR lack the highly specific ligand binding site found with other steroid hormone receptors.

Indirect evidence has been obtained using transfection experiments whereby a number of structurally diverse compounds have been demonstrated to activate PPARα, including fibric drugs, phthalates and lipid derivates (Göttlichner *et al,* 1992, Isseman *et al*, 1993; Keller *et al,* 1993; Varanasi *et al,* 1996; Lapinskas and Corton, 1997). As activation of the PPARα by PxP

occurs at 10 times lower concentrations than those required by other PPAR subtypes, it has been suggested that PxP may be direct ligands for the PPARα (Kleiwer *et al,* 1994). However, other studies have shown that structurally diverse PxP produce similar conformational changes within mouse PPARα which may underlie the molecular basis of these compounds to activate PPAR (Dowell *et al*, 1997).

*1.5.1.3 What are the endogenous ligands for the PPAR?*

The existence of the PPAR strongly suggests the presence of an endogenous ligand. Studies utilising *in vitro* expression systems have shown that certain polyunsaturated fatty acids and their derivatives, including leukotrienes and prostaglandins which may act as endogenous ligands (Göttlichner *et al,* 1992; Schmidt *et al* , 1992; Kliewer *et al*, 1995; Krey *et al,* 1997; Lapinskas and Corton, 1997).

*1.5.1.4 Does variation in the PPAR underlie species differences?*

The knowledge that the PPAR mediates some, if not all the effects of PxPs (Lee *et al,* 1995) suggests that non-responsive species may have differences in the PPAR compared to responsive rodent species. It is unlikely that the RXR or its ligand *cis*-9-retinoic acid is absent or present in lower quantities in nonresponsive species as *cis*-9-retinoic acid and RXR are involved in the activation of other nuclear receptor systems (Ashby *et al,* 1994). But still, there could be undiscovered factor(s) required for PPAR action which are lacking in nonresponsive species or in some species specific represser of receptor activated transcription may be present. The activation threshold for mouse PPAR is similar to that of human PPAR indicating that a similar level of agonist binding should initiate the same response (Tugwood *et al,* 1996). While there is considerable intraindividual variation, the gene for PPARα is expressed at very low levels in humans (Tugwood *et al,* 1996) and these low expression levels have been speculated to result in the lack of PxP response in humans (Lapinskas and Corton, 1997).

### 1.5.2 A role for substrate overload in peroxisome proliferation?

While it is possible that certain PxP directly activate the PPAR, it is also logical that a PxP-mediated increases in intrahepatic levels of endogenous ligands also increase peroxisome numbers. This is supported by the observations that increased influx of fatty acids into the liver caused by dietary, physiological or pathological conditions cause peroxisome proliferation (Neat *et al*, 1980; Ishii *et al* , 1980a; Osmundsen, 1982).

Histological studies have shown an increase in fatty droplet accumulation indicating a transient increase in neutral lipid content in the liver shortly after administration of certain PxP including di (2-ethyl hexyl) phthalate (DEHP) (Lock *et al,* 1989). This suggests an

inhibition of fatty acid metabolism, causing a pooling of unmetabolised lipid in the cytoplasm, thus resulting in an activation of the PPAR.

Fatty acid metabolism involves a number of biochemical steps and any of these could be inhibited, ultimately causing an increase in the amount of unmetabolised fatty acids (Elcombe and Mitchell, 1986). For example, the first step of fatty acid metabolism involves the formation of a fatty acid acyl CoA ester. It follows that a decrease in the availability of acyl CoA would inhibit fatty acid metabolism. A number of carboxylic acid PxPs are able to form acyl CoA esters which are unable to undergo ß-oxidation and decrease the amount of acyl CoA in the hepatocyte (Becker and Harris, 1983; Vamecq *et al*, 1985; Bronfman *et al,* 1986). Also, as fatty acid ß-oxidation is shared between the mitochondria and the peroxisome, perturbation in cellular fatty acid level may originate from the mitochondria. Inhibitors of mitochondrial fatty acid oxidation cause peroxisome proliferation (Becker and Harris, 1983), yet only in responsive species (Mitchell *et al,* 1985; Elcombe and Mitchell, 1986).

Alterations in intracellular handling and storage may also provide potential increases in fatty acid levels. For example, the PxP, carbon tetrachloride ($CCl_4$) is believed to inhibit the synthesis of apolipoprotein by $CCl_4$, resulting in decreased lipid transport out of the hepatocyte and fat accumulation (Becker *et al*, 1987). Also fatty-acid binding protein has a high affinity for long chain fatty acids thus providing intracellular storage for fatty acids (Mishkin *et al,* 1975). Some PxP have been reported to bind to the fatty acid binding site thereby displacing fatty acid and increasing cellular fatty acid concentration (Cannon and Eacho, 1991).

It is clear that there are multiple loci at which PxP can increase fatty acids in the hepatocyte, namely by increasing influx into the liver either via diet or from extrahepatic stores; inhibiting metabolism of fatty acids; reducing transport out of the hepatocyte or by displacing fatty acids from intracellular storage sites. It is quite possible that both the receptor mediated and substrate overload theories are not mutually exclusive, with both theories participating in the overall proliferation produced by chemicals. It is possible that such a combination of effects may account for the variations in the time course of induction, potency and the lack of structure activity relationships between the PxP.

### 1.6 The effects of exposure to PxPs

Short term treatment shows that PxP are well tolerated and nonnecrogenic at doses producing peroxisome proliferation and other characteristic biochemical alterations within the cell (Reddy *et al,* 1980). The long term effects of chronic exposure to PxP involve a massive increase in the incidence of hepatocarcinogenesis. For example, the hypolipidemic drug, clofibrate has been reported to cause hepatocarcinogenesis in over 90% of exposed rats

(Reddy and Qureshi, 1979). As with the occurrence of extensive peroxisome proliferation, the high incidence of hepatocarcinogenesis has only been identified in rats and mice (Ashby *et al,* 1994). An extensive study which critically assessed data from both responsive and nonresponsive species showed a good but incomplete 81% correlation between the short term ability to proliferate peroxisomes and the long term incidence of hepatocarcinogenesis (Ashby *et al*, 1994). In general, it appears that doses of PxP that initiate a 10 fold increase in peroxisomes within 14 days of treatment, will ultimately lead to an 80% or greater incidence of hepatic cancer. The exceptions, whereby peroxisome proliferation and hepatocarcinogenecity appear not to be related, could be explained by differences in dose and study design, sex, species, strain or other unidentified factors (Ashby *et al*, 1994).

### 1.6.1 Long term exposure: Hepatocarcinogenesis

PxPs are both specific and complete hepatocarcinogens requiring simply sustained exposure in the absence of an initiator. It has been well established that PxP belong to a class of epigenic (or nongenotoxic) carcinogens. A number of different tests have investigated the potential interactions of PxPs with DNA including *in vitro* assays such as the Ames test, covalent binding of radiolabelled PxP to DNA, DNA adduct determination by $^{32}$P-postlabelling, alkaline elution assays and *in vivo* studies with all these tests having yielded negative results, verifying that direct DNA damage is not a feature of the carcinogenic action of PxPs (Warren *et al*, 1980; Butterworth *et al* 1984; Goel *et al*, 1985; Zeiger *et al*, 1985; Bentley *et al*, 1987; Elliott and Elcombe, 1987; Cattley *et al*, 1988; Lefevre *et al*, 1994).

Two mechanisms have been suggested to explain the hepatocarcinogenecity of PxP, both of which may result from PxP activation of the PPAR. These are the sustained induction of cell proliferation and the biochemical induction of an intracellular oxidative stress.

#### *1.6.1.1 Induction of cell proliferation*

Following administration of most PxP, there are 2 distinct phases of cell proliferation. An initial burst of mitotic activity and cell replication occurs in the first few days of exposure followed by sustained stimulation of DNA synthesis and cell division which persists for as long as the PxP is administered (Marsman *et al,* 1988; Eacho *et al,* 1991; Price *et al,* 1992; Lake *et al,* 1993). Although it has been suggested that the latter phase has greater importance in the development of carcinogenesis (Wada *et al*, 1992), the early phase could result in the formation of an initiated cell, which is amplified and sustained during the ensuing cell replication phase (Ashby *et al,* 1994). In support of this theory as a mechanism of hepatocarcinogenesis, a comprehensive study investigating cell replication during PxP exposure revealed a good correlation between stimulation of S-phase of cell division and the incidence of hepatic tumours (Ashby *et al,* 1994).

The activation of PPARα has been implicated in the mitogenic process and while the genes involved have not been identified, this activation is a significant component of tumour progression (Cattley and Preston, 1995). In addition to stimulation of mitogenesis, PxP treatment also inhibits apoptosis, or programmed cell death (Bayly *et al,* 1993; Bayly *et al,* 1994). Apoptosis normally identifies defective cells, such as those with damaged DNA suggesting a mechanism where initiated cells would be removed prior to division and attaining their carcinogenic potential. This suggests that with long term PxP treatment there could be an enhanced population of defective cells which may provide the starting point for carcinogenesis.

*1.6.1.2 Oxidative Stress Theory*

The other mechanism proposed to be involved in the initiation of hepatocarcinogenesis is the oxidative stress theory. Oxidative stress, or an overall cellular increase in reactive oxygen species has been implied as an important part in the promotion of hepatic tumours (Cerutti, 1985). The origin of this theory is related to disproportionate increase of peroxisomal enzymes involved in the production compared to the degradation of $H_2O_2$ which links shortterm peroxisome proliferation to the onset of hepatocarcinogenesis. To damage nuclear DNA, $H_2O_2$ would have to leave the peroxisome, transverse the cytoplasm where it may or may not be converted to other oxyradicals and enter the nucleus whilst avoiding any intracellular defences in the form of both degradative enzymes and antioxidants. Investigation of this theory has involved a number of different strategies for direct or indirect evidence for an increase in oxidant species however interpretation has been plagued by conflicting results.

In support of an increase in intracellular $H_2O_2$, an over 30 fold induction of $H_2O_2$ was found in peroxisomes purified from PxP treated compared to untreated controls (Fahl *et al,* 1984). Also, an increase in hydroxyl radical formation determined by electron spin resonance studies was reported in liver microsomes prepared from PxP treated rats (Elliot *et al,* 1986). However, in both studies, the inhibition of catalase via cyanide addition to prevent mitochondrial fatty acid ß-oxidation would give an artefactually high result. Other measurements of $H_2O_2$ in homogenates have found a relatively small increases when compared to the extent of peroxisome proliferation (Tomaszewski *et al,* 1986; Tamura *et al,* 1990). However, a recent study observed an 89% increase in steady state $H_2O_2$ following 2 weeks of PxP treatment (Lores-Arnaiz *et al,* 1997). In addition, the formation of superoxide anion has also been reported to be 4 fold higher in mouse leukemia cells exposed to clofibrate in culture for 3 days (Lawson and Gwilt, 1993). Also, there have been some *in vitro* studies that suggest an increase in oxidative species following peroxisome proliferation after treatment with PxP is possible. For example, when transfected rat peroxisomal acyl CoA oxidase was exposed to its

substrate, linoleic acid, an overproduction of $H_2O_2$ occurred, and when transplanted into mice resulted in enhanced tumour formation (Chu *et al* , 1995).

*o Evidence of oxidative stress following exposure to PxP?*

A major cellular target of oxyradicals is unsaturated fatty acids in the cellular membranes. At these sites, hydrogen abstraction by a reactive species initiates an autocatalytic cascade involving lipid peroxyl radicals (Halliwell and Gutteridge, 1989). Numerous studies have yielded conflicting results following exposure to PxPs with increases (Goel *et al,* 1986; Tamura *et al,* 1991), decreases (Tomaszewski *et al,* 1986; Huber *et al,* 1991) and no alteration (Perera *et al,* 1986; Elliot and Elcombe,1987) in the formation of either malondialdehyde or conjugated dienes, both of which are markers of lipid peroxidation. Thus the expected increase in lipid peroxidation does not seem to be conspicuous following PxP treatment. Indeed, in the studies in which an increase was seen, the magnitude of the reported increases were not great, typically less than 50% over control values (Lake *et al,* 1981; Goel *et al,* 1986; Tamura *et al,* 1990).

In addition to lipid peroxidation products, the 8-hydroxyguanine (8OHG) adduct of DNA has been widely used as a marker for oxidative stress. While some studies have shown no change in 8OHG, an up to 2.5 fold increase in 8OHG levels has also been reported (Kasai *et al,* 1989; Hegi *et al,* 1990; Takagi *et al*, 1990; Cattley and Glover, 1993). However, separation of the nuclear material from the mitochondrial fraction showed that the 8OHG levels in the nucleus were unchanged (Cattley and Glover, 1993). As the normal level of 8OHG in mitochondria is 16 times higher than that observed in the nucleus (Richter *et at,* 1988), the observed increase in 8OHG levels may reflect the around 1.4 fold increase mitochondrial content that occurs with PxP treatment (Sausen *et al,* 1995). Interestingly, other studies have found that PxP treatment enhances the susceptibility of DNA to damage by other agents (Lawson and Gwilt, 1993; Hayashi *et al,* 1995). This suggests that PxP, while not being directly genotoxic, could amplify either spontaneous or chemically-induced DNA damage leading to an increased risk of initiation of carcinogenesis. Finally, If PxPs cause an intracellular oxidative stress, then coadministration of an antioxidant would be expected to ameliorate long term carcinogenesis. Again conflicting results have been reported, with a decrease in ciprofibrate-induced hepatic tumour size and number observed upon coadministration of the antioxidants 2(3)-*tert*-butyl-4-hydroxyanisole or ethoxyquin (Rao *et al,* 1984), while an increased number and size of individual tumours was observed after 2 years of coadministration of α-tocopherol (Glauert *et al,* 1990).

*1.6.1.3 A combination of cell proliferation and oxidative stress ?*

While the oxidative stress theory is conceptually feasible, there is no conclusive evidence that oxidative stress is involved in the hepatocarcinogenic response of PxP. It is possible that the

mechanism of hepatocarcinogenesis of PxP involves both cell replication and oxidative stress. During enhanced replication, the cell has reduced time available for DNA repair and thus a higher probability of uncorrected oxidative DNA damage becoming fixed as mutations. Another possibility is that oxidative stress occurs after the development of tumour nodules. As the nodule grows, the blood supply alters such that cells are supplied by the hepatic artery. The increased oxygen levels from the enhanced hepatic artery blood supply could enhance the potential for oxidative stress within the nodule. Such an increase in oxidative stress could be important in the transition from promotion to progression phases in the process of hepatocarcinogenesis (Bentley *et al,* 1993). As a final possibility, the accumulation of spontaneously initiated cells as a result of age related increases in basal oxidative damage may be increased by PxP exposure. Coupled with the PxP related increase in cell replication, there would be an overall increase in hepatocarcinogenesis. In support of this premise, nafenopin more readily promotes tumour formation in the livers of old compared to young rats (Kraupp-Grasl *et al,* 1991).

In conclusion, the proliferation of the actual peroxisome organelle may not be the only factor involved in the carcinogenic effect of PxPs. It is possible that PxPs produce cancer in rodents via a mechanism totally distinct from and independent of an increase in peroxisomal number.

### 1.6.2 Short term exposure: Peroxisome proliferation

Although the proliferation of peroxisomes is a unifying and easily identifiable parameter, short term (less than 1 month) pretreatment with PxPs induces a number of other characteristic changes at different levels of cell and tissue organisation.

#### *1.6.2.1 Morphological effects*

*o Whole organ - Liver*

The most obvious effect produced by PxPs is a marked increase in liver size or hepatomegaly involving both hyperplasia (increase in cell number) and hypertrophy (increase in cell size) (Caythew *et al,* 1997). Hepatomegaly can be detected as an increase in liver weight within a few days of commencement of PxP administration with steady state reached within 10-14 days and maintained for the duration of PxP administration (Reddy *et al,* 1976). In the first few days, stimulation of cell division results in a hyperplastic response which peaks at day 3 (Moody *et al,* 1977; Reddy *et al,* 1979; Elcombe *et al,* 1985). The liver continues to slowly increase in size due largely to hypertrophy caused by an increased synthesis of both smooth endoplasmic reticulum and peroxisomes (Carthew *et al,* 1997).

*o The liver lobule*

Following PxP treatment, the number of peroxisomes within parenchymal cells becomes uneven across the lobule, with greater numbers observed in the centrilobular region (Just *et al,*

1989; Bell *et al,* 1991). In addition, the hepatomegalic effects of PxPs on hepatocytes is spatially heterogenous with hyperplasia predominantly observed in periportal hepatocytes and hypertrophy occurring in the centrilobular region (Marsman *et al,* 1988; Barass *et al,* 1993; Soames and Foster, 1995).

*o The hepatocyte*

While the peroxisomes are by far the most responsive organelle to PxPs, various changes also occur in other organelles including the endoplasmic reticulum, mitochondria, lysosomes and plasma membranes (Kurup *et al,* 1970; Gear *et al,* 1974; Conway *et al*, 1989; Bartles *et al,* 1990; Malki *et al*, 1990; Sausen *et al,* 1995; McGuire *et al,* 1995).

*o The peroxisome*

PxPs seem to induce the synthesis of a different type of peroxisome to those normally present in hepatocytes. Two distinct populations of peroxisomes were isolated following PxP treatment, one of which was not present in untreated livers (Hayashi *et al*, 1975). PxP-induced peroxisomes are larger, with a 25% increase in diameter (Leighton *et al,* 1975). They also differ in the size of their crystalloid cores, the types of inclusion bodies in the matrix, shape, and ability to form clusters (Hruban *et al,* 1966; Novikoff and Edelstein, 1977; Pollera *et al*, 1983).

*1.6.2.2 Biochemical changes as a result of peroxisome proliferation.*

In addition to altering the morphology and cellular numbers of peroxisomes, PxPs also induce a number of characteristic biochemical changes both within the peroxisome and in other cellular organelles.

*o Within the peroxisome*

Since not all the enzymes contained in peroxisomes are induced to the same extent, the enzyme profile within newly synthesised peroxisomes can differ quite markedly from that of the parent organelle. For example, depending on the PxP and the duration of exposure, acyl CoA oxidase activities can be induced from 5 to over 25 fold (Elcombe, 1985; Elcombe *et al,* 1985; Tomaszewski *et al,* 1986; Berge *et al,* 1988; Ikeda *et al,* 1988; Keith *et al,* 1992). Other enzymes, for example carnitine acyl transferase, exhibit more modest changes of around 3 fold (Moody and Reddy, 1978; Reddy and Lalwani 1983). For enzymes such as catalase, the induction is much less pronounced as seen in the 1.3 to under 2-fold increase in catalase levels despite greater increases in peroxisome number (Lazarow and de Duve, 1976; Osumi and Hashimoto, 1978; Elcombe *et al,* 1985; Tomaszewski *et al,* 1986; Rao and Reddy, 1987; Berge *et al,* 1988; Ikeda *et al,* 1988). Finally, urate oxidase levels are not induced above those found in the normal liver (Leighton *et al,* 1975; Berge *et al*, 1988; Usuda *et al*, 1988). Overall,

this indicates that the amount of catalase and urate oxidase is actually lower in peroxisomes from PxP treated compared to control animals (Leighton *et al*, 1975).

From a toxicological perspective, the imbalance between the extent of induction of fatty acid ß-oxidation enzymes and catalase is most significant. Following PxP treatment, the disproportionate levels of the 2 enzymes could cause an increase in peroxisomal $H_2O_2$ levels. $H_2O_2$ can diffuse through the highly permeable peroxisomal membrane (Cohen and Grasso, 1981) leading to an increase in the level of cytosolic $H_2O_2$ which forms the basis of the oxidative stress theory of carcinogenesis discussed in 1.6.1.

*o Extraperoxisomal Changes*

Biochemical alterations as a result of PxP exposure also occur in other organelles, including the mitochondria and lysosomes (Conway *et al*, 1989; Hakkola *et al,* 1994). The increase in smooth endoplasmic reticulum is accompanied by an increase in CYP4A1 activity which can be measured by determining lauric acid hydroxylase activity and despite being a microsomal enzyme, is routinely used as a marker for peroxisome proliferation (Ikeda et al, 1988; Lake *et al,* 1989a). Other examples of extraperoxisomal alterations will be discussed in Section 1.7.

In general, exposure to PxP induces peroxisomal enzymes involved in the metabolism of fatty acids while other peroxisomal enzymes which are not directly involved with fatty acid metabolism are only slightly increased or not affected. There is also a specific induction of microsomal CYP4A1, which is involved in fatty acid hydroxylation. Overall, the enzymatic and cofactor profile induced following PxP administration is appropriate for the metabolism of fatty acid excess.

## 1.7 The effects of short term PxP pretreatment on biotransformation.

In addition, PxPs alter the activity of a number of enzymes and the level of various factors known to be involved in drug metabolism and the manifestation of cell injury. In general, the process of biotransformation converts an xenobiotic into metabolites that are more hydrophilic, thereby facilitating excretion from the body. Xenobiotic metabolism is composed of 2 phases. In the majority of cases, the activation phase, Phase I, involves the insertion of a new functional group into the molecular structure while the detoxication phase, or Phase II, deactivates the products of Phase I metabolism. A generalised scheme for biotransformation is given in Figure 1.4.

### 1.7.1 Alterations in Phase I pathways

The activation phase is catalysed by Phase I enzymes, the main function of which is to provide or expose groups for further metabolism. The majority of Phase I oxidative metabolism involves CYP450 system located in the endoplasmic reticulum. CYP450 is

composed of a number of different isoforms with differing and overlapping substrate specificities enabling metabolism of a broad range of chemicals. Repeated administration of some xenobiotics can induce the CYP450 isoforms involved in its metabolism, thus promoting clearance of the xenobiotic. It is obvious that the toxicity of other xenobiotics metabolised by the induced isoform(s) would also be altered.

In rat liver, the major CYP4 induced by PxP is CYP4A1, an isoform which exhibits high specificity for the ω-hydroxylation of fatty acids and arachidonic acid (Gibson and Lake, 1991). While the level of CYP4A1 is around 4% of total CYP450, it can be increased 5 to 10 fold by treatment with PxP (Sharma *et al,* 1988). Any xenobiotic metabolised by CYP4A1 will have enhanced clearance or toxicity depending on the types of intermediates produced and affinity for subsequent detoxication reactions. However, while CYP4A1 has an important physiological role catalysing hydroxylation of fatty and arachidonic acids, it appears to play a limited role in the metabolism of other chemicals (Orton *et al,* 1984; Gonzales, 1989).

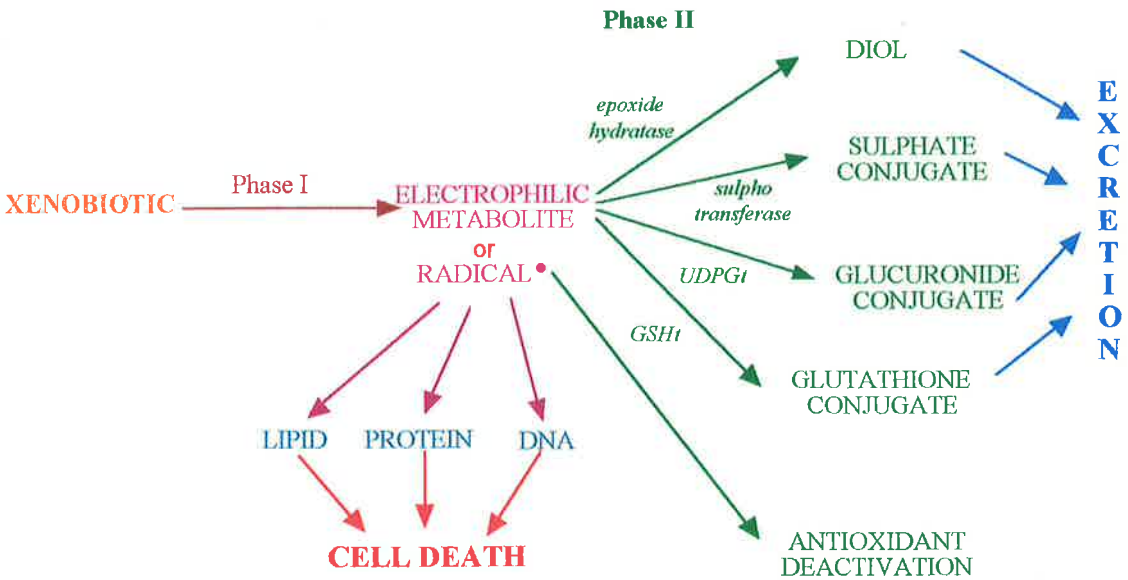



**Figure 1.4.** Generalised scheme of xenobiotic metabolism. Phase I enzymes can transform the xenobiotic into a reactive species, which if not deactivated by Phase II enzymes or antioxidants which facilitate excretion, have the potential to interact with cell macromolecules that can lead to cell death.

### 1.7.2 Alterations in Phase II conjugation pathways

Prior to interaction with cell macromolecules, electrophilic intermediates are usually detoxified in conjugation reactions catalysed by Phase II enzymes. For a cell to avoid toxicity, there must be a balance between the activation and detoxication phases of xenobiotic metabolism. The Phase II enzymes are situated in various cellular locations and usually composed of isoforms which differ in physiochemical properties. The action of Phase II enzymes is dependent on the both the affinity of the substrate and also th availability of the conjugate. As with Phase I, Phase II enzymes can be modified by xenobiotics, with either an

induction or inhibition of enzyme levels and/or activity leading to an exacerbation or amelioration of xenobiotic toxicity.

*1.7.2.1 Glutathione S-transferases*

Glutathione S-transferases (GSHt) are multifunctional cytosolic enzymes whose major cellular role is to catalyse the nucleophilic conjugation of glutathione (GSH) to electron deficient species and comprise a major detoxication pathway. While the majority of GSH conjugates are readily excretable, some are more toxic than the parent compound, for example, episulphinium ions derived from dihaloalkanes (Elfarra and Anders, 1984; Inskeep *et al,* 1986). There are a number of GSHt isozymes, which can be categorised into subgroups on the basis of substrate specificities and physicochemical properties (Ketterer *et al,* 1988).

A number of studies have been conducted on GSHt following pretreatment with PxP, with no clear unifying action on the activity of this enzyme family. In general, PxP have been found to cause a decrease in GSHt activity of up to 80% in rats and mice (Goel *et al,* 1986; Lundgren and DePierre, 1989). However, other studies have observed either no change or an increase in GSHt activity (Moody *et al,* 1991; Demoz *et al,* 1993; McIntosh *et al,* 1993; Thomas *et al,* 1994). When a number of different substrates for GSHt were tested, GSHt activity was increased, decreased or remained the same, depending on the GSHt substrate used (Foliot *et al,* 1986; Thomas *et al,* 1989a). Similarly, measurement of individual isozyme subunit levels or activity of individual isozymes indicated differential effects by PxP (Nicholls *et al,* 1983; Awasthi *et al,* 1984; Schramm *et al,* 1989; Foliot and Beaune 1994; Voskoboinik *et al,* 1996). Overall, although GSHt activity can be modified, it appears independent of PxP initiated transcription events and is probably related to the chemical characteristics of the individual PxP itself.

*1.7.2.2 UDP-glucuronyl transferase*

Uridine diphosphate glucuronide transferases (UDPGt) are a family of isozymes which catalyse the conjugation of UDP glucuronic acid to a variety of xenobiotics with constituent groups including hydroxyl, amino and sulphydryl groups. UDPGt are capable of induction and this usually occurs concurrently with induction of Phase I metabolism (Hayes, 1989).

All PxP have the common property of stimulation of the glucuronidation of bilirubin which results from an increase in mRNA for the specific bilirubin UDPGt isoform (Magdalou *et al,* 1993). However, there appears to be no correlation between the extent of UDPGt induction and the potency of the PxP to either increase the number of peroxisomes or induce CYP4A1 activity (Thomas *et al,* 1989b; Arand *et al,* 1991).

*1.7.2.3 Sulphotransferases*

Sulphotransferases (SOt) catalyse the transfer of sulphate groups from 3'-phospho-adenosine-5'-phosphosulphate (PAPS) to hydroxyl groups of xenobiotics or Phase I metabolites. The enzyme is present in most eukaryotic species and there are at least 5 different subgroups of SOt isoforms. Unlike GSHt and other Phase II enzymes, SOt isoforms are not induced by classic inducers of Phase I activity (Hayes, 1989).

Only a limited number of studies have investigated the effects of PxP on SOt activity. While pretreatment with DEHP and clofibrate had no effect on SOt activity, perfluorocarboxylic acid caused a significant reduction in the level of 3 SOt isoforms (Witzmann *et al,* 1996). Also, the direct effects of PxP on SOt activity, SOts have been suggested to be involved in the instigation of the peroxisome proliferative effect of dehydroepiandrosterone (Waxman, 1996).

*1.7.2.4 Epoxide Hydralase*

Epoxide hydralases (EHs) occur in different isoforms in both the microsomal (mEH) and cytosolic (cEH) fractions of the cell and can be distinguished on the basis of their substrate specificities (Moody *et al,* 1985). In animals, EHs catalyse the hydration of epoxides derived from Phase I metabolism to the corresponding *trans*-diols.

A number of PxP have been reported to cause a 2 to 13 fold increase in the activity of cEH (Moody *et al,* 1985; Moody and Hammock, 1987; Lundgren *et al,* 1988; Lundgren and DePierre 1989; Moody *et al,* 1991; Sohlenius *et al,* 1993). In contrast, PxPs have little or no effect on mEH activity (Meijer and DePierre, 1988; Moody *et al*, 1991). In addition to the effects of chemical PxP, peroxisome proliferation induced by both diabetes and starvation caused a 2 fold increase in cEH, but a 60% reduction in mEH activity (Thomas *et al,* 1989a). It has been established in a study investigating the liver protein profile, that one of the over 100 proteins induced by PxP treatment was cEH (Anderson *et al,* 1996). This suggests that the increased cellular content of this enzyme results from a direct activation of the PPAR.

Collectively, the effects of PxP on this selected group of four Phase II detoxication enzymes indicate that each enzyme is differentially affected and that consideration must be given to the effects of PxP on individual isoforms within the cell milieu.

### 1.7.3 Alteration in antioxidant enzyme defences

Along with the Phase II enzymes, another group of important detoxication enzymes are the antioxidant enzymes, specifically catalase, superoxide dismutase (SOD) and glutathione peroxidase (GSHPx), which protect against the adverse effect of reactive oxygen species or their products. Decreased levels of catalase, GSHPx and SOD would increase the potential for oxidative stress to occur in the hepatocyte. Catalase activity, which is peroxisomal in origin,

has been found to be induced approximately 1.5 fold (Leighton *et al,* 1975; Elcombe *et al,* 1985; Berge *et al,* 1988; Ikeda *et al,* 1988), however, as the level of peroxisomes is increased 5 fold, the actual catalase protein content in each individual organelle is decreased (Leighton *et al,* 1975).

*1.7.3.1 Glutathione peroxidase*

Any endogenously produced $H_2O_2$ can be metabolised by either catalase in the peroxisome or GSHPx found in the cytoplasm. Unlike catalase, which metabolises only $H_2O_2$, GSHPx metabolises a variety of peroxides including fatty acid hydroperoxides formed during the lipid peroxidation cascade. Oxidised GSH (GSSG) is a product of the action of GSHPx and is considered a marker of intracellular oxidative stress. There are two forms of glutathione peroxidase, selenium (Se) and non-Se dependent GSHPx (Lawrence and Burk, 1976). $H_2O_2$ is preferentially metabolised by the Se-dependent form which constitutes 65% of the total GSHPx activity (Halliwell and Gutteridge, 1989). The non-Se GSHPx forms part of the α-subclass of multifunctional glutathione S-transferase (GSHt) family which can detoxicate various peroxides including cumene hydroperoxide.

The activity of GSHPx has been found to be decreased by 30 to 80% of control activity after PxP treatment (Furukawa *et al,* 1985; Goel *et al,* 1986; Perera *et al,* 1986, Cattley *et al,* 1987; Elliot and Elcombe 1987; Conway *et al,* 1989). The decrease in GSHPx activity was observed not to be related to a decrease in the amount of enzyme, but a direct effect on enzymatic activity (Makowska, *et al,* 1990). GSSG is reduced back to GSH by the action of glutathione reductase (Gred). Gred has been found to be inhibited by PxP (Glauert *et al,* 1992), suggesting that in PxP treated animals, GSH concentrations would be lower, thereby increasing the potential for intracellular oxidative stress.

*1.7.4.2 Superoxide dismutase*

Superoxide dismutases (SOD) are a group of metalloenzymes, comprising a copper:zinc form in the cytoplasm and a manganese form in mitochondria. Both SOD isoforms catalyse the dismutation and therefore degradation of the superoxide anion which is generated during normal cellular metabolism.

Short term treatment with PxP was found to decrease SOD activity however, the decrease was modest 20% (Ciriolo *et al*, 1982; Elliot and Elcombe, 1987). A longer term treatment of 78 weeks was found to cause either no change or a slight increase in activity (Glauert *et al,* 1992). SOD has been identified in the peroxisomal matrix and activity in this organelle was found to be increased following 3 weeks of treatment with ciprofibrate (Dhaunsi *et al,* 1994).

In general, each of these three antioxidant enzymes have been reported to be decreased by pretreatment with PxP. The reduction in GSHPx, catalase and SOD activities would suggest that liver cells are less able to detoxify the active forms of oxygen generated during oxidative stress, including lipid hydroperoxides which are formed during lipid peroxidation.

### 1.7.4 Alterations in cellular antioxidants

Other than radicals resulting from Phase I activation, redox cycling of various xenobiotics can produce the superoxide anion which has the capacity to react with hydrogen peroxide to produce the reactive hydroxyl radical, an overproduction of which can lead to cellular oxidative stress. Endogenous antioxidants, such as GSH, α-tocopherol and ascorbic acid, are involved in the metabolism of these radicals and thus protection of the hepatocyte against oxidative damage.

#### *1.7.4.1 Glutathione*

GSH is an important cytoplasmic constituent which protects cells by participating in several non-enzymatic and enzymatic reactions. GSH can function as a nucleophile in drug conjugation, reductant in peroxide metabolism where it is converted to GSSG, a reductant of dehydroascorbate and α-tocopherol thereby maintaining functional levels of these antioxidants, a scavenger of singlet oxygen and the hydroxy radical, in addition to maintaining the thiol groups of intracellular proteins such as dihydrolipoate and coenzyme A (Halliwell and Gutteridge, 1989; Meister, 1991).

Following pretreatment with PxP, GSH concentration have been reported to be either unchanged or slightly increased compared to untreated controls (Agarwal *et al,* 1982; Goel *et al,* 1986; Perera *et al,* 1986; Lake *et al,* 1989b; Tamura *et al,* 1990; Glauert *et al,* 1992). Interestingly, the studies that report unchanged GSH levels are those involving long term (over 6 months) PxP treatment while increased GSH is reported in studies of 10 weeks or less. Treatment with PxP has been found to increase the levels of GSSG in both *in vivo* and *in vitro* models (Lake *et al,* 1989b; Tamura *et al,* 1990) which provide indirect support for the oxidative stress theory. PxP treatment appears to modify the levels of GSH and GSSG, however the extent and direction of the changes must be examined in individual studies considering the potential influence of species, dose, duration of treatment and PxP utilised in the study.

#### *1.7.4.2 α-tocopherol and ascorbic acid*

The chain breaking lipophilic antioxidant, α-tocopherol is able to donate an electron to a lipid peroxide to deactivate the lipid peroxidation cascade. The resultant α-tocopherol radical is more stable, hence less reactive, than the lipid peroxide and is reduced back to α-tocopherol in a reaction involving GSH and ascorbic acid (Halliwell and Gutteridge, 1989). Ascorbic acid is

able to reduce the superoxide anion, hydroxyl radical and singlet oxygen, reduces thiyl radical derived from GSH and deactivates hypochlorous acid, a powerful oxidant that is produced during inflammation (Halliwell and Gutteridge, 1989). The levels of both α-tocopherol and ascorbic acid have been reported to decrease in rodent liver during chronic treatment with PxP (Conway et al, 1989; Lake *et al,* 1989b; Glauert *et al,* 1992).

*1.7.4.3 Metallothioneins and other antioxidants*

There have been limited studies on the effects of PxPs on other antioxidants. Metallothioneins are cytosolic sulhydryl rich (33%) metalloproteins which are believed to provide some defence against oxidants. The high sulphydryl capacity makes these molecules excellent scavengers of singlet oxygen and hydroxyl radicals (Abel and deRuiter, 1989). In addition, metallothioneins strongly bind heavy metals, including univalent copper which is known to initiate lipid peroxidation (Halliwell and Gutteridge, 1989). Metallothionein genes are transcriptionally activated within tissues undergoing oxidative stress (Bauman *et al,* 1991). Treatment of rats with clofibrate was found to repress the expression of the metallothionein gene (Motojima *et al,* 1992). However, pretreatment with DEHP was shown to increase metallothionein levels up to 11-fold in a course of treatment spanning 11 weeks (Waalkes and Ward, 1989).

Ubiquinone is a relatively poor antioxidant when compared to α-tocopherol, the major lipophilic antioxidant in cellular membranes (Burton and Traber, 1990). However, unlike α-tocopherol which has decreased levels following PxP treatment, ubiquinone has been reported to be increased up to four fold in the liver by PxP (Aberg *et al,* 1996). The effects of PxP on the content of antioxidants, such as uric acid or bilirubin have not been examined.

In summary, treatment with PxPs appears to have a diverse action on cellular antioxidant levels being dependent on the potency and duration of administration of the specific PxP.

### 1.7.5 Conclusion: Potential effects of PxP on xenobiotic metabolism

The toxicity of xenobiotics is usually related to the formation of quantitatively minor, highly reactive intermediates by Phase I metabolism. The activity of Phase II and radical metabolising enzymes, cellular levels of conjugates and antioxidants are vital to hepatocyte defence. Other variables such as: species, strain, sex, age, genetic predilection, lifestyle and environmental modifiers (eg diet, occupational exposures, therapeutic and recreational drug use) can influence the levels and/or activities of detoxication defences (Hayes, 1989; Timbrell, 1994; Casarett and Doull, 1995). It is apparent that alterations in either Phase I, Phase II or other defence systems can change the handling of specific xenobiotics by the cell. Depending on the nature and extent of the alteration involved, this can result in either

increased or decreased toxicity. The work described in this thesis investigates these possibilities using a number of well established toxicants.

The PxP can also have a number of effects on xenobiotic metabolism. While it is evident that there is a number of different loci whereby PxP can sway the toxicity of other compounds (Figure 1.5), yet little is known about the consequences of prior exposure to PxPs on the toxicity of other chemicals. In addition, there is no consistently expressed biochemical alteration which could provide a clue as to the mechanism by which PxP could change hepatotoxicity.

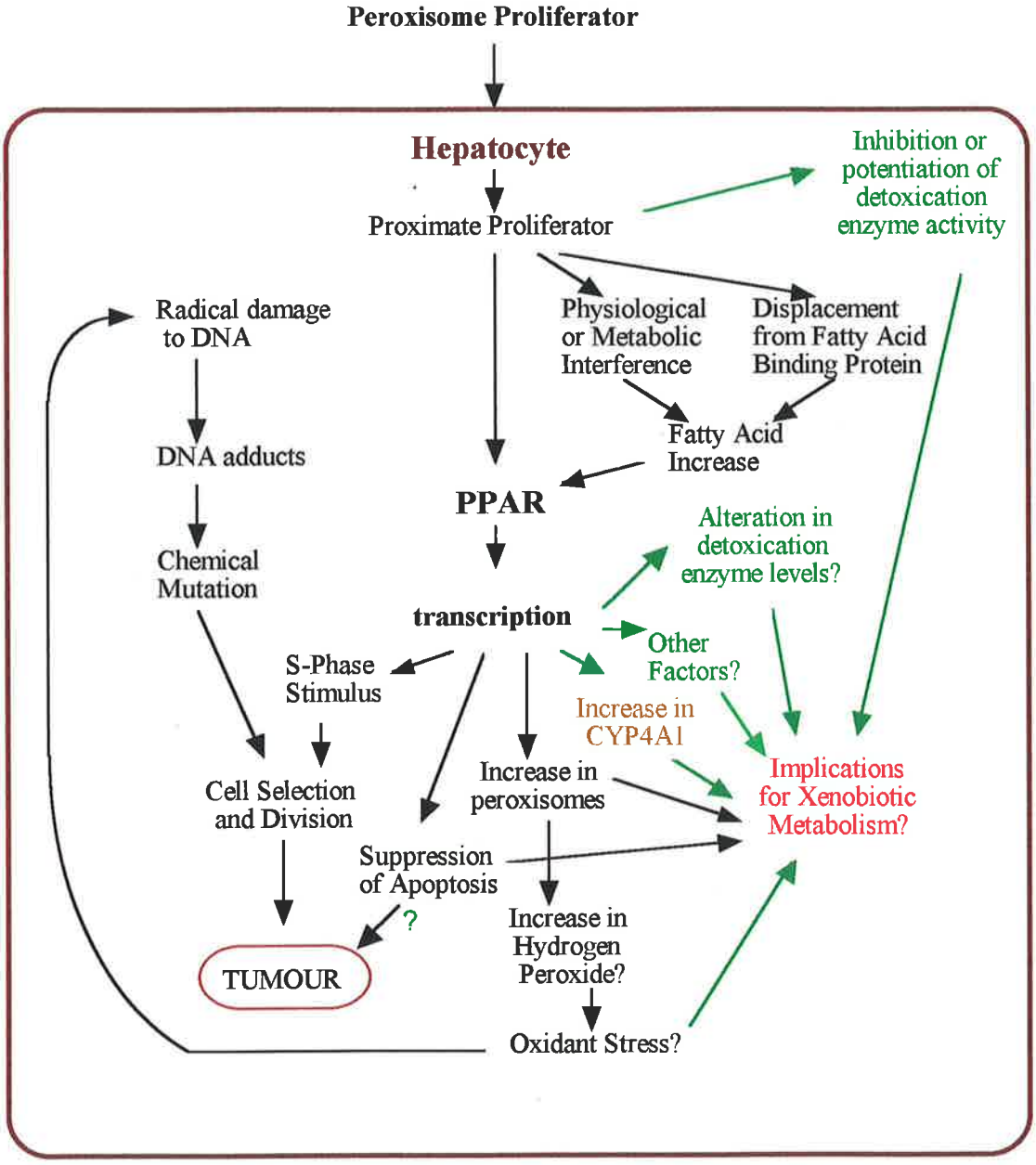



**Figure 1.5:** Diagram of the action of PxP in causing both peroxisome proliferation and hepatocarcinogenesis and the possible interaction of peroxisome proliferation on drug metabolism

### 1.8 Evidence that PxP alter susceptibility to hepatotoxicity?

When given alone, short term administration of PxP indicates that this class of chemical is extremely well tolerated with no necrosis or other indications of liver toxicity either *in vivo* (Reddy *et al,* 1986) or loss of cell viability in *in vitro* cell culture (Gray *et al,* 1983). The effect of prior exposure to PxP on the toxicity of other xenobiotics has received limited attention in literature. Of the studies done, a common feature is using PxPs as a tool to manipulate a specific biochemical event within the cell to determine the involvement of this event in the toxicity of another chemical. It thus follows that any conclusions were based on the PxP-induced alteration utilised in the investigation.

The earliest study investigating the effect of a PxP on the toxicity of another compound employed cerium chloride ($CeCl_3$), a rare earth metal that causes both liver necrosis and steatosis following administration to rats (Salas *et al,* 1976). On the basis of the hyperlipidemic response and the massive increase in plasma triglycerides accompanying $CeCl_3$ toxicity, the hypolipidemic, nafenopin, was administered prior to $CeCl_3$ challenge. Nafenopin was observed to protect rats against the lethality and hepatotoxicity induced by $CeCl_3$, which was speculated to be due to the nafenopin induced reduction of hepatic triglycerides (Tuchweber and Salas, 1978).

Akin to $CeCl_3$, tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity is associated with fat accumulation within the liver. Pretreatment of rats with the PxP, DEHP, reduced the increase in cholesterol and triglyceride thus decreasing fatty liver in TCDD treated animals. Also observed in this study was a reduction in the level of body weight loss and mortality associated with TCDD toxicity (Tomaszewski *et al,* 1988). The protection afforded by DEHP was proposed to result from either enhanced hepatic peroxisomal ß-oxidation and inhibition of triglyceride synthesis in the liver or perhaps a non-specific effect of DEHP on tissue distribution of TCDD (Tomaszewski *et al,* 1988). Similarly, pretreatment with clofibrate was also shown to prevent the ethanol related increase in plasma triglycerides and increase the clearance of ethanol. This was suggested to be a result of an increase in the size and overall metabolic capacity of the liver or as a result of the 2 fold increase in hepatic mitochondrial aldehyde dehydrogenase activity, thus promoting the clearance of the ethanol metabolite, acetaldehyde (Hawkins *et al,* 1974; Kramer and Kresmer, 1984).

In contrast to these studies where a decrease in plasma triglycerides was suspected of being involved in hepatoprotection, pretreatment with clofibrate was shown to protect rats against the acute toxicity of hypoglycin, a hypoglycaemic agent (Van Hoof *et al*, 1985). Clofibrate was used as a tool to enhance the number of hepatic peroxisomes which had been observed to be decreased by 75% following administration of hypoglycin. However, while the peroxisomal number was normalised by clofibrate treatment, protection did not appear to result directly from the proliferation of peroxisomes (Van Hoof *et al,* 1985).

Other than alteration of lipid or triglyceride levels, other studies have utilised PxP-induced alterations in various enzyme levels to investigate the toxicity of other xenobiotics. While investigating the role of CYP450 activation in uroporphyria induced by iron dextran in mice, nafenopin was utilised as a specific inducer of the CYP4 family of CYP450 isoforms. In stark contrast to other CYP450 inducers, nafenopin was shown to protect against iron induced uroporphyria *in vivo* in mice (Smith *et al,* 1990). Although the mechanism of protection was not ascertained, it was speculated that the induction of CYP4A1 could in some way interfere with the inhibition of uroporphyrinogen decarboxylase activity or even be the result of induction of some protective system associated with PxP treatment (Smith *et al,* 1990).

As well as being used to alter Phase I metabolism, PxP have been used to alter detoxication enzymes such as catalase. In a study investigating the effect of enhanced $H_2O_2$ catabolism on the pulmonary toxicity of paraquat, rats were pretreated with clofibrate to increase catalase levels in the lung. Although the use of this PxP did not actually alter the activity of antioxidant enzyme including catalase, SOD or GSHPx in the lung, a striking 70% reduction in mortality was observed (Frank *et al,* 1982). As there was no change in either the level of peroxidation prone polyunsaturated fatty acids in the lung or in the uptake or clearance of paraquat from the blood stream, the authors reasoned that clofibrate may have increased the level of some other antioxidant, thus protecting the lungs against the toxicity of paraquat (Frank *et al,* 1982). This conclusion was also drawn in part, from a study investigating the potential involvement of $H_2O_2$ in the carcinogenic process. Hepatocytes isolated from nafenopin pretreated rats were resistant against externally applied $H_2O_2$ induced cytotoxicity, as measured by cell detachment in culture (Garberg *et al,* 1992). As this protection was not seen following the indirect increases in $H_2O_2$ which occur during the metabolism of either adriamycin or hydroquinone, it was concluded that it was associated with an alteration in the intracellular metabolism of $H_2O_2$ to a pathway less dependent on GSH or that the liver had developed some kind of resistance that prevented cytotoxicity (Garberg *et al,* 1992).

Collectively, these studies into the consequences of exposure to PxPs on the toxicity of other xenobiotics have consistently revealed a cytoprotective response in a range of target tissues. Possible explanations for PxP protection observed in these studies have included alterations in triglyceride and cholesterol levels; in both Phase I and II enzymes; cellular antioxidants and other enzymes involved in xenobiotic metabolism and also physiological changes such as an increase in liver size or in the number of hepatic peroxisomes. Although it appears that pretreatment with PxP protects against the toxicity of a variety of xenobiotics, PxP did not protect against the cytotoxicity of chloroacetates in isolated hepatocytes (Bruschi and Bull, 1993). It is clear that despite some indication of the effects of PxPs on various components involved in xenobiotic metabolism, the overall effect of prior exposure to PxP on the manifestation of toxicity of various xenobiotics cannot be easily predicted.

The aim of this thesis is to investigate the effect of pretreatment with PxPs on the hepatotoxicity of various xenobiotics. Due to the potential alteration of Phase I, II and oxidant-metabolising enzymes and also antioxidant factors by PxP pretreatment, a model hepatotoxicant whose toxicity is influenced by these alterations was sought and thus the hepatotoxicant paracetamol (AAP), was used as a model toxicant in initial studies.

### 1.9 Paracetamol - model hepatotoxicant

Paracetamol (acetaminophen; *N*-acetyl-*p*-aminophenol; 4-hydroxyacetanilide) is a commonly used analgesic for the treatment of mild to moderate pain. The therapeutic mechanism of action of AAP is not well understood, although is believed to involve reversible noncompetitive inhibition of cyclooxygenase (Lands, 1981). A weak acid with a pKa of 9.5, orally administered AAP is rapidly absorbed reaching peak plasma concentrations in 30 to 60 minutes. AAP has a half life of between 45 minutes to 3 hours and at therapeutic doses, is cleared from the body. However, when taken in sufficient quantities, AAP exhibits hepatotoxicity characterised by fulminating centrilobular hepatic necrosis which can extend out to the periportal regions of the liver lobule (Mitchell *et al,* 1973a; Plancke *et al,* 1987). In addition to the hepatotoxic response, AAP toxicity also is manifested as opification of the cornea, necrosis of the nasal epithelium, renal necrosis and CNS depression (Jeffery and Hascheck, 1988; Hart *et al,* 1995).

AAP itself is not cytotoxic. It contains a ring hydroxyl group which allows direct enzymatic conjugation to glucuronide and sulphate in reactions involving UDPG-transferase and sulphotransferase, respectively. These 2 conjugates form the majority of excreted metabolites although following saturation of these metabolic pathways during overdose, an enhanced proportion of AAP is metabolised by an alternative pathway involving bioactivation via CYP450 (Mitchell *et al*, 1973a). In this pathway, the putative reactive intermediate, *N*-acetyl-*p*-benzoquinoneimine (NAPQI) can interact with cellular targets initiating biochemical events resulting in cell death (Figure 1.6).

The bioactivation of AAP to NAPQI involves several CYP450 isoforms including; 2E1, 1A1 and to a lesser extent 3A4 (Patten *et al,* 1993). Due to the high hepatic CYP450 content, the formation of NAPQI in the liver is believed to primarily result from the activity of this enzyme, however, *in vitro* studies have shown that the peroxidase activity of prostaglandin H synthetase (PGHS), which utilises a peroxide substrate such as $H_2O_2$ can activate AAP to NAPQI 100 times faster than CYP450 (Boyd and Eling 1981; Moldéus *et al*, 1982; Potter and Hinson, 1987; Harvison *et al,* 1988). In renal tissue with comparatively lower levels of CYP450, bioactivation of AAP via PGHS in the kidney medulla initiates nephrotoxicity

(Moldeus *et al,* 1982). Whether the formation of NAPQI in the liver is exclusive to CYP450 activity or also includes a PGHS component is not entirely clear.

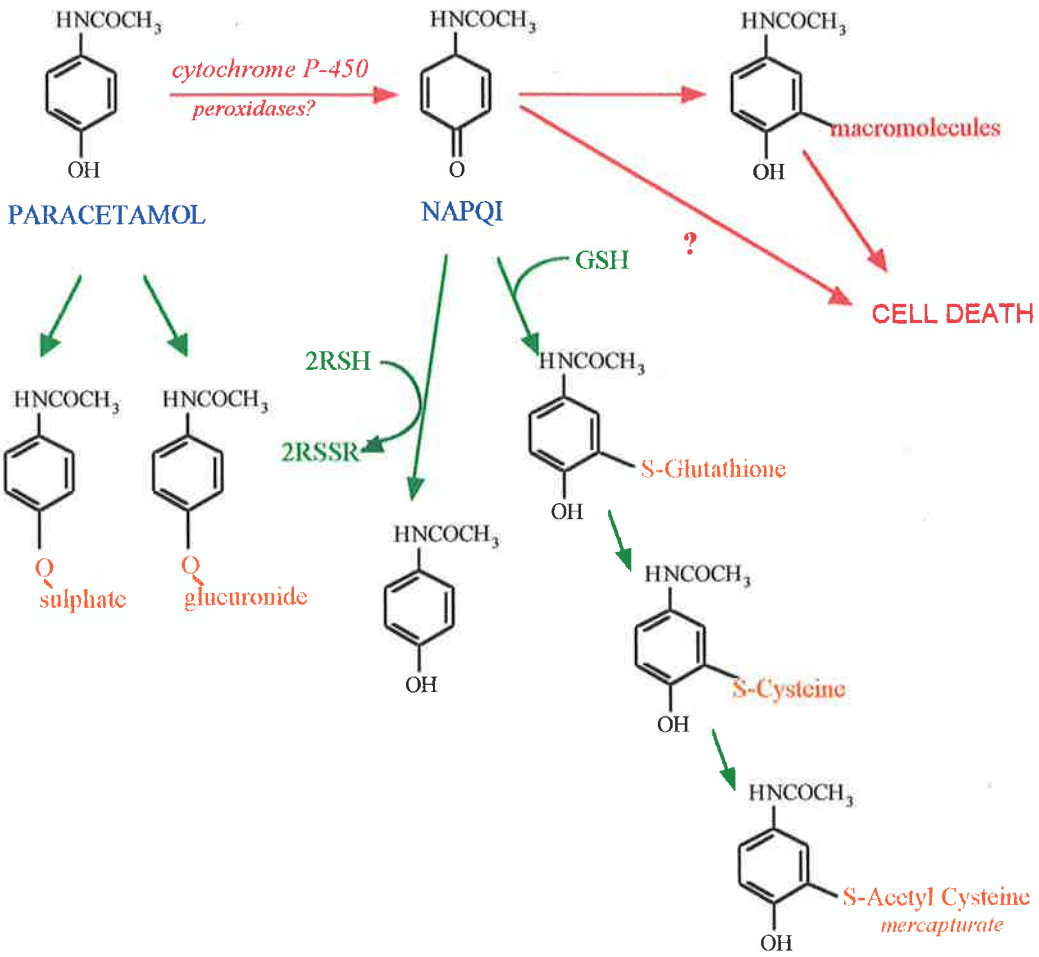



**Figure 1.6:** Proposed mechanism of AAP metabolism. Hepatotoxicity is dependent of the formation of a reactive intermediate (NAPQI) which interacts with critical cellular targets to produce toxicity and ultimately cell death.

Regardless of the mechanism of its formation, synthetically produced NAPQI has the same profile of biochemical action as the reactive metabolite of AAP including *in vitro* binding to protein thiols, reacting with GSH to form 3-glutathionyl-AAP, *in vitro* binding to proteins including bovine serum albumin and casien, lethality to isolated mouse hepatocytes and inhibition of mitochondrial function ((Dahlin *et al,* 1984; Holme *et al,* 1984; Streeter *et al,* 1984; Tee *et al*, 1987; Burcham and Harman, 1991). Collectively, these studies show that the concentration of NAPQI required to exhibit similar toxicity is only 1 to 5% of that of AAP and thus only a small proportion of AAP would have to be converted to NAPQI to observe toxicity.

NAPQI has a high affinity for sulphydryl groups and therefore conjugation to the ubiquitous cellular nucleophile GSH, is the major detoxication pathway. This reaction minimises interaction with cellular targets and the glutathione:paracetamol (GSH:AAP) conjugate is safely excreted from the body as cysteine and mercapturate metabolites. However when the

supply of GSH is exhausted during overdose, the reactive intermediate NAPQI has an enhanced ability to interact with other sulphydryls or suitable binding sites.

Following formation of NAPQI, the critical molecular events that lead to cell death following AAP overdose are unclear. Proposals have included; binding to critical cellular proteins, disruption to calcium homeostasis, mitochondrial dysfunction and oxidative damage.

### 1.9.1 Possible mechanisms of AAP hepatotoxicity

*1.9.1.1 Protein binding*

The evidence supporting a role for covalent binding is largely circumstantial, although it has been reported by some as a more satisfactory explanation than other theories of AAP toxicity (Gibson *et al,* 1996). The highest levels of covalently bound AAP is observed in the centrilobular region where necrosis was evident (Jollow *et al,* 1973; Roberts *et al,* 1991). Subsequent studies revealed that covalent binding to protein is an early event in centrilobular necrosis, occurring only 30 minutes after AAP administration in cells most proximal to the central vein (Bartelone *et al,* 1989) and binding occurred prior to the development of GSH depletion and centrilobular necrosis (Roberts *et al,* 1991). However, other studies have revealed that the relationship between the extent of protein binding and AAP toxicity is not entirely correlated. The amount of covalent binding of AAP adducts in mouse hepatocytes isolated from young mice was greater than that observed for older mice despite equivalent cell lethality (Harman and Self, 1986). Likewise, treatment with substances which prevent AAP-induced liver necrosis did not significantly modify the extent of the binding of AAP to liver proteins including 3-O-methyl-(+) catechin, dithiothreitol, desferrioximine (DFO) or *in vivo* using α-mercaptopropionyglycine (Labadarios *et al,* 1977; Devalia *et al* 1982; Albano *et al,* 1985; Gerson *et al,* 1985; Tee *et al,* 1986).

Comparison of the level of covalent binding between AAP and its nonhepatotoxic regioisomer, 3'-hydroxyacetanilide showed that despite similar amounts of total binding, subcellular fractionation revealed differing protein binding profiles, especially in the mitochondria (Tirmenstein and Nelson, 1989; Myers *et al,* 1995). Similar binding differences were observed during piperonyl butoxide protection (Brady *et al,* 1990) which together suggest that AAP toxicity is related to the selective binding of NAPQI. Even so, binding to a cytosolic 58 kDa selenoprotein, which together with the 44 kDa, glutamine synthetase constitutes 85% of the total NAPQI binding (Bartelone *et al,* 1989; Bartelone *et al,* 1992; Pumford *et al,* 1992) has been shown to also be specifically adducted by 3-hydroxyacetanilide yet exhibit no toxicity (Pumford *et al,* 1990). As GSH levels decrease, the initial binding of NAPQI is likely to be to protein targets with high levels of accessible sulphydryl groups, thus preventing binding and providing protection to critical protein targets and eventually the cell would be overwhelmed and unable to maintain vital functions (Pumford *et al,* 1997). Proteins

targetted by NAPQI include many from the cytosol, nuclear and mitochondrial fractions, however only a few have been identified. These include *N*-10-formyl tetrahydrofolate dehydrogenase, glutamine synthetase and glutamate dehydrogenase, yet the involvement, if any, in the development of AAP cytotoxicity is unknown (Bulera *et al,* 1995; Halmes *et al,* 1996; Pumford *et al,* 1997). Identification of the specific protein targets for NAPQI and elucidation of their role in cell homeostasis, should provide important information concerning the critical biochemical events involved in the manifestation of AAP toxicity and also the role of protein binding in AAP toxicity (Myers *et al,* 1995; Pumford *et al,* 1997).

*1.9.1.2 Disruption of intracellular calcium homoeostasis*

Calcium ($Ca^{++}$) is involved in a number of cellular processes including; cell division, maintenance of cytoskeleton, protein synthesis and enzyme activity including proteases, phospholipases and endonucleases which can lead to stimulation of autolysis, membrane damage and DNA fragmentation respectively (Nicotera *et al,* 1988; Timbrell, 1994). Under normal conditions, there is a 10,000 fold difference in the concentration of $Ca^{++}$ in the extracellular compared to intracellular fluid and thus the concentration of intracellular $Ca^{++}$ is kept under tight regulation via ATP-dependent pumps in the plasma membrane and sequestration into mitochondrial and endoplasmic reticulum stores (Nicotera *et al,* 1988).

In support of the calcium dyshomeostatis theory, an up to 18 fold increase in cytosolic $Ca^{++}$ occurred following AAP exposure in rat liver (Landen *et al*, 1986). The increase in $Ca^{++}$ concentration was shown not only to be correlated to the extent of necrotic damage, AAP toxicity was prevented by coadministration of either $Ca^{++}$ channel blockers or calmodulin inhibitors in both *in vitro* and *in vivo* models, as did incubation of isolated rat hepatocytes in $Ca^{++}$-free medium (Landen *et al*, 1986; Thibault *et al,* 1991; Satorres *et al,* 1995).

The steep extracellular to intracellular concentration gradient is maintained by transport of $Ca^{++}$ out of the cell via the action of ATPases and also by compartmentalisation into sequestering organelles, namely the endoplasmic reticulum and the mitochondria. It can therefore be postulated that the $Ca^{++}$ dyshomeostatis is the result of NAPQI binding to thiol groups on membrane ATPases involved in $Ca^{++}$ movement or sequestration (Orrenius *et al*, 1989; Tirmenstein and Nelson, 1989). However, other studies interpreted an increase in intracellular $Ca^{++}$ as indicating $Ca^{++}$ influx associated with the loss of cell membrane integrity prior to or at death (Lemasters *et al*, 1987). In spite of this, it is still possible that $Ca^{++}$ dyshomeostatis, rather than being associated with an increase in intracellular $Ca^{++}$ may have more directed action in altering $Ca^{++}$ homoeostasis within specific organelles, such as the mitochondria (Harman and Maxwell, 1995).

Other than possible mitochondrial effects, an early accumulation of $Ca^{++}$ in the nucleus, which was correlated to the extent of cytotoxicity, was observed in isolated mouse hepatocytes exposed to AAP (Shen *et al,* 1991). Equally in the *in vivo* situation, a 3.6 fold increase in nuclear $Ca^{++}$ levels in AAP-intoxicated mice, and a 2.5 fold increase in DNA fragmentation likely due to endonuclease activation, was evident 2 to 6 hours after AAP administration (Ray *et al*, 1992). The nuclear alterations and AAP toxicity were prevented by $Ca^{++}$ channel blockers even with administration as late as 6 hours after the hepatotoxic dose of AAP (Ray *et al*, 1992; Satorres *et al,* 1995). With the activation of $Ca^{++}$-dependent endonucleases implicated in AAP toxicity and inhibitors of either $Ca^{++}$ -dependent proteases or phospholipases delaying the onset of AAP cytotoxicity, it seems likely that increases in $Ca^{++}$ in particular organelles, is involved in the development of AAP toxicity (Nicotera *et al*, 1988; Shen *et al,* 1991; Ray *et al*, 1992).

*1.9.1.3 Mitochondrial dysfunction*

Another possible mechanism of NAPQI related cytotoxicity is impairment of mitochondrial activity, especially oxidative phosphorylation. The most important factor of disruption to mitochondrial function is the loss of cellular ATP essential for the continuation of all active processes within the cell, including compartmentalisation of various ions (eg; $Na^{+}$, $Ca^{++}$), synthesis of protein, lipids and bile and active transport.

Ultrastructural changes in hepatic mitochondria are clearly evident following AAP intoxication (Plancke *et al,* 1987). In addition, other studies have shown alterations in the normal biochemical function of the mitochondria isolated from the livers AAP intoxicated animals. For example, a reduction in both the ability of the mitochondria to sequester $Ca^{++}$ and also a decrease in the amount of mitochondrial GSH, which comprises around 15% of the total hepatic GSH, occurs during AAP intoxication (Masini *et al,* 1986; Tirmenstein and Nelson, 1989). As well as an inability to sequester $Ca^{++}$, the mitochondria of AAP intoxicated mice have impaired oxidative phosphorylation, as evidenced in both *in vivo* and *in vitro* studies (Meyers *et al,* 1988; Esterline and Ji, 1989; Burcham and Harman, 1991; Strubelt and Younes, 1992; Donnelly *et al,* 1994). The interference with mitochondrial oxidative phosphorylation appears to be due to specific interaction of the reactive metabolite NAPQI, to certain sites in the mitochondrial transport chain (Burcham, 1990; Burcham and Harman, 1991; Donnelly *et al,* 1994). An inhibition of mitochondrial oxidative phosphorylation occurs prior to the loss of cell membrane integrity and also precedes overt hepatic necrosis thus suggesting that mitochondria are an early target during the development of AAP toxicity (Donnelly *et al,* 1994).

*1.9.1.4 Oxidative damage*

The final mechanism by which NAPQI has been proposed to initiate toxicity is via oxidative stress, which is indicated by the profound hepatoprotection against AAP toxicity provided by coadministration of a number of different antioxidants including ascorbic acid, α-tocopherol butylated hydroxyanisole, dimethylsulphoxide and the iron chelator DFO (Lake *et al,* 1981; Fairhurst *et al,* 1982; Rosenbaum *et al,* 1984; Harman, 1985; Park et al, 1988; Ito *et al,* 1994; Sakaida *et al,* 1995). Moreover, an increase in the activity of two enzymes involved in the metabolism of reactive oxygen species, namely superoxide dismutase and catalase has also been found to protect against AAP toxicity (Kyle *et al,* 1987).

While a number of studies have shown an absence of oxidative stress or indices of lipid peroxidation following AAP intoxication (Thorgeirsson *et al,* 1976; Adams *et al,* 1983; Lauterburg *et al,* 1984), others have observed increases in the lipid peroxidation byproduct, malondialdehyde (MDA) in the liver (Muriel *et al,* 1992; Saikaida *et al,* 1995) and also increases in breath alkanes in AAP intoxicated mice (Wendel and Feuerstein, 1981). As this latter parameter was only detected after prior depletion of GSH by starvation or induction of CYP450, it was suspected that lipid peroxidation following AAP intoxication was an indirect result of the consequences of NAPQI-induced GSH depletion and dying cells, rather than a direct mediator of hepatic necrosis (Wendel and Feuerstein, 1981). Depletion of cellular GSH would render the hepatocyte susceptible to the deleterious effects of oxygen metabolites, by reducing the availability of both GSH for both the metabolism of peroxide compounds by GSHPx and the maintenance of endogenous antioxidants in a reduced (and active form). In general support of this concept, antioxidants have been shown to protect against AAP toxicity despite no alteration in either the extent of either GSH depletion or protein binding associated with AAP toxicity (Gerson *et al,* 1985; Younes *et al,* 1988; Amimoto *et al,* 1995; Saikaida *et al,* 1995).

While oxidative damage may be an indirect result of GSH depletion, there has been some evidence that the metabolism of AAP may generate reactive oxygen species. A 72% increase in steady state $H_2O_2$ levels and a 240% increase in superoxide anion formation was measured 15 minutes after a hepatotoxic dose of AAP (Lores-Arnaiz *et al,* 1995). As the intermediate NAPQI does not appear to undergo redox cycling to produce superoxide anion (Powis *et al,* 1984), the increase of these reactive oxygen species (ROS) could well be due to inhibition of the peroxide metabolising enzymes, catalase and GSHPx (Tirmenstein and Nelson, 1989; Lores-Arnaiz *et al,* 1995). As speculation, an increase in superoxide anion is believed to result in release of ferritin from cell membranes (Thomas and Aust, 1985) which could result in an increase in $Fe^{++}$ in the locale of the endoplasmic reticulum initiating lipid peroxidation. The involvement of $Fe^{++}$ in AAP toxicity is supported by the protective effect of $Fe^{++}$ chelators, such as DFO (Saikaida *et al,* 1995). Regardless of the mechanism of formation of the ROS, whether it be direct or indirect via AAP or the interference of NAPQI with cellular

processes, a variety of antioxidants with a diverse range of action have been shown to protect against the hepatotoxicity of AAP and seems likely that an oxidative stress element is involved in the sequences of events leading to cell death.

In summary, while it has been established that the CYP450 derived electrophile, NAPQI, mediates the toxicity of AAP, the actual mechanism remains obscure. It is quite possible that the postulated mechanisms are not mutually exclusive, with each forming part of the overall sequence of events that lead to the death of the cell. For example, the specific protein targets to which NAPQI binds could be implicated in the loss of mitochondrial calcium regulation.

### 1.10 Conclusion

The aim of the work described in this thesis was to investigate the effect of pretreatment with PxPs on the acute toxicity of various hepatotoxicants, with most attention directed to towards the classic bioactivation dependent hepatotoxicant, AAP. AAP toxicity can be altered via modification of Phase I activation, modification of Phase II enzyme activity or availability of Phase II conjugates or via the availability of antioxidants (Mitchell *et al,* 1973a; Mitchell et al, 1973b; Lake *et al,* 1981; Dahlin *et al,* 1984; Harman, 1985; Jaeschke 1990). As all of these parameters can be modified by PxP pretreatment, AAP provides an appropriate choice as a model hepatotoxicant for studies on the effect of prior short term pretreatment with PxPs.

# CHAPTER 2

## ESTABLISHMENT OF A MURINE MODEL OF PEROXISOME PROLIFERATION

### 2.1 INTRODUCTION

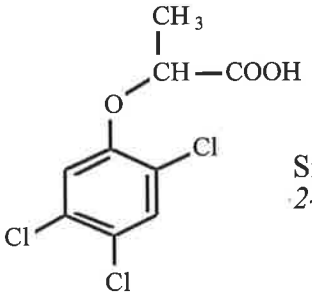
The aim of the studies described in this thesis was to investigate the effect of pretreatment with PxPs on the acute toxicity of various hepatotoxicants. To achieve this aim, a method of peroxisomal proliferation had to be established and verified as producing the characteristic biochemical alterations associated with PxP treatment. Although morphometric techniques have been used to quantify cellular peroxisomes, biochemical analysis of peroxisomal marker enzymes has been found to be the most useful technique for verifying peroxisome proliferation. In rodents, the hepatic activity of key peroxisomal enzymes such as acyl CoA oxidase is highly correlated to the extent of peroxisome induction (Lock *et al,* 1989; Ashby *et al*, 1994).

The mouse was chosen as the experimental animal since this species is particularly responsive to PxPs (Elcombe *et al,* 1985, Lundgren *et al,* 1987, Rao *et al,* 1988). Although rats are also responsive to these agents, the mouse exhibits greater sensitivity to AAP, the first hepatotoxicant that was investigated (discussed further in Chapter 3). Thus the main aim of the experiments described in this chapter was to establish a treatment protocol for the induction of peroxisome proliferation in the mouse. Three different routes of administration (intraperitoneal, oral with food and oral gavage) of the PxP silvex were tested and also three different PxP (clofibrate, silvex and DEHP) were compared for their capacity to induce proliferation after intraperitoneal administration. Although other more potent PxP have since been identified, the three PxP chosen for investigation were well characterised and readily available. The full chemical names of these compounds and their molecular structures are shown in Figure 2.1.

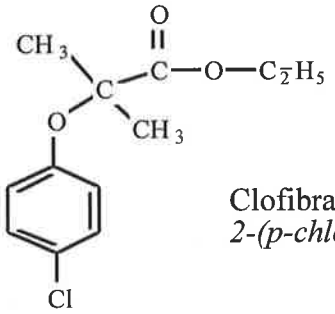
Two experimental protocols were used to accomplish these aims. In the first experiment, the herbicide, silvex, was administered either in the feed, via intraperitoneal injection or via oral gavage. In the second experiment, the peroxisome proliferation produced by silvex was compared to that produced by the hypolipidemic agent clofibrate and the plasticiser, DEHP (Figure 2.1). The intraperitoneal route of administration was used in this particular study.

In both experiments, peroxisome proliferation was biochemically assessed by measuring the activity of two peroxisomal marker enzymes; palmitoyl CoA oxidase and catalase. The biochemical rationale for selecting these enzymes as markers of peroxisome proliferation was

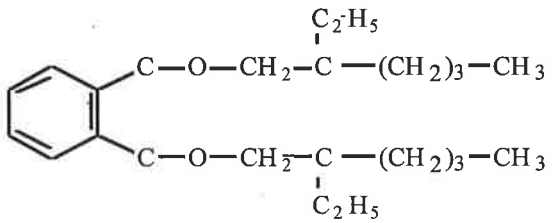
discussed in the Introduction (Section 1.6). To assess hepatomegaly induced by the various treatments, liver weight was also measured. To enable histological verification of peroxisome proliferation, liver samples from both the clofibrate treated and control mice were also analysed by light microscopy. Finally, the general health of the animals was monitored by assessing their general appearance, food consumption and body weight throughout the experiments.



Silvex
*2-(2,4,5-trichlorophenoxy)propionic acid*



Clofibrate
*2-(p-chlorophenoxy)-2-methylpropionic acid ethyl ester*



DEHP
*Di (2-ethyl hexyl) phthalate*

**Figure 2.1:** Structures of the peroxisome proliferators used in this study.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Chemicals

All chemicals were of the highest grade commercially available. For a listing of chemicals and suppliers, see Appendix 1.

### 2.2.2 Animals

Male Laca Swiss white mice (30 - 40 g) were obtained from the Waite Animal House, University of Adelaide. The mice were housed in the University of Adelaide Medical School Animal House in a standard 12 hour light/dark cycle at 22° C. Mice were kept in groups of no more than 6 per cage with water and food (standard laboratory rodent chow), unless otherwise indicated, supplied *ab libitum*.

### 2.2.3 Preparation of mouse feed containing Silvex

Mouse feed preparation was based on a method outlined in Appendix 2. Mice received fresh feed every 3 days.

### 2.2.4 Animal Treatment

To examine the effect of different routes of silvex administration, 20 mice were randomly allocated into four groups comprising one control and 3 treated groups. The treated mice received silvex for 10 days by one of three methods:

a) 100 mg/kg/day in 0.1 ml of olive oil by oral gavage;

b) 100 mg/kg/day in 0.1 ml of olive oil via i.p. injection;

c) 0.15 % (w/w) in feed.

Control mice were untreated (no olive oil administration) and fed standard laboratory chow. Mice that received silvex via oral gavage or i.p. injection were treated between 17.00 and 18.00 hour each day. All mice were weighed at the start and finish of the treatments and the amount of food consumed was measured every 3 days.

For the experiment where different PxPs were compared, 16 mice were randomly allocated into four groups; a) vehicle treated controls, b) silvex treated (100 mg/kg/day), c) clofibrate treated (500 mg/kg/day) or d) DEHP treated (2 g/kg/day) (n = 4). All treatments were administered for 10 days. These doses were chosen on the basis of literature reports concerning the effects of each agent in mice (Hess *et al,* 1965; Ashby *et al,* 1994; Voskoboinik *et al,* 1996). Each PxP was administered intraperitoneal injection in olive oil in a volume of 0.1 ml /40 g.

### 2.2.5 Tissue Sampling

On the day following the administration of the final dose of PxPs (day 11), mice were weighed then killed by cervical dislocation between 9.00 and 10.00 am. Livers were removed, weighed and placed in 10 ml of ice cold 0.1 M potassium phosphate buffer (pH 7.4) and homogenised for 10 seconds using an Ultra Turrax homogeniser. The volume of the homogenate was adjusted with buffer to give a 10 % w/v solution. This was divided into 1.0 ml aliquots in Eppendorf tubes and stored at -80° C until enzymatic analyses were performed.

During the second experiment, a 1 mm slice was obtained from the midsection of 2 lobes from the livers of control and clofibrate-treated mice. The slices were immediately fixed in 10 % phosphate buffered formalin for histological determination of peroxisome levels.

### 2.2.6 Enzyme Assays:

*2.2.6.1 Palmitoyl CoA Oxidase*

The activity of this peroxisomal marker was measured by following the cyanide insensitive palmitoyl CoA dependent reduction of $NAD^+$ as reported by Bronfman *et al* (1979). An aliquot of homogenate was centrifuged in a benchtop microfuge for 1 minute at 3,000 x g. A 0.75 ml volume of supernatant was taken and added to 0.75 ml of ice cold 2% Triton-X 100 in 0.1 M phosphate buffer, pH 7.4 and stored on ice. A 20 µl aliquot of sample was added to 0.75 ml of reaction mix containing 50 µM palmitoyl CoA, 50 µM coenzyme A, 0.12 mM FAD, 94 mM nicotinamide, 2.8 mM dithiothreitol, 2.0 mM potassium cyanide in 60 mM Tris hydrochloride buffer, pH 8.3. The presence of cyanide ensured that the activity detected was peroxisomal and not mitochondrial. The reaction was started by the addition of 30 µl of 12.3 mM solution of $NAD^+$. The formation of NADH was monitored at 340 nm using a Hitachi U-2000 spectrophotometer. Results were expressed as µmol $NAD^+$ reduced/min/g wet weight liver.

*2.2.6.2 Catalase*

The method for measurement of catalase activity involved permanganate determination of $H_2O_2$ remaining in solution following timed incubations and was adapted from the method of Cohen *et al* (1970). Briefly, aliquots of homogenate were centrifuged using a benchtop microfuge for 1 minute at 3,000 x g to pellet cell debris. A 0.5 ml volume of resultant supernatant was then added to an Eppendorf tube containing 5 µl of absolute ethanol, vortexed and stored on ice for 30 minutes. This step degraded any catalase complex II present in the samples. Following addition of 50 µl of 10% Triton-X100, the samples were left on ice for a further 5 minutes. The homogenates were diluted 1:1000 with ice cold 0.01 M potassium phosphate buffer (pH 7.0) and after thorough mixing a 0.25 ml aliquot was placed in a cold glass test tube. Catalase induced degradation of $H_2O_2$ was started by the addition of 2.5 ml cold 6 mM $H_2O_2$, the mixtures were vortexed and then returned to ice. After exactly 3 minutes, the reaction was stopped by adding 0.5 ml of cold 6 M sulphuric acid. The $H_2O_2$ remaining in solution was determined by adding 3.5 ml of 10 mM potassium permanganate and measuring the absorbance at 480 nm exactly 30 seconds later. The catalase activity was determined as the first order rate constant (k) using the following equation (Cohen *et al,* 1970). Results were expressed as k/mg wet weight liver.

$$k = \log \frac{S_t - S_b}{S_t - Abs} \times \frac{2.3}{t}$$

where:
$S_t$ = absorbance of spectrophotometric standard
$S_b$ = absorbance of reaction system blank
Abs = absorbance of sample
t = time

### 2.2.7 Histological identification of peroxisomes

Peroxisomes were histologically assessed in liver slices based on a benzidine visualisation procedure for peroxidase activity (Novikoff and Goldfischer, 1969). Liver slices were fixed for 4 hours in phosphate buffered formalin prior to the procedure. Immediately after fixing, the liver slices were rinsed with 1% gum acacia : 30 % sucrose solution, cut to 10 µm sections on a cryostat microtome and floated onto a 0.9 % saline solution. The slices were carefully transferred to peroxisome visualisation medium (5.5 mM 3,3'-diaminobenzidine and 0.6 M $H_2O_2$ solution buffered with 0.04 M 2-amino-2-methylpropanediol, pH 9.0) and then incubated for 30 minutes at 37° C. The slices were then washed 3 times using saline and finally placed onto gelatinised microscope slides. The slides were fixed in 10% phosphate buffered formalin, washed in 0.1 M phosphate buffer, pH 7.4, and counterstained for 30 seconds in Lillie-Mayer's Haemotoxylin solution. The slides were again washed in buffer, alkalinised with 0.2 % potassium carbonate in 2 % magnesium sulphate before a final wash and mounting on glycerol jelly covered glass slides. The slides were dried, covered with glass coverslips and examined under the light microscope.

### 2.2.8 Statistical Analysis

All treatment groups were compared to either the untreated control (Protocol 1) or the olive oil vehicle (Protocol 2), using unpaired Students t-test (significance level, $P<0.05$).

## 2.3 RESULTS

The effect of different routes of administration of silvex on peroxisome proliferation in mice are shown in Table 2.2.

| | %Liver Wt as Body Wt *(%)* | Palmitoyl CoA Oxidase *µmol NADH/min/g* | Catalase *k/mg* | Feed Consumed *g/mouse/day* | Weight Change *g/mouse/ 10days* |
|---|---|---|---|---|---|
| Untreated | 5.55 ± 0.20 | 0.288 ± 0.037 | 4.75 ± 0.55 | 4.37 | +0.6 ± 0.5 |
| Feed *0.15% w/w* | 8.61 ± 0.27 ** | 1.760 ± 0.084 **** | 7.44 ± 1.14 | 2.99 | -6.3 ±0.2 |
| Oral Gavage *100 mg/kg/day* | 7.46 ± 0.07 ** | 0.967 ± 0.057 *** | 9.69 ± 1.22 * | 4.14 | +0.8 ±0.4 |
| Intraperitoneal *100 mg/kg/day* | 7.26 ± 0.24 ** | 0.783 ± 0.077 ** | 5.86 ± 0.63 | 4.09 | -0.1 ±0.5 |

**Table 2.2:** Indices of peroxisome proliferation and physical parameters in groups of Swiss mice pretreated for 10 days with silvex via three different routes of administration (Results expressed as mean ± s.e.m, n=5, *=P<0.05. **=P<0.01, ***=P<0.001, ****=P<0.0001)

In keeping with its known properties as a PxP, silvex treatment increased liver weight, palmitoyl CoA oxidase and catalase activity in comparison to the control group. The increased liver weight as a percent of body weight and palmitoyl CoA oxidase activities were most pronounced when silvex was administered in the feed, although catalase was induced more strongly after oral gavage. In mice that received dietary silvex, a suppression of feed consumption and weight gain occurred (Table 2.2). Their general condition and appearance also deteriorated throughout the experiment, indicating that this route of administration was unlikely to be suitable for future experiments.

Since the i.p. route of administration proved to be more convenient than oral gavage, in a subsequent experiment investigating three different PxP, only this route of administration was used. Table 2.3 shows the effects of different PxP on marker enzymes and liver weights following i.p administration of silvex, clofibrate and DEHP.

| | Liver Weight % Body Weight | Palmitoyl CoA Oxidase *µmolNADH/min/g* | Catalase *k/mg* |
|---|---|---|---|
| Olive Oil Control *0.1 ml/day* | 5.30 ± 0.34 | 0.30 ± 0.04 | 5.03 ± 1.04 |
| Clofibrate *500 mg/kg/day* | 6.92 ± 0.46 ** | 1.06 ± 0.28 * | 10.14 ± 1.92 |
| DEHP *2 g/kg/day* | 5.80 ± 0.30 | 0.36 ± 0.04 | 6.12 ± 0.90 |
| Silvex *100 mg/kg/day* | 6.60 ± 0.23 * | 0.78 ± 0.14 ** | 8.21 ± 3.05 |

**Table 2.3**: Indices of peroxisome proliferation in the livers of Swiss mice following 10 days of i.p. treatment with three different PxP, namely clofibrate, DEHP and silvex by i.p route (Results expressed mean ± s.e.m, n=4, * =P<0.05, ** = P<0.01).

Both clofibrate and silvex produced similar increases in each of the three indices measured. DEHP administration produced only minor increases that were not statistically significant. Since clofibrate was very well tolerated by the mice, it was decided to investigate the peroxisome proliferating potential of this agent using a histochemical procedure.

The results obtained during the histochemical analysis of control and clofibrate-treated mouse livers are shown in Figure 2.4. Peroxisomes can be clearly seen as dark brown cytoplasmic spots produced upon treatment with benzidine, a staining procedure specific for peroxidase

activity, present in high levels within peroxisomes (Novikoff and Goldfischer, 1969; Just *et al,* 1989). Erythrocytes were also detected due to their high endogenous peroxidase levels. As indicated in Figure 2.4, there was a substantial increase in peroxidase staining of the clofibrate-treated livers compared to the control livers, providing qualitative confirmation of the biochemical data that suggested extensive hepatic peroxisome proliferation in these animals. Since the 500 mg/kg/day dose of clofibrate consistently produced extensive peroxisome proliferation and was well tolerated by the animals, this dose was routinely used in subsequent experiments to achieve hepatic peroxisome proliferation.

