EFFECT OF THE DRILLING FLUIDS IPAR AND NEODENE ON BIOTRANSFORMING ENZYMES IN RATS

HUI WANG

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EFFECT OF THE DRILLING FLUIDS IPAR AND NEODENE ON BIOTRANSFORMING ENZYMES IN RATS

By

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ABSTRACT

Drilling fluid, as a rich source of petroleum compounds, is a potential environmental pollutant after disposal. The effects of two drilling fluids: IPAR and NEODENE on biotransforming enzyme activities in rats were examined following administration of 1 to 4 doses (i.p. 1ml/dose). These activities measured included hepatic and renal mixed function oxidases, glutathione S-transferases, and peroxisomal enzymes.

IPAR specifically induced two isoforms of cytochrome P450: CYP 1A1 and CYP2B1 in liver. Induction of CYP 1A1 protein (100%) as well as its associated EROD activity (46%) was significant 24 hours after IPAR administration. Although these elevations returned to normal by 72 hours, further administration of IPAR caused EROD activity and CYP 1A1 protein levels to increase by about 30% in both the 2 dose and 4 dose treatment groups. Moreover, IPAR increased hepatic PROD activity (CYP 2B1 associated) 9-fold in the 24 hour (1 dose) group, 3-fold in the 72 hour (1 dose) and 6 day (2 doses) groups, and 1.5-4 fold in the 12 day (4 doses) group. The associated CYP 2B1 protein levels were also increased correspondingly, but the extent of increase was not as much as that of PROD activity. However, IPAR had no effect on glutathione S-transferases activities.

In contrast, NEODENE significantly inhibited hepatic microsomal cytochrome P450 levels (25 to 30%) and the dependent EROD activity (20 to 45%), along with glutathione S-transferase activity (DCNB substrate, 14 to 40%) in all four treated groups. However, Western blot analysis showed that individual protein levels did not correlate well with the associated enzyme activity. The CYP 1A1 protein concentration was slightly increased as opposed to the decreased EROD activity. The Ya protein of glutathione S-transferase was decreased only in the 12 day (4 doses) group and remained unchanged in other treated groups. The Yb subunit of glatathione S-transferase was not altered corresponding to the decreased GST activity. There was no change in PROD activity after NEODENE administration.

Overall, IPAR and NEODENE have the potential to cause metabolic dysfunction. A significant weight loss was observed after NEODENE administration in every treated group. The inhibition of xenobiotic metabolizing enzyme activities would lead to a slower elimination of NEODENE which may contribute to its toxicity. However, the alteration of enzyme activity is not dose-dependent. Therefore, these endpoints as biomarkers of drilling fluid exposure need to be further examined.

The peroxisomal enzymes, palmitoyl CoA oxidase and carnitine transferase along with microsomal lauric acid hydroxylase and serum cholesterol and

triglycerides levels were not significantly altered suggesting that neither IPAR nor NEODENE is likely to cause peroxisomal proliferation.

Overall, it appears that both IPAR and NEODENE have significant effects on biotransforming enzymes and NEODENE appears to be more toxic than IPAR.

To

My Family

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LIST OF ABBREVIATIONS

SBF(s):	synthetic based drilling fluid(s)
OBF(s):	oil based drilling fluid(s)
SD:	standard deviation
NADPH:	β–nicotinamide adenine dinucleotide phosphate, reduced form
NADPH:	β–nicotinamide adenine dinucleotide phosphate
FAD:	flavin adenine dinucleotide
DNBT:	5',5'-dithio-bis-nitrobenzoicacid
EDTA:	ethylenediamine tetraacetic acid
DMSO:	dimethyl sulfoxide
Tween-20:	poly oxyethylenesoibitan monolaurate
BSA:	bovine serum albumin
CYP:	cytochrome
EROD:	7-ethoxyresorufin-O-deethylase
PROD:	7-pentoxyresorufin-O-depentylase
ER:	ethoxyresorufin
PR:	pentoxyresorufin
CDNB:	1-chloro-2,4-dinitrobezene
DCNB:	1-chloro-2,4-dinitrobenzene

GSH:	glutathione, reduced form
GST(s):	glutathione S-transferase(s)
SDS:	sodium dodecyl sulphate
CAPS:	3-cyclohexylamino-1-propanesulfonic acid
TTBS:	Tris-tween buffered saline buffer
rpm:	rotation per minute
EPA:	Environmental Protection Agency
BCIP:	5-bromo-4-chloro-3-indolylphosphate
NBT:	nitro blue tetrazolium

C H A P T E R 1

1. Introduction

Accompanying the development of modern industry, more and more chemicals that include industrial products and solvents, fuels, agricultural chemicals, drugs, and consumer products are being released into the environment. Chronic exposures of large populations to these chemicals may cause environmental pollution and other consequences such as exerting teratogenic effects, affecting the aging process, or causing cell mutations and inducing cancer directly (Ames, 1979; Crow, 1973; Freese, 1973; De Serres, 1975; Thomas and Michael 1980). Because of the toxic, mutagenic or carcinogenic properties of some chemicals, environmental contamination has become a significant public health problem. More and more research is being carried out to assess the health and ecological effects of these toxic substances.