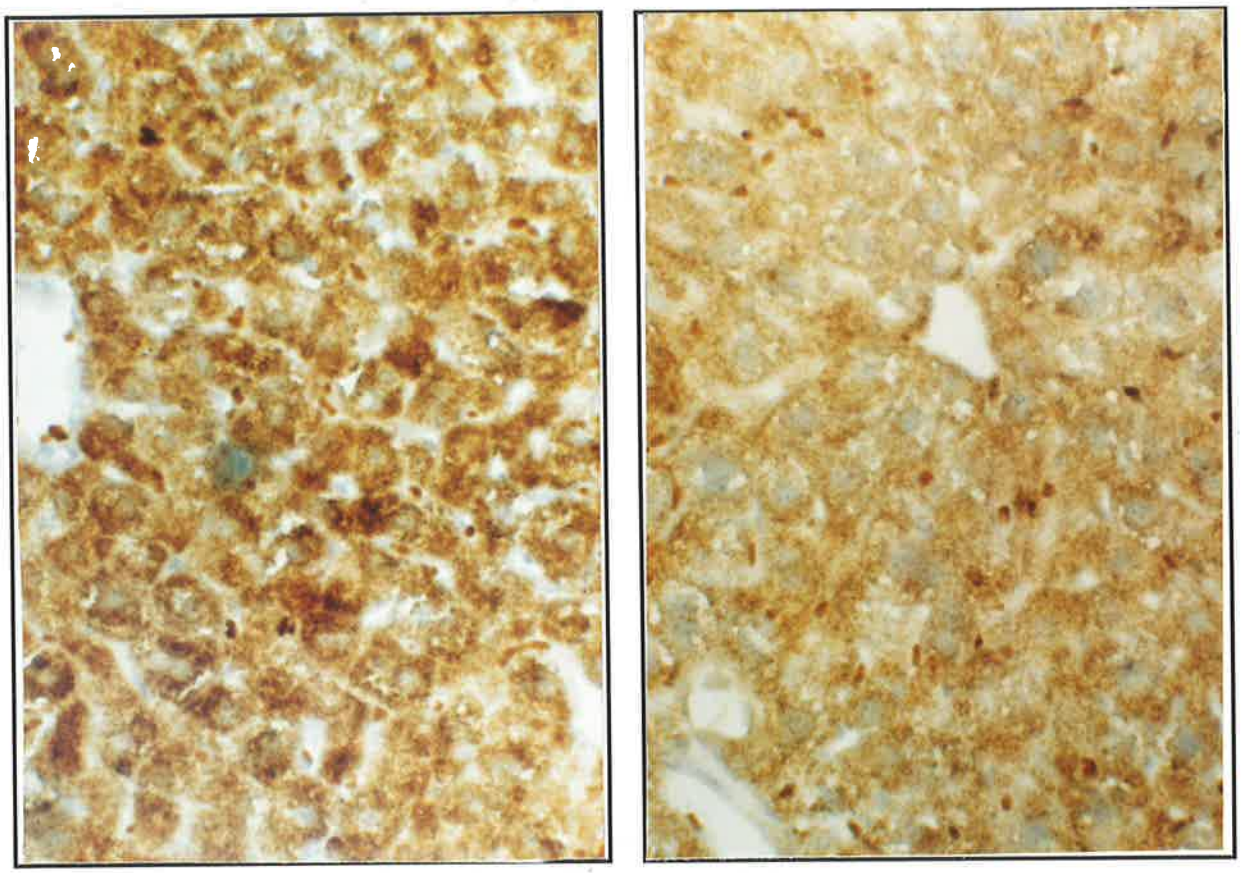


**Figure 2.4:** Histochemical analysis of mouse livers following staining of liver slices with diaminobenzidine. **A:** (left panel) liver slice prepared from mouse treated with clofibrate 500 mg/kg/day for 10 days. **B:** (right panel) liver slice from mouse treated with olive oil vehicle 0.1 ml/day for 10 days (x100)

## 2.4 DISCUSSION

Peroxisome proliferation is characterised by a marked increase in liver size as well as both morphological and biochemical changes (Reddy and Lalwani, 1983). These observations were confirmed during the present experiments since it was found that treatment with three classical PxP, regardless of the route of administration, increased liver weights and also hepatic palmitoyl CoA oxidase and catalase activities. While treatment with either clofibrate or silvex caused substantial increases in palmitoyl CoA activity, the increases in catalase levels were more modest, illustrating an uneven level of induction between the two different

enzymes. Similar patterns of induction of these two peroxisomal markers have been observed by others (Moody and Reddy, 1978; Rao and Reddy, 1987).

The present findings are consistent with the opinion that cyanide insensitive palmitoyl CoA oxidase is the marker of choice for peroxisome proliferation (Reddy *et al*, 1986; Lake *et al,* 1993). Catalase is less useful since the extent of induction often correlates poorly to the actual increase in peroxisome numbers (Lazarow and de Duve, 1976; Rao and Reddy, 1987). Other widely used peroxisomal marker enzymes include microsomal lauric acid ω-hydroxylase (Lake *et al,* 1987) and carnitine acetyltransferase (Bieber *et al*, 1981). However, the latter enzyme is less peroxisome specific since it is also present in mitochondria (Bieber *et al*, 1981). As liver weight increase also accompanied peroxisome proliferation, this parameter expressed as %LBW, was useful as a nonspecific indicator of peroxisome proliferation.

The majority of studies on peroxisome proliferation in rodents have used dietary supplementation to administer PxP to the animals (Ashby *et al,* 1994). However, as the animals are housed in groups the actual dose administered to each mouse cannot be determined under these conditions. Also, the physiological status of the animal can be compromised if the mouse finds the food unpalatable and chooses to eat only for survival and not *ab libutum*. This is an important consideration with chlorinated phenoxyacetic acid derivatives, such as silvex and clofibrate as the chemical odour often renders the food nonpalatable to rodents. Such factors probably contributed to the present findings since lower feed consumption and diminished weight gain occurred in animals that received silvex in their diet (Table 2.1). It is known that starvation can also cause peroxisome proliferation (Ishii *et al,* 1980b). Since the extent of palmitoyl CoA oxidase induction was greatest in the silvex fed mice who actually lost weight during the experiment, it is likely that starvation contributed to the overall level of peroxisome proliferation observed. Due to the lack of dosage control, loss of physiological condition and the potential stress of feed administration, this method was not used in subsequent experiments.

The plasticiser DEHP has also been used to achieve peroxisome proliferation in a number of studies (Lake *et al,* 1986; Short *et al,* 1987; Sharma *et al,* 1988). In our experiments, however, DEHP only produced a modest increase in palmitoyl CoA oxidase activity when administered via i.p. injection (Table 2.3). Moreover, the DEHP treated mice had extensive diarrhoea and were dehydrated when assessed by a skin reflex test. On the basis of these findings, and with the exception of the PxP comparison experiment outlined in Chapter 3, it was decided not to use DEHP in future studies.

Since PxP treated mice were to be used in subsequent experiments that used high doses of hepatotoxic substances, it was important that the physiological status of the animals was not compromised by the PxP pretreatment. Clofibrate was well tolerated by the mice and the fact

that it was readily solubilised in the olive oil vehicle ensured this drug was chosen as the PxP for further experimentation.

In conclusion, the work outlined in this chapter has shown that treatment of mice with PxPs initiates peroxisome proliferation which can be detected by measuring liver weight and by histochemical means and is readily quantitated by measuring palmitoyl CoA oxidase activity. Clofibrate administered by intraperitoneal injection was chosen to produce peroxisome proliferation in future experiments that investigated the susceptibility of pretreated mice to chemically induced organ toxicity.

# CHAPTER 3

## EFFECT OF PEROXISOME PROLIFERATION ON THE ACUTE TOXICITY OF THE MODEL HEPATOTOXICANT, PARACETAMOL.

### 3.1 INTRODUCTION

The results outlined in the previous chapter revealed that clofibrate given by the i.p. route provides a reliable means of achieving peroxisome proliferation in mice. This was readily quantitated via measurement of the activity of the specific marker enzyme, palmitoyl CoA oxidase. In the work described in the present chapter, this treatment regimen was used to investigate the effect of hepatic peroxisome proliferation on the susceptibility of mice to paracetamol (AAP) induced hepatotoxicity.

As discussed in the general Introduction (Section 1.9), while AAP is well tolerated at low doses it causes extensive hepatic necrosis when given in overdose (Mitchell *et al,* 1973a). The extent of toxicity produced by a given dose of AAP can be modified by many factors, such as induction or inhibition of CYP450 activity or alterations in the activity of various Phase II enzymes, for example UDP-glucuronosyl transferase (Schnell *et al,* 1988; Speck and Lauterberg, 1990). In addition, changes in the cellular supply of Phase II cofactors and antioxidants also alter the susceptibility of rodent livers to AAP-induced toxicity (Mitchell *et al,* 1973a; Mitchell *et al,* 1973b; Harman, 1985; Uhlig and Wendel, 1990). Since changes in each of these parameters occur following treatment with PxP, it seems likely that the susceptibility of PxP-pretreated animals to AAP toxicity will be different from control animals. AAP thus seemed an appropriate choice as a model hepatotoxicant for studies on the interactions of prior peroxisome proliferation and the hepatotoxicity of xenobiotics.

The phenomenon of peroxisome proliferation is most pronounced in rats and mice. The results presented in the preceding chapter demonstrated that mice are an excellent toxicological model for achieving chemically induced peroxisome proliferation. Although AAP toxicity is well characterised in both species, rats are relatively resistant to this hepatotoxicant, as indicated in the $LD_{50}$ for orally administered AAP being 777 mg/kg in mice and 3763 mg/kg in rats (Rhodes *et al,* 1995). The interspecies differences in susceptibility to AAP are largely related to the extent of bioactivation of AAP by CYP450 to NAPQI (Green *et al,* 1984; Tee *et al*, 1987). Pharmacokinetic studies using a number of specific CYP2E1 inducers and inhibitors have revealed that mice contain 3-6 times more hepatic 2E1 activity than rats, the major isoform involved in AAP activation (Rose *et al* 1994; Dekant *et al,* 1995; Hart *et al,*

1995; Seaton *et al,* 1995). Consequently, the susceptibility of mice to AAP toxicity and peroxisome proliferation makes this species an ideal experimental animal for these studies.

To investigate the effect of peroxisome proliferators on the susceptibility of mice to AAP, it was necessary to identify an appropriate indicator of hepatic damage. The hepatotoxicity of xenobiotics can be determined by both morphological assessment and biochemical analysis (Mitchell *et al,* 1973a; Mitchell *et al,* 1973b; Hayes, 1989; Timbrell, 1994). The most common method for the confirmation of AAP toxicity is the measurement of hepatic enzymes in animal serum. Since these enzymes are normally present in hepatocyte cytosol, they are released into the blood following the loss of cell membrane integrity which occurs with toxicant damage. Since the activity of such enzymes in plasma is directly correlated to the extent of hepatic damage, plasma enzymes provide a reliable and sensitive means of detecting hepatic damage (Mitchell *et al,* 1973a). Three enzymes, namely alanine aminotransferase (ALAT), sorbitol dehydrogenase (SDH) and lactate dehydrogenase, (LDH) are all cytosolic enzymes found in the liver that can be used as markers of necrotic cell injury following AAP administration (Liu *et al,* 1992, Peterson and Brown, 1992; Liu J *et al,* 1993; Speck *et al,* 1993; Woo *et al,* 1995). In this chapter, each of these enzymes was investigated for its suitability as an indicator of AAP induced hepatic damage in mice. This information was then used to determine whether the susceptibility of PxP pretreated mice to hepatic injury differed from that of control mice.

Three main aims were pursued:

a) to identify a marker enzyme for use in the quantitation of hepatic toxicity produced by AAP
b) to determine the effect of pretreatment with three PxPs on the toxicity produced by a single hepatotoxic dose of AAP
c) to investigate the effect of the model PxP clofibrate, on the dose:response curve of AAP hepatotoxicity.

The first two aims were investigated in an extension of the study described in the previous chapter. Although these findings suggested that clofibrate was the optimal compound for the induction of peroxisomes in this strain of mice, silvex and DEHP were included to enable confirmation of any observed response as being related to pretreatment with PxPs as a group in general, rather than a specific response to clofibrate itself. Following pretreatment with one of the three different PxP, mice were administered a single hepatotoxic dose of AAP (250 mg/kg). The extent of toxicity was then determined by measuring the activity of ALAT, SDH and LDH in the serum of mice after 24 hours.

The third aim was investigated in a subsequent study involving measurement of hepatotoxicity of increasing doses of AAP, following i.p administration of clofibrate. Histological analysis of individual livers was performed to confirm and elaborate on any enzymatic findings.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Chemicals

All chemicals were of the highest quality commercially available. For a listing of the chemicals and suppliers, see Appendix 1.

### 3.2.2 Animal treatment

In the first experiment (Study 1), peroxisome proliferation was achieved following intraperitoneal injection of one of three PxP as described in Chapter 2. At 10 am on the day following the final PxP administration, mice were injected with a single i.p injection of 250 mg/kg AAP, dissolved in saline and injected at a dose volume of 20 ml/kg. Due to the circadian variations in AAP toxicity, all AAP treated mice described in this thesis were injected with AAP at 10 am when murine AAP lethality was reported to be at its nadir (Schnell *et al,* 1983).

In the second experiment (Study 2), 50 mice were randomly allocated into 2 groups of 25. One group was pretreated for 10 days with 500 mg/kg/day clofibrate i.p. while the other group (control) received 0.1 ml/day of olive oil vehicle. At 10 am on the day following the final PxP administration, each group was divided into 5 groups (n=5) which received one of the following AAP doses: 200, 300, 400 or 500 mg/kg AAP. Mice in the control group (0 mg/kg dose) received saline alone.

### 3.2.3 Collection of blood for plasma enzyme analysis

After 24 hours, all mice were anaesthetised by an i.p injection of 0.1 ml of 0.06 % nembutal. Following attainment of adequate anaesthesia, 0.8 to 1ml of blood was collected from each mouse by closed cardiac puncture into a syringe that had been previously flushed with heparinised saline. Blood was stored in an Eppendorf tube on ice until centrifuged at 3,000 x g for 1 minute. The plasma supernatant was collected and stored at -20° C. Serum enzyme analyses were completed within the next 24 hours. In Study 1, each plasma sample had ALAT, SDH and LDH activity determined. In study 2, only SDH activity was determined for each sample.

### 3.2.4 Microscopic analysis of liver samples

In Study 2, samples for histological analysis were taken from livers from control and AAP treated mice in the control and clofibrate treated groups. For this, a 1mm slice was cut from the large anterior and the triangular posterior lobes of each liver. Slices were fixed in 10% phosphate buffered formalin for a few days, then haemotoxylin and eosin stained sections were prepared for the assessment of the extent of necrosis.

### 3.2.5 Plasma Enzyme Assays

In Study 1, the activity of three plasma enzymes was measured in each plasma sample. In the event of rapid curvilinear reaction rates, samples were diluted in physiological saline and reassayed.

#### *3.2.5.1 Alanine Aminotransferase*

This assay was based on the method of Wroblewski and LaDue (1956). A 0.1 ml aliquot of mouse plasma was added to 0.9 ml of reagent mix containing 0.4 M l-Alanine, 12 mM α-ketoglutarate, 2 U/ml lactate dehydrogenase in 0.25 M phosphate buffer, pH 7.4. The reaction was initiated by the addition of 7 mM $NAD^+$. The change in absorbance was measured in a Hitachi U-2000 spectrophotometer for 1 minute at 340 nm. Results are expressed in units of enzyme activity per litre of plasma (U/L), where 1 U is defined as the amount of enzyme required to produce 1 μmol NADH per minute at 25° C.

#### *3.2.5.2. Sorbitol Dehydrogenase (SDH)*

The determination of plasma SDH activity is based on the method of Asada and Galambos (1963). A 0.1 ml aliquot of mouse plasma was mixed with 0.5 ml of 1.4 mM NADH in 0.1 M Tris buffer, pH 7.5 and left at room temperature for 10 minutes to ensure removal of interfering ketoacids present in plasma. The reaction was started by the addition of 100 μl of 4 M fructose in buffer. The decrease in absorbance due to NADH oxidation was followed at 340 nm and 25° C using a Hitachi U2000 spectrophotometer. Results are expressed in U/L, where 1 U is defined as the amount of enzyme required to produce 1 μmol NAD+ per minute at 25° C.

#### *3.2.5.3. Lactate Dehydrogenase (LDH)*

The method for plasma LDH determination is based on the method of Richards *et al*, (1975). A 50 μl aliquot of mouse plasma was mixed in a cuvette with 0.5 ml of 0.05 M l-lactic acid and 7mM NAD+ in 0.25 M tris buffer, pH 8.9. The cuvette was placed immediately in a Hitachi U-2000 spectrophotometer and the change in absorbance was measured for up to 1 minute at 340 nm. The results are expressed in U/L, where 1 U is defined as the amount of enzyme required to produce 1 μmol NADH per minute at 25° C.

### 3.2.6 Statistical Analysis

Plasma enzyme data was analysed by the Alternate Welch t-test ($P<0.05$). This method was used due to the considerable variance observed within the data obtained from the different treatment groups. In the dose response experiment, data was analysed by ANOVA, which was followed by a Bonferroni post hoc test for analysis of specific pairs of data ($P<0.05$).

## 3.3 RESULTS

### 3.3.1 Effect of pretreatment with three different PxP on the acute toxicity of a single dose of AAP.

The activities of three different hepatic enzymes were measured in plasma to quantitate the hepatotoxicity of a single dose of AAP.

No increase in any of the three plasma enzymes of interest were observed in the control (no AAP) mice which received PxP treatment indicating that no hepatotoxicity was associated with these pretreatments *per se.* Since any PxP-induced toxicity could have confounded detection of liver damage caused by AAP, this finding confirmed that the dose schedules used for various peroxisome proliferators were appropriate.

In mice pretreated with olive oil vehicle, there was a marked increase in each of the plasma enzymes 24 hours after the administration of 250 mg/kg AAP. The increases were statistically significant for both ALAT and SDH, but not for LDH, because of marked interindividual variability.

Pretreatment with each of the three PxPs protected mice against the acute hepatotoxicity of a single toxic dose of AAP and hepatoprotection was clearly apparent with each of the three plasma enzymes examined. There were no increases in enzyme activity in PxP pretreated mice, whether or not they had been challenged with AAP. The hepatoprotection was also obvious at the macroscopic level. Independent evaluation of the macroscopic appearance of the livers confirmed characteristic necrosis in the AAP intoxicated control mice, but no deviation from normal appearance was observed in the PxP pretreated mice.

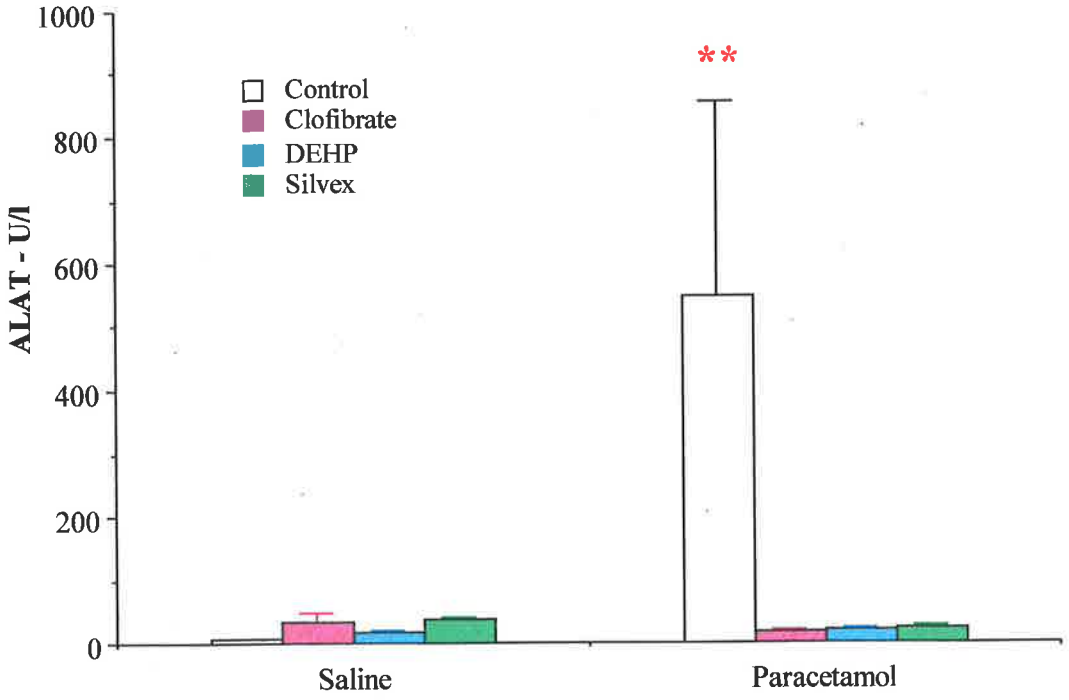



**Figure 3.1:** Plasma ALAT activity from mice pretreated with PxP; clofibrate, silvex or DEHP (for doses see text) i.p. for 10 days, with or without 250 mg/kg AAP. Mice were sampled after 24 hours (Results expressed as; mean ± s.e.m, n=4, **= $P < 0.01$, Alternate Welch test).

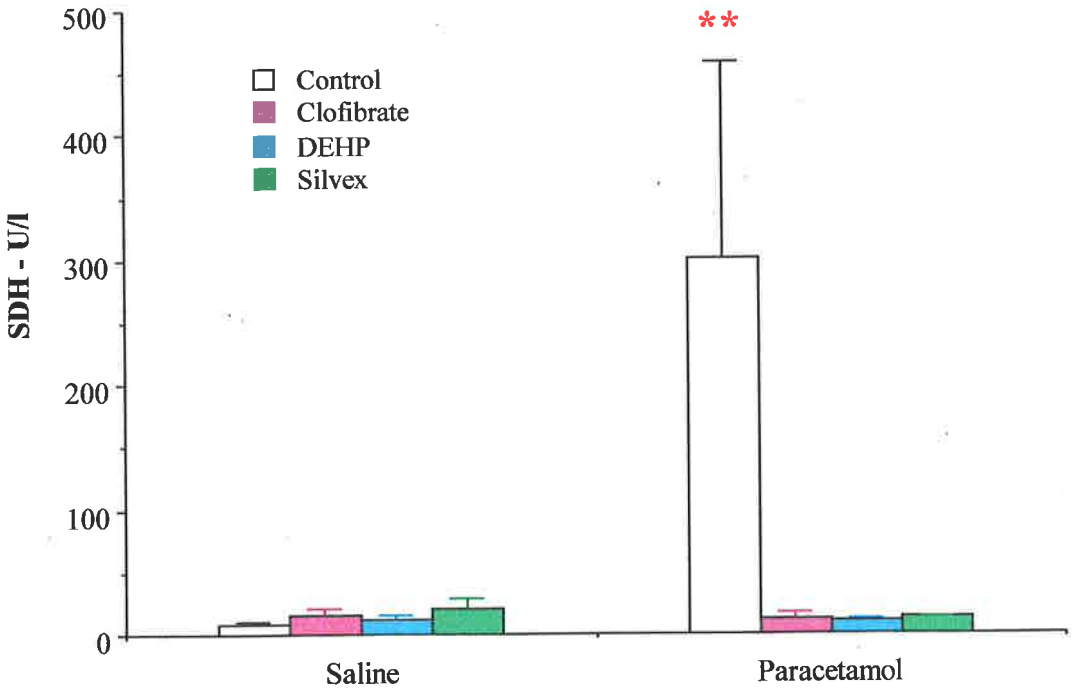



**Figure 3.2:** Plasma SDH activity from mice pretreated with PxP; clofibrate, silvex or DEHP (for doses see text) i.p. for 10 days, with or without 250 mg/kg AAP. Mice were sampled after 24 hours (Results expressed as; mean ± s.e.m, n=4, **= $P < 0.01$, Alternate Welch test).

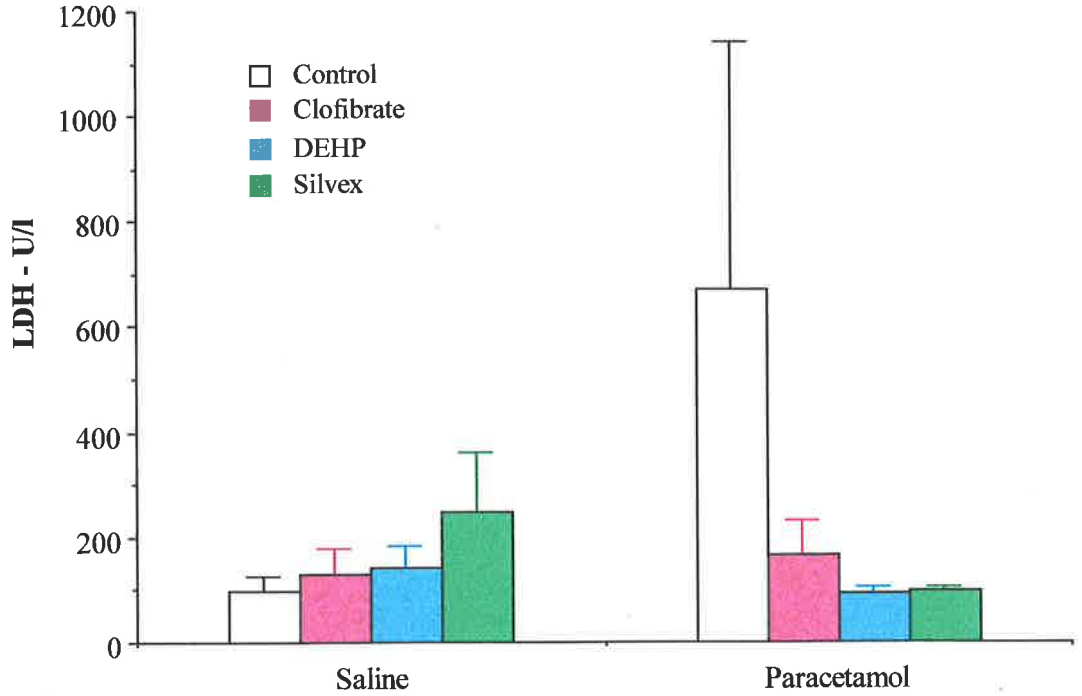



**Figure 3.3:** Plasma LDH activity from mice treated with 3 different PxP being clofibrate, silvex and DEHP (for doses see text) i.p. for 10 days, with or without 250 mg/kg AAP. Mice were sampled after 24 hours (Results expressed as; mean ± s.e.m, n=4).

### 3.3.2 Effect of pretreatment with the peroxisome proliferator clofibrate on the dose-response curve of AAP toxicity.

To investigate the effect of PxP pretreatment on the dose-response relationship of AAP, the toxicity of AAP in control and clofibrate pretreated mice was assessed over a range of doses. In this experiment, the toxicity of AAP at doses up to 500 mg/kg was investigated by both plasma enzyme activity and histological analysis. Following consideration of the results in 3.3.1 (see Discussion), plasma SDH activity was chosen as the marker enzyme of choice and was used as an index of hepatotoxicity in subsequent experimentation.

Results obtained during the investigation of the effects of clofibrate pretreatment on the dose-response relationship for AAP-toxicity are shown in Figure 3.4. Hepatotoxicity in the form of increased SDH activity was evident in the control group at 300 mg/kg doses of AAP and above with increases in SDH activity above control level (Figure 3.4). Pretreatment with clofibrate has caused profound hepatoprotection against the acute hepatotoxicity of AAP at all doses. This is evident by the lack of hepatotoxicity, as measured by plasma SDH activity even at the highest dose of AAP (500mg/kg).

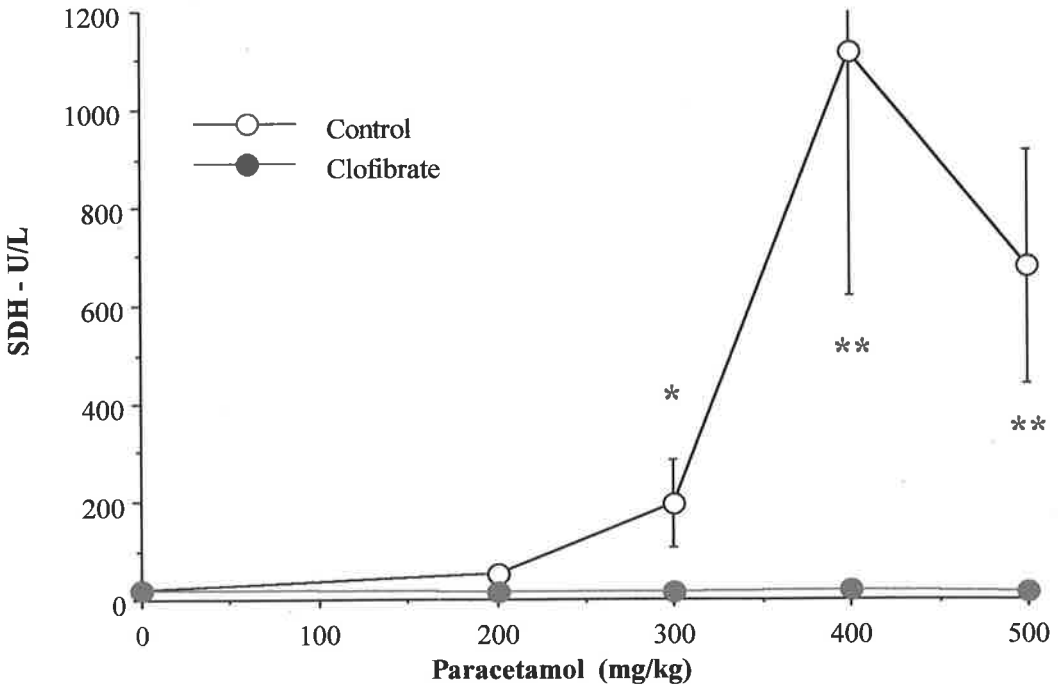
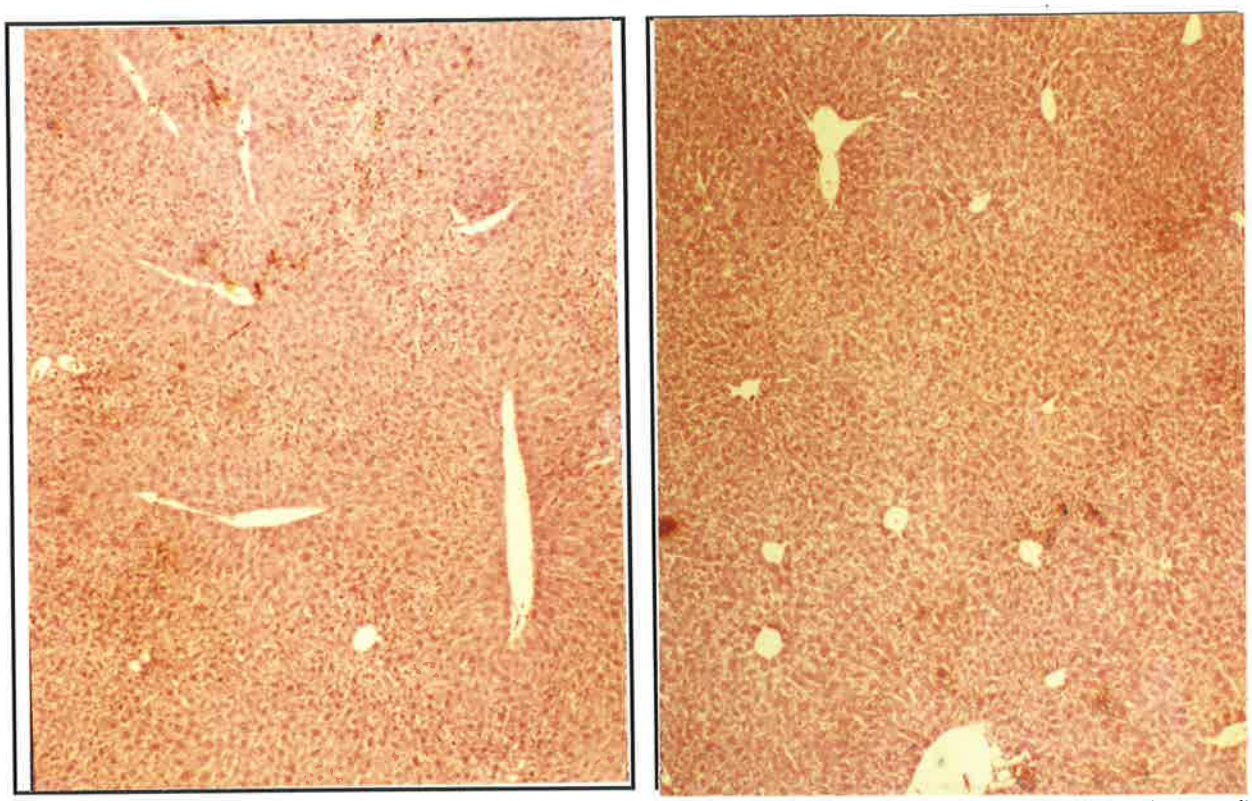
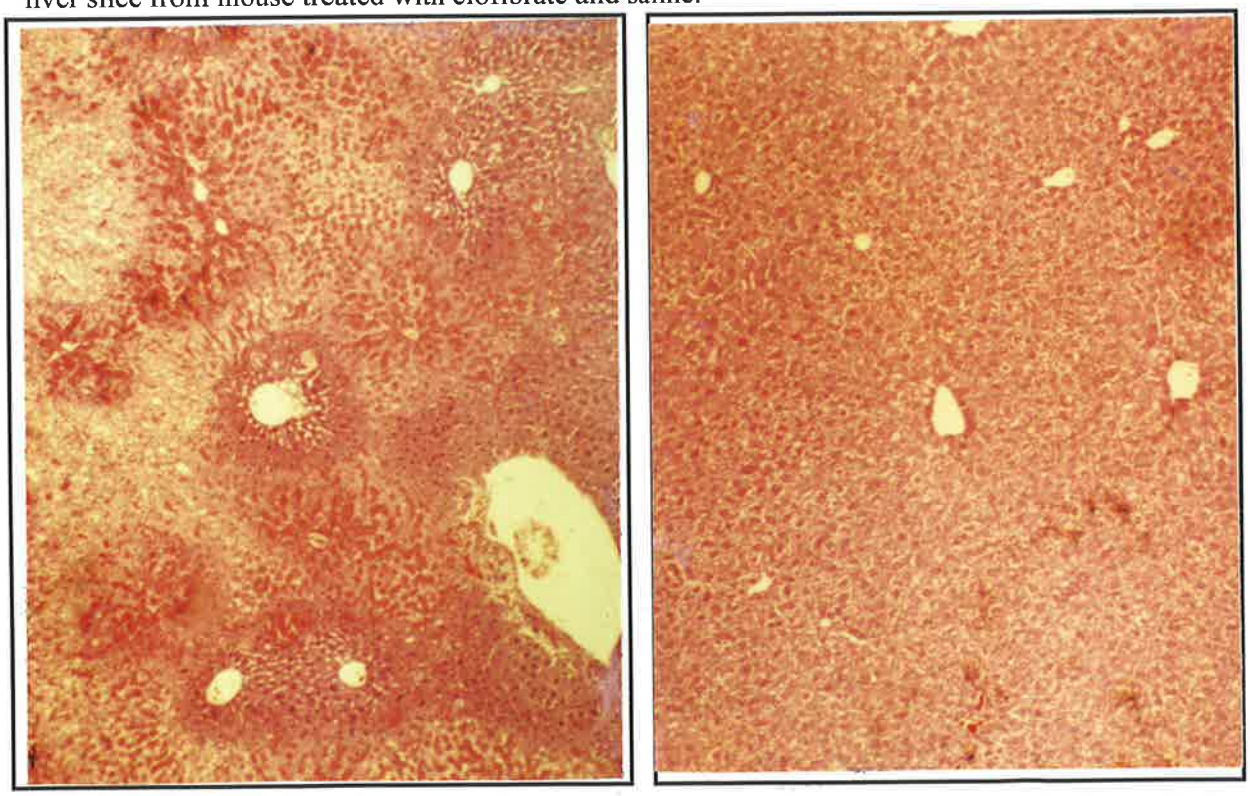



**Figure 3.4:** Plasma SDH levels in control and clofibrate (500 mg/kg/day for 10 days i.p.) treated mice at 24 hours following challenge with 0 to 500 mg/kg AAP (Results expressed as mean ± s.e.m., n=5, *=P<0.05, **=P<0.01; Bonferroni post-hoc test)

The plasma SDH activity results were supported by histological analysis of AAP treated livers in control and clofibrate treated mice. Centrilobular necrosis of dose related severity was observed in vehicle pretreated mice, while no change in morphology was noted in any clofibrate pretreated mouse. Representative photographs of microscopic fields for vehicle control and clofibrate pretreated mice, with and without 500 mg/kg AAP challenge are presented in Figures 3.5A to D. Normal lobule morphology was observed in control mice that received saline, with the limited portal vacuolation presumably reflecting preparation artefacts (Figure 3.5A). Pretreatment of mice with clofibrate caused little alteration in basic morphology, with the exception of the appearance of midzonal mitoses and increased levels of binucleate cells (Figure 3.5 B). The latter effect is indicative of cell proliferation characteristic of treatment with PxPs. Administration of 500 mg/kg AAP produced massive focal haemorrhagic necrosis over large areas of the whole lobule. The necrosis was maximal in the centrilobular region and spared of the portal areas (Figure 3.5 C). In contrast, 500 mg/kg of AAP administered to clofibrate pretreated mice showed no centrilobular necrosis (Figure 3.5 D) with no significant differences seen between these livers and those of non-intoxicated control mice (Figure 3.5 A).



**3.5 A:** (left panel) liver slice prepared from control pretreatment and saline vehicle **B:** (right panel) liver slice from mouse treated with clofibrate and saline.



**3.5 C:** (left panel) liver slice prepared from control mouse treated with AAP **D:** (right panel) liver slice from mouse treated with clofibrate and AAP

**Figures 3.5A to D:** Photographs of representative slides prepared from mice given either control (olive oil vehicle, 0.1 ml/day) or clofibrate (500 mg/kg/day for 10 days) pretreatment then administered with either alkalinised saline vehicle or 500 mg/kg AAP (x40). Samples was taken 24 hours after administration.

## 3.4 DISCUSSION

It is obvious from both the plasma enzyme activity and morphological assessment data that pretreatment with PxP provides profound protection against the acute hepatotoxicity of AAP. Doses that were observed to cause extensive toxicity in control mice had no effect following clofibrate pretreatment. As the hepatoprotective effect was observed with all 3 PxP investigated, it seems likely that this effect is related to the class of PxPs, rather than a specific phenomenon associated with clofibrate alone.

### 3.4.1 Selection of a plasma marker enzyme for quantitation of hepatotoxicity

The first aim of the work described in this chapter was to identify a plasma enzyme marker for routine use during quantitation of AAP hepatotoxicity. Three different enzymes were analysed, namely ALAT, SDH and LDH.

ALAT is a widely distributed enzyme found in liver, kidney and heart (Kachmer, 1976) Increased plasma levels of this enzyme are usually associated with hepatic damage as it is not greatly increased in conditions of renal or myocardial toxicity (Kachmer, 1976). ALAT is a robust enzyme which can be stored at -20°C for a number of weeks without loss of activity. However, results are falsely elevated in haemolysed samples due to the high concentration of ALAT in erythrocytes (Richterich, 1969). This poses a potential problem due to the difficulty in obtaining non-haemolysed blood samples from AAP-intoxicated mice.

In a similar manner, the high LDH content in erythrocytes restricts analysis to nonhaemolysed samples. Furthermore, LDH exists in many isozymic forms which exhibit differing tissue distributions. The assay used in this study did not differentiate between the various isoforms. An increase in LDH activity indicates that a toxic response has occurred, but determination of the site of toxicity requires identification of the LDH isoform(s), in particular the $LDH_5$ form which is specific to the liver (Hayner *et al,* 1984).

In contrast, SDH is found only in the liver and seminal vesicles (Richterich, 1969) and thus raised levels in plasma would indicate hepatocellular damage. SDH can be used in haemolysed samples often obtained in the presence of severe liver damage. In consideration of both these factors, SDH was considered the marker enzyme of choice in future *in vivo* experiments.

### 3.4.2 Effect of pretreatment with clofibrate on the hepatotoxicity of AAP

The protective effect of PxP on the acute hepatotoxicity of AAP was further investigated by determination of the dose response curve to AAP toxicity following pretreatment with clofibrate. Hepatotoxicity was determined both by plasma SDH activity and morphological assessment. It was revealed that pretreatment with clofibrate had an extensive

hepatoprotective effect, as evidenced by the lack of toxicity seen at AAP doses up to 500 mg/kg (Figure 3.4). Histological analysis of livers from control mice administered high dose AAP confirmed the presence of centrilobular necrosis. However, there was no evidence of any necrotic changes in liver samples from mice administered AAP following pretreatment with clofibrate. The protection from AAP hepatotoxicity is in general agreement with earlier studies that pretreatment with PxP provides resistance against xenobiotic toxicity including $CeCl_3$ (Tuchweber and Salas, 1978) paraquat (Frank *et al,* 1982) and iron (Smith *et al,* 1990).

The hepatoprotective effect of PxP pretreatment toward AAP toxicity is an intriguing observation. AAP is bioactivated to the reactive intermediate, NAPQI, which is detoxified by conjugation to GSH. AAP also forms conjugates with glucuronide and sulphate, which constitute the majority of excreted AAP metabolites. When the balance of bioactivation to detoxication favours excessive formation of NAPQI, toxicity becomes evident. Thus the metabolism and toxicity of AAP can be influenced by alteration of CYP450 bioactivation, of the availability of conjugates for phase II conjugation, of Phase II enzyme activities and is also altered by administration of antioxidants. Pretreatment with PxP is known to cause induction of a specific CYP450 isoform, alteration in the activity of detoxication enzymes and in the levels of endogenous antioxidants.

As the hepatoprotection was observed with three distinct PxP, it appears that the protection is related to the class of PxP, rather than a particular effect attributable to clofibrate alone. Exposure to PxP initiates a plethora of biochemical changes within the cell. The protective effect could be due to one specific change or involve a number of changes and therefore be multifactorial in origin. Are the biochemical changes involved in hepatoprotection directly related to the increase in peroxisomal numbers produced or is the protection due to some secondary biochemical event that occurs as a remote consequence of the peroxisome induction?

One way to address this complex issue is to examine the time course of the hepatoprotection upon initiation of clofibrate treatment in comparison to the onset of peroxisome proliferation. Such knowledge should indicate whether there is a close association between protection and proliferation, or whether the occurrence of these two phenomena is purely coincidental. The correlation between the proliferation of the peroxisome and the onset of hepatoprotection toward AAP should be investigated and forms the basis of the experiments described in Chapter 4.

# CHAPTER 4

# INVESTIGATIONS INTO THE DOSE-RESPONSE AND TEMPORAL RELATIONSHIPS BETWEEN CLOFIBRATE INDUCED PEROXISOME PROLIFERATION AND PROTECTION FROM PARACETAMOL-INDUCED HEPATOTOXICITY.

## 4.1 INTRODUCTION

The data presented in Chapter 3 established that pretreatment with the PxP, clofibrate, protects against the acute hepatotoxicity of AAP in mice. However, these experiments did not assess whether the hepatoprotection is directly related to the increased number of peroxisomes. One way of gaining insight into the involvement of the peroxisome is to examine the relationship between the time course of peroxisome proliferation and the development of hepatoprotection.

The experiments in this chapter investigated the timing of the onset of hepatoprotection in relation to the time course of the increase in peroxisome numbers. Peroxisome proliferation was achieved by treating mice for 10 days with 500 mg/kg clofibrate. It is not known if either a shorter amount of time or a lower dose will provide equivalent levels of peroxisome proliferation and/or hepatoprotection and therefore the dose response relationship and alteration of the duration of treatment was investigated. The effects of cessation of clofibrate treatment upon the return of the liver to normal peroxisome numbers and sensitivity to hepatotoxicity was also investigated in order to further clarify the relationship between these two phenomena.

Investigation of the temporal relationship between the extent of peroxisome proliferation and the degree of protection from AAP toxicity required that 2 separate evaluations be made in each group of mice given clofibrate in different dosing regimens. The first evaluation required that a group of biochemical parameters associated with PxP treatment be monitored. The level of cellular peroxisomes was determined by measurement of the activity of the specific marker enzyme, palmitoyl CoA oxidase. As well, microsomal CYP4A1 activity, determined by lauric acid ω-hydroxylation, and % liver weight to body weight (%LBW) as a measurement of hepatomegaly, provided information about effects of treatment with the PxP which were not directly related to the induction of the peroxisome itself. The second evaluation involved administration of a toxic dose of AAP to mice to determine the extent of hepatoprotection provided by their respective clofibrate treatments. A dose of 500 mg/kg of AAP was chosen for this purpose, having been established in Chapter 3 to be profoundly toxic to control mice, while having no adverse effect on clofibrate pretreated mice.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Materials

All materials used in this study were the highest quality commercially available. For details of chemicals and suppliers, see Appendix 1.

### 4.2.2 Clofibrate dosing protocols

In the first experiment (Study 1) , the time required for the onset of peroxisome proliferation and the dose response relationship for clofibrate were investigated. Swiss White male mice (35-40g) were obtained and housed as specified in Chapter 2 and divided into 9 groups (10 to 15 mice per group). They then received one of the treatments outlined below. Groups 1A to E were intended to provide information on the dose-response relationships for the induction of PxP by clofibrate, while groups 1E to I were designed to investigate the timing of the onset of hepatoprotection and peroxisome proliferation.

1A) 0.1 ml/day olive oil (injection vehicle, i.p.) for 10 days
1B) 20 mg/kg/day clofibrate (i.p.) for 10 days
1C) 50 mg/kg/day clofibrate (i.p.) for 10 days
1D) 200 mg/kg/day clofibrate (i.p.) for 10 days
1E) 500 mg/kg/day clofibrate (i.p.) for 10 days*
1F) 500 mg/kg/day clofibrate (i.p.) for 6 days
1G) 500 mg/kg/day clofibrate (i.p.) for 3 days
1H) 500 mg/kg/day clofibrate (i.p.) for 1 day
1I) Untreated

** used as a standard treatment to achieve peroxisome proliferation*

Measurements were made approximately 16 hours after the last dose of clofibrate or vehicle.

In the second experiment (Study 2), the effects of cessation of PxP treatment on hepatoprotection and peroxisome proliferation were investigated. Male Swiss White mice (35-40 g) were allocated to one of the treatment groups (n=10). Mice had staggered treatment commencement dates to ensure all animals were ready for use on the same day. All mice in the 2A to F groups were treated with 500 mg/kg clofibrate in olive oil by intraperitoneal route for 10 days then treatment ceased for varying times from zero to up to 3 weeks following the final clofibrate dose. Groups 2F and 2G comprised mice that received the standard treatments with clofibrate or injection vehicle. Measurements were made approximately 16 hours after the final dose in Groups 2F and G, or in accordance to the cessation scheme for groups 2A to E.

2A) 3 weeks off treatment
2B) 2 weeks off treatment
2C) 1 week off treatment
2D) 3 days off treatment
2E) 1 day off treatment
2F) 500 mg/kg/day clofibrate for 10 days - treatment control - 0 days off treatment
2G) 0.1 ml/day olive oil vehicle for 10 days

At the end of the pretreatment phase, mice in both Groups 1A to 1I and 2A to 2G were allocated into two subgroups, with one (Subgroup A) used for the assessment of peroxisome proliferation and the other (Subgroup B) for the assessment of hepatoprotection against AAP.

### 4.2.3 Animal sampling

*4.2.3.1 Subgroup A: Quantitation of peroxisome proliferation*

Mice in subgroup A were anaesthetised with 0.6 % nembutal, weighed and then blood sample collected by closed cardiac puncture. Blood was centrifuged at 3,000 x g for 5 minutes and plasma stored at -20°C until plasma SDH activity was determined typically within 24 hours of plasma collection. Livers were removed, weighed and 10% (w/v) homogenate prepared in 0.1 M potassium phosphate buffer, pH 7.4. For Study 1, homogenate was stored at -80°C prior to the determination of palmitoyl CoA oxidase or lauric acid ω-hydroxylase activities. In Study 2, an additional 0.5 ml of homogenate was added to 0.5 ml of 0.4 M trichloroacetic acid vortexed and stored at -80°C, prior to the measurement of GSH.

*4.2.3.2 Subgroup B: Investigation of AAP toxicity*

Mice were injected with 500 mg/kg AAP dissolved in alkalinised saline. After 24 hours, blood was collected from the surviving mice as outlined in 4.2.3.1 for determination of plasma SDH levels. Livers were removed, weighed and examined by an independent assessor for the macroscopic determination of toxicity. In Study 2, due to the mortality associated with AAP administration, mice were inspected at 8 hours. If any mouse appeared unlikely to survive to 24 hours were killed at 8 hours and blood samples were taken for SDH analysis.

### 4.2.4 Biochemical Assays

Hepatic palmitoyl CoA oxidase and plasma SDH activity were determined according to the methods outlined in Section 2.2.6.1 and Section 3.2.5.2., respectively.

*4.2.4.1 Lauric Acid ω-hydroxylation.*

The formation of hydroxylauric acid was determined in Study 1 by autoradiography using a modification of the assay of Mitchell *et al* (1985). Homogenate samples (0.5 ml) were diluted

1:2 with cold 0.1 M potassium phosphate buffer, pH 7.4. A 1.5 ml volume of 0.127 mM lauric acid, 0.75 mM NADPH in 66 mM Tris buffer, pH 7.4, spiked with 1.7 μ Cu [$^{14}$C]-lauric acid was preincubated at 37° C for 5 minutes. A 0.5 ml volume of diluted homogenate was added and incubated for a further 10 minutes and the reaction was then stopped by the addition of 1 ml of 1 M hydrochloric acid. To extract lauric acid and its metabolites, 3 ml of diethyl ether was added to each tube and placed on a gyratory rotor for 1 hour. The tubes were centrifuged for 5 minutes at 3000 rpm at 4° C. A 2 ml volume of the upper ether layer was transferred to a fresh glass tube and evaporated to dryness under nitrogen using a heating block. Samples were redissolved in 100 μl of methanol and the entire amount spotted onto reverse phase thin layer chromatography plates (Whatman KC18F, 5 x 20cm). Lauric acid and its metabolites were separated using a methanol:water:glacial acetic acid (80:19.5:0.5) mobile phase. Plates were dried, covered with Amersham Hyperfilm ß-max photographic film and stored in total darkness for 48 hours.

Lauric acid and its metabolites were visualised by developing the photographic film using standard procedures. A lauric acid standard was run on a separate plate prior to observation under UV light and was found to have a Rf value of 0.28. In a microsomal incubation using $^{14}$C-lauric acid, an intense spot was seen at this Rf value, in addition to a distinct band at Rf = 0.76 which was over 20 times more intense in microsomes isolated from clofibrate treated mice. This band is likely to be the hydroxylated metabolite of lauric acid. The lauric acid and the hydroxylated metabolite were identified from the photographic film and scraped into separate scintillation vials. Following the addition of 0.5 ml of 1.8 M glacial acetic acid, a 10 ml volume of Beckman Readyvalue cocktail was added and samples counted for 10 minutes in a Beckman LS 3801 liquid scintillation counter. Results are expressed in nmol hydroxylauric acid/min/g liver.

*4.2.4.2 Glutathione level*

The method for the analysis of GSH was based on the method for the determination of non-protein sulphydryls of Saville (1958). A 0.5 ml volume of 10 % w/v homogenate was added to 0.5 ml of 0.4 M trichloroacetic acid and centrifuged at 3,000 x g for 1 minute. Deproteinated supernatant (0.25 ml) was added to 1.25 ml of 1 mM sodium nitrite in 0.54 M sulphuric acid and left at room temperature for 10 minutes. A 0.25 ml volume of 44 mM ammonium sulphamate was added and then the tubes were vortexed and incubated for a further 2 minutes. Following this, 2.5 ml of 9.3 mM mercuric chloride in 0.15 M sulphanilamide was added just prior to 2 ml of 0.1 % *N*-1-naphthyl ethylenediamine dihydrochloride. The absorbance of the samples was measured at 540 nm 10 minutes later using a Hitachi U-2000 spectrophotometer. The GSH content of the samples was determined from a standard curve prepared over a range of 0 to 1 mM GSH. Results were expressed as mmol GSH/g liver.

### 4.2.5 Statistics

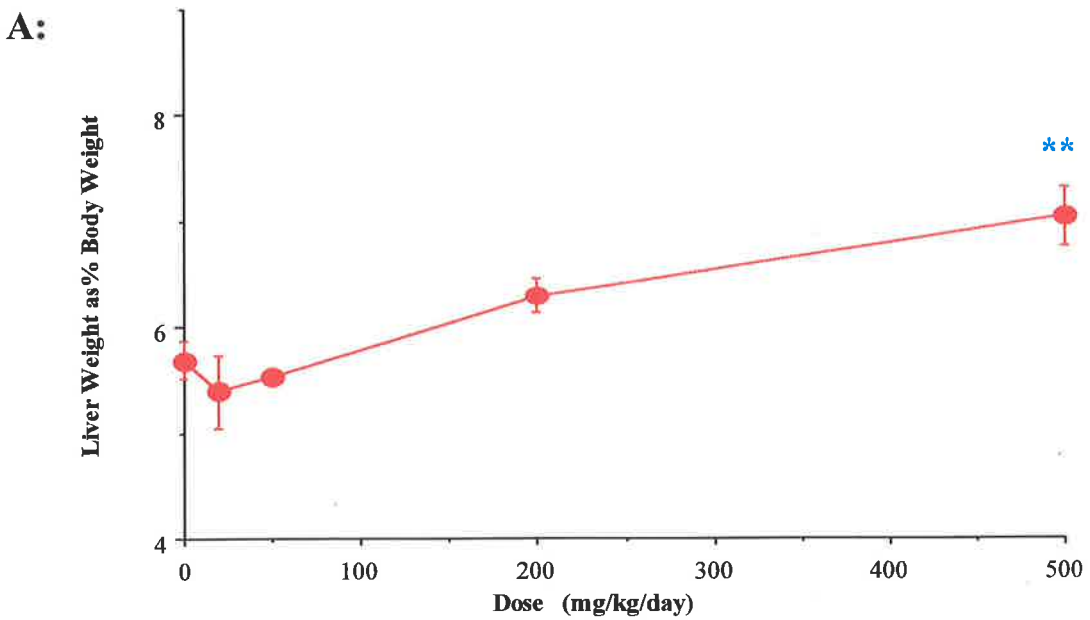
All data obtained in this study were analysed by ANOVA. When significant differences were observed ($P<0.05$), post hoc tests were performed. In Study 1, the control group for the duration of treatment study was 1I, being the untreated group. For the clofibrate dose study, 1A formed the control group having received olive oil vehicle control for a full 10 days. Similarly, the vehicle treated control group 2G, formed the control group for comparison in the cessation of treatment experiment. Thus the post hoc test for Study 1 was the Dunnett's test was used for comparison of all groups to their respective controls. For the SDH assay in both studies, controls were available at each time or dose point, therefore the Bonferroni Post Hoc test was used for comparison of specific pairs of data.

## 4.3 RESULTS

### 4.3.1 Time and dose dependency of peroxisome proliferation by clofibrate

*4.3.1.1 Dose response relationship*

In these experiments, the effects of clofibrate treatment were monitored by the peroxisomal marker enzyme, palmitoyl CoA oxidase, microsomal lauric acid ω-hydroxylase as a marker of CYP4A1 activity and %LBW. Treatment of the mice for 10 days with 20 and 50 mg/kg of clofibrate showed no statistically significant increase in any of the indicators of peroxisome proliferation when compared to the control group (Figure 4.1 A, B and C).



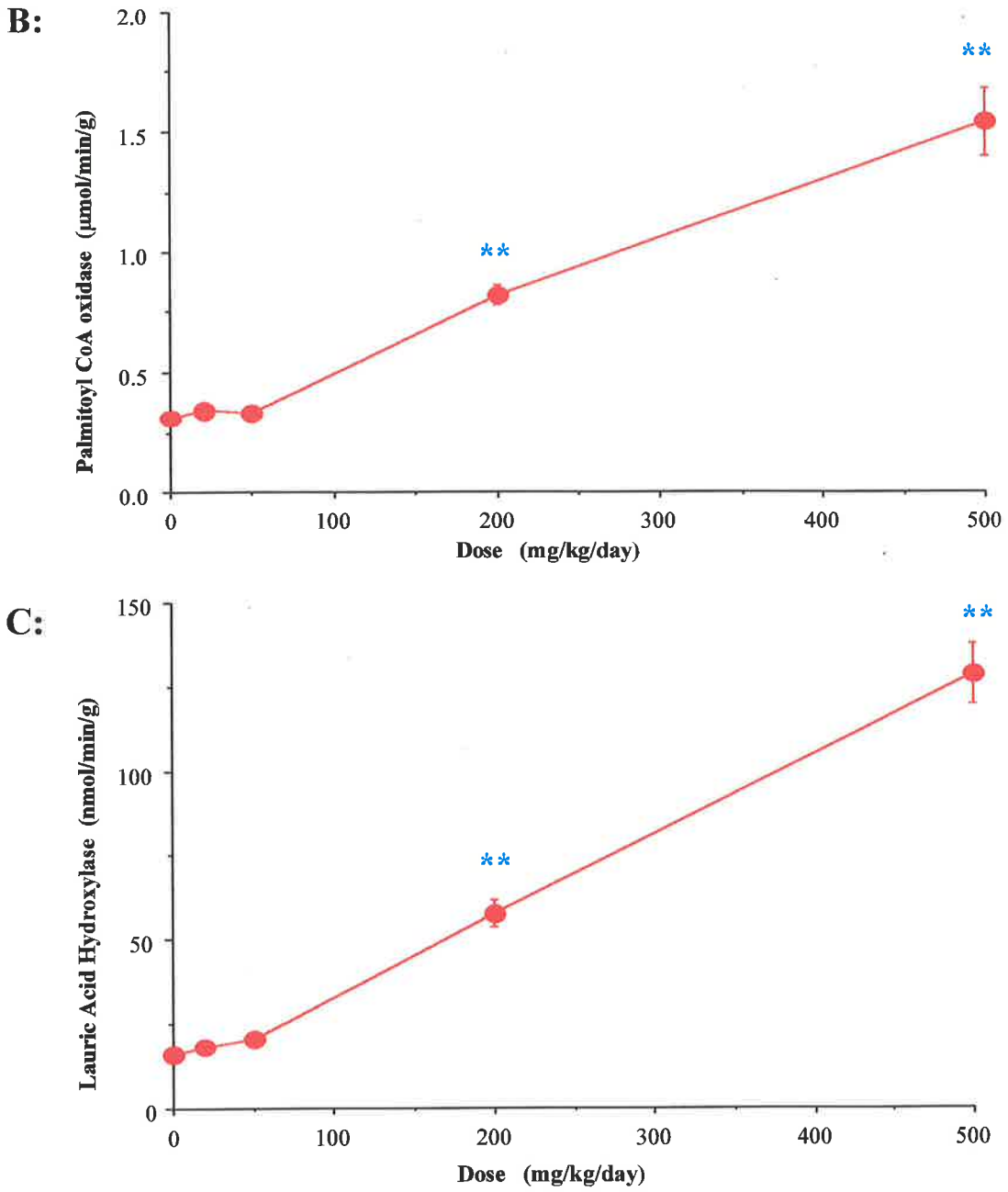



**Figure 4.1.:** Indicators of peroxisome proliferation following treatment with 0, 20, 50 or 500mg/kg/day clofibrate i.p for 10 days. **A:** Liver weight as a percent body weight **B:** Palmitoyl CoA oxidase activity (measured as µmolNADH/min/g) **C:** Lauric acid hydroxylase (measured as nmol/min/g). (Results were expressed as, mean ± s.e.m, n=4, **=P<0.01 for Dunnett's post hoc test)

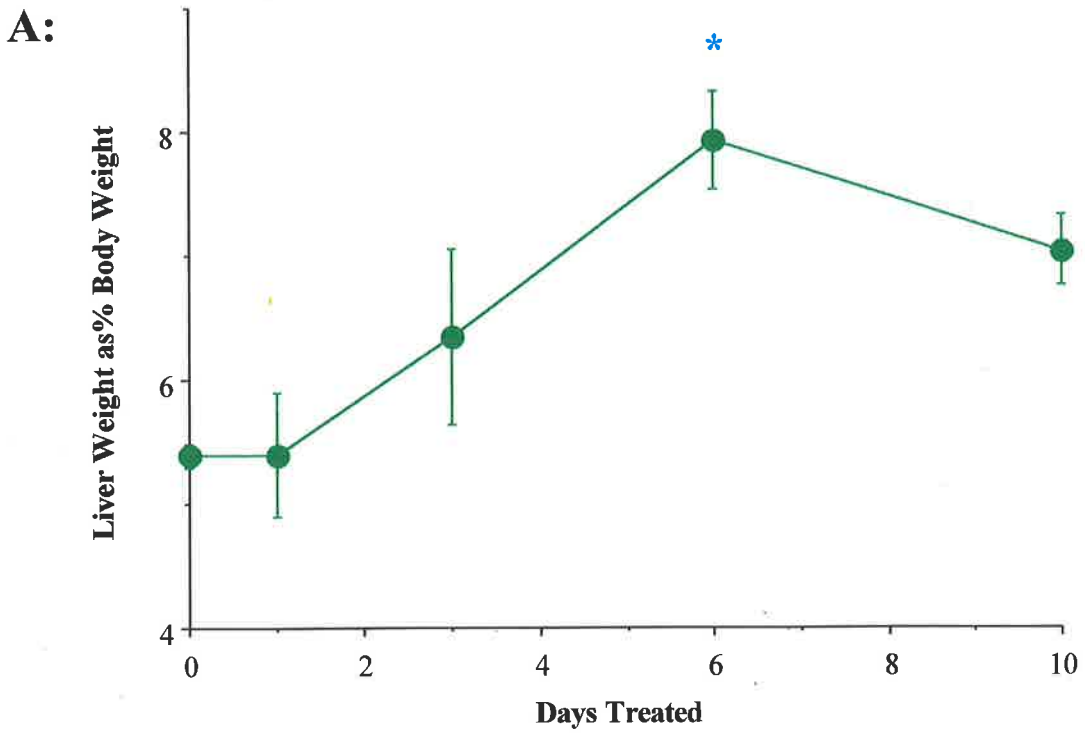
The 200 mg/kg dose provided intermediary results between those obtained for the control and 500 mg/kg dose. The 500 mg/kg dose led to the expected increases associated with peroxisome proliferation with namely a 4-fold increase in palmitoyl CoA oxidase activity, a 7-fold increase in lauric acid ω -hydroxylase and a 30 % increase in %LWB. (Figures 4.1 A, B and C). As the biochemical effects of PxP treatment are due to activation of the PPAR, these results suggest that a dose of between 50 mg/kg and 200 mg/kg reaches the intracellular concentrations required to activate the PPAR and thus initiate the biochemical and macroscopic changes associated with peroxisome proliferation.

*4.3.1.2 Time course of peroxisome proliferation*

Comparison of the onset of the changes in the three parameters associated with PxP treatment during administration of clofibrate at 500mg/kg/day for less than 10 days each revealed subtle differences in the response of each marker.

The earliest observed response was a 100% increase in lauric acid ω-hydroxylase activity, occurring after just a single dose of clofibrate (Figure 4.2.C). This enzyme reached a maximal level of induction at 3 days then the response plateaued at subsequent time points. In contrast, the hypertrophic response, indicated by measurements of %LBW, reached a maximal effect at 6 days with a subsequent plateau (Figure 4.2.A).

The induction of palmitoyl CoA oxidase activity occurred still more slowly with a maximum 4 fold increase evident at 10 days (Figure 4.2.B). Whether this represents the maximum extent of induction that could be achieved with this dose of clofibrate is unknown. Nonetheless, since the activity of palmitoyl CoA oxidase is directly related to the level of peroxisomes, this finding clearly indicates that there is a steady increase in peroxisomal numbers over the 10 days of treatment.



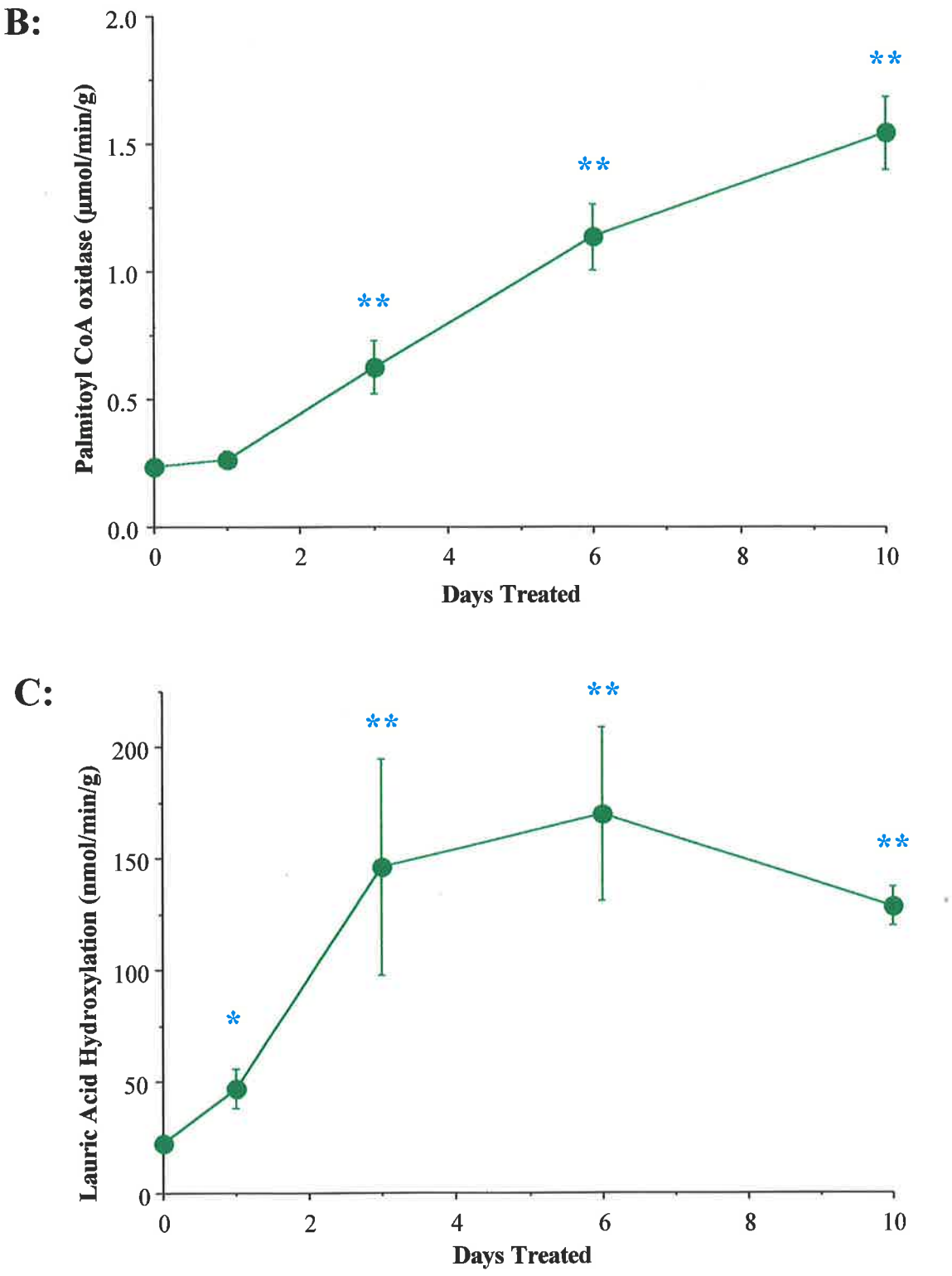



**Figure 4.2:** Indicators of peroxisome proliferation following treatment with 500mg/kg/day clofibrate i.p for 0, 1, 3, 6 or 10 days. **A:** %LBW **B:** Palmitoyl CoA oxidase activity (measured as µmol NADH/min/g) **C:** Lauric acid hydroxylase (measured as nmol/min/g). (Results expressed as mean ± s.e.m, n=4, *=P<0.05, **=P<0.01 for Dunnett's Post Hoc test).

To gain further information about the nature of the cellular events involved in hepatoprotection (Section 4.3.2) the time course of the loss of protection was compared to the reversion to a

non-proliferative state following cessation of clofibrate treatment. This study was done using the standard clofibrate regimen of 500 mg/kg/day for 10 days regimen.

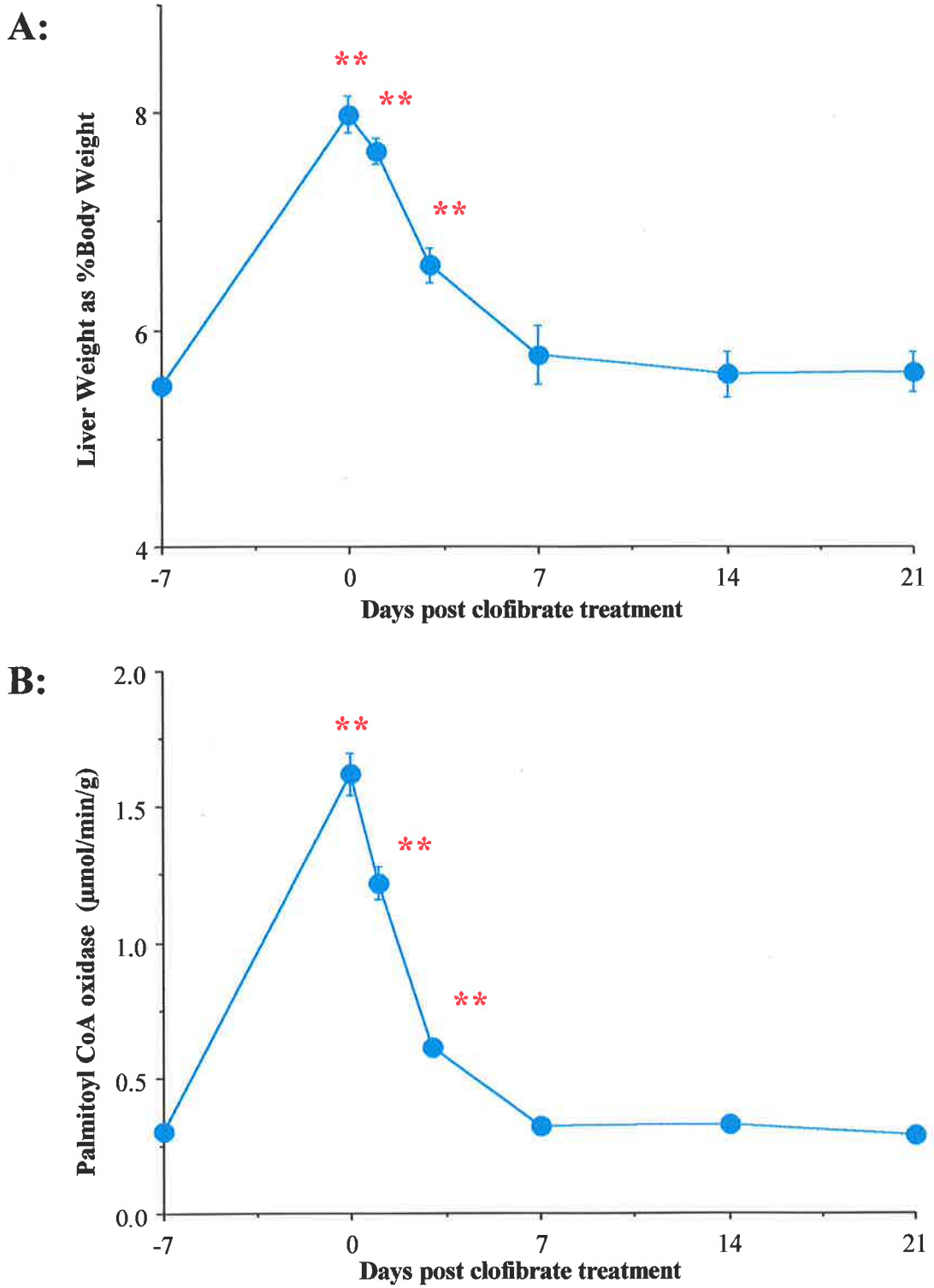



**Figure 4.3:** Palmitoyl CoA oxidase activity and %LWB in mice at various times following cessation of 500mg/kg clofibrate i.p. for 10 days. The value on the *y* axis is vehicle pretreated controls. The 0 day value is obtained after 10 days pretreatment with clofibrate. **A**: %LBW **B:** Palmitoyl CoA oxidase activity (µmolNADH/min/g) (Results are expressed as mean ± s.e.m, n=4, **=P<0.01, compared to oil vehicle control indicated on *y* axis).

After one day of withdrawal following the final clofibrate dose, the palmitoyl CoA oxidase activity had dropped by 30%. This rapid decline continued, with a 70% decrease in activity of this marker after three days and a return to control rates within 1 week of discontinuation of treatment (Figure 4.3.B). Similar results were observed for the hepatomegalic response, with control %LBW being regained within 1 week of cessation of the drug treatment (Figure 4.3.A).

In addition to the PxP parameters previously investigated during the onset study, the hepatic level of cellular GSH was investigated in Study 2 and was found to have increased by 35% following clofibrate treatment (Figure 4.4). This level was maintained for 3 days after discontinuation of treatment, returning to control levels within one week.

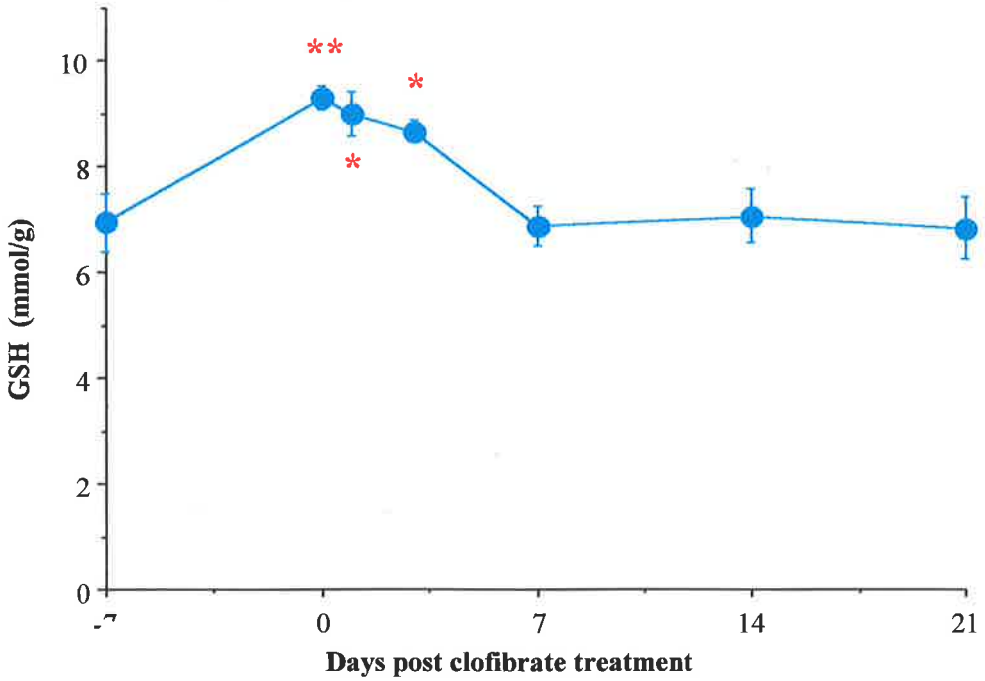



**Figure 4.4:** Hepatic GSH levels at various times following cessation of treatment with 500mg/kg/day clofibrate i.p. for 10 days. The value on the *y* axis are vehicle pretreated controls. The 0 day value is the value obtained after 10 days pretreatment with clofibrate. Results are expressed as mmol GSH/ gram of wet weight liver, mean ± s.e.m (*=P<0.05,**=P<0.01, compared to oil vehicle control as indicated on *y* axis).

Overall, it was observed that the onset of peroxisome proliferation, as measured by the peroxisome-specific marker enzyme, palmitoyl CoA oxidase was evident within 3 days of the start of administration. For a 10 day treatment, a dose of approximately 200 mg/kg clofibrate was required to increase palmitoyl CoA activity. However, while the onset of palmitoyl CoA oxidase activity was comparatively slow, there is a rapid offset, with a 70% return to control values after 3 days and complete return within 1 week of cessation of treatment. Neither the %LBW nor the lauric acid ω-hydroxylation followed the same time course of induction or offset following cessation of treatment, indicating that cellular changes pertaining to PPAR activation are not strictly correlated to each other.

### 4.3.2 Dose and time dependency of clofibrate induced hepatoprotection against AAP.

The second evaluation addressed in this chapter was the determination of the onset of hepatoprotection following administration of 500 mg/kg AAP. This dose of AAP had been previously shown in Chapter 3 to cause extensive toxicity in the control group. Hepatotoxicity was assessed by measuring plasma SDH activity 24 hours after AAP administration. Once again the effects of different doses of clofibrate as well as alteration in the duration of treatment were investigated, including the effect of cessation of clofibrate treatment on hepatoprotection.

Pretreatment of mice with either 20 or 50 mg/kg/day of clofibrate for 10 days provided no protection against AAP hepatotoxicity as assessed by measurements of plasma SDH activity (Figure 4.5).

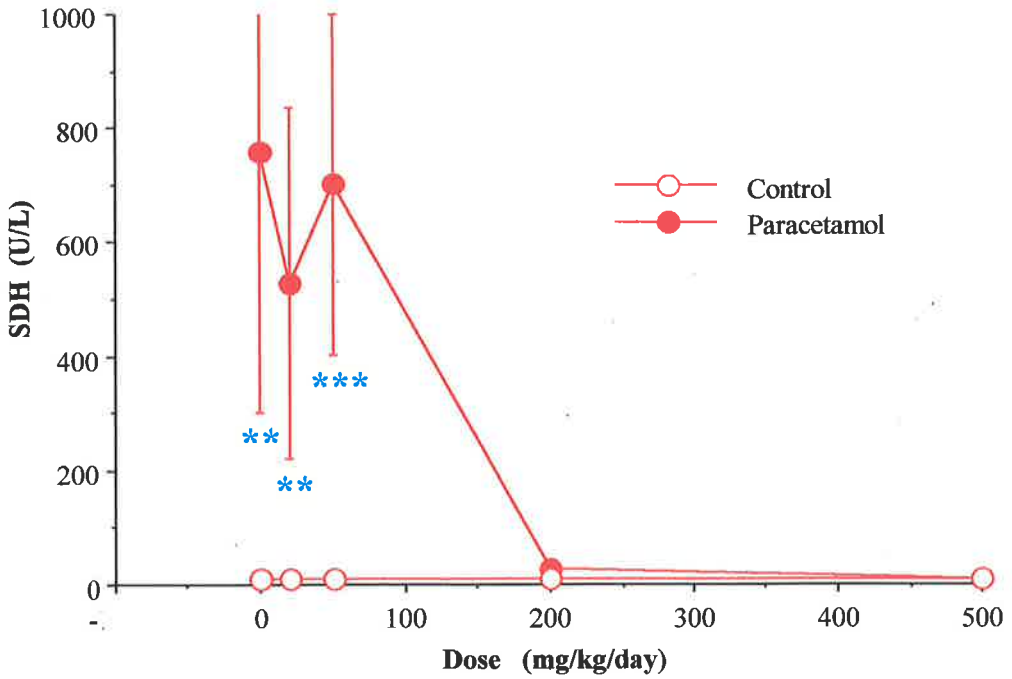



**Figure 4.5:** Plasma SDH activity in mice 24 hours after administration of either alkalinised saline control or 500mg/kg AAP. Mice had been pretreated with either 0, 20, 50, 200 or 500 mg/kg clofibrate prior to AAP challenge (Results are expressed as mean ± s.e.m, n=4-10, **=P<0.01, ***=P<0.001, Bonferroni post hoc test).

At these doses and for the vehicle pretreated control mice, the mortality was between 20 and 50% (data not shown). In contrast, the 200 mg/kg daily dose of clofibrate appeared to provide substantial protection against AAP in terms of enzyme effects, and although 33% mortality was observed in this treatment group, all the surviving mice exhibited plasma SDH levels within the control range. It appears that at this dose of clofibrate, the same one that consistently achieved peroxisome proliferation in the preceding experiments, an "all or none" protection was provided against AAP toxicity. In keeping with the data presented in Chapter 3, the 500 mg/kg/day treatment regimen afforded complete protection against AAP induced hepatotoxicity (Figure 4.5).

To determine the time course of the onset of hepatoprotection, mice that had received daily doses of 500 mg/kg clofibrate for 1, 3, 6 and 10 days were given a hepatotoxic dose of 500 mg/kg AAP. The 0 day timepoint consisted of untreated mice and formed the control group. Plasma SDH activity was measured 24 hours after AAP administration to determine the extent of hepatotoxicity. A single clofibrate dose of 500 mg/kg clofibrate provided no protection against the acute hepatotoxicity of AAP. However repeated administration for 3 or more days of clofibrate treatment afforded complete hepatoprotection (Figure 4.6). In the 0 and 1 day treatment groups, 24 hour mortality rates of 37 and 16% respectively were observed. There were no deaths in any of the other groups. The hepatoprotective effect is therefore related to a cellular alteration that takes between 1 and 3 days to become effective.

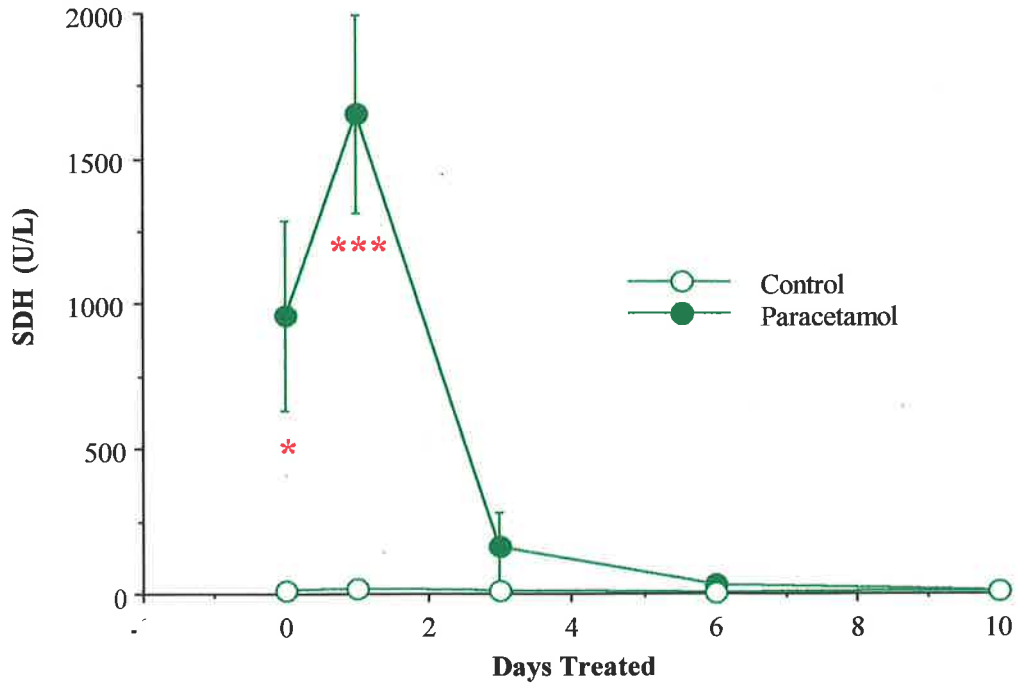



**Figure 4.6:** Plasma SDH activity 24 hours after administration of either saline control or 500mg/kg AAP. Mice had been treated with 500mg/kg clofibrate treatment for different number of days. (Results are expressed as mean ± s.e.m, n=4-10, *=P<0.05, ***=P<0.001, Bonferroni post hoc test).

In addition to the time of onset, the effects of cessation of clofibrate treatment on the loss of hepatoprotection were compared to the return of the peroxisomal induction markers to control levels. In this experiment, it was observed that the protective effect persisted for 3 days, as seen by the maintenance of control plasma SDH values. However after 3 days, the protective effect was rapidly lost so that no protection was observed at 1 week or any time point thereafter (Figure 4.7). At 1 and 3 days after cessation of treatment, no mortality was observed, however when the cessation time reached 7 days and beyond a 60% mortality was observed. This data suggests that following removal of the PxP, a loss of the cellular factors

responsible for hepatoprotection occurs, with a degradation half life such that the minimum level required for protection is lost within 7 days.

Collectively these results reveal that complete hepatoprotection is evident after treatment with 200mg/kg clofibrate for 10 days in about 67% of mice while the remaining 33% died. Protection was obtained after 3 days of clofibrate administration at the standard dose of 500 mg/kg and was maintained for at least 3 days after the cessation of treatment. Of the three parameters associated with PxP treatment investigated, none totally correlated with this time course of protection. Most importantly, the palmitoyl CoA oxidase activity indicative of peroxisome presence, appears to correlate with the onset of hepatoprotection, in being evident after 3 days of 500 mg/kg/day and also 200 mg/kg/day for 10 days, yet the offset is delayed with hepatoprotection evident after a rapid reduction in peroxisomal number.

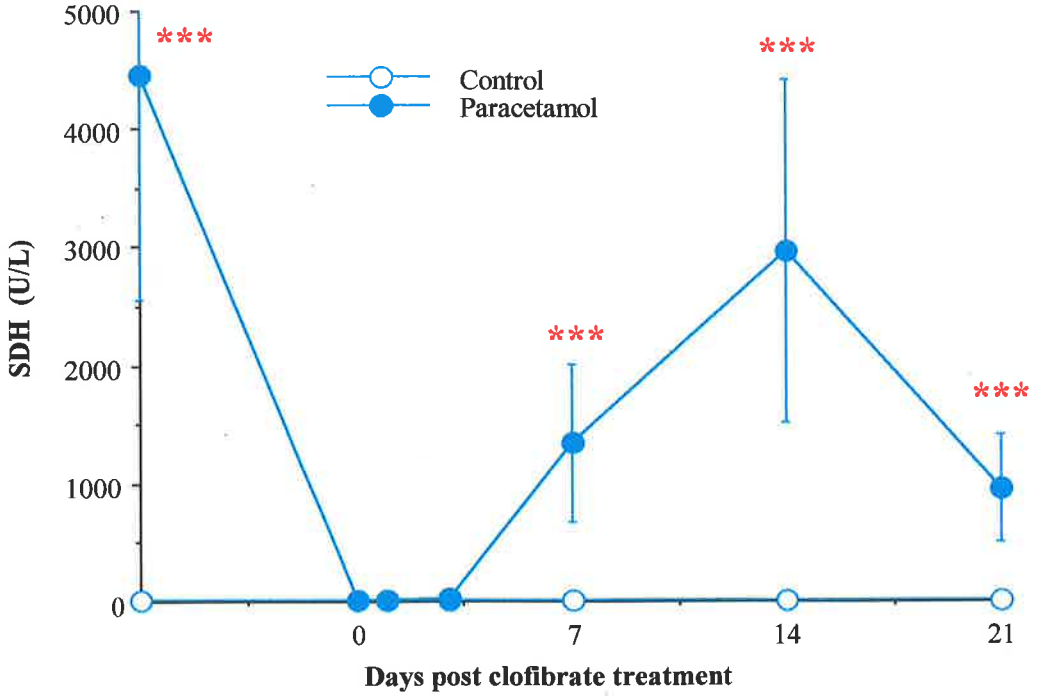



**Figure 4.7:** Plasma SDH activity in mice 24 hours after administration of either alkalinised saline or 500mg/kg AAP. Mice were administered 500 mg/kg/day clofibrate for 10 days activity then treatment ceased for the times indicated. The value on the *y* axis is for vehicle pretreated controls. (Results are expressed U/L, mean ± s.e.m , n=6, ***=P<0.001 compared to oil vehicle control indicated on *y* axis).

## 4.4 DISCUSSION

The experiments described in this chapter provided important insights into the correlation between the onset and decline of clofibrate induced peroxisome proliferation and hepatoprotection against AAP. Peroxisome proliferation was assessed by measuring the activity of an enzyme involved in ß-oxidation of fatty acids, palmitoyl CoA oxidase, which is known to correlated closely with the number of peroxisomes present in liver cells (Moody and Reddy, 1976). However, an increase in cellular peroxisomes is only one of the many responses mediated by activation of the PPAR receptor. The hypertrophic response to PxPs,

as measured by %LWB and microsomal CYP4A1 activity by determination of lauric acid hydroxylase activity are both characteristic of exposure to PxPs but do not reflect actual changes in peroxisome number.

The determination of onset of peroxisome proliferation involved both adjustment of dose while keeping the treatment time of 10 days constant and adjustment of treatment duration while keeping the dose at 500 mg/day. There was a dose response relationship, in that while the 2 lower doses did not produce any measurable alteration in PxP parameters, the 200 mg/kg and 500 mg/kg produced dose related changes in PxP parameters.

In the study involving use of a 500 mg/kg dose of clofibrate for differing times up to 10 days, it was observed that the earliest event was induction of CYP4A1 as determined by lauric acid hydroxylase activity. The increase in liver weight was evident from three days onward. Palmitoyl CoA oxidase activity increased more slowly with time. These results agree with previous studies investigating the onset of PxP effects which have also shown that the induction of lauric acid hydroxylase preceded increases in palmitoyl CoA oxidase activity (Lake *et al,* 1984b; Bell *et al,* 1991; Bieri *et al,* 1991; Hofstra *et al,* 1997). In addition, pretreatment with clofibrate gave a 9-fold induction of lauric acid hydroxylase activity (Sharma *et al,* 1988) with only 3 days of dosing able to result in the same level of lauric acid hydroxylase induction as 14 day exposure (Gibson and Lake, 1991). Both studies are in agreement with the results reported in this chapter. Morphological studies have provided support for these findings by showing that treatment with clofibrate produced a 6 fold increase in peroxisomal area in 4 days (Meijer *et al,* 1993) and that increases in smooth endoplasmic reticulum and peroxisome volume was evident in 3 days, reaching steady state after 2 weeks (Moody and Reddy, 1976). The increases in peroxisomal volume and endoplasmic reticulum are reflected in enhanced palmitoyl CoA oxidase activity and lauric acid ω hydroxylation respectively.

Withdrawal of clofibrate treatment produced a rapid decline of palmitoyl CoA oxidase activity with a 70% decrease from induced values 3 days after cessation. Since control levels were regained within one week, it appears that this enzyme has a half life of around 2 days. A similar half life estimate was reported following 14 days of treatment with DEHP (Muyazawa *et al,* 1980) and coincides with the estimated half life of the peroxisome from morphological studies (Leighton *et al,* 1975). In addition, it appears that palmitoyl CoA oxidase activities follow a similar pattern with onset being slower than withdrawal, as also observed with other enzymes including epoxide hydrolase. After 7 days of PxP treatment, a maximal 8-fold increase in mRNA for epoxide hydratase was observed, which returned to control levels within 2 days after cessation of treatment (Johansson *et al,* 1995), however, the activity of the enzyme returned to normal within 7 days (Moody *et al,* 1985). The rate of decline is related to the degradation halflife of the excess enzyme following cessation of PxP exposure. It remains

a distinct possibility that activation of the PPAR has resulted in enhanced cellular levels of a protein with nucleophilic or antioxidant properties which provides protection against AAP toxicity or induction of enzymes involved in AAP metabolism, for example UDP glucuronyl transferase.

The hepatomegalic response to clofibrate started to decline at 3 days after cessation of drug treatment and returned to control levels by 7 days. Other investigators have reported similar observations during morphological studies (Moody and Reddy, 1976; Meijer *et al,* 1993). The decrease in liver size is believed to result from a massive induction of apoptosis within the liver. It has been observed that another PxP, nafenopin causes a 16-fold reduction in apoptosis, however upon cessation of exposure this rate increases up to 200-fold causing rapid cell removal (Roberts *et al,* 1995). In addition, after 4 days there is an apparent increase in cellular lysosome content, probably associated with cellular removal of internal membranes (Moody and Reddy, 1976). It is apparent that the biochemical events occurring in the liver during the onset phase are very different to those occurring following the cessation of treatment.

Having confirmed the dose and time dependence of clofibrate induced peroxisome proliferation, the correlation between peroxisome proliferation and hepatoprotection was investigated. Such a correlation could result in at least three possible scenarios; with protection preceding, accompanying or occurring after the proliferation of the organelle. If protection precedes the increase in peroxisomes, it would appear unlikely that the protection was directly associated with the organelle. Alternatively, if the onset of protection closely coincides with the increase in peroxisomal numbers, then the organelle may well be directly involved in the protective process. Finally, if the protection lags behind the increase in peroxisomes, then the protection could be associated with some other event occurring in response to the increase in peroxisome number. Hence the possibility exists that the relationship between the protective effect and the increase in peroxisome number is purely coincidental. In general, the present findings indicate that the onset of peroxisome proliferation coincided with the hepatoprotective effect suggesting the organelle may well be directly involved in the protective process. However, the cessation study revealed that the protection was sustained longer than increased numbers of peroxisomes suggesting induction of protective factor(s) separate from the peroxisome.

At least three days of treatment with 500 mg/kg clofibrate was required to achieve hepatoprotection (Figure 4.6). This coincided with an increase in all of the 3 measured parameters (Figure 4.2 A, B &C). Investigation of the dose response relationship indicated that at doses where no PxP related effects were observed (20 and 50 mg/kg) there was no hepatoprotection against AAP toxicity. In contrast, the 500 mg/kg dose group produced both peroxisome proliferation and hepatoprotection. Therefore, both the duration of treatment and

the dose study suggested an association between the onset of hepatoprotection and the proliferation of peroxisomes. Interestingly, the mice in the 200 mg/kg group were either completely protected or succumbed to AAP lethality, with no gradation of effect observed. It is tempting to speculate that the 200 mg/kg dose is very close to the threshold for activation of the PPAR for clofibrate, which subsequently initiates hepatoprotection.

In this study, a single dose of clofibrate provided no protection indicating that this effect is not due to a direct action of clofibrate, such as an inhibition of CYP2E1 catalysed formation of NAPQI. In addition, activation of the PPAR would involve dissociation of a 70 kDa heat shock protein chaperone (Huang *et al,* 1994) which belongs to a class of proteins with antioxidant capability (Donati *et al,* 1990). While one could speculate that this antioxidant protein might be involved, the lack of any protection observed following 1 day of treatment indicates that any increase in the cellular level of the chaperone protein is insufficient to produce a chemoprotective effect. In contrast to the findings in this chapter, a recent study reported that mice treated with a single dose of 500 mg/kg clofibrate followed by an 18 hour fast, prior to an 800 mg/kg dose of AAP, showed a 60% decrease in plasma SDH levels (Manautou *et al,* 1996). In this study, the 500mg/kg of AAP produced profound hepatotoxicity in control mice yet no toxicity in PxP treated animals. Manautou and colleagues routinely used a very high dose of 800 mg/kg AAP following an 18 hour fast in order to induce AAP toxicity (Manautou *et al,* 1994; Manautou *et al,* 1995; Manautou *et al,* 1996). The starvation phase is intended to reduce hepatic GSH levels, thus enhancing AAP toxicity (Wendel and Jaeschke, 1983). One reason Manautou needed such extreme steps could be that the AAP vehicle, 50% propylene glycol, has recently been shown to be a powerful inhibitor of CYP2E1 activity and protect against AAP toxicity (Snawder *et al*, 1993; Thomsen *et al*, 1995). The presence of CYP2E1 inhibition and starvation induced GSH depletion could well complicate the overall hepatoprotective effect observed. Also, in the event of very high levels of hepatic damage, a 60% decrease in plasma SDH does not necessarily indicate extensive hepatoprotection. For example, in the cessation study reported in this chapter, AAP administration to mice 2 weeks after withdrawral of clofibrate treatment resulted in a mean SDH of 2900 IU. In contrast, the 3 weeks off clofibrate treatment recorded a mean SDH activity of 950 IU, a reduction of almost 70%, however both of these groups recorded identical lethality and extent of necrosis.

Following clofibrate treatment, GSH levels were raised by 35% and remained raised for 3 days following cessation of treatment (Figure 4.4). This was the same pattern as observed for the hepatoprotection profile observed in the withdrawal study (Figure 4.7). GSH forms a conjugate with the active metabolite, NAPQI and thus provides cellular protection against AAP toxicity. Although the changes in GSH levels produced by PxP treatment were not marked, it is possible that alterations in GSH turnover, regeneration and thus availability are

responsible for the hepatoprotection against AAP. This possibility will be further investigated in Chapter 6.

Finally, hepatoprotection was evident at i) 200mg/kg/day for 10 days, ii) at 3 days of treatment of 500 mg/kg/day and iii) after 3 days of withdrawal. At all of these time points, there is an approximate 100% increase in palmitoyl CoA oxidase activity. If the peroxisome itself was involved in hepatoprotection, then this extent of increase in peroxisomes may be all that is required for hepatoprotection. It was noted that 2 g/kg/day DEHP, a classic weak PxP produced only a 20% increase in palmitoyl CoA oxidase activity (Chapter 2) yet provided hepatoprotection (Chapter 3). This also suggested the requirement for only a modest increase in peroxisomal number, while not negating other consequences of activation of the PPAR.

In conclusion, the onset study failed to dissociate the time and dose dependence of hepatoprotection and peroxisome proliferation. In the experiment investigating cessation of treatment there was a rapid decrease in peroxisomal number while protection was maintained. Protection could thus be the result of a PPAR initiated protein(s) with a degradation half life such that protection was still observed 3 days after cessation of treatment. Another possibility emerging from the present experiments is that GSH could be involved in the hepatoprotective response. If so, one would predict to see differences in the extent of formation of GSH-derived metabolites of AAP in clofibrate-pretreated mice to controls. To clarify the role of GSH-dependent and other biotransformation processes in the hepatoprotective effect, it is clear that the effects of clofibrate pretreatment on the metabolic fate of AAP need to be determined. In the next 2 chapters, the fate of AAP is considered further, with Chapter 5 investigating the role of bioactivation and the metabolic fate of NAPQI, while Chapter 6 concentrates on the role of detoxication by GSH in the hepatoprotective response.

# CHAPTER 5

## THE FATE OF PARACETAMOL IN MICE PRETREATED WITH CLOFIBRATE.

### 5.1 INTRODUCTION

The hepatoprotective effect of PxP against AAP toxicity does not appear to be directly associated with the occurrence of an increased number of peroxisomes (Chapter 4). The cellular modification(s) that provided protection were present for at least three days after cessation of PxP treatment, while the induction of activity of the marker enzyme for the peroxisome, palmitoyl CoA oxidase, had declined by 70% toward control levels. The nature of the protective mechanism is unknown but an obvious possibility is that the capacity of the liver cell to metabolise AAP is altered as a consequence of PxP exposure.

Pretreatment with PxPs is known to alter the activity of a number of xenobiotic metabolising enzymes (Ashby *et al,* 1994) and also alter the protein profile of hepatocytes due to effects on *de novo* protein synthesis (Anderson *et al,* 1996). Thus it is possible that PxP-induced changes in levels of one or more proteins involved in the metabolism, detoxication or activation of AAP may alter the cellular handling of the drug or its reactive metabolite, NAPQI. To address this possibility, the experiments described in this chapter used a combination of *in vivo* and *in vitro* approaches to assess the effects of clofibrate pretreatment on the fate of AAP within the hepatocyte.

The first experiment investigated the level of CYP450 bioactivation of AAP to the reactive metabolite NAPQI, to ascertain whether the ability to bioactivate AAP is compromised in the livers of clofibrate treated mice. Early AAP metabolism studies showed that induction of CYP450 by phenobarbitone enhanced, while inhibition by piperonyl butoxide or cimetidine reduced AAP toxicity (Mitchell *et al,* 1973a; Mitchell *et al*, 1984). Specific inhibition of CYP2E1, the major bioactivating isoform of CYP450, by pretreatment with compounds such as propylene glycol protects against AAP toxicity (Snawder *et al*, 1993; Thomsen *et al*, 1995). Treatment with clofibrate is known to increase the level of CYP450, specifically the CYP4 family of isoforms induced by PxPs, which will alter the overall CYP450 profile and may have ramifications for the extent of AAP bioactivation. The level of AAP bioactivation was determined by *in vitro* measurement of the formation of the AAP:GSH conjugate, which requires the intermediary step of NAPQI formation. This method can be readily manipulated for comparison of protein, time and concentration dependence of AAP:GSH formation by microsomes isolated from both control and clofibrate pretreated mice.

To attain *in vivo* data on the level of AAP:GSH conjugate formation, a second complementary experiment was undertaken measuring the level of the GSH derived cysteine and mercapturate metabolites in the urine of mice treated with AAP. The urinary metabolites of AAP are readily quantitated by HPLC and therefore simultaneous determination of the urinary metabolite profile for glucuronide and sulphate conjugates also provides information on the Phase II conjugation capability of the liver of control compared to the clofibrate pretreated mice. Both the glucuronide and sulphate metabolites do not require bioactivation and form the majority of urinary metabolites following AAP dose. An increase in hepatic glucuronidation has been postulated as a mechanism for AAP hepatoprotection (Schnell *et al*, 1988; Speck and Lauterburg, 1990; Madhu and Klassen, 1991; Liu *et al*, 1992), and due to PxP alteration of this pathway (Magdalou *et al,* 1993), may also contribute to the protective effect of clofibrate.

Both the microsomal and the urinary experiments enabled comparison of the extent of bioactivation of AAP in control versus clofibrate pretreated mice. The quantity of GSH derived metabolites in the urine provided some indication of the level of detoxication of NAPQI by GSH, as did determination of the localisation of AAP *in vivo*, following administration of radiolabelled AAP to mice. The measurement of the extent of binding within the hepatocyte is a reflection on the balance between bioactivation and detoxication mechanisms. Also, following administration of radiolabelled AAP, its systemic fate can be determined by measurement of radioactivity associated with various tissues. As treatment with PxP increases %LBW, the systemic distribution and metabolism of AAP may be modified. It is also possible to fractionate the liver into various subcellular components and thereby determine alterations in the extent of NAPQI binding within the hepatocyte. It is known that NAPQI targets specific proteins and while it is not possible to identify the actual target protein by this method, measurement of NAPQI binding to subcellular fractions provided information on whether protection is associated with a decrease in binding in a particular organelle.

The toxicity of AAP is associated with the binding of NAPQI to critical cellular targets in the cytosol, mitochondria or endoplasmic reticulum. In addition, the formation of 3-cysteinyl-AAP protein adducts, occurs in the centrilobular region prior to GSH depletion and necrosis (Roberts *et al*, 1991), seemingly indicating a causal relationship for protein binding in the development of hepatotoxicity. Only a few of the proteins have been characterised and their potential involvement in the hepatotoxicity of AAP is subject to speculation. It is possible that PxP-induced alteration of cellular protein profiles have modified the level of critical proteins involved in either the quenching of NAPQI or the subsequent manifestation of toxicity. It has been found that 85% of AAP protein binding is associated with two proteins, namely a 44 kDa and a 58 kDa protein (Beierschmitt *et al,* 1989). The 44 kDa protein has subsequently been identified as microsomal glutamine synthetase (Bulera *et al,* 1995). To determine

whether the extent of damage to this target is altered in the livers of clofibrate-pretreated mice the activity of glutamine synthetase was measured following a hepatotoxic dose of AAP.

As well as microsomal interactions, both *in vivo* and *in vitro* experiments have revealed that AAP toxicity is associated with a decrease in mitochondrial respiration and disruption of hepatocellular energy homeostatis (Esterline *et al,* 1989; Burcham, 1990; Burcham and Harman, 1991; Donnelly *et al,* 1994). These mitochondrial effects result from NAPQI interaction with specific sites in the respiratory chain (Burcham, 1990; Burcham and Harman, 1991; Donnelly *et al,* 1994) which raise the possibility that pretreatment with clofibrate decreases the level of AAP induced damage to the mitochondrial function. To investigate this, rates of oxygen utilisation in mitochondria isolated from AAP-intoxicated livers of control and clofibrate pretreated mice were determined in the presence of various respiratory chain substrates. The experiments measuring glutamine synthetase activity and mitochondrial respiration were designed to clarify the possible mechanisms involved in clofibrate-induced hepatoprotection. In all experiments described in this chapter, plasma SDH activity (or ALAT for the mitochondrial experiment) was used as confirmation of hepatotoxicity in the control group and protection in the clofibrate pretreated group.

It was anticipated that the results of the microsomal incubation, urinary metabolite analysis, radiolabel binding and target molecule activity studies would provide some indication as to the fate of AAP when it enters the hepatocyte and by comparison with control animals, provide information about to the hepatoprotection afforded by PxP pretreatment.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Materials

All chemicals used were of the highest quality commercially available. For details of chemicals and suppliers see Appendix 1.

### 5.2.2 Animal treatment regimen

For all experiments reported in this chapter, male Swiss White mice (35-40g) were obtained from the University of Adelaide Waite Animal House and allocated into 1 of 2 treatment groups: a) control group receiving equivolume olive oil vehicle at 0.1 ml/mouse/day for 10 days by i.p. injection or b) 500 mg/kg/day clofibrate in olive oil for 10 days. All mice were injected between 17.00 and 18.00 hours each day.

### 5.2.3 Microsomal study:Metabolism of AAP

*5.2.3.1 Microsome preparation and incubation*

A 10 % w/v liver homogenate was prepared in 0.1 M potassium phosphate buffer, pH 7.4, from the pooled livers of 6 mice. The homogenate was centrifuged at 12,000 x g for 20 minutes at 4° C then the post-mitochondrial supernatant was recentrifuged at 100,000 x g for 1 hour. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7.4 containing 20 % glycerol to form a 1g liver/ml microsomal suspension and stored at -80° C until use. The protein content of the microsomal fractions for both control and clofibrate fractions was determined by the method of Hartree (1972) and samples were diluted to 5 mg protein/ml prior to use.

In the study comparing the ability of control or clofibrate microsomes to metabolise AAP, a 125 µl volume of prewarmed 0.05 M potassium phosphate buffer, pH 7.0 was added to 50 µl microsomes (up to a final protein concentration of 1 mg/ml), 25 µl 2.5 mM GSH and 25 µl varying concentrations of AAP to give a final AAP concentration range from 0.1 to 2 mM. The concentration of the reactants and time of incubation varied according to the requirements of the experiment. The formation of the AAP:GSH conjugate was started by the addition of 25 µl of cofactor solution, containing 1 mM EDTA, 3 mM magnesium chloride, 1 mM ADP, 5 mM glucose-6-phosphate and 1 U/ml glucose 6-phosphate dehydrogenase. The reaction was stopped by the addition of 0.5 ml ice cold methanol, the mixture was vortexed and stored at -20° C (total volume 0.75 ml) until analysis.

*5.2.3.2 HPLC Analysis of the GSH:AAP conjugate*

The quantitation of GSH:AAP conjugate was based on the method of Madhu and Klassen, 1991. Samples were centrifuged at 3000 x g for 5 minutes to pellet any flocculant microsomal protein. A 20 µl volume of microsomal incubation was injected onto a $C_{18}$ spherisorb column following equilibration with a 1.5 % w/v glacial acetic acid: methanol (6:1) mobile phase at a flow rate of 1 ml/min. The GSH:AAP metabolite was detected at 254 nm via a Jasco UVI DEC-100-V detector and quantitated on a Shimadzu integrator. Retention times were found to be 4.8 to 5 minutes for AAP and 10.5 to 11.5 minutes for GSH:AAP conjugate, with no interfering peaks. The GSH:AAP conjugate was quantitated using the AAP standard curve and expressed as µmole GSH:AAP produced under the specified conditions of the assay.

### 5.2.4 Analysis of urinary AAP metabolite formation

*5.2.4.1 Sample collection*

In the urinary metabolite study, 2 different doses of AAP were investigated. A dose of 200mg/kg AAP (low dose, n=8) has been found to be well tolerated causing minimal toxicity in mice and thus was used to avoid the complication of hepatic blood engorgement and

necrotic cell death which could alter the metabolic profile. The second dose of 500mg/kg AAP (high dose, n=6) had been previously observed to cause extensive liver necrosis in control mice, while having no toxicity in clofibrate treated mice (Chapter 3). Urine sample blanks were obtained from mice injected with physiological saline alone and urine collected over 4 hours.

Control and clofibrate pretreated mice were injected with AAP in alkalinised saline by i.p. injection and placed on plastic funnels with wire grid base. After 4 hours, urine samples were collected either by lower abdominal palpation for the low dose or aspiration of the bladder for the high dose groups. Due to the expected high mortality in the control group given high dose AAP, a blood sample was also collected at this time for SDH analysis. The funnel was rinsed with 10 ml of 1:1 methanol:water and the volume of urine made up to 20 ml, centrifuged at 3000 x g for 10 minutes at 4°C to remove any debris and the supernatant stored. Blood samples for were collected 24 hours after low dose AAP administration in control and clofibrate treated mice to determine the total extent of hepatic damage. All samples were stored at -20°C until analysis was performed.

*5.2.4.2 HPLC analysis of urinary AAP metabolites*

The HPLC method for determining urinary metabolites of AAP was based on the method of Miners *et al*, (1984). A 0 to 1 mM AAP standard curve was prepared in using a 0.02M potassium phosphate buffer, pH 4.7: acetonitrile (97.5:2.5) mobile phase with minor variations in equipment used in the 200 mg/kg and the 500 mg/kg study (for details see Appendix 3).

### 5.2.5 Radiolabelled AAP experiment

*5.2.5.1 Preparation and administration of $^{14}$C-AAP.*

Radiolabelled AAP ($^{14}$C located at the C-1 position of the benzene ring) was repurified by ethylacetate extraction, freeze drying and suspension in physiological saline. Purity of AAP was confirmed by TLC and UV scan. Control and clofibrate pretreated mice (n=5), were injected with a 500 mg/kg dose of AAP, containing 6.7 x $10^6$ DPM of $^{14}$C-AAP. The animals were kept in a wirebottom cage for the next 4 hours then anaesthetised with 0.6% nembutal in preparation for tissue collection and radiolabel counting. The methods are based on those reported by Corcoran *et al,* (1985) and the instructions of the manufacturer of the scintillation products.

5.2.5.3 Tissue sampling, preparation and radioactivity counting.

*o Blood and extrahepatic tissue collection and analysis*

To determine the distribution of AAP in extrahepatic tissues, various tissues including blood, were collected. In general, tissues were solubilised in Soluene with a volume of 20% benzyl peroxide and also glacial acetic acid added prior to the addition of 10 ml Beckman organic scintillation fluid. The volumes and incubation times differed for the individual tissues being in accordance with specifications of the manufacturer of the scintillation products used. Samples were counted in a Beckman LS2 ß-scintillation counter.

A 1ml blood sample was taken by closed cardiac puncture. A 100 µl aliquot was prepared for counting and results are expressed in DPM/ml blood. To determine the level of AAP associated with the erythrocyte fraction, a 100 µl volume of whole blood was added to 0.9 ml of physiological saline, centrifuged at 2,000 x g for 5 minutes and the supernatant discarded. The erythrocyte pellet was washed twice with saline, then after the final wash, the cells were lysed with 100 µl of distilled water and counted following the solubilisation procedure. Results are expressed in DPM/ml blood. The plasma from the remaining blood was collected for SDH activity as outlined in Section 3.2.5.2.

A 100-150 mg slice was taken from the lung, kidney, spleen and brain of each mouse, weighed and lightly minced in 0.2 ml of distilled water then counted following specific solubilisation procedure. Results are expressed as DPM/g tissue.

*o Urine collection and analysis*

Since no urine collected in the bottom of any of the wire bottomed cages during the experiment, the abdominal cavity of the mouse was opened via a longitudinal incision and the entire urine contents removed from the bladder via aspiration. The volume was adjusted to 2 ml with saline, then the sample was vortexed and a 1 ml aliquot added to 10 ml of Beckman Readyvalue aqueous scintillation fluid for counting. Results are expressed as DPM per total 4 hour sample.

*o Preparation of liver homogenate and subcellular fractions*

Liver homogenates (25% w/v) were prepared in 0.1M potassium phosphate, pH 7.4 ("buffer"). To measure total AAP content in the homogenate, 0.5 ml aliquots were digested with 1 ml of soluene, then processed in accordance with the method for tissue slices outlined above. In addition, covalent binding to homogenate proteins was determined following trichloroacetic acid precipitation of homogenate proteins. A 1 ml aliquot of homogenate was precipitated by adding 3 ml of 0.6 M TCA. After centrifugation at 3,000 x g for 5 minutes, the resulting pellet was washed twice in TCA then twice in 80% ethanol. The pellet was then resuspended in 2 ml of 1.0 M sodium hydroxide and a 1ml aliquot counted using Readyvalue aqueous scintillation fluid.

The remainder of the liver homogenate was centrifuged at 3,000 x g for 5 minutes at 4° C. The supernatant was saved and the resulting pellet was resuspended and washed 4 times in buffer. The final 3,000 x g pellet was resuspended in 5 ml buffer then 0.5 ml was solubilised for counting. To prepare the mitochondrial fraction, the supernatant of the 3,000 x g spin was centrifuged at 15,000 x g for 10 minutes. The resulting supernatant was collected and the mitochondrial pellet was resuspended and washed via centrifugation 3 times in buffer. After the final wash, the remaining mitochondrial pellet was resuspended in 0.5 ml of buffer and solubilised prior to counting.

For preparation of the microsomal and cytosolic fractions, the remaining post 15,000 xg supernatant was centrifuged at 100,000 x g for 1 hour. The resulting microsomal pellet was washed once prior to resuspension in 1 ml of buffer and a 0.5 ml aliquot solubilised and counted. The total and protein-bound AAP content of the cytosol was determined in the same manner as for the homogenate fraction. Protein for the subcellular fractions were determined using the method of Hartree (1972). Protein content was determined from a standard curve prepared from bovine serum albumin standards.

### 5.2.6 Effect of AAP on mitochondrial oxidative phosphorylation.

For determination of the effect of clofibrate pretreatment on oxidative phosphorylation following AAP intoxication, control and clofibrate pretreated mice (n=5-8) were treated with either 500 mg/kg AAP or alkalinised saline vehicle. After 4 hours, liver and blood were collected following nembutal anaesthesia. Blood plasma was prepared and stored at -80°C until analysed for ALAT activity as described in Section 3.2.5.1 as a measure of hepatotoxicity. ALAT was chosen to determine the extent of hepatotoxicity due to its ability to maintain activity during extended (up to 2 weeks) sample storage.

#### *5.2.6.1 Mitochondrial preparation*

Following removal, livers were weighed and the gall bladders were carefully excised. Livers were then placed in 5 ml of icecold 5 mM Tris buffer, pH 7.4, containing 0.25M sucrose and volume was adjusted to make a 20% w/v solution. The individual livers were lightly minced and homogenised by 2 passes of a PE homogeniser. The homogenate was centrifuged at 1,000 x g at 4 °C for 10 minutes to pellet nuclei and cell debris. The supernatant was carefully collected and recentrifuged at 10,000 x g for 12 minutes. The mitochondrial pellet was resuspended in 5 ml of buffer, washed by centrifugation under the same conditions and resuspended in 1 ml of tris-sucrose buffer

#### *5.2.6.2 Mitochondrial analysis*

Determination of mitochondrial respiration was performed using an oxygen electrode with 3 different substrates. The electrode was enclosed in a water jacketed chamber of 2.7 ml capacity maintained at 30° C. A 2.5 ml volume of respiration buffer (containing 1 mM EDTA,

50 mM Tris acid, 100 mM potassium chloride, 5 mM magnesium chloride and 10 mM potassium dihydrogen orthophosphate) was placed in the chamber together with 50 µl of resuspended mitochondria. After 1 minute, 5 µl of 1 M ATP was added followed about 1 minute later by addition of substrate. Three different substrates were used to investigate respiration at each of the three energy coupling (EC) sites associated with the electron transport chain; for EC site 1, the Complex I substrate, 5 mM glutamate and 5 mM maleate; for EC site 2 the Complex II, 5 mM succinate and for EC site 3, the Complex IV substrate, 0.2 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) in 2 mM ascorbic acid. All substrate concentrations are expressed as final concentration in the electrode cell and were investigated separately. About 30 seconds after the administration of substrate, 5 µl of 1 M ADP was added (1 µl for TMPD experiments) and state 3 respiration measured. When all the ADP had been consumed, state 4b was measured for 15 seconds, then a further 5 µl of ADP added. Experiments using succinate as substrate included prior addition of 4 µg rotenone. Respiratory control ratios (RCR) were determined according to the following equation:

$$RCR = \frac{\text{rate of State 3 respiration}}{\text{rate of State 4b respiration}}$$

where State 3 respiration is the maximal respiration rate attained in the presence of both ADP and substrate, while State 4b is the low respiration rate associated with exhausted ADP levels that directly follows State 3.

### 5.2.7 Effect of AAP intoxication on microsomal enzyme activity

To further survey the effects of clofibrate pretreatment on *in vivo* AAP intoxication, the activity of the microsomal enzyme glutamine synthetase was investigated. Control and clofibrate pretreated mice (n=6) were administered either 500 mg/kg AAP or alkalinised saline vehicle 4 hours prior to liver collection. Liver homogenates (10% w/v) were prepared in 0.1 M tris hydrochloride buffer, pH 7.4. The glutamine synthetase assay was derived from the method of Meister (1985) which utilises the glutamine synthetase catalysed formation of L-γ-glutamyl hydroxamate and ammonia from glutamine and hydroxylamine. This reaction is accompanied by inorganic phosphate release from ATP which was measured by the method of Napapetian and Bassiri (1975). For this, a 25µl sample of post 1,000 x g supernatant was combined with 0.7 ml of prewarmed reagent mix containing 0.025 M mercaptoethanol, 0.02 M magnesium chloride, 0.125 M hydroxylamine hydrochloride, 0.05 M glutamate and 0.01 M ATP in 0.1 M imidazole buffer pH 7.2 with 75 µl buffer to make a total volume of 0.8 ml. Background levels of inorganic phosphate formation were determined by reaction blanks prepared without enzyme, glutamate and also ATP. The samples were incubated for 2 minutes in a 37° C heating block and stopped by 0.6 ml 1.2 M perchloric acid. The precipitated samples were centrifuged at 3,000 x g for 5 minutes and 1 ml of supernatant was combined with 0.75 ml 0.95 % ammonium molybdate in 0.75 M sulphuric acid and 0.1 ml

2 % ascorbic acid. After 20 minutes, absorbance was determined at 660 nm. Phosphate levels were quantitated by a standard curve (0-10 mM $PO_4^{-3}$) after glutamate free buffer correction. Protein levels in the homogenate were determined by the Pierce BCA protein in accordance to the instructions of the manufacturer. Results were expressed as mM $PO_4$ generated/min/mg protein.

### 5.2.8 Statistical Analysis

All results in the radiolabelled AAP experiments and the urinary metabolite analysis were analysed for differences between the control and clofibrate groups using unpaired Students t-tests ($P<0.05$). For all other experiments, data were analysed by ANOVA. When significance was observed, the Bonferroni post-hoc test was used for comparison of specific pairs of data.

## 5.3 RESULTS

### 5.3.1 Microsomal formation of GSH:AAP conjugate in vitro.

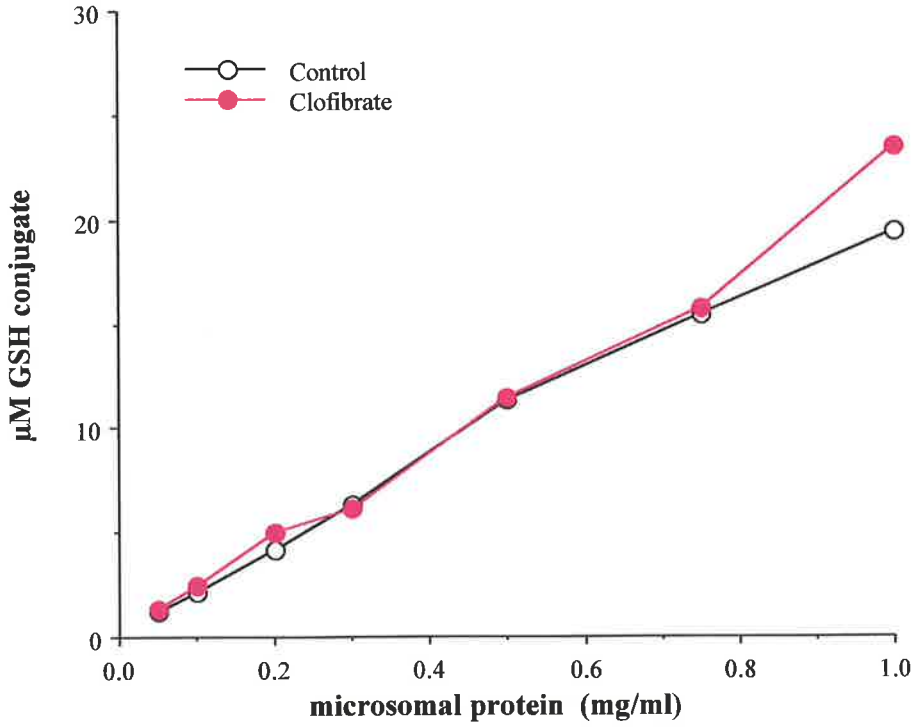



**Figure 5.1:** Formation of GSH:AAP conjugate at differing protein concentrations in the range 0.05 to 1.0 mg/ml microsomal protein. Microsomes were derived from either control (olive oil vehicle) or clofibrate treated (500mg/kg/day for 10days). Experiments were done in the presence of 1mM AAP and 0.25 mM GSH following incubation for 30 minutes at 37°C.

In this experiment, microsomes isolated from either control or clofibrate treated mice were incubated in a NADPH regenerating system and the formation of the AAP:GSH was measured. Due to the induction of endoplasmic reticulum that accompanies clofibrate pretreatment, microsomal protein was adjusted to compensate for each experiment as indicated.

There was no difference in the amount of AAP:GSH conjugate formation between control and clofibrate treated microsomes in relation to either microsomal protein concentration (Figure 5.1) or time of incubation (Figure 5.2).

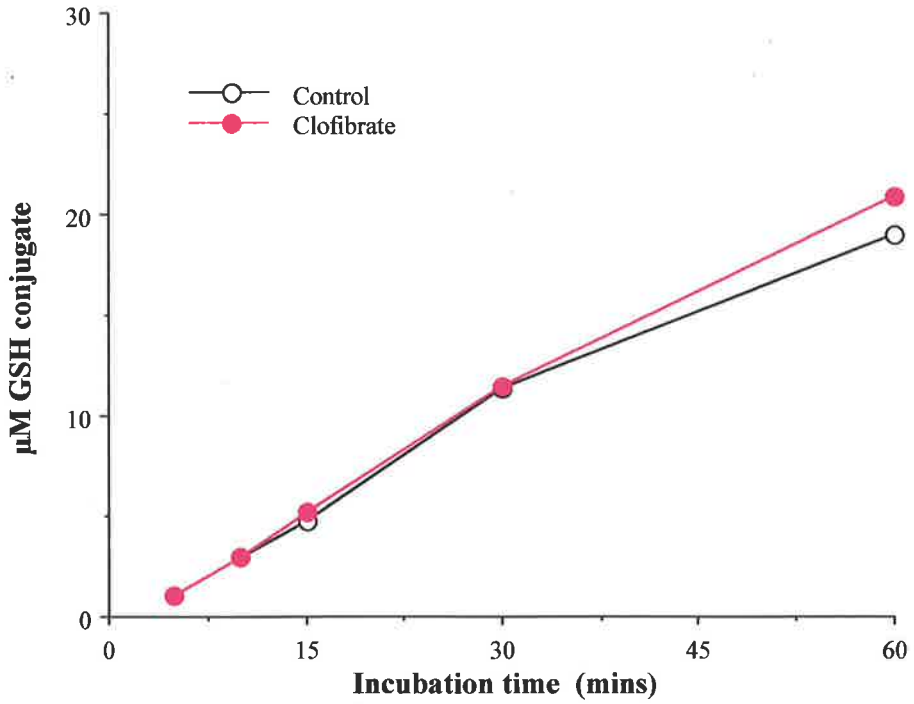



**Figure 5.2:** Microsomal formation of GSH:AAP conjugate using 1mM AAP and 0.25 mM GSH following incubation for various times from 5 up to 60 minutes at 37°C in the presence of 0.5 mg/ml microsomal protein.

In addition, there was no difference between groups in the amount of AAP:GSH formation at all concentrations of AAP from 10µM up to 2 mM (Figure 5.3). It is apparent that the microsomes derived from control and clofibrate treated mice were not different in terms of their respective abilities to form the AAP:GSH conjugate.

The experiments described here were done without the addition of a GSHt source in the incubation. The conjugation of GSH to NAPQI has been reported to be dependent on GSHt (Miners *et al*, 1984). The GSHt isoform which catalyses this reaction is the cytosolic GSHt π (Ketterer *et al,* 1988) and not microsomal GSHt. In a separate study, individual microsomal fractions were isolated from both control and clofibrate pretreated mice (n=6) and individual cytosols prepared. Similar incubations to those described (30 minute incubation at 37°C in the presence of 0.25 GSH and 1mM AAP) both with and without the addition of 100µl of cytosol revealed that 60% of AAP:GSH formation is nonenzymatic and that overall level of AAP:GSH formation in the control and clofibrate groups was equivalent.

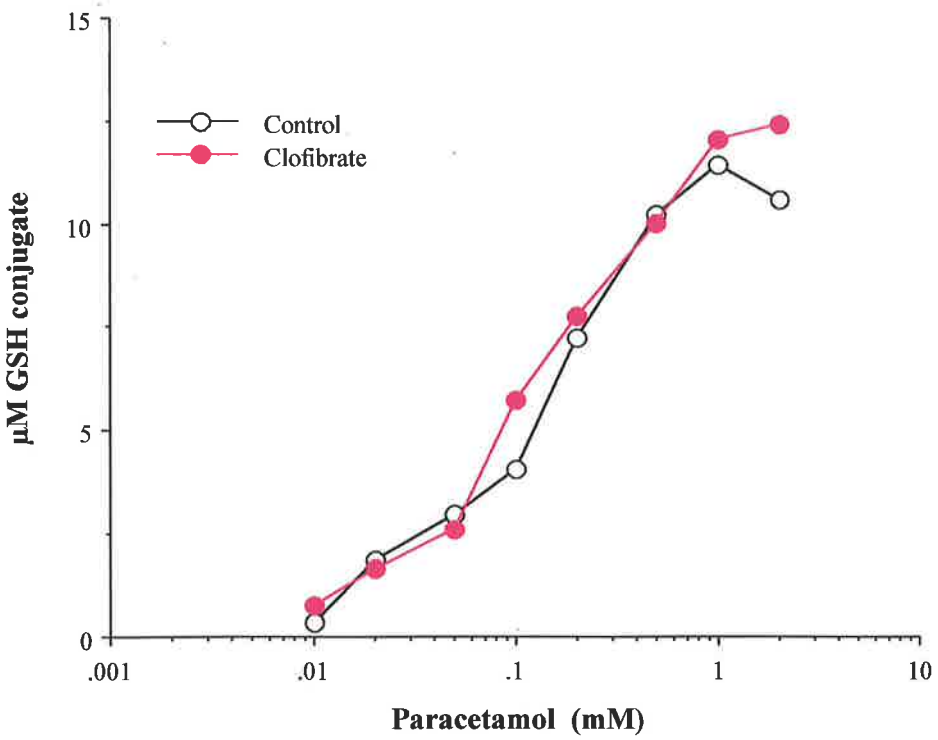



**Figure 5.3:** Formation of GSH:AAP conjugate using various concentrations of AAP (0.01 to 2 mM final concentration in incubation) and 0.25 mM GSH following incubation at 37°C for 30 minutes in the presence of 0.5 mg/ml microsomal protein.

### 5.3.2 Analysis of urinary metabolites of AAP following clofibrate treatment.

In the analysis of mouse urine following AAP administration, the conjugates of AAP can be determined as mg AAP equivalents from the AAP standard curve as the molar extinction coefficient for AAP conjugates are essentially the same as AAP (Howie *et al,* 1977). Urine samples were collected 4 hours after AAP administration. This time point was chosen due to the potential lethality of the 500mg/kg dose in control mice, from 4 hours onward. The major metabolite of AAP, in quantitative terms was found to be the glucuronide, followed by the sulphate and oxidative metabolites (Figures 5.4 & 5.5) The proportions of each metabolite was in agreement with previously reported urinary metabolite studies in the mouse (Gregus *et al*, 1988).

After administration of the 200 mg/kg (low) dose of AAP, there were no statistically significant differences between the control and the clofibrate pretreated groups in the amount of AAP metabolites (Figure 5.4) or unchanged AAP excreted. Analysis of plasma SDH levels revealed a raised but non significant increase in SDH in control animals of 28.9±11.7 IU compared to 9.8±0.9 IU in the clofibrate group. In contrast, the 500 mg/kg (high) dose mice showed a statistically significant decrease in the glucuronide, sulphate and oxidative metabolites in the control compared to clofibrate treated mice (Figure 5.5). There was no difference in the excretion of unchanged AAP.

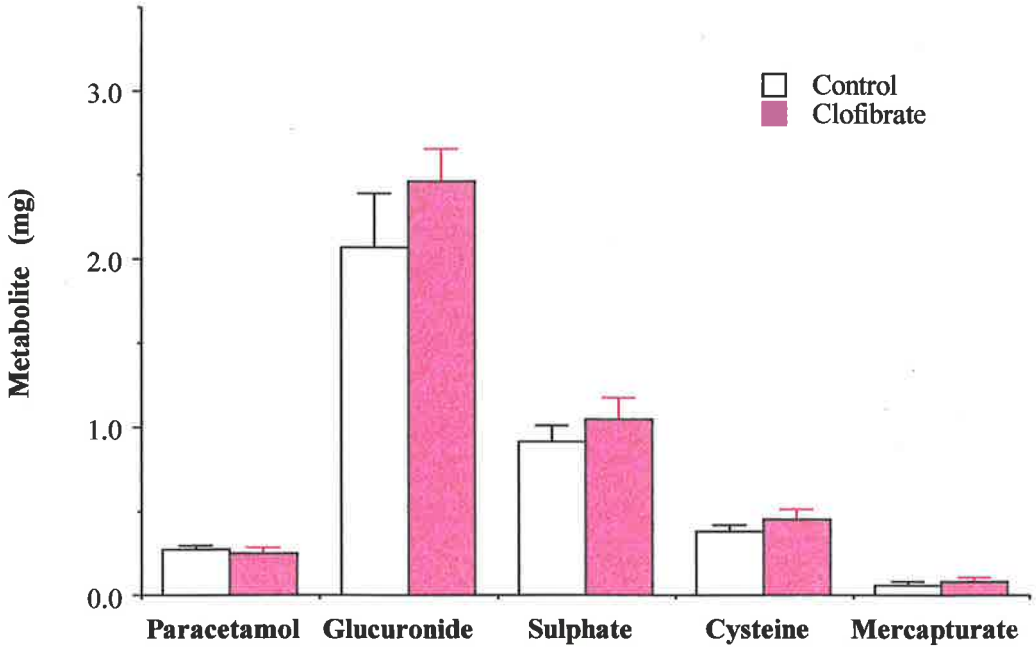



**Figure 5.4:** Measurement of urinary metabolites after a 200mg/kg i.p. dose of AAP following pretreatment with either clofibrate 500mg/kg/day for 10 days control. Results are expressed as AAP mg equivalents (no statistical significance between the groups; mean ± s.e.m, n=8).

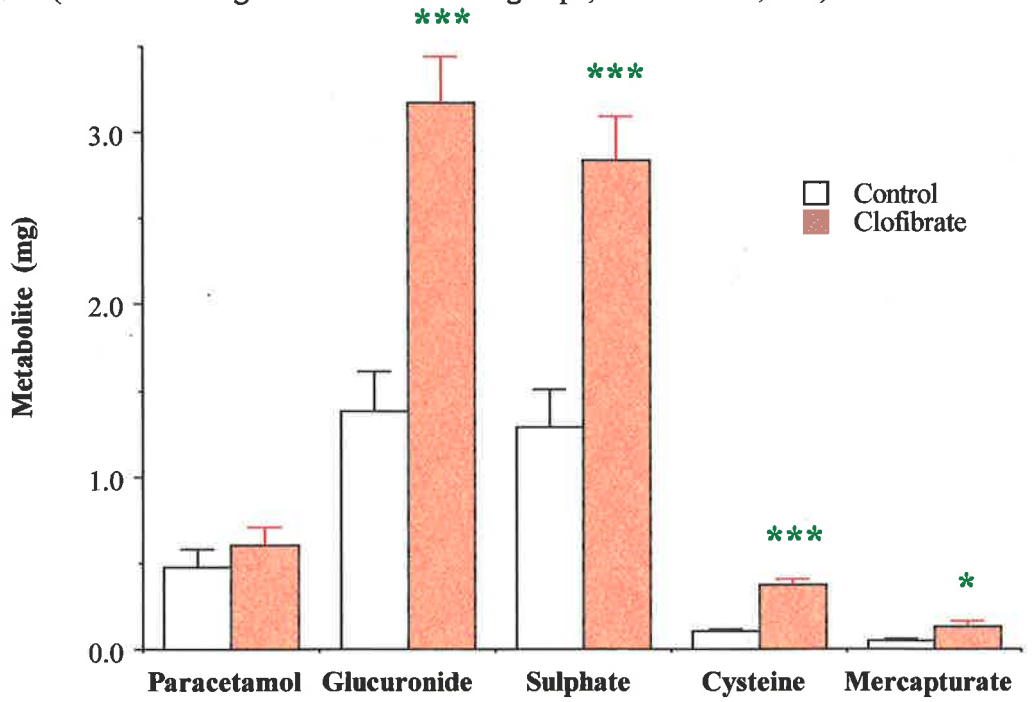



**Figure 5.5:** Measurement of urinary metabolites after a 500mg/kg i.p. dose of AAP following treatment with clofibrate 500mg/kg/day for 10 days or vehicle control. Results are expressed as AAP mg equivalents (*=P<0.05; ***=P<0.001; results expressed as mean ± s.e.m, n=5)..

Comparison of the clofibrate pretreated mice given the different doses of AAP showed that while a greater proportion of AAP was excreted as the sulphate metabolite in the 500mg/kg clofibrate group, there was little difference in the total amount of conjugate excreted in 4 hours between the low and high dose groups. This could indicate a saturation of enzymatic

conjugation. The control mice administered high dose AAP had lower amounts of urinary AAP metabolites than the control mice given the lower dose. On this basis, it appears that the decrease in AAP metabolites in the control mice following a 500mg/kg dose is due to a reduced capacity to form metabolites as a result of lethal intoxication by AAP.

### 5.3.3 Investigation of the systemic fate and cellular binding of AAP using radiolabelled $^{14}$C-AAP.

The systemic and subcellular fate of AAP was monitored following administration of 500 mg/kg AAP spiked with $^{14}$C-AAP. In control mice, 500 mg/kg AAP given 4 hours previously caused hepatotoxicity after 4 hours, as evidenced by both an increase in plasma SDH level (745 ± 453 IU) and the macroscopic appearance of the liver where intrahepatic vascular congestion was evident. No increase in plasma SDH levels was observed for clofibrate treated mice (15 ± 2.8 IU) nor was there any apparent alteration in morphology, confirming the hepatoprotective effect of clofibrate pretreatment observed in earlier experiments.

*5.3.3.1 Urinary excretion of total AAP following clofibrate pretreatment in the mouse.*

Urine from clofibrate treated mice contained over 70% more radioactivity than control mouse urine ($1.35 \times 10^6 \pm 0.27 \times 10^6$ DPM compared to $0.79 \times 10^6 \pm 0.10 \times 10^6$ DPM ($P<0.05$)), which is consistent with the difference in the amount of metabolites found in the urine of mice assayed in 5.3.2. The enhanced clearance observed in the clofibrate group could be the result of maintenance of cell viability. The plasma SDH levels indicate that 4 hours after AAP administration, substantial cell death has occurred in the control treated group which would reduce cellular metabolic activity and resultant metabolite formation.

*5.3.3.2 Effect of clofibrate treatment on the distribution of AAP to extrahepatic tissue.*

The distribution of AAP following a hepatotoxic dose to extrahepatic tissues following pretreatment with clofibrate was also investigated. An aliquot of blood and washed erythrocytes as well as small samples of kidney, brain, lung and spleen were dissolved in Soluene and resuspended in organic scintillation fluid. The amount of AAP binding was determined as either DPM/ml of blood or DPM/g tissue.

Analysis of whole blood revealed that despite the higher counts in the whole blood sample from control mice, there was no statistically significant difference in AAP binding in either the blood or the erythrocyte fraction in control compared to the clofibrate pretreated mice (Table 5.6). The major extrahepatic location for AAP toxicity is the kidney where intrarenal biotransformation of AAP contributes to renal necrosis (Hart *et al,* 1995). In contrast, the brain, lung and spleen are not commonly considered targets for AAP toxicity. Due to the

variation within groups, there were no statistically significant differences between tissues taken from either control or clofibrate pretreated mice (Table 5.6).

| | Control | Clofibrate |
|---|---|---|
| Whole blood<br>*DPM/ml* | 13268<br>±3806 | 9416<br>± 295 |
| Erythrocytes<br>*DPM/ml* | 6970<br>±520 | 7170<br>±230 |
| Kidney<br>*DPM/g* | 60405<br>±18207 | 35513<br>±11140 |
| Brain<br>*DPM/g* | 1507<br>±293 | 1124<br>±87 |
| Lung<br>*DPM/g* | 10333<br>±3511 | 7780<br>±1291 |
| Spleen<br>*DPM/g* | 11711<br>±1435 | 9848<br>±1388 |

**Table 5.6:** Measurement of AAP associated with blood and tissues following treatment with clofibrate 500 mg/kg/day for 10 days or equivolume olive oil vehicle control. Results are expressed as DPM/ml of blood (for whole blood and erythrocytes) or DPM/g tissue (Results expressed as mean ± s.e.m, n=5)

*5.3.3.3 Subcellular localisation of binding in the liver*

It was not possible to make a DPM/g wet weight tissue analysis of the liver, unlike other body organs, due to the pathological effects of AAP hepatotoxicity. AAP intoxication results in intrahepatic haemorrhage and vascular congestion with liver engorgement and organ weight gain as a result of acute protein and fluid accumulation (Corcoron *et al,* 1985) causing up to 1.2 fold increase in liver weight in the control treatment group. Coupled with hypertrophic responses to clofibrate pretreatment (as observed in Chapter 4), DPM/g wet weight liver provides an unreliable method of normalising data between individual animals. All results in the subcellular fractionation experiment are therefore expressed in terms of DPM/g protein content.

Homogenate samples prepared from clofibrate pretreated mice were found to have 44% less DPM/g than the control group (Table 5.7). Similar decreases were observed in the analysis of the mitochondrial (-33%), microsomal (-39%) and cytosolic (-48%) compartments. A reduction in the 3000 x g pellet containing nuclei, plasma membranes and cell debris was also observed in the clofibrate compared to the control group. However, considerable variation was seen in the control group due to gross blood contamination caused by early haemorrhagic damage that accompanies acute necrosis.

| | Control<br>*DPM/g protein* | Clofibrate<br>*DPM/g protein* |
|---|---|---|
| Homogenate | 99456<br>±16047 | 53386<br>±4567<br>* |
| 3000g pellet | 96698<br>±30852 | 33035<br>±3408 |
| Mitochondria | 87472<br>±11568 | 58593<br>±3661<br>* |
| Microsomes | 105834<br>±14610 | 64403<br>±3874<br>* |
| Cytosol | 88035<br>±13437 | 46287<br>±8348<br>* |

**Table 5.7:** Measurement of AAP binding in various subcellular fractions following treatment with clofibrate 500mg/kg/day for 10 days or equivolume olive oil vehicle control. Results are expressed as DPM/g protein (*=P<0.05; results expressed as mean ± s.e.m, n=5).

In addition to measurement of DPM associated with the subcellular fraction, the amount of protein binding in these fractions was also investigated. It was found that clofibrate pretreatment resulted in a 40% and 50% decrease in AAP binding to homogenate and cytosol protein, respectively (Table 5.8) being equivalent to the reduction observed as total binding. It was of interest to note that around 85% of the cytosol DPM was associated with the protein fraction, indicating the presence of a NAPQI binding protein(s) in the hepatic cytosol.

| | Control<br>*DPM/g protein* | Clofibrate<br>*DPM/g protein* |
|---|---|---|
| Homogenate | 52917<br>±5606 | 31612<br>±4425<br>* |
| Cytosol | 78072<br>±13516 | 38259<br>±6774<br>* |

**Table 5.8:** Measurement of AAP binding associated with the protein fraction after TCA precipitation of the homogenate and cytosol following treatment with clofibrate 500 mg/kg/day for 10 days or equivolume olive oil vehicle control. Results are expressed as DPM/g protein (*=P<0.05; results expressed as mean ± s.e.m, n=5)

Collectively, the results of the radiolabel experiments indicate a 60% increase in urinary clearance in the clofibrate treated mice. This was associated with a reduction in AAP counts

in the liver, with intracellular compartments recording a 30-50% decrease in binding. There was no difference in the extrahepatic distribution of AAP. It is apparent that the increase in urinary counts was offset by a decrease in hepatic binding in the clofibrate pretreated mice.

### 5.3.4 Investigation of mitochondrial effects of AAP.

The effects of *in vivo* AAP intoxication on mitochondrial respiration was investigated following either control or clofibrate pretreatment. The hepatotoxicity of AAP at a 500mg/kg dose after 4 hours of treatment was confirmed by an over 10-fold increase in plasma ALAT activity, and as expected on the basis of previous results, there was no difference between the clofibrate pretreated groups (Figure 5.9).

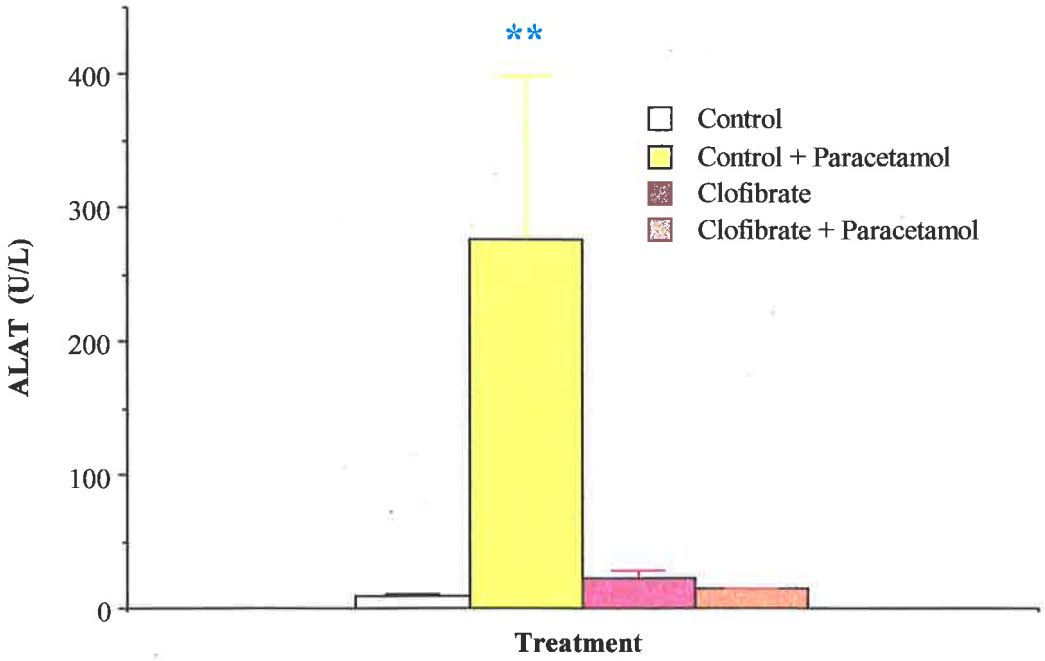
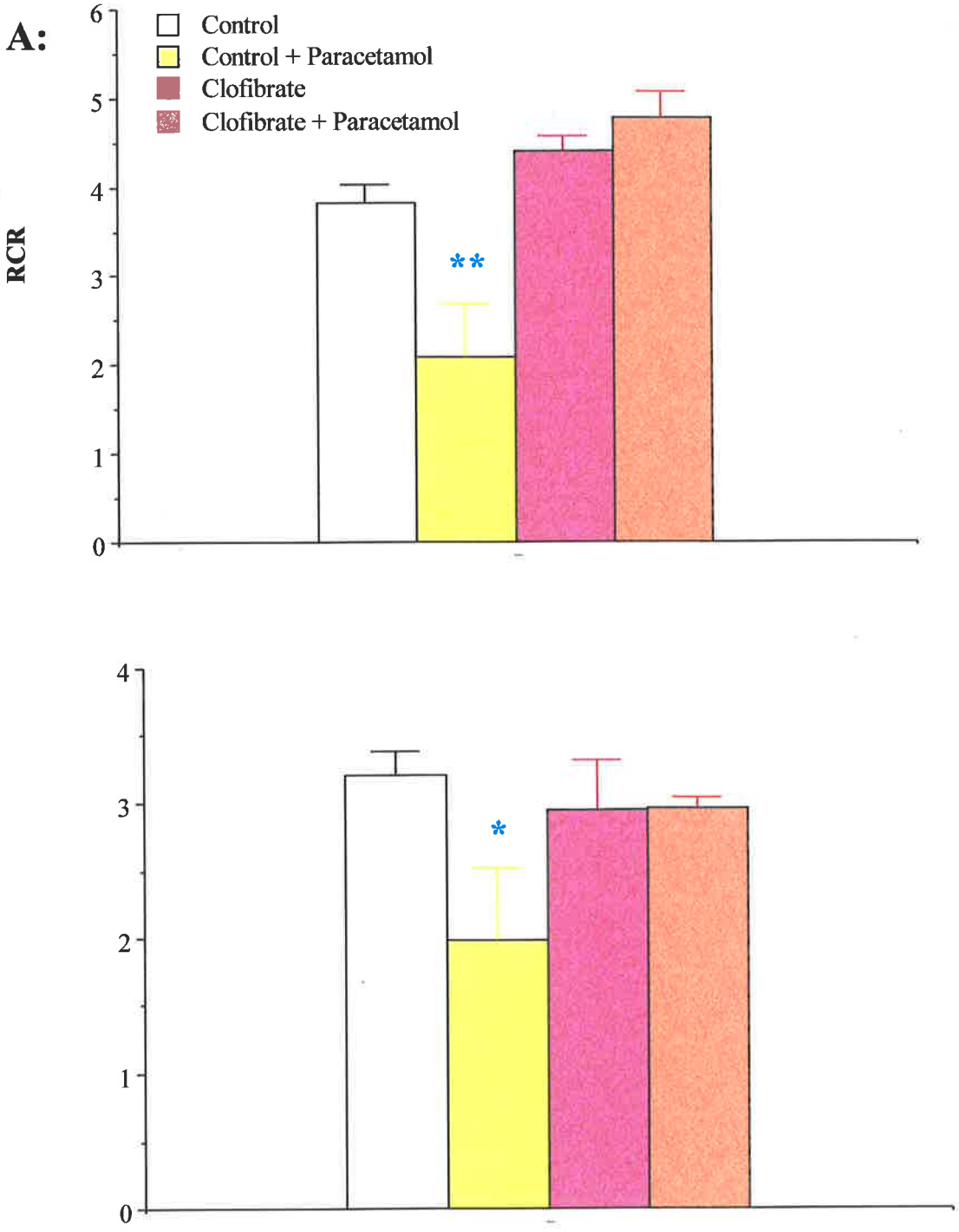



**Figure 5.9:** Level of plasma ALAT activity in mice 4 hours after 500mg/kg AAP challenge, following pretreatment with either clofibrate (500mg/kg/day for 10 days) or vehicle pretreated control. (Results are expressed as mean±s.e.m, n=5-8. **=P<0.01)

Three different substrates were used for each of the 3 sites associated with the electron transport chain; for EC site 1, equimolar glutamate and maleate, for EC site 2, succinate and for EC site 3, TMPD in ascorbate solution. Using the State 3 and State 4b rates, the RCR was calculated.

Similar site specific toxicity following AAP intoxication have been reported by others (Burcham and Harman, 1991; Donnelly *et al,* 1994). Mitochondria isolated from mice pretreated with clofibrate showed no alteration in RCR using any of the substrates in the presence of AAP intoxication. Pretreatment with the PxP clofibrate appears to have completely protected the mitochondria from AAP induced damage.



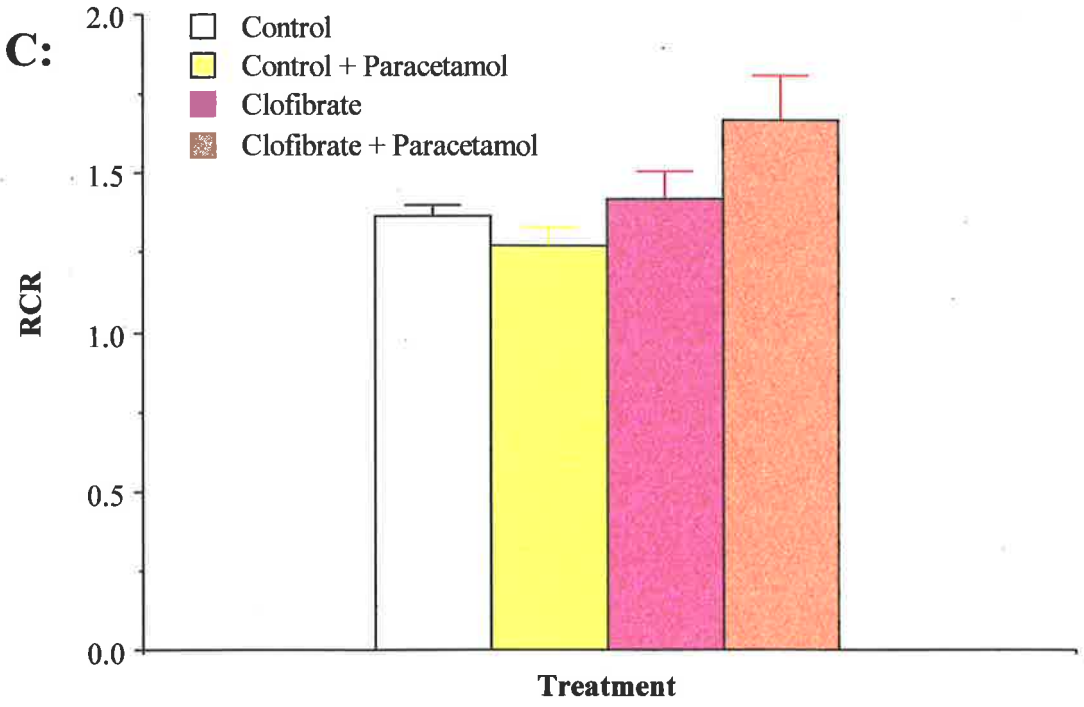



**Figure 5.10:** The effect of clofibrate pretreatment (500mg/kg/day for 10 days) on mitochondrial respiratory control ratios (RCR) in hepatic mitochondria 4 hours after 500mg/kg AAP challenge using substrates for three different complexes. **A:** Complex I. *substrate* glutamate:maleate **B:** Complex II *substrate* succinate **C:** Complex IV *substrate* TMPD. (Results expressed as mean±s.e.m, n=5-8. *=P<0.05, **=P<0.01).

### 5.3.5 Glutamine synthetase

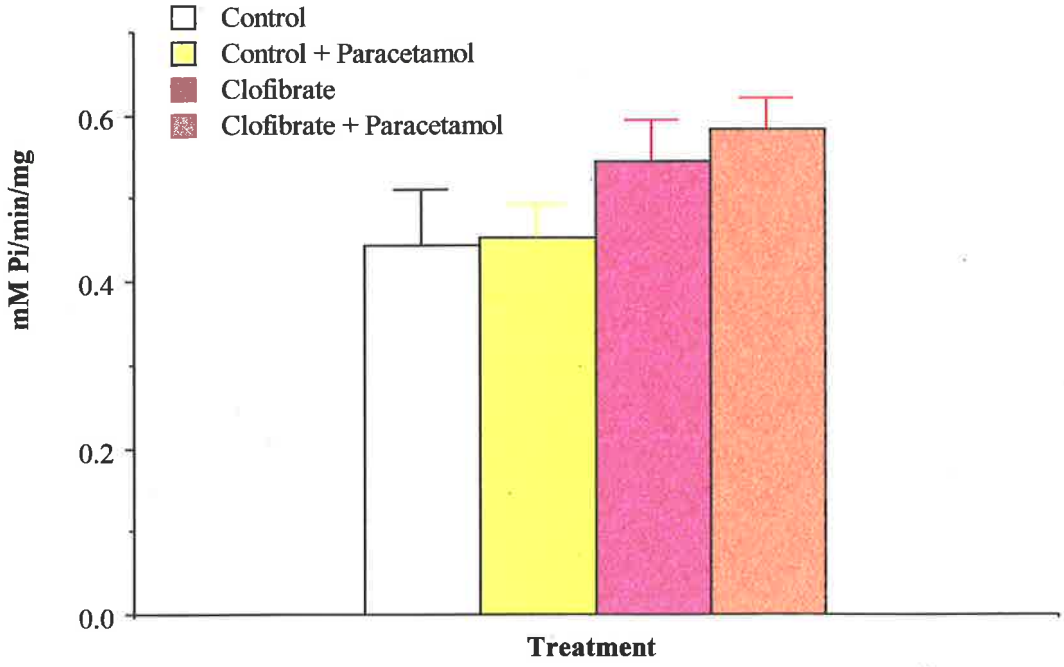



**Figure 5.11:** Level of homogenate glutamine synthetase activity in mice 4 hours after 500mg/kg AAP challenge, following pretreatment with either clofibrate (500mg/kg/day for 10 days) or control. (Results are expressed as mean±s.e.m, n=6).

Microsomal glutamine synthetase activity was determined by measuring the level of inorganic phosphate released during hydroxamate formation. Pretreatment with clofibrate led to a non-significant 23% increase in glutamine synthetase activity (Figure 5.11). In addition, administration of AAP had no effect on glutamine synthetase activity in either the control or clofibrate treated groups suggesting that binding of NAPQI to the enzyme does not interfere with its function. The lack of significant increase in the amount of glutamine synthetase indicates that hepatoprotection as a result of an increase in the 44 kDa NAPQI target is unlikely.

## 5.4 DISCUSSION

The aim of the experiments described in this chapter was to ascertain any differences in the *in vivo* fate of AAP following clofibrate pretreatment. Due to the number of biochemical effects resulting from pretreatment with PxP, the cellular fate of AAP could be influenced at a number of potential loci. The experiments in this chapter investigated bioactivation of AAP and some of the effects of NAPQI in the hepatocyte.

### 5.4.1 Investigation of the bioactivation of AAP by hepatic microsomes

The use of microsomal incubations for the *in vitro* analysis of metabolite formation for various xenobiotics, including AAP is well established (Lake *et al,* 1981; Madhu and Klassen, 1991; Jaw and Jeffery, 1993). Various aspects of AAP metabolism can be investigated using this method to provide further information on *in vivo* observations. Induction of CYP450 by phenobarbitone is known to increase the toxicity of AAP (Mitchell *et al,* 1973a) and similarly pretreatment with phenobarbitone prior to microsome isolation, increases AAP:GSH formation by 33% in a reconstituted microsomal system (Jaw and Jeffery, 1993). Coadministration of both *N*-acetyl cysteine and *l*-ascorbic acid have been found to decrease the hepatotoxicity of AAP and such protection is carried over to the *in vitro* situation with a decrease in hepatic binding observed in the presence of *N*-acetyl cysteine and *l*-ascorbic acid treated microsomes (Tredger *et al,* 1980; Lake *et al,* 1981). In the experiments outlined in this chapter the microsomal formation of the GSH:AAP metabolite exhibited time, microsomal protein and AAP dose dependence.

The microsomal activation of AAP to NAPQI is believed to be catalysed by CYP2E1 as the toxicity of AAP has been found to be modified in the presence of specific substrates of CYP2E1 including caffeine and ethanol (Tredger *et al,* 1986; Gale *et al,* 1987). There is also a high correlation between the covalent binding of AAP with the localisation of CYP2E1 in tissues exhibiting AAP toxicity (Hart *et al*, 1995). While 1A2 also catalyses NAPQI formation, it has a lower affinity for AAP than 2E1 and catalyses AAP bioactivation at high doses only (Rose *et al,* 1994). In addition to 2E1 and 1A2, CYP3A4 has been shown to have some activity toward AAP (Patten *et al*, 1993). Pretreatment with PxPs causes a characteristic

increase in the levels of CYP4A1, the CYP450 isoform associated with fatty acid metabolism. Clofibrate treatment was observed to have no effect on the activity of CYP450 isoforms 2A1, 1A1 or 3A4 while decreasing the activity of others (Kojo *et al,* 1996). The profile of effects on these CYP450 activities were not entirely shared by the PxP gemfibrozil, indicating a non-PxP related alteration, specific to clofibrate treatment itself. Furthermore, there were no major changes in the CYP450 isoforms known to be involved in AAP metabolism (Gonzalez, 1989; Kojo *et al,* 1996). The activity of 2E1 isoform is not altered by treatment with the PxP, DEHP (Austin *et al,* 1995). Also while treatment with ciprofibrate caused an increase in CYP2E1 mRNA and protein in the kidney, there was no alteration in the liver (Zanger *et al,* 1996). Also, with consideration of the CYP4 altered by PxP treatment, there are no identified xenobiotics metabolised by these isoforms (Parkinson, 1995).

The lack of alteration of the AAP metabolising isoforms by clofibrate pretreatment is reflected in the results where the microsomal formation of NAPQI and its subsequent conjugation with GSH, (both with or without the addition of GSHt source) was the same in both control and clofibrate treated mice. Consequently, a decrease in the CYP450-catalysed bioactivation of AAP cannot explain the observed protective effect of clofibrate pretreatment .

### 5.4.2 Urinary metabolites of AAP

An enhanced conjugation of AAP to glucuronide or sulphate, or of the reactive intermediate NAPQI to GSH or finally conversion of NAPQI back to AAP could, potentially explain the protection against AAP toxicity. For example, an increase in glucuronidation and sulphation of AAP has been implicated in the strain dependent resistance of the diabetic rat model to AAP toxicity (Price and Jollow, 1986). Thus analysis of the profile of urinary AAP metabolites following *in vivo* exposure can indicate whether alterations have occurred in any of these parameters following clofibrate pretreatment.

#### *5.4.2.1 Glucuronide and sulphate metabolites*

The conjugation of both the glucuronide and sulphate metabolites are dependent on the activity of their respective conjugation enzymes and the availability of the substrate. In mice, the glucuronide conjugate is the major metabolite of AAP. Alteration in glucuronide formation or the availability of the conjugate have been implicated in various pretreatments resulting in hepatoprotection including sodium selenite, fish oil diet and fulvomentisides, where a 25 % increase in glucuronide formation was observed (Schnell *et al,* 1988; Speck and Lauterburg, 1991; Liu *et al,* 1992). However, butylated hydroxyanisole increased the rate of glucuronide formation 7 fold due to an increase in UDPGt activity and hepatic UDPG-acid content with only a marginal protection toward AAP toxicity in mice (Hazelton *et al,* 1986).

While an increase in urinary sulphate metabolite excretion was observed following protective dithiothreitol treatment, this was not considered the sole mechanism of hepatoprotection

(Harman, 1985). Similarly, *D*-N-acetyl cysteine increased excretion of AAP-$SO_4$ yet provided no protection against hepatotoxicity, while its regioisomer, *L*-N-acetyl cysteine, resulted in no change in AAP-$SO_4$ yet provided protection against AAP toxicity (Wong *et al,* 1986). Thus an increase in sulphation seems less likely than an increase in glucuronidation in providing protection against AAP toxicity.

There was no increase in the extent of glucuronidation or sulphation in the clofibrate pretreated compared to control group administered a 200 mg/kg (low) dose of AAP (Figure 5.4). However, an increase in both conjugates was observed in clofibrate treated mice given 500 mg/kg (high) dose AAP when compared to the respective control group where a 50% reduction in both conjugates occurred (Figure 5.5). The control group exhibited considerable hepatotoxicity indicating a decreased capacity for the intoxicated hepatocytes to undertake AAP metabolism.

*5.4.2.2 Glutathione-derived metabolites*

Unlike the glucuronide and sulphate conjugates, GSH derived metabolites require AAP bioactivation. Mice excrete 7% of the total dose as oxidised metabolites (combined cysteine and mercapturate) in 2 hours compared to less than 1% in the rat (Gregus *et al*, 1988), reflecting the higher CYP450 bioactivation of AAP in mice. The pathway for the GSH conjugate involves excretion into the bile, resorption, and finally disposition in urine as cysteine and mercapturate metabolites. In a study following the fate of GSH:AAP conjugate in mice, 3 hours after a dose of AAP, 13.9% of the total GSH:AAP was in the bile while 41.2% was in the urine (Wong *et al,* 1983). In the present study, urine was collected after 4 hours which gives both an indication of the metabolic ratio of metabolites and also allows collection of samples from fatally intoxicated mice, which usually succumb between 4 and 8 hours after AAP administration.

Conjugation to the sulphydryl GSH provides a safe excretion pathway ultimately as either cysteine or N-acetyl cysteine metabolites or 2 thiols can reduce NAPQI back to AAP. Modification of either CYP450 catalysed bioactivation of AAP or levels of GSH will alter the amount of GSH derived conjugates excreted. Ethanol pretreatment, which induces CYP2E1, caused a 52% increase in mercapturate following a 400 mg/kg dose of AAP (Tredger *et al,* 1986). With reference to GSH, an increase in GSH synthesis, induced for example by prednisolone, will not only provide protection against AAP toxicity, but result in increased oxidised urinary metabolites (Speck *et al,* 1993). Similarly, provision of another sulphydryl, eg N-acetyl cysteine, cysteine or methionine can also provide protection against AAP toxicity (Miners *et al,* 1984; Corcoran and Wong, 1986). The lack of alteration in GSH metabolites at low dose AAP is supportive of there being no difference in the *in vivo* bioactivation of AAP following clofibrate treatment.

Clofibrate pretreatment has been reported to increase biliary GSH:AAP conjugate content at 2 hours after administration of 800 mg/kg AAP but not at subsequent time points (Manautou *et al,* 1996). In this study, at a low non-toxic dose no alteration in urinary GSH derived conjugates were observed. At the higher dose of 500 mg/kg AAP, a decrease in GSH:AAP formation would be expected in the nonprotected control livers due to the substantial cell toxicity and death that has occurred. It is possible that the clofibrate induced increase in GSH:AAP conjugate observed by Manautou and colleagues, was purely artefactual due to the lower metabolic capacity of the lethally intoxicated control livers.

*5.4.2.3 Unchanged parent compound*

The final component of the urinary profile is unchanged AAP. It is possible that the urinary concentration of AAP would be influenced by the conversion of NAPQI back to AAP. Compounds that can initiate this conversion include GSH and ascorbic acid which reduce NAPQI back to AAP, in a reaction catalysed by NADPH CYP450 reductase (Dahlin *et al,* 1984) without any effects on hepatic GSH levels (Peterson and Knodell, 1984). An example of a xenobiotic that protects against AAP by this mechanism is the thiol dependent antioxidant ebselen. The profound protective effect afforded by ebselen involves the oxidation of its metabolite ebselen selenol to ebselen diselenol, with concurrent conversion of NAPQI back to AAP (Li *et al,* 1994). In addition, AAP has antioxidant capability (Halliwell and Gutheridge, 1989) and has been reported to inhibit the lipid peroxidation that accompanies high dose AAP administration (Garrido *et al,* 1991). This raises the possibility that if lipid peroxidation is important in AAP toxicity, the presence of high intracellular AAP concentrations, the drug may protect against its own toxicity. However, no alteration in the levels of unchanged AAP was observed following clofibrate treatment.

Overall, the results of the urinary study indicate that there is no difference in the urinary metabolite profile between the control and the clofibrate groups at a dose (200 mg/kg) which causes no or minimal hepatotoxicity. This suggests that neither an increase in glucuronidation or sulphation nor conversion of NAPQI back to AAP can adequately explain the hepatoprotective effect. The level of bioactivation of AAP to NAPQI appears equivalent due to the lack of alteration in GSH derived metabolites. The hepatotoxicity evident in the high dose AAP control group possibly explains the overall decrease in AAP metabolites observed in this group.

### 5.4.3 The fate of radiolabelled $^{14}$C-AAP

The reduction in AAP and its metabolites in the urine of control mice administered a high dose (500 mg/kg) of AAP was confirmed in the radiolabel experiment, with a reduced number

of AAP counts was found in the urine. The decrease in urinary AAP counts was shown to coincide with higher liver counts when compared to clofibrate treated animals.

There were no statistically significant differences between extrahepatic tissues taken from the control and the clofibrate treated groups. While high counts were obtained in the kidney, comparatively low counts were found in the brain, an organ not prone to AAP toxicity. The kidney is able to bioactivate AAP to NAPQI by both CYP450 and PGHS activities resulting in renal necrosis during AAP intoxication (Moldeus *et al*, 1982) which may involve macromolecular binding (Hart *et al,* 1995; Manautou *et al,* 1995). Although there was no significant difference between the groups possibly due to the presence of considerable within group variation, there was a 41% decrease in radiolabel in the kidneys of the clofibrate group compared to the control.

In the blood, AAP is irreversibly bound to haemoglobin, which occurs as a result of bioactivation of AAP in the liver (Axworthy *et al,* 1988). The lack of difference in erythrocyte binding between the control and clofibrate groups indicates equivalent bioactivation of AAP to NAPQI, an observation in support of both the microsomal and urinary data previously discussed in the chapter.

There was high levels of binding in the subcellular fractions in control mice, including the microsomes, mitochondria and the 3000 *xg* pellet containing plasma membranes. These results are in agreement with previous studies (Corcoran *et al,* 1985; Pumford *et al,* 1990). NAPQI forms 3-(cystein-S-yl) AAP adducts with protein of which only a few have been positively identified including cytosolic *N*-10-formyl tetrahydrofolate dehydrogenase, nuclear lamin-A, microsomal glutamine synthetase and mitochondrial glutamate dehydrogenase synthetase (Hong *et al,* 1993; Bulera *et al,* 1995; Halmes *et al,* 1996; Pumford *et al,* 1997). The involvement or role of any of these proteins in the manifestation of hepatotoxicity of AAP is as yet unknown.