In the petroleum industry, mainly aliphatic and aromatic hydrocarbons and a few nonhydrocarbons (Atlas and Bartha, 1973) are the major pollutants in the environment. Their toxic effects such as growth delay (Gundersen et al., 1996), immune system impairment (Tahir and Secombes, 1995) and neoplasia (Max, 1986) have been well documented in different species. Drilling fluid, essential for the successful drilling operation of an oil well, is a rich resource of petroleum

based compounds. Thus, it may be a potential toxicant to the environment and to humans after disposal. With the increasing number of oil wells and new leases for oil exploration, the components and types of drilling fluid are being carefully selected and examined. Furthermore, toxicity tests of drilling fluids have received more serious consideration in order to minimize the possible environmental impact from their disposal.

1.1 Drilling fluid

1.1.1 Function of drilling fluid

Drilling fluid, also referred to as mud, is pumped and recirculated through the borehole of oil wells. It lubricates the drill, cleans the hole of drilling debris, cools the drilling head, weights the column to prevent blow-outs, seals the surrounding surface, and serves other beneficial functions (Logan and Sprague, 1979).

1.1.2 Classification of drilling fluid

According to the International Association of Drilling Contractors (IADC) and the American Petroleum Institute (API), drilling fluids are categorized into

four basic mud systems: water-based systems, low solid systems, oil-based systems, and air, gas, mist systems (Drilling fluid file, 1974).

Historically, most drillings have been performed with water-based muds along the Canadian continental shelf. However, oil-based muds tend to be more efficient and better lubricants. The use of oil-based muds has thus led to a steady decline in the usage of water-based muds since 1983. By 1987, 58% of exploratory wells were drilled with oil-based muds (Chenare et al., 1989).

Since about 1990, the oil and gas extraction industry has developed new synthetic base materials from which to formulate high performance drilling fluids named synthetic-based drilling fluids (SBFs). The synthetic-based fluids have the drilling performance characteristics of traditional mineral oil and diesel oil-based fluids (OBFs), but have lower environmental impacts and greater worker safety.

1.1.3 Composition of drilling fluid

Drilling fluid began as mud---just clay and water. It is a complex mixture and its chemical composition is only partly known. Most muds consist of a 0.5% slurry of bentonite, an organic material such as lignite or lignosulfonate to stabilize the slurry in water. Sodium hydroxide is added as a dispersant and a density-increasing material and, usually, barite ($BaSO_4$) is added to help float out rock particles (Miller and Pesaran, 1980).

Nowadays, modern muds are designed for a wide range of drilling conditions of the hole. The properties of drilling fluid can be modified by a variety of additives, such as pH control additives, bactericides, calcium removers, corrosion inhibitors, defoamers, emulsifiers, filtrate reducers, foaming agents, lost circulation materials, lubricants, shale control inhibitors, surface active agents, thinners, dispersants, viscocifiers, and weighting materials. Many factors are carefully addressed on environmental safety.

In synthetic-based muds, the synthetic materials are produced by reaction of specific purified chemical feedstock, as opposed to the oil-based fluids that are derived from crude oil solely through physical separation processes. These oleaginous (oil-like) materials include vegetable esters, poly alpha olefins, internal olefins, synthetic paraffins, ethers, alkyl benzene, and others. Typically, synthetic-based fluids are free of polycyclic aromatic hydrocarbons (PAHs) which are known to be toxic to the environment (www.epa.gov).

The two drilling fluids used in my research are IPAR and NEODENE. They are synthetic-based muds. IPAR and NEODENE differ significantly in their chemical components as can be seen from the HPLC graphs (Figures 1.1 and 1.2). However, other properties are unknown due to little information released from the oil company.