### 5.4.4 Effects of AAP intoxication on cellular targets

In this study, the effects of intoxication with 500 mg/kg AAP was investigated in 2 subcellular targets, microsomal glutamine synthetase and the mitochondrial respiratory chain.

#### *5.4.4.1 Mitochondria*

Interaction with the mitochondria appear to be an early event in AAP toxicity. While a decrease in mitochondrial respiration was reported after 1 and 1.5 hours, an increase in plasma ALAT was not observed until 4 hours following AAP administration indicating that mitochondrial damage precedes overt hepatic necrosis (Donnelly *et al,* 1994). Both *in vivo* and *in vitro* studies have revealed that administration of AAP interferes with oxidative

phosphorylation following site specific interaction of NAPQI, observed as a decrease in RCR values (Burcham and Harman, 1991; Donnelly *et al,* 1994). As a decrease in RCR indicates an impairment of mitochondrial oxidative phosphorylation, these observations explain the 50% decrease in hepatic ATP content prior to parenchymal cell damage following a 500mg/kg dose of AAP (Jaeschke, 1990) presumably enhancing the onset of hepatotoxicity.

In this study, a 500mg/kg dose of AAP administered to control mice reduced the RCR of EC Site 1 (glutamate:maleate supported) and EC Site 2 (succinate supported) by 45 and 40% respectively with no alteration in RCR at EC Site 3 (TMPD supported) site. These results are in agreement with those of Burcham (1990), where NAPQI derived site specific damage resulted in mitochondrial dysfunction. Pretreatment with clofibrate appears to have entirely protected the mitochondria from such damage with no alteration in RCR observed using any of these substrates.

This lack of toxicity could be due to deactivation of NAPQI before it reaches the mitochondria or to modification of mitochondrial functions providing resistance against NAPQI. Pretreatment with peroxisome proliferators is known to alter the cellular mitochondrial population. Clofibrate has been reported to cause a 40% increase in the number of mitochondria which because they are smaller in size than mitochondria isolated from control mice does not result in any change in mitochondrial volume (Lundgren *et al,* 1990). The amount of NAPQI interacting with the mitochondria was shown in the present radiolabel study to be decreased by 33% in clofibrate treated mice. It would appear that this decrease protected against the attainment of a particular level of binding which results in dysfunction. In untreated mice, the almost 3-fold lower doses of 175 mg/kg and also 350 mg/kg AAP significantly decreased mitochondrial RCR following *in vivo* AAP exposure (Burcham, 1990). Clofibrate treatment has protected mice against mitochondrial dysfunction, although whether clofibrate treatment has provided some form of resistance to the events following NAPQI binding in the mitochondrial pool could not be ascertained from the results of this chapter.

*5.4.4.2. Microsomal glutamine synthetase.*

The effect of AAP intoxication on the activity of glutamine synthetase, recently identified as one of the key NAPQI binding proteins (Bulera *et al,* 1995), was investigated. Glutamine synthetase is a dodecamer enzyme being composed of 12 identical 44 kDa subunits. The subunits have been identified as the 44 kDa target, which together with a cytosolic 58 kDa selenoprotein, account for 85% of the protein binding by AAP following *in vivo* administration (Beierschmitt *et al,* 1989). However, glutamine synthetase is only a target for NAPQI following *in vivo* and not *in vitro* exposure to AAP (Bartelone *et al,* 1989). Recent studies have revealed that the mRNA for glutamine synthetase is expressed only in cells in the

immediate vicinity of the terminal hepatic venule (Chen *et al,* 1994) and thus glutamine synthetase appears to be a NAPQI target solely due to its location close to the site of bioactivation in the centrilobular region. Covalent binding in the centrilobular region is present only 30 minutes after AAP administration in cells most proximal to the centrilobular vein and therefore NAPQI binding to glutamine synthetase is an early event in AAP hepatotoxicity (Bartelone *et al,* 1989). In this study, no difference in glutamine synthetase activity was observed as a result of either clofibrate pretreatment or AAP toxicity. Not only does it appear that NAPQI does not bind to the active site of the enzyme, but also indicates that hepatoprotection cannot be explained on the basis of an increase in this target acting as a protein sink for NAPQI.

The other major NAPQI target is a 58 kDa selenoprotein found in the cytosol (Bartolone *et al,* 1992). Pretreatment with clofibrate has been shown to decrease the binding of NAPQI to the 58 kDa protein, which was not associated with decreases in the amount of this protein in the cell (Manautou *et al,* 1994). This observation is in agreement with the findings of the present radiolabel study, where a 48% decrease in AAP associated binding to cytosolic protein was observed in clofibrate treated mice, and these findings strongly suggest that NAPQI is deactivated before reaching critical intracellular targets. Although the role of neither the 44 kDa nor the 58 kDa proteins in the manifestation of AAP hepatotoxicity is known, it appears unlikely that either play a role in clofibrate induced hepatoprotection.

### 5.4.5 Conclusion

The results reported in this chapter have investigated the fate of AAP in the body to clarify the role of biotransformation in the hepatoprotection accompanying pretreatment with the PxP, clofibrate. It has been previously established that clofibrate protects against AAP toxicity and this could potentially involve alterations to the bioactivation, detoxication or toxication responses following AAP exposure.

There was no difference in the comparative level of bioactivation of AAP to NAPQI in microsomes isolated from the livers of either control or clofibrate pretreated mice. In support of this observation, there was no alteration of GSH derived urinary metabolites, which are products of oxidative metabolism of AAP. In addition, there was no alteration in the other conjugated metabolites indicating that a potential increase in glucuronidation or sulphation was not a component of hepatoprotection. Use of radiolabelled AAP indicated enhanced urinary excretion of AAP in clofibrate treated mice following administration of a AAP dose that was found to be hepatotoxic in control mice. In contrast, control mice had high hepatic and lower urinary counts suggesting a decreased ability to excrete AAP due to a loss of cell viability. In clofibrate pretreated livers, regardless of the mechanism of hepatoprotection, biotransformation can proceed without the complication of cell death which should be given

due consideration when considering the role of metabolism in the protective effect of clofibrate.

Protection against the mitochondrial dysfunction accompanying AAP intoxication was observed following pretreatment with clofibrate. Although it is possible that the newly synthesised mitochondria which have resulted from clofibrate exposure are in some way resistant to NAPQI toxicity, the logical explanation appears to be that NAPQI is deactivated as it traverses from the site of activation to the mitochondrial as well as other cellular targets. This postulated hepatoprotective effect would be similar to that of the hepatoprotectant, ascorbyl palmitate, which decreased covalent binding and prevented AAP mortality by an apparent destruction of NAPQI and provided a sparing effect to hepatic GSH (Jonker *et al,* 1988). Many cytoprotective thiols also work via this mechanism, however, the obvious first choice for the deactivation of NAPQI is GSH.

GSH is the most abundant cellular sulphydryl and the toxicity of AAP can be dramatically altered by modification of cellular GSH levels (Mitchell *et al,* 1973b). GSH mostly protects against AAP hepatotoxicity by direct reaction with NAPQI to form an AAP:GSH adduct. The present findings tend to rule this out as a mechanism of clofibrate hepatoprotection since there was no increase in GSH-derived metabolites in the urine of clofibrate-pretreated, compared to vehicle-pretreated mice. However, GSH could be influencing the development of hepatotoxicity in other ways, such as by reducing NAPQI back to AAP, by regenerating antioxidants or by protecting against reactive oxygen species. In the light of these possibilities, it is clear that the effect of clofibrate treatment on hepatocellular GSH homeostasis needs to be analysed in order to better ascertain the role of GSH in clofibrate hepatoprotection. This goal forms the basis of experiments described in Chapter 6.

# CHAPTER 6

## INVOLVEMENT OF GLUTATHIONE IN THE PROTECTIVE EFFECT OF CLOFIBRATE TOWARD THE ACUTE HEPATOTOXICITY OF PARACETAMOL.

### 6.1 INTRODUCTION

In the previous chapter, it was found that while there was no difference in the CYP450 catalysed bioactivation of AAP to NAPQI, there was a reduction in the level of NAPQI reaching target molecules in clofibrate compared to control pretreated mice. This indicates that NAPQI is deactivated prior to target molecule interaction and this deactivation appears likely to be involved in the hepatoprotective effect of clofibrate. The most likely candidate for this deactivating role is the ubiquitous tripeptide, glutathione (GSH), which is known to conjugate to NAPQI and thus protect the cell from injury. This is illustrated with treatments such as N-acetylcysteine that conserve the GSH pool and protect against AAP toxicity while those which decrease the store of cellular GSH, such as diethylmaleate (DEM) potentiate AAP hepatotoxicity (Mitchell *et al,* 1973b).

GSH is found in most cells and has been proposed to have evolved as a molecule to protect eukaryotic cells against the toxicity of aerobic metabolism (Fahey and Sundquist, 1991). It is the most abundant of the intracellular sulphydryls and has a number of roles vital to cell survival including maintaining the reducing milieu of the cell, preservation of thiol groups of intracellular proteins and also maintenance of endogenous antioxidants, such as ascorbate (Martensson and Meister, 1991) and α-tocopherol (Niki *et al,* 1982). GSH plays a critical role in the detoxication of electrophiles generated during xenobiotic metabolism in conjugation reactions catalysed by glutathione S-transferases (GSHt) (Habig *et al,* 1974) and reduction of reactive oxygen intermediates, including $H_2O_2$ and lipid peroxides. This latter reaction is catalysed by glutathione peroxidase (GSHPx) which produces as a byproduct oxidised glutathione (GSSG) which is subsequently reduced back to GSH by glutathione reductase (Gred). The balance between the level and activity of GSHPx and Gred is essential to the maintenance of high intracellular concentrations of GSH (Figure 6.1).

As cells do not absorb GSH, cellular GSH must be synthesised within the cell (Hahn and Oberrauch, 1978). GSH synthesis involves the action of 2 enzymes, γ-glutamyl cysteine synthetase, which regulates its own synthesis by feedback inhibition and also glutathione synthetase (Richman and Meister, 1975). The three constituent amino acids; glutamate, glycine and cysteine, are all nonessential amino acids that can be synthesised in the body or obtained from the diet. While the availability of key amino acids is important for the synthesis

of GSH, cysteine appears to be the most important due to its provision of the sulphydryl group through which GSH gains its reducing and conjugating actions (Thor *et al,* 1979). Cysteine is also used to maintain the cellular pool of PAPS, the sulphate conjugate used in sulphotransferase reactions which like GSH is required in protection against the toxicity of AAP (Figure 6.1).

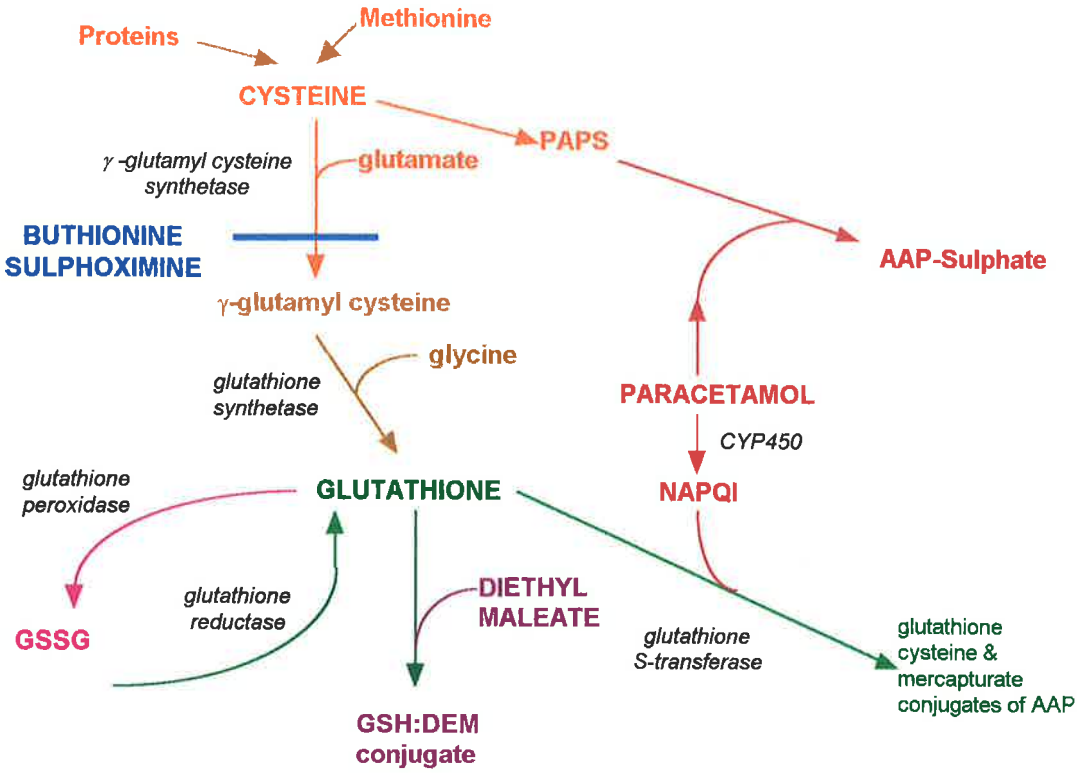



**Figure 6.1:** Generalized diagram showing the synthesis of GSH from 3 constituent amino acids and the interaction points of paracetamol, diethylmaleate and buthionine sulphoximime as depleting agents of hepatic GSH.

In order for the cell to function efficiently, the amount of cellular GSH must be maintained in the millimolar range (Meister, 1991). Cellular GSH is in a dynamic state. It must address losses resulting from efflux, catabolism or conjugate formation, maintain distinct intracellular pools and also deal with extracellular influences including diurnal rhythms, intralobular variations and physiological condition of the animal eg, starvation (Tateishi *et al,* 1974; Isaacs and Binkley, 1977; Smith *et al*, 1979). In order to maintain an adequate intracellular concentration of GSH, cells rapidly increase the rate of GSH synthesis. Without a high concentration of GSH, the cell has a decreased ability to respond to endogenous and exogenous stresses.

The overall aim of this chapter is to investigate the role of GSH in the hepatoprotective effect of clofibrate on the acute toxicity of AAP. To investigate this aim, three series of experiments were performed following pretreatment with the PxP clofibrate. These were:

1) determination of the effect of clofibrate treatment on the amount of GSH in the liver and the extent of GSH depletion following administration of AAP

2) investigation of the activity of GSH dependent enzymes known to be involved in GSH turnover (GSHPx and Gred) and conjugation to xenobiotics (GSHt).

3) following comparison of the ability of two GSH depleting agents to lower cellular GSH, determine of the effect of prior GSH depletion on the hepatic toxicity of AAP.

For the first experiment, the total amount of GSH was determined in the livers of both control and clofibrate pretreated mice to ascertain if clofibrate pretreatment had increased the amount of GSH. However, clofibrate pretreatment may have altered the ability of hepatocytes to maintain GSH levels during exposure to electrophiles so to investigate this possibility both GSH depletion following various doses of AAP and the time course of GSH depletion by a single dose of AAP were also investigated. To further investigate the maintenance of GSH, the effect of clofibrate pretreatment on the activities of 3 GSH dependent enzymes was also considered. GSHPx and Gred are involved in protection against cellular oxidative stress and regeneration of utilised GSH. Also, the GSHt, a multi-functional group of isoforms with different substrate specificities were considered. The GSHt isozyme profile can vary between tissues, the strain, species, age and sex of the animal and by deliberate, dietary or environmental exposure to xenobiotics which can either induce GSHt isoforms or inhibit their activity. Modification of the activity of a particular isoform will affect all associated cellular reactions of that isoform. Three major multigene classes of cytosolic GSHt activity have been characterised in human, rat and mouse; the $\alpha$ , $\mu$ and $\pi$ classes. More recently, since performing these experiments, a fourth class, namely the $\theta$ (theta) family has been identified (Hiratsuka *et al,* 1995). As the conjugation of NAPQI is catalysed by the $\pi$ and to a lesser extent, the $\alpha$ families, it was important to investigate the effect of clofibrate pretreatment on the activity of these enzymes. GSHt activity was determined using 5 different substrates to investigate the 3 main GSHt isoform families.

The final series of experiments involved measurement of the toxicity of AAP following GSH depletion. These experiments were designed to conclusively ascertain a role of GSH in clofibrate associated hepatoprotection. For these experiments, 2 different agents were used to deplete GSH prior to the administration of AAP, namely diethylmaleate (DEM) and buthionine sulphoximine (BSO). In the first study, the amount of hepatic GSH was measured after administration of increasing doses of DEM and BSO. This was intended to establish whether the livers of clofibrate pretreated mice are less prone to GSH depletion in addition to identifying a suitable dose for the subsequent AAP toxicity experiment.

DEM forms a direct conjugate with GSH resulting in a rapid decrease in cellular GSH content (Figure 6.1). In contrast, both BSO and AAP have indirect mechanisms of GSH depletion.

BSO depletes GSH by specific inhibition of GSH synthesis by irreversible inhibition of γ-glutamylcysteine synthetase. Due to the inability of cells to take up GSH from the extracellular fluid, and the inability of synthesis to replenish cellular stores, GSH decreases over a few hours. AAP requires prior bioactivation to NAPQI which binds to GSH causing a comparatively slower rate of decrease in GSH levels than DEM, being dependent on both the rate of NAPQI formation and alternate conjugation pathways. Thus DEM, BSO and AAP all act to deplete cellular GSH but by different mechanisms (Figure 6.1). GSH was depleted by both DEM and BSO prior to administration of AAP with hepatotoxicity quantitated 24 hours later by measurement of plasma SDH levels.

As an adjunct experiment, the nephrotoxicity of AAP was also investigated in the experiment measuring AAP toxicity following GSH depletion by BSO. The extent of renal injury observed following AAP administration is of a far lower magnitude than that of the liver with hepatotoxic doses of AAP exhibiting no nephrotoxicity (observations this chapter). It has been previously observed that clofibrate pretreatment causes extrahepatic protection against the acute toxicity of paraquat in the lung (Frank *et al,* 1982). AAP intoxication causes damage to the convoluted proximal tubule cells of the kidney (Hart *et al,* 1994). The renal toxicity of AAP is GSH dependent and the kidney is known to be sensitive to GSH depletion by BSO (Griffith and Meister, 1979). Thus the potential exacerbation of the renal toxicity of AAP by BSO provided the ideal opportunity to investigate the effects of clofibrate pretreatment on the toxicity of AAP in another organ. AAP nephrotoxicity was determined by measurement of blood urea nitrogen (BUN) levels, a known marker of kidney damage (Talke and Schubert, 1965). Morphological elaboration of any SDH or BUN changes was obtained by collecting liver and kidney samples for independent microscopic evaluation.

It is anticipated that the combined results of the experiments described in this chapter will definitively address the involvement of GSH in the protective effect of clofibrate against AAP toxicity.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Chemicals

All chemicals used in studies reported in this chapter were of the highest quality commercially available. For a list of chemicals and suppliers, see Appendix 1.

### 6.2.2 Animals and treatment regimen

Swiss white male mice (35-40g) were used in all experiments in this chapter with the exception of the study of GSH depletion by buthionine sulphoximine (BSO) which used inbred balb-c mice (25-30g). Mice were obtained and housed in accordance to specifications outlined in Section 2.2. Clofibrate pretreatment involved administration of a 500mg/kg/day

dose of clofibrate i.p. for 10 days while the control group received equivolume olive oil vehicle alone. AAP was administered at the specified dose in alkalinised saline with 0 mg/kg AAP receiving equivolume vehicle.

### 6.2.3 Measurement of hepatic GSH following clofibrate treatment

In order to determine the effect of clofibrate pretreatment on the amount of hepatic GSH, livers from both control and clofibrate pretreated mice were homogenised to a 10% w/v solution in 0.1 M potassium phosphate buffer, pH 7.4 and GSH level was determined in accordance to the method outlined in Section 4.2.4.2

### 6.2.4 Depletion of hepatic GSH by AAP

To investigate the depletion of hepatic GSH with time, mice from both the control and clofibrate pretreated groups were administered a 250 mg/kg dose of AAP and sacrificed 3, 6 and 24 hours later (n=3-6). In a second study investigating dose related decrease in hepatic GSH, mice were injected with 200 to 500 mg/kg AAP and sampled 3 hours later. At each specified time endpoint, livers were homogenised to a 10 % w/v homogenate in 0.1 M potassium phosphate buffer, pH 7.4.

### 6.2.5 Assays for various GSH related enzymes

A 10% (w/v) homogenate was prepared from vehicle and clofibrate pretreated mice (n=6) in 0.1 M potassium phosphate buffer, pH 7.4. Following centrifugation at 12,000 x g for 20 minutes at 4° C, the mitochondria-free supernatant was collected and centrifuged at 100,000 x g for 1 hour. The resultant supernatant (cytosol) was collected and stored in individual aliquots at -80° C until use.

#### *6.2.5.1 Glutathione S-transferase determination*

The assay for the measurement of GSHt activity is based on the method of Habig *et al*, (1974). In all, 5 substrates were used to give a broad indication of the effects of clofibrate pretreatment on individual GSHt isoforms. The biochemical parameters for each substrate differed for concentration of substrate and GSH, buffer pH, assay temperature and wavelength and are summarised with the extinction coefficient, in Table 7.2. Assays were performed in a Hitachi U-2000 spectrophotometer fitted with a water circulated temperature control unit.

In the GSHt assay, 2.015 ml of GSH in 0.1 M potassium phosphate buffer and 75 µl of substrate were combined and preincubated for 1 minute. The reaction was initiated by the addition of 10 µl of cytosol and the reaction rate was measured for either 1 or 5 minutes at the specified wavelength. Due to the nonenzymatic reaction rate of each substrate, activity was

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measured against an appropriate blank. Enzyme activity was expressed as mmol product produced/ min/ g.

| Substrate | λ nm | Reaction pH | [GSH] mM | [Substrate] mM | time min | ε /mmol/min |
|---|---|---|---|---|---|---|
| Chlorodinitrobenzene | 340 | 6.5 | 1.0 | 1.0 | 1 | 9.6 |
| Dichloronitrobenzene | 344 | 7.5 | 1.0 | 1.0 | 1 | 8.5 |
| t-phenylbutenone | 290 | 6.5 | 0.25 | 0.05 | 5 | -24.8 |
| ethacrynic acid | 270 | 6.5 | 0.25 | 0.2 | 5 | 5.0 |

**Table 6.2:** Biochemical assay parameters for the measurement of hepatic GSHt activity in control and clofibrate treated mice using different substrates.

*6.2.5.2 Glutathione peroxidase determination*

The method for both selenium and non-selenium dependent GSHPx activity was based on the method of Lawrence and Burk (1976). A 0.8 ml volume of reagent mix containing 1.25 mM EDTA, 1.25 mM sodium nitrite, 1.25 mM GSH, 1.25 IU Gred and 2.5 mM NADPH in 0.05 M potassium phosphate buffer, pH 7.0 was added to 0.1 ml of a 10 fold dilution of cytosol. After 5 minutes, the reaction was started by the addition of 0.1 ml of the peroxide source, being either 1.5 mM $H_2O_2$ or cumene hydroperoxide for Se dependent and non-Se dependent activities respectively. The Se-dependent GSHPx assay also contained 1.5 mM sodium azide to prevent interference of catalase. The reaction was monitored for the change of absorbance between 30 and 60 seconds after the addition of peroxide substrate, at 340 nm, 25° C. Results are expressed in U/L where 1 U is the amount of enzyme for the oxidation of 1 mol NADPH/min/g.

*6.2.5.3 Glutathione reductase activity determination*

The assay for hepatic Gred was based on the method of Carlberg and Mannervik (1985). A 0.9 ml volume of 0.1 M potassium phosphate buffer containing 1 mM EDTA and 50 µl of oxidised glutathione (GSSG) was warmed to 30° C. A 50 µl volume of 2 mM NADPH in 0.05 M potassium phosphate buffer, pH 7.0 was added and the reaction was started by the addition of 10 µl of cytosol. The change in absorbance at 340 nm was determined and reaction rate was expressed in units, where 1 unit is the amount of enzyme required for the oxidation of 1 mol NADPH/min/g.

### 6.2.6 Depletion of hepatic GSH by various xenobiotics

*6.2.6.1 Depletion of hepatic GSH by DEM*

Both control and clofibrate pretreated mice were administered either 0 to 0.9 ml/kg DEM suspended in olive oil in 0.3 ml/kg increments (n=3). Liver homogenates were prepared 30 minutes later as a 10% homogenate in 0.1M phosphate buffer, pH 7.4. The dose chosen for use in the subsequent GSH depletion experiments was 0.9 ml /kg DEM (see Section 6.3.4.1).

*6.2.6.2 Depletion of hepatic GSH by BSO*

Both control and clofibrate pretreated mice were administered either 0 to 1.2 g/kg BSO in saline in 0.2 g/kg increments (n=3). Liver and kidney homogenates were prepared 3 hours later as outlined above. The dose chosen for use in the subsequent GSH depletion experiments was 0.8g/kg BSO (see Section 6.3.5.1).

### 6.2.7 Determination of AAP toxicity following prior GSH depletion.

*6.2.7.1 Depletion of GSH by DEM with AAP challenge*

Clofibrate and vehicle pretreated mice were administered 0.9 ml/kg DEM followed 30 minutes later by either 0, 100 or 200mg/kg AAP dissolved in saline (n=4 per dose treatment group). After 24 hours, a blood sample was taken for plasma SDH determination.

*6.2.7.2 Depletion of GSH by BSO with AAP challenge*

Clofibrate and vehicle pretreated mice were administered 0.8 g/kg BSO, followed 3 hours later by either 0, 100, 200 or 400 mg/kg AAP (n=5 per dose treatment group). After 24 hours, a blood sample was taken for plasma SDH and BUN determination. The liver and the right kidney were dissected from each mouse. A 1 mm slice across the major anterior lobe of the liver and along the major axis of curvature in the kidney was taken for microscopic analysis. Samples were fixed in 10% formalin until histological slides were prepared between 3 and 7 days post collection.

### 6.2.8 Indices of toxicity

*6.2.8.1 Hepatotoxicity: SDH*

Plasma SDH activity was determined using the method outlined in Section 3.2.5.2.

*6.2.8.2 Renal toxicity: BUN*

Determination of renal toxicity was performed by measurement of blood urea nitrogen (BUN) levels based on a method of Talke and Schubert (1965). A 0.5 ml volume of reagent mix containing 8 mM α-ketoglutarate, 50 U/ml urease, 1.5 U/ml glutamate dehydrogenase and 0.25 mM NADH in 0.1 M potassium phosphate buffer, pH 8.0 was added to 50 µl of plasma,

previously diluted 10-fold in physiological saline. The change in absorbance at 340 nm was measured immediately. The resulting change in absorbance was compared to a standard curve prepared from 0 to 30 mM Urea ($r^2$ = 0.9995 to 1).

### 6.2.9 Statistical Analysis

In GSH content and GSH enzyme analysis, control and clofibrate samples were compared by Students t test for unpaired data ($P<0.05$). All other results reported in this chapter were analysed by ANOVA. In the event of statistical significance ($P<0.05$), Bonferroni post hoc test was performed on specific pairs of data.

## 6.3 RESULTS

The aim of the experiments described in this chapter was to determine the involvement of GSH in the hepatoprotective effect of clofibrate. The first consideration was whether clofibrate pretreatment itself had altered the amount of GSH present in the liver. Clofibrate treatment is known to cause an increase in liver size due to an increase in both cell size and number (Moody *et al,* 1977; Reddy *et al,* 1979; Elcombe *et al,* 1985). During the course of the entire studies described in the thesis, it was observed that hypertrophic effect on the liver after clofibrate pretreatment followed seasonal circadian variations, with peak levels attained in summer.

Investigations of alterations of hepatic GSH by PxP have yielded inconsistent conclusions with various studies reporting no change in hepatic GSH levels (Conway *et al,* 1989; Agarwal *et al,* 1982), increases of around 20% (Tamura *et al,* 1990; Goel *et al,* 1986) or up to 50% (Perera *et al,* 1986). GSH levels are also known to undergo circadian variations which could underlie the disparity in the results reported from different laboratories. Therefore, to gain a more useful indication as to the effect of clofibrate treatment on the amount of GSH in the liver, the results of GSH measurements evenly spaced over one year were collated.

It was observed that there was no difference in the final body weight of the mouse from either the control or clofibrate pretreated groups. However, in mice from the clofibrate pretreated group, there was an average 22% increase in liver size (Table 6.3). Determination of hepatic GSH revealed an average 15% increase in clofibrate compared to control pretreated mice.

This increase in GSH/g liver together with the 22% increase in size, results in an average 40% increase in the total level of GSH in the liver (range for individual studies included in the analysis being 25 to 55% increase in hepatic GSH). This increase in hepatic GSH indicates that the clofibrate pretreated mice would have an enhanced ability to deactivate NAPQI and thus protect the cell from toxicity. To investigate this potential protective mechanism further, the effect of clofibrate pretreatment on the depletion of GSH by AAP was investigated.

| | Control | Clofibrate |
|---|---|---|
| Liver Weight (g) | 2.18 ± 0.09 | 2.66 ± 0.09<br>122%<br>*** |
| Body Weight (g) | 39.1 ± 0.68 | 38.4 ± 0.73<br>98% |
| GSH (mmol/g) | 7.44 ± 0.27 | 8.60 ± 0.37<br>115%<br>* |
| Total GSH/liver (mmol) | 16.2 ± 0.86 | 22.7 ± 1.18<br>140%<br>**** |

**Table 6.3:** Measurement of total hepatic GSH with consideration of both liver size and GSH content after pretreatment with either clofibrate (500 mg/kg/day for 10 days) or olive oil vehicle control. Results are expressed in units as indicated. Clofibrate column indicates % difference between clofibrate followed by the significance level between groups. (Results expressed as mean ± s.e.m, n=28, *=P<0.05, ***=P<0.001, ****=P<0.0001)

### 6.3.2 Depletion of hepatic GSH by AAP

*6.3.2.1 Time based analysis*

With the higher amount of GSH in the liver, it would be anticipated that the loss of GSH upon AAP intoxication would be less pronounced in the livers of clofibrate pretreated mice. This possibility was investigated in the experiment for which the results are presented in Figure 6.4.

In this experiment, all mice were given a 250 mg/kg dose of AAP and livers sampled after 3,6 or 24 hours. This dose was chosen as the maximum dose causing no lethality in control mice. Control mice were observed to have a 75% depletion of hepatic GSH after 3 hours, characteristic of the acute administration of AAP (Figure 6.4). By 6 hours, GSH levels were returning to control levels which were regained by 24 hours. It is clearly seen in the clofibrate treated mice that the extent of GSH depletion at both 3 and 6 hours is less than that observed in the control group with only 30% depletion of hepatic GSH observed after 3 hours.

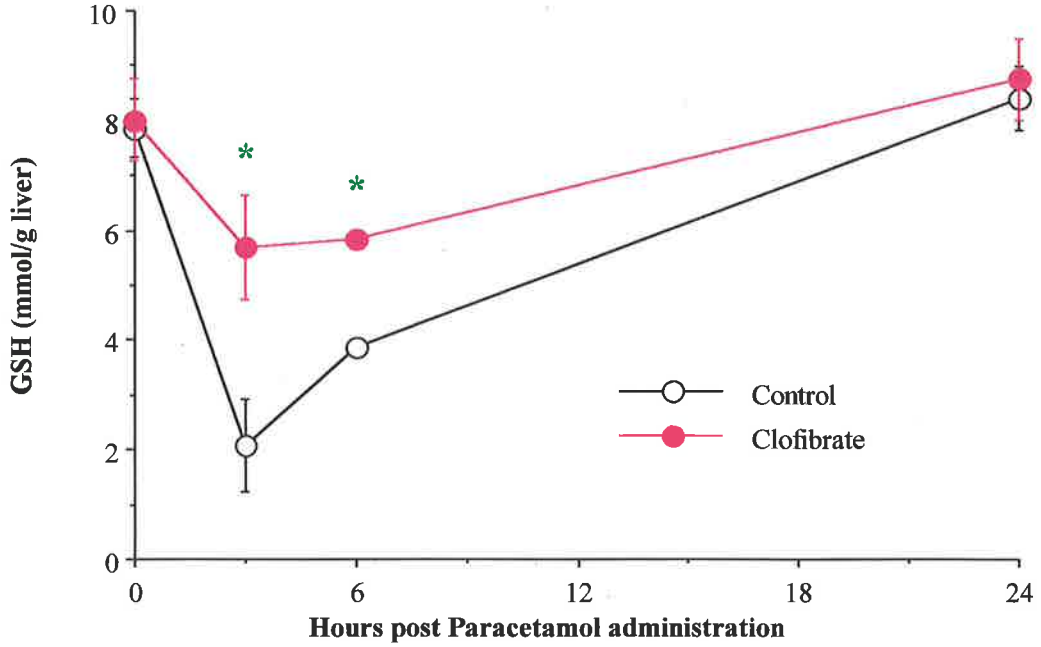



**Figure 6.4:** GSH levels in control (olive oil vehicle) and clofibrate (500 mg/kg/day for 10 days) treated mice following 250 mg/kg AAP. Samples were taken at 0, 3, 6 or 24 hours post AAP administration. (Results expressed as mean ± s.e.m, n=3-6 in each group,* = P<0.05).

### *6.3.2.2 Dose based analysis*

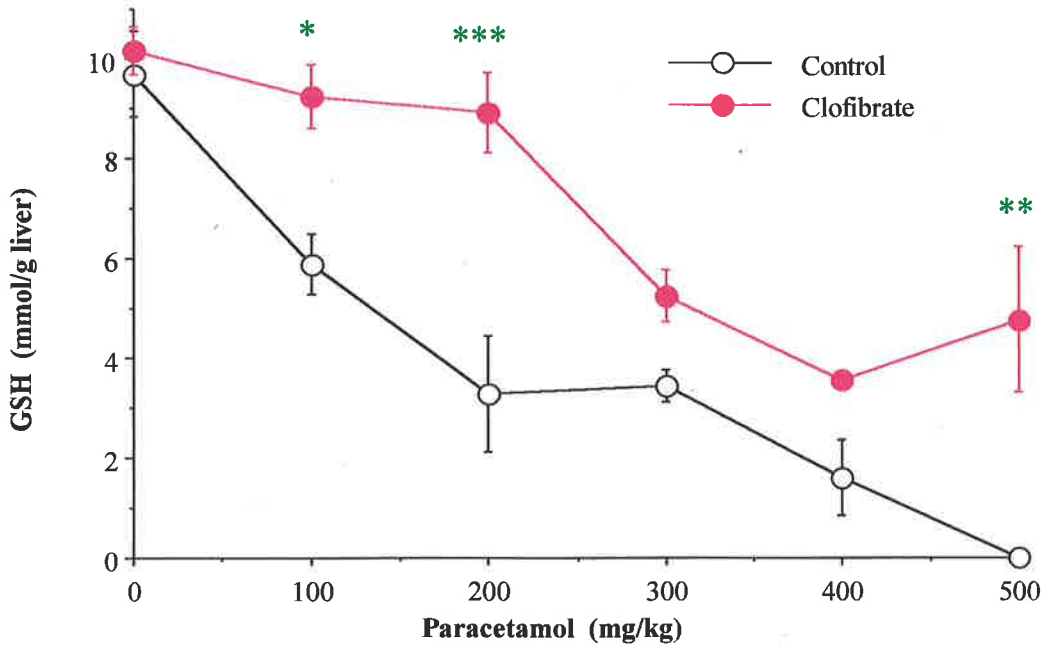



**Figure 6.5:** Measurement of GSH levels in both control and clofibrate treated mice following AAP - 0 to 500 mg/kg - sampled 3 hours post administration. (Results expressed as mean ± s.e.m, n=3-6, * = P<0.05, **= P<0.01, ***=P<0.001).

The preceding experiment (Figure 6.4) confirmed that the livers of clofibrate treated mice were less prone to GSH depletion upon adminstration of a single dose of 250 mg/kg AAP.

However, whether this diminished propensity to GSH loss also occurred when the mice were exposed to higher doses of AAP that produce more pronounced toxicity needs to be considered. In order to address this issue, control and clofibrate pretreated mice were administered between 100 to 500 mg/kg AAP and livers sampled 3 hours later for hepatic GSH content.

A clear dose dependent decrease in hepatic GSH content occurred in control mice, with almost complete depletion observed in mice that received 500mg/kg AAP (Figure 6.5). The pattern of dose related GSH depletion was quite different in the clofibrate pretreated mice. At low doses of AAP (100 and 200 mg/kg), there was no loss of hepatic GSH, despite this dose reducing GSH levels in control livers by 40 and 65 % respectively (Figure 6.5). Following the 300 mg/kg AAP dose, a 50% decrease in hepatic GSH was observed, which was not significantly different to the 65% decrease this dose produced in vehicle pretreated controls (Figure 6.5). The extent of hepatic GSH depletion produced by the 400 mg/kg dose of AAP was also similar in the two groups of mice, although there was a clear resistance to GSH depletion in the clofibrate pretreated livers following the 500 mg/kg dose of AAP. Indeed, the data in Figure 6.5 suggests that the extent of GSH depletion in clofibrate pretreated livers was not the same as that in control pretreated mice. This raises a number of possibilities including that a GSH store resistant to depletion exists in clofibrate pretreated mouse livers, that the increase in total amount of GSH in clofibrate pretreated mice has created a buffer to GSH depletion, that rates of GSH synthesis following depletion are higher in these mice or that there is enhanced enzymatic replenishment of GSH.

Taken together, the data obtained from both the time and the dose based studies (Figures 6.4 and 6.5) indicate that the levels of hepatic GSH in clofibrate treated mice are in some way preserved after AAP challenge. Such resistance to depletion during exposure to AAP could well explain the hepatoprotection against AAP toxicity seen in the previous chapters (Chapters 3, 4 and 5).

### 6.3.3. Effect of clofibrate treatment on GSH related enzymes

In this series of experiments, the effects of clofibrate treatment on the activity of the enzymes involved in protection against cellular oxidative stress and also GSH recycling were investigated. In addition, the activity of the 3 major GSHt isoform families was also investigated following clofibrate pretreatment. The conjugation of NAPQI to GSH is mostly catalysed by the $\pi$ and to a lesser extent, the $\alpha$ family of GSHt isoforms (Ketterer *et al*, 1988). Overall, these experiments investigated the possibility that an enhanced capacity to metabolise xenobiotics via GSH dependent pathways underlies the hepatoprotection associated with clofibrate pretreatment.

| Substrate | GSHt Family | Control | Clofibrate |
|---|---|---|---|
| Chlorodinitrobenzene | Universal | 18.5 ± 1.4 | 15.9 ± 1.8<br>86% |
| Dichloronitrobenzene | μ | 4.75 ± 0.49 | 6.39 ± 0.55<br>134%<br>* |
| t-phenylbuteneone | μ | 0.17 ± 0.2 | 0.23 ± 0.02<br>138%<br>* |
| ethacrynic acid | π | 12.71 ± 0.96 | 7.28 ± 0.74<br>57.3%<br>** |
| cumene hydroperoxide<br>(Non Se GSHPx) | α | 0.38 ± 0.01 | 0.31 ± 0.02<br>82%<br>** |
| hydrogen peroxide<br>(Se-dependent GSHPx) | NA | 0.42 ± 0.01 | 0.35 ± 0.02<br>84%<br>** |
| Gred | NA | 0.93 ± 0.04 | 1.08 ± 0.05<br>117%<br>* |

**Table 6.6** Measurement of hepatic GSHt activities using different substrates in both control (olive oil vehicle) and clofibrate (500 mg/kg/day for 10 days) pretreated mice. Results are expressed as mmol/min/g. Clofibrate column indicates % difference between clofibrate and control and indication of significance level between groups. (NA= Not Applicable. Results expressed as mean ± s.e.m, n=6 in each group, * = $P<0.05$, **= $P<0.01$).

The measurement of GSHt involved the use of 4 substrates, which together with the measurement of non-Se dependent GSHPx activity encompassed the 3 major families of GSHt isoforms (Table 6.6). The GSHt activity with the universal substrate, 1-chloro 2,4-dinitrobenzene (CDNB) was decreased by 14% following clofibrate pretreatment. Interestingly, the use of substrates with high specificity to particular families of GSHt isozymes yielded different results. The activity of the μ family, represented by both *t*-phenylbuteneone and 3,4-dichloronitrobenzene substrates, were both increased by 35 to 40%. In contrast, the π family which catalyses GSH conjugation to ethacrynic acid, was decreased by 43%. The α family was decreased to an equivalent level to that observed with CDNB. Overall, the combined effects of inhibiting the α and π families and induction of the μ family, results in a slight reduction observed when using CDNB as substrate.

GSHPx activity was analysed via measurement of both selenium and nonselenium dependent activities, which are differentiated by the peroxide substrate used. Since GSHPx is important in protecting cells against oxidative stress and lipid peroxidation products, it was possible that

an induction in its activity might account for the hepatoprotection. Both forms of GSHPx were similarly effected with an approx 15-20% decrease in activity while an increase of 17% in Gred activity was observed following clofibrate treatment. As the magnitude of the changes are not great, it appears unlikely that the reduction in the ability of the cell to deactivate peroxides would provide a survival advantage.

### 6.3.4 Effect of GSH depletion by DEM on the toxicity of AAP

*6.3.4.1 Depletion of hepatic GSH by DEM*

Drawing the conclusion that the hepatic GSH pool of clofibrate pretreated mice is less susceptible to depletion than that of control mice is complicated by the fact that AAP is not a direct-acting GSH depleting agent but instead requires CYP450-catalysed bioactivation in order to react with GSH. There could be subtle *in vivo* differences between control and clofibrate pretreated animals with regard to bioactivation and thus subsequent experiments were designed to investigate the susceptibility of the GSH pool of control and clofibrate pretreated livers to a direct acting GSH depleting agent, DEM.

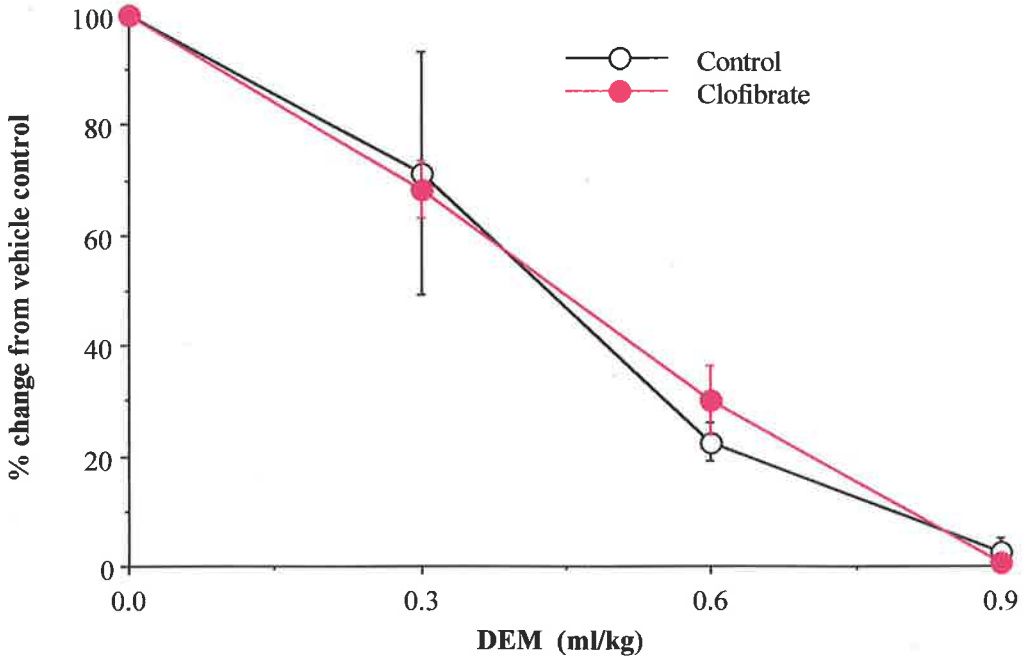



**Figure 6.7:** Measurement of GSH levels in control (olive oil) and clofibrate (500 mg/kg/day for 10 days) treated mice following DEM treatment at 0, 0.3, 0.6 or 0.9 mg/kg and sampled 30 minutes post DEM administration. (n=3 in each group, mean ± s.e.m ).

Vehicle and clofibrate pretreated mice were injected with 0.3 to 0.9 ml/kg of the direct GSH depleting agent DEM and sacrificed 30 minutes later prior to determination of hepatic GSH as described in the methods section. The data shown in Figure 6.7 shows that DEM causes a rapid, dose dependent decrease in hepatic GSH in control mice, with GSH levels reduced to negligible levels by the 0.9 ml/kg dose of DEM (Figure 6.7). Importantly, it was found that

the GSH pool in the livers of clofibrate pretreated mice was equally sensitive to DEM, since no difference between the level of GSH was seen at any DEM dose in the control versus clofibrate pretreated livers (Figure 6.7). Contrary to initial expectations, this suggested that the hepatic pool of clofibrate-pretreated mice was not inherently resistant to GSH-depleting agents, casting doubt on any explanation that solely invoked GSH-dependent mechanisms to account for clofibrate-induced hepatoprotection. A dose of 0.9 ml/kg DEM, was chosen for GSH depletion prior to the administration of AAP.

### *6.3.4.2 Effect of DEM on GSH depletion and AAP hepatotoxicity in mice following treatment with clofibrate*

To address the role of GSH in clofibrate hepatoprotection against AAP, a dose of DEM that achieved almost complete GSH depletion was administered to control and clofibrate pretreated mice prior to the administration of AAP. If the protective mechanism was solely due to GSH dependent pathways, complete abolition of the GSH pool should remove the hepatoprotection in clofibrate pretreated livers, restoring the sensitivity to AAP to that seen in vehicle-pretreated mice.

For this experiment, control and clofibrate pretreated mice were given a 0.9 ml/kg dose of DEM and then 30 minutes later the mice received either 0, 100 or 200 mg/kg AAP. Because this treatment was expected to potentiate the toxicity of AAP, these doses of AAP were deliberately chosen to be lower than those that produced hepatotoxicity (Chapter 3). Blood samples were collected 24 hours later for determination of plasma SDH activity. The results obtained in this experiment are shown in Figure 6.8.

Treatment with DEM was shown to increase the hepatotoxicity of AAP in control mice with increases in SDH activity produced at both the 100 and 200 mg/kg doses of AAP (Figure 6.8). In addition to the increase in plasma SDH, a 25% mortality in the 100 mg/kg and a 75% mortality in the 200 mg/kg AAP doses was observed in the control mice. In contrast, clofibrate pretreated mice retained their resistance to AAP hepatotoxicity even when their livers were depleted of GSH by prior treatment with diethylmaleate. Not only was there no mortality in any of the AAP-intoxicated clofibrate pretreated groups, but also plasma SDH activities were not different from the clofibrate pretreated mice which did not receive AAP. The obvious conclusion to be drawn from these findings is that GSH independent mechanisms must also be involved in the hepatoprotection associated with chronic exposure to clofibrate, since protection was maintained even when mice had been pretreated with the direct acting GSH-depleting agent, DEM.

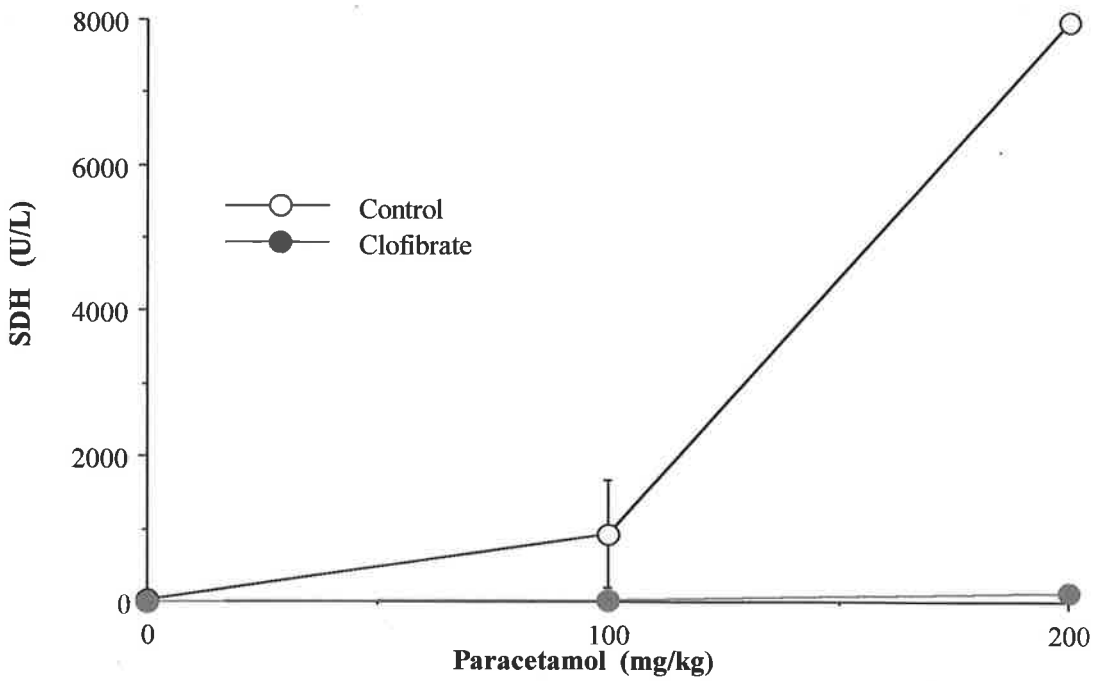



**Figure 6.8:** Measurement of plasma SDH levels in control and clofibrate treated mice following 0.9 ml/kg DEM pretreatment, 30 minutes prior to administration with either 100 or 200 mg/kg AAP. Plasma samples were collected 24 hours later. Control (0 mg/kg) received alkalinised vehicle alone (n=4 in each group, mean ± s.e.m).

Overall, these results show that prior GSH depletion by DEM enhances AAP toxicity and lethality in control mice, however despite the apparent complete depletion of GSH by DEM, there is no major alteration in the susceptibility of clofibrate pretreated mice.

### 6.3.5 Effect of GSH depletion by BSO on hepatic and renal GSH levels and toxicity of AAP in mice following treatment with clofibrate

*6.3.5.1 Depletion of hepatic GSH by DEM*

Having found that the direct removal of GSH from the liver by DEM did not alter the hepatoprotective effect of clofibrate, the next experiment was conducted to establish whether clofibrate pretreated mice had an enhanced ability to resynthesise GSH. For this BSO was used to specifically inhibit γ-glutamylcysteine synthetase, one of the enzymes involved in GSH synthesis. As the cell cannot replenish GSH by increasing synthesis or by reuptake, the intracellular concentration gradually decreases as GSH is lost via efflux (Meister, 1991). Thus, compared to DEM, the depletion of GSH by BSO is slower in onset, yet more persistent.

In order to obtain a dose of BSO to be used for GSH depletion prior to administration of AAP, control and clofibrate treated mice were given 0.2 to 1.2 mg/kg BSO and liver and kidney samples for GSH level analysis were collected 3 hours later.

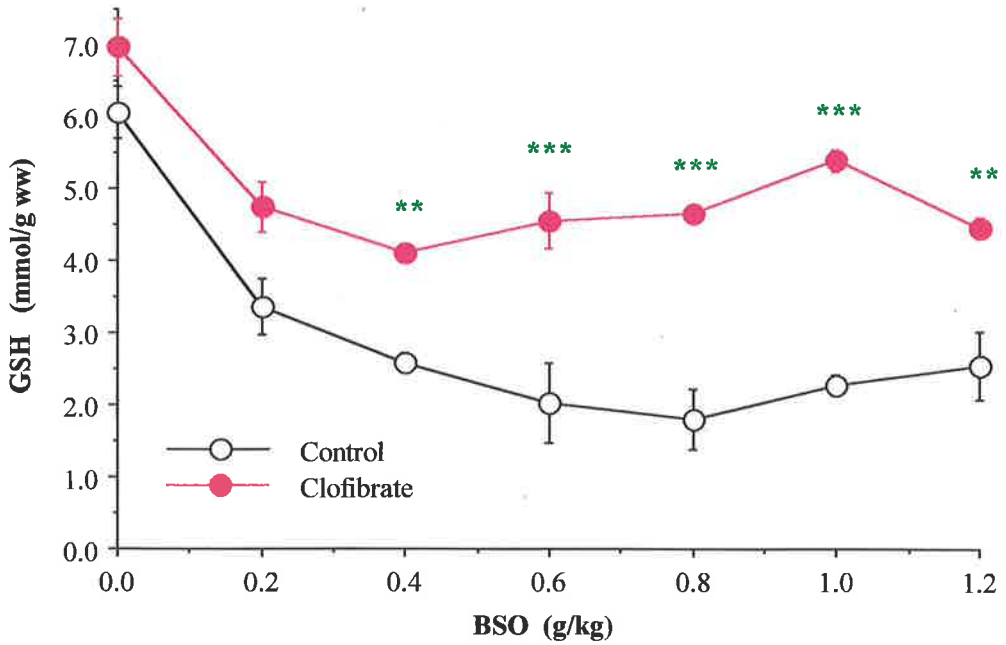



**Figure 6.9:** GSH levels in the livers of control (olive oil vehicle) and clofibrate (500 mg/kg/day for 10 days) treated mice following 0 to 1.2 g/kg BSO. Livers were sampled 3 hours post BSO administration. (Results expressed as mean ± s.e.m, n=3-4, **= P<0.01, ***=P<0.001)

Pretreatment with clofibrate was found to cause a 15% increase in basal hepatic GSH content, which was not statistically significant (Figure 6.9). Treatment with BSO caused a dose dependent decrease in GSH to a plateau indicative of a 60 to 70 % reduction from control GSH levels with doses of 0.6 g/kg BSO and above. In contrast, the GSH depletion by BSO in clofibrate treated animals still showed a plateau response but at an amount considerably higher than that of control mice. From the 0.2 g/kg dose onward a 25 to 40 % reduction of GSH from control levels was observed. Thus overall there was about a 30% more hepatic GSH at all doses of BSO in the clofibrate pretreated as compared to the control pretreated mice (Figure 6.9).

The renal GSH level was found to be reduced by about 20 % in the kidney following clofibrate pretreatment (Figure 6.10). Administration of BSO was observed to cause a depletion in renal GSH down to a plateau at a 80% reduction from control levels. In contrast to the depletion pattern observed in the liver, there was no difference in the level of GSH depletion at any dose of BSO, between the control and the clofibrate pretreated mice (Figure 6.10).

A dose of 0.8 g/kg was well tolerated and chosen as the dose of BSO for further experimentation. The level of GSH depletion was at plateau for both liver and kidney, indicating enzyme inhibition was maximal for this time point.

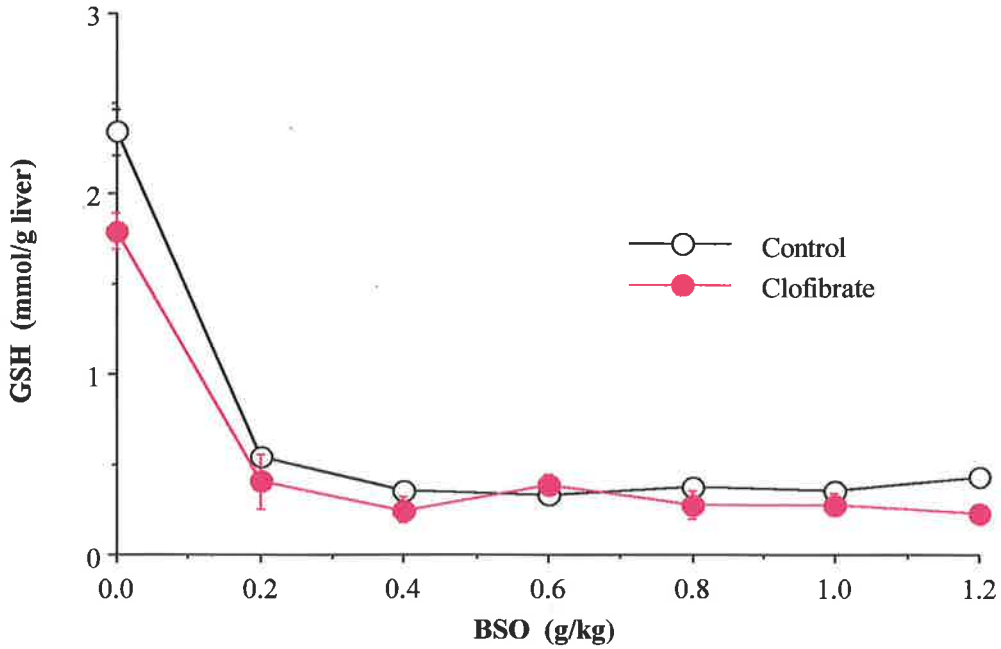



**Table 6.10:** GSH levels in kidneys of control (olive oil vehicle) and clofibrate (500 mg/kg/day for 10 days) treated mice following BSO, 0 to 1200 mg/kg sampled 3 hours post BSO administration. (Results expressed as mean ± s.e.m, n=3-4)

### *6.3.5.2 Effect of BSO on AAP toxicity in mice following treatment with clofibrate*

In order to determine the effect of GSH depletion by BSO on the toxicity of AAP, 0.8 g/kg BSO was administered 3 hours before injection of either 0, 50, 100, 200 or 400 mg/kg AAP.

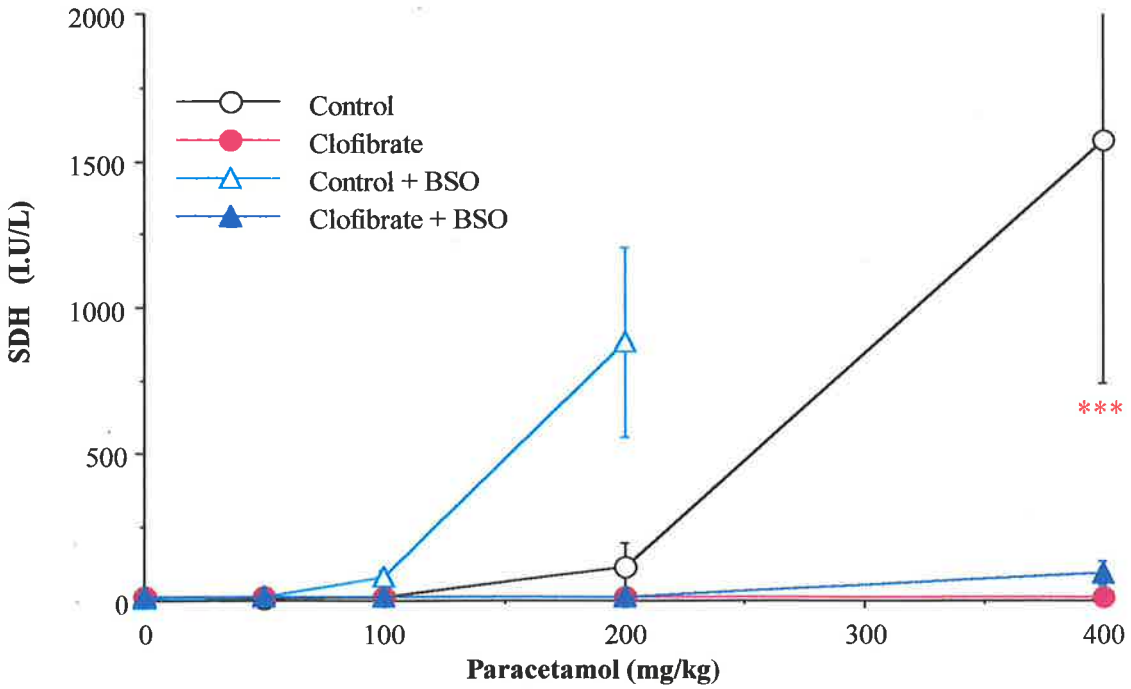



**Figure 6.11:** Measurement of plasma SDH levels following 0.8 g/kg BSO treatment, with or without 50 to 400 mg/kg AAP challenge in control (olive oil vehicle) and clofibrate (500 mg/kg/day for 10 days) treated mice. (n=5, mean ± s.e.m, ***=P<0.001- red stars = difference control versus clofibrate)

Pretreatment with BSO was found to cause a shift of the plasma SDH curve to the left indicating an enhancement of hepatotoxicity in control mice (cyan compared to black line - Figure 6.11). In addition to enhanced SDH levels, there was an increase in mortality with 20% mortality in control mice given 400 mg/kg AAP alone while 100% mortality was observed at the same 400 mg/kg dose following BSO treatment.

Akin with earlier reported results (Chapters 3, 4 & 5)), no hepatotoxicity was observed in clofibrate pretreated mice administered AAP at the highest dose of 400 mg/kg. Administration of BSO did not influence the hepatotoxicity of clofibrate pretreated mice until 400 mg/kg AAP, where a slight increase in plasma SDH was observed (navy compared to magenta line- Figure 6.11). At the 400 mg/kg dose, there was no mortality nor obvious centrilobular necrosis in the clofibrate pretreated groups. This is in direct contrast to the effects in control pretreated mice with 400 mg/kg AAP causing extensive hepatic damage, and if administered BSO prior to AAP, a 100% mortality was observed (for histological observations, see Table 6.13).

The GSH level in control pretreated mice without either BSO or AAP was 6.2 μmole/g liver, while the amount of GSH in clofibrate pretreated mice administered with BSO plateaued at level of 4.6 μmole/g or 25% lower (Figure 6.9). However, control pretreated mice yielded a mean SDH value of around 1500 IU following AAP intoxication, while the clofibrate pretreated mice that were also administered BSO exhibited only 70 IU SDH activity after AAP administration (Figure 6.11). This indicates that despite having lower GSH than control mice, clofibrate pretreatment still provided protection against AAP hepatotoxicity. This supports the conclusions obtained in the determination of AAP toxicity following depletion of GSH by DEM, that the protective effect of clofibrate does not exclusively involve GSH. In data not plotted from this study, administration of an 800 mg/kg dose of AAP to BSO and clofibrate pretreated mice produced a plasma SDH level of 315±120 IU/l (n=4), equivalent to those in control mice that received between 100 to 150 mg/kg AAP with BSO. This indicates that pretreatment with clofibrate provides a protection such that 6 times the dose of AAP is required to exhibit the same level of hepatotoxicity.

Other than hepatotoxicity, the renal damage caused by AAP was evaluated using plasma BUN as an index of nephrotoxicity. This was of interest as AAP does cause renal damage and renal GSH levels are quite sensitive to BSO induced depletion (Meister, 1991). Also, this adjunct study allowed inspection as to whether the protection associated with clofibrate pretreatment is exhibited in extrahepatic organs.

AAP was not nephrotoxic at doses up to 200 mg/kg in control mice while a relatively modest 70 % increase in BUN occurred at the highest dose of 400 mg/kg AAP. This shows that the renal toxicity of AAP is not of the same magnitude as that in the liver considering the

extensive hepatic necrosis that accompanies administration of this dose. Prior administration of BSO caused a dramatic increase in nephrotoxicity, with 3 fold and 7 fold increase in plasma BUN observed at 100 and 200 mg/kg respectively (cyan compared to back line - Figure 6.12). In contrast to control mice, a 400 mg/kg dose of AAP had no effect on plasma BUN in clofibrate treated mice, indicating protection against the nephrotoxicity of AAP. Prior administration of BSO resulted in a 3 fold increase in BUN levels only at the 400 mg/kg dose of AAP, with no alteration in toxicity at lower doses. As this increase in plasma BUN was observed at 100mg/kg AAP in control mice, it appears that 4 times the dose of AAP was required to produce equivalent nephrotoxicity in clofibrate treated mice.

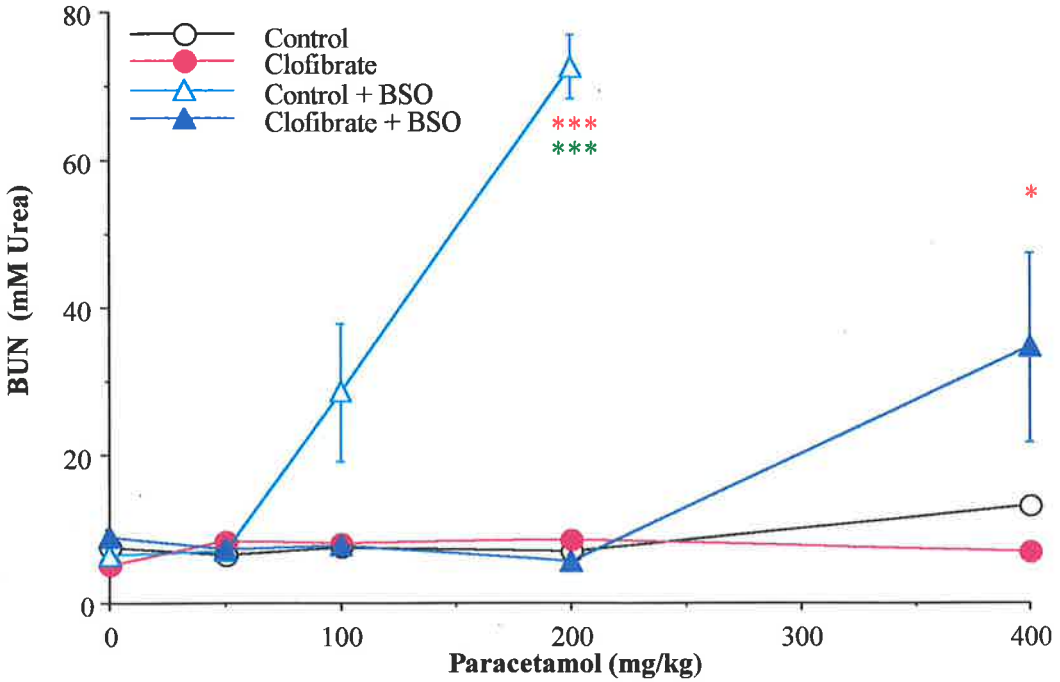



**Figure 6.12:** Plasma BUN levels following BSO treatment, with or without 50 to 400 mg/kg AAP challenge in control and clofibrate treated mice. (n=5, mean ± s.e.m, *=P<0.05. ***=P<0.001- green stars = difference related to BSO treatment, red stars = difference related to clofibrate treatment)

In addition to providing hepatoprotection, clofibrate pretreatment clearly protected the kidney against the toxicity of AAP. As there was no difference in the level of GSH depletion in control and clofibrate pretreated mice, it is obvious that clofibrate is protective against the acute nephrotoxicity of AAP in a reaction that is not dependent on the availability of cellular GSH.

Histological samples were taken from the liver and kidneys for independent microscopic assessment. The results confirmed the SDH and BUN data obtained with the 400 mg/kg dose of AAP, either with or without BSO in both the control and clofibrate pretreated groups (Table 6.13). Comparison of the 400 mg/kg AAP doses indicate that no cellular damage was present in the clofibrate pretreated animals, in contrast to control mice. The prior

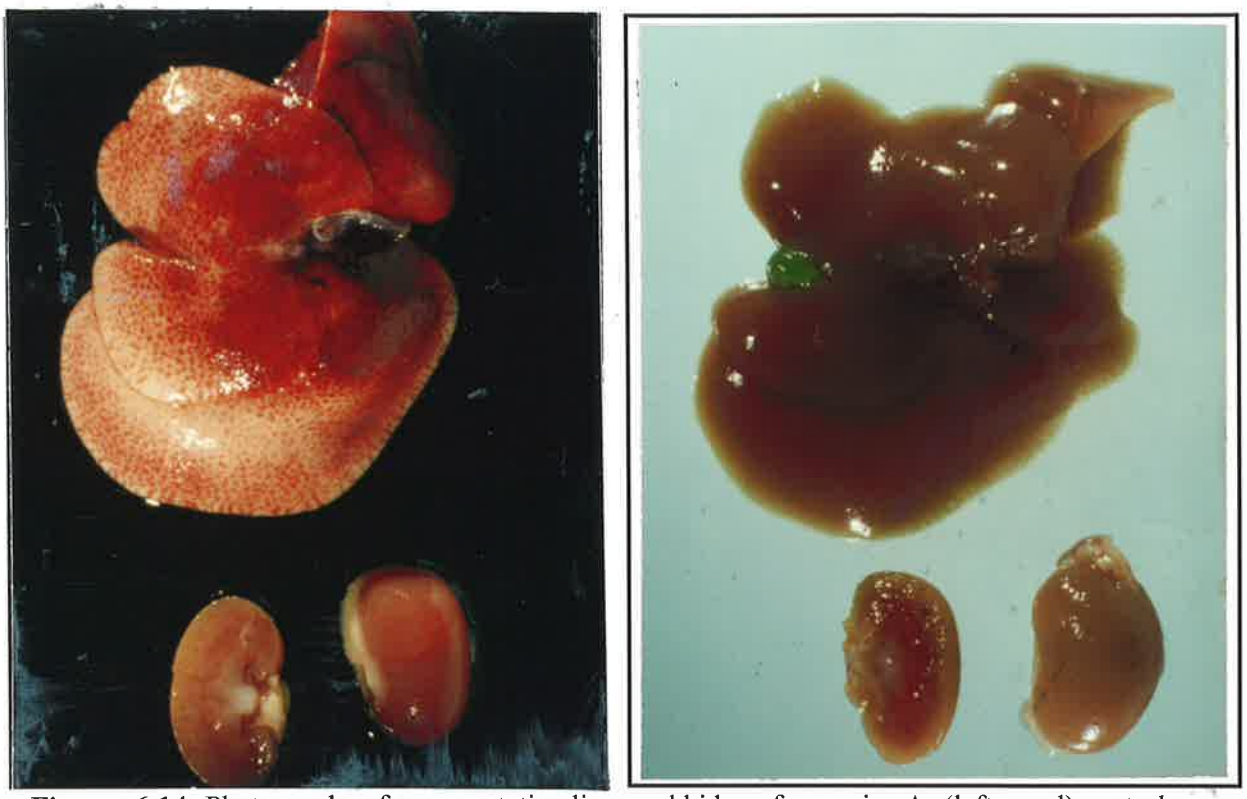
administration of BSO enhanced AAP hepatic and renal necrosis in control pretreated mice, but only caused a slight increases in damage in clofibrate pretreated mice.

| | Liver | Kidney |
|---|---|---|
| Olive Oil<br>Saline<br>Saline | Normal | Normal |
| Olive Oil<br>BSO<br>Saline | Near normal, some altered cells | Very little change, near normal |
| Olive Oil<br>Saline<br>400 mg/kg AAP | Centrilobular necrosis - some samples had severe confluent panlobular necrosis with congestion | Near normal, some samples with mild tubular damage |
| Olive Oil<br>BSO<br>400 mg/kg AAP | No survivors | No survivors |
| Clofibrate<br>Saline<br>Saline | Normal, some samples with individual mitosis | Normal |
| Clofibrate<br>BSO<br>Saline | Near normal, some individual hepatocyte damage | Normal |
| Clofibrate<br>Saline<br>400 mg/kg AAP | Very little change, some mitosis occurring, some individual hepatocyte damage | Near normal |
| Clofibrate<br>BSO<br>400 mg/kg AAP | No obvious confluent necrosis, individual hepatocyte necrosis and mild centrilobular hepatic generation | Tubular damage |
| Clofibrate<br>BSO<br>800 mg/kg AAP | Centrilobular degeneration, early necrosis - mild and patchy | Moderate to severe tubular damage with casts |

**Table 6.13:** Histological analysis of liver and kidney samples from mice treated with control (olive oil vehicle) or clofibrate (500 mg/kg/day for 10 days) followed by 0.8 g/kg BSO or saline vehicle and 3 hours later by either 400 mg/kg AAP or saline vehicle.

To illustrate the extent of hepatoprotection of clofibrate pretreatment on the acute hepatotoxicity of AAP, representative photographs are displayed in Figure 6.14. On the left is control mouse liver and kidney, after a dose of 400 mg/kg AAP (with no other chemical modification). The liver has the characteristic strawberry appearance, a red inflamed exterior with pale necrotic lesions, indicative of severe hepatic damage and blood engorgement and congestion (Figure 6.14A). In contrast, on the right is the liver and kidney from a representative clofibrate pretreated mouse given BSO prior to 800 mg/kg AAP, that is twice the dose received by the control mouse liver (Figure 6.14A) and in the presence of GSH depletion by BSO. The liver exhibits an almost normal morphology with little evidence of hepatotoxicity. The kidney of this mouse showed some nephrotoxicity with an oedematous cortex and reddened medulla. It

should be noted that the clofibrate pretreated liver and kidney sample (Figure 6.14B) was representative of a group of mice where no mortality was observed. In fact, there was no survivors in the control group given half this dose of AAP with prior BSO administration. The visual comparison of the control and clofibrate pretreated livers reveals the extensive hepatoprotection as a result of clofibrate pretreatment.



**Figures 6.14:** Photographs of representative liver and kidney from mice **A:** (left panel) control mouse treated with 400 mg/kg AAP **B:** (right panel) liver slice from mouse treated clofibrate (500 mg/kg/day for 10 days), 0.8 g/kg BSO 3 hours prior to 800 mg/kg AAP (twice the AAP dose of the control mouse). Tissues were collected 24 hours after AAP administration

Of interest was the emerald green colour of the gall bladder in the clofibrate pretreated mice given both BSO and AAP. This unusual, atypical colour could be the result of a high concentration of biliverdin, the green pigment which is an oxidised metabolite of bilirubin, in the bile. When bilirubin is utilised as a cellular antioxidant, it is converted to biliverdin and subsequently converted back to bilirubin by the action of biliverdin reductase (McDonagh, 1990). Bilirubin is an effective chain breaking antioxidant and also quenches the highly reactive species, singlet oxygen (Halliwell and Gutteridge, 1989). Biliverdin itself is known to have antioxidant properties (Wu *et al,* 1991; Stocker and Peterhans, 1989). If the green pigment is indeed biliverdin, then its occurrence in the bile of clofibrate pretreated mice could indicate a high usage of bilirubin in protecting against an oxidative stress caused by the combined administration of BSO and AAP.

## 6.4 DISCUSSION

In this chapter, the involvement of GSH in the hepatoprotective effect of clofibrate on the acute toxicity of AAP was investigated. For this, a number of separate studies were performed including; measurement of the amount of GSH, GSH enzyme activities, level of GSH depletion and finally determination of the effect of prior depletion of GSH by DEM and BSO on the hepatotoxicity of AAP.

### 6.4.1 Effect of clofibrate treatment on amount of hepatic GSH, both before and after AAP administration

It is well established that supplementation with sulphydryl compounds protects against AAP toxicity if administered in a time frame allowing effective conjugation of the reactive intermediate (Mitchell *et al,* 1973b). As GSH cannot cross plasma membranes, either administration of GSH pro-drugs, such as GSH-esters (Uhlig and Wendel, 1990) or supplementation with cysteine or related sulphydryl precursors (Kitamura *et al,* 1988; Butterworth *et al,* 1992), would increase GSH availability. If GSH is involved in clofibrate induced hepatoprotection, then an equivalent increase in GSH or sulphydryl replenishment should provide the equivalent protection as that observed in this study.

#### *6.4.1.1 Effect of clofibrate pretreatment on the amount of hepatic GSH*

Investigation of the effect of clofibrate pretreatment on both liver GSH content (clofibrate pretreatment results in increases from 0 to 35%) and hypertrophy (range from 20 to 40% increase in %LBW) revealed that these mice have approximately 40 % more hepatic GSH than do control mice. At this point, it should be noted that during the course of studies in this thesis, doses of up to 800 mg/kg AAP (administered in saline) exhibited no hepatotoxicity in clofibrate pretreated mice. In a study investigating the effect of increasing cellular GSH levels, administration of 500 mg/kg glutathione isopropyl ester raised hepatic GSH levels by 47% and reduced the toxicity of a 400 mg/kg dose of AAP as measured by a decrease in plasma SDH from 800 to 100 U of SDH (Uhlig and Wendel, 1990). Although pretreatment with the GSH-ester provided extensive protection, a low level of hepatotoxicity was still observed.

An increase in GSH synthesis is another strategy for increasing the level of hepatic GSH. This can be achieved by supplying the cell with sulphydryl compound. The sulphydryl prodrug cystathionine, enhances cellular cysteine levels and GSH synthesis, attenuated ALAT levels from 4132 U to 616U and 24 hour mortality from 54 to 14% following a 760 mg/kg dose of AAP (Kitamura *et al,* 1988). Similarly, administration of 150 mg/kg cysteine both before and after AAP challenge, was observed to reduce the microscopic occurrence of necrotic damage from 97% to 16% following administration of a 375 mg/kg dose of AAP (Mitchell *et al,* 1973b). Another sulphydryl, S-adenosylmethionine has been reported to maintain the GSH

pool providing equivalent protective power as other sulphydryls including N-acetyl cysteine and methionine, and was shown to reduce the macroscopic toxicity of a 500 mg/kg dose of AAP by half (Bray *et al,* 1992). Collectively, these studies show that modification of cellular GSH either directly or via supplementation of sulphydryl intermediates, provides considerable protection against AAP toxicity and mortality, however, each study revealed incomplete protection. In contrast, clofibrate pretreatment provided complete protection at all of the doses used in the studies reported in this chapter.

*6.4.1.2 Effect of clofibrate pretreatment on GSH depletion by AAP*

In experiments described in this chapter, clofibrate pretreatment was shown to enhance the amount of cellular GSH. As NAPQI is detoxified by GSH, an enhanced level of GSH would provide some protection against hepatotoxicity. The results in the preceding section suggest a role for GSH in the hepatoprotection of clofibrate against AAP hepatotoxicity. AAP was observed to cause both a time and dose dependent depletion of GSH. However, in clofibrate treated animals, the extent of depletion was less marked at all of the time points studied. At high doses of AAP, GSH depletion appears to have plateaued at around 40% of original levels in clofibrate mice. It is also of interest that the 200mg/kg dose of AAP caused minimal depletion of GSH in the clofibrate treated mice in contrast to the control mice, yet the level of GSH metabolites in the urine at this dose was equivalent (Chapter 5). Collectively, these observations indicate a possible increase in GSH synthesis and GSSG turnover to replenish and maintain GSH levels, and it is therefore important to measure the effect of clofibrate treatment on the activity of relevant GSH dependent enzymes.

### 6.4.2 Effect of clofibrate pretreatment on the activity of GSH dependent enzymes

The protective properties of GSH are associated with its dual role as a cellular reductant and conjugate. GSHt activity, which catalyses the conjugation of GSH to reactive intermediates, is composed of 4 separate isoform families. Of these, the $\pi$ family is involved in the conjugation of NAPQI to GSH which if enhanced in activity would indicate a greater potential deactivation by this pathway. Also, investigation of activities of both Gred and GSHPx as well as GSHt following clofibrate pretreatment will provide information on the capability of the liver to undergo xenobiotic metabolism.

*6.4.2.1 GSH recycling - Glutathione peroxidase and Glutathione reductase*

Two of the major enzymes influencing the level of hepatic GSH are GSHPx and Gred. The protective actions of GSHPx utilises GSH forming GSSG as a byproduct. GSSG is reduced back to GSH, thus aiding in the maintenance of GSH levels without the direct involvement of GSH synthesis. A decrease in the activity of GSHPx would lead to an increase in the level of damaging peroxides, while a decrease in Gred would increase the intracellular concentration of GSSG, which when actively transported out of the cell reduces the crucial sulphydryl pool

(Meister, 1991). In the opposite direction, an increase in both GSHPx and Gred could potentially provide a faster GSH turnaround time during oxidative stress or chemically induced toxicity. In support of the involvement of these enzymes in chemoprotection, an increase in both GSHPx and Gred activity has been suggested as the mechanism by which 2 week old mice are resistant to AAP, while inhibition of these enzymes increased the susceptibility of these mice to AAP (Adamson and Harman, 1989).

GSHPx has both non-Se dependent and Se dependent activities against peroxide substrates (Lawrence and Burk, 1976). The non-Se dependent GSHPx is also an isoform of GSHt from the α family and constitutes around 30% of the total GSHPx in mouse liver (Halliwell and Gutteridge, 1989). Although the *in vivo* substrates for GSHPx includes hydroperoxides such as those derived from steroids (eg cholesterol and progesterone) and also linoleic and linolenic acids, the major cellular substrate is $H_2O_2$. As $H_2O_2$ is metabolised by both catalase and GSHPx and since catalase is increased by PxP treatment, sodium azide was added to the enzyme assay to inhibit catalase and thereby specifically measure alterations to GSHPx activity. From the results of this experiment, both forms of GSHPx were reduced by around 15% in activity compared to the control mice (Table 6.3). Similar decreases in GSHPx activity have been reported following pretreatment with other PxP including ciprofibrate (Glauert *et al,* 1992) DEHP (Perera *et al,* 1986; Tomaszewski *et al,* 1986) and nafenopin (Lake *et al,* 1989b; Tomaszewski *et al,* 1986).

In contrast, clofibrate pretreatment enhanced Gred activity by 17%, which is opposite to the reduction others have reported in rats following ciprofibrate treatment (Glauert *et al,* 1992). An increase in Gred activity has been implicated in protection against AAP toxicity, when treatment with oltipraz was shown to increase cellular GSH due to enhanced Gred activity, thus protecting hamsters against the acute toxicity of AAP (Davies *et al,* 1991). Also, the protection afforded by lobenzarit against AAP hepatotoxicity is believed to be related to a 30% increase in Gred activity (Armesto *et al,* 1993). Although an increase in Gred was observed following pretreatment with clofibrate, it is unlikely that the 17% increase provides the basis for hepatoprotection. Consideration of enzymatic rates reveals the maximal GSSG reduction rate by Gred to be 8-10 μmol/min/g (Pinto and Bartley, 1969) while the maximal rate of GSH oxidation by GSHPx is 40 μmol/min/g (Jones *et al,* 1981). The 4 fold greater rate of GSHPx activity is indicative of why GSSG formation during oxidative stress can overwhelm Gred activity leading to GSSG leakage, and thus a 17% increase in Gred activity coupled with a 15% decrease in GSHPx would provide little alteration to the balance between the two enzymes.

Overall, the results of this study show that pretreatment with clofibrate is associated with minimal change in the activity of either GSHPx or Gred activities. It thus seems unlikely that

alterations in GSH turnover due to enhanced GSHPx/Gred cycling are involved in clofibrate induced hepatoprotection.

*6.4.2.2 Glutathione S-transferase*

In the experiments described in this chapter, GSHt activity was determined using 5 different substrates; 1-chloro 2,4-dinitrobenzene (CDNB), a substrate with appreciable activity with the 3 main GSHt classes investigated; ethacrynic acid, substrate for the π class and *t*-phenyl butenone and dichloronitrobenzene which are both substrates for the μ class. The activity for the α-class was investigated by measuring non-selenium dependent GSHPx activity using cumene hydroperoxide as a substrate. Although each substrate is conjugated to some degree by isoforms from the other 2 GSHt families, activity towards each of the aforementioned substrates is greatest with the respective isoforms shown, enabling construction of an overall profile of GSHt isoforms in mouse liver. Recently a fourth class of mouse GSHt, the θ class, was identified which like the α-class, has high GSH peroxidase activity (Hiratsuka *et al*, 1995)

Most studies investigating the effects of PxP treatment have utilised the universal substrate, CDNB which has almost equivalent activity with the 3 major GSHt isoforms (Ketterer *et al,* 1988). In general, decreases in GSHt activity are observed in the livers of PxP-treated rodents. For example, a 15% decrease in GSHt catalysed CDNB conjugation was reported in the livers of clofibrate pretreated mice (Manautou *et al,* 1994) and in rats following exposure to clofibrate analogues (Foliot *et al*, 1986) while up to 35% decreases were seen using other PxP including ciprofibrate, perfluorodecanoic acid, nafenopin, silvex and DEHP (Glauert *et al,* 1992; Voskobonik *et al*, 1996). Since overexpression of one isozyme can mask the inhibition or down regulation of another, use of a non-isoform specific substrate such as CDNB does not provide any information about the effects of PxP treatment on individual isoforms. For example, pretreatment with various xenobiotics including butylated hydroxyanisole, phenobarbitol and *trans*-stilbene oxide was found to alter the levels of various individual GSHt isoforms in the mouse, a finding which was confirmed via measurement of GSHt activity using specific substrates (Di Simplico *et al.,* 1989). As the conjugation of NAPQI to AAP is catalysed by the π-class and to a lesser extent the α-class (Ketterer *et al,* 1988), it was of interest to determine the effect of PxP treatment on these isoforms. Clofibrate is known to decrease the enzymatic activity of GSHt-μ isoforms in rats and hamsters, as well as GSHt-α in rats (Foliot and Beaune, 1994), indicating possible species differences in response to PxP. Whether changes in individual mouse GSHt isoforms occur upon exposure of mice to clofibrate was unknown until the present experiments were performed.

There was a 14% decrease in GSHt activity observed in the livers of mice pretreated with clofibrate when CDNB was used as substrate. A similar decrease was observed when an α

specific substrate (cumene hydroperoxide) was used, however, the other 2 classes were differently effected. The π class had an almost 50% decrease in activity as measured by ethacrynic acid. This observation is in agreement with the suppressed synthesis of the GSHt-π class observed in rats after treatment with PxPs (Tsuchida *et al,* 1993) In contrast, the μ class exhibited a 35-40 % increase in activity, observed when dichloronitrobenzene and *t*-phenylbutenone were used as GSHt substrates. It thus seems that the increase in one form have been masked by a decrease in another when CDNB was used as substrate. This is especially important with regard to the changes of the GSHt isoforms that participate in the detoxication of NAPQI. The fact that a marked (50%) decrease in the activity of the main π-class which are involved in NAPQI conjugation was observed in the livers of clofibrate pretreated mice indicates that an enhanced ability to form the AAP:GSH conjugate is unlikely to account for the hepatoprotection against AAP described in the previous chapters.

### 6.4.3. GSH depletion by DEM and BSO

If the hepatoprotective effect of clofibrate is solely due to an enhanced availability of GSH, then it follows that GSH depletion should substantially lessen or revert AAP toxicity to that observed in control mice. To investigate this, two different approaches to GSH depletion were used. Administration of DEM, a carbonyl compound that can nonenzymatically conjugate GSH, causes a rapid depletion in cellular GSH. DEM induced GSH depletion is short lived, lasting around 2 hours (Younes *et al,* 1980). This is due to the stimulation of GSH synthesis to counteract the sudden decrease in GSH (Deneke and Fanburg, 1989). The second method of GSH depletion involved administration of the GSH synthesis inhibitor, BSO which causes a comparatively slower decrease in GSH levels, due to the net efflux of GSSG and the inability to resynthesise the lost GSH. BSO causes a 65 to 80% drop in GSH within 2 to 3 hours, which is followed 8 hours later, by a slower recovery phase (Griffith and Meister, 1979). The sustained depletion plateau in the kidney is maintained for over 16 hours prior to commencement of the recovery phase (Drew and Miners, 1984). In order to obtain an appropriate dose of both DEM and BSO for use in subsequent AAP toxicity studies, the amount of GSH depletion over a range of doses was determined.

There was no difference in the extent of hepatic GSH depletion achieved by different DEM doses in clofibrate pretreated and control mice, with negligible levels of GSH remaining 30 minutes after a dose of 0.9 ml/kg DEM. In contrast, BSO was unable to deplete hepatic GSH by more than 40% in clofibrate pretreated mice while in control mice a decline of approximately 70% was achieved. These results suggest that clofibrate pretreated mice have a higher nonprotein sulphydryl capacity. Possibilities for increased GSH capacity include; an increase in the GSH content of another cellular compartment; an increase in a non-GSH sulphydryl that chemically interferes with the GSH assay; or an inhibition of GSH transport

out of the cell. The possibility that an increase in GSH occurs in BSO and clofibrate pretreated livers is unlikely due to BSO inhibition of γ-glutamylcysteine synthetase.

In isolated hepatocytes, DEM has been observed to deplete cytosolic GSH while having no effect on mitochondrial GSH. The latter comprises a discrete pool of GSH with a half life of 30 hours, while that of the cytosolic pool is just 2 hours (Meredith and Reed, 1982). The mitochondrial pool is an important source of GSH and comprises 10 to 15% of the total cellular GSH (Wahlländer *et al,* 1979). In contrast, in the *in vivo* situation, the cytosolic and mitochondrial pools of GSH are depleted at equivalent rates by DEM (Lauterburg *et al,* 1984) which could explain the near complete depletion of GSH observed in this study. As BSO is impermeable to the mitochondrial membrane (Meister, 1991), it can only deplete cytosolic GSH in the 3 hour time frame leaving the mitochondrial pool intact. Also, BSO has been observed to cause a 75% depletion of cytosolic GSH 3 hours after administration while only a 20% decrease in nuclear GSH indicating yet another discrete pool of GSH in the cell (Edgren and Révész, 1987).

A 3 hour exposure to BSO in the control group resulted in a decrease in GSH that plateaued around 40% control levels (Figure 6.9), which is in agreement with previously reported results (Griffith and Meister, 1979). On the basis of results obtained in clofibrate pretreated mice, it appears that both AAP and BSO were unable to deplete a "non-protein sulphydryl" pool that DEM was able to access. Clofibrate treatment does increase the number of cellular mitochondria, but as AAP can deplete mitochondrial GSH *in vivo* (Lauterburg *et al,* 1984), it appears unlikely that this extra pool is purely mitochondrial. Also, as there was no consistent alteration in the amount (ranging from 0 to 35% increase) of hepatic GSH following clofibrate administration, the difference in GSH depletion between clofibrate pretreated and control mice appears not to be related to a simple increase in the level of this sulphydryl. Overall, no firm conclusion can be reached as to the origin of the approximate 40% increase in "nonprotein sulphydryl" identified in the combined BSO and AAP depletion experiments.

### 6.4.4 Effect of prior GSH depletion by BSO and DEM on hepatotoxicity of AAP

The determination of AAP toxicity following GSH depletion in clofibrate pretreated mice should indicate whether the protective effect was purely due to increased in GSH availability. Should GSH be exclusively involved, then depletion should abolish the hepatoprotection. The doses of DEM and BSO chosen caused maximal GSH depletion in the preliminary dose response experiments. DEM caused complete GSH depletion in both control and clofibrate mice. An enhancement of AAP toxicity by GSH depletion was clearly evident with lethal hepatotoxicity produced by previously non-toxic doses of AAP in DEM pretreated control mice. In contrast, the complete lack of toxicity in the 100mg/kg group and the minimal

toxicity in the 200 mg/kg AAP groups pretreated with clofibrate supports the conclusion that clofibrate hepatoprotection is not exclusively due to enhanced GSH availability.

GSH was depleted down to approximately 40 % of normal values by BSO in the control pretreated mice. This level of GSH depletion greatly exacerbated the hepatotoxicity of AAP (Figure 6.11). Although the reduction of hepatic GSH was not as pronounced in the clofibrate pretreated group, BSO had little effect on the hepatotoxicity of AAP in these mice. The final concentration of GSH in the livers of clofibrate treated mice following 0.8 g/kg BSO was 4.6 mmol/g, which was less than the control value of 6.1 mmol/g (Figure 6.9). Even with adjustment for liver weights, the total amount of GSH in the liver of clofibrate mice given BSO was less than in the control mouse. However, it is apparent in Figure 6.11, that the toxicity of AAP in control mice is far greater than in the clofibrate and BSO pretreated mice (black line compared to navy line). Although GSH obviously plays a role in protection of the cell against AAP toxicity, as revealed by the enhancement observed in the GSH depleted control mice, it does not adequately explain the hepatoprotective effect of clofibrate.

In a study by another group investigating the role of GSH in clofibrate protection against AAP, it was suggested that the hepatoprotection was a direct result of an overall increase in liver size which together with an increase in cellular GSH results in a substantial increase in the amount of hepatic GSH available in each mouse (Manautou *et al,* 1994). Indeed, in this study, the amount of GSH in the liver was estimated to increase by 40% (Table 6.2). However, it should be noted that due to circadian variations in both %LBW and GSH, while the amount of GSH present in mouse liver can vary over the course of a year, there was no alteration in the extent of hepatoprotection as a result of clofibrate pretreatment. Also, Chapter 4 revealed no increase in %LBW after 3 days of treatment, yet hepatoprotection was evident at this time, indicating that the onset of protection preceded the increase in liver size. Also, an initial 50% increase in biliary GSH:AAP conjugate formation was reported in clofibrate pretreated mice, and it was proposed that the increased clearance of AAP by this route protects the liver against hepatotoxicity (Manautou *et al,* 1996). As prior administration of DEM depleted GSH to negligible levels in clofibrate pretreated mice, it would be expected that the inability to clear AAP as a GSH conjugate through the biliary route would enhance toxicity. However, it was found in this chapter, that there was no increase in hepatotoxicity observed in clofibrate pretreated mice also administered DEM. It therefore seems unlikely that an increase in GSH conjugation is exclusively responsible for the hepatoprotection following PxP pretreatment.

### 6.4.5 Protection against the renal toxicity AAP by clofibrate

In order to determine whether clofibrate affords protection against AAP toxicity in extrahepatic tissue, the renal toxicity of AAP was also investigated. While not of the same

magnitude as that observed in the liver, the kidneys do exhibit biochemical changes following treatment with PxPs (Sharma *et al,* 1988). At a dose of 400 mg/kg AAP, some renal damage was evident in the control group, but not in the clofibrate treated group. Unlike the liver, the level of GSH depletion by BSO in the kidney was the same in both control and clofibrate treated mice, with both being reduced to 20% of the normal GSH level. The renal toxicity of AAP in control mice, observed as tubular necrosis, was greatly exacerbated by GSH depletion by BSO. In stark contrast, the equivalent depletion of GSH by BSO did not greatly influence AAP renal toxicity in clofibrate pretreated mice and it was estimated that around 4 times the dose of AAP was required to exhibit equivalent renal damage. After the liver, the greatest content of PPARα is found in kidney (Jones *et al,* 1995). As the proximal tubule cells are involved in AAP metabolism and toxicity (Hart *et al,* 1994), it seems that the biochemical changes associated with PxP hepatoprotection have also transpired in the kidney.

### 6.4.6 Conclusion

The aim of the experiments described in this chapter was to investigate the involvement of GSH in the hepatoprotective effect of clofibrate pretreatment. Collectively, the results of the chapter indicate that although GSH probably plays a role in protection against the acute toxicity of AAP, it is unlikely to be the sole mechanism by which clofibrate exerts its hepatoprotective effects. Despite the overall increase in the amount of GSH in the liver, depletion of GSH did not greatly modify the hepatotoxicity of AAP. Interestingly, the protective effect of clofibrate is not exclusive to the liver, with GSH-independent protection observed toward the renal toxicity of AAP. It seems probable that the deactivation of NAPQI involves interaction with some other as yet unidentified nucleophile other than GSH. In order to clarify this further, the *in vivo* toxicity of both a GSH dependent and also a prooxidant hepatotoxicants were investigated in experiments described in the next chapter.

# CHAPTER 7

# EFFECT OF PRETREATMENT WITH CLOFIBRATE ON THE TOXICITY OF OTHER TOXICANTS: *IN VIVO* STUDIES WITH CARBON TETRACHLORIDE AND BROMOBENZENE.

## 7.1 INTRODUCTION

Studies outlined in the previous chapters demonstrated that extensive hepatoprotection was afforded against AAP toxicity by pretreatment with clofibrate. Due to the equivalent level of microsomal activation (Chapter 5), the protective effect was not simply due to a diminished capacity to bioactivate AAP to its toxic metabolite, although there was a decrease in the amount of NAPQI available within the cell to interact with cellular targets. This was indicated by the lower level of GSH depletion occurring in the AAP-poisoned livers of clofibrate pretreated mice and also in the lower level of protein binding and the lack of mitochondrial dysfunction in these animals.

In order to define the hepatoprotection further, the effect of pretreatment with clofibrate on the *in vivo* toxicity of two hepatotoxic halogenated solvents, bromobenzene (BrB) and carbon tetrachloride ($CCl_4$) was investigated. This experiment would establish whether the hepatoprotective effect of clofibrate is extended to chemicals other than AAP. BrB and $CCl_4$ were chosen because of their similarity to AAP in relation to their ability to cause centrilobular necrosis following CYP450 catalysed conversion to reactive metabolites. However, the dissimilarities between the mechanisms by which these xenobiotics are thought to produce toxicity may also provide insights into the nature of clofibrate hepatoprotection. Before describing these experiments, it is necessary to discuss the essential features of $CCl_4$ and BrB induced hepatotoxicity.

Exposure to BrB causes both hepatotoxicity and nephrotoxicity. The metabolism and toxicity of BrB is dependent both on the hepatic levels of specific CYP450 isoforms and on the subsequent detoxication by Phase II enzymes. BrB can be bioactivated to either a 2,3 or a 3,4 epoxide intermediate (Figure 7.1). The CYP1A1 activated 2,3 epoxide spontaneously rearranges to produce 2-bromophenol, a metabolite which has been implicated in the nephrotoxicity associated with exposure to BrB (Monks and Lau, 1988). In contrast, the conversion of BrB to the 3,4 epoxide of BrB is catalysed by CYP2A6 and is associated with the development of hepatotoxicity. At non-toxic doses, over 70% of the 3,4-epoxide is deactivated by conjugation to GSH, and excreted as the GSH derived metabolite, 4-

bromophenyl mercapturic acid (Zampaglione *et al,* 1973). This GSHt catalysed reaction is extremely efficient but it is dependent on the availability of GSH (Zampaglione *et al,* 1973).

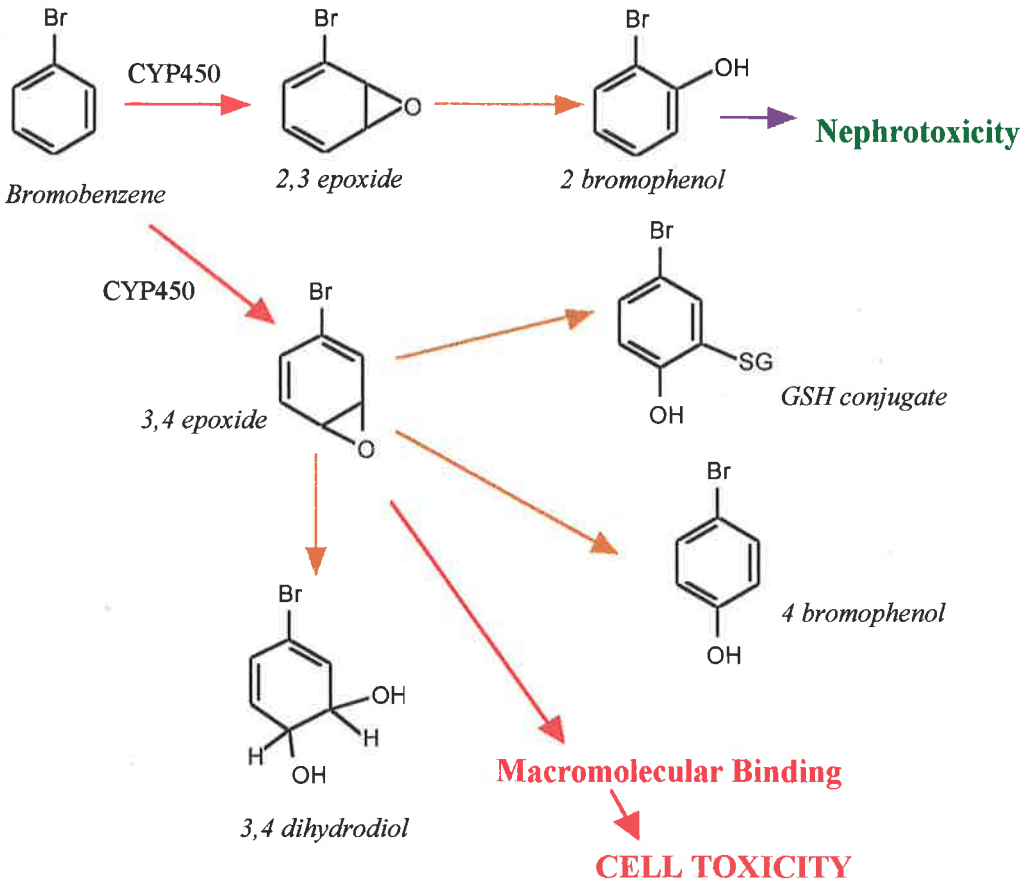



**Figure 7.1:** Schematic diagram of the proposed metabolism of BrB (only the major metabolites are shown). Activation by distinct CYP450 isoforms produces different epoxide intermediates that are thought to produce their toxicity in different organs (derived from Timbrell *et al,* 1994)

The hepatotoxicity produced by BrB is known to be altered by a number of factors that disrupt the balance between Phase I activation and Phase II detoxication. For example, pretreatment with 3-methylcholanthrene, a specific inducer of CYP1A1 ameliorates the hepatotoxicity of BrB by reducing the relative formation of the hepatotoxic 3,4 epoxide compared to the 2,3 epoxide (Lau and Zannoni, 1981). Alternatively, the extent of hepatotoxicity can be diminished by coadministration of agents that increase the thiol status of the liver. Increasing the sulphydryl capacity of the cell gives protection against BrB toxicity, as exemplified by coadministration of the cysteine prodrug, *L*-2-oxothiazolidine-4-carboxylic acid, which enhanced the urinary excretion of the mercapturate metabolite of BrB (Brodeur and Goyal, 1987). Collectively, such data indicate that changes to the levels of individual CYP450 isoforms or the concentration of GSH will alter the hepatotoxicity of BrB in a similar way to that observed with AAP.

Like AAP and BrB, high doses of $CCl_4$ result in centrilobular necrosis however unlike these hepatotoxicants, the metabolism of $CCl_4$ does not involve a decrease in cellular GSH and also

involves more extensive lipid peroxidation (Timbrell, 1994). Furthermore, fat accumulation is also a conspicuous early feature of $CCl_4$ hepatotoxicity which may result from an inhibition of the synthesis of a fatty acid carrier protein or lipoprotein synthesis (Becker *et al,* 1987). The bioactivation of $CCl_4$ to a hepatotoxic intermediate involves homolytic cleavage of a carbon-chloride bond to form the trichloromethyl radical ($CCl_3$•) (Figure 7.2).

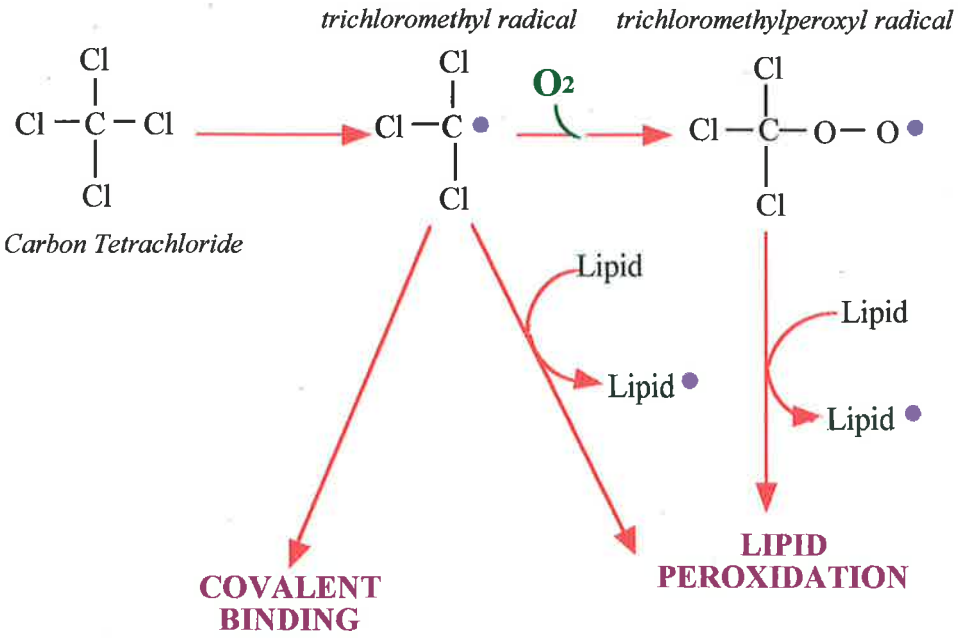



**Figure 7.2:** Proposed biochemical events involved in $CCl_4$ toxicity. The putative ultimate toxic metabolite, the trichloromethylperoxyl radical, is formed by the reaction of the CYP450 generated $CCl_3$• with molecular oxygen (derived from Timbrell *et al,* 1994).

The CYP450 isoform involved in the activation of $CCl_4$ is CYP2E1, which is also the key isoform involved in AAP bioactivation. Once formed, $CCl_3$• can be involved in a number of interactions. $CCl_3$• can deactivate CYP2E1 (Dai and Cederbaum, 1995), which explains the protective effects of low doses against subsequent high doses of $CCl_4$ (Sesardic *et al,* 1989). In addition, $CCl_3$• can covalently bind to cellular macromolecules such as proteins (Reynolds, 1967). Alternatively, it can be reduced to chloroform by the abstraction of a methylene hydrogen from a polyunsaturated lipid to initiate a lipid peroxidation cascade (Recknagel and Glende, 1973). In an aerobic environment, $CCl_3$• can interact with molecular oxygen to form the aggressive trichloromethylperoxyl radical ($CCl_3OO$•). Due to the aerobic nature of the hepatocyte, $CCl_3OO$• would be rapidly formed and predominate over $CCl_3$• (Halliwell and Gutteridge, 1989).

As with $CCl_3$•, $CCl_3OO$• would initiate a chain reaction of lipid peroxidation ultimately resulting in the destruction of cellular membrane structures and is therefore the likely mediator of the lipid peroxidative effects of $CCl_4$. As the toxicity of $CCl_4$ is mediated by a radical intermediate, prior administration of radical scavenging antioxidants, such as α-tocopherol

tocopherol (Biasi *et al,* 1991), ascorbic acid (Maelloro *et al,* 1994) and various flavonoids (Cholbi *et al,* 1991) have been reported to diminish the extent of lipid peroxidation and also the level of toxicity observed. The fact that free radicals play a key role in the induction of hepatotoxicity by $CCl_4$ is a major reason why this compound was chosen for use in the present studies into the hepatoprotection associated with peroxisome proliferation. Its use will provide information on whether an enhanced ability to detoxicate free radicals is associated with the hepatoprotection, while the use of BrB (like AAP) provides information on the ability of these livers to cope with electrophilic metabolites as compared with radicals.

The aim of the experiments described in this chapter was to determine the effect of pretreatment with the PxP, clofibrate, on the acute *in vivo* toxicity of BrB and $CCl_4$ and to compare the results obtained with those described in previous chapters. The 2 hepatotoxicants were each administered to vehicle and clofibrate pretreated mice at 5 different doses and their hepatotoxicity was assessed 24 hours later via measurements of plasma SDH activity and histological analysis of individual livers. In addition, BrB nephrotoxicity was determined by measuring plasma BUN levels.

## 7.2 MATERIALS AND METHODS

### 7.2.1 Materials:

All chemicals used in studies reported in this chapter were of the highest quality commercially available. For a list of chemicals and suppliers, see Appendix 1.

### 7.2.2 Animal treatment and sampling

Swiss White male mice (n = 92) were randomly allocated into 2 subgroups of 46 mice. One group received the standard clofibrate pretreatment protocol of 500mg/kg/day i.p injection for 10 days, while the control group received olive oil vehicle alone. On day 11, 6 mice from each subgroup served as the treatment control. The remaining mice formed 10 groups of n = 4, with each group assigned one of 5 doses of either $CCl_4$ or BrB. Different concentrations of $CCl_4$ (dose range of 0.3 to 1.5g/kg $CCl_4$, in 0.3g/kg increments) and BrB (dose range of 0.3 to 0.9g/kg BrB in 0.15g/kg increments) were prepared as olive oil suspensions for injection at 10ml/kg. The treatment control group (0 mg/kg) received olive oil vehicle alone. All mice were injected between 9.00 and 10.00 am and blood samples were collected 24 hours later. Liver samples were obtained for histological analysis as outlined in Chapter 3. Plasma was collected following centrifugation at 3 000 xg for 5 minutes and stored at -20°C. SDH and BUN enzyme analysis was completed within 36 hours of collection.

### 7.2.3 Plasma Assays

Plasma SDH activity and BUN level were determined using methods outlined in Section 3.2.5.2 and Section 6.2.8.2, respectively.

### 7.2.4 Statistical Analysis

All data were analysed by ANOVA. If a significant variance was observed ($P<0.05$), the data was further analysed using the Bonferroni Post Hoc test for pairs of data.

## 7.3 RESULTS

### 7.3.1 Effect of clofibrate pretreatment on BrB toxicity

The first hepatotoxicant to be investigated was BrB, whose toxicity involves the formation of CYP450 mediated metabolites (Figure 7.1). Administration of BrB was found to cause acute hepatotoxicity in control mice as illustrated by the increased plasma SDH activities observed 24 hours after BrB administration (Figure 7.3). The toxic response was not strictly dose related as toxicity seemed attenuated at intermediate doses of 0.6 and 0.75 g/kg (Figure 7.3).

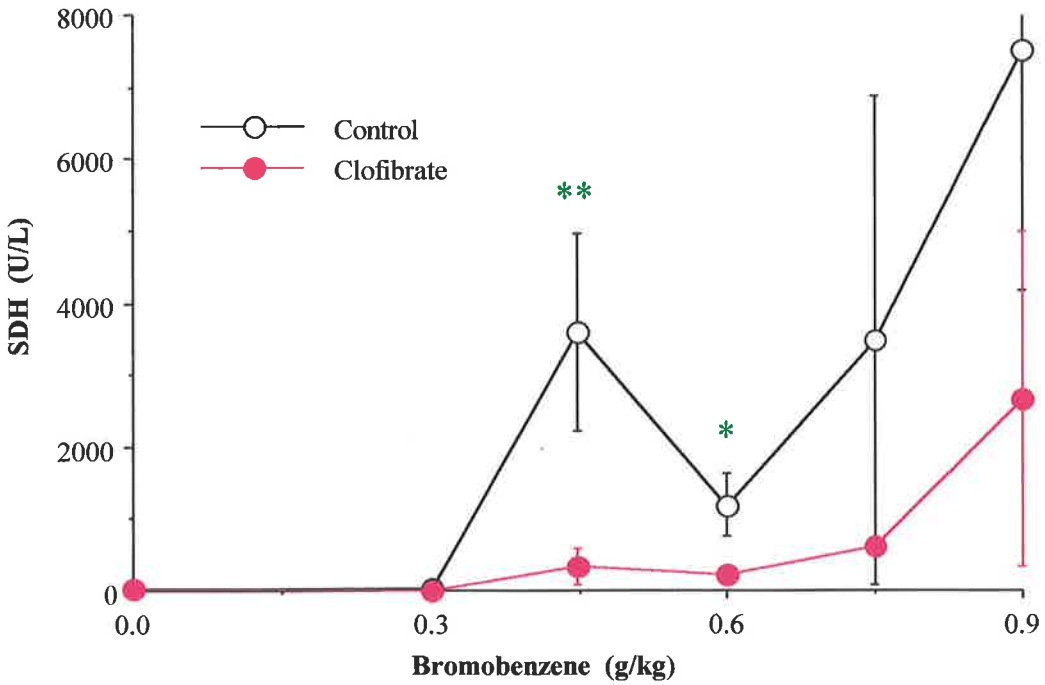



**Figure 7.3:** Plasma SDH levels in control and clofibrate pretreated mice 24 hours after the administration of 0 - 0.9 g/kg BrB (Results expressed as mean±s.e.m, n=2-4, *=P<0.05, **=P<0.01, Bonferroni Post Hoc test)

Importantly, the livers of clofibrate pretreated mice seemed resistant to BrB induced toxicity, with a statistically significant 80 % reduction in plasma SDH activities evident in the plasma of the clofibrate versus vehicle pretreated mice following the 0.45 and 0.6 g/kg doses. At the 2 highest doses of 0.75 and 0.9 g/kg, there was no difference in plasma SDH activities of mice

between the two pretreatment groups, where a 50% mortality in both groups was noted. The hepatoprotection against BrB afforded by clofibrate pretreatment did not seem as great as previously seen with AAP (Chapter 3), were no cell damage or mortality was observed in clofibrate pretreated mice given doses lethal to control mice. However, this conclusion is tempered by the fact that the lethality accompanying the use of high doses introduced considerable variation into the data. As noted in the preceding chapter, constraints imposed on this work by the University of Adelaide Animal Ethics Committee precluded the option of using larger animal numbers in an effort to offset the effects of this variability. Representative histological slides obtained from mice that received 0.6g/kg BrB are shown in Figures 7.4A and 7.4B. Fat containing vacuoles were observed within periportal parenchymal cells in the livers of BrB intoxicated mice. In contrast to the centrilobular toxicity induced by BrB, there appeared to be no difference in the extent of this adverse effect between control and clofibrate treated groups.

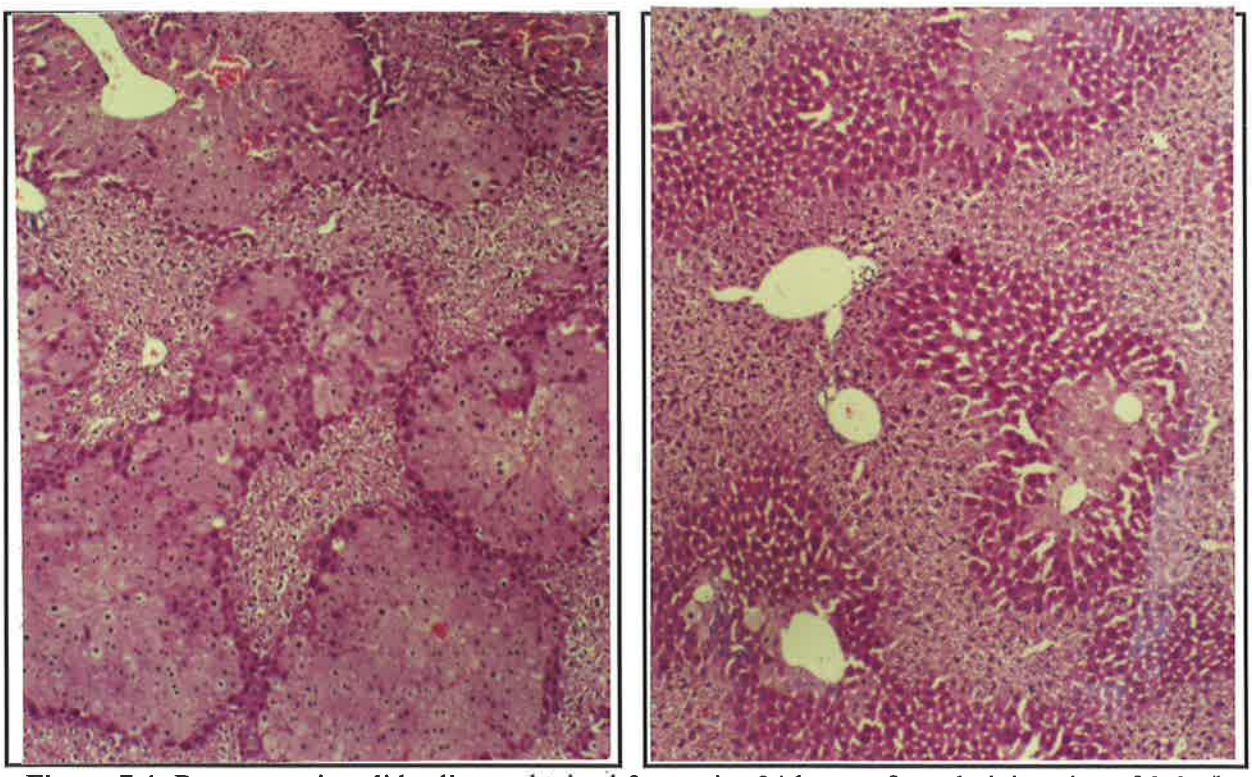


**Figure 7.4:** Representative slides livers obtained from mice 24 hours after administration of 0.6 g/kg BrB. **Panel A):** (left) control mouse **Panel B):** (right) corresponding liver slice prepared from a clofibrate pretreated mice (x40).

Morphological assessment of livers from BrB poisoned, vehicle pretreated mice revealed both centrilobular and periportal alterations. Loss of cell definition in the centrilobular region was indicative of necrosis, thus confirming the hepatotoxicity suggested by the plasma SDH activity measurements. In addition, the livers of clofibrate pretreated mice appeared less damaged than those of control mice. A reduction of necrotic area was evident in the centrilobular regions of clofibrate pretreated mice that received both 0.45 and 0.6 g/kg BrB

doses in clofibrate treated mice when compared to control mice that received the same dose of toxicant.

In addition to hepatotoxicity, there appeared to be dose related renal toxicity occurring in control mice that received BrB, as evidenced by the increases in the plasma levels of BUN (Figure 7.5). None of the clofibrate pretreated mice exhibited any nephrotoxicity at any dose of BrB, which was suggestive of a protective effect of clofibrate against BrB nephrotoxicity.

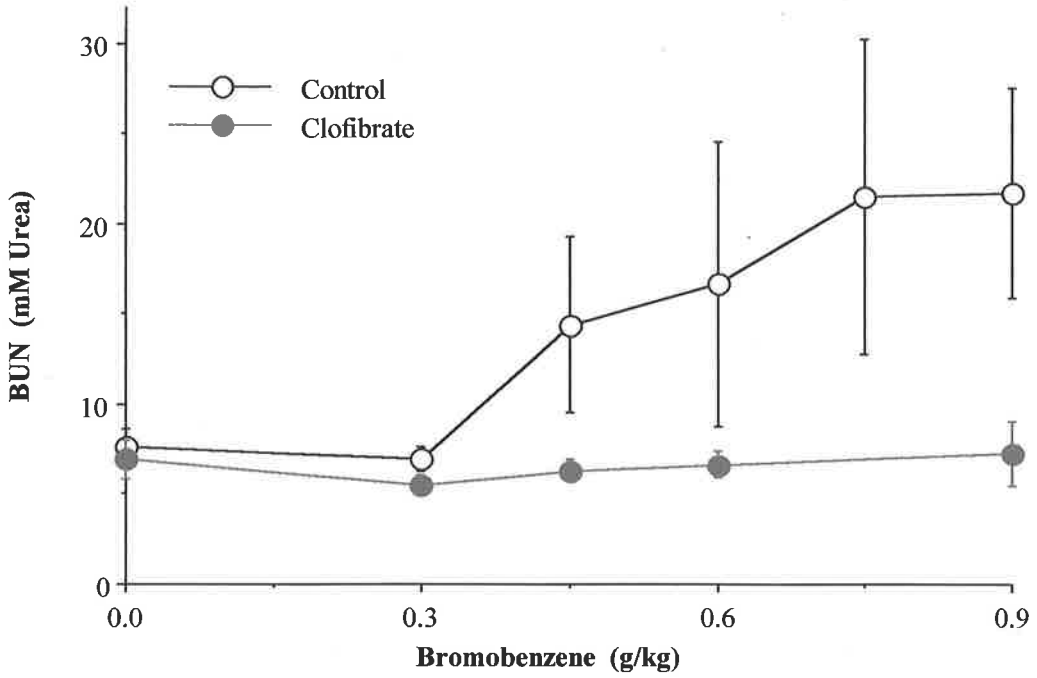



**Figure 7.5:** Plasma BUN levels in samples collected 24 hours after administration of BrB 0 - 0.9 g/kg in control and clofibrate pretreated mice. (Results expressed as mean ± s.e.m, n=2-4).

### 7.3.2 Effects of clofibrate pretreatment on $CCl_4$ toxicity

The second hepatotoxicant investigated was the chlorinated solvent $CCl_4$, administration of which causes a characteristic steatosis, lipid peroxidation and centrilobular necrosis (Becker *et al,* 1987; Timbrell, 1994). As with BrB, a degree of protection was afforded against the hepatotoxicity of $CCl_4$ in clofibrate pretreated mice (Figure 7.6) and similarly, the effect was not as clear as was observed with AAP (Figure 3.4). In control mice, $CCl_4$ produced dose dependent hepatotoxicity as evidenced by an increase in plasma SDH activity (Figure 7.6). Maximum plasma SDH values were obtained with the 0.6 g/kg dose of $CCl_4$, with higher doses not increasing the SDH activity over these values.

Pretreatment with clofibrate produced a clear shift of the plasma SDH curve to the right, although a statistically significant decrease was only observed at the 0.6 g/kg dose (Figure 7.6). Comparison of the $CCl_4$ doses required to cause a half maximal elevation in plasma SDH

activity in the control compared to clofibrate pretreated mice shows that a more than 2 fold greater dose was required to produce an equivalent level of toxicity.

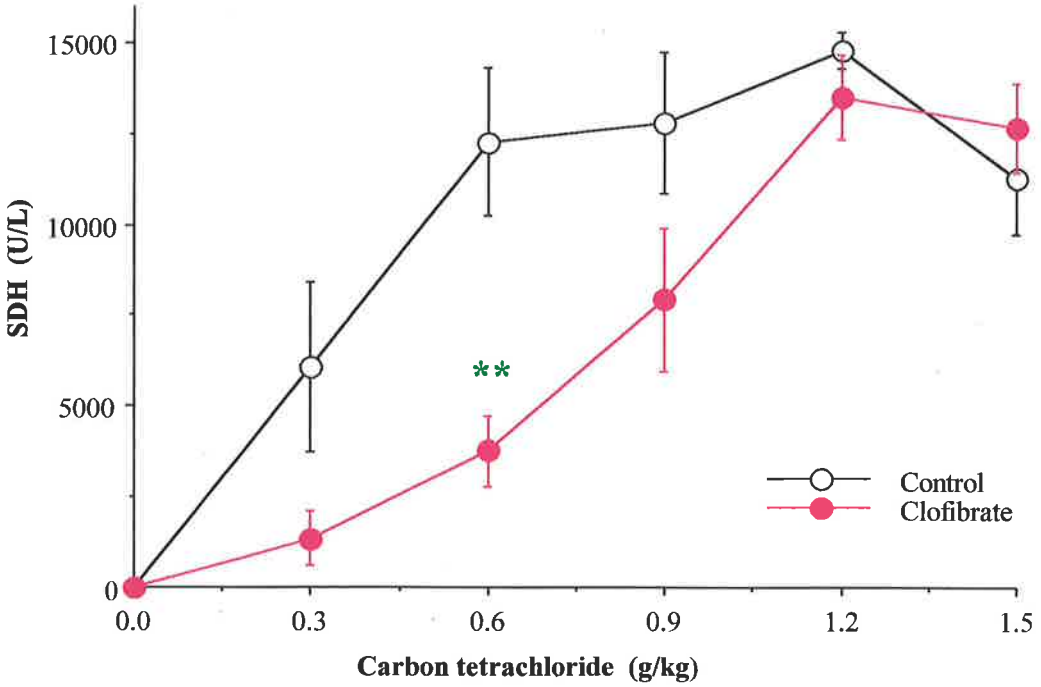



**Figure 7.6:** Plasma SDH levels in control and clofibrate pretreated mice 24 hours after intoxication with 0-1.5g/kg $CCl_4$. Results are expressed as mean±s.e.m, n=4, **=P<0.01 Bonferroni Post Hoc test).

The hepatoprotective effect against $CCl_4$ was confirmed by histological analysis of the livers, with a clear reduction in the extent of necrosis evident in the centrilobular region in clofibrate pretreated compared to control livers (Figure 7.7 A & B). The necrosis occurring in the centrilobular region of clofibrate treated mice also included the presence of swollen "balloon" cells. This type of specific cell alteration has been reported in both AAP and also $CCl_4$ centrilobular necrosis (Miller *et al,* 1978) and has an unknown aetiology. Evidence of steatosis was observed at higher concentrations in both groups of mice.

## 7.4 DISCUSSION

The experiments outlined in this chapter investigated the effect of pretreatment with the PxP, clofibrate, on the acute hepatotoxicity of BrB and $CCl_4$. Like AAP, both these xenobiotics produce centrilobular hepatotoxicity secondary to bioactivation by CYP450. In general, the results obtained suggest that clofibrate protects against hepatotoxicants whose mechanisms of action involve similarities to AAP, although the extent of protection observed for either $CCl_4$ or BrB was not as pronounced as that observed with AAP.

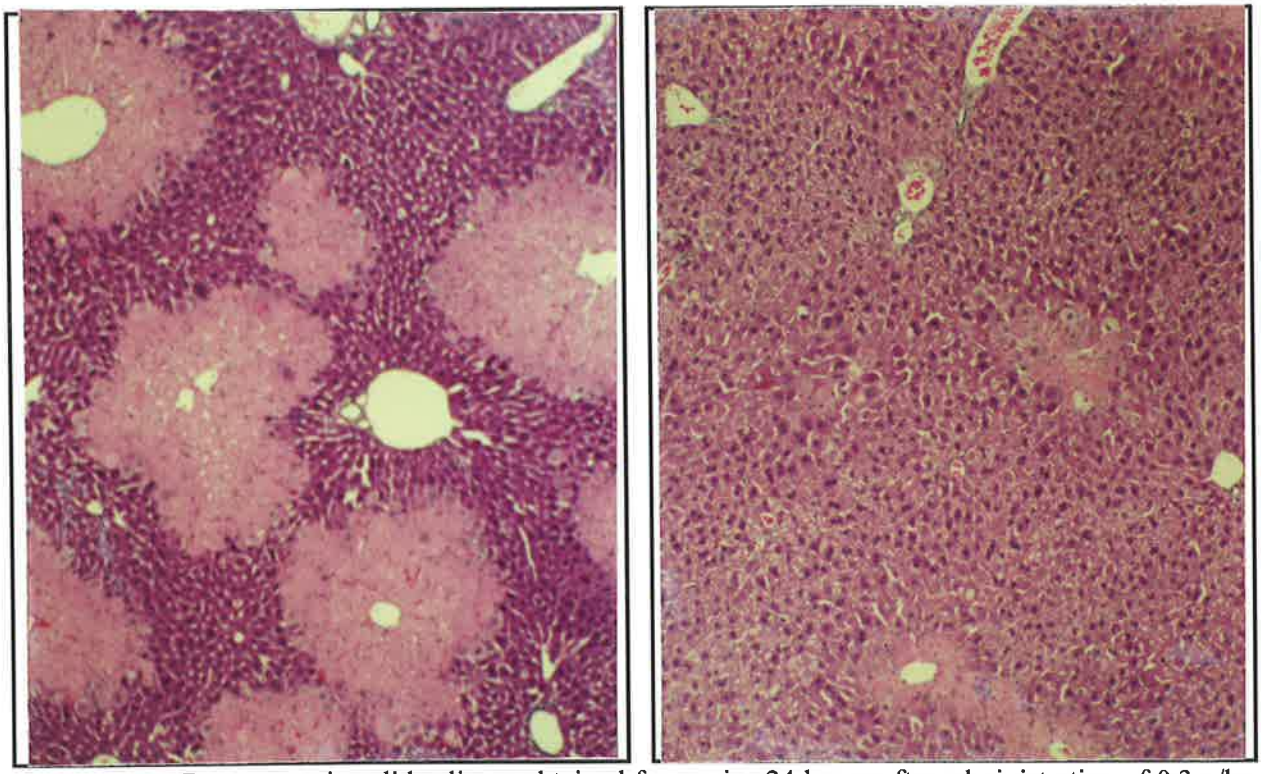


**Figure 7.7:** Representative slides livers obtained from mice 24 hours after administration of 0.3 g/kg $CCl_4$. **Panel A):** (left) control mouse **Panel B):** (right) corresponding liver slice prepared from a clofibrate pretreated mice (x40).

### 7.4.1 Effect of clofibrate pretreatment on the toxicity of BrB

There are certain similarities in the hepatotoxicity produced by AAP and BrB. For example, both undergo CYP450 catalysed bioactivation to electrophilic intermediates that alkylate cellular macromolecules (Timbrell, 1994). Furthermore, the toxicity of both compounds can be modified by CYP450 induction (Zampaglione *et al,* 1973; Jollow *et al,* 1974; Lau and Zannoni, 1981; Hetu *et al,* 1982) and by altering the sulphydryl status of the liver (Jollow *et al,* 1974; Thor *et al,* 1978; Thor *et al,* 1979; Monks *et al,* 1982; Brodeur and Goyal 1987). In this present study, pretreatment with clofibrate was observed to protect against BrB-induced elevations in plasma SDH activities at low but not high BrB doses (Figure 7.3). At higher BrB doses, despite the clofibrate groups exhibiting lower SDH values, the large degree of variability as a result of the low sample size (due to mortality), hindered detection of statistical significance. The hepatoprotective effect at lower doses was confirmed by histological analysis where a reduction in necrotic area was seen in the centrilobular but not the periportal regions of the liver lobule (Figure 7.4).

A number of explanations can be suggested to account for the mild hepatoprotection against BrB toxicity that was afforded by clofibrate. The metabolism and precise mechanism of BrB toxicity remains somewhat obscure. It is known to involve formation of a number of different metabolites of varying organ toxicities, and this is probably reflected in the lack of a clear

dose to toxicity relationship (Figure 7.3). Due to the complex metabolism of BrB *in vivo*, and the uncertainty surrounding the exact mechanisms whereby BrB causes toxicity, it is difficult to decide which of these mechanisms is the most plausible.

One obvious possibility is that the metabolic fate of BrB is altered in the livers of clofibrate pretreated mice, with a shift in the relative balance between the production of hepatotoxic versus nonhepatotoxic metabolites to a less toxic combination. Treatment of rats with the PxP, silvex, caused a 3.5 fold increase in CYP1A1 activity (Ahokas *et al,* 1995). Although this extent of induction is less than that observed by classic CYP1A1 inducers, it is possible that such induction could alter the balance of metabolites by increasing the formation of the less hepatotoxic 2,3-epoxide (Zampaglione *et al,* 1973), and thus account for the slight hepatoprotection observed. If this was the case, then it would be expected that an enhanced nephrotoxicity would accompany the increased formation of the 2,3-epoxide. However, contrary to such expectation, clofibrate pretreatment provided protection against the nephrotoxicity of BrB. Consequently, it seems unlikely that a shift in the production of the CYP450 metabolites toward the nephrotoxic 2,3-epoxide and away from the hepatotoxic 3,4-epoxide accounts for the hepatoprotection produced by clofibrate pretreatment.

A second possible protective mechanism could be that the ability of clofibrate pretreated livers to detoxicate hepatotoxic metabolites formed from BrB is enhanced. Since the major pathway involved in the detoxication of the hepatotoxic 3,4-epoxide involves GSHt-catalysed GSH conjugation, it is conceivable that this route of detoxication is enhanced in clofibrate pretreated livers. However, given that marked changes in the activity of various GSHt isoforms did not accompany clofibrate administration (Section 6.3.3), this explanation also seems unlikely.

A further argument against a role for GSH in clofibrate associated hepatoprotection relates to the role that this tripeptide plays in the nephrotoxicity of BrB. In contrast to the role of GSH in detoxifying the hepatotoxic 3,4-epoxide metabolite, GSH is actually involved in the bioactivation of BrB to nephrotoxic metabolites (den Besten *et al,* 1994). The 2,3 epoxide formed from BrB is converted to 2-bromophenol which then forms a quinone derivative which reacts with GSH to form the nephrotoxic species, 2-bromo-(diglutathionyl-S-yl) hydroquinone (Monks *et al,* 1985). This contribution of GSH to the bioactivation of BrB to nephrotoxic metabolites explains why coadministration of GSH depleting agents, such as DEM, typically enhances BrB hepatotoxicity while protecting against nephrotoxicity (Rush *et al,* 1984). The decreased hepatotoxicity and nephrotoxicity was contrary to expectation if the protection observed was a result of GSH oversupply.

The covalent binding of the reactive metabolite(s) of BrB to critical protein targets have been implicated in the generation of BrB toxicity (Manataou *et al,* 1995) and therefore clofibrate pretreatment may have modified the pattern of protein interactions. It is of interest to note that one of the major protein targets of NAPQI, the reactive intermediate of AAP, is a 58 kDa cytosolic protein (Bartelone *et al,* 1992, Pumford *et al*, 1992) that has also been observed to be a major protein binding target of the reactive intermediate of BrB (Manataou *et al,* 1995). Although any potential role of this protein in the development of necrosis is yet to be established, pretreatment with clofibrate was found not to alter the cellular amount of the 58 kDa protein (Manataou *et al,* 1994).

Another possibility for the protective mechanism in clofibrate pretreated livers could involve an enhanced ability to cope with oxidative stress and lipid peroxidation. A number of studies have suggested that oxidative mechanisms may be more important in BrB toxicity than covalent binding to tissue macromolecules. For example, a 2.8 fold increase in microsomal $H_2O_2$ production has been observed *ex vivo* following BrB intoxication in rats (Behyl and Meyer, 1980). In addition, a number of antioxidants have been found to protect against BrB toxicity. These include; the α-tocopherol analogue, trolox (Casini *et al,* 1985), 7,8-dihydroflavone (Paya *et al,* 1993) and zinc-metallothionein polymer (Koterov and Kon, 1995) and both DPPD and DFO which protect against the toxicity of BrB without altering the extent of BrB covalent binding (Coleman *et al,* 1990). Administration of BrB has also been shown to decrease cellular α-tocopherol levels prior to an increase in lipid peroxidation (Maellaro *et al,* 1990). Such findings concerning a possible role for oxidative stress in BrB toxicity suggest that an enhanced ability to detoxicate oxidative stress mediators might be associated with pretreatment with PxPs and therefore could account for the moderate protection against BrB seen in the present experiments.

It remains possible that an enhanced production of oxidative stress mediators, such as $H_2O_2$ seen during BrB toxicity is occurring as a secondary consequence of the depletion of cellular GSH. It is well established that GSH normally participates in the detoxication of such species, so a decrease in its intracellular concentrations can shift the balance of oxidative stress occurring in the tissue (Timbrell, 1994). For example, the GSH-depleting agent, DEM, which is neither bioactivated nor covalently bound to cellular proteins, has been reported to enhance lipid peroxidation simply as a secondary consequence of GSH depletion (Casini *et al*, 1985). In a similar fashion, increased production of oxidative stress byproducts as a secondary consequence of GSH depletion, rather than an active over-production of reactive intermediates may mediate BrB toxicity.

Whether the degree of oxidative stress that occurs in response to a loss of GSH is sufficient to explain the hepatotoxicity of BrB is open to question. Protection by various antioxidants

against the toxicity of xenobiotics including allyl alcohol (Miccadei *et al,* 1988a; Silva and O'Brien, 1989), diquat (Petry *et al,* 1992), methylmethanesulfonate (Mizumoto *et al,* 1993) and naphthazine (Öllinger and Brunk, 1995) occurs without preventing the loss of cellular GSH that accompanies exposure to these compounds. Although not excluding the possibility that GSH depletion and lipid peroxidation are independent events, these studies indicate that antioxidants provide protection via GSH independent means and thus lipid peroxidation is likely to be a relatively late event in the biochemical toxicity of these xenobiotics.

In summary, the data obtained using BrB as a model hepatotoxicant suggests that clofibrate pretreatment provided mild protection against the toxicity of this xenobiotic, although the effect was less pronounced than with AAP. While the nature of the biochemical alteration underlying hepatoprotection remains unclear, the conclusion that changes in GSH homeostatis are not exclusively responsible for clofibrate induced hepatoprotection was supported.

### 7.4.2 Effect of clofibrate on $CCl_4$ toxicity

As discussed in the Introduction of this chapter, the hepatotoxicity of $CCl_4$ is dependent upon the CYP450 catalysed formation of a trichloromethyl radical. This radical rapidly adds $O_2$ to form a peroxyl radical which has the capacity to undergo one electron reactions with tissue macromolecules, in contrast to the 2 electron transfer reactions of the electrophilic intermediates formed from AAP and BrB. The toxicity of $CCl_4$ can be enhanced by treatment with CYP450 inducers including phenobarbitone (Garner and MacLean, 1969) and ethanol (Hasumura *et al,* 1974) and decreased by CYP450 inhibitors such as SKF-525A. In addition, the lipid peroxidation and toxicity of $CCl_4$ is ameliorated by antioxidants, such as DFO, that limit the availability of iron (Younes and Siegers, 1985) and the chain breaking $\alpha$-tocopherol (Biasi *et al,* 1991). Also, unlike AAP or BrB, the concentration of cellular GSH is not a major factor during the hepatotoxicity of $CCl_4$. Some *in vitro* studies have shown that a product of $CCl_4$ metabolism, phosgene can be conjugate by GSH, yet this probably represents only a minor pathway (Timbrell, 1994). In addition, lipid peroxides, appear to be comparatively poor substrates for GSHPx and therefore there is no marked increase in GSSG during $CCl_4$ intoxication (Lauterburg *et al,* 1984). However, some studies have suggested a role for GSH due to a potentiation of $CCl_4$ toxicity following pretreatment of mice with GSH depleting agents such as DEM (Ruch *et al,* 1986). Overall, the hepatotoxic effects of $CCl_4$ appears related to the formation of radical intermediates. Consequently, protection against centrilobular necrotic damage can be achieved by inhibition of bioactivation to the radical or coadministration of antioxidants to deactivate the radical prior to interaction with cellular macromolecules.

The contribution of free radicals to its hepatotoxic effects made $CCl_4$ a useful tool for studying the effects of PxP pretreatment on the livers susceptibility to xenobiotics. It was

found that pretreatment with clofibrate provided some protection against the acute *in vivo* toxicity of $CCl_4$, as indicated by both plasma SDH levels and morphological alterations. The dose response curve for plasma SDH activity appeared to shift to the right, although a significant difference between the 2 treatment groups was only observed with the 0.6 g/kg dose of $CCl_4$ (Figure 7.5). This level of protection is notable, given that GSH is commonly thought to be less effective in preventing toxic reactions induced by $CCl_4$ than those caused by either AAP or BrB. This provides additional evidence for the lack of an exclusive involvement by GSH in clofibrate induced hepatoprotection.

As there have been reports of antioxidants protecting against AAP, BrB and $CCl_4$ toxicity, it is possible that PxP pretreatment has increased hepatocellular levels of an endogenous molecule with antioxidant properties. However, all endogenous antioxidants so far investigated have been shown to be either marginally increased or even reduced by PxP pretreatment (Chapter 1). Therefore, if overproduction of an antioxidant does account for the hepatoprotection produced by clofibrate, its identity has yet to be established.

### 7.4.3 Conclusion

In this chapter, the toxicity of the centrilobular hepatotoxicants, $CCl_4$ and BrB were compared to those of AAP following pretreatment with the PxP, clofibrate. A mild degree of protection was seen against both hepatotoxicants, although the effect was not as strong as was observed with AAP. These experiments provided only limited insights into the possible protective mechanisms involved, although they did further suggest that GSH is not the sole mediator of protection.

On the basis of these findings, and in order to more carefully study the role of GSH and other antioxidants in hepatoprotection, it was decided to conduct subsequent investigations using an isolated hepatocyte model. A major purpose of these experiments was to establish whether the protective mechanism is retained in hepatocytes obtained from clofibrate pretreated mice subjected to isolated cell culture. This will address the question as to whether the hepatoprotection following clofibrate pretreatment is strictly an *in vivo* phenomenon. In addition, since far greater manipulation of experimental variables is possible with isolated hepatocytes, this model will enable the role of GSH in protection to be more carefully examined.

# CHAPTER 8

# THE USE OF ISOLATED MOUSE HEPATOCYTES TO INVESTIGATE THE ACUTE TOXIC RESPONSE OF PARACETAMOL FOLLOWING CLOFIBRATE PRETREATMENT.

## 8.1 INTRODUCTION

Although the use of other *in vivo* hepatotoxicants revealed that pretreatment with clofibrate provided some protection against toxicants with different metabolism to that of AAP, the nature of the protective effect remained unclear. Further elaboration would be obtained by increasing the survey of toxicants which cause hepatic damage, however such experimentation requires that *in vitro* methodologies be considered. As an example, the *in vivo* toxicity of paraquat is characterised by fatal lung toxicity in addition to a number of other systemic effects, including hepatic and renal necrosis (Haley, 1979). It is impossible to observe and investigate the hepatotoxicity of paraquat *in vivo*, without the pathological complications associated with lung damage. This situation can be overcome by investigating the lethality of such toxicants using isolated hepatocyte methods.

Isolated hepatocytes provide a useful alternative to *in vivo* investigations by allowing delineation in toxic responses not achievable in the whole animal. They retain the essential biochemical properties of the intact liver and contain oxidative and conjugative enzymes in the correct cellular locations (Berry *et al,* 1991). The external environment of the cell (incubation media) is easy to manipulate and readily assessable for metabolite studies. Cytotoxicity parameters including thiol depletion, protein and nucleic acid alkylation and lipid peroxidation can also be readily quantitated. Furthermore, hepatocytes from one animal can be subcultured in small batches, providing the opportunity to investigate the interrelationships between a number of cellular events simultaneously. Finally, the use of isolated hepatocytes removes complicating pharmacokinetic factors, such as absorption and disposition, that influence the amount of xenobiotic that reaches the cell. As a potential mechanism of hepatoprotection, PxP pretreatment may have altered the systemic handling of xenobiotics and thus the hepatoprotection could have simply been due to *in vivo* dispositional changes. Study of AAP toxicity in hepatocytes isolated form clofibrate pretreated mice should either support or dismiss this possibility.

The aims of experiments described in this chapter are;

1) to establish a method for the short term incubation of primary cultures of mouse hepatocytes obtained from clofibrate pretreated mice.
2) to compare the acute lethality of AAP in isolated hepatocytes from control and clofibrate pretreated mice
3) to provide further information on conclusions drawn from earlier studies concerning AAP metabolism and the role of GSH in AAP toxicity in clofibrate induced hepatoprotection.

The first aim requires finding a method for producing peroxisome proliferated hepatocytes in culture for comparison with control hepatocytes. This can be achieved by either administering clofibrate to the animal prior to hepatocyte isolation, or adding the PxP to the culture media following their isolation. The latter approach requires exposure of cells to PxP for a number of days (Gray *et al,* 1983; Kocarek and Feller, 1989; Tomaszewski *et al,* 1990). However, long term culture of hepatocytes alters the level and activity of enzymes involved in xenobiotic metabolism. Since investigation of xenobiotic toxicity following PxP treatment is a major aim of these experiments, it is preferable to use hepatocytes that are most like those in the *in vivo* situation. Also, acute toxicity studies require relatively short incubations and it is anticipated that the AAP toxicity studies will be completed within 8 hours. Therefore it was decided to use hepatocytes that were isolated by collagenase digestion, from animals that had already been pretreated with and biochemically altered by clofibrate.

To investigate toxicant lethality a method for determination of cell death needed to be established. Due to the vast array of biochemical events that occur prior to cell death, an actual definition of what constitutes cell death is difficult. A useful cytotoxic endpoint is measurement of the intactness of the cell membrane, which loses integrity either as a result of cytotoxicity or as a consequence of cell death. Cell membrane integrity can be measured either by exclusion assays, such as permeability to the nuclear dye, Trypan blue or by measuring the leakage of cytosolic enzymes into the surrounding media. The most commonly used enzyme is lactate dehydrogenase, a robust cytosolic enzyme that is easy to quantitate via biochemical assay procedures.

By devising a method for the isolation and incubation of clofibrate pretreated hepatocytes, it will be possible to investigate toxicants deemed unsuitable for *in vivo* investigation. However, it must first be established that clofibrate protection is in fact observed *in vitro* and thereby dispel the possibility that the hepatoprotection is simply due to alterations in *in vivo* toxicant disposition. A concentration:lethality experiment was thus performed where the extent of cell viability was assessed by measurement of %LDH leakage into the culture media at certain time points following the commencement of exposure to AAP.

Finally, due to the flexibility of the isolated hepatocyte model, further repetition of previous studies were possible. The ability of clofibrate cells to replete GSH following acute depletion with DEM and the depletion of GSH by various concentrations of AAP enabled investigation of the cells abilities to replenish GSH. The ability to use a number of time and dose points from plates prepared from one animal overcame the problems due to individual variation in GSH levels. In addition, experiments performed in Chapter 5 suggested that decreases in urinary metabolites at high AAP doses in control mice compared to clofibrate pretreated mice was due to acute liver toxicity. This possibility was directly investigated in hepatocytes by measurement of the formation of both the glucuronide and GSH metabolite of AAP with concurrent determination of cell lethality. This study was extended by determining both metabolite formation and cellular lethality following depletion of hepatocyte GSH by DEM prior to AAP application. It was anticipated that on the basis of conclusions drawn in Chapters 5 and 6, GSH depleted clofibrate pretreated cells would exhibit resistance to AAP induced cell killing.

## 8.2 MATERIALS AND METHODS

### 8.2.1 Materials

All chemicals used in this chapter were the highest quality commercially available. For a full list of chemicals and suppliers, refer to Appendix 1.

### 8.2.2 Animal Treatment

In all experiments reported in this chapter, adult male Swiss mice (35-40g) were treated with either the PxP clofibrate 500mg/kg i.p for 10 days or equivolume olive oil vehicle alone for control mice and housed in accordance to conditions specified in Chapter 2.

### 8.2.3 Isolation of mouse hepatocytes

Hepatocytes were isolated by collagenase digestion and plated onto collagen coated petri dishes, by a method based on the procedure of Adamson and Harman (1989). Plated cells were maintained at 37°C in 5% $CO_2$ at all times during the experiment. Cell viability was assessed by measuring lactate dehydrogenase (%LDH) leakage into the culture media.

#### *8.2.3.1 Solution preparation for isolation of hepatocytes and incubation procedures*

The isolation of hepatocytes requires the use of 3 separate modified Krebs Henseleit buffers. The *standard* Krebs-Henseleit buffer contains 116 mM sodium chloride, 5.4 mM potassium chloride, 0.8 mM magnesium sulphate, 0.4 mM potassium dihydrogen orthophosphate, 0.3 mM disodium hydrogen orthophosphate, 26 mM sodium bicarbonate and 20 mM HEPES, pH 7.3. The buffer is stored at 4°C until use.

The 3 Krebs Henseleit buffers involved in the isolation procedure were prepared fresh on the day of use.

| | Description | Additions |
|---|---|---|
| A | Calcium-free buffer | 1 mM EDTA |
| B | Digesting buffer | 1 mM $CaCl_2$, 0.2 mg/ml collagenase, 10 µg/ml DNase |
| C | Washing buffer | 1 mM $CaCl_2$, 10 µg/ml DNase |

*8.2.3.2 Preparation of RPMI-1640*

RPMI-1640 media were prepared in accordance to the manufacturer. For these experiments, 10 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin was added to RPMI-1640 solution and the pH was adjusted to 7.2. RPMI-1640 was sterilised by filtration through a sterile 0.22 µm Millipore filter unit and stored in autoclaved bottles, at 4°C until use.

*8.2.3.3 Preparation of Hank's buffered salt solution*

Hank's buffered salt solution containing 0.1 M sodium chloride, 5.4 mM potassium chloride, 0.33 mM disodium hydrogen orthophosphate and 0.44 mM potassium dihydrogen orthophosphate, was prepared at pH 7.3. Phenol red (10 mg/l) is included for ongoing pH indication. The prepared solution is stored at 4°C in an Oxford dispenser bottle for use.

*8.2.3.4 Preparation of acid soluble rat tail collagen*

The tails from 10 rats (>400g) were collected after euthanasia and frozen at -20°C until use. Tails were skinned and white ligamentous tendons stripped free. The combined tendons were added to 1 l of 0.2% glacial acetic acid and then stirred gently for 3 days at 4°C. The resulting solution was filtered to remove insoluble material. The soluble collagen was precipitated by addition of sodium chloride and collected by centrifugation at 3 000 x g for 15 minutes. The collagen was redissolved in 0.2% glacial acetic acid and stored at 4° C until use. Each batch of collagen varies in its ability to both coat plates and adhere cells. For this reason, a trial experiment using different dilutions of collagen stock was always performed prior to use. In general, it was found that a 1 in 3 dilution of collagen stock into 0.2 % glacial acetic acid was required.

*8.2.3.5 Preparation of plates for cell culture*

Plastic ethylene dioxide sterilised petri dishes of 5 cm diameter were used in these experiments. Plates were prepared 12 to 16 hours before use. Approximately 10 ml of diluted

rat tail collagen was poured into a petri dish and then poured into subsequent dish, leaving behind around 0.5 ml of collagen solution. After 10 minutes drying time, the plates were vigorously shaken in a horizontal direction to ensure the plate was evenly coated with collagen. The plates were then air dried overnight. The plates were exposed to 5 minutes of UV light for decontamination, prior to use.

### 8.2.4 Isolation of mouse hepatocytes by collagenase perfusion and preparation of hepatocyte monolayers.

Following pretreatment, on day 11, control and clofibrate mice were anaesthetised with 30 mg/kg nembutal containing 1250 U of heparin by i.p. injection. A longitudinal midline incision was made in the abdomen and the inferior vena cava was cannulated. The thoracic cavity was opened and central vein clamped prior to commencement of perfusion. The hepatic vein was snipped to allow outflow. The liver is perfused *in situ* for 2 minutes with Buffer A at 4 ml/min, followed by perfusion with Buffer B up to a maximum of 5 minutes duration. Extended perfusion at either of these steps resulted in lower cell viability.

The liver was then carefully excised and placed in a petri dish filled with Buffer C. The remainder of the procedure was completed in a laminar flow cabinet. Hepatocytes were released by gently teasing apart the liver with forceps, and filtered through 400 μm followed by 100 μm nylon mesh gauze. Cells were placed in sterile 50 ml centrifuge tubes and centrifuged at 60 xg for 2 minutes. The supernatant (containing nonviable cells) was aspirated off, cell pellet washed with two additional rounds of centrifugation in fresh buffer C. After the final wash, hepatocytes were resuspended in up to 75 ml of RPMI-1640 and their viability was checked by Trypan Blue exclusion (routine viability was >85%). Cells were plated in 3 ml aliquots onto collagen coated petri dishes (maximum of 24 plates prepared in any one experiment) and immediately placed in a 37°C 5% $CO_2$ incubator. After approximately 90 minutes adhesion time, the medium was aspirated and the attached cells were gently washed at least twice with 3 ml aliquots of Hank's buffer. A 3 ml aliquot of RPMI-1640 (with or without toxicant) was applied to individual plates and then they were returned to the $CO_2$ incubator for the duration of the experiments.

### 8.2.5 Dose versus lethality relationship of AAP.

To investigate AAP cytotoxicity, a 3 ml aliquot of RPMI-1640 containing 0 to 3 mM AAP was applied to hepatocyte monolayers and incubation was commenced. At 1, 2, 4 and 8 hours after AAP administration, 60 μl aliquots were taken for the determination of LDH release. After the 8 hour aliquot, 0.24 ml of 2 % Triton-X-100 was added to each plate and the cells were lysed by rubbing the plate with the bevelled end of a piece of latex tubing. A 1 ml volume of lysate was centrifuged at 3,000 xg for 2 minutes to pellet cellular debris prior to

assay. This lysed sample constitutes the "total-LDH" activity of the sample. Cell viability was determined by measurement of % LDH leakage into the incubation media for each time point, expressed as a percentage of total LDH activity. The dose-lethality experiment was repeated 6 times. Complete graphical results for this experiment are shown in Appendix 4. Statistical analysis was performed on 4 hour results combined with those from 2 other studies performed under identical conditions (n=15) and are illustrated in Figure 8.1.

*8.2.5.1 % LDH leakage for cell viability determination*

LDH activity was determined based on the method described by 3.2.5.3. From the collected aliquot, a 50 µl volume was added to 0.5 ml of reagent containing 50 mM lactic acid and 7 mM NAD+ in 0.25 M tris hydrochloride buffer, pH 8.9. The change in absorbance at 340 nm was recorded in a Hitachi U-2000 spectrophotometer. In the event that the reaction was curvilinear, the assay was repeated with a suitable dilution. All LDH assays were completed within 2 hours of sample collection.

**8.2.6 Determination of GSH depletion by AAP.**

To determine the extent of GSH depletion following application of AAP, in a separate experiment, 0.1 to 3 mM AAP was added to individual hepatocyte coated plates. The incubation was stopped either 1 or 4 hours later by aspiration of the media and the plates were then washed three times with Hanks solution before 1ml of 6.5% TCA added to precipitate protein. Cells were scraped from the bottom of the plate and transferred to an Eppendorf tube. After centrifugation of the collected sample, the acidic supernatant was analysed for GSH content and the pellet for protein content. Results are expressed as nmol GSH/ mg protein. The experiment was repeated 4 times.

*8.2.6.1 Glutathione Assay*

Cellular GSH level was determined in accordance to the method outlined in Section 4.2.4.2 with the exception that the standard curve was prepared in the range of 0 to 100 µM GSH.

*8.2.6.2 Protein determination of pellet*

The protein pellet was dissolved in 0.5 ml of 0.5 M sodium hydroxide, incubated at 40°C for 1 hour. Protein concentration was determined using the Pierce BCA Protein determination kit according to the instructions of the manufacturer. The standard curve was prepared using bovine serum albumin dissolved in 0.5 M sodium hydroxide in the range of 0 to 1 mg/ml.

**8.2.7 Determination of GSH repletion following acute depletion by DEM.**

To determine the ability of the cells to replenish GSH following acute GSH depletion, hepatocyte monolayers were exposed to 0.5 mM DEM for 30 minutes. The media containing

DEM was aspirated, the plates washed and then the medium was replaced with fresh RPMI-1640 containing no toxicant. At 1, 2 and 4 hours after replacement of media, the incubation was stopped using the TCA acid procedure outlined in 8.2.6. GSH was determined as nmol/mg protein. The experiment was repeated on 4 separate occasions using hepatocytes from different mice.

### 8.2.8 Determination of the glucuronide and GSH metabolites of AAP.

In order to measure the formation of both glucuronide and GSH metabolites of AAP, hepatocyte monolayers were exposed to concentrations of 0.01 to 3 mM AAP. After an 8 hour incubation, 200µl aliquots of media were placed into Eppendorf tubes containing 200µl of ice cold methanol. The samples were stored at -20°C until HPLC analysis was performed.

The HPLC method for determination of AAP metabolites was based on the method of Madhu and Klassen (1991). Briefly, a Waters phenyl µBondapak column was equilibrated with a mobile phase of water : methanol: acetic acid (17.75: 3.00: 0.27) at a flow rate of 1 ml/minute. A 200µl volume of sample comprising culture media: methanol (1:1) was injected and the metabolites were detected using a Waters 490 Programmable multiwavelength detector set at 254 nm. The retention times were 4.2 minutes for the glucuronide and 15.8 minutes for the GSH conjugate with the unchanged AAP parent having a retention time of 7.1 minutes. Results expressed as metabolite Peak Area/mg protein. The experiment was repeated to a total of 8 individual observations. These experiments were also repeated using hepatocytes that had been exposed for 30 minutes to 0.5 mM of the GSH depleting agent, DEM.

### 8.2.9 Statistical Analysis

All results in this chapter were compared by ANOVA. In the event of $P<0.05$, Bonferroni post hoc tests were preformed on specific pairs of data between control and clofibrate treatment groups.

## 8.3 RESULTS

### 8.3.1 Isolation of mouse hepatocytes

The experiments performed in this present work required the development of a method for short term incubation of primary cultures of hepatocytes isolated from mice previously pretreated with clofibrate. The aim of any isolation procedure is to obtain high yields of single viable cells. Other than influences directly related to the housing of the donor animal (eg diet, light cycle and temperature), prior exposure to xenobiotics can decrease the yield of viable cells (Dr J. Phillips, Flinders Medical Centre, personal communication).

The separation of viable from the less dense nonviable cells can be achieved by centrifugation, either at low speed or through a Percoll density gradient (Berry *et al,* 1991). However, the use of collagen covered plates also allows the removal of nonviable cells and substantially reduces mechanical stress to the cells. During the preliminary incubation (cell attachment) phase, only viable hepatocytes adhere to the collagen coated plates leaving floating, non viable cells that are easily removed by aspiration. An advantage of this method is that hepatocyte monolayers can be successfully used for over 24 hours, while free suspensions of hepatocytes are only suitable for experiments of up to a 4 or 5 hour duration.

The isolation procedure described in the Section 8.2.3 provided individual plates of viable hepatocytes which routinely had less than 5% LDH leakage after 8 hours incubation in RPMI-1640. Such cultures enabled further investigations into the protective effects of clofibrate treatment on the toxicity of AAP and other toxicants.

### 8.3.2 Concentration:Lethality relationship of AAP

The first experiment investigated whether the protective effects of clofibrate were retained in isolated hepatocytes. AAP produced a concentration and time-dependent loss of cell viability in control cells, as assessed by % LDH leakage into the culture media (Figure 8.1 & Appendix 4).

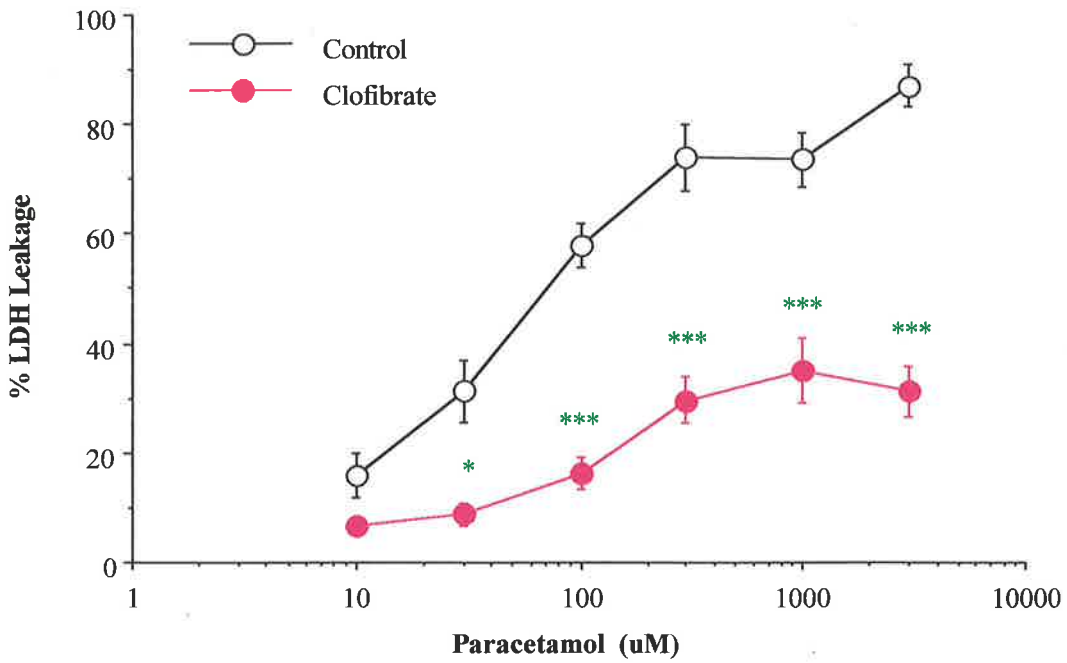



**Figure 8.1:** The effect of clofibrate pretreatment on the 4 hour lethality of AAP (10 to 3000 μM) in primary cultures of mouse hepatocytes. Cells were isolated from either control or clofibrate pretreated mice. Viability was determined by %LDH leakage. (Results expressed as mean±s.e.m, n=15 for each AAP concentration, *<0.05, ***P<0.001 Bonferroni post hoc test).

At all concentrations from 10 up to 3000 μM AAP there was a significant decrease in AAP lethality in cells from clofibrate treated animals. The maximum extent of cell killing in cells isolated from clofibrate pretreated mice was 35% compared to the 90% LDH leakage in the control group. It is obvious that the biochemical alteration associated with the *in vivo* hepatoprotective effect of clofibrate pretreatment is conserved in the *in vitro* situation. It is also apparent that hepatoprotection is related to an alteration within the hepatocyte itself and not due to dispositional changes in the handling of AAP as a result of clofibrate pretreatment.

### 8.3.3 Effect of clofibrate pretreatment on GSH depletion and repletion

*8.3.3.1 Effect of clofibrate pretreatment by AAP induced GSH depletion*

To determine the effects of AAP on GSH depletion, hepatocyte monolayers were incubated with concentrations of AAP from 0.1 to 3 mM with plates collected either 1 or 4 hours later. At the commencement of the experiment, there was no difference in the amount of GSH/mg protein between the control (40.2±7 nmol/mg) compared to clofibrate pretreated (38.7±7 nmol/mg) hepatocytes. Furthermore, there was no statistically significant difference between the control or clofibrate pretreated cells at any subsequent time points following incubation with a range of AAP concentrations (Figure 8.2). This contrasts with the depletion of GSH *in vivo,* where the extent of GSH depletion induced by AAP was less pronounced in the livers of clofibrate pretreated mice (Figure 6.4).

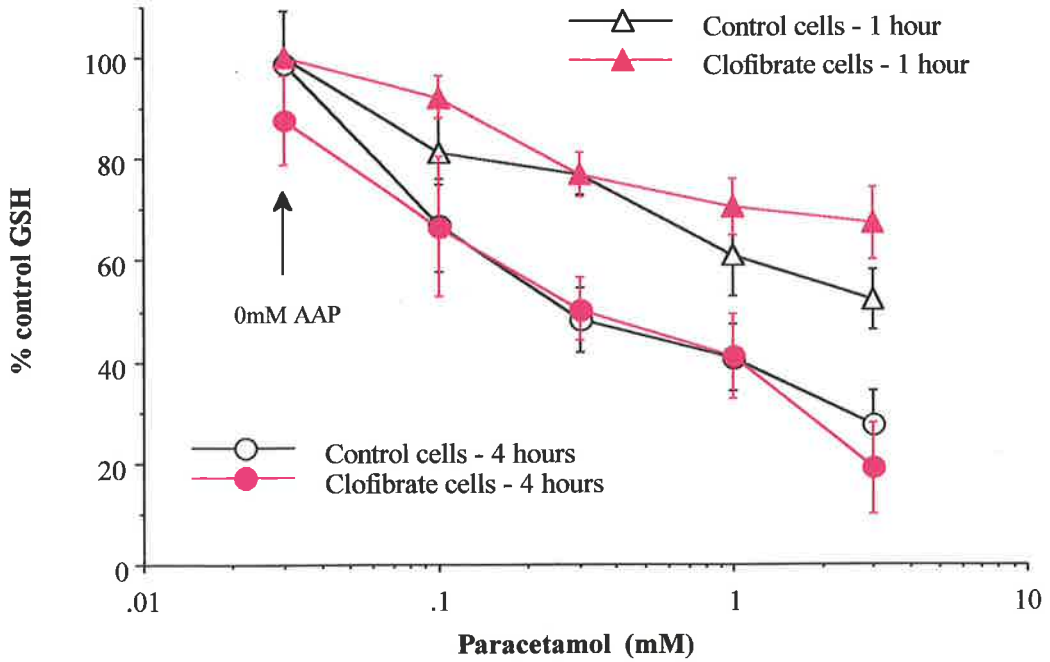



**Figure 8.2:** The level of GSH depletion in hepatocytes at both 1 and 4 hours post administration of 0.1 to 3 mM AAP. Results are expressed as a percentage of pre-AAP GSH level. Cells were isolated from either control or clofibrate pretreated mice. (Results expressed as mean±s.e.m, n=4).

*8.3.3.2 Effect of clofibrate pretreatment on GSH repletion following depletion by DEM*

Conclusions drawn from the GSH-dependent enzyme data in Chapter 6 suggest that there were no differences in GSH turnover between clofibrate pretreated mice compared to control mice. To investigate this further, hepatocyte monolayers were exposed to 0.5 mM DEM for 30 minutes prior to the addition of fresh media. Cellular GSH levels were determined either 1, 2 or 4 hours later. This concentration of DEM caused a depletion of GSH to between 30 and 40% of controls GSH in cells from both treatment groups (Figure 8.3). GSH levels stayed low for the next 2 hours then slowly recovered from 20% at 2 hours to 40% of control levels at 4 hours. Sampling at later time points was not possible due to a loss of cell viability in the control cell preparations. There were no statistically significant differences observed between the control and the clofibrate treated cells at any of the time points investigated.

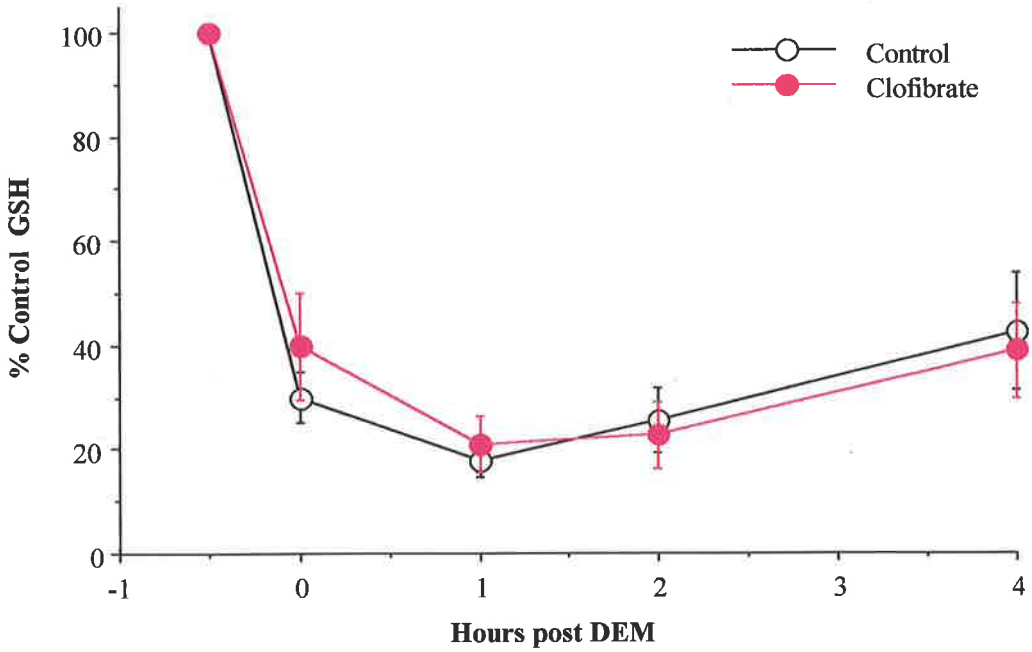



**Figure 8.3:** GSH repletion in mouse hepatocytes following 30 minute exposure to 0.5 mM DEM. Cells were isolated from either control or clofibrate mice. Results are expressed as a % of GSH determined prior to DEM administration (Results expressed as mean±s.e.m, n=4).

### 8.3.4 Effect of clofibrate pretreatment on AAP metabolism in isolated hepatocytes: Measurement of glucuronide and GSH metabolites.