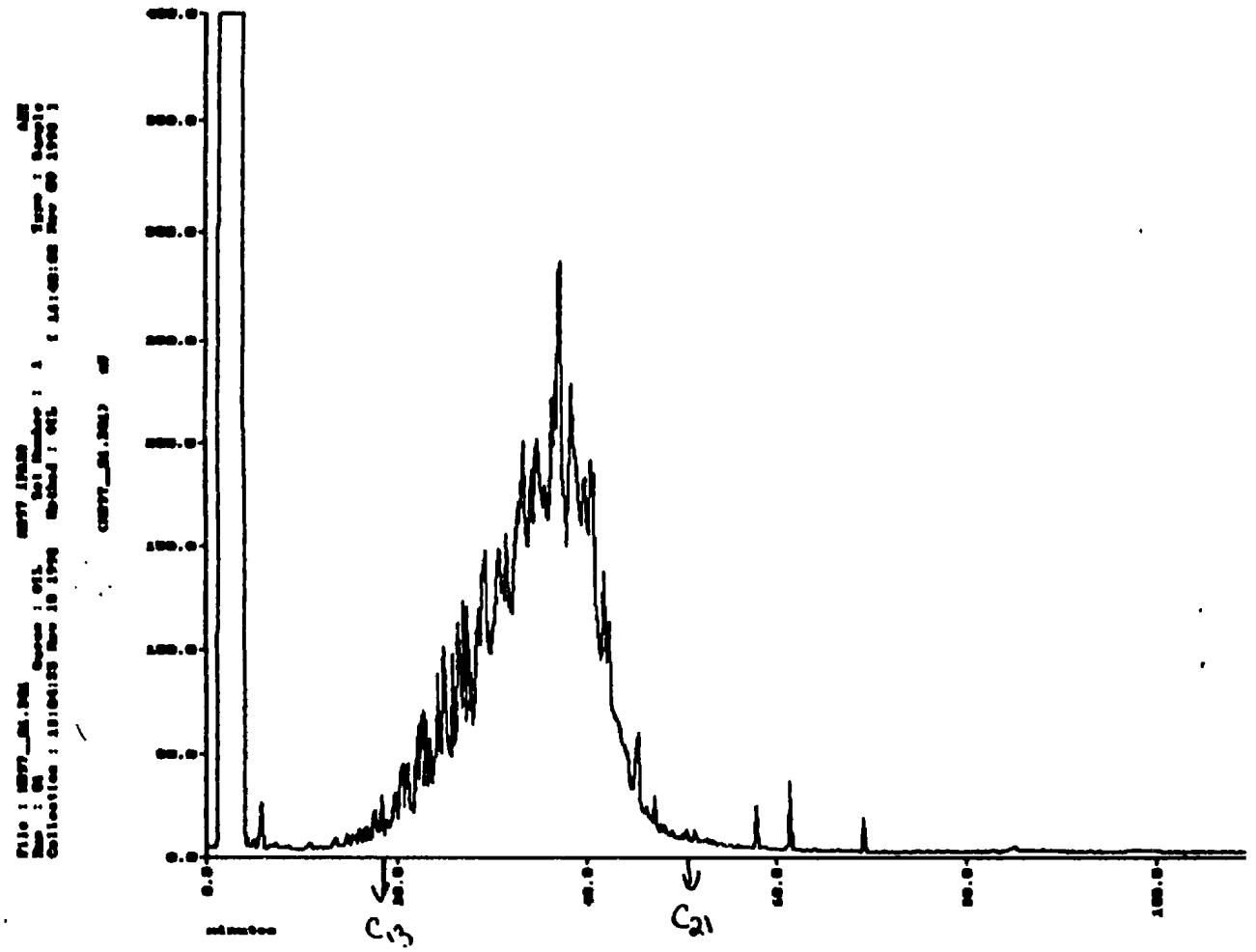


Figure 1.1 HPLC analysis* presenting C_{13} to C_{21} components in IPAR

* Conditions of HPLC analysis are not released from the oil company

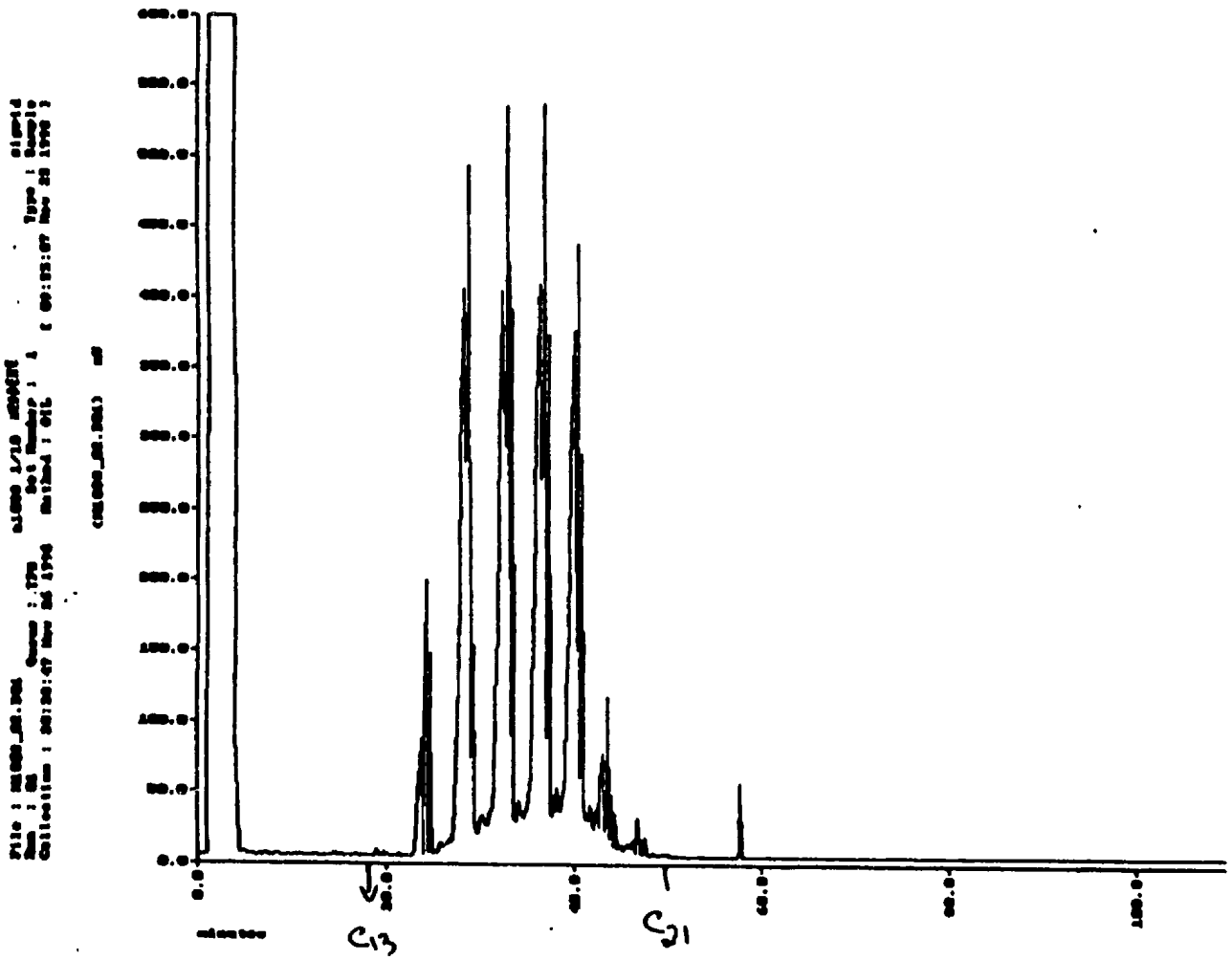


Figure 1.2 HPLC analysis* presenting C_{13} to C_{21} components in NEODENE

* Conditions of HPLC analysis are not released from the oil company

1.1.4 Disposal of drilling fluid

Since drilling fluids are rich in petroleum based compounds some of which could be toxic or mutagenic to living organisms, a great concern is given to its disposal. Nesbitt and Sandes (1981) presented a set of disposal methods with approximate cost to serve as an initial guide for disposal of drilling fluids. The Canada-Newfoundland Offshore Petroleum Board (C-NOPB), the Canada-Nova Scotia Petroleum Board (C-NSOPB) and The National Energy Board (NEB) co-published a review of the Offshore Waste Treatment Guidelines (OWTG) that described minimum standards for the treatment and/or disposal of waste associated with routine operations of drilling and production installations offshore in September, 1996 (http://www.neb.gc.ca). Disposal of drilling fluid from land-based operations is usually into temporary or permanent pits (Collins, 1971). As for offshore or lake drilling, methods of disposal are based upon careful consideration of mud formulation and specific environmental conditions at the site. Water-based muds may be discharged from offshore installations without treatment if fluids are not contaminated with oil, heavy metals, or other bioaccumulating substances. Oil-based or synthetic-based muds are often reconditioned or recycled, transferred to shore and disposed in a manner approved by authorities (http://www.grida.no). The Environmental Assessment Division (EAD) pointed out that synthetic-based drilling fluids are preferred for more

complex drilling situations as long as the drilling cuttings can be discharged on site rather than hauled to shore for disposal (http://www.ead.anl.gov). Toxicity tests of drilling fluids are performed to estimate the maximum amount of drilling fluid that can be discharged without having a direct, toxic effect on the environment or a predicted impact on living organisms.

1.1.5 Toxicity of drilling fluid

The environmental effects of drilling fluid have been evaluated on soil, plants, bacterial and marine organisms since early 1980's. The degree of impact drilling fluids have on the environment depends on the type of mud used and the prevailing environmental conditions. Water-based mud is generally less damaging compared to oil-based mud. Drilling muds contaminated with petroleum hydrocarbons from oil bearing formations could be more toxic than uncontaminated muds. Despite the large scale of inputs of drilling fluids, all the field studies around the North Sea platforms showed that the major deleterious biological effects occurred within 500 m of the platform. In the surrounding area, approximately within 400-1000 m of the platform, subtle biological effects could be detected (Davies et al., 1984). This is mainly because discharge of drilling mud into the ocean results in substantial dilution of potentially toxic components with natural seawater (Audredge et al., 1986).

When an oil-based drilling fluid is dispersed in water, the oil components are present in three phases: a water phase, a particle phase and a mineral phase. The dissolved and particle oil has toxic effects, which results in the growth delay of mussels. The mineral particles are usually biologically inert, but in high concentration, they would cause stress to the test organisms and might have a negative effect on growth (Stromgren and Reiersen, 1988). The oily cuttings do not disperse as much as water-based muds and so may deposit on the seabed. High concentrations of organic materials can have profound effects on plants and animals living on the seabed. Laboratory data showed that a number of macrobenthic invertebrates were significantly suppressed after exposure for different periods to different drilling fluids (Weber et al., 1992). The productivity of seagrass was also reduced by drilling fluid exposure in response to seasonal changes (Price et al., 1986). The Allium test, which tests the toxicity in plant systems, demonstrated that in spite of different organic and inorganic components in the drilling fluids, they all have toxic effects on plants (Vidalovic et al., 1993). Similarly, not only the individual fluid components, but also the complete drilling fluid mixture can cause poor plant growth (Miller and Pesaran, 1980). The organic materials accumulated on the seabed also result in the generation of anaerobic conditions because rapid bacterial activity uses up the available oxygen in an area.