Previous experiments showed a decrease in the level of GSH derived metabolites in the urine of control compared to clofibrate pretreated mice following high dose AAP intoxication (Chapter 5). As the amount of AAP excreted in control mice administered an hepatotoxic 500 mg/kg dose was lower than the amount excreted by mice that received a non-hepatotoxic 200 mg/kg dose, it appeared likely that the diminished urinary excretion of metabolites was an indirect result of hepatic damage. The use of hepatocyte monolayers provided a simplified method for analysis of GSH metabolite formation while simultaneously measuring cell lethality. To complement measurement of the GSH metabolite, the glucuronide metabolite was

measured as a metabolite that does not require prior bioactivation. Sulphate metabolite was unable to be measured due to spectral interference by the parent peak in the HPLC method used. Results were expressed as Peak Area/mg protein, while the lethality produced by each AAP concentration was determined by measuring the %LDH leakage.

After an 8 hour incubation with varying concentrations of AAP, a concentration dependent increase in excretion of the AAP-glucuronide conjugate was observed, with metabolite production appeared to plateau at 0.3 mM and higher concentration (Figure 8.4). While there appeared to be an increase in the level of AAP-glucuronide excreted by clofibrate pretreated cells at 0.3 and 1.0 mM concentrations, there was no statistically significant difference at any of the concentrations investigated.

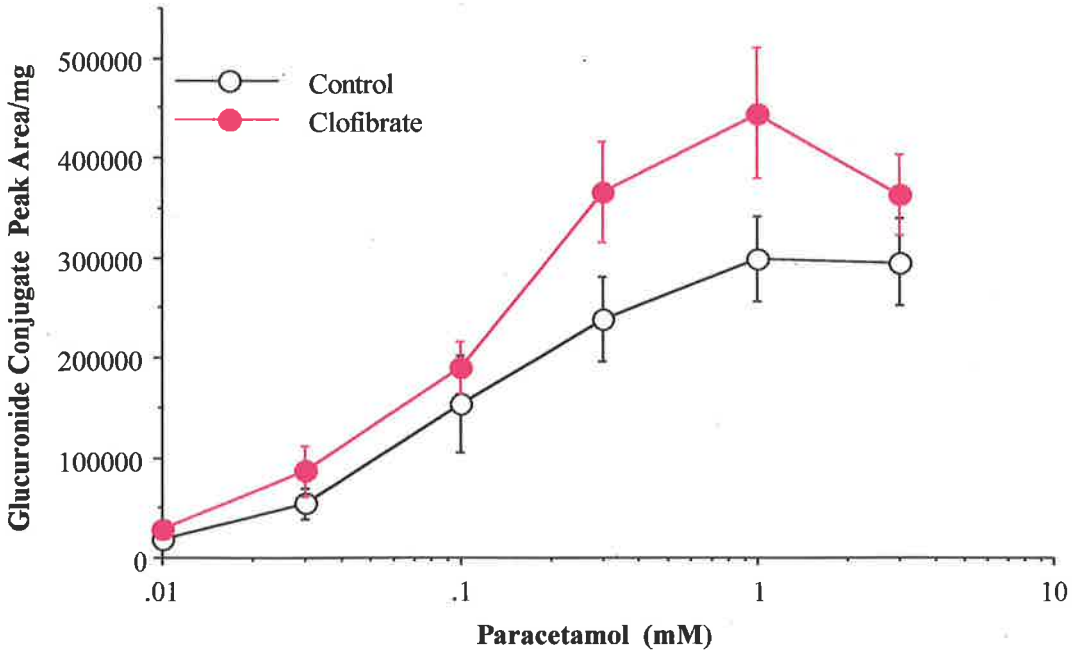



**Figure 8.4:** The effect of clofibrate pretreatment on the formation of the AAP:glucuronide conjugate in mouse hepatocytes after 8 hours exposure to AAP (10 to 3000 μM). Cells were isolated from either control or clofibrate pretreated mice. The amount of AAP:glucuronide metabolite was determined by HPLC integration and normalised for protein content. (Results expressed as mean±s.e.m, n=6 for each AAP concentration).

In contrast, there were clear differences in the formation of the AAP-GSH conjugate in control compared to clofibrate pretreated cells (Figure 8.5). There was a concentration dependent increase in AAP-GSH conjugate formation in clofibrate pretreated cells with a similar plateau response from 0.3 mM AAP as observed with the glucuronide metabolite. However, at AAP concentrations of 30 μM and above, there was no further increase in the level of GSH metabolite produced by control cells. At the three highest concentrations investigated (0.3, 1 and 3 mM) there was an over 2 fold increase in GSH-AAP metabolite observed in clofibrate pretreated cells. This suggests that the clofibrate cells have an enhanced capacity to metabolise AAP by the GSH pathway. However, consideration should be given to the role of cell lethality

in these findings. After an 8 hour incubation, a concentration dependent increase in %LDH leakage was observed in both control and clofibrate treated cells yet the %LDH leakage was typically over 40 % lower in clofibrate pretreated compared to control cells (Figure 8.6).

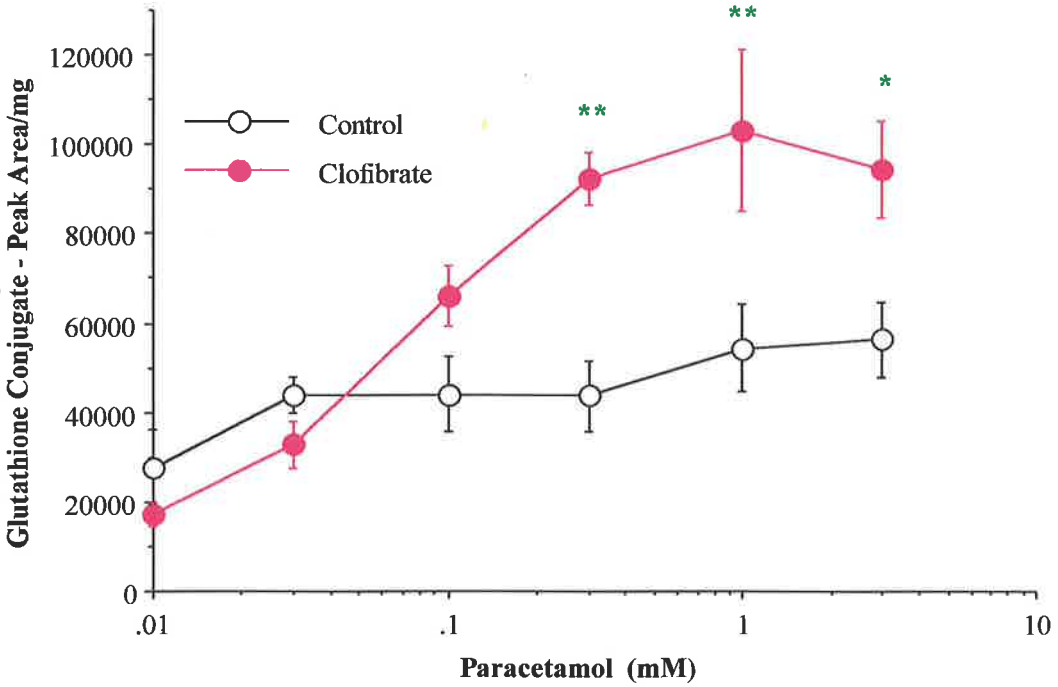



**Figure 8.5:** The effect of clofibrate pretreatment on the formation of the AAP:GSH conjugate in mouse hepatocytes after 8 hours exposure to AAP (10 to 3000 μM). Cells were isolated from either control or clofibrate pretreated mice. (Results expressed as mean±s.e.m, n=6, results obtained from same cells as Figures 8.4; *<0.05, **P<0.01 Bonferroni post hoc test).

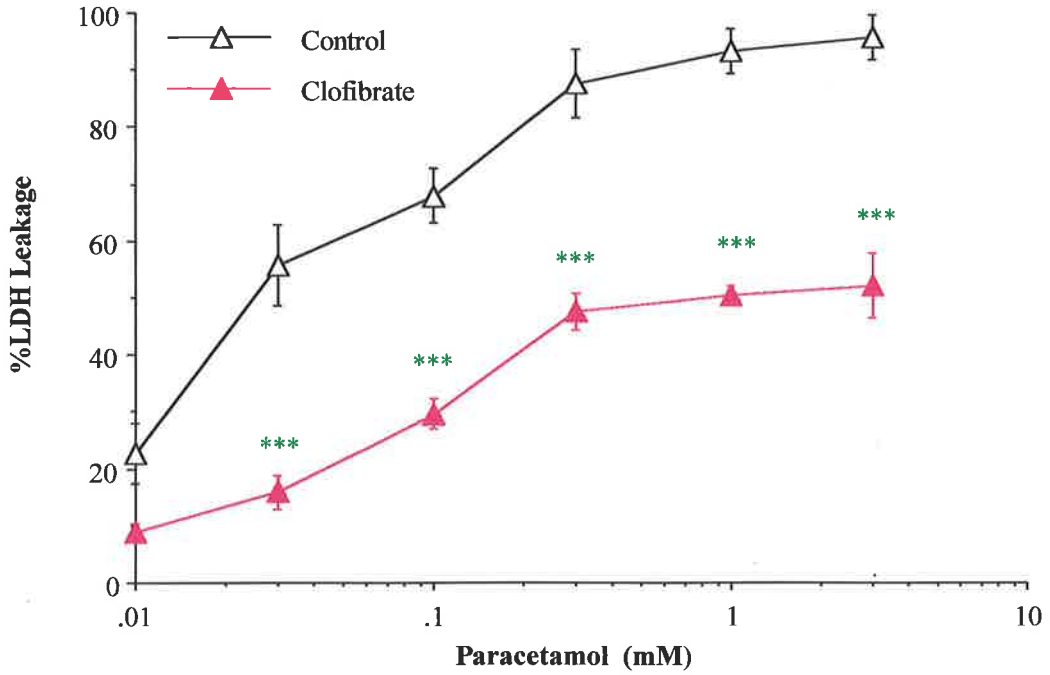



**Figure 8.6:** The effect of clofibrate pretreatment on the 8 hour AAP lethality (10 to 3000 μM) in primary cultures of mouse hepatocytes. Cells were isolated from either control or clofibrate pretreated mice. Viability was determined by %LDH leakage. (Results expressed as mean±s.e.m, n=6, results obtained from same cells as Figures 8.4 and 8.5, ***P<0.001 Bonferroni post hoc test).

A maximum of *approx.* 50% LDH leakage was observed in the clofibrate pretreated cells at 0.3 mM and up to 3 mM concentrations of AAP. In contrast, this degree of cell killing was produced by just 30 μM AAP in control cells, with a 90% LDH leakage evident at 0.3 mM AAP. Comparison of the cell viability with the GSH metabolite data reveals that little AAP-GSH metabolite is produced once cell lethality exceeds *approx.* 60%.

Considering that CYP450 mediated activation of AAP to NAPQI requires NADPH, a cofactor produced only by viable hepatocytes, cell death would dramatically effect the extent of NAPQI formation. Also, analysis of cell culture medium after a 24 hour incubation when almost 100% lethality was evident in AAP treated control cells, revealed that negligible amounts of AAP-GSH metabolite (data not shown). It seems possible that the release of lysosomal or other enzymes from nonviable cells, which are capable of degrading the GSH metabolite, may result in artefactually low levels in preparations with extensive cell lethality.

Overall, the present data implies that the metabolism of AAP was compromised in control hepatocytes during exposure to highly toxic concentrations of AAP. It could be suggested that as clofibrate pretreated hepatocytes remain viable, they continue to metabolise AAP even when exposed to concentrations that are lethal to control cells. A maintenance of cell viability and also of detoxication capability could well be interpreted as enhanced GSH availability in the clofibrate pretreated cells. However, the results described in 8.3.3 indicate that there were no differences in GSH availability or regeneration capability in hepatocytes isolated from clofibrate pretreated compared to control mice livers.

### 8.3.5 Effect of clofibrate pretreatment on AAP metabolism in isolated hepatocytes following intracellular depletion of GSH by DEM.

The flexibility of the hepatocyte monolayer model allows an extension of the AAP metabolite study, in which short term exposure to DEM is used to remove GSH prior to application of AAP. It is expected from the *in vivo* results obtained in Chapter 6, that the removal of GSH by DEM should enhance the toxicity of AAP in control cells while clofibrate pretreated cells will be resistant to lethality, in spite of equivalent GSH depletion.

The 4 hour formation of GSH and glucuronide metabolites of AAP at concentrations ranging from 10 μm to 1 mM were investigated in cells previously exposed to 0.5 mM DEM (Figure 8.7). In section 8.3.2, it was found that this concentration of DEM resulted in about a 60% decrease in the amount of cellular GSH.

As observed with the non-GSH depleted hepatocytes, there was no statistically significant differences in clofibrate and control cells in the level of glucuronide metabolite formation

(magenta compared to black line - Figure 8.7). Addition of DEM resulted in no change in the ability of the control (yellow compared to black line - Figure 8.7) or the clofibrate pretreated cells (red compared to magenta line - Figure 8.7) to form the AAP-glucuronide metabolite. However, there appeared to be a trend toward a reduction in the level of glucuronide in the control cells, which was potentiated by DEM pretreatment. This seems consistent with the proposal that decreasing cell viability will effect the ability of the cells to metabolise AAP.

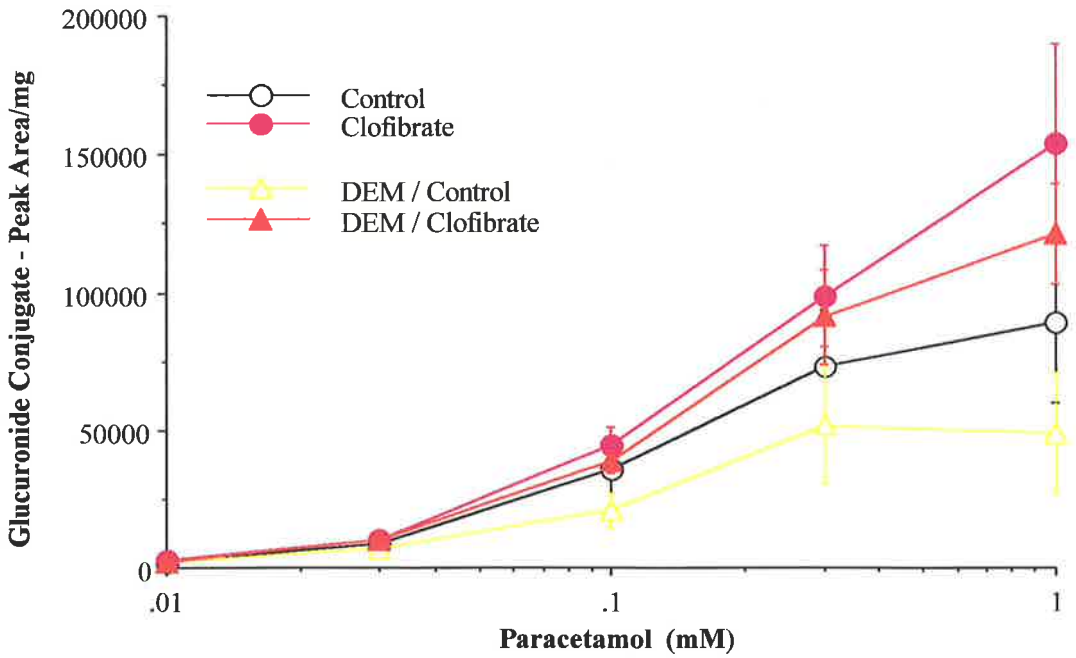



**Figure 8.7:** The effect of clofibrate pretreatment on the formation of the AAP:glucuronide conjugate in mouse hepatocytes after 4 hours exposure to AAP (10 to 1000 μM) following prior depletion of GSH by 0.5 mM DEM. Cells were isolated from either control or clofibrate pretreated mice. (Results expressed as mean±s.e.m, n=4 for each AAP concentration).

There was no statistically significant difference in the formation of GSH-AAP metabolite in control compared to clofibrate pretreated cells, following a 4 hour incubation. At concentrations of 0.1 mM AAP and above, there was no further increase in the level of GSH-AAP metabolite consistent with the earlier suggestion in Section 8.3.4, that at high levels of cell lethality, formation of this metabolite is decreased. The depletion of GSH by prior incubation with DEM was shown to decrease the level of GSH metabolite formed in both control and clofibrate pretreated mice by approximately 60%, which was statistically different from their respective treatment group control at the same AAP concentration (Figure 8.8).

The % LDH leakage for AAP induced cell killing in DEM pretreated cells, provided some further insights into the lack of involvement of GSH in the hepatoprotective effect. First, it is quite clear that DEM treatment has potentiated AAP lethality in control cells (yellow compared to black line - Figure 8.9).

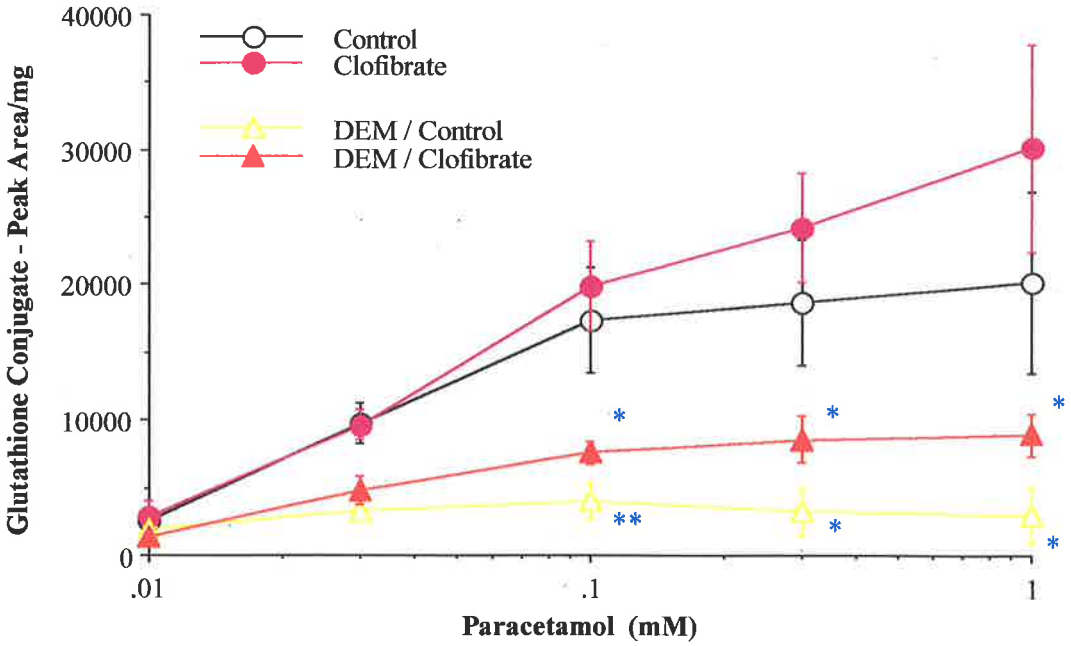



**Figure 8.8:** The effect of clofibrate pretreatment on the formation of the AAP:GSH conjugate in mouse hepatocytes after 4 hours exposure to AAP (10 to 1000 μM) following prior depletion of GSH by 0.5 mM DEM. Cells were isolated from either control or clofibrate pretreated mice. (Results expressed as mean±s.e.m, n=4 for each AAP concentration, results obtained from same cells as Figures 8.7; *<0.05, **P<0.01 Bonferroni post hoc test- for differences related to DEM depletion within the treatment group).

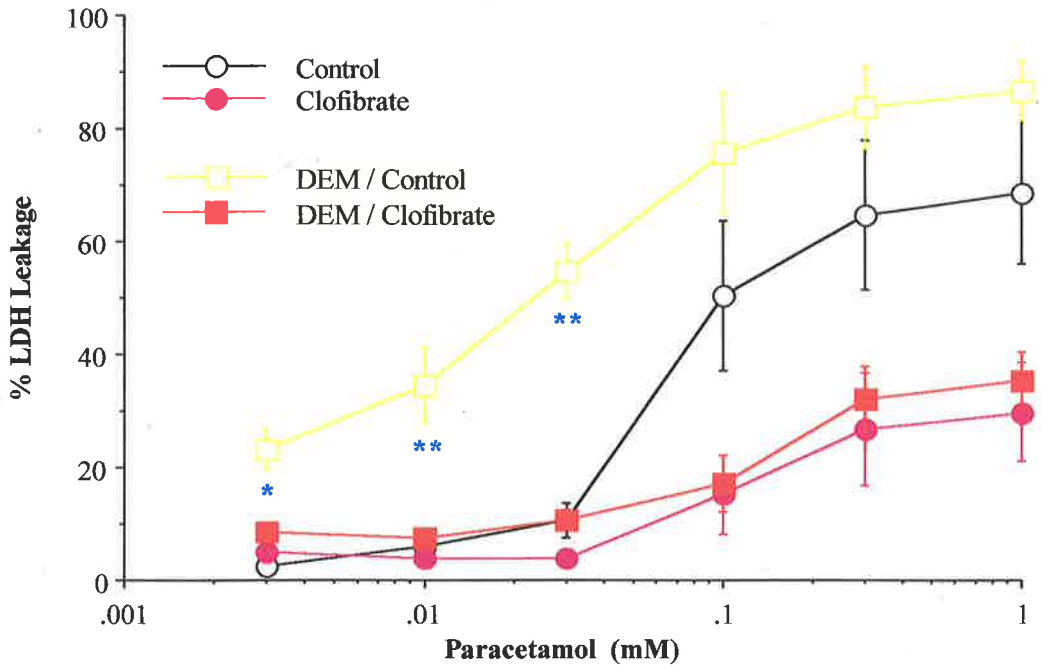



**Figure 8.9:** The effect of clofibrate pretreatment on the 4 hour AAP lethality (10 to 1000 μM) in primary cultures of mouse hepatocytes following prior GSH depletion by 0.5 mM DEM. Cells were isolated from either control or clofibrate pretreated mice. Viability was determined by %LDH leakage. The %LDH value on the left hand side is the 0 mM value, subjected only to the prior treatment and 0 mM AAP for the 4 hour incubation (Results expressed as mean ± s.e.m, n=4 for each AAP concentration, results obtained from same cells as Figures 8.7 and 8.8).

Moreover, the DEM treatment itself caused a 20% increase in lethality in cells containing no AAP. Close inspection of %LDH release at both the 10 and 30 μM AAP concentrations show that when the cytotoxicity associated with AAP and also DEM are combined, a synergistic rather than an additive effect was present (Figure 8.9). This is not observed at higher concentrations presumably due to the high lethality of AAP alone.

There was no difference in the toxicity of AAP in clofibrate pretreated cells, regardless of whether GSH had been depleted or not (magenta compared to red line - Figure 8.9). The level of GSH in clofibrate pretreated cells given 0.5 mM DEM is approximately 70% lower than those measured in non-depleted control cells (Figure 8.3). However, the extent of lethality was far greater in control compared to the DEM and clofibrate pretreated cells (black compared to red line -Figure 8.9). Clearly, these results strongly support the conclusion that while GSH is important in prevention against AAP toxicity, it is not the only factor involved in the protective effect provided by pretreatment with clofibrate

## 8.4 DISCUSSION

The experiments described in this chapter have further defined the effect of clofibrate pretreatment on the acute hepatotoxicity of AAP using primary hepatocyte cultures isolated from clofibrate pretreated mice. It is apparent from the results of the present work, that the protective effect of clofibrate against AAP hepatotoxicity persists in this *in vitro* model, as well as the whole animal (Chapter 3).

### 8.4.1 Verification of a method of short term cultures of isolated hepatocytes from clofibrate pretreated mice and investigation of AAP cytotoxicity.

The first aim of this chapter was to establish a method for the isolation and maintenance of primary cultures of mouse hepatocytes. Preparations with high viability (> 85%) and yield suitable for the culture of 24 individual plates were routinely obtained. Hepatocytes isolated from clofibrate, as well as control pretreated cells, maintained excellent viability under control conditions for up to 24 hours.

These preparations were used to compare the concentration:lethality relationship of AAP between the control and clofibrate pretreated mice. It was observed that 3 mM AAP caused 90% lethality in controls but produced only 30% death in clofibrate cells after a 4 hour incubation (Figure 8.1 and Appendix 4). This suggests that the protection involves biochemical changes in the liver cells and is not merely due to *in vivo* alterations in AAP disposition.

### 8.4.2 DEM induced GSH depletion and repletion in clofibrate pretreated hepatocytes

The retention of protection in this *in vitro* model provided the opportunity to investigate the protective effect at the cellular level. The first experiments investigated the decrease in amount of cellular GSH at increasing concentrations of AAP. There was no difference in the levels of GSH depletion between the control and clofibrate pretreated cells (Figure 8.2). A 1 mM concentration of AAP resulted in a 30% decrease in GSH within 1 hour and a 60% decrease after 4 hours in both groups of cells. These observations were comparable to previous studies investigating the depletion of GSH by AAP in hepatocytes isolated from untreated mice (Moldeus, 1978; Massey and Racz, 1981). However, the equivalent GSH depletion in the control and the clofibrate pretreated cells contrasts with data obtained in the *in vivo* experiments performed in Chapter 6, where the decrease in hepatic GSH as a result of AAP exposure was reduced in clofibrate pretreated compared to control livers (Figure 6.5). This anomaly is likely to be related to intrinsic differences between the *in vivo* and *in vitro* models used in this study.

The isolated hepatocyte model was also used to investigate the ability of hepatocytes to replenish GSH after acute exposure to 0.5 mM DEM. This concentration of DEM was observed to decrease GSH by 60 to 70 % within 30 minutes which, after removal of the DEM, was followed by a slow repletion phase. The slow repletion phase has also been observed in studies with rat hepatocytes where a 0.7 mM concentration of DEM caused a 90% drop in GSH with only 10% recovery after 90 minutes (Fischer-Nielsen *et al*, 1995). There was no difference between the control and clofibrate treated cells with regards to either the extent of GSH depletion or the rate of GSH repletion. Overall it is evident that the ability of the hepatocyte to replete GSH by resynthesis in this model, has not been altered by clofibrate pretreatment.

### 8.4.3 Effect of clofibrate pretreatment on AAP metabolite formation

Having established that there was no difference in the response of the control and clofibrate pretreated hepatocytes to GSH depletion by DEM, it was of interest to investigate the metabolism of AAP while monitoring cell viability. It had been suggested from the results of earlier experiments that the maintenance of viability in clofibrate pretreated livers caused high levels of urinary AAP metabolites in clofibrate pretreated compared to control mice (Chapter 5). To address this issue, both GSH and glucuronide metabolites of AAP were determined 8 hours after administration of AAP with simultaneous determination of cell viability by measurement of %LDH leakage.

In this present study, an approximate 4 to 1 ratio of glucuronide to GSH metabolite was observed (Figure 8.4). This 4:1 ratio was also seen in other studies measuring AAP metabolite formation by isolated mouse hepatocytes (Moldeus, 1978; Massey and Racz, 1981, Harman

and Self, 1986). It has been reported that AAP causes a rapid decrease in GSH during the first 30 minutes of exposure, which is accompanied by an increase in GSH conjugate excretion. The GSH conjugate continues to form at a slower rate, levelling off after 2 hours at which time the cell viability is rapidly decreasing (Moldeus, 1978). Similarly, the results in the present study indicate that at %LDH leakage of around 60% and greater (Figure 8.6), there is no further alteration in the level of GSH conjugate formation while clofibrate cells are still able to form this metabolite (Figure 8.5). It appears likely that the loss of cell viability has comprised bioactivation, a process requiring essential cellular cofactors.

### 8.4.4 Effect of GSH depletion on AAP metabolite formation in clofibrate pretreated hepatocytes

As previous results suggested that GSH is not exclusively responsible for the hepatoprotection, it was decided to investigate the metabolism and also the lethality of AAP in hepatocyte monolayers following GSH depletion. A 0.5 mM concentration of DEM was used to deplete cellular GSH and this was reflected in the over 60% decrease in GSH metabolite in both control and clofibrate pretreated hepatocytes, when compared to their respective controls (Figure 8.8). The results of the cell viability measurements during this experiment highlight the difference in ability of the control and the clofibrate pretreated cells to cope with AAP intoxication after GSH depletion. While DEM exacerbated AAP cytotoxicity in control cells, no such potentiation was seen in cells isolated from clofibrate pretreated mice. Indeed, despite having lower levels of GSH than control cells, less AAP cytotoxicity was observed in clofibrate pretreated cells which had prior DEM exposure (Figure 8.9). This provides further evidence for the lack of exclusive involvement of GSH in the hepatoprotection associated with pretreatment of mice with PxP.

### 8.4.5 Conclusion

The results in this present study have shown that hepatocytes isolated from clofibrate pretreated mice were able to be used in short term primary culture. Importantly, the hepatoprotection observed as a result of clofibrate pretreatment was conserved in this *in vitro* model, providing a useful tool for further investigation of the hepatoprotective effect. Collectively, the studies reported here suggest that an increase in GSH is not the sole reason for the hepatoprotective effect of clofibrate. As suggested in Chapter 7, use of a wide range of toxicants might provide a better indication as to the mechanism of hepatoprotection. Consequently, primary cultures of isolated hepatocytes were used to investigate the toxicity of a diverse range of toxicants during experiments outlined in Chapter 9.

# CHAPTER 9

# EFFECT OF PRETREATMENT WITH CLOFIBRATE ON THE TOXICITY OF OTHER AGENTS: *IN VITRO* STUDIES WITH VARIOUS CHEMICAL TOXICANTS.

## 9.1 INTRODUCTION

In Chapter 7, it was found that clofibrate pretreatment provided protection against the *in vivo* hepatic toxicity of BrB and $CCl_4$, but to a lesser extent than the protection observed following AAP intoxication. Although changes in GSH homeostatis have been ruled out as the primary hepatoprotective mechanism, little else is known of the mechanism underlying this phenomenon. As protection was seen when the radical generating hepatotoxicant, $CCl_4$ was administered, an increased antioxidant capacity in the liver of clofibrate pretreated mice appears a distinct possibility. It was anticipated that by examining the lethality of a number of diverse acting chemicals, further information as to the nature of the hepatoprotective effect following clofibrate pretreatment would become evident.

The use of primary cultures of isolated mouse hepatocytes provides a simple experimental system where xenobiotics unsuitable for *in vivo* analysis can be investigated. In this chapter, the acute lethality of 9 different toxicants was investigated, all of which have been reported to cause toxicity in this experimental model (Table 9.2). All in all, these toxicants act via a variety of different mechanisms, including bioactivation to electrophiles, GSH depletion, induction of lipid peroxidation and oxidative stress. For simplicity, these compounds are combined into 3 general groups: 1) generators of reactive oxygen species, 2) GSH depleting agents by means other than redox cycling and 3) a miscellaneous group whose proposed mechanism of toxicity is not primarily dependent on the availability of GSH.

The first group of toxicants to be considered were those whose mechanism of toxicity involves the generation of reactive oxygen species via redox cycling, namely paraquat, nitrofurantoin and menadione. In these reactions, a one electron reduction typically catalysed by NADPH CYP450 reductase forms a radical species that reduces molecular oxygen to form of superoxide anion ($O_2o^-$) and regenerate the parent compound. The xenobiotic is able to repeat this same futile cycle, thus generating considerable quantities of $O_2o^-$ at the expense of NADPH (Figure 9.1). The $O_2o^-$ is converted to $H_2O_2$, either by the action of SOD or spontaneous dismutation. The $O_2o^-$ and $H_2O_2$ thus generated can, via the Haber-Weiss reaction, react together to form the highly reactive hydroxyl radical (Figure 9.1). The

oxyradicals formed during redox cycling can damage polyunsaturated lipids in cell membranes, initiating the self propagating lipid peroxidation cascade that also generate toxic species. As with lipid peroxides, $H_2O_2$ is metabolised by GSHPx with the formation of GSSG. The reduction of GSSG back to GSH by Gred places further demands on the cellular NADPH pool and ultimately, these xenobiotics lead to a decrease in both cellular NAPDH and GSH. Due to multiple passes through the redox cycle, these toxicants stimulate higher levels of biliary GSSG excretion than that produced by direct substrates for GSHPx, such as *tert*-butylated hydroperoxide (*t*-BHP) (Lauterburg *et al,* 1984). This was supported by the 4 and 15-fold increases in biliary GSSG in rats following treatment with either *t*-BHP or the redox cycling agent, diquat respectively (Jaeschke, 1990).

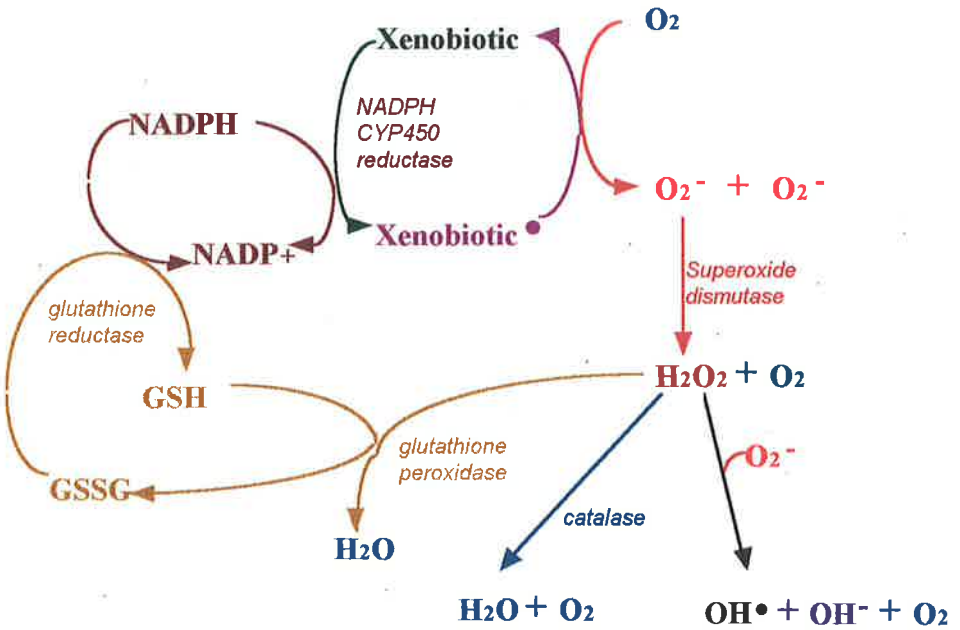



**Figure 9.1:** Proposed biochemical events involved in the mediation of toxicity by xenobiotics which act as generators of reactive oxygen species including paraquat, nitrofurantoin and menadione (*derived from:* Timbrell *et al,* 1994; Casarett and Doull, 1995).

The next group of toxicants also deplete cellular GSH, but not through the redox cycling pathway. The toxicity of *t*-BHP is dependent on metabolism by 2 pathways, namely GSHPx reduction to tert-butyl alcohol with formation of GSSG and consumption of NADPH (Sies *et al,* 1972; Plummer *et al,* 1981; Bellomo *et al,* 1982) and also metabolism to reactive peroxy and alkoxy radicals (Davies, 1989). The formation of GSSG is also prominent in mechanism of action of diazene dicarboxylic acid *bis*-*N,N*-dimethylamide (diamide) and 1,3-*bis*-(2-chloroethyl)-1-nitrosurea (BCNU). Diamide, a specific sulphydryl oxidising agent, rapidly oxidises intracellular GSH to GSSG which is exported from the cell (Kosower and Kosower, 1989; Halliwell and Gutteridge, 1989). BCNU is an irreversible inhibitor of Gred and prevents the cell from maintaining an adequate GSH pool (Bellomo *et al,* 1982). The final toxicant investigated in this group was DEM, which in contrast to the other 3 agents, depletes GSH without a concurrent increase in GSSG. DEM conjugates directly to GSH causing a rapid drop in the amount of cellular GSH (Miccadei *et al,* 1988b). In summary, each of the

four toxicants considered in this group decrease GSH but via different mechanisms (Table 9.2).

| Toxicant | Mechanism of Toxicity | Reference |
|---|---|---|
| Paraquat | Generator of oxidants via redox cycling. Toxicity believed to result from NADPH depletion and lipid peroxidation. | Witschi *et al,* 1977<br>Bus *et al,* 1976 |
| Nitrofurantoin | Generator of oxidative stress by redox cycling. Induces lipid peroxidation/mitochondrial dysfunction. | Rossi *et al,* 1988<br>Lim *et al,* 1986 |
| Menadione | Generator of oxidative stress by redox cycling. GSH depletion. Does not induce lipid peroxidation. | Di Monte *et al,* 1984<br>Powis *et al,* 1987 |
| BCNU | Irreversible inhibition of glutathione reductase - results in accumulation of GSSG and oxidative stress | Adamson & Harman, 1993<br>Meredith & Reed, 1983 |
| Diamide | Thiol oxidant - causes rapid GSH loss due to enhanced GSSG formation and excretion from cell | Kosower & Kosower, 1989<br>Mirabelli *et al* , 1988 |
| Diethylmaleate | Direct conjugation to GSH causing rapid GSH depletion | Miccadei *et al,* 1988a<br>Maellaro *et al,* 1994 |
| *t*- BHP | Metabolism via GSHPx results in GSSG formation, forms alkoxy & peroxyl radicals, lipid peroxidation | Rush *et al,* 1985<br>Glascott *et al,* 1995 |
| Frusemide | Bioactivated by CYP450 to a reactive epoxide intermediate? GSH independent toxicant. | Massey *et al,* 1987 |
| Iodoacetate | Alkylating agent. Inhibits glycolysis resulting in decrease in ATP formation | Imberti *et al,* 1992<br>LeMasters *et al,* 1987 |

**Table 9.2:** Proposed mechanisms of toxicity for each of the toxicants used in the present chapter in the isolated rodent hepatocyte model.

The 2 remaining toxicants differ in having mechanisms of toxicity that do not involve redox cycling or pronounced decreases in the amount of cellular GSH. Frusemide causes hepatic necrosis when administered in high doses *in vivo* (Mitchell *et al,* 1974) which has been hypothesised to be related to the CYP450 mediated generation of a reactive arene intermediate (Wirth *et al,* 1976). Finally, iodoacetate is a metabolic inhibitor that causes rapid cell death by direct inhibition of glycolysis, impairing ATP synthesis and thus affecting energy metabolism within the cell (LeMasters *et al,* 1987).

The toxicants investigated in this Chapter encompass a range of different biochemical mechanisms of toxicity including lipid peroxidation, GSH depletion and bioactivation as well as other actions specific to the individual toxicant. Each toxicant was investigated in short term primary cultures of isolated mouse hepatocytes, with aliquots of media taken at specific time points for measurement of toxicant induced cell lethality via %LDH leakage determination.

In addition, the results of Chapter 7 suggested that the decrease in the *in vivo* toxicity of $CCl_4$, and to a lesser extent BrB, may result from an increase in the antioxidant capacity of the liver and therefore the extent of lipid peroxidation in free suspensions of isolated mouse

hepatocytes was determined following exposure to 2 prooxidants namely, ferrous sulphate and *t*-BHP.

The overall aim of experiments described in this chapter was to further investigate the protective effect of clofibrate by examining the lethality of a diverse range of toxicants in primary cultures of isolated mouse hepatocytes. It is anticipated that the results of this chapter will provide further understanding into the possible mechanism of hepatoprotection observed following clofibrate pretreatment.

## 9.2 MATERIALS AND METHODS

### 9.2.1 Chemicals

All chemicals used in this chapter were of the highest quality commercially available. For a complete list of chemicals and suppliers, refer to Appendix 1.

### 9.2.2 Animals

Swiss white male mice (35-40 g) were obtained and housed as outlined in Section 2.2. Mice were pretreated with either olive oil vehicle (control) or 500 mg/kg/day clofibrate for 10 days, prior to hepatocyte isolation.

### 9.2.3 Investigation of toxicant lethality using isolated mouse hepatocytes

Primary cultures of mouse hepatocytes were prepared by collagenase digestion and adhesion to collagen coated sterile plastic plates, as described in Section 8.2.3. Following cell adhesion, plates were washed twice with Hank's Buffer then media replaced with 3 ml aliquots of RPMI-1640 medium containing a range of concentrations of toxicants specified in Table 9.3.

#### *9.2.3.1 Preparation of toxicants for addition to isolated cell culture*

The required range of toxicant concentrations was obtained by appropriate dilutions of a stock solution prepared in RPMI-1640 which was prepared at the highest required concentration. The majority of the toxicants were freely soluble in the RPMI-1640 medium, however BCNU and nitrofurantoin required a prior dissolution step. For BCNU, a measured amount of compound was dissolved in minimal 70% ethanol. Nitrofurantoin was dissolved in 0.2 ml of RPMI-1640, alkalinised by addition of 0.5 M sodium hydroxide. For both, a suitable aliquot was added to the required volume of medium resulting in an overall 100 fold dilution. Suitably prepared blank plates revealed no toxicity or interference in the LDH assay by either of the solvents added to aid toxicant dissolution. Both *t*-BHP and DEM solutions were diluted 1:9 at least twice prior to measurement the required aliquot for stock preparation. This was to avoid the inherent errors in the measurement of minute liquid volumes.

*9.2.3.2 Assessment of cell lethality*

A 60 µl aliquot of medium was removed at each of the specified time points for measurement of LDH activity (Table 9.3). After the final time point, cells were lysed by the addition of 240 µl of 2 % triton X-100 to each individual plate and total LDH activities were determined in accordance to the method outlined in Chapter 8.

| Toxicant | [conc] Range (µM) | Sampling Times (Hours) |
|---|---|---|
| Paraquat | 100 - 3000 | 1, 2, 4, 8 |
| Nitrofurantoin | 10 - 1000 | 1, 2, 4, 8 |
| Menadione | 10 - 1000 | 0.5, 1, 2, 3, 4 |
| BCNU | 30 - 1000 | 1, 2, 4, 8 |
| Diamide | 30 - 1000 | 1, 2, 4, 8 |
| Diethylmaleate | 200 - 4000 | 1, 2, 4, 8 |
| *tert*- Butyl hydroperoxide | 10 - 3000 | 1, 2, 4 |
| Frusemide | 3 - 100 | 1, 2, 4, 8 |
| Iodoacetate | 30 - 3000 | 0.5, 1, 2, 3, 4 |

**Table 9.3:** Concentration ranges and sampling times for measurement of associated cell lethality for each of the 9 toxicants investigated in this chapter.

Concentration response curves for the lethality (as measured by % LDH leakage) for all toxicants were prepared at each time point. For the purpose of simplicity, data obtained at the 4 hour time point only is presented in the present chapter, with the exception of iodoacetate where data for the 1 hour time point is displayed. For graphical presentation of all results incorporating each sampling time point, see Appendix 5. Using the 4 hour graph (or 8 hour for DEM and paraquat), the $LC_{30}$ concentration, defined as the concentration causing 30% cell lethality, for each toxicant was determined for both control and clofibrate pretreated cells. The control to clofibrate ratio for $LC_{30}$ values was then calculated to enable comparison of the extent of protection afforded by clofibrate pretreatment between each of the toxicants investigated. A value of 1 indicated no difference between the lethality of the toxicant in control and clofibrate pretreated cells.

### 9.2.4 Lipid peroxidation in isolated mouse hepatocytes

A general indication of the extent of lipid peroxidation occurring in hepatocytes can be obtained by measuring the levels of malondialdehyde (MDA), a byproduct of the fragmentation of cyclic endoperoxides formed during the lipid peroxidation cascade. MDA is a small dicarbonyl compound which is able to crosslink 2 thiobarbituric acid molecules under

hot acidic conditions producing a chromophore with a peak absorbance at 532 nm. As this method is not specific for malondialdehyde, the results are typically presented as thiobarbituric acid reactive substances (TBARS) (Halliwell and Gutteridge, 1989). The TBARS method used to assess lipid peroxidation in free hepatocyte suspensions was based on the procedure of Stacey and Priestly (1978). For this, a 1 ml aliquot of freshly isolated hepatocyte suspension containing 1 x $10^6$ cells, was combined with 1 ml of either 0.1 M potassium phosphate buffer, pH 7.4 or one of two prooxidants, namely 2 mM ferrous sulphate or 2 mM *t*-BHP. The samples were incubated at 37°C for 30 minutes when the reactions were stopped by the addition of 2 ml of 10% trichloroacetic acid. Following centrifugation for 5 minutes at 1000 x g at room temperature, 1 ml of supernatant was added to 2 ml of 0.375% thiobarbituric acid and 20 µl 2% butylated hydroxytoluene in glass tubes capped with teflon lined caps. Tubes were then incubated at 100°C in a water bath for 20 minutes. Approximately 10 minutes after removing the tubes from the water bath, the caps were loosened and tubes cooled to room temperature. Absorbance was measured at 532 nm and using an extinction coefficient of 1.56 x $10^5 M^{-1}$ $cm^{-1}$, results were expressed in nmol TBARS/$10^6$ cells.

### 9.2.5 Statistical Analysis

Data obtained for dose response curves presented in this chapter were investigated by ANOVA for the 4 hour time point alone (or at 1 hour for iodoacetate). If a significant variance ratio was found, the data was further analysed using the Bonferroni Post Hoc test for specific pairs of data. The lipid peroxidation study utilised the Dunnett's Post Hoc test, for comparison of results to control (buffer alone, no prooxidant) treatment.

## 9.3 RESULTS

In the first series of experiments, the acute lethality of various toxicants was measured at set time points in hepatocytes from vehicle and clofibrate pretreated mice. This was followed by investigation of the extent of lipid peroxidation in free suspensions of mouse hepatocytes following exposure to prooxidants.

### 9.3.1 Determination of %LDH leakage from control and clofibrate pretreated isolated mouse hepatocytes exposed to various toxicants

In these experiments, the lethality of a number of different toxicants was investigated in primary cultures of mouse hepatocytes.

#### *9.3.1.1 Generators of reactive oxygen species by redox cycling*

In all, 3 different chemicals from this group were investigated namely paraquat, nitrofurantoin and menadione. Each of these toxicants exhibited time and concentration dependent toxicity

(Appendix 5 A, B & C respectively) with the cytotoxicity of each toxicant showing differences in hepatocytes isolated from clofibrate pretreated mice. After 4 hours exposure, lethality at each concentration of paraquat (0.1 to 1 mM) was more pronounced in control cells than in those from clofibrate pretreated group mice, although the difference was only significant at the 3 mM concentration (Figure 9.4). At 8 hours the difference was even more pronounced with a 80% LDH leakage observed in the control cells exposed to 3 mM paraquat, compared to just 30% in the clofibrate pretreated group (Appendix 5A). Determination of the $LC_{30}$ ratio (Table 9.13) showed that over 6 times the concentration of paraquat was required to produce equivalent toxicity in clofibrate pretreated compared to control cells.

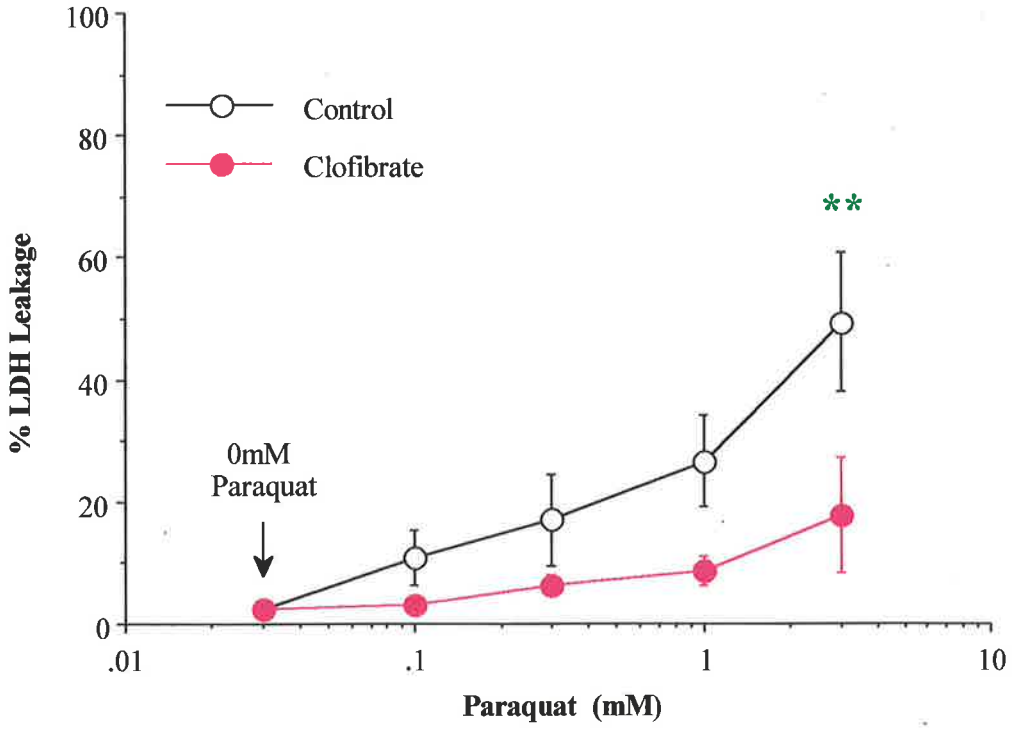



**Figure 9.4:** The effect of clofibrate pretreatment on the 4 hour lethality of paraquat (0.1 to 3 mM) in primary cultures of hepatocytes. Cells were isolated from either control or clofibrate pretreated mice. Viability was determined by %LDH leakage. (Results expressed as mean±s.e.m, n=5, **<0.01, Bonferroni post hoc test).

Nitrofurantoin exhibited greater potency as a cytotoxicant than paraquat, with the 1 mM concentration producing 80% lethality in control cells within 4 hours, compared to 30% for paraquat (Figures 9.4 *cf* 9.5). Although clofibrate pretreatment causes a statistically significant decrease in the level of lethality at 0.3 mM, clofibrate pretreatment also produced less marked protection against nitrofurantoin than was the case observed with paraquat (Figure 9.5). Determination of the $LC_{30}$ ratio revealed that approximately twice the concentration of nitrofurantoin was required to exhibit the same toxicity in clofibrate compared to control pretreated cells (Table 9.13; Appendix 5B).

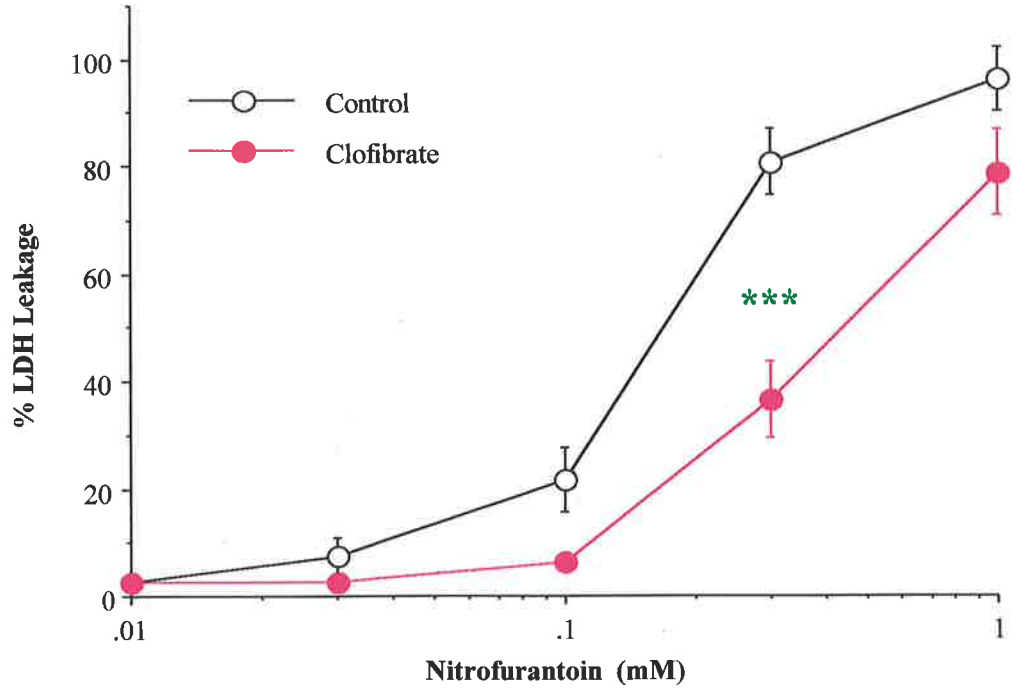



**Figure 9.5:** The effect of clofibrate pretreatment on the 4 hour lethality of nitrofurantoin (0.01 to 1mM) in mouse hepatocytes. Cells were isolated from either control or clofibrate pretreated mice. (Results expressed as mean±s.e.m, n=5, ***P<0.001 Bonferroni post hoc test).

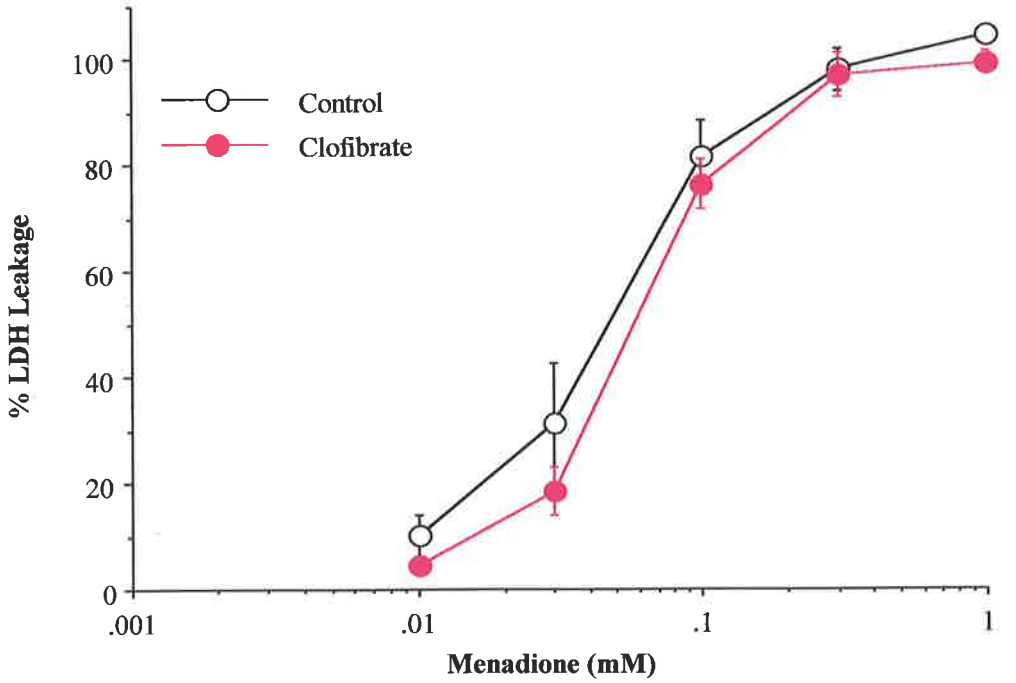



**Figure 9.6:** The effect of clofibrate pretreatment on the 4 hour lethality of menadione (0.01 to 1mM) in mouse hepatocytes. Cells were isolated from either control or clofibrate pretreated mice. (Results expressed as mean±s.e.m, n=6-10).

Menadione was found to be the most potent cytotoxicant of the three redox cycling agents investigated, with the 1 mM concentration producing 100% LDH leakage within 4 hours (Figure 9.6), compared to 80% and 30% recorded for nitrofurantoin and paraquat respectively. As with the other cycling agents, menadione exhibited dose and time dependent toxicity

(Appendix 5C), however in direct contrast to paraquat and nitrofurantoin, clofibrate pretreatment did not provide any protection against the lethality of menadione. Menadione toxicity is associated with GSH availability via both direct (GSH conjugation) and indirect (GSSG formation) mechanisms (Di Monte *et al,* 1984; Maellaro *et al,* 1994). This result further supports the conclusion derived in Chapters 6, 7 and 8, that increased availability of GSH is not involved in the hepatoprotective effect of clofibrate pretreatment.

*9.3.1.2 GSH depleting agents (non- redox cycling)*

The second group of toxicants used in the present study all deplete cellular GSH by non redox cycling mechanisms. Each of the 4 toxicants were protected by clofibrate pretreatment but as with the results of the precious section, there was considerable variation in the degree of hepatoprotection afforded by clofibrate pretreatment.

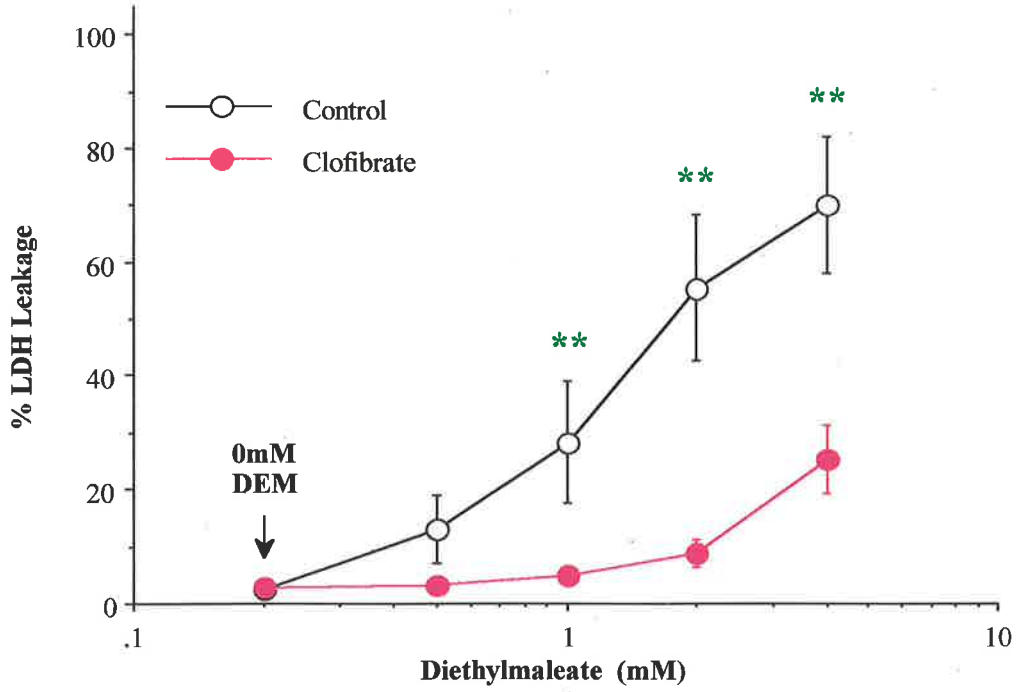



**Figure 9.7:** The effect of clofibrate pretreatment on the 4 hour lethality of DEM (0.05 to 5 mM) in primary cultures of hepatocytes isolated from either control or clofibrate pretreated mice. Viability was determined by %LDH leakage. (Results: mean±s.e.m, n=4, **P<0.01 Bonferroni post hoc test).

Previous experiments described in Chapter 8 revealed that application of DEM results in a rapid decrease in cellular GSH. It was found that a 0.5 mM concentration of DEM produced a 70-80% decrease of GSH within 30 minutes in hepatocytes isolated from both control and clofibrate pretreated mice (Figure 8.3). In the present experiments DEM, in concentrations from 0.5 to 4 mM, was maintained in culture for up to 8 hours. Under these conditions, DEM caused time and concentration dependent toxicity in both control and clofibrate pretreated cells, however there was a marked reduction in the amount of cell death observed in the clofibrate treated group (Appendix 5.D). After 4 hours incubation with DEM, 70% lethality was produced by 1 mM DEM in the control cells compared to only 23% in the clofibrate pretreated cells. The $LC_{30}$ ratio was calculated to be of 6.4 (Table 9.13), which was

comparable to the degree of protection afforded against paraquat. This clearly highlights the extent of protection afforded by clofibrate pretreatment. During extended incubation in a GSH deficient state, hepatocytes isolated from clofibrate pretreated mice were able to remain viable, unlike hepatocytes isolated from control treated mice.

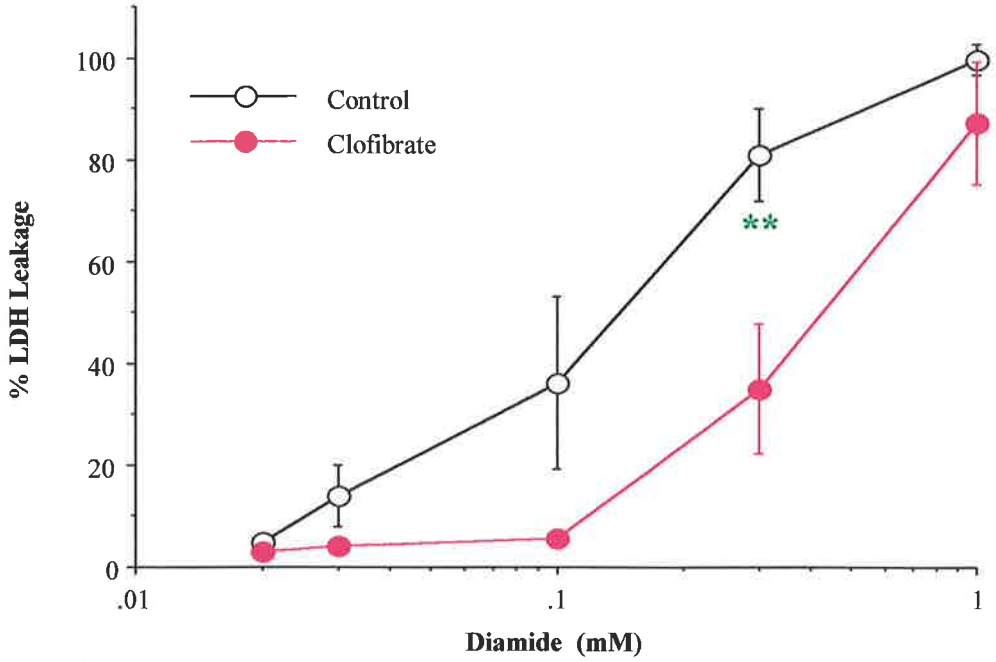



**Figure 9.8:** The effect of clofibrate pretreatment on the 4 hour lethality of diamide (0.02 to 1 mM) in hepatocytes isolated from either control or clofibrate pretreated mice. Viability was determined by %LDH leakage. (Results expressed as mean±s.e.m, n=4, **P<0.01 Bonferroni post hoc test).

As with DEM, diamide initiates a non-enzymatic decrease in cellular GSH. Diamide is a thiol specific oxidant, catalysing the oxidation of GSH to GSSG. GSSG leaves the cell by energy dependent efflux, causing a rapid drop in GSH levels. Diamide proved more toxic than DEM, with 1 mM diamide causing 100% lethality after a 4 hour incubation (Figure 9.8). For complete concentration versus lethality data for diamide, refer to Appendix 5E. Although hepatoprotection was evident in cells isolated from clofibrate treated animals, statistical significance was seen only at the 0.3 mM concentration, with an $LC_{30}$ ratio of 3.6 (Figure 9.8).

The other two toxicants in this group deplete GSH via enzyme based mechanisms, thus disrupting cellular defences against oxidative stress. In addition to the proposed formation of oxyradicals (Davies, 1989), *t*-BHP is metabolised by GSHPx to *tert*-butyl alcohol which utilises GSH to form GSSG as byproduct of the enzymatic reaction. In contrast, BCNU irreversibly inhibits Gred, resulting in inhibition of the regeneration of GSH from GSSG

The results of earlier studies (Chapter 5), indicated that the activity of Gred is slightly increased by clofibrate treatment. In addition, there was no difference between control and clofibrate cells in the extent of Gred inhibition after 4 hour exposure to BCNU at any of the

concentration investigated (data not shown). Since it is dependent on the prior conversion of GSH to GSSG by endogenous oxidants, GSH depletion by BCNU develops more slowly than is the case with direct acting GSH depleting agents such as DEM or diamide. The results of this study show that clofibrate protected against BCNU induced toxicity, with statistically significant protection evident at 0.1 mM BCNU (Figure 9.9). The $LC_{30}$ was 1.9, indicating that the protection provided was not as pronounced for this toxicant as either diamide or DEM.

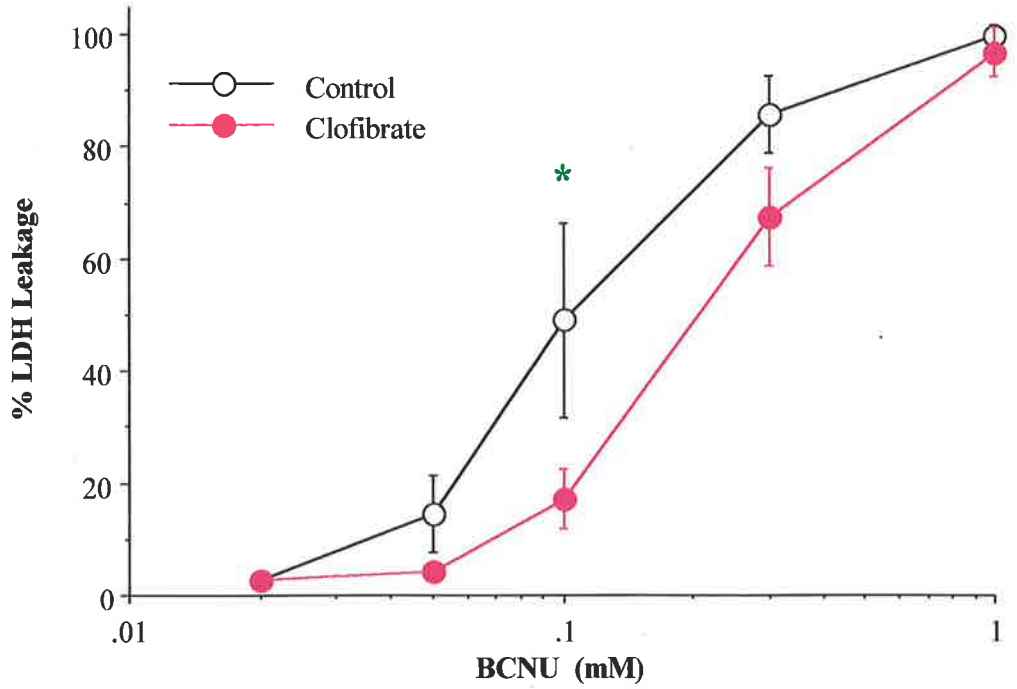



**Figure 9.9:** The effect of clofibrate pretreatment on the 4 hour lethality of BCNU (0.03 to 1mM) in primary cultures of mouse hepatocytes. Cells were isolated from either control (vehicle) or clofibrate (500 mg/kg/day for 10 days) mice. Viability was determined by %LDH leakage. (Results expressed as mean±s.e.m, n=5, *<0.05, Bonferroni post hoc test).

The final GSH depleting toxicant investigated was *t*-BHP which, like BCNU, causes a cellular oxidative stress via an accumulation of GSSG. In addition to its GSHPx metabolism to *tert*-butyl alcohol, *t*-BHP is also converted to radical species (Davies, 1989). The concentration response curves to *t*-BHP revealed that while there were a concentration related effect, the changes with time was not as pronounced as with other toxicants (Figure 9.10 and Appendix 5.G). At the 3 highest doses investigated (0.3, 1.0 and 3.0 mM), there was no difference between the control and clofibrate pretreatment groups (Figure 9.10). However, at lower concentrations, protection against lethality was seen in the clofibrate pretreated groups with statistical significance at the 0.1 mM concentration. The $LC_{30}$ ratio for *t*-BHP was 2.7 (Table 9.13).

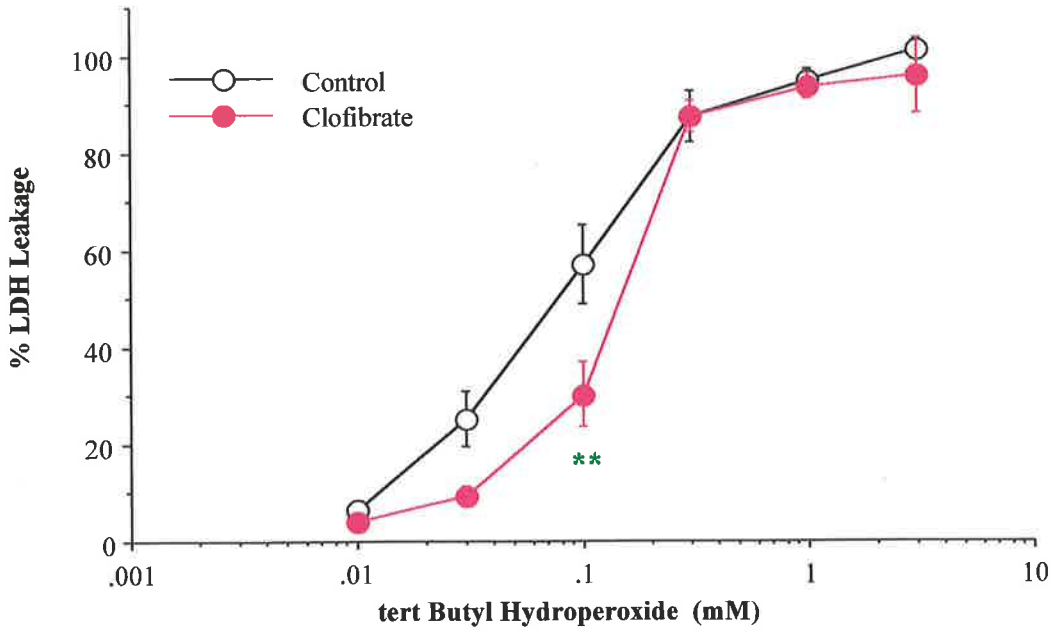



**Figure 9.10:** The effect of clofibrate pretreatment on the 4 hour lethality of tert-butyl hydroperoxide (0.01 to 3 mM) in primary cultures of mouse hepatocytes. Cells were isolated from either control (vehicle) or clofibrate (500 mg/kg/day for 10 days) mice. Viability was determined by %LDH leakage. (Results expressed as mean±s.e.m, n=7-14, **<0.01, Bonferroni post hoc test).

*9.3.1.3 Toxicants with cytotoxicity associated with mechanisms other than GSH depletion.*

The final two chemicals investigated in this study were toxicants whose cytotoxicity is believed to result primarily from a mechanism other from GSH depletion. The diuretic, frusemide has been reported to be bioactivated to a CYP450 reactive epoxide intermediate (Wirth *et al,* 1976). Prior treatment with clofibrate was shown to provide no protection against frusemide lethality at concentrations up to 0.1 mM (Figure 9.11, Appendix 5.H), thus recording an $LC_{30}$ ratio close to 1.

In this model, the 0.1 mM concentration of frusemide produced maximum toxicity. This situation of equivalent or decreased toxicity compared to lower doses has also been reported by another group (Massey *et al,* 1987). However, part of the problem in accurately determining frusemide lethality at concentrations greater than 0.1 mM was due to the inherent absorbance of frusemide itself at 340 nm which interfered with the LDH assay.

Iodoacetate is an inhibitor of cellular glycolysis which reduces the supply of reduced cofactors for oxidative phosphorylation, ultimately causing rapid cell death by suppression of ATP synthesis and inhibition of energy dependent pathways (Le Masters *et al,* 1987). Iodoacetate was found to be the most rapidly acting toxicant used in this study, which is reflected in the presentation of 1 hour (Figure 9.12) compared to 4 (or more) hour lethality data with the other toxicants discussed in this chapter. Pretreatment with clofibrate had no effect on the lethality of

iodoacetate in isolated mouse hepatocytes at any of the time points investigated (Figure 9.12, Appendix 5.I).

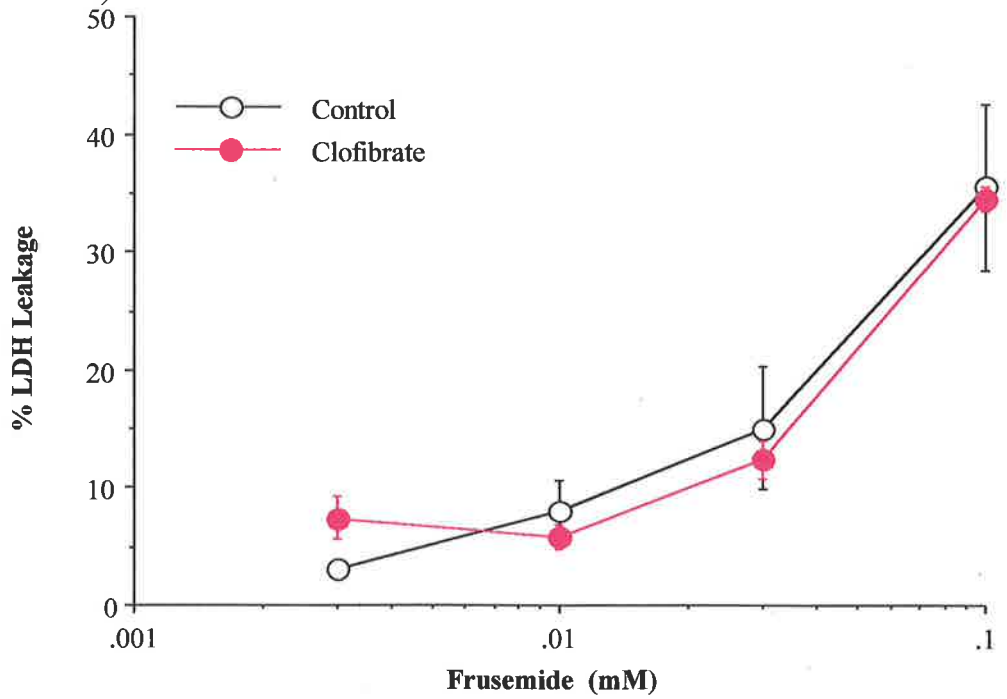



**Figure 9.11:** The effect of clofibrate pretreatment on the 4 hour lethality of frusemide (0.003 to 0.1 mM) in primary cultures of mouse hepatocytes isolated from either control or clofibrate pretreated mice. Viability was determined by %LDH leakage. (Mean±s.e.m, n=6).

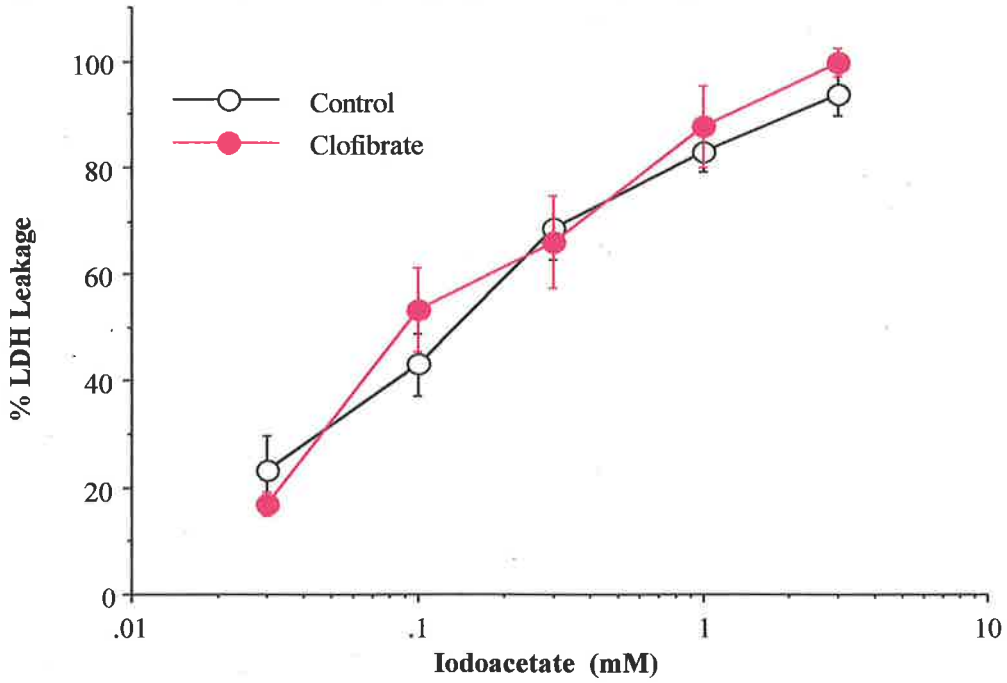



**Figure 9.12:** The effect of clofibrate pretreatment on the 1 hour lethality of iodoacetate (0.03 to 3 mM) in primary cultures of mouse hepatocytes. Cells were isolated from either control (vehicle) or clofibrate pretreated mice. Viability was determined by %LDH leakage. (Mean±s.e.m, n=4-8).

On the whole, the extent of cytotoxicity observed for the control cells with each of the toxicants are in general agreement with previous studies (references as per Table 9.1). The

calculation of the $LC_{30}$ ratio as the concentration of toxicant causing cell death in the clofibrate compared to the control cells revealed that there was no obvious common pattern to the extent of protection observed when considering the mechanism of action for example; oxyradical formation, NADPH depletion or GSSG formation.

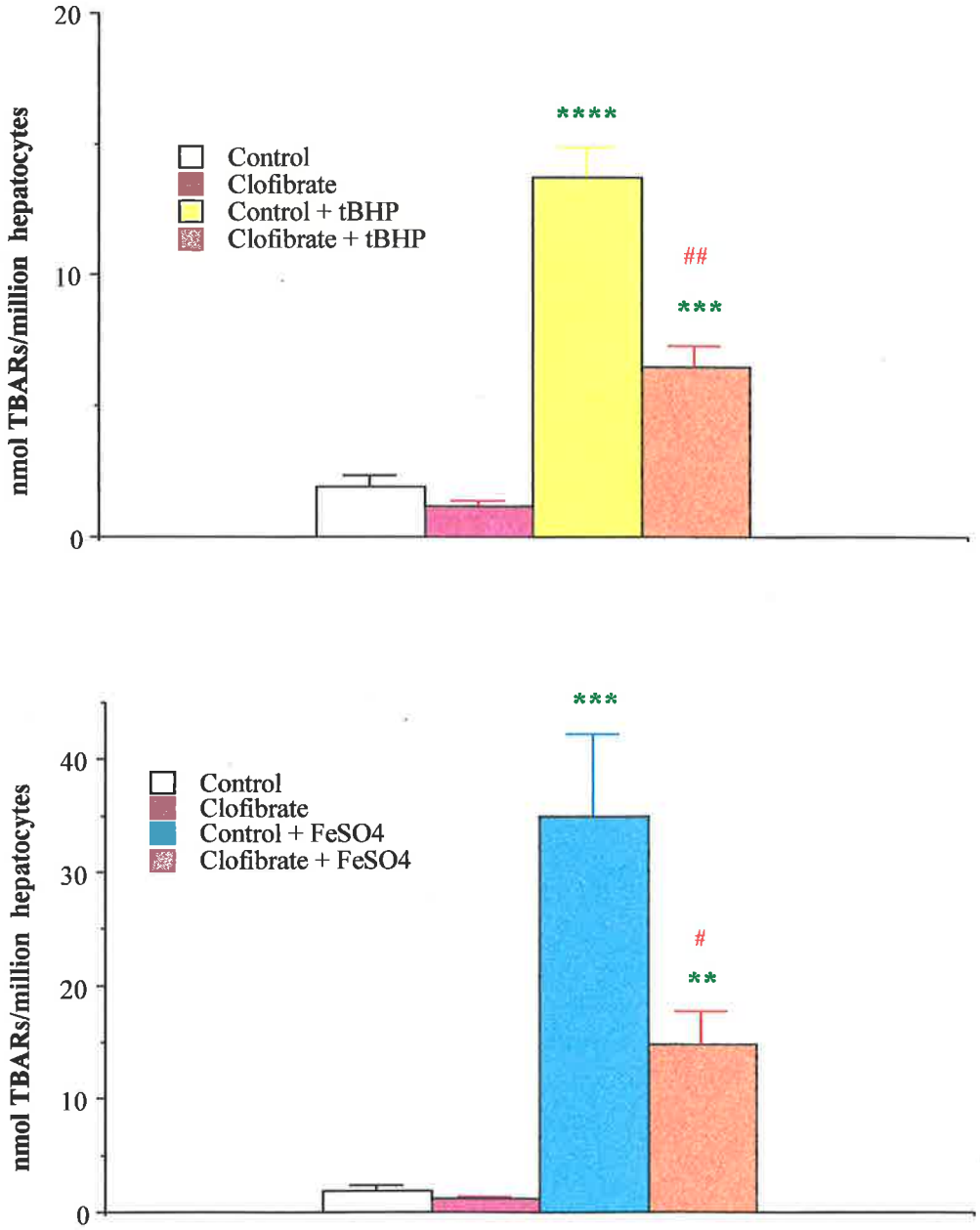
| Toxicant | $LC_{30}$ Control (mM) | $LC_{30}$ Clofibrate (mM) | Ratio | time (hour) |
|---|---|---|---|---|
| Paraquat | 0.44 | 3.0 | 6.8 | 8 |
| Nitrofurantoin | 0.12 | 0.23 | 1.9 | 4 |
| Menadione | 0.028 | 0.038 | 1.3 | 4 |
| BCNU | 0.079 | 0.14 | 1.8 | 4 |
| Diamide | 0.076 | 0.27 | 3.6 | 4 |
| Diethylmaleate | 0.5 | 3.2 | 6.4 | 8 |
| *tert*- Butyl hydroperoxide | 0.037 | 0.10 | 2.7 | 4 |
| Frusemide | 0.072 | 0.074 | 1.02 | 4 |
| Iodoacetate | 0.047 | 0.047 | 1.0 | 1 |
| Paracetamol | 0.018 | 0.18 | 10 | 4 |

**Table 9.13:** $LC_{30}$ values for the lethality of various toxicants in both control and clofibrate pretreated mouse hepatocytes. Values were determined at the 4 hour time point or as indicated. The AAP data was taken from Figure 8.1.

### 9.3.2 Effect of clofibrate pretreatment on prooxidant induced lipid peroxidation in suspension of isolated mouse hepatocytes

As a final experiment in this chapter, the level of TBARs formation, both with and without exposure to prooxidants was investigated in free suspensions of mouse hepatocytes. This was to ascertain if hepatocytes from clofibrate pretreated mice exhibited resistance to prooxidant damage, thus investigating a tentative conclusion drawn in Chapter 7 that clofibrate pretreatment may have increased the antioxidant capacity of the liver.

There was no statistically significant difference between the control and clofibrate groups with regard to TBARS formation in the absence of prooxidant. Both of the prooxidants ($FeSO_4$ and *t*-BHP) initiated lipid peroxidation which was measured as 17 and 6.5 fold respective increases in TBARs formation in control cells (Figure 9.14). However, while $FeSO_4$ and *t*-BHP also increased lipid peroxidation in clofibrate pretreated cells, the levels of TBARS formed were 55 - 60 % lower than in control cells. This supports the hypothesis that the clofibrate pretreated cells have an increased resistance to prooxidants due to higher antioxidant capacity, which may explain the protective effect observed towards $CCl_4$ (Chapter 7) and indeed some of the toxicants investigated in this chapter.




**Figures 9.14 A&B** Level of lipid peroxidation observed in isolated hepatocytes from either control or clofibrate pretreated mice. Hepatocytes were incubated at 37°C for 30 minutes with either buffer or one of two different prooxidants **A:** (upper) 1mM *t*-BHP **B:** (lower) 1 mM $FeSO_4$. (Results expressed as mean±s.e.m, n=4-5, ## = P<0.01, ***=P<0.001, ****=P<0.0001). Green = difference between prooxidant treated and control, Red = difference between clofibrate and control groups.

## 9.4 DISCUSSION

The work described in this chapter utilised primary cultures of isolated mouse hepatocytes to investigate the lethality of various toxicant following pretreatment of the donor mice with clofibrate. This model allowed investigation of toxicants for which hepatotoxicity is secondary to toxicity in other organs in the *in vivo* model. For example, paraquat enters Type I

and Type 2 alveolar epithelial cells to ultimately cause alveolar collagen accumulation and death due to lung failure *in vivo*, yet it also causes necrotic lesions in the liver, kidney and muscle (Haley, 1979).