Such a condition leads to the almost total elimination of bottom dwelling organisms very close to the rig (http://www.l.slb.com).

The potential adverse effects after discharge of drilling fluids have been examined in a variety marine species, such as fish, lobster, crab, and mussels. Intraperitoneal administration of an extract from a diesel oil-based drilling mud to rainbow trout caused both stimulatory and inhibitory effects of the immune system (Tahir and Secombes, 1995). However, it is difficult to extrapolate these findings to the consequences of immunomodulation of fish in drilling fluid-contaminated areas, because of the diversity of components involved in the test system.

In order to assess the environmental effects of drilling fluids properly, some sensitive stages of potentially affected organisms are usually chosen. In assessing the developmental toxicity of drilling fluids in marine species, it is important to observe effects on fertilization, embryonic development, and early larval life. Studies on the embryo development in Fundulus showed clearly that drilling fluid contained toxic materials which affected the development of teleosts and the fertilization and development of sand dollars (Crawford and Gates, 1981).

Although some investigations have shown that drilling fluids have little or no effect on adult marine organisms, larvae and juvenile invertebrates are believed to be sensitive to the exposure of drilling fluids. Bookhout et al. (1984) focused on the effects of a low density lignosulfonate type drilling fluid on the complete

larval development of two species of crabs. The aqueous fraction and suspended particle fraction were nontoxic to the development of crabs from the time of hatching to the first crab stage. However, a decline in swimming speed in general was observed with both fractions of drilling fluid which indicated sublethal stress on crabs.

Moreover, drilling fluids have been demonstrated to induce sublethal effects on animal behavior. In the American lobster, burrowing behavior and food detection capabilities were changed because of exposure to drilling fluid (Atema et al., 1982). The survival metabolism, energy utilization and other biochemical effects in lobsters were also altered by exposure to drilling fluids (Derby and Capuzzo, 1984).

The toxicity of drilling fluids varies widely mainly because the components present in them are not completely known. Because of interaction between components, the toxicity of total drilling fluids may not be the simple additive toxicity of individual components. Logan and Sprague (1979) found that an additive effect was only observed in three used drilling fluids out of seven. Less-than-additive toxicity or antagonism was also observed. Due to the complexity of interactive effects, it is important to emphasize that research should be carried out on whole drilling fluids rather than on partial fluids or single components so that relatively comprehensive biological effects could be assessed.

On the other hand, synthetic-based fluids have a lower environmental impact because of their lower toxicity, lack of polycyclic aromatic hydrocarbons, fast biodegradability, lower bioaccumulation potential and, in some drilling situations, less drilling waste volume according to current Environmental Protection Agency (EPA) data (http://www.epa.gov). The use of synthetic-based fluids would thus be environmentally preferable to the use of oil-based fluids.

1.2 Biotransformation and toxicity

Xenobiotics present in an animal must undergo biotransformation to more hydrophilic derivatives which are easy to excrete. Without biotransformation, lipophilic compounds would bioaccumulate in organisms and eventually overwhelm and kill the cells. Generally, the overall sequence may be considered as a detoxification or deactivation since the metabolite is usually less toxic than the parent compound. However, not all biotransformtions are beneficial to the organisms. Some components could become more toxic or biologically active than the parent compound after individual reactions; in these cases, biotransformation becomes a sequence of toxication or activation. A great number of chemicals have been studied for their toxicities that are closely related to their biotransformation in organisms. The hepatotoxicity of the anesthetic halothane is due to its

biotransformation either to a free radical that initiates lipid peroxidation or to a reactive electrophile that covalently binds to hepatic proteins and/or DNA (Reynolds and Moslen, 1980). Chemicals such as aflatoxin B_1, benzo(a)pyrene, dimethynitrosamine, safrole, benzidine, etc., are activated to their ultimate carcinogenic forms through a number of metabolic reactions (Henry and Yvonne, 1996). Therefore, biotransformation may be an important determinant of the toxicity of certain chemicals.

Biotransformation is carried out by Phase I enzymes, mainly cytochromes P450, which usually results in a small increase in hydrophilicity of chemicals and phase II enzymes such as glutathione S-transferases, glucuronosyl transferases and sulfotransferases which leads to a large increase in hydrophilicity of chemicals. In most species, exposure of an animal to certain foreign organic chemicals or drugs for a period of time can either induce or inhibit the activities of these biotransforming enzymes. Because of this, the rate at which an animal can metabolize and excrete xenobiotics is altered correspondingly. This perturbation may eventually influence the potential toxicity of chemicals. In most cases, induction of enzyme activity causes polar metabolites to be formed and excreted more quickly, therefore, it decreases the toxicity of chemicals. However, since certain xenobiotics are metabolized to more toxic intermediates, an increase of enzyme activity may exaggerate the toxic effects to an animal, especially when

other pathways for further metabolism and elimination of the toxic intermediates are not also induced or possibly inhibited.