The results presented in this chapter reveal that clofibrate pretreatment provides varying degrees of protection against the acute lethality of a variety of toxicants in isolated hepatocytes. Of the 10 toxicants investigated (including AAP from Chapter 8) and using the $LC_{30}$ ratio as an index of protection, the greatest protection was seen against AAP, paraquat and DEM with over 6 times higher concentrations required to produce equivalent toxicity in clofibrate treated cells compared to control cells. Menadione, frusemide and iodoacetate showed no statistically significant differences in lethality at any of the concentrations investigated, which was reflected in the $LC_{30}$ values being close to 1. An intermediate degree of protection was seen with the other toxicants.

On initial scrutiny of the results, there appeared no obvious mechanism by which clofibrate pretreatment protects against hepatotoxicity. The 3 toxicants whose mechanism of action is primarily through redox cycling exhibited a wide range of $LC_{30}$ ratios, with paraquat, nitrofurantoin and menadione having $LC_{30}$ values of 6.8, 1.9 and 1.3 respectively. Similarly, toxicants with GSH depletion as a major component of toxicity (or increases in GSSG) showed no consistent response, exhibiting a range of degrees of protection.

### 9.4.1 Analysis of the cytotoxicity of menadione in clofibrate pretreated hepatocytes

The use of the menadione as a toxicant allows critical consideration of various potential mechanisms of protection, since it was the only redox cycling compound that was not protected against by clofibrate pretreatment. Menadione generates toxic oxygen radicals via the NADPH CYP450 reductase catalysed formation of a semiquinonimine radical. In the presence of dioxygen, the semiquinone radical can be reoxidised to menadione with the concurrent formation of the superoxide anion radical (Thor *et al,* 1982). The enzymatic or spontaneous dismutation of $O_2o^-$ yields $O_2$ and $H_2O_2$. The latter, in the presence of certain metals, reacts with $O_2o^-$ to form more reactive oxygen species such as the OH-o and singlet $O_2$ (Halliwell and Gutteridge, 1989).

#### *9.4.1.1 Involvement of DT-diaphorase?*

Other than the futile redox cycling, menadione also undergoes a 2 electron reduction catalysed by NADPH:quinone reductase (DT-diaphorase) to a toxicologically inert metabolite and can thus be considered a detoxication pathway (Thor *et al,* 1982). However, this reaction also utilises NADPH, placing further stresses on the cellular supply of this reducing agent. The activity of DT-diaphorase has been observed to be minimally affected by PxP pretreatment

(Glauert *et al,* 1992; Cai *et al,* 1995), and as menadione was not protected by clofibrate pretreatment, DT-diaphorase is not likely to be involved in the hepatoprotective effect.

*9.4.1.2 Involvement of a depletion of NADPH?*

During menadione metabolism, the cellular reductant NADPH is consumed in both the toxication (redox cycle) and detoxication (DT-diaphorase) pathways. In addition to its involvement in these capacities, NADPH is also involved in reducing GSSG to GSH via Gred, as well as a being a key cofactor in a number of other cellular processes (Conway *et al,* 1983). Due to its involvement in maintaining a reducing milieu within the cell, NADPH is kept at a concentration of about 100 times higher than NADP+ (Krebs and Eggleston, 1974).

Due to the important roles played by NADPH, a rapid consumption of this factor could be implicated in the development of xenobiotic toxicity. A decrease in NADPH due to GSSG reduction was thought to be a minor route of NADPH consumption during exposure to menadione with most decreases in NADPH due to redox cycling via activation by NADPH CYP450 reductase (Smith *et al,* 1987). Of the three redox cycling agents used, menadione has the highest affinity for NADPH CYP450 reductase (Adam *et al,* 1990), which may account for the greater lethality of this agent when compared to the other redox cycling agents used in this study, paraquat and nitrofurantoin. The activity of NADPH CYP450 reductase, which was measured by the reduction of cytochrome c, was found to be increased by 100% in the mouse liver following clofibrate pretreatment (data not shown). It thus appears possible that the increase in cellular levels of NADPH CYP450 reductase may counteract any clofibrate related protective effect, especially with toxicants with a high affinity for this enzyme.

*9.4.1.3 Involvement of GSH depletion?*

Exposure to menadione is associated with a rapid decrease in cellular GSH (Di Monte *et al,* 1984; Maellaro *et al,* 1994) which coincides with an increase in GSSG and a loss of protein thiols (Kyle *et al,* 1989a). In addition to the indirect loss of GSH via redox cycling, menadione forms a direct conjugate to GSH which constitutes about 15% of the total GSH loss (Di Monte *et al,* 1984). The toxicity of menadione can therefore be exacerbated or attenuated by altering the cellular concentration of GSH. For example, thiol depleting agents such as DEM (Nicotera *et al,* 1988) or the Gred inhibitor, BCNU (Di Monte *et al,* 1984) increase the toxicity of menadione, while in contrast, hepatocytes from newborn mutant c14CoS/c14CoS mice with three times the GSH content of wild type controls are resistant to menadione toxicity (Liu R*et al,* 1993; Shertzer *et al,* 1994). The lack of protection associated with clofibrate pretreatment for this toxicant is further evidence that it is unlikely that GSH plays a major role in clofibrate hepatoprotection.

*9.4.1.4 Involvement of antioxidants?*

Despite being a redox cycling agent, menadione does not appear to initiate lipid peroxidation (Sandy *et al,* 1988). Also, while paraquat initiated a two-fold increase in lipid peroxidation in isolated liver cells, menadione inhibited lipid peroxidation (Dicker and Cederbaum, 1991). In addition, *in vivo* studies revealed that a synergistic amount of ethane expiration, a indicator of lipid peroxidation, in mice coadministered $Fe^{++}$ and either paraquat or nitrofurantoin, while no change was seen in mice coadministered $Fe^{++}$ and menadione (Younes *et al,* 1985).

Investigations into the effects of antioxidants on menadione toxicity reveal that the toxicity is unaltered by exposure to either ascorbic acid (Maelloro *et al*, 1994) or DPPD (Starke and Farber, 1985). Also, there is no depletion of the membrane located antioxidant, α-tocopherol during menadione exposure (Sandy *et al,* 1988), which would be expected if lipid peroxidation was indeed occurring in the membranous portion of these cells. While these antioxidants appear to offer no protection against menadione toxicity, the antioxidant, butylated hydroxyanisole, was found to protect against menadione toxicity. However this antioxidant also causes a 13 fold increase in NADPH:quinone reductase activity which would enhance detoxication of menadione via its alternate metabolic route. Unlike menadione, the toxicity of both paraquat (Bus *et al,* 1976; Di Monte *et al,* 1986) and nitrofurantoin (Buc-Calderon and Roberfroid, 1989) have been found to be decreased by coadministration of antioxidants and therefore differ substantially and importantly, from menadione in this regard.

*9.4.1.5 Involvement of lobular location? - Is a discrete population of hepatocytes targeted?*

Finally, it is of interest to note that menadione toxicity exhibits lobular selectivity, with cell death occurring in the oxygen rich periportal regions of the isolated perfused rat liver (Bahr *et al,* 1989; Ganey *et al,* 1990). As the biochemical alterations associated with peroxisome proliferation occur mainly in the centrilobular region (Bell *et al,* 1991), it seems possible that the protective effect is specific to a discrete population of liver cells. This concept was supported by the protective effect afforded by clofibrate pretreatment toward AAP and $CCl_4$ (Chapter 3 & 7), both of which exhibit centrilobular necrosis and are protected against by clofibrate pretreatment. The lobular specificity of the other toxicants used is largely unknown, since most are used only in *in vitro* experimentation. GSH is known to be present in higher concentrations in the periportal regions (Timbrell, 1994). This suggests that the centrilobular hepatocytes would be more sensitive to GSH depleting agents, such as DEM, the toxicity of which was protected against by clofibrate pretreatment. Whether the lobular selectivity is maintained in the *in vitro* situation where all original cell contact and organisation is lost is not clear, however, if the biochemical alterations are maintained, which appears likely due to the carryover *of in vivo* protection of AAP to an *in vitro* model (Chapter 8) then the possibility of a discrete population of protected cells seems feasible.

*9.4.1.6 Conclusion for the effects of clofibrate pretreatment on the cytotoxicity of menadione*

Overall, pretreatment of mice with clofibrate prior to hepatocyte isolation provided no protection against the acute toxicity of the periportal toxicant, menadione. As the toxicity of menadione is reduced by increased GSH availability, this provides further evidence against GSH dependent pathways. Other studies have shown that despite all three redox cycling agents causing a rapid loss of GSH, only nitrofurantoin and paraquat cause lipid peroxidation. This may be significant in light of the observation that clofibrate pretreatment only protected against nitrofurantoin and paraquat toxicity in the present chapter. In contrast, menadione appears to decrease lipid peroxidation and thus the lethality of this agent is insensitive to the protective actions of antioxidants. Collectively, these considerations may suggest that an increase in a cellular antioxidant other than GSH is involved in clofibrate hepatoprotection.

### 9.4.2 A role for increased antioxidant capacity providing hepatoprotection following pretreatment with clofibrate

Support for the antioxidant theory comes from the experiments involving the incubation of isolated hepatocytes in the presence of either $FeSO_4$ or *t*-BHP. Control hepatocytes produce around 2 nmol TBARs per million cells (Figure 9.14), which closely corresponds to values obtained in other studies (Smith *et al*, 1982; Rush *et al,* 1985). Incubation with either $FeSO_4$ or *t*-BHP increased the yield of TBARs in both control and clofibrate pretreated cells, although the response was clearly attenuated in the clofibrate pretreated group, suggesting a greater antioxidant capacity in these cells. From these results it would appear that the ability of antioxidants to diminish the lethality of the individual toxicants are important considerations in the assessment of protection observed.

The second group of toxicants all deplete GSH as part of their profile of cellular biochemical interactions. Regardless of the mechanism of depletion, a lowering of cellular GSH renders the cell more susceptible to toxicity via chemotoxicity and oxidative stress. This susceptibility to toxicity could well be ameliorated by an increase in cellular antioxidant capacity.

*t-BHP*

The metabolism of *t*-BHP by GSHPx to *tert*-butylalcohol is accompanied by the oxidation of GSH to GSSG (Jaeschke, 1990). As the reduction of GSSG back to GSH consumes NADPH, there is a rapid depletion of both NADPH and GSH during the course of *t*-BHP metabolism (Sies *et al,* 1972; Bellomo *et al,* 1982). As well as the enzymatic formation of an alcohol, *t*-BHP is also metabolised to reactive peroxy and alkoxy radicals (Davies, 1989) which places further burden on cellular GSH levels (Halliwell and Gutteridge, 1989). Administration of *t*-BHP is known to be associated with lipid peroxidation, which can be ameliorated by coadministration of antioxidants including; α-tocopherol, disulfuram, desferrioximine, DPPD and ascorbic acid (Glascott *et al,* 1992; Kyle *et al,* 1989b; Glascott *et al,* 1995). Clofibrate

pretreated hepatocytes in free suspension were resistant to lipid peroxidation induced by incubation with this toxicant (Figure 9.14) and thus it appears likely that clofibrate pretreatment has in some way increased the antioxidant capacity of the cell providing protection against cell lethality.

*Diamide and BCNU*

Similar to *t*-BHP, both diamide and BCNU cause a corresponding increase in GSSG levels. Diamide rapidly oxidises internal GSH to GSSG causing a reduction in cytoskeletal sulphydryl groups (Mirabelli *et al* , 1988) and export of GSSG into the surrounding media (Halliwell and Gutteridge, 1989). In contrast, BCNU initiates an oxidative stress via an accumulation of GSSG due to the inhibition of Gred (Adamson and Harman, 1989). Both these toxicants have been less studied compared to *t*-BHP, being mainly used as chemical tools for the induction of oxidative stress (Adamson and Harman, 1989; Kosower and Kosower, 1989) or in GSH homeostasis studies (Farber *et al,* 1988; Nakae *et al,* 1988). In a study investigating AAP toxicity following GSH depletion by BCNU, coadministration of the antioxidants DPPD and desferrioximine protected against AAP lethality without altering the decline in GSH levels (Nakae *et al,* 1988). This indicates that an increase in antioxidant level or capacity in the cell can protect against the lethality of toxicants via a GSH independent manner.

*DEM*

DEM conjugates directly to GSH in isolated hepatocytes (Miccadei *et al,* 1988a) and can decrease GSH levels to lower than 20% without cell lethality (Gerard-Monnier *et al,* 1992; Maelloro *et al,* 1994). While a decrease in GSH would limit cellular defence against lipid peroxidation, it has been suggested that lipid peroxidation associated with DEM exposure is not due to GSH depletion but rather other cellular effects of DEM (Reiter and Wendel, 1982). Protection against DEM induced lipid peroxidation is afforded by antioxidants such as disulfuram and ascorbic acid (Kyle *et al,* 1989b). Also, administration of ascorbic acid prevented lipid peroxidation related cell death in α–tocopherol deficient cells treated with DEM despite pronounced GSH depletion (Maellaro *et al,* 1994). This observation further supports that antioxidants can protect against toxicant lethality in a manner independent of GSH status.

*Frusemide and Iodoacetate*

The remaining 2 toxicants used, frusemide and iodoacetate are known not to induce GSH depletion as their primary mechanism of toxicity. Only a small decrease in GSH was observed during frusemide toxicity at very high nonphysiological doses *in vitro* (Massey *et al,* 1987). Moreover, the *in vivo* hepatic toxicity of frusemide is unaltered by prior GSH depletion by DEM (Mitchell *et al,* 1973c). Frusemide causes hepatic necrosis when administered in high

generation of a reactive arene intermediate (Wirth *et al,* 1976). There is no known reports of antioxidants, including GSH, protecting against frusemide toxicity.

The metabolic inhibitor, iodoacetate is a puissant alkylating agent causing rapid cell death. The mechanism of iodoacetate toxicity is related to an inhibition of glycolysis causing a decrease in ATP synthesis and disruption of ionic balance within the mitochondria (LeMasters *et al,* 1987; Casarett and Doull, 1995). Iodoacetate was the most potent of the toxicants used in this study (Figure 9.12 and Appendix 5.I). It could be speculated that the rapid reduction in cellular ATP indicates that the hepatoprotective effect of PxP pretreatment is an active process. Also, it is of interest to note that the only toxicant previously reported not to be protected by PxP pretreatment are the comparatively less toxic, chloroacetates (Bruschi and Bull, 1992). This suggests that halogenated acetates are in some way unresponsive to the hepatoprotective effects of PxP pretreatment. Finally, whether an increase in a cellular antioxidant would protect against the toxicity of iodoacetate is not currently known.

### 9.4.3 Conclusion

In conclusion, this chapter investigated the acute lethality of a range of toxicants with diverse mechanisms of action including oxidant stress, GSH and NADPH depletion, metabolic activation and lipid peroxidation. Clofibrate pretreatment provided no protection of isolated mouse hepatocytes against menadione, frusemide or iodoacetate toxicity. It was noted that each of the toxicants which were to some degree protected against by clofibrate pretreatment, all exhibit toxicity that can be ameliorated by the administration of antioxidants. Collectively, on the basis of results reported in the present and the 3 preceding chapters, induction of an antioxidant other than GSH remains the most plausible hypothesis for the hepatoprotection followings exposure to clofibrate.

# CHAPTER 10

# INVESTIGATION OF CLOFIBRATE PRETREATMENT ON LIPID PEROXIDATION, FATTY ACID PROFILE AND PROTEIN CARBONYL FORMATION: POSSIBLE RESISTANCE TO OXIDATIVE STRESS?

## 10.1 INTRODUCTION

In the previous chapter, assessment of the lethality of a number toxicants in isolated mouse hepatocytes suggested that pretreatment with clofibrate could be associated with an increase in a cellular antioxidant(s). This was supported by the observation that hepatocytes isolated from clofibrate pretreated mice were resistant to lipid peroxidation initiated by exposure to two different prooxidants. It appears likely that this resistance is in some way involved in the diminished response to hepatotoxicants.

If the explanation for the decreased formation of MDA, as determined by TBARS, involves the PxP induced synthesis of an intracellular antioxidant that scavenges mediators of oxidative stress, lower levels of oxidised macromolecules (eg DNA, protein or lipids) in the livers of PxP mice would be expected. Unfortunately, there is no consensus as to the effect of PxP pretreatment on the basal levels of lipid peroxidation in the rodent liver (Elliott and Elcombe, 1987; Perera *et al,* 1986; Tomaszewski et al, 1986). Likewise, the literature concerning levels of DNA oxidation in the livers of PxP-treated rodents is equally ambiguous (Kasai *et al,* 1989; Hegi *et al,* 1990; Takagi *et al*, 1990; Cattley and Glover, 1993; Ashby *et al.,* 1994). Another method of assessing oxidative macromolecular damage in tissues is to measure oxidised residues in proteins using an assay for carbonyl groups (Levine *et al,* 1990). Whether PxP-treatment alters the carbonyl content of hepatocellular proteins is not known.

However a simpler explanation for the diminished susceptibility of hepatocytes from clofibrate pretreated mice to lipid peroxidation could be that clofibrate, a hypolipidemic drug, has altered the lipid composition of the liver. It has been well established that MDA is mainly derived from poly unsaturated fatty acids (PUFAs) during the process of lipid peroxidation (Slater 1988) and that the PUFA content of hepatocellular membranes strongly influences hepatocyte responses to oxidative stress (Cogrell *et al.*, 1993, Sugihara *et al.*, 1994). As an example, transformed rat liver hepatocytes are resistant to lipid peroxidation, as measured by TBARS, and have 8 times lower PUFA levels than those measured in control cells (Cogrell *et al.,* 1993). A lowering of the relative levels of peroxidation-prone PUFAs and increase in peroxidation resistant saturated and monounsaturated fatty acids could well account for the

diminished yield of MDA upon challenge of clofibrate liver extracts with prooxidants *in vitro,* without requiring any induction of cellular antioxidants.

The overall aim of the experiments described in this chapter was to investigate the effects of PxP pretreatment on parameters of oxidative damage for both cellular protein and lipids in order to confirm the existence of an enhanced antioxidant capacity in clofibrate pretreated mice. However, it must be first ruled out that the resistance to lipid peroxidation is not related to an alteration in fatty acid profile. Thus the hepatic profile of saturated and unsaturated fatty acids was determined by gas liquid chromatography in both control and mice pretreated with either of two different PxP namely clofibrate and Silvex. Silvex and clofibrate were both used to ensure that any observed alterations were characteristic of the class of PxP and not a unique action of clofibrate itself. Both these PxP were also used to determine the extent of protein oxidation by measurement of basal protein carbonyl level, following the standard 10 day pretreatment protocol.

It was also anticipated that the resistance to lipid peroxidation observed in isolated hepatocytes would be also detected in homogenate fractions and that various biochemical manipulation would permit further characterisation of the suspected antioxidant. Following investigation of the amount of TBARS formed with respect to prooxidant concentration, a number of other *in vitro* studies were performed to determine if there were any differences between homogenates prepared from the livers of control compared to clofibrate pretreated mice.

Finally, the effect of clofibrate pretreatment on TBARS formation following *in vivo* administration of a hepatotoxic dose of AAP to mice was measured to determine if the resistance to lipid peroxidation was also seen in the whole animal model. An increase in TBARS formation has been previously reported following administration of hepatotoxic doses of AAP to rodents (Dimova and Stoychev, 1990; Woo *et al,* 1995). Furthermore, coadministration of antioxidants such as DMSO (Park *et al,* 1988) and DFO (Ito *et al,* 1994) diminish the *in vivo* hepatotoxicity of AAP. Consequently, it is of interest to see if there is a reduction in lipid peroxidation in clofibrate pretreated, AAP intoxicated mice.

## 10.2 MATERIALS AND METHODS

### 10.2.1 Materials

All chemicals used in this chapter were of the highest quality commercially available. For a complete list of chemicals and suppliers, refer to Appendix 1.

### 10.2.2. Animal treatment

For all experiments in this chapter, Swiss white male mice (35-40 g) were treated for 10 days by intraperitoneal injection with either the PxP clofibrate (500mg/kg/day) or equivolume olive oil vehicle. Both the fatty acid analysis and protein carbonyl experiments included a third group of mice which were treated with Silvex (200mg/kg/day, as an olive oil suspension) for 10 days.

### 10.2.3 Determination of hepatic fatty acid profile following PxP pretreatment

*10.2.3.1 Sample preparation for fatty acid analysis*

Control, clofibrate and Silvex pretreated mice (n=5 or 6 each group) were anaesthetised with 0.15 ml of 0.6 % nembutal. Following cannulation of the central vein, ligation of the aorta and excision of the hepatic vein, livers were perfused *in situ* with 20 ml of phosphate buffered saline to clear the liver of blood. The liver was excised and a 0.10 - 0.15g slice from the anterior lobe was placed in 1ml of icecold methanol in a Kontes eppendorf tube and stored at -20°C for fatty acid analysis.

*10.2.3.2 Fatty Acid analysis by gas liquid chromatography*

The determination of lipid composition of the livers was based on the method of Raynor and Howe (1995). Liver slices were carefully homogenised in the Kontes eppendorf tube using a polyethylene pestle. The homogenate was transferred to a glass culture tube, containing 2 ml of chloroform and shaken vigorously for 30 seconds. A 0.5 ml volume of 0.1 M hydrochloric acid was added to each tube, shaken for a further 30 seconds before centrifugation at 2,000 xg for 10 minutes. The upper aqueous layer was removed by aspiration and the lower organic fraction was evaporated to dryness under vacuum in a 60° C water bath. A 1.5 ml volume of 1 % sulphuric acid was added, vortexed and incubated at 100° C for 45 minutes. After cooling to room temperature, 3 ml of glass distilled water and 5 ml of petroleum spirit was added and the mixtures were shaken vigorously for 30 seconds. The upper phase was collected and the lower aqueous phase reextracted with petroleum spirit twice more. The combined petroleum spirit fractions were evaporated to dryness under a stream of nitrogen and resuspended in 1.5 ml of hexane. The samples were transferred to 2 cm glass columns containing activated magnesium silicate and the methyl esters extracted with 2 ml of 10 % diethylether in hexane, with eluate collected in chromacol vials. The methyl esters were evaporated to dryness, then resuspended in 50 µl iso-octane and stored at -20° C until analysed by GLC. The levels of individual fatty acids were expressed as % of the total fatty acids.

### 10.2.4. Lipid Peroxidation

Control and clofibrate pretreated mice (n=6 for each group, for all experiment described) were killed by cervical dislocation and the liver was rapidly removed. A 10 % (w/v)

homogenate was prepared in 0.1 M potassium phosphate buffer, pH 7.4 (buffer). Homogenates where centrifuged at 1,000 xg for 5 minutes to pellet debris prior to use. Lipid peroxidation was measured by TBARS formation using the same general protocol outlined in Section 9.2.4, with some modifications according to the requirements of the individual experiments. Results were expressed in nmol TBARS/g (liver), unless otherwise indicated.

*10.2.4.1 Basal level of TBARS formation following extended aerobic incubation.*

To determine if clofibrate homogenates had a higher basal antioxidant capacity during extended aerobic incubations, homogenate samples were incubated exposed to air at 37°C for 1, 2 or 3 hours. The reaction was stopped by the addition of 1 ml of 10% TCA. The level of TBARS in the samples were then determined as outlined in 9.2.4.

*10.2.4.2 Concentration response curve for ferrous sulphate and tert-butyl hydroperoxide.*

For determination of the TBARS formation associated with increasing prooxidant concentrations, 1 ml aliquots of individual homogenates were incubated in the presence of either 1 ml of $FeSO_4$ (0.1 to 10 mM in buffer - final concentration) or 1 ml of *t*-BHP (0.3 to 10 mM in buffer - final concentration) at 37°C for 30 minutes. The reactions were stopped by the addition of 2 ml of 10% TCA and TBARS determined.

*10.2.4.3 Effect of GSH depletion by DEM on basal TBARS formation.*

To ascertain the effect of GSH depletion on the basal level of TBARS, a 1 ml aliquot of individual 12.5% (w/v) homogenates (n = 6) prepared from control and clofibrate pretreated mice was combined with a 0.25 ml of 0.04 M DEM in buffer and incubated exposed to air for 30 minutes at 37°C. The reaction was stopped by the addition of 1.25 ml of 10 % TCA, and TBARS determined as described in Section 9.2.4. A set of concurrent incubations were stopped after the initial DEM depletion step for analysis of GSH levels using the method outlined in Section 4.2.4.2.

*10.2.4.4 Effect of exogenous GSH on ferrous sulphate initiated lipid peroxidation*

In this experiment, GSH was added to homogenate samples to compare the resistance observed in clofibrate samples to that produced by various concentrations of this antioxidant. A 1 ml aliquot of homogenate was combined with 0.25 ml of GSH in buffer (final GSH concentrations ranged from 0.1 to 5 mM) and samples were incubated at 37°C for 5 minutes. A 1.25 ml volume of 2 mM $FeSO_4$ was added, vortexed and incubated for a further 30 minutes. The reaction was stopped by the addition of 2.5 ml 10% TCA. A 2 ml volume of stopped reaction was added to 2 ml of 0.375% thiobarbituric acid and TBARS determined.

*10.2.4.5 Effect of cytosol addition on TBARS formation in microsomal samples following incubation with ferrous sulphate.*

This study was performed to investigate whether the proposed antioxidant is in the cytosol or other portion of the cell. For this, control and clofibrate pretreated homogenates were centrifuged at 12,000 xg for 20 minutes, supernatant collected and recentrifuged at 100,000 xg for 1 hour. The individual microsomal pellets from the control group only were resuspended in buffer to 0.25 g of liver per ml. The cytosolic fractions from the control group were pooled, as were those from the clofibrate group. Four 1 ml aliquots of each microsomal sample were incubated with 1 ml of buffer (2 tubes) or cytosol (control and clofibrate) for 5 minutes then 2 ml of 2 mM $FeSO_4$ was added to one buffer and the cytosol tubes, vortexed and incubated at 37°C for 30 minutes. A 2 ml volume of buffer was added to the remaining buffer tube to measure basal TBARS level. The reaction was stopped by the addition of 4 ml 10 % TCA. A 2 ml volume of stopped incubation was added to 2 ml of 0.375 % thiobarbituric acid and TBARS determined. The amount of microsomal protein was determined by Pierce BCA protein method. Results were expressed in nmol TBARS/mg protein.

*10.2.4.6 Is the antioxidant providing resistance to lipid peroxidation a protein?*

To investigate the proteinaceous character of the suspected antioxidant, the effect of enzymatic protein digestion on the resistance of clofibrate pretreated homogenates was determined by prior incubation with proteinase K, before prooxidant challenge with $FeSO_4$. To four 1 ml aliquots of homogenate was added either buffer or 500 μg/ml of Proteinase K (2 tubes each). They were then incubated at 37°C for 10 minutes. A 2 ml volume of either buffer or 2 mM $FeSO_4$ was added and the tubes were incubated for a further 30 minutes. The reaction was stopped by the addition of 4 ml 10 % TCA and TBARS were determined.

In a second experiment, three 1 ml aliquots of each sample were plunging into boiling water for 2 minutes to heat denature the homogenate. To 2 tubes was added either buffer or 2 mM FeSO4 and incubated for a further 30 minutes at 37°C. To the third tube was added 1 ml of 6.5 % TCA and then GSH was determined in accordance to methods outlined in Section 4.2.4.2.

*10.2.4.7 Measurement of in vivo hepatic lipid peroxidation following AAP intoxication.*

Control and clofibrate pretreated mice (n=12) were each divided into 2 groups of 6. One group of mice were administered 500 mg/kg AAP while the other received injection vehicle alone. After 4 hours, the livers were excised and a 10 % (w/v) homogenate produced in buffer. A 1 ml aliquot was deproteinated by the addition of 1 ml of 10 % TCA and the level of TBARS determined. Results were expressed in nmol TBARS/ g.

### 10.2.5 Protein carbonyl determination

*10.2.5.1 Sample preparation*

Control, clofibrate and Silvex pretreated mice were perfused with phosphate buffered saline as outlined in 10.2.4.1. The liver was excised and homogenised to 20%w/v in 0.1M potassium phosphate buffer, pH 7.4. A 0.5 ml aliquot was added to 0.5 ml of buffer containing 0.6 mM phenyl methylsulfonyl fluoride and vortexed well. A 0.5 ml aliquot of 10% w/v homogenate was precipitated with 0.5 ml of 6.5 % trichloroacetic acid and stored at -80°C until analysis. In addition, to determine whether the resistance to lipid peroxidation was observed in homogenates derived from mice pretreated with a different PxP, homogenates were diluted to 10 % (w/v) in buffer, incubated with either buffer or 1 mM $FeSO_4$, and TBARS level determined.

*10.2.5.2 Protein carbonyl formation*

The method for determination of carbonyl content of homogenate proteins was based on the method of Levine *et al.*, (1990). Into 2 separate eppendorf tubes, 200 µl of resuspended precipitated homogenate sample was added to 0.8 ml of 6.5 % TCA, vortexed and centrifuged at 1,000 xg for 2 minutes. The supernatant was aspirated and either 1 ml of 0.1 % 2,4-dinitrophenylhydrazine in 2 M hydrochloric acid or 1 ml of 2 M hydrochloric acid (tissue blank) was added. The samples were vortexed, incubated for 1 hour at 37° C with vortexing every 5 minutes. The samples were centrifuged at 2 000 xg for 2 minutes, with pellets washed at least three times with 1 ml of 1:1 ethanol:ethylacetate mix. After the final wash, the pellets were dissolved in 1 ml of 8 M urea in 20 mM tris hydrochloride buffer, pH 8.0 containing 20 mM EDTA, after incubation at 40° C for 30 minutes. The absorbance of each sample was measured at both 370 nm being the hydrazine derivative λ maxima and 520 nm, to allow correction for background interference. The protein concentration of each sample was determined by the Pierce BCA assay, performed in accordance to the instructions of the manufacturer. Following correction by tissue blank values, results were expressed as nmol/mg protein, using an extinction coefficient of $2.1 \times 10^4\ M^{-1}\ cm^{-1}$.(Levine, 1990)

### 10.2.6 Statistical Analysis

All lipid peroxidation studies were analysed by ANOVA. In the event of statistical significance, either the Dunnett's post hoc test for comparison of treatment groups to a control or Bonferroni post-hoc tests which compares specific pairs of data, were used. In the fatty acid profile, data was compared using the unpaired Students t-test ($P<0.05$).

## 10.3 RESULTS

The overall aim of the experiments described in this chapter was to investigate the resistance of clofibrate pretreated livers to the effects of lipid peroxidation. In the first experiment, the hepatic fatty acid profile was determined to ensure that the resistance were not due to a change in the ratio of saturated to non-saturated fatty acids.

### 10.3.1 Fatty Acid Analysis

The determination of 14 individual fatty acids in the livers of control, clofibrate and Silvex pretreated mice was performed by GLC, with results shown in Table 10.1.

| Fatty Acid | Control<br>% total | Clofibrate<br>% total | Silvex<br>% total |
|---|---|---|---|
| 14:0 | 0.20 ± 0.04 | 0.15 ± 0.02 | 0.16 ± 0.05 |
| 16:0 | 23.29 ± 0.67 | 25.58 ± 0.58 * | 23.68 ± 0.37 |
| 16:1 | 2.21 ± 0.52 | 2.96 ± 0.48 | 2.71 ± 0.36 |
| 18:0 | 11.75 ± 1.08 | 8.65 ± 0.61 * | 8.98 ± 0.57 * |
| 18:1 | 20.08 ± 2.65 | 22.25 ± 1.12 | 22.80 ± 0.70 |
| 18:2 | 14.97 ± 0.93 | 12.72 ± 0.82 | 13.91 ± 0.30 |
| 20:0 | 0.37 ± 0.2 | 0.09 ± 0.04 *** | 0.05 ± 0.03**** |
| 20:1 | 0.39 ± 0.04 | 0.38 ± 0.02 | 0.41 ± 0.05 |
| 20:2 | 0.30 ± 0.05 | 0.75 ± 0.09** | 0.60 ± 0.07 |
| 20:3 | 1.94 ± 0.13 | 3.96 ± 0.17**** | 4.16 ± 0.29*** |
| 20:4 | 9.46 ± 1.01 | 7.69 ± 0.52 | 8.28 ± 0.35 |
| 20:5 | 0.96 ± 0.09 | 1.41 ± 0.09** | 1.18 ± 0.13 |
| 22:5 | 1.19 ± 0.08 | 1.91 ± 0.14 ** | 1.57 ± 0.10 * |
| 22:6 | 10.75 ± 0.61 | 10.04 ± 0.54 | 9.93 ± 0.47 |

**Table 10.1:** Individual fatty acid content of livers (expressed as a percentage of total fatty acid content) from mice pretreated for 10 days with either olive oil vehicle, clofibrate (500 mg/kg/day) or silvex (200 mg/kg/day). Results expressed as mean ± s.e.m, n=5-6, * =P<0.05, ** =P<0.01, *** =P<0.001, **** =P<0.0001, Students unpaired t-test).

There was no change in 7 of the fatty acids investigated. Contrary to the hypothesis that levels of peroxidation-prone PUFAs are lowered in PxP livers, significant increases in the levels of two peroxidisable PUFAs, namely 20:3 and 22:5, were observed. In addition, there were significant increases in 20:2 and 20:5 fatty acids in clofibrate pretreated mouse livers, which although raised in Silvex pretreated livers, were not statistically significant. There were also significant decreases (P<0.05) in the levels of both the saturated fatty acids 18:0 (stearic acid) and 20:0 (arachidic acid) in PxP-treated livers when compared to controls (Table 10.1). Collectively, these findings indicate that decreased levels of peroxidation prone PUFAs do not

account for the diminished yield of TBARS upon treatment of clofibrate hepatocytes with prooxidants.

### 10.3.2 TBARS formation in homogenates during aerobic incubation

In the event that clofibrate pretreatment had increased the amount of a cellular antioxidant, then the basal level of TBARS formation should be lower than control during extended aerobic incubations. In this scenario, the homogenate will naturally degrade at a rate dependent on the extent of prooxidant stress and homogenate antioxidant content. In this experiment, homogenates were incubated for up to 3 hours at 37°C (Figure 10.2).

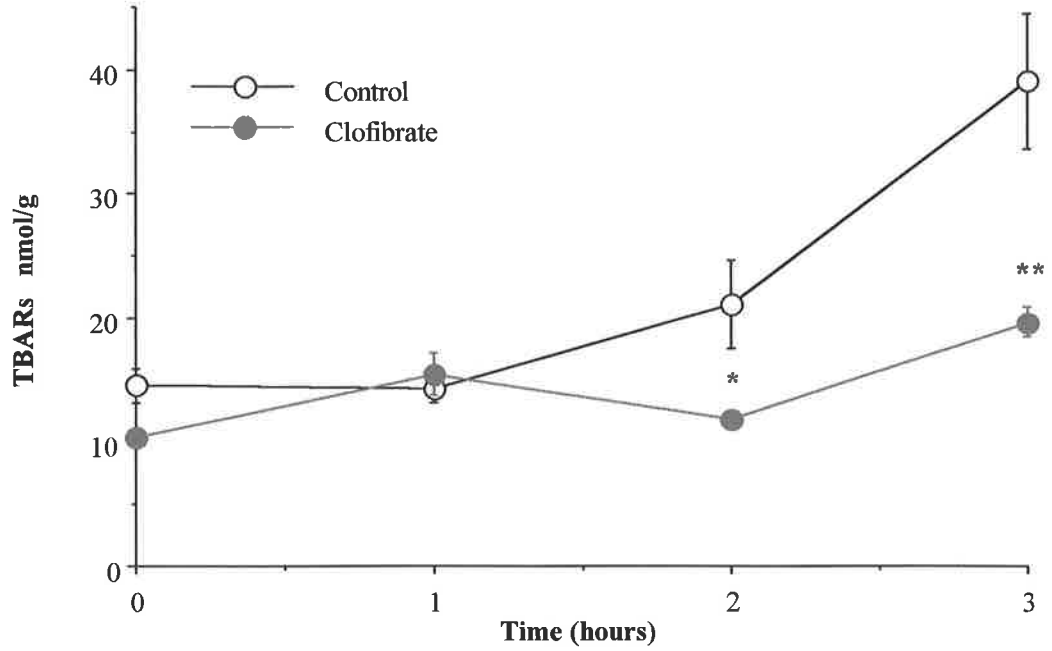



**Figure 10.2:** Measurement of lipid peroxidation in 10% mouse liver homogenates incubated for up to 3 hours at 37°C following control or clofibrate pretreatment. Lipid peroxidation was determined by nmol TBARS/ g liver. Results are expressed as mean ± s.e.m., n=6, *=P<0.05, **=P<0.01, Bonferroni Post Hoc test).

There was no difference in the level of TBARS in control or clofibrate pretreated homogenates at either the 0 or 1 hour time point. However after 2 hours, there was a significant 70% increase in TBARS in control homogenates, which had risen to 300% by 3 hours. In contrast there was no significant increase in TBARS in clofibrate homogenates over the 0 hour basal level. This indicates that the clofibrate pretreatment has increased the antioxidant capacity of the liver.

### 10.3.3 Prooxidant concentration versus extent of lipid peroxidation

The next experiment to be done was to ascertain whether the increased antioxidant capacity provided resistance to prooxidant TBARS formation similar to that observed in the isolated mouse hepatocytes (Chapter 9). For this, the concentration response relationship for two

different prooxidants, $FeSO_4$ and *t*-BHP, were investigated. For both these studies, the amount of basal level TBARS were not different in control or clofibrate pretreated homogenates following a 30 minute incubation (Figure 10.3 and 10.4).

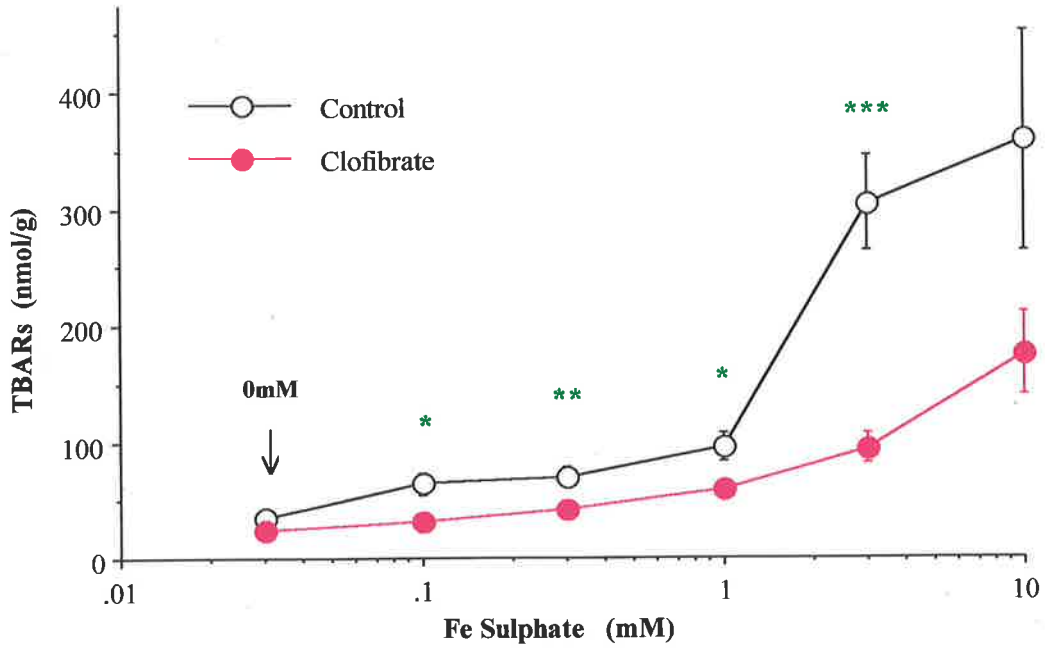



**Figure 10.3:** Measurement of lipid peroxidation in 10% w/v mouse liver homogenates incubated with ferrous sulphate (0.1 to 10 mM) for 30 minutes at 37°C following control or clofibrate pretreatment. Lipid peroxidation was determined by nmol/g TBARS formation. Results are expressed as mean ± s.e.m., n=6, *=P<0.05, **=P<0.01, ***=P<0.001, Bonferroni Post Hoc test).

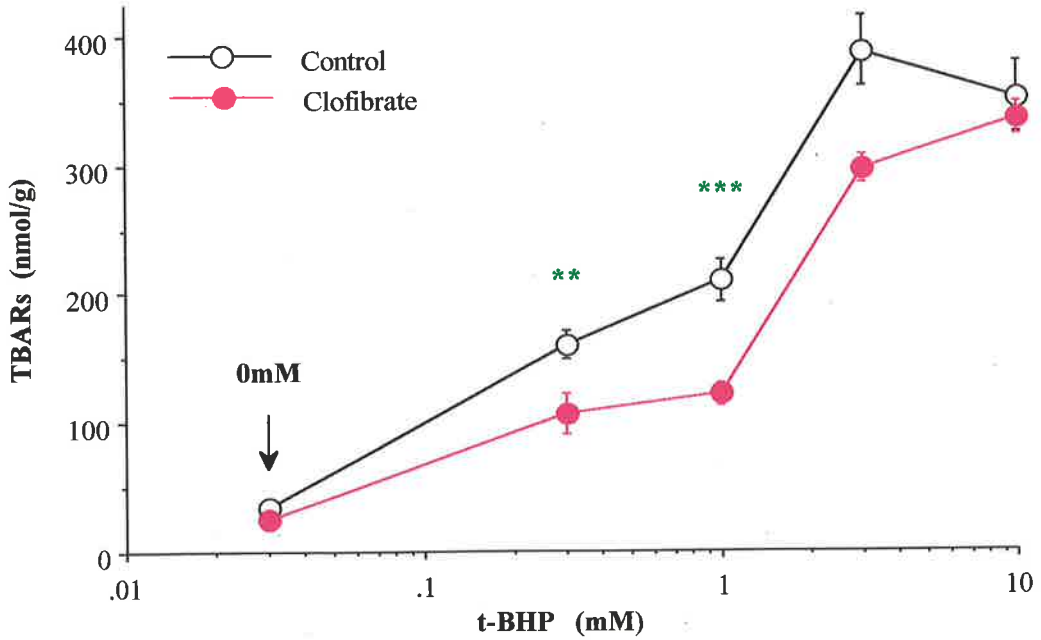



**Figure 10.4:** Measurement of lipid peroxidation in 10% w/v mouse liver homogenates incubated with *tert*-butyl hydroperoxide (0.3 to 10 mM) for 30 minutes at 37°C following control or clofibrate pretreatment. Lipid peroxidation was determined by nmol/g TBARS formation. Results are expressed as mean ± s.e.m., n=6, **=P<0.01, ***=P<0.001, Bonferroni Post Hoc test).

Investigation of amount of TBARS formed with increasing $FeSO_4$ concentration revealed that from 0.1 up to 3 mM $FeSO_4$, there was a significantly lower yield of TBARS in clofibrate compared to control treated mice (Figure 10.3). Similarly, when using 0.3 or 1 mM *t*-BHP as a prooxidant, there was a significant resistance observed in liver homogenates from clofibrate compared to control mice (Figure 10.4). There appeared to be an upper plateau reached at around 400 nmol/g TBARS in both control and clofibrate treated homogenates, which could indicate the maximum capacity for TBARS formation in this experimental system. Incubations using homogenates can be easily biochemically manipulated and therefore the resistance to lipid peroxidation in liver homogenates from clofibrate pretreated mice, allowed some characterisation of the antioxidant.

### 10.3.4 Involvement of GSH in prooxidant resistance of clofibrate homogenates

Although it had been established that it is unlikely that alteration of GSH availability is the sole reason behind the protective effect of clofibrate against xenobiotic toxicity, it was of interest to confirm these conclusions by comparing the effect of GSH depletion and also GSH supplementation on the yield of TBARS in liver homogenates.

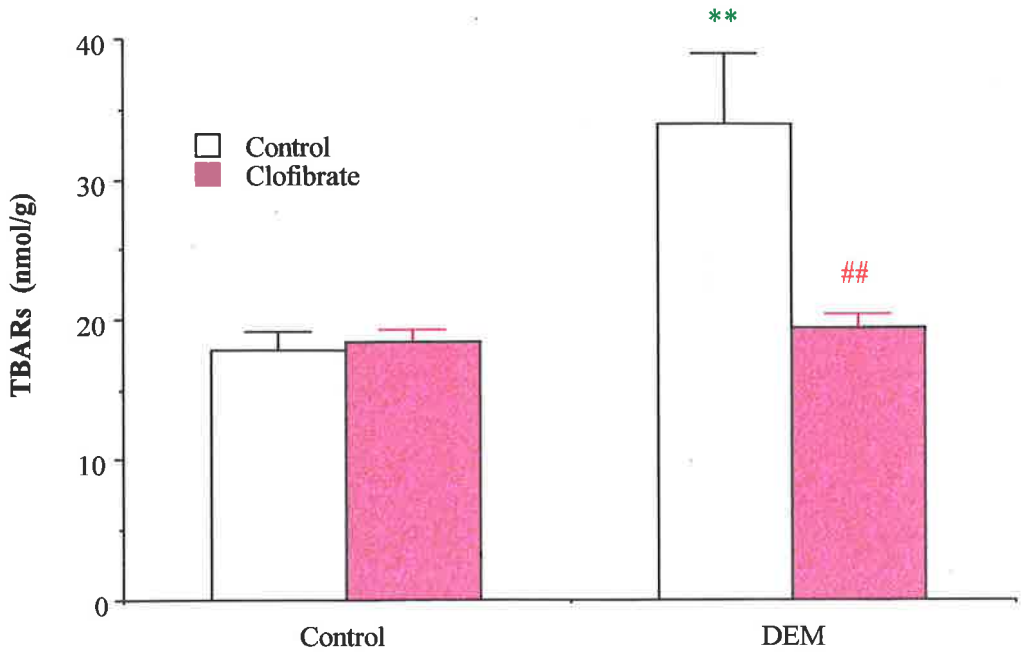



**Figure 10.5:** Measurement of lipid peroxidation in 10% w/v mouse liver homogenates incubated for 30 minutes at 37°C, either with or without prior incubation with 8.0 mM DEM following control or clofibrate pretreatment. Lipid peroxidation was determined as nmol/g TBARS formation. Results are expressed as mean ± s.e.m., n=6, **=P<0.01, Dunnett's Post Hoc test. Green stars = difference between control homogenates as a result of DEM treatment, red crosshatches = difference between control and clofibrate pretreatment).

In the first experiment, the effect of GSH depletion on basal levels of TBARS formation was determined. For this, the direct GSH conjugating agent DEM was added to both control and clofibrate treated homogenate samples. A brief incubation with DEM resulted in an over 98% decrease in available GSH. As per earlier studies, there was no significant difference in basal

TBARS in control or clofibrate pretreated homogenates. The depletion of GSH by DEM caused a 100% increase in basal TBARS in control homogenates, an effect not observed in the clofibrate homogenates (Figure 10.5). In fact the clofibrate homogenates were resistant to aerobic oxidative damage despite almost entire removal of GSH, suggesting the presence of a non-GSH antioxidant in the homogenate of clofibrate pretreated mice.

This experiment was followed by a study where GSH was added to control homogenate incubations to estimate the amount of GSH required to achieve the same antioxidant capacity seen in clofibrate treated homogenates. For this 10% homogenates from both control and clofibrate pretreated mice were incubated with 0.1 to 5 mM GSH (final concentration) prior to addition of $FeSO_4$. The actual level of endogenous GSH in the homogenate, and thus already present in the incubation was an average of 0.59 and 0.70 mM for control and clofibrate pretreated homogenates, respectively.

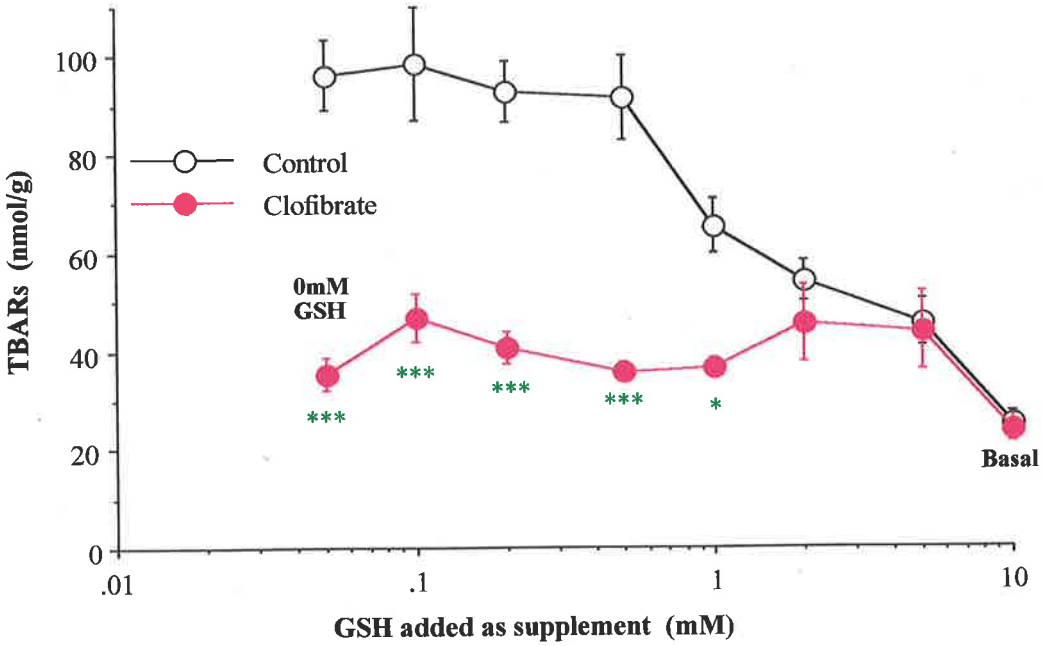



**Figure 10.6:** Effect of GSH supplementation on the level of TBARS formation as a result of exposure to 1 mM $FeSO_4$. Homogenates were prepared from either control (olive oil vehicle) or 500 mg/kg/day clofibrate for 10 days. Samples were incubated for 30 minutes at 37°C, either with or without addition of a specified amount of GSH supplement (0.1 to 5 mM GSH). The extreme right is the basal level of TBARS formation without either $FeSO_4$ or GSH. The extreme left value (0 mM GSH) is incubated only in the presence of 1 mM $FeSO_4$, clearly showing the protective effect of clofibrate pretreatment. Lipid peroxidation was determined by nmol/g TBARS as an indication of MDA formation. Results are expressed as mean ± s.e.m., n=6, *=P<0.05, ***=P<0.001, Bonferroni Post Hoc test).

In agreement with all previous studies in this chapter, there was no difference in the level of basal TBARS after a 30 minute incubation at 37°C (no added prooxidant - extreme right value, Figure 10.6). When compared with values on extreme left, where 1 mM $FeSO_4$ was added to

the incubation, a clear difference in the extent of prooxidation was observed. While a 440% increase in peroxidation was observed in control homogenates, only a 70 % increase was evident in clofibrate pretreated homogenates. Addition of up to 0.5 mM GSH to control homogenate incubations provided no protection against $FeSO_4$ induced lipid peroxidation. It was found that a supplementation of 2 mM GSH was required to suppress the differences between the control and the clofibrate pretreated homogenates. Considering that the homogenates are present as 10% (w/v) solutions, this supplementation would correspond to a nonphysiological GSH level of 20 mM, which is highly unlikely in an *in vivo* situation.

### 10.3.5 Effect of cytosol addition on $FeSO_4$-initiated peroxidation in mouse liver microsomes.

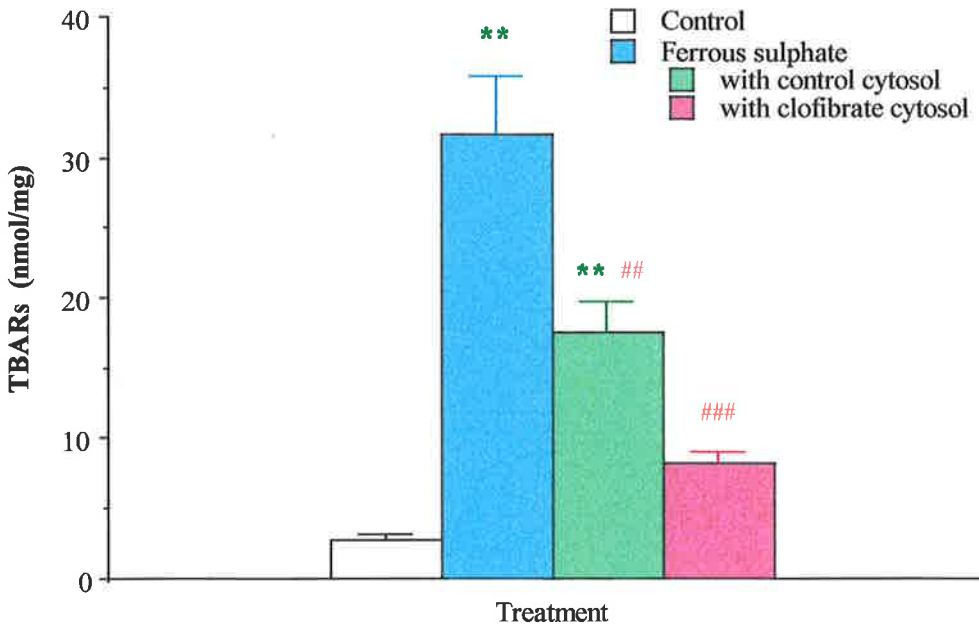



**Figure 10.7:** TBARS formation in mouse liver microsomes isolated from control mice. Microsomes were incubated either with or without 1.0 mM $FeSO_4$, the former with or without cytosol addition for 30 minutes at 37°C. Lipid peroxidation was determined by nmol TBARS/mg protein as an indication of MDA formation. Results are expressed as mean ± s.e.m., n=6, **=P<0.01, ***=P<0.001, Dunnett's Post Hoc test. green stars = difference compared to control incubation, red crosshatches = difference compared to $FeSO_4$ incubation).

The suspected antioxidant can be either hydrophilic and exist in the cytosol of the cell or hydrophobic and thereby reside in the cell membranes. Experiments to date have utilised partially purified homogenate preparations. To investigate the location of the antioxidant, microsomal and cytosolic fractions were prepared from control and clofibrate pretreated homogenates. It was anticipated that if the antioxidant was present in the cellular membranes, then the microsomal fractions would be resistant to prooxidant damage, while if in the cytosol, then addition of cytosol from clofibrate pretreated mouse livers to microsomal incubations should provide protection to $FeSO_4$ damage.

In this experiment, microsomes prepared from control mice were incubated with either buffer or cytosol prior to the addition of $FeSO_4$. Incubation with $FeSO_4$ caused a 10 fold increase in TBARS formation, which was reduced by the addition of either control or clofibrate cytosol obtained from a 10% (w/v) homogenate. However, the extent of reduction was greater for clofibrate cytosol, with an 82% decrease in TBARS levels compared to a 49% decrease for control cytosols (Figure 10.7). The results for TBARS formation were the same regardless of whether microsomes were isolated from either control (as illustrated) or clofibrate pretreated mice. Thus from the results of this experiment it appears that the antioxidant factor associated with clofibrate pretreatment is present within the soluble fraction of the cell.

### 10.3.6 Is the proposed protective antioxidant a protein?

The final experiment investigated whether the antioxidant effect involves the induction of a cellular protein. For this, homogenate proteins were denatured both by enzymatic digestion and heat.

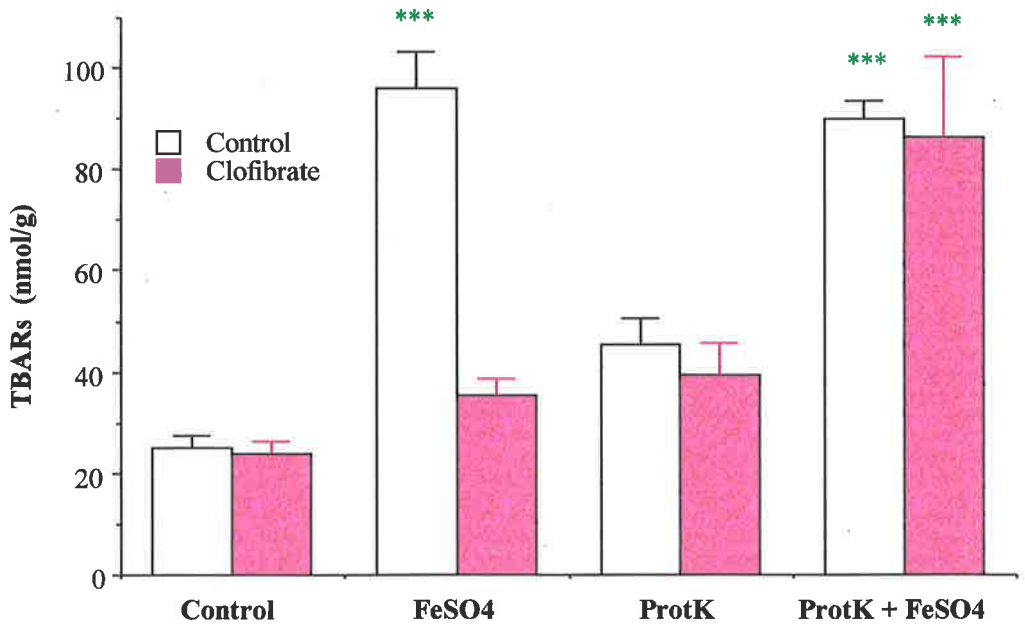



**Figure 10.8:** Measurement of lipid peroxidation in 10% w/v mouse liver homogenates incubated for 30 minutes at 37°C, with or without either 1 mM $FeSO_4$ or 500 µg/ml Proteinase K. Homogenates were prepared from either control or clofibrate for 10 days. Lipid peroxidation was determined by nmol/g TBARS as an indication of MDA formation. Results are expressed as mean ± s.e.m., n=6, **=P<0.01, Bonferroni Post Hoc test).

For the first experiment, control and clofibrate pretreated homogenates were incubated in the presence of proteinase K, a serine protease which cleaves and digests native proteins at the carboxylic sides of aromatic, hydrophobic and aliphatic amino acids. There was a 4 fold increase in TBARS as a result of $FeSO_4$ incubation in control homogenate, which was observed as only a 40% increase in clofibrate pretreated samples (Figure 10.8). Prior

incubation with proteinase K, caused a proportional 70% increase in TBARS in both control and clofibrate pretreated homogenates. However, the protective effect of clofibrate treatment is lost in these samples when challenged with $FeSO_4$. This finding suggests that the protective effect involves induction of a proteinase K sensitive antioxidant protein.

In the second experiment, homogenate proteins were heat denatured by plunging the samples into boiling water prior to the addition of a prooxidant. There was no difference in the level of TBARS in control and clofibrate pretreated samples incubated with buffer (268 ± 17 nmol/g compared with 258 ± 4 nmol/g), however, the protective effect of clofibrate was not seen following $FeSO_4$ incubation (434 ± 16 nmol/g compared with 396 ± 13 nmol/g, for control and clofibrate groups respectively). There was no alteration in the level of GSH in these samples as a result of heat denaturement. From the results of the proteinase digestion and heat denaturement it appears that the induction of an unknown protein is associated with the resistance to lipid peroxidation following clofibrate pretreatment.

### 10.3.7 Lipid peroxidation after AAP challenge

While there is still controversy concerning the role of oxidative stress in AAP toxicity, it has been established by various research groups that AAP toxicity is associated with an increase in liver TBARS (Dimova and Stoytchev, 1990; Ozdemirler *et al,* 1994; Woo *et al,* 1995). For this experiment, mice were administered a 500mg/kg dose of AAP, which has been previously shown to cause extensive hepatotoxicity in control mice, while having no effect on the livers of clofibrate pretreated mice (Chapters 3, 4, and 5). Administration of AAP was shown to cause a 4 fold increase in MDA formation in the control group as measured by an increase in TBARS (Figure 10.9). There is no AAP related increase in lipid peroxidation in the livers of clofibrate treated mice. This finding is consistent with the previous experiments described in this chapter that suggest that an antioxidant induced by clofibrate protects cells from the development of cell damage.

### 10.3.8 Protein Carbonyl formation following pretreatment with PxP

Cellular proteins, as well as lipids, are another cell constituent that are damaged by oxidative stress situations. Carbonyl groups are introduced into proteins as a result of oxidant damage and therefore exposure to reactive oxygen species during oxidative stress can be quantitated by measuring the level of carbonyl groups in liver proteins. While there was no difference in the overall protein content, hepatic protein carbonyl levels were found to be decreased by more than 45% in clofibrate pretreated and by 36% in Silvex pretreated mice (Figure 10.10). This result is indicative of substantial protection of cellular protein against oxidation and is supportive of an induction of a potent intracellular antioxidant by PxP pretreatment.

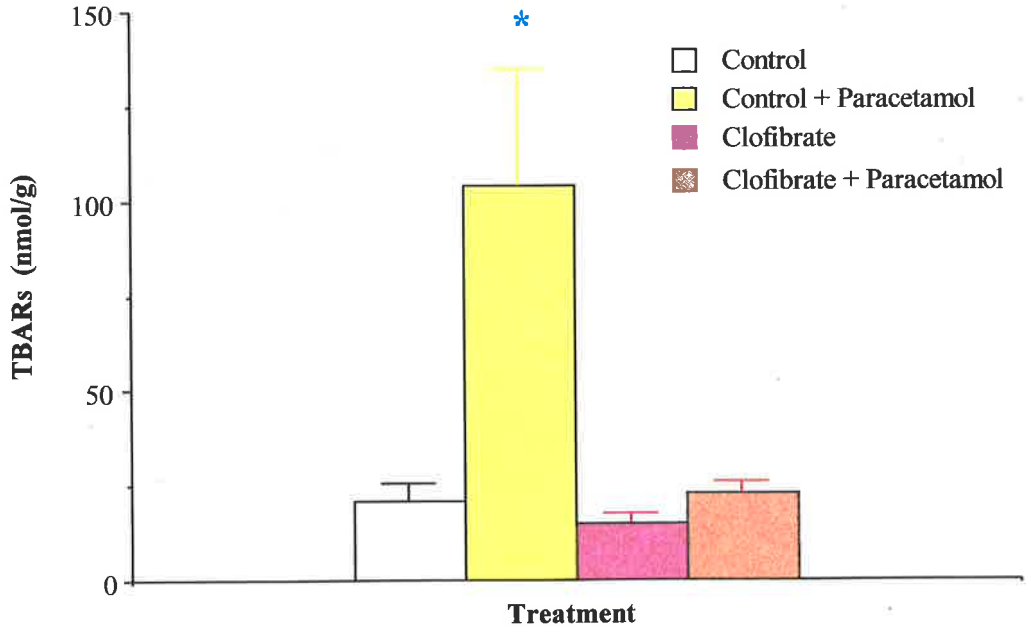



**Figure 10.9:** Assessment of lipid peroxidation by measurement of TBARS in mice 4 hours after 500mg/kg AAP challenge, following pretreatment with either control (olive oil vehicle) or clofibrate (500mg/kg/day for 10 days). (Results are expressed as mean±s.e.m, n=6. *=P<0.05)

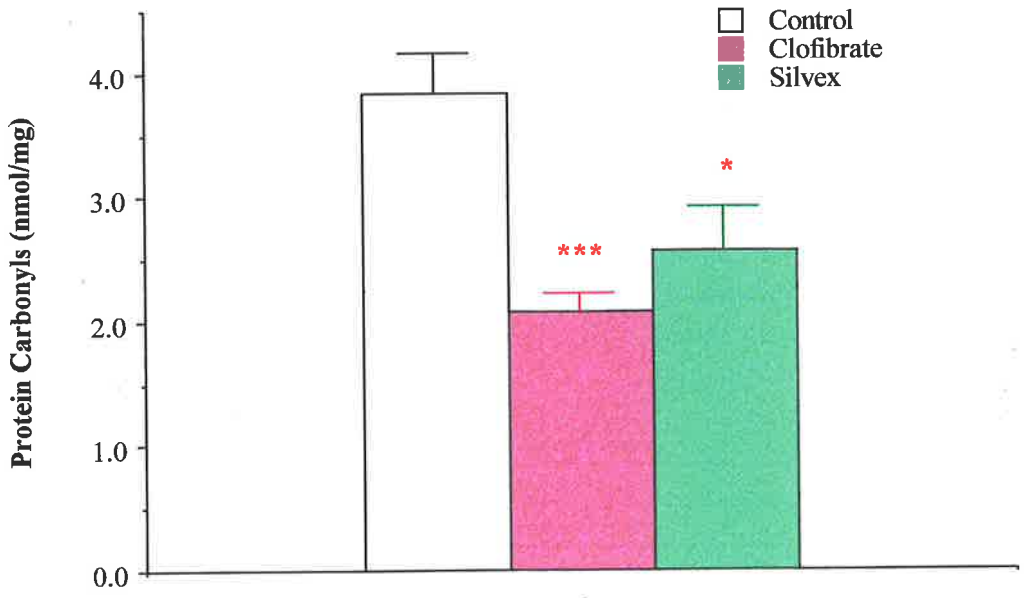



**Figure 10.11:** Measurement of protein carbonyl content in 10 % (w/v) mouse liver homogenate prepared from mice treated with either control (olive oil vehicle), 500 mg/kg/day clofibrate or 200 mg/kg/day Silvex for 10 days. Results are expressed as mean ± s.e.m., n= 13 for control, 14 for clofibrate and 5 for Silvex, *=P<0.05, ***=P<0.001, Dunnett's Post Hoc test).

## 10.4 DISCUSSION

The results reported in this chapter further elaborate on the resistance to prooxidant damage observed in hepatocytes isolated from clofibrate treated mice described in Chapter 9. They

also helped clarify the potential involvement of an antioxidant induced by clofibrate in the protective effect against various toxicants.

### 10.4.1 Fatty acid analysis

The first experiment measured the relative abundance of 14 fatty acids in the livers of control and PxP pretreated mice. This was performed to rule out the possibility that the apparent resistance to lipid peroxidation due to an alteration in the ratio of saturated to non-saturated fatty acids. It is known that the majority of MDA determined in the TBARS procedure is derived from peroxidising fatty acids with 4 or more double bonds (Halliwell and Gutteridge, 1989). Arachidonic acid (20:4) and decosahexaenoate (22:6) are the most affected in iron induced prooxidant stress in tissue homogenates (Slater, 1988). Monounsaturated and diunsaturated fatty acids give less colour yield in the TBARS assay (Halliwell and Gutteridge, 1989). Therefore, an increase in the amount of saturated or mono-unsaturated fatty acids would cause a decrease in MDA formation which would be reflected in a decreased amount of TBARS formed during prooxidant exposure.

PUFA deficiency has been implicated in the resistance of various cells to both of the prooxidants used in this present study. Transformed rat hepatocytes (Cogrell *et al* , 1993) and keratinocytes (Wey *et al,* 1993) are resistant to $Fe^{++}$ and *t*-BHP induced lipid peroxidation, respectively. Also, an alteration in fatty acid ratio has been implicated in drug metabolism and toxicity, with a resistance to AAP hepatotoxicity observed in rats fed a diet high in PUFAs (McDanell *et al,* 1992) and also fish oils (Speck and Lauterburg, 1990). An increase in the PUFA content of cell membranes increases membrane fluidity which alters the mobility and orientation of membrane bound proteins and these have been proposed to enhance AAP clearance via the glucuronidation pathway (Speck and Lauterburg, 1990). However, the results of this present study revealed only very subtle changes in fatty acids and it is unlikely that alterations in fatty acid profile were involved in the hepatoprotective effect of PxP pretreatment.

As a whole, there was little difference in the fatty acid profiles between the control and PxP treated mice. In fact, the changes observed suggest that TBARS formation would increase in PxP pretreated mice, with significant increases in the peroxidation prone 20:3 and 22:5 fatty acids (Table 10.1). The decrease in the saturated 18:0 and 20:0 fatty acids could be explained in that both are substrates for peroxisomal fatty acid ß-oxidation which is enhanced following PxP treatment, and it is possible that the increased metabolism would lead to lower cellular concentrations of long chain saturated fatty acids.

The results of this investigation are in general agreement with other studies that have measured the amount of some fatty acids following pretreatment with PxP. In the microsomal

fractions of rats pretreated with clofibrate and other fibric drugs, increases in 16:0, 16:1 and 18:1 fatty acids and decreases in 18:0, 22:5 and 22:6 were observed and reported to be specific to treatment with PxP (Vazquez *et al,* 1995). In addition, the levels of the peroxidation prone linoleic (18:2) and docosahexaenoic (22:6) fatty acids were decreased in rats fed with nafenopin (Huber *et al,* 1991). In this study the levels of these PUFAs were similarly affected, but in some cases, the changes were not statistically different from controls. It was therefore of interest to investigate other explanations of the prooxidant resistance of clofibrate pretreated samples toward lipid peroxidation observed in Chapter 9.

### 10.4.2 Evidence for the induction of a proteinaceous antioxidant.

In this present study, the method used to detect lipid peroxidation was the colorimetric measurement of TBARS which detects increases in the amount of MDA and probably other byproducts of lipid peroxidation. Unfortunately, the TBARS assay is not specific to MDA and a number of endogenous compounds including biliverdin, methionine and homocysteine are also detected by this test (Halliwell and Gutteridge, 1989). MDA is believed to be cleaved from cyclic endoperoxides formed as part of the lipid peroxidation cascade initiated by radical species (Halliwell and Gutteridge, 1989). MDA release thus occurs as a relatively late event in the lipid peroxidation cascade. Ideally, thorough investigation of antioxidant interaction with lipid peroxidation products should include a number of different biochemical assays with more sensitive endpoints. However, it has been concluded that when compared to other simple methods, TBARS analysis provided a useful indication of the extent of peroxidation in biological samples (Smith *et al,* 1982). As the majority of the experiments described in this chapter compared TBARS level after *in vitro* incubation, it is assumed that any increases in TBARS above basal level resulted from MDA release during lipid peroxidation. This allowed comparison of the relative resistance to lipid peroxidation of homogenate samples prepared from the livers of control versus clofibrate pretreated mice.

On the whole, the basal level of TBARS was not statistically different between vehicle and clofibrate pretreated liver homogenates in any of the short term incubations performed. This is in agreement with other studies where PxP was administered on a short term basis (Agarwal *et al,* 1982; Elliott and Elcombe, 1987). However, when the homogenates were exposed to prooxidant conditions, clear differences between the control and the clofibrate pretreated samples were observed. PxP (both clofibrate and Silvex) pretreated mouse livers were resistant to prooxidant damage initiated by incubation with $FeSO_4$, which is in agreement with observations made in Chapter 9 using suspensions of isolated hepatocytes.

The enhanced endogenous antioxidant capacity in clofibrate pretreated homogenates was confirmed by extended aerobic incubation (Figure 10.2) and also by the distinct shift of the prooxidant concentration response curve to the right when incubated with either $FeSO_4$

(Figure 10.3) or *t*-BHP (Figure 10.4). The prooxidant resistance of clofibrate pretreated homogenates was able to be further investigated by biochemical modification of the basic incubation procedure. Despite virtual complete removal of GSH by DEM, clofibrate pretreated homogenates were resistant to lipid peroxidation compared to control (Figure 10.5) suggesting the existence of a non GSH protective factor. Also, supplementation with an equivalent to 20 mM GSH was required in control homogenates to observe the same degree of protection provided by clofibrate pretreatment, against $FeSO_4$-induced lipid peroxidation (Figure 10.6). Experiments utilising microsomal and cytosolic fractions prepared from control and clofibrate pretreated livers suggested that the antioxidant was cytosolic in location. If the antioxidant was present in cell membranes then microsomes prepared from clofibrate pretreated mice should be resistant to prooxidant exposure compared to control microsome. However, there was no difference between the 2 treatment groups in the basal amount of microsomal TBARS, nor were there differences following incubation with either $FeSO_4$ or *t*-BHP (data not shown). The addition of cytosol from either the control or clofibrate pretreated mice to microsomal incubations decreased TBARS formation although the decrease produced by the clofibrate cytosol was much greater than that produced by control cytosols (Figure 10.7). As a final experiment, the homogenates from both control and clofibrate pretreated mice were subjected to protein denaturement either by enzymatic digestion or heat. In both cases, while GSH levels were maintained, the protective effect observed in the clofibrate pretreated homogenates was lost (Figure 10.8).

The results of the *in vitro* lipid peroxidation studies suggest that pretreatment with PxP induces the formation of a cytosolic antioxidant protein. With consideration of the conclusions drawn from Chapter 9, it appears likely that this antioxidant provides protection against the lethality of a diverse range of toxicants.

### 10.4.3 TBARS formation in AAP poisoned mice

Since the above conclusions are drawn largely on the basis of *in vitro* findings, whether the same decrease in TBARS formation was seen *in vivo* was also performed. It was of interest to see if the proposed antioxidant was able to prevent lipid peroxidation in AAP intoxicated mice. For this, an hepatotoxic dose of AAP was administered 4 hours prior to collection of the livers. A 4-fold increase in TBARS level was observed in AAP poisoned control mice, which is in agreement with results from other studies where 3 to 6 fold increases in TBARS were detected (Dimova and Stoytchev, 1990; Ozdemirler *et al,* 1994; Woo *et al,* 1995). As what would be predicted from the *in vitro* observations presented earlier, there was no increase in TBARS level above basal levels in clofibrate pretreated mice which also received AAP (Figure 10.9). Regardless of the course of events leading to the increase in TBARS, this result indicates that an increase in lipid peroxidation occurs as a consequence of AAP administration and that it is prevented in clofibrate pretreated mice.

Coadministration of antioxidants have been found to both reduce the level of lipid peroxidation, as measured by TBARS formation and also modify the extent of cell damage following administration of AAP. Treatment with the chain breaking antioxidant, α - tocopherol produced an 87% decrease in the TBARS formation following *in vivo* exposure of mice to a 400 mg/kg dose of AAP (Amimoto *et al,* 1995). Similarly, the iron chelating antioxidant, DFO was found to prevent both AAP-induced TBARS formation and also decrease the mortality following AAP intoxication in mice (Saikaida *et al,* 1995).

Collectively, the results of the lipid peroxidation studies in this present chapter suggest that clofibrate pretreatment has increased the level of a potent antioxidant. This yet to be identified protein may play a role in protecting against the liver damage and mortality associated with AAP toxicity.

### 10.4.4 Protein carbonyls

As a final investigation to this series of experiments, the protein carbonyl content of liver homogenates from both control and PxP pretreated mice was determined. Protein carbonyls are formed as a result of oxidative modification to proteins and as such, protein carbonyl levels have been used to confirm and quantitate systemic oxidative stress (Witt *et al,* 1992). In this present study, it was observed that pretreatment with PxP resulted in a 40% decrease in protein carbonyl level. This is indicative of a lowering of steady state level of oxidative stress to which the cell is normally exposed and in light of the results of the previous study, is supportive of an increase in a cellular antioxidant. Other than an increase in antioxidant capacity, it could be argued that as pretreatment with PxP is associated with hypertrophy and hyperplasia of the liver, the decrease in protein carbonyl formation could be a direct result of an increase in *de novo* hepatic protein synthesis, effectively diluting the cellular protein pool. However, following consideration of the liver weight increases that occurred during this experiment, the magnitude of the decrease in the levels of protein carbonyls cannot be completely explained by this factor.

It seems likely that the difference in protein carbonyl contents between control and PxP pretreated livers must be purely related to some biochemical change that has resulted from PxP pretreatment. A possible explanation is an increase in protease activity resulting in an enhanced ability of the cell to remove modified or damaged proteins. For example, alkaline proteases which are involved in the proteolysis and removal of oxidised proteins show an age related decrease in activity which is linearly related to an increase protein carbonyl content (Agarwal and Sohal, 1994). Other possibilities include a decrease of endogenous protease inhibitors, or an intracellular increase in the degradative organelle, the lysosome, all of which could increase the efficiency of removal of damaged proteins from the cell.

This study has shown that the protein and indirectly, lipid fractions of PxP treated mice appear protected against oxidative damage. Confirmation of DNA damage following PxP treatment has not been conclusively established. Studies reported by various groups measuring 8-OHG levels as an indicator of oxidative DNA damage have found either no change (Hegi *et al,* 1990) or slight increases (Kasai *et al,* 1989, Cattley and Glover, 1993) in 8-OHG levels in PxP treated rats. However, subcellular fractionation revealed that the damaged DNA was associated with the mitochondrial and not the nuclear DNA pool (Cattley and Glover, 1993). Other methods of analysis of DNA damage have yielded equally ambiguous results (Ashby *et al*, 1994). Interestingly, the pattern of age-associated accrual of 8-OHG has been found to be virtually identical to that of protein carbonyl content (Agarwal and Sohal, 1994). Perhaps oxidised DNA damage has not been observed as a result of PxP pretreatment because DNA is protected against *in vivo* oxidative damage?

### 10.4.5 Conclusion

Pretreatment with PxP appears to result in a reduction in intracellular oxidative stress in the livers of treated mice. This was seen in a decrease in protein carbonyl levels and in a resistance to prooxidant stress as measured by lipid peroxidation in homogenate fractions. It is likely that there is some kind of biochemical alteration present in PxP treated livers that has provided the protection observed.

In support of an increase in antioxidant associated with PxP treatment, a recent study has shown that PxP treated rats were resistant to *in vivo* hepatic lipid peroxidation (Huber *et al,* 1997). Indeed, due to the broad spectrum of antioxidant action, the previous studies which used PxPs as biochemical tools to investigate the mechanism of toxicity of other compounds, routinely observed a protective effect which could well be explained by an increase in cellular antioxidants. These include both *in vitro* and *in vivo* studies, where decreases in $H_2O_2$ cytotoxicity in isolated rat hepatocytes (Garberg *et al,* 1992), paraquat induced pneumotoxicity (Frank *et al,* 1982), $CeCl_3$ induced hepatic necrosis (Tuchweber and Salas, 1978), $Fe^{++}$-induced uroporphyria (Smith *et al,* 1990) were observed after pretreatment with PxP.

On the basis of evidence herein described, it appears likely that the resistance to oxidative stress and/or hepatotoxicity following clofibrate pretreatment is due to the induction of a cellular antioxidant. However, whether this antioxidant is a radical scavenger, a metal chelator or acts in another antioxidant capacity is yet to be elucidated.

# CHAPTER 11

## GENERAL DISCUSSION

Repeated administration of PxPs to rodents, results in a characteristic increase in cellular peroxisomes as well as a number of biochemical alterations within liver cells. While many, if not all, of the effects of PxPs involve the activation of the PPAR, the actual mechanism of carcinogenesis following long term exposure to these compounds or the consequences of a shorter duration of exposure remains essentially unknown. Since the latter includes alterations in Phase I, Phase II and other detoxication enzyme activities, it seems likely that exposure to PxPs will alter the metabolic fate and toxicological properties of other xenobiotics that undergo biotransformation. The aim of the experiments described in this thesis was to thus investigate the effects of short term treatment with PxP on the acute toxicity of various toxicants, with particular attention focussed on the classic hepatotoxicant, AAP.

Having established a murine model for peroxisome proliferation using the hypolipidemic clofibrate (Chapter 2), the toxicity of AAP following pretreatment with this PxP was investigated. It was found that a 10 day pretreatment with 500 mg/kg clofibrate profoundly protected mice against the acute hepatotoxicity of a single toxic dose of AAP (Chapter 3). During numerous repeats of this experiment, no increase in plasma SDH was ever measured in clofibrate pretreated mice administered 500 mg/kg AAP. In stark contrast, up to 1000-fold increases in plasma SDH were observed in control (olive oil vehicle pretreated) mice administered the same hepatotoxic dose of AAP, which was accompanied by gross centrilobular necrosis and an overall 30% mortality. As the same protective effect was observed following Silvex and DEHP pretreatment, this suggested that the hepatoprotective effect was a result of pretreatment with PxPs in general and was not simply a unique property of clofibrate itself. In addition to AAP, pretreatment with clofibrate protected against the toxicity of $CCl_4$ (*in vivo*) , DEM (*in vitro*), paraquat (*in vitro*) and to a lesser extent BrB (*in vivo*), diamide, BCNU and nitrofurantoin (*in vitro*), amongst other toxicants (Chapters 7 and 9). The use of diverse acting toxicants in addition to AAP, provided further information about the nature of the cytoprotection afforded by pretreatment with clofibrate.

### 11.1 Potential protective mechanisms of PxP on AAP toxicity

The major toxicological consequence following the administration of high doses of AAP is hepatic damage. Although there is strong evidence for AAP toxicity being mediated by the highly reactive CYP450 generated metabolite NAPQI, the critical molecular events that lead to cell death following AAP overdose are unclear. Due to the lack of knowledge about the precise intracellular targets for NAPQI, it is difficult to identify a single protective mechanism

that could account for the hepatoprotection afforded by clofibrate. For example, a reduction in the formation of NAPQI, deactivation of NAPQI before it reaches its target site or interference with the ensuing cytodestructive events would all constrain AAP toxicity. The diversity of physiological, cellular and biochemical changes that accompany short term treatment with PxPs raise the possibility that the hepatoprotective effect could be the result of multiple biochemical alterations. With a view to reviewing the strengths and weaknesses of various explanations of the protective effect, potential protective mechanisms of pretreatment with PxP on the acute hepatotoxicity of AAP are discussed in the following sections (11.1.1 to 11.1.6).

### 11.1.1 Alteration in the toxicokinetics of AAP

When considering the time course of AAP hepatotoxicity from the commencement of exposure to the completion of cell death, the first point at which protection could occur could be at the level of AAP disposition. One of the most obvious effects of exposure to PxP is hepatomegaly. In these studies, a 30% increase in %LBW was routinely observed in mice following clofibrate pretreatment (Chapter 3 and 4). One possibility could be that this increase in %LBW increases hepatic blood flow and enhances the supply of blood borne nutrients that are important in the maintenance of biotransformation reactions, thus increasing the ability of the liver to cope during conditions of increased exposure to xenobiotics. However, hepatotoxicity was observed in control mice administered 250 mg/kg AAP, while no toxicity was observed in clofibrate pretreated mice administered twice this dose (Chapter 3), suggesting that a 30% increase in liver size would not completely explain PxP induced hepatoprotection. Furthermore, it could be argued that the increased blood supply to the liver would result in individual hepatocytes within PxP livers being exposed to lower AAP concentrations than in control mice. The key prediction of this explanation is that the protection is strictly due to *in vivo* toxicokinetic factors, and that it would not be retained in an *in vitro* setting in cells that were cultured from the livers of PxP pretreated mice. Since the experiments described in Chapters 8 and 9 showed that short term culture of mouse hepatocytes from clofibrate pretreated mice were resistant to killing by AAP and other toxicants, this explanation of the protective mechanism could be easily dismissed. Thus it was evident that the protective effect is a result of specific biochemical changes within the hepatocyte itself.

### 11.1.2 An increase in peroxisomal number provided protection against AAP toxicity

The main characteristic of PxP treatment is an increase in the number of hepatic peroxisomes. Since the peroxisomes contain enzymes such as catalase and superoxide dismutase that influence the metabolism and toxicity of xenobiotics, one explanation for the hepatoprotection could invoke these intraperoxisomal changes as the fundamental protective mechanism. Such an explanation is certainly consistent with some findings in the present work. For instance, it

known that the induction of peroxisomes is not even across the liver lobule, with the greatest peroxisome proliferation occurring in hepatocytes in the centrilobular region (Just *et al,* 1989; Bell *et al,* 1991). It was interesting to note that the centrilobular toxicants AAP (Chapter 3) and $CCl_4$ (Chapter 7) were well protected by clofibrate pretreatment. In contrast BrB, a toxicant with periportal and centrilobular effects, was less protected against (Chapter 7) while minimal protection was provided against menadione cytotoxicity (Chapter 9), a toxicant which produces toxicity mainly in the oxygen rich periportal area (Bahr *et al,* 1989; Ganey *et al,* 1990). Such findings suggest that the hepatocytes in the centrilobular region of clofibrate pretreated livers have obtained a specific resistance to xenobiotic toxicity, perhaps as a direct consequence of the increased peroxisome numbers in this zone of the liver.

Nonetheless, a key factor that mitigates the strength of this argument is the fact that while the onset of the hepatoprotective effect was accompanied by increases in cellular peroxisomes, there appeared to be no such correlation between the 2 parameters following cessation of clofibrate treatment (Chapter 4). Thus it seems unlikely that the protective effect of clofibrate would be due to changes in peroxisomal components that do not correlate with the absolute numbers of the organelles. It seems more likely that hepatoprotection was the result of an extraperoxisomal biochemical alteration that was initiated by peroxisome proliferation, but persists longer upon the cessation of exposure to PxPs.

### 11.1.3 Increase in metabolic clearance of AAP via Phase II pathways

An additional mechanism that might be involved in PxP induced protection against AAP could involve an enhancement of the capacity of the liver to clear AAP via Phase II conjugative pathways. The glucuronide and sulphate conjugates represent the main fate of AAP in mice. As these metabolites do not require CYP450 catalysed bioactivation of AAP, an increase in formation of these conjugates by increasing either cofactor concentrations or the activities of key Phase II enzymes could diminish the amount of AAP that undergoes bioactivation to NAPQI. In addition, AAP in its unchanged form, can be excreted without bioactivation thereby reducing a potential source of NAPQI. However, there was no difference in the urinary levels of glucuronide or sulphate metabolites in clofibrate compared to control pretreated mice (Chapter 5). In addition, there was no difference in the rates of AAP-glucuronide metabolite formation in isolated hepatocytes from control and clofibrate pretreated mice, at least at sublethal AAP concentrations in the control cells (Chapter 8). Furthermore, another possible protective mechanism could involve enhanced renal clearance of the unchanged parent drug. In this regard, various *in vitro* studies have shown NAPQI can be reduced back to AAP (Li *et al*, 1994) however analysis of urinary metabolites showed no alteration in the amount of unchanged AAP following administration of PxP (Chapter 5). All in all, these results suggest that enhanced excretion of nontoxic metabolites of AAP is not involved in PxP hepatoprotection.

### 11.1.4 Diminished CYP450 catalysed activation of AAP.

Since the toxicity of AAP is due to the Phase I dependent formation of a reactive intermediate (NAPQI), the protective effect following pretreatment with PxPs could be related to an inhibition of CYP450 bioactivation. Therefore the extent of bioactivation of AAP was indirectly assessed by measuring the capacity of PxP-treated livers to bioactivate AAP, by firstly measuring of the formation of GSH derived metabolites and secondly by determining the extent of covalent binding of AAP to cellular protein.

There was no increase in the level of urinary GSH derived metabolites in control compared to clofibrate pretreated mice (Chapter 5). In addition, *in vitro* studies showed that there was no difference in AAP-GSH conjugate formation by microsomes (Chapter 5) or hepatocytes (Chapter 8) isolated from control compared to clofibrate pretreated mice. This suggested that the ability of PxP pretreated livers to oxidatively bioactivate AAP was not compromised. However, a decrease in the amount of radiolabelled AAP bound to hepatic protein was seen in the livers of clofibrate treated mice at hepatotoxic doses of AAP. These seemingly contradictory findings suggest that while the amount of NAPQI formed in PxP-pretreated livers is the same as in controls, by some as yet unidentified mechanism, NAPQI undergoes deactivation before it reaches its critical intracellular targets. The most likely candidate for fulfilling such a role is GSH.

### 11.1.5 Enhanced deactivation of NAPQI by GSH

The endogenous electrophile scavenger GSH plays a major protective role during exposure to AAP, since it protects protein sulphydryls against adduction by NAPQI. It is evident that either an increase in GSH availability, via either an increased hepatic GSH pool or by enhanced rates of synthesis, would provide hepatoprotection against AAP toxicity. A number of studies were thus performed to investigate the involvement of GSH in the hepatoprotective effect afforded by clofibrate pretreatment.

It was established that the hepatic GSH level was either unchanged or slightly increased by clofibrate pretreatment (Chapters 4, 6, 8 and 10). When combined with the increased liver weight that accompanied PxP administration, the extent of protection could not be solely explained on the basis of an increase in the absolute amount of GSH present in the liver (Chapter 6).

The availability of GSH can also be altered by enzymes involved in GSH turnover. The activity of the detoxication enzyme, GSHPx, which metabolises peroxides to form GSSG, was decreased by less than 20% while the GSH regenerating enzyme, Gred was increased by 17%. These are relatively minor alterations which were considered to have no major impact on the

GSH availability within the PxP pretreated cell (Chapter 6). Also, there was no increase in the activity of the GSHt-α and GSHt-π isoforms which are involved in the enzymatic conjugation of GSH to NAPQI (Chapter 6). Collectively, these findings are consistent with the observation that no significant change in GSH-AAP metabolite formation was seen either *in vivo* or *in vitro* during the present work (Chapters 5 and 8).

In further investigations of the involvement of GSH in PxP hepatoprotection, studies using a variety of toxicants other than AAP revealed no consistent role for GSH. For example, clofibrate pretreated mice were protected against $CCl_4$ hepatotoxicity (GSH independent) (Chapter 7), while contrary to predictions if GSH was solely responsible for protection, no resistance was provided against the cytotoxicity of menadione (GSH dependent) in hepatocytes from clofibrate pretreated mice (Chapter 9). However, the most compelling evidence for the lack of exclusive involvement of GSH in the hepatoprotective effect of clofibrate pretreatment was an investigation of AAP toxicity following GSH depletion. The *in vivo* treatment of mice with the GSH depleting agents, either DEM, which conjugates directly to GSH, or BSO, a GSH synthesis inhibitor, resulted in a massive potentiation of AAP toxicity in control mice. No such increase in toxicity was seen in clofibrate pretreated mice (Chapter 6). Similarly, no increase in AAP cytotoxicity was observed in primary cultures of mouse hepatocytes isolated from clofibrate pretreated mice which were depleted of GSH by DEM, unlike control cells (Chapter 8).

While it is obvious that any increase in cellular GSH would provide some protection against AAP toxicity, the various findings described above strongly indicate that an increase in GSH availability is not the sole factor underlying the hepatoprotection afforded by pretreatment with PxP. Rather, it appears that NAPQI is deactivated via a GSH independent pathway before it reaches subcellular targets.

### 11.1.6 An increase in the level of a cellular antioxidant?

A final possible explanation for the hepatoprotective effect of PxP pretreatment that was examined in this thesis pertained to an increase in the antioxidant capacity of clofibrate pretreated livers. While the participation of oxidative stress in AAP toxicity remains controversial (Thorgeirsson *et al,* 1976; Adams *et al,* 1983; Lauterburg *et al,* 1984), several lines of evidence point to such an involvement. For example, AAP toxicity has been reported to be accompanied by the generation of ROS (Lores-Arnaiz *et al,* 1995), while coadministration of antioxidants have been shown to protect against AAP toxicity (Lake *et al,* 1981; Harman, 1985; Park *et al,* 1988; Jaeschke 1990; Ito *et al,* 1994).

An induction of antioxidant capacity in the livers of clofibrate pretreated mice was suggested by a number of separate observations. Firstly, the basal level of oxidatively modified proteins

was decreased in the livers of clofibrate pretreated mice suggesting that proteins in these livers are protected against damage by oxidants produced during routine cellular metabolism (Chapter 10). Secondly, the *in vivo* hepatotoxicity of the free radical generating $CCl_4$ was attenuated by clofibrate pretreatment (Chapter 7), which also suggested of an increase in the level of an antioxidant(s) other than GSH. Thirdly, during study of the toxicity of a range of diverse acting toxicants in isolated hepatocytes, it was consistently seen that those whose toxicities are known to be attenuated by antioxidants that were protected against by clofibrate pretreatment (Chapter 9). It was also shown that both hepatocytes (Chapter 9) and homogenates derived from the livers of clofibrate pretreated mice (Chapter 10) were resistant to lipid peroxidation when compared to control mice. This was not the result of a decline in peroxidisable fatty acids in the liver, but rather appeared to be due to the induction of a cytosolic protein with antioxidant capabilities (Chapter 10).

Pretreatment with PxP has been proposed to produce an cellular oxidative stress due to the increase in $H_2O_2$ by peroxisomal enzymes, although the evidence for this proposal remains somewhat tenuous. The present findings would suggest that if any such increase in oxidative stress is to occur, it may be accompanied by an increase in the antioxidant capacity of the cell in an effort to maintain homeostasis. This altered antioxidant capacity of the liver could then account for the increased resistance to xenobiotics that produce toxicity via oxidative pathways. Although this is an attractive hypothesis, it not supported either by measurements of the levels of "known" cellular antioxidants or of the activities of enzymes involved in the metabolism of reactive oxygen species (outlined in Chapter 1). The increased antioxidant capacity could well explain the protective effect of PxP pretreatment observed in other studies (Tuchweber and Salas, 1978; Frank *et al,* 1982; Smith *et al,* 1990; Garberg *et al,* 1992).

Collectively, the results of this thesis have suggested that the hepatoprotective effect of clofibrate and other PxP is not solely due to an alteration in GSH levels and is probably associated with the induction of a cytosolic protein with either or both antioxidant capability and electrophile scavenging ability. Some further speculation on the possible identity of such a PxP inducible antioxidant protein follows in Section 11.3.

### 11.2 Other uninvestigated possible mechanisms of hepatoprotection

While the induction of an antioxidant protein following pretreatment with PxP is supported by the data presented in this thesis, there are other potential mechanisms by which AAP has been postulated to cause toxicity, which will be considered below. The discussion will be of a speculative nature, since the lack of time prevented direct investigation of each mechanism. They are included here because each of the possibilities are consistent with some of the data presented in this thesis.

### 11.2.1 Involvement of lysosomes?

The lysosome is a single membrane bound organelle containing both non-haem iron and hydrolytic enzymes (Sakaida *et al,* 1990; Timbrell, 1994). While destabilisation of lysosomes is commonly observed during the late stages of cell toxicity, it has not been established whether it is a cause or simply a result of the cell reaching the "point of no return" in the sequence of events leading to cell death (Timbrell, 1994; Öllinger and Brunk, 1995). However, for some toxicants, lysosomal damage plays a central role in the pathogenesis of toxicity. In the case of naphtharin toxicity in hepatocytes, for example, although naphtharin induces thiol and intracellular $Ca^{++}$ dyshomeostasis as well as a depletion of ATP stores, cell death does not occur so long as the integrity of lysosomal membranes remain intact (Öllinger and Brunk, 1995). Antioxidants that protect against lysosomal destabilisation thus prevent naphtharin toxicity (Öllinger and Brunk, 1995).

The involvement of lysosomes in AAP toxicity was suggested by a study which reported that high concentrations of AAP are distributed to the lysosomal fraction in AAP poisoned mice (Studenberg and Brouwer, 1993). Following AAP intoxication, an increase in hepatic lysosomal enzyme activity occurred which is suggestive of lysosome rupture (Khandkar *et al,* 1996). In addition, a decrease in biliary excretion of iron accompanies AAP toxicity in rats, which was suspected to be the result of decreased lysosomal exocytosis of iron (Gupta *et al,* 1994). Lysosomal destabilisation would increase both the activity of lysosomal enzymes and the amount of hepatic iron, the latter of which is reflected in the lower biliary iron excretion (Gupta *et al,* 1994).

It is well known that the hepatic lysosomal compartment is altered upon exposure to PxPs, with an increase occurring in organelle numbers as well as the activity of various lysosomal enzymes dependent on the duration of exposure and the potency of the PxP (Conway *et al,* 1989; Malki *et al,* 1990). While it is possible that these lysosomal changes enhance the resistance of the organelles to AAP and other toxicities, further work is needed to directly address this possibility. Alternatively, the possibility that the lysosomes are somehow protected by the yet to be identified antioxidant that is induced in PxP treated livers could also be examined. By virtue of decreasing lipid peroxidation or chelating of lysosomal iron, such a protein may have prevented cytotoxicity by stabilising the lysosomal membranes.

### 11.2.2 Involvement of Kupffer cells?

In addition to AAP causing damage to parenchymal cells, it has also been suggested that cytotoxic mediators released from non-parenchymal hepatic macrophages known as Kupffer cells may participate in the pathogenesis of AAP toxicity (Laskin *et al,* 1995). Upon activation, Kupffer cells have been reported to release superoxide anion radicals and $H_2O_2$ (Matsuo *et al,* 1985), cytotoxic eicosaniods and cytokines (Edwards *et al,* 1993; Blaszka *et al,*

1995). A role for cell damage by such Kupffer cell derived cytotoxicants in AAP toxicity is suggested by the observation that the stabilisation of Kupffer cells completely blocks the hepatotoxicity of AAP (Laskin *et al,* 1995) and also $CCl_4$ (Sipes *et al,* 1991; Edwards *et al,* 1993; el Sisi *et al,* 1993). On the basis of this finding, the hepatoprotective effect of PxP could involve stabilisation of the Kupffer cell population thus reducing hepatic damage as a result of toxicant exposure.

Nonetheless, whether such a stabilisation of Kupffer cells occurs upon treatment with PxPs seems unlikely, given that others have shown that these cells are actually activated in rodents upon administration of clofibrate (Bojes and Thurman, 1996). However, the relationship between Kupffer cell activation and hepatotoxicity is quite complex, since one would predict that it would enhance susceptibility to toxicity, under some circumstance a protection against toxicity can accompany treatment with Kupffer cell activators. For example, others have shown that administration of the Kupffer cell activator, *Corynebacterium parvum*, 5 days prior to AAP challenge attenuates the subsequent hepatotoxicity (Raiford and Thigpen, 1994). An explanation for this observation could be that certain activators alter the balance between the release of cytodestructive Kupffer cell products relative to that of less toxic mediators. Prior priming with low dose lipopolysaccaride has been shown to confer protection against subsequent lethal doses of lipopolysaccaride via this type of Kupffer cell activation (Hafenrichter *et al,* 1994). In a similar fashion, it could be that prior activation of Kupffer cells by PxP has conferred resistance to toxicants by altering the type of mediator released following a secondary challenge with toxicants. The requirement for 2 to 3 days of PxP pretreatment for hepatoprotection to become evident (Chapter 4) lends support to a conclusion of this nature. However, the fact that the protective effect was observed *in vitro* in cultured parenchymal cells (Chapter 8), would suggest that an intracellular alteration in these cells is responsible for hepatoprotection, rather than a change in secondary cells that are much less abundant in cultured systems. It thus seems unlikely that prior activation of the non-parenchymal Kuppfer cells population would completely explain the hepatoprotective effect observed.

### 11.2.3 Alteration of prostaglandin and/or other eicosanoids?

A number of lines of evidence indicate that oxygenated derivatives of fatty acids play an important role in AAP toxicity. For example, an increased level of cytodestructive eicosanoids such as thromboxane and certain leukotrienes have been observed in AAP intoxicated rodent livers (Guarner *et al,* 1988; Ben-Zvi *et al,* 1990; Horton and Wood, 1991a; Horton and Wood, 1991b; Rénic *et al*, 1993; Culo *et al,* 1995). Similarly, the administration of certain cytoprotective PGs can protect against the biochemical and physiological symptoms of AAP toxicity as well as that caused by other toxicants. For example, administration of exogenous prostacyclin ($PGI_2$) has been shown to protect against AAP, $CCl_4$ and BrB (Bursch and

Schulte-Hermann, 1986; Guarner *et al,* 1988). In addition, the prostacyclin analogue, ilioprost, abolishes the cytotoxicity of AAP at just picamolar concentrations, far lower than the concentrations required for activation of the PG receptors (Nasseri-Sina *et al*, 1992). Also, $PGE_2$ and its stable analogue, 16,16-dimethyl prostaglandin $E_2$, have been reported to decrease the toxicity of a number of diverse acting hepatotoxicants including $CCl_4$, BrB and AAP (Stachura *et al,* 1981; Funck-Brentano *et al,* 1984; Monto *et al,* 1994). The eicosatrienoic PG, $PGE_1$, has been observed to be protect against hepatic ischemia (Ueda *et al,* 1989) as well as microvascular injury, an early event in AAP hepatotoxicity (Lim *et al,* 1995). On the basis of these findings, it could be argued that pretreatment with peroxisome proliferators has in some way altered the production of eicosanoids so that the production of cytoprotective eicosanoids outweighs that of cytotoxic forms. That such changes could occur upon PxP treatment is feasible, given that PxPs alter the levels of at least 2 enzymes that oxidise arachidonic acid, namely CYP4A1 and cyclooxygenase-2 (Leung and Glauert, 1996; Ledwith *et al,* 1997; Leung and Glauert, 1997). At present, further research is required to determine whether such changes are implicated in the hepatoprotective response.

### 11.2.4 Alteration in calcium distribution?

According to one theory of chemical toxicity, a loss of control over cytosolic calcium concentrations results in an activation of $Ca^{++}$ dependent enzymes. Some evidence suggests that events of this nature may occur in AAP toxicity, since marked changes in intracellular $Ca^{++}$ have been observed in AAP intoxicated livers (Landen *et al*, 1986). This includes an increase in nuclear $Ca^{++}$ levels, which have been shown to cause DNA fragmentation due to endonuclease activation (Ray *et al*, 1992). A role for $Ca^{++}$ dyshomeostasis in AAP toxicity is further indicated by the finding that the $Ca^{++}$ channels blocker, verapamil and the calmodulin inhibitor, chlorpromazine, attenuate necrosis following AAP intoxication, presumably by decreasing $Ca^{++}$ accumulation in the nucleus and by reducing DNA hydrolysis (Ray *et al*, 1992). On the basis of such data suggesting a role for $Ca^{++}$ in cell damage by AAP, the possibility emerges that pretreatment with PxP's somehow attenuates intracellular $Ca^{++}$ fluxes, leading to a protection against toxicity.

Studies investigating the effect of PxPs on intracellular $Ca^{++}$ homeostasis have revealed that PxPs such as clofibrate, cause a concentration dependent increase in cytosolic $Ca^{++}$, probably due to a redistribution of subcellular $Ca^{++}$-pools (Shackleton *et al,* 1995) and may be due to a transient decrease in microsomal $Ca^{++}$-ATPase activity (Bennett and Williams, 1992). These findings suggest that activation of the PPAR causes an increase in cytosolic $Ca^{++}$ levels (Gibson, 1996). This is supported by the observations that PxP induced increases in the formation of mRNA for CYP4A1 and acyl CoA oxidase, the increase in DNA synthesis, and also the increase in peroxisome numbers are all blocked by $Ca^{++}$-channel antagonists (Bennett and Williams, 1993; Ram and Waxman, 1994; Gibson, 1996). However, while such findings

indicate that an increased mobilisation of intracellular $Ca^{++}$ occurs upon treatment with PxPs, it is difficult to see how these changes would have hepatoprotective consequences. To recall, according to the $Ca^{++}$ dyshomeostasis theory, an uncontrolled increase in free $Ca^{++}$ is a critical event in cell damage. Given that PxPs themselves seem to increase cytosolic $Ca^{++}$, it is clear that a prevention of uncontrolled fluxes in cytosolic $Ca^{++}$ are unlikely to be the mechanism whereby PxPs afford protection against hepatotoxicity. Consequently, it seems unlikely that a protection against $Ca^{++}$ dyshomeostasis represents the major hepatoprotective action of PxPs.

### 11.2.5 Changes in the final events leading to cell death?

A final possible mechanism whereby PxPs might protect the liver against toxicity could involve an interference with the cellular signalling in the biochemical events that result in cell death. There are two forms of cell death that are morphologically and biochemically distinct from each other, namely apoptosis and necrosis. Apoptosis is an active form of cell death, involving the removal of single "unwanted" (eg damaged) cells in a series of controlled cellular biochemical events, ultimately resulting in the phagocytosis of the cell. As the integrity of the cell membrane is maintained throughout, there are no gross tissue changes such as inflammation due to the release of intracellular mediators, in apoptotic cell death. Necrosis on the other hand, never occurs under physiological conditions being a result of severe toxic chemical assault and involves the collapse of the internal homeostasis of the cell leading to membrane lysis and tissue inflammation. As extensive necrosis can damage the structure of the tissue, apoptosis appears to be more advantageous for the removal of injured cells than necrosis (Bursch *et al,* 1992).

Various studies performed by others suggest that apoptosis and necrosis are not mutually exclusive processes, with a given chemical causing death via a combination of the two pathways often in a dose dependent manner. Thus at low levels of cellular insult by toxicants or some physiological conditions where there is only minor damage to the cell, the major form of cell death is apoptosis while at higher doses, necrotic cell death occurs (Daoust and Morais, 1986; Lennon *et al,* 1991; Ray *et al,* 1996; Zeid *et al,* 1997). Thus both apoptosis and necrosis occur in the liver following toxicant challenge with a range of hepatotoxicants including AAP (Bursch *et al*, 1992; Pritchard and Butler, 1989; Ledda - Columbano *et al,* 1991; Ray *et al,* 1992; Ray *et al,* 1996). On the basis of these and other findings, the possibility thus emerges that the hepatoprotective effect of PxPs may involve an inhibition of the apoptotic component of cell death initiated by AAP and other toxicants.

A role for suppression of apoptotic cell death in the protective effect of PxPs seems possible given that others have shown that pretreatment with PxP inhibits hepatic apoptosis in both *in vitro* and *in vivo* models (Bayly *et al,* 1994; Hofstra *et al,* 1997; James and Roberts, 1996). Such effects would explain why higher doses of hepatotoxicants are required to damage

hepatocytes from PxP treated mice to those required to injure cells from untreated mice. It is also consistent with other experimental results obtained in this thesis which suggest that the hepatoprotective effect of PxPs involves a resistance to oxidative stress. Various studies have indicated that there is a close relationship between oxidative stress and apoptotic cell death. For example, a range of oxidative stress mediators, including, $H_2O_2$, lipid peroxides and superoxide anion radical have been shown to cause apoptosis in certain tissues (Lennon *et al,* 1991; Kazzaz *et al,* 1996). Also, other studies have shown that addition of exogenous antioxidants can block apoptotic liver cell death triggered by a range of toxicants including bile acids and arachidonic acid (Chen *et al,* 1997; Patel and Gores, 1997). Similarly, endogenous antioxidants, such as α-tocopherol, ascorbic acid and GSH play a role in determining whether a cell undergoes apoptotic cell death (Fernandes and Cotter, 1994; Haendeler *et al,* 1996). This is exemplified in AAP toxicity, in which the depletion of intracellular GSH facilitates a change in the predominant form of cell death from apoptosis to necrosis (Fernandes and Cotter, 1994; Ellouk-Achard *et al,* 1995). Collectively, these studies show that the process of apoptosis can be initiated by ROS and prevented by antioxidants. Given that the studies described in this thesis have shown that an increase in the antioxidant capacity of the liver seems to be involved in the hepatoprotective effects of PxP pretreatment, it could be that the unidentified antioxidant somehow prevents the apoptotic component of cell death initiated by AAP and other toxicants. Clearly, future work should actively address this possibility.

### 11.3 Some speculation on the PxP related antioxidant.

As was discussed in 11.1.6, the data presented in this thesis suggests that pretreatment with PxP caused the induction of a cytosolic antioxidant protein, raising the possibility that this compound is involved in the protection against AAP and other toxicants. Unfortunately, although the levels of various antioxidants have been measured in the livers of PxP treated rodents, none of them were found to be sufficiently elevated over control values to be likely explanations of the hepatoprotective response (Lake *et al,* 1989b; Glauert *et al,* 1992; Aberg *et al,* 1994; Ashby et al, 1994; Amimoto *et al*, 1995). The list of antioxidants measured was not exhaustive, so future work should be directed towards examining the effects of PxP pretreatment on other oxidant scavengers. This task is made somewhat easier by the fact that it was found in the present study that the antioxidant is proteinaceous in nature. This conclusion was drawn on the basis of the finding that the antioxidant was labile upon treatment with either heat or proteinase K (Chapter 10). These observations narrow the field considerably and rule out low molecular weight antioxidants such as α-tocopherol, ubiquinone, ascorbate and uric acid (Halliwell and Gutteridge, 1989).

At least two antioxidant proteins present themselves as possible mediators of PxP induced hepatoprotection against AAP. The first candidate is the cytosolic 58kDa selenoprotein. Due

to its high sulphydryl content, NAPQI binds to this protein and it has thus been postulated to have a cytoprotective role in AAP toxicity (Bartelone *et al,* 1989; Bartelone *et al,* 1992). However, the fact that the extent of NAPQI binding to this protein does not always correlate closely with the severity of toxicity raises questions about the toxicological importance of this protein (Beierschmitt *et al,* 1989). Another factor that minimises the likelihood of this protein being involved in PxP induced hepatoprotection is that pretreatment with clofibrate has been shown not to increase the amount of this protein in mouse livers (Manataou *et al,* 1994).

A second endogenous protein that could possibly participate in the protective effect afforded by PxP pretreatment is metallothionein, a 6.5 kDa sulphydryl rich cytosolic protein which plays an important role in protecting against oxidative stress (Halliwell and Gutteridge, 1989). Due to its high sulphydryl content, this protein seems a likely target for NAPQI. In support of this protein having a hepatoprotective action, administration of metallothionein has been shown to protect against $CCl_4$ toxicity (Cagen and Klaassen, 1979). Also, prior exposure to the inflammogen, *Mycobacterium tuberculosis*, increased both hepatic metallothionein levels and provided protection against the hepatotoxicity of AAP (Wright, 1987). The induction of metallothionein is rapid and extensive, with a 30-50 fold increase in its levels observed within 24 hours of DEM administration with lesser fold induction reported with other toxicants including AAP, $CCl_4$, DEM, paraquat diamide and *t*-BHP (Oh *et al,* 1978; Bauman *et al,* 1991; Sato, 1991; Bauman *et al,* 1992).

In addition to the above treatments causing an increase in metallothionein expression, an induction of an oxidative stress has also been observed to increase the levels of this protein in rodent liver (Bauman *et al,* 1991; Bauman *et al,* 1992). It is thus possible to speculate that the increased flux of $H_2O_2$ that is thought by some researchers to occur during a state of peroxisome proliferation could lead to an overexpression of metallothionein genes. At present, the data concerning the effect of PxP on metallothionein expression is ambiguous, with some workers reporting a decrease in metallothionein mRNA following a week of PxP treatment (Motojima *et al,* 1992), while others reported an 11-fold increase in the amount of metallothionein after a 11 week exposure to DEHP (Waalkes and Ward, 1989). It is not known whether metallothionein is induced by PxP in the 2 to 3 day pretreatment time frame required for hepatoprotection observed in Chapter 4. Consequently future studies should be directed at clarifying the time course of the induction of metallothionein in mouse livers under the PxP exposure conditions used in the present study. Such knowledge would indicate whether this protein is a likely contributor to the hepatoprotective effect of PxPs.

### 11.4 Further experimentation

A number of future studies to further clarify the mechanisms involved in the hepatoprotective effect of PxP pretreatment have been suggested in the preceding discussion. Effort should

initially be directed towards determining whether metallothionein levels are increased under the PxP treatment regimen used in this study, since this protein appears to have characteristics which closely resemble PxP hepatoprotection. Whether this protein plays a role in the regulation of apoptotic cell death could also be examined.

A further question which should be addressed in greater detail pertains to the role of zonal heterogeneity in PxP induced hepatoprotection since it was observed that the extent of hepatoprotection was the greatest for those toxicants with essentially centrilobular actions, where peroxisome proliferation also takes place. Addressing the role of zonal heterogeneity should determine whether the hepatoprotection targets a specific population of hepatocytes. In addition, the possible involvement of alteration in PG levels or the impact of apoptotic suppression by PxP on hepatoprotection could be investigated. An analysis of the cytotoxicity of AAP using hepatocytes isolated from species other than mice may answer some of these questions. For example, a recent study showed that nafenopin is able to suppress apoptosis in not only mouse and rat, but also in hamster and guinea pig hepatocytes, species that are usually thought to show little or no increase in peroxisome numbers upon treatment with PxPs (James and Roberts, 1996). Thus if PxPs produce hepatoprotection by suppressing apoptosis and not by increasing the peroxisome numbers, then it would be especially useful in these experiments since they exhibit similar AAP hepatotoxicity to the mouse. Due to time and other constraints, such as the restriction on importation of hamsters into Australia, investigation of these proposals was not possible during this study.

### 11.5 Final conclusion

The results outlined in this thesis have shown that pretreatment with the PxP, clofibrate, protects mice against the acute toxicity of AAP as well as a number of other toxicants with diverse mechanisms of action. While the exact mechanism was not elucidated, the induction of a cytosolic antioxidant protein appears likely to be important in the extensive hepatoprotection afforded following pretreatment with PxP. Since few prior studies have examined the effect of pretreatment with peroxisome proliferators on the toxicity of other xenobiotics, the current work has provided some unexpected insights into the toxicological consequences of exposure to this diverse class of compounds. Further studies are required to determine whether the results obtained here can be generalised to species other than mice.

# APPENDICES

## APPENDIX 1 - Chemicals and Suppliers

| CHEMICAL | SUPPLIER |
|---|---|
| acetonitrile | Mallinckrodt Chemicals, Paris, Ky, USA |
| ADP | Bohringer Mannheim, Mannheim, Germany |
| alanine | Sigma Chemical Co, St Louis, Mo, USA |
| albumin (bovine serum) | Sigma Chemical Co, St Louis, Mo, USA |
| 2-amino-2-methylpropanediol | Sigma Chemical Co, St Louis, Mo, USA |
| ammonium molybdate | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| ammonium sulphamate | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| ascorbic acid | Sigma Chemical Co, St Louis, Mo, USA |
| ATP | Bohringer Mannheim, Mannheim, Germany |
| benzyl peroxide | ICN Pharmaceutical Inc, Cleveland, Ohio, USA |
| bromobenzene | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| *tert*-butyl hydroperoxide | Sigma Chemical Co, St Louis, Mo, USA |
| butylated hydroxytoluene | Sigma Chemical Co, St Louis, Mo, USA |
| calcium chloride | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| calcium hydrogen orthophosphate | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| carbon tetrachloride | Sigma Chemical Co, St Louis, Mo, USA |
| *bis*-carboxynitrosurea | Gift : Dr A. Harman, Uni of Western Australia |
| chlorodinitrobenzene | Sigma Chemical Co, St Louis, Mo, USA |
| chloroform | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| choline chloride | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| chromium potassium sulphate | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| clofibrate | Serva Feinbiochemica, Heidelburg, Germany |
| coenzyme A | Bohringer Mannheim, Mannheim, Germany |
| collagenase | Bohringer Mannheim, Mannheim, Germany |
| copper sulphate | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| cumene hydroperoxide | Sigma Chemical Co, St Louis, Mo, USA |
| diethylhexylphthalate | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| diamide | Sigma Chemical Co, St Louis, Mo, USA |
| 3,3'-diaminobenzidine | Sigma Chemical Co, St Louis, Mo, USA |
| 3,4-dichloronitrobenzene | Sigma Chemical Co, St Louis, Mo, USA |
| diethylether | May and Baker, Dagenham, England, UK |
| diethylmaleate | Merck Biochemicals, Munich, Germany |
| 2,4-dinitrophenylhydrazine | Sigma Chemical Co, St Louis, Mo, USA |
| dipotassium hydrogenorthophosphate | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| disodium hydrogen orthophosphate | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| dithiothreitol | Sigma Chemical Co, St Louis, Mo, USA |
| DNase | Bohringer Mannheim, Mannheim, Germany |
| EDTA | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| ethacrynic acid | Sigma Chemical Co, St Louis, Mo, USA |
| ethanol | Merck Biochemicals, Munich, Germany |
| ethylacetate | Mallinckrodt Chemicals, Paris, Ky, USA |
| FAD+ | Sigma Chemical Co, St Louis, Mo, USA |
| ferric citrate | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| ferrous sulphate | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| fructose | Sigma Chemical Co, St Louis, Mo, USA |

| | |
|---|---|
| frusemide | ICN Pharmaceutical Inc, Cleveland, Ohio, USA |
| glacial acetic acid | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| glucose 6-phosphate dehydrogenase | Bohringer Mannheim, Mannheim, Germany |
| glucose-6-phosphate | Bohringer Mannheim, Mannheim, Germany |
| glutamate | Sigma Chemical Co, St Louis, Mo, USA |
| glutamate dehydrogenase | Bohringer Mannheim, Mannheim, Germany |
| glutathione-oxidised | Sigma Chemical Co, St Louis, Mo, USA |
| glutathione-reduced | Sigma Chemical Co, St Louis, Mo, USA |
| gum acacia | Sigma Chemical Co, St Louis, Mo, USA |
| heparin | Fisons, Thornleigh, NSW, Australia |
| HEPES | ICN Pharmaceutical Inc, Cleveland, Ohio, USA |
| hexane | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| hydrochloric acid | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| hydrogen peroxide | Sigma Chemical Co, St Louis, Mo, USA |
| hydroxylamine hydrochloride | Sigma Chemical Co, St Louis, Mo, USA |
| imidazole | ICN Pharmaceutical Inc, Cleveland, Ohio, USA |
| iodoacetate | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| iso-octane | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| α-ketoglutarate | Sigma Chemical Co, St Louis, Mo, USA |
| lactate dehydrogenase | Bohringer Mannheim, Mannheim, Germany |
| lactic acid | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| lauric acid | Sigma Chemical Co, St Louis, Mo, USA |
| $^{14}$C-lauric acid | ICN Pharmaceutical Inc, Cleveland, Ohio, USA |
| magnesium chloride | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| magnesium oxide | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| magnesium silicate | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| magnesium sulphate | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| maleate | Sigma Chemical Co, St Louis, Mo, USA |
| manganous sulphate | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| menadione | Sigma Chemical Co, St Louis, Mo, USA |
| mercaptoethanol | Aldrich Chemical Co Ltd, Milwaukee, Wi, USA |
| mercuric chloride | May and Baker, Dagenham, England, UK |
| methanol | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| *N*-1-naphthylethylenediamine | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| NAD+. | Bohringer Mannheim, Mannheim, Germany |
| NADH | Bohringer Mannheim, Mannheim, Germany |
| NADPH | Bohringer Mannheim, Mannheim, Germany |
| nicotinamide | Sigma Chemical Co, St Louis, Mo, USA |
| nitrofurantoin | Aldrich Chemical Co Ltd, Milwaukee, Wi, USA |
| nitrogen gas | CIG, Northfield, SA Australia |
| olive oil | Faulding,Pharmaceuticals, Salisbury, SA, Australis |
| organic scintillation fluid | Beckman Industries, Palo Alto, Ca, USA |
| palmitoyl CoA | Sigma Chemical Co, St Louis, Mo, USA |
| paracetamol | Sigma Chemical Co, St Louis, Mo, USA |
| $^{14}$C-AAP | New England Nuclear, USA |
| paraquat | Gift - Dr L Smith, CTL |
| penicillin | ICN Pharmaceutical Inc, Cleveland, Ohio, USA |
| perchloric acid | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| petroleum spirit | May and Baker, Dagenham, England, UK |
| phenol red | May and Baker, Dagenham, England, UK |
| *tert*-phenylbutenone | Sigma Chemical Co, St Louis, Mo, USA |

| | |
|---|---|
| Pierce BCA assay | Progen Industries Ltd, Darra Qld, Australis |
| PMSF | Sigma Chemical Co, St Louis, Mo, USA |
| potassium chloride | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| potassium citrate | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| potassium cyanide | Sigma Chemical Co, St Louis, Mo, USA |
| potassium dihydrogenorthophosphate | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| potassium iodate | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| potassium permanganate | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| potassium sulphate | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| proteinase K | Bohringer Mannheim, Mannheim, Germany |
| Readyvalue scintillation fluid | Beckman Industries, Palo Alto, Ca, USA |
| rotenone | Sigma Chemical Co, St Louis, Mo, USA |
| RPMI-1640 | ICN Pharmaceutical Inc, Cleveland, Ohio, USA |
| Silvex | Gift: Dr R Drew, Flinders Medical Centre, SA. |
| sodium azide | Sigma Chemical Co, St Louis, Mo, USA |
| sodium bicarbonate | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| sodium chloride | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| sodium hydroxide | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| sodium nitrite | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| sodium selenite | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| soluene | Beckman Industries, Palo Alto, Ca, USA |
| streptomycin | ICN Pharmaceutical Inc, Cleveland, Ohio, USA |
| succinate | Sigma Chemical Co, St Louis, Mo, USA |
| sucrose | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| sulphanilamide | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| sulphuric acid | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| tetramethyl-p-phenylenediamine | Sigma Chemical Co, St Louis, Mo, USA |
| thiobarbituric acid | ICN Pharmaceutical Inc, Cleveland, Ohio, USA |
| trichloroacetic acid | BDH Chemicals (Australia), Kilsyth, Vic, Australia |
| tris hydrochloride | Sigma Chemical Co, St Louis, Mo, USA |
| triton X-100 | Ajax Australia Pty Ltd, Auburn, NSW, Australia |
| Trizma base | Sigma Chemical Co, St Louis, Mo, USA |
| urea | Progen Industries Ltd, Darra Qld, Australis |
| urease | Bohringer Mannheim, Mannheim, Germany |
| zinc chloride | BDH Chemicals (Australia), Kilsyth, Vic, Australia |

## APPENDIX 2: Preparation of mouse feed containing PxP.

The composition of this diet is based on the rodent diet used by the Human Nutrition research section, Royal Melbourne Hospital (Mrs Merryn Steele, Prof Andrew Sinclair-Personal Communication)

2.0 kg of wholemeal plain flour
400 g of skim milk
84.0 g of mineral mix
24.0 g of vitamin mix
3.5 g of choline chloride.
(20% protein, 5% fat, 75% flour)

Mineral mix

| | |
|---|---|
| 632.4g | calcium hydrogen orthophosphate |
| 74.0g | sodium chloride |
| 220.0g | potassium citrate |
| 52.0g | potassium sulphate |
| 24.0g | magnesium oxide |
| 6.0g | ferric citrate |
| 5.1g | manganous sulphate |
| 2.7g | zinc chloride |
| 0.78g | chromium potassium sulphate |
| 606mg | copper sulphate |
| 10mg | potassium iodate |
| 6.6 mg | sodium selenite |

Chemicals (<1 g) were weighed and then ground together to a fine powder using a mortar and pestle, before being mixed with the other chemicals in the mineral mix.

The required amount of peroxisome proliferator was added to the dry feed components, and these are mixed and bound together with minimal water. Small handfuls are squeezed into approx 3 x 2 x 2cm cubes, and placed on a drying oven rack covered in aluminium foil. The tray is loosely covered with aluminium foil (to guard against too much evaporation) and left in the drying oven at 45°C overnight. The dryness of the feed was assessed by snapping open a pellet from the centre of the tray.

Dried feed was weighed and stored in plastic bags at -20°C.

**APPENDIX 3: Parameters for HPLC analysis of urinary metabolites of AAP**

| | 200mg/kg | 500mg/kg |
|---|---|---|
| Column | Column $C_{18}$ spherisorb | Waters RadialPak<br>Novapak phenylbonded phase 4μm, 100 mm cartridge |
| Sample volume | 20μl - manual | 20μl -Waters 717 Autosampler |
| Flow rate | 1.0 ml/min | 1.2 ml/min |
| Detector | Jasco UVI DEC-100-V | Waters 490 programmable multiwavelength detector |
| Integrator | Schimatzu | Waters computer programmable |

Retention times (minutes):

| | |
|---|---|
| glucuronide | 4.0 - 4.2 |
| cysteine | 11.3 - 12.3 |
| sulphate | 19.7 - 21.7 |
| paracetamol (parent) | 23.4 - 25.1 |
| mercapturate | 45.6 - 51.0 |

Variation of retention times was the result of pH fluctuations due to slight changes in temperature.

**200 mg/kg AAP study**

Standard curve for AAP (0-1 mM).

Coefficient of variation = 6.5% , $r^2$=0.999.

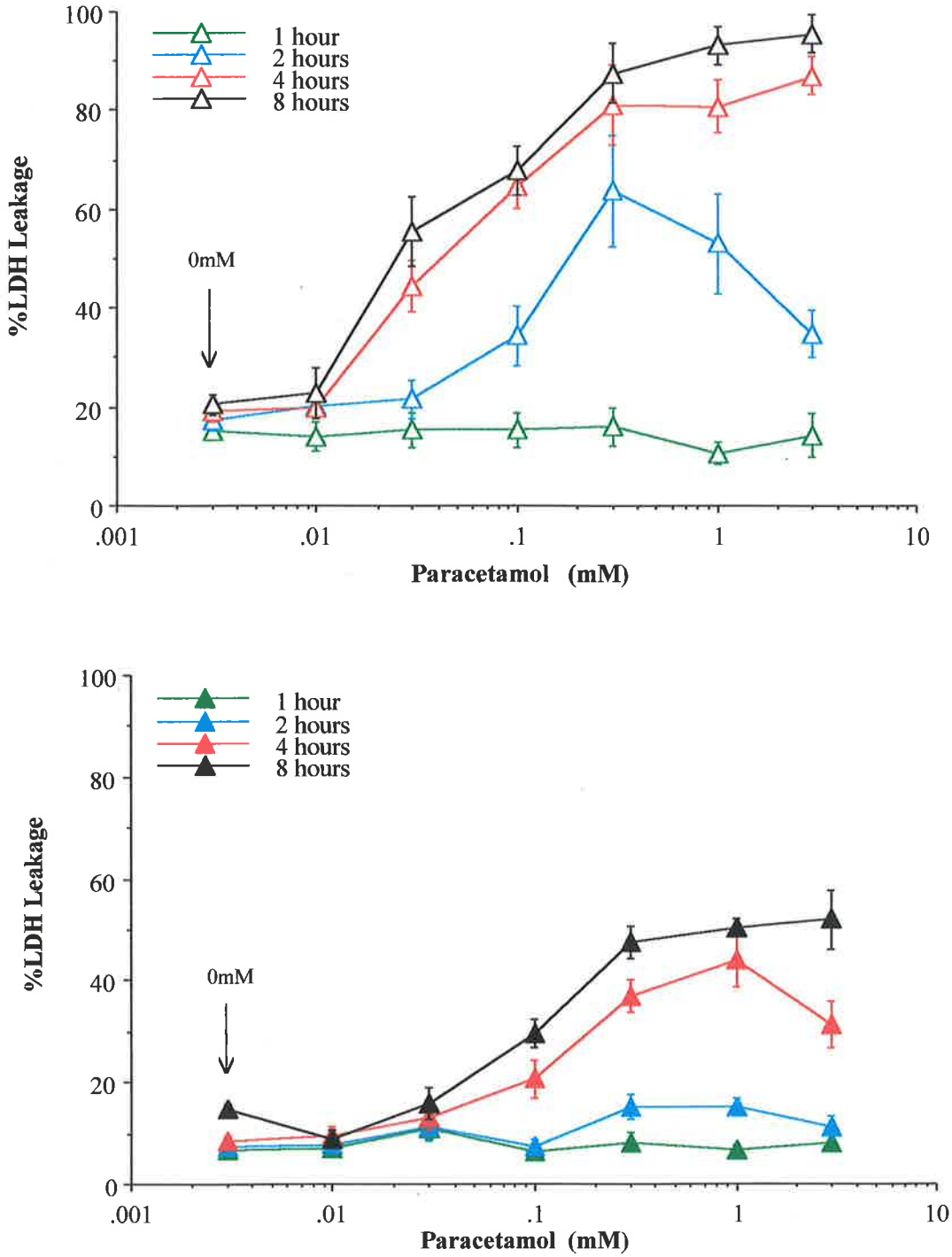
**500 mg/kg AAP study**

Standard curve for AAP (0-1 mM).

Coefficient of variation = 4.3%, $r^2$=0.999.

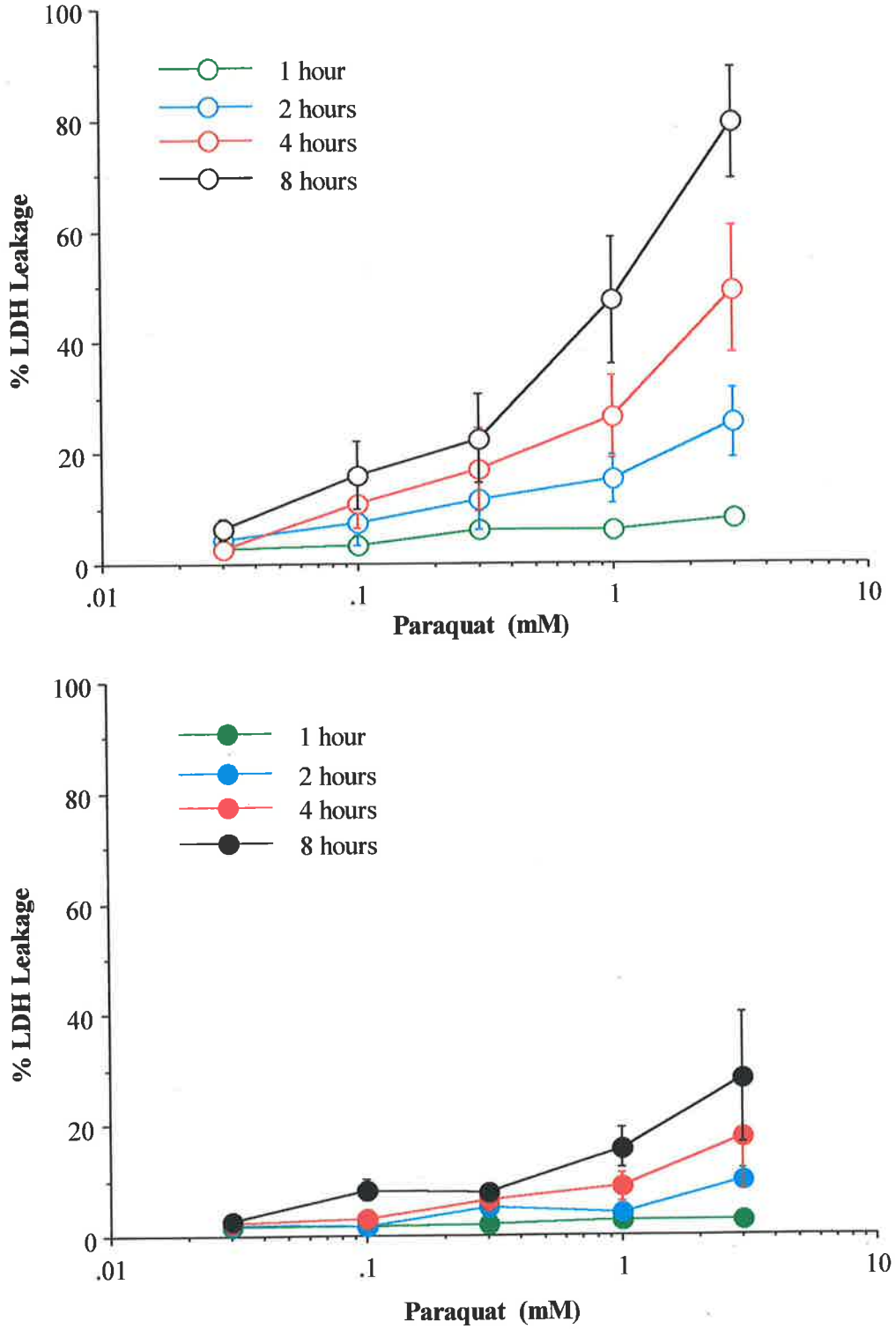
(lowest standard on linear curve 10μM).

## APPENDIX 4 : AAP lethality following clofibrate pretreatment in isolated mouse hepatocytes

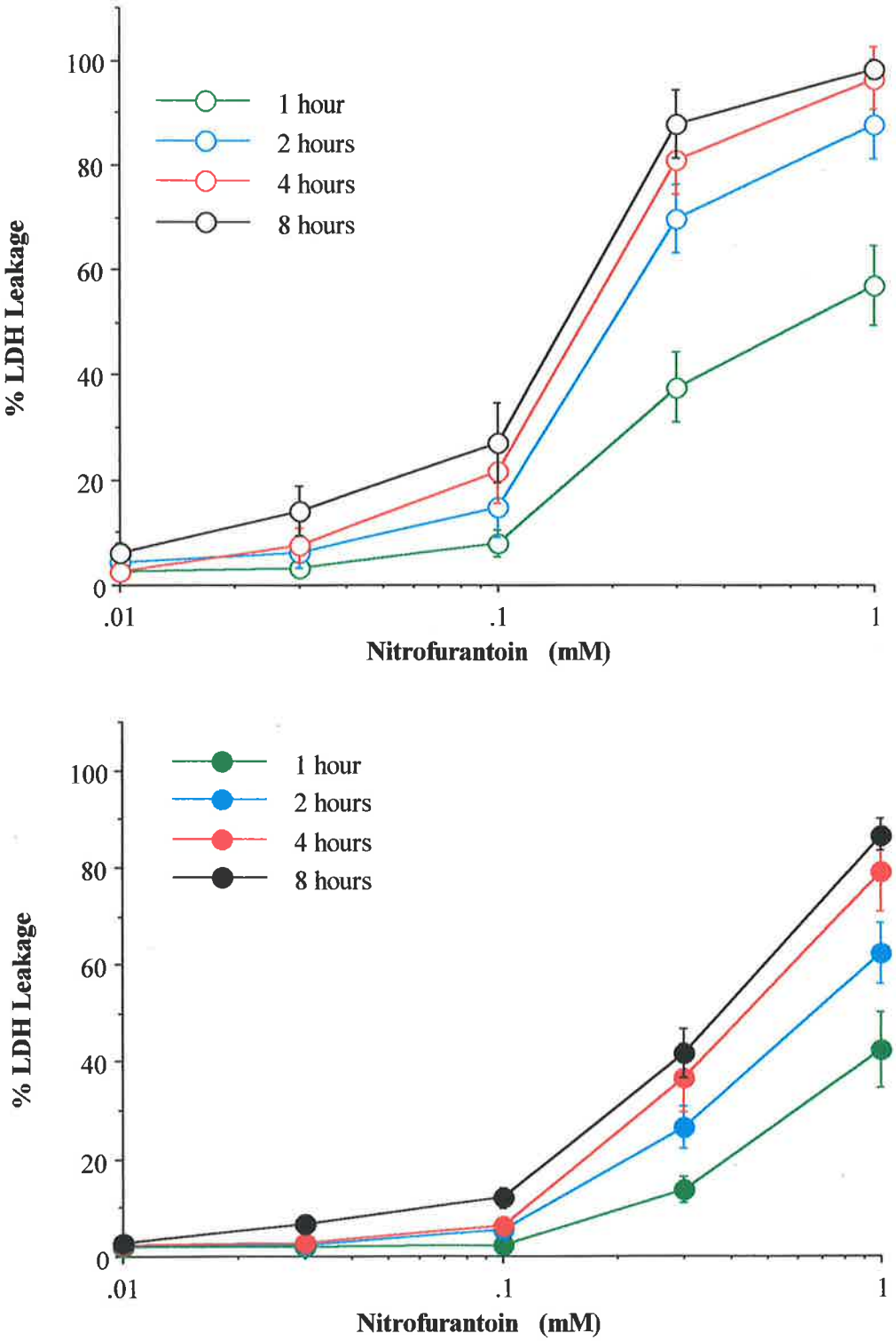



**Appendix 4:** AAP mediated LDH leakage from isolated hepatocytes prepared from control and clofibrate pretreated mice. Upper panel (open circles) shows data obtained from control (olive oil pretreated) hepatocytes while the lower panel (closed circles) show data from clofibrate pretreated mice. Samples were collected at 1,2,4 and 8 hours after the commencement of the experiment for the determination of LDH leakage. (Results expressed as mean ± s.e.m, n=6).

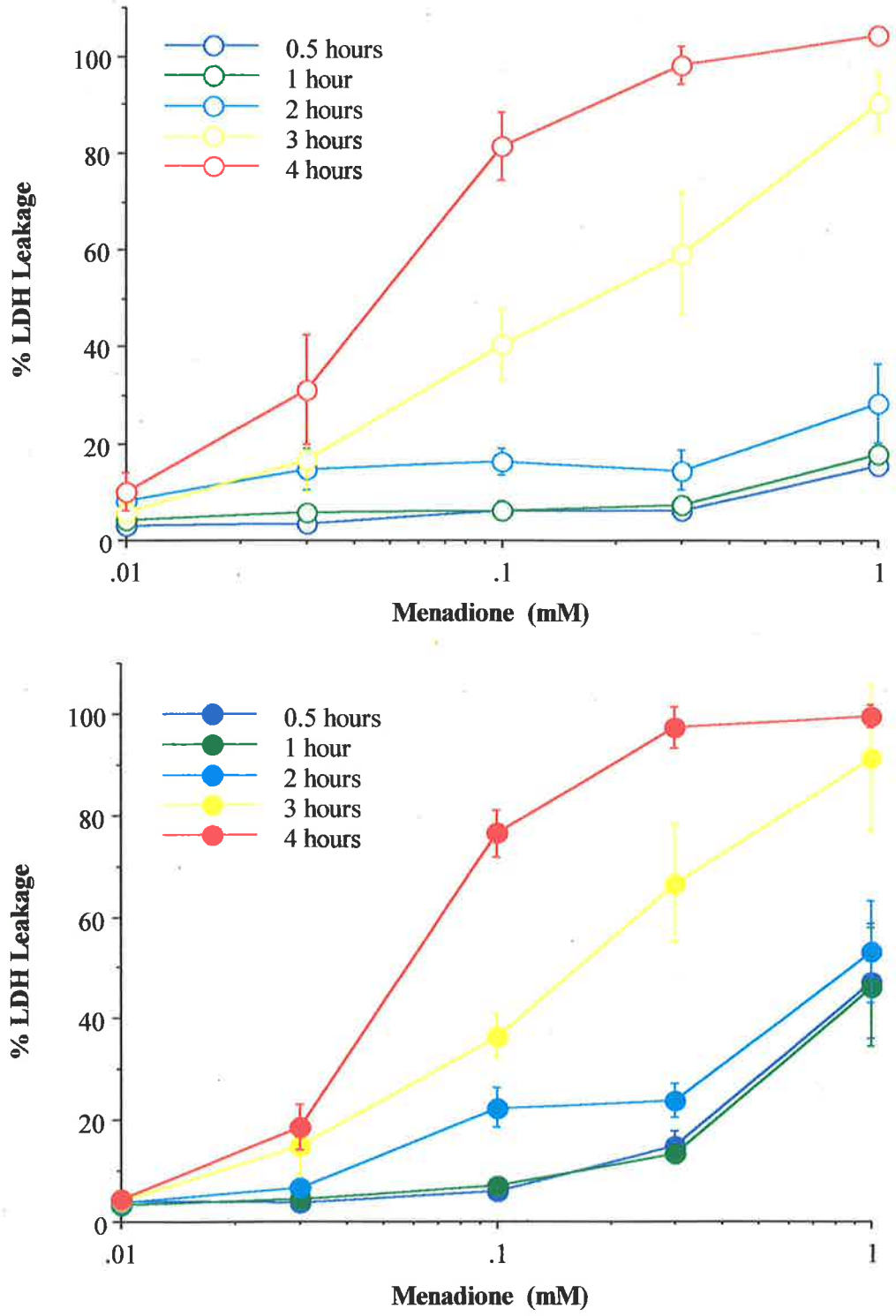
**APPENDIX 5 : Lethality of various toxicants following pretreatment with clofibrate in isolated mouse hepatocytes**



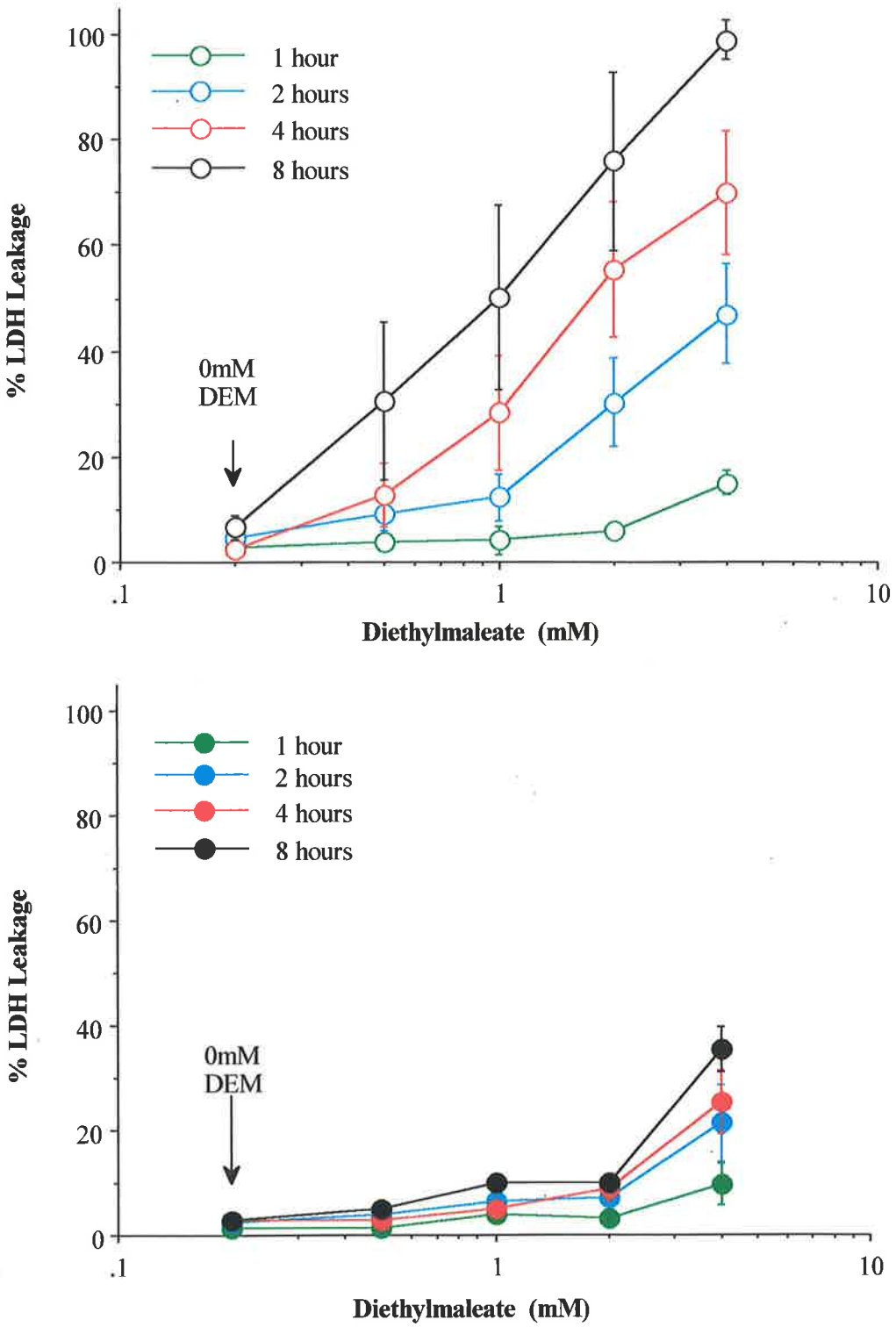

**Appendix 5A:** Paraquat mediated LDH leakage from isolated hepatocytes prepared from control and clofibrate pretreated mice. Upper panel (open circles) shows data obtained from control (olive oil pretreated) hepatocytes while the lower panel (closed circles) show data from clofibrate pretreated mice. Samples were collected at 1, 2, 4 and 8 hours after the commencement of the experiment for the determination of LDH leakage. (Results expressed as mean ± s.e.m, n=6-10).



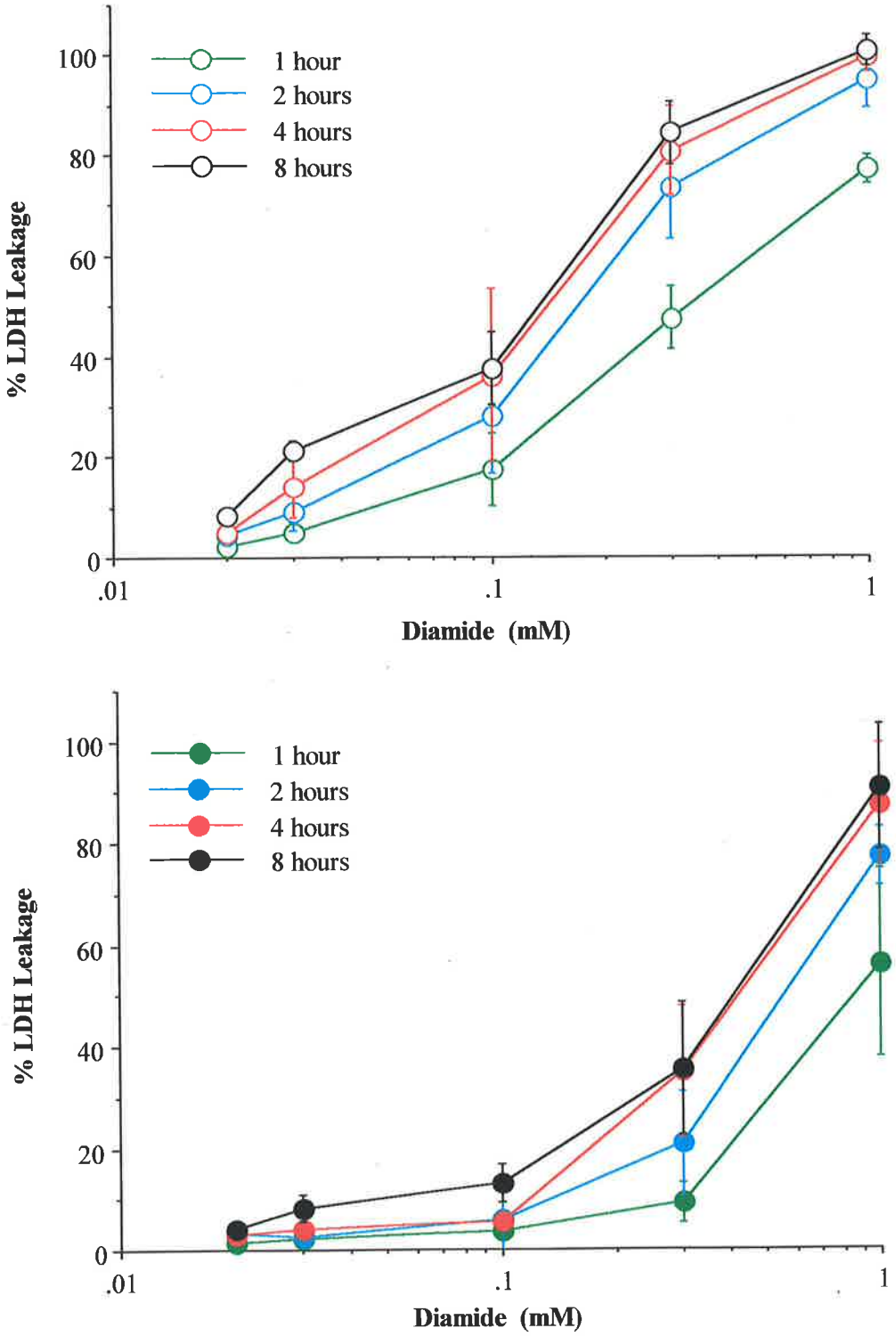

**Appendix 5B:** Nitrofurantoin mediated LDH leakage from isolated hepatocytes prepared from control and clofibrate pretreated mice. Upper panel (open circles) shows data obtained from control (olive oil pretreated) hepatocytes while the lower panel (closed circles) show data from clofibrate pretreated mice. Samples were collected at 1, 2, 4 and 8 hours after the commencement of the experiment for the determination of LDH leakage. (Results expressed as mean ± s.e.m, n=5).



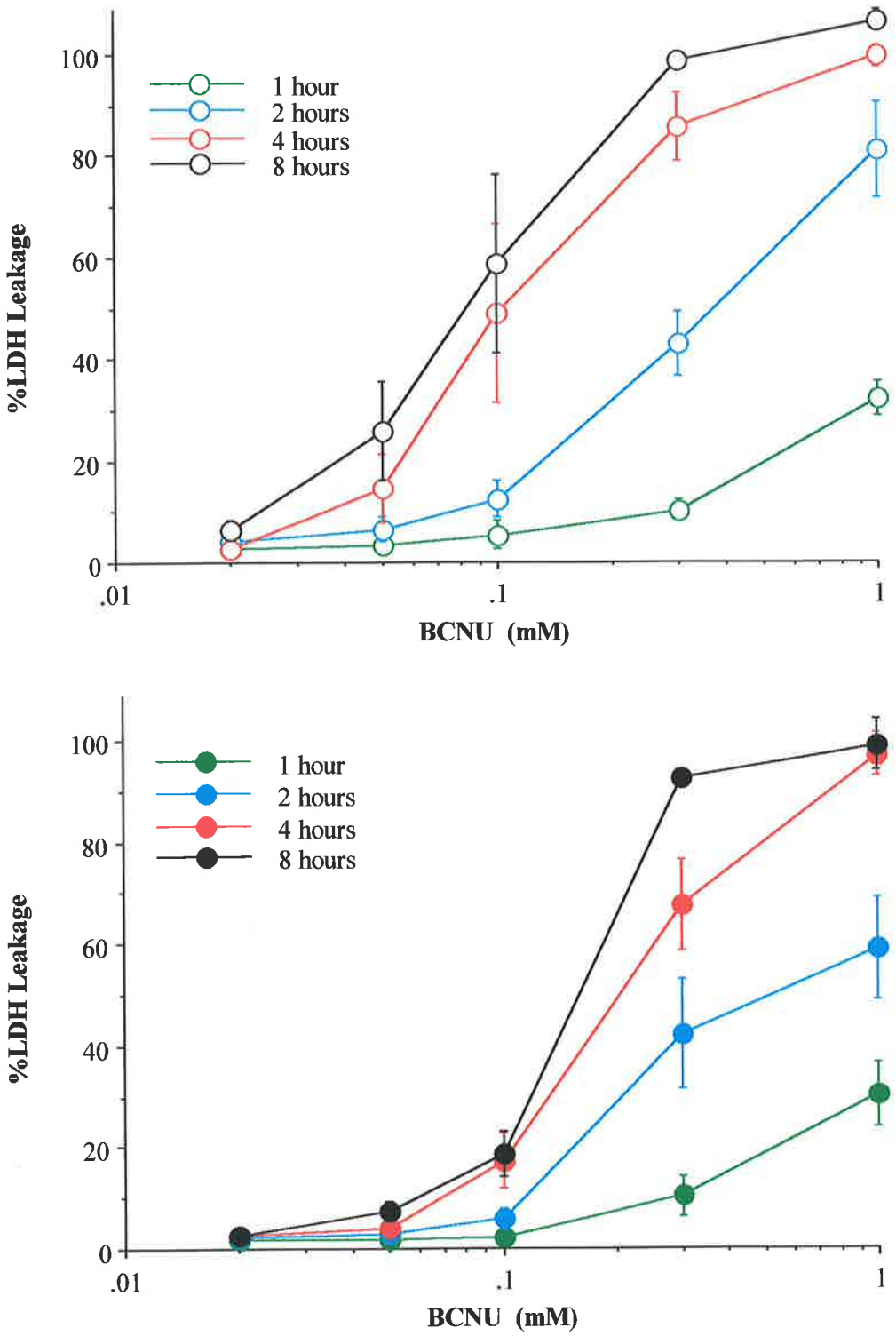

**Appendix 5C:** Menadione mediated LDH leakage from isolated hepatocytes prepared from control and clofibrate pretreated mice. Upper panel (open circles) shows data obtained from control (olive oil pretreated) hepatocytes while the lower panel (closed circles) show data from clofibrate pretreated mice. Samples were collected at 0.5, 1, 2, 3 and 4 hours after the commencement of the experiment for the determination of LDH leakage. (Results expressed as mean ± s.e.m, n=6-10).



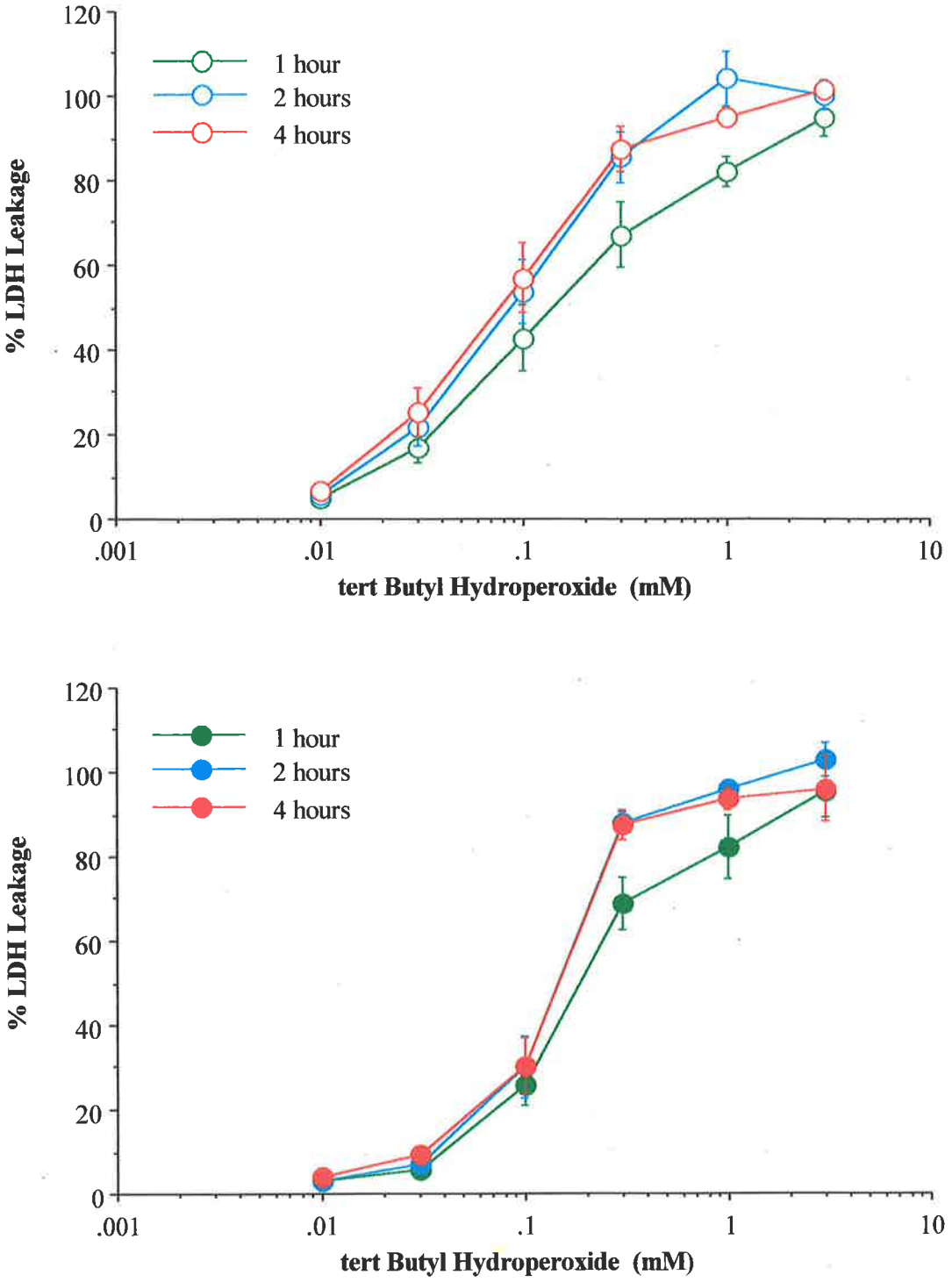

**Appendix 5D:** Diethylmaleate mediated LDH leakage from isolated hepatocytes prepared from control and clofibrate pretreated mice. Upper panel (open circles) shows data obtained from control (olive oil pretreated) hepatocytes while the lower panel (closed circles) show data from clofibrate pretreated mice. Samples were collected at 1,2,4 and 8 hours after the commencement of the experiment for the determination of LDH leakage. (Results expressed as mean ± s.e.m, n=4).



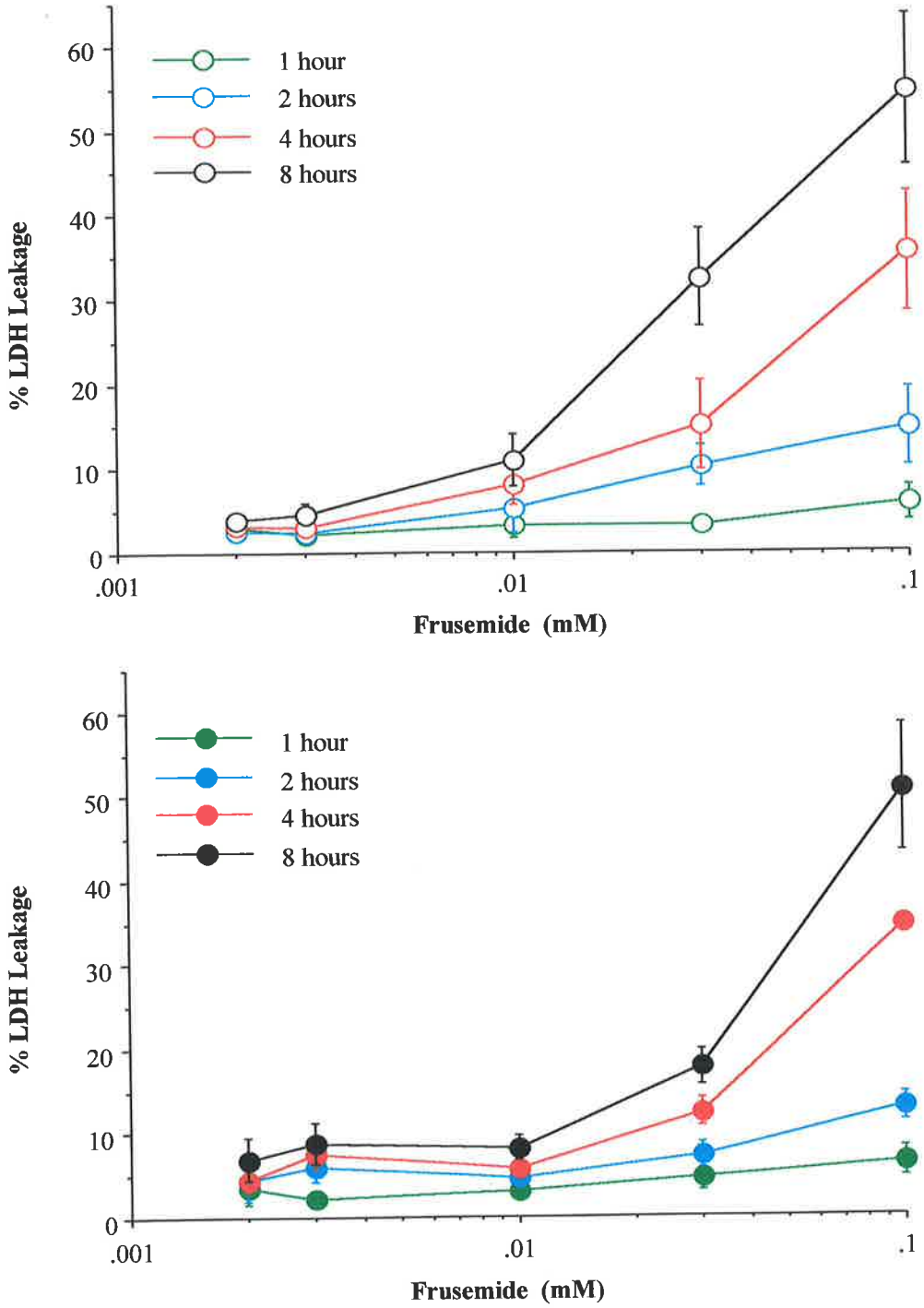

**Appendix 5E:** Diamide mediated LDH leakage from isolated hepatocytes prepared from control and clofibrate pretreated mice. Upper panel (open circles) shows data obtained from control (olive oil pretreated) hepatocytes while the lower panel (closed circles) show data from clofibrate pretreated mice. Samples were collected at 1,2,4 and 8 hours after the commencement of the experiment for the determination of LDH leakage. (mean ± s.e.m, n=4).



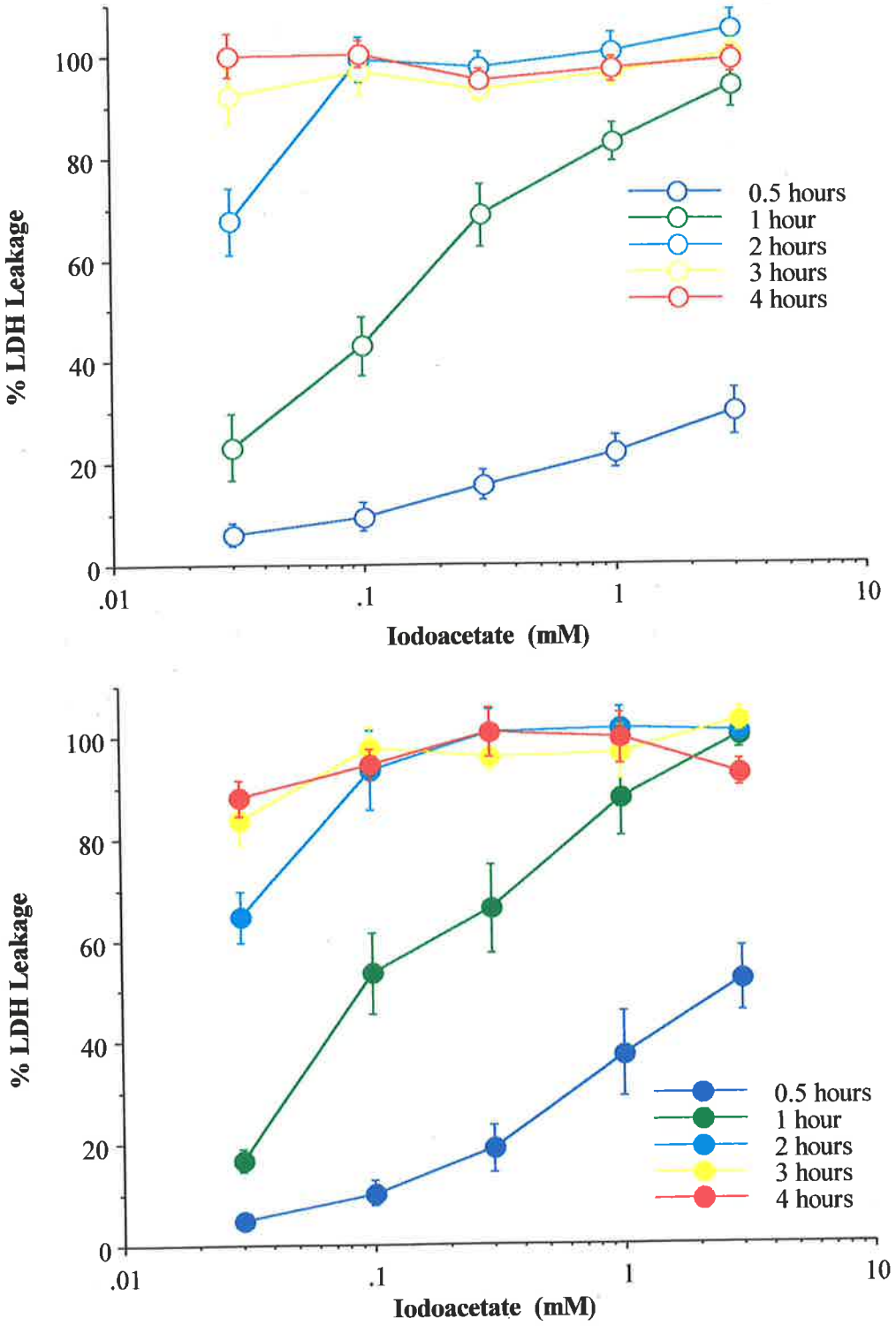

**Appendix 5F:** 1,3-*bis*-(2-chloroethyl)-1-nitrosurea (BCNU) mediated LDH leakage from isolated hepatocytes prepared from control and clofibrate pretreated mice. Upper panel (open circles) shows data obtained from control (olive oil pretreated) hepatocytes while the lower panel (closed circles) show data from clofibrate pretreated mice. Samples were collected at 1,2,4 and 8 hours after the commencement of the experiment for the determination of LDH leakage. (Results expressed as mean ± s.e.m, n=5).




**Appendix 5G:** tert-butyl hydroperoxide mediated LDH leakage from isolated hepatocytes prepared from control and clofibrate pretreated mice. Upper panel (open circles) shows data obtained from control (olive oil pretreated) hepatocytes while the lower panel (closed circles) show data from clofibrate pretreated mice. Samples were collected at 1, 2 and 4 hours after the commencement of the experiment for the determination of LDH leakage. (Results expressed as mean ± s.e.m, n=7-14).




**Appendix 5H:** Frusemide mediated LDH leakage from isolated hepatocytes prepared from control and clofibrate pretreated mice. Upper panel (open circles) shows data obtained from control (olive oil pretreated) while the lower panel (closed circles) show data from clofibrate-pretreated mice. Samples were collected at 1, 2, 4 and 8 hours after the commencement of the experiment for the determination of LDH leakage. (Results expressed as mean ± s.e.m, n=6).




**Appendix 5I:** Iodoacetate mediated LDH leakage from isolated hepatocytes prepared from control and clofibrate pretreated mice. Upper panel (open circles) shows data obtained from control (olive oil pretreated) hepatocytes while the lower panel (closed circles) show data from clofibrate pretreated mice. Samples were collected at 0.5, 1, 2, 3 and 4 hours after the commencement of the experiment for the determination of LDH leakage. (Results expressed as mean ± s.e.m, n=4-8).

**APPENDIX 6: Retention times of hepatic fatty acid by Gas-Liquid chromatographic analysis**

The peaks of 20:5 and 22:0, in addition to 22:5 and 24:0 have very close retention times. Following incubation of the samples in the presence of bromine, the peaks were identified as the unsaturated fatty acids; 20:5 and 22:5.

Retention Times:

| Fatty Acid | Retention Time (mins) |
|---|---|
| 14:0 | 3.20 |
| 16:0 | 4.93 |
| 16:1 | 5.06 |
| 18:0 | 6.78 |
| 18:1 | 6.98 |
| 18:2 | 7.43 |
| 20:0 | 8.62 |
| 20:1 | 8.90 |
| 20:2 | 9.33 |
| 20:3 | 9.56 |
| 20:4 | 9.79 |
| 20:5 | 10.41 |
| 22:5 | 12.21 |
| 22:6 | 12.5 |

Peak retention times could vary on individual traces by ± 0.06 minutes

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*This is not the end,*

*It is not even the beginning of the end*

*But it is perhaps,*

*the end of the beginning.*

*Winston Churchill - 1945*