1.2.1 The role of cytochrome P450 in metabolism and toxicity

1.2.1.1 Induction of cytochrome P450

Cytochrome P450 enzymes are heme-containing proteins which are important in the oxidative, peroxidative, and reductive metabolism of a wide range of foreign chemicals as well as endogenous compounds. Certain isoforms of cytochrome P450 are inducible by a variety of xenobiotics such as drugs, pesticides, and industrial chemicals, resulting in increased levels of the associated catalytic activities as well as increased expression of RNA and protein. The inducing ability varies mainly depending on the nature of the chemicals. Nims and Lubet (1995) categorized the potential environmental contaminants into four classes: polycyclic aromatic hydrocarbons, polyhalogenated aromatic hydrocarbons, organochlorine pesticides and phthalate plasticizers because of their specific patterns of cytochrome P450 induction. In a structural study of PCB mixtures, chemicals with 2,2'-dichloro substitution and additional substitution especially at the *para* position of biphenyl are predominantly CYP 2B inducers, while chemicals which lack substitution at the *ortho* position are usually CYP1A

inducers (Safe, 1984). However, other structurally dissimilar compounds such as industrial plasticizers, di(2-ethylthexyl)phthalate (DEHP) and hypolipidemic drugs such as clofibrate and nafenopin induce lauric acid ω–hydroxylation, which is catalyzed by the cytochrome P450 4A family (Orton and Parker, 1982).

Many drugs have the potential to induce cytochrome P450 activity (Frotschl et al., 1998). This induction is clinically important especially in explaining or predicting drug interactions and drug side effects. Usually, the induction of cytochrome P450 accelerates the elimination of foreign chemicals. Therefore, it may lower plasma concentrations of simultaneously administered drugs and decrease their therapeutic activity. For example, aminoglutethimide, a drug used in the treatment of advanced breast cancer, can accelerate the clearance of warfarin, theohylline and digitoxin about 3- to 5-fold (Kvinnsland et al., 1986). The combined use of isoniazid and rifampicin in the treatment of tuberculosis can sometimes, cause fulminant hepatitis which is due to the inductive effect of rifampicin (Pessayre et al., 1963).

Not only drugs, but also many environmental pollutants have been found to exert their toxic effects via activation by cytochrome P450. For example, acrylonitrile is known to be a carcinogen in animals and a suspected human carcinogen whose toxicity is associated with its metabolism by cytochrome P450 (Felten et al., 1998). Under these circumstances, the induction of cytochrome

P450 leads to a number of adverse consequences. Nyarko et al. (1997) found that the induction of P450 enhanced the metabolism of N-(3,5-dichlorophenyl) succinimide (NDPS), an agricultural fungicide and suggested that the induction of cytochrome P450 is closely correlated to NDPS-induced nephrotoxicity. Lehman-McKeemanld et al. (1997) observed that musk xylene, a synthetic nitromusk perfume ingredient, was capable of inducing cytochrome 2B protein which caused liver tumors in mice. The hepatotoxicity of carbon terachloride and acetaminophen is largely increased in rats by inducing hepatic cytochrome 2E1 after alcohol exposure (Wang et al., 1999).

Exposure to certain chemicals such as polycyclic aromatic hydrocarbons causes cytochrome P450 induction in many species. For example, elevated cytochrome P450 activities were observed in fish after exposure to petroleum hydrocarbons (Payne and Denrose, 1975). Although there is no causal relationship between induction and exposure, this inducing response provides a way to monitor environmental pollution and predict potential health hazards.

1.2.1.2 Inhibition of cytochrome P450

Cytochrome P450 activities can also be inhibited by many chemicals (Reidy, 1990). As a consequence, the inhibition of cytochrome P450 delays the metabolism, detoxification and elimination of xenobiotics and causes overload of

chemicals in the body which turns into adverse effects in organisms. However, in a study of developing chemopreventive compounds, 2-(allylthio) pyrazine (2-AP) was found to reduce rat hepatoxicity by inhibiting the cytochrome P450 mediated metabolic activation and protein expression (Kim and Kim, 1999).

Inhibition of cytochrome P450 is also an important issue in the study of drug metabolism and drug interaction because it can cause a rapid and profound increase in blood levels of a drug, which can lead to toxic effects and symptoms of drug overdose (Walkins, 1990). Blobner et al. (1999) reported that inhibition of cytochrome P450 led to a decreased sensitivity to and a decreased elimination of vecuronium, which causes inflammatory liver dysfunction.

1.2.2 The role of glutathione S-transferase in metabolism and toxicity

1.2.2.1 Isoforms of glutathione S-transferase

The glutathione S-transferases (GSTs) are a family of proteins that conjugate glutathione on the sulfur atom of cysteine to various electrophilic xenobiotics or electrophilic metabolites. GSTs are composed of several isoforms which are arranged into four gene families designated A, M, P, and T (which refer to alpha, mu, pi, and theta or α, μ, π and θ). In rat liver, the most important

isoforms belong to the α and μ families. The α family includes principally Ya and Yc subunits and the μ family includes the Yb subunit (Cecil, 1989).

1-Chloro-2,4-dinitrobenzene (CDNB) is a general substrate for the reactions catalyzed by all glutathione S-transferase isoenzymes, while other substrates are fairly specific for one class of enzymes. Table1.1 lists substrate specificities for the major glutathione S-transferases in rat (Bengt, 1985).

1.2.2.2 Induction of glutathione S-transferase

Glutathione S-transferases are believed to play an important protective role in living organisms by metabolizing electrophiles. Otherwise, some toxic xenobiotics and metabolites would bind to critical macromolecules, such as proteins and nucleic acids and cause cellular damage, mutation and even cancer. Glutathione S-transferases are differentially induced by xenobiotics, carcinogens and other drugs. This induction is usually associated with increased levels of mRNA and protein as well as glutathione S-transferase activity (Marcelo and Vivianna, 1999; Viviana and Marcelo, 1998). Similar to the induction of cytochrome P450, the induction of glutathione S-transferase is usually beneficial to the animals because it facilitates the clearance of active metabolites. Bishayee et al. (1999) showed that elevation of glutathione S-transferase led to a reduced intracellular concentration of carcinogen-derived reactive intermediates and

Table 1.1 Substrate specifities of major rat glutathione S-transferases

Substrate	YaYa	YaYc	YcYc	Yb_1Yb_1	Yb_1Yb_2	Yb_2Yb_2
1-Chloro-2,4-dinitrobenzene	100(38)	100(28)	100(19)	100(38)	100(28)	100(18)
1,2-Dichloro-4-nitrobenzene	<0.2	<0.2	0.2	10	9	1
Ethacrynic acid	0.4	3	7	0.3	1	3
Trans-4-phenyl-3-buten-2-one	ND	ND	ND	0.2	2	9
1,2-Epoxy-3-(p-nitrophenoxy)-propane	0.4	0.3	0.3	1.3	1.6	5

I. Relative specific activity (umol/min/mg) is given as a percentage of the specific activity determined with 1-chloro-2,4-dinitrobenzene as substrate

II. ND=not detectable activity under assay conditions used

III. From Bengt, 1985

resulted in an inhibitory response against rat liver carcinogenesis. The induction was also considered as a protective response and can be used as a bioindicator of chemical exposure (Egaas et al., 1999).

1.2.2.3 Inhibition of glutathione S-transferase

Over-expression of glutathione S-transferase isoenzymes such as α, μ, π have been found in many cultured cells after exposure to various chemotherapeutic drugs such as cisplastin (Teicher et al., 1987) and chlorambucil (Schisselbauer et al., 1990). These responses have been found to play a significant role in cell resistance to these drugs. Therefore, GST inhibitors as well as GSH depletors are receiving more attention to overcome the GST mediated drug resistance in cancer chemotherapy. Nakanishi et al. (1997) showed that some glutathione derivatives enhanced the sensitivity of tumors to acriamycin by inhibiting GST activity. Thus, these glutathione derivatives are tested as sensitizers in chemotherapy. However, inhibition of glutathione S-transferases may delay the excretion of toxic substances and thus enhance the potential toxicity. In a study of the neurotoxic effects of the organophosphate, phosphamidon on GST activity, a significantly lower level of GST activity was observed in various CNS regions of phosphamidon intoxicated rat (Naqui and Hasan, 1991).

1.3 Biomarker and toxicity test of drilling fluid

Toxicity tests are performed to predict the impact of different xenobiotics on the receiving environments or organisms. The most common method is to determine LD_{50} after exposure of animals to crude drilling fluid or diluted drilling fluids. However, besides the increasing concern for the welfare and protection of laboratory animals, LD_{50} can be influenced by many factors so that it may not reflect the environmental risks properly. Also, it is a crude measure of toxicity and does not take into account subtle, sublethal effects. Some studies have also detected the chemical concentration present in animal tissues, but it seemed hard to establish a close relationship between exposure to chemicals and environmental effects of drilling fluid. In addition, the complex chemical composition of drilling fluid makes the assessment of its toxic effect more difficult.

Currently, EPA is collecting a variety of toxicity test data about biodegradation, bioaccumulation and biotranformation of drilling fluids in order to improve effluent limitation guidelines and to better regulate the discharge of drilling fluids. In this case, the use of biological markers, which are defined as xenobiotically induced alterations in cellular or biochemical components or processes, structures or functions that are measured in a biological system or samples is receiving more interest. Biomarkers serve as endpoints of natural toxic responses. These include alterations in cytochrome P450 enzyme activity,

immunoresponse, stress protein levels, and DNA adduction. These changes establish a potential relationship between health impairment in an organism and exposure to contaminants. Among many endpoints, it is a challenge to identify which endpoint is more sensitive. Ngui and Bandiera (1999) found that induction of cytochrome CYP 2B to monitor exposure to a polychlorimated biphenyl mixture is more sensitive than the induction of CYP 1A. In a study monitoring the toxicity of 1,3 butadiene, a hazardous air pollutant, a series of endpoints were compared in order to find out a useful biomarker of exposure in which quantitative linkages between exposure and its carcinogenicity could be established (Osterman and Bond, 1996). However, a single endpoint may be affected by other potentially complicating factors. Leave et al. (1988) found a seasonal variation of increased cytochrome P450 EROD and AHH activities in fish after exposure to drilling muds. Thus, it is necessary to use a suite of biomarkers to reflect the responses to exposure more completely and accurately so that the interactions of other potentially complicating factors could be avoided. Stein et al. (1992) demonstrated that usage of the biomarkers EROD, GST, and DNA adduction concurrently provided a clearer assessment of the impact of contaminants in fish. Together with other parameters: chemical content, sediment toxicity, rate of biodegradation, the environmental risk of drilling fluids could be well assessed.

1.4 Objective of the thesis

The objective of my research is to investigate the effects of administration to rats of two drilling fluids, IPAR and NEODENE, on activities of some isoforms of cytochrome P450, glutathione S-transferases and of peroxisomal enzymes. The purpose of these experiments is to establish the primary patterns of enzymatic alternation related to IPAR and NEODENE exposure, respectively. These responses would further help to shed light on the possible mechanism(s) of their impact on the environment and could be considered as useful biomarkers to assess the adverse effects of these two drilling fluids.

C H A P T E R 2

2. Methods

2.1 Materials

Drilling fluids: IPAR and NEODENE were obtained from Dr. Jerry Payne, Department of Fisheries and Oceans (DFO), St. John's. Tris (enzyme grade), sodium dithionite, BSA (bovine serum albumin), ethoxyresorufin, pentoxyresorufin, DL-isocitric acid, isocitric dehydrogenase, resorufin, methanol, DMSO (dimethyl sulfoxide), cholesterol (Sigma diagnostics procedure No.352), triglyceride (Sigma diagnostics procedure No.336), NADPH (β–nicotinamide adenine dinucleotide phosphate, reduced form), NADP (β–nicotinamide adenine dinucleotide phosphate), ether, hexane, Sintiverse II, palmitoyl-CoA, FAD (flavin adenine dinucleotide), scopoletin, peroxidase, Triton X-100, borax, 30% H_2O_2, acetyl-CoA, DL-carnitine, EDTA (ethylenediamine tetraacetic acid), ethacrynic acid, DNBT (5',5'-dithio-bis-nitrobenzoicacid), CDNB (1-chloro-2,4-dinitrobenzene), GSH (glutathione, reduced form), DCNB (1-nitro-2,4-dichlorobenzene), trans-4-phenyl-3-buten-2-one, SDS (sodium dodecyl sulphate), CAPS (3-cyclohexylamino-1-propanesulfonic acid), acylamide, N, N'-methylene-bis-acrylamide, sodium azide, glycine, glycerol, brilliant blue R, bromophenol blue, AP (ammonium persulfate), TEMED (N, N, N', N'-tetramethyl-

ethylenediamine), BCIP (5-bromo-4-chloro-3-indolylphosphate), NBT (nitro blue tetrazolium) and Tween-20 (poly oxyethylenesoibitan monolaurate) were purchased from Sigma Chemical Co. (St. Louis, MO). Lauric acid was from BDH Canada (Toronto, Ontario). ^{14}C Lauric acid in hexane (specific activity 2.15 Gbq/mmol, 58.0 mCi/mmol) was purchased from Amersham Life Sciences (Buckinghamshire, England). Prestained protein molecular weight markers, polyclonal goat anti-rat GST Yb, polyclonal goat anti-rat GST Ya and alkaline phosphatase-conjugated anti-goat IgG were purchased from Oxford Biomedical Research (Oxford, MI). Anti-rat CYP2B1 serum and anti-rat CYP1A1 serum were purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan).

2.2 Method

2.2.1 Animal treatment

Male Sprague-Dawley rats weighing 190 g - 220 g were divided into 3 groups of 6 rats each: control group, IPAR group, and NEODENE group. One ml IPAR or NEODENE was administered intraperitoneally to each rat every time. Control rats received an equivalent amount of 0.9% NaCl. Additional dosing(s) were given at 3 day intervals after the first treatment. Rats were sacrificed 24

hours or 3 days after the final administration. The treatment regimen is illustrated below (Table 2.1):

Rats were sacrificed by cervical dislocation. Blood was collected from the heart of each ether-anesthetized rat with the aid of a 10 ml plastic disposable syringe. The individual blood samples were centrifuged at 9,000 rpm for 10 min to obtain serum (about 1 ml). Sera were stored in -70°C for one week prior to measurement of cholesterol and triglyceride concentrations.

Each liver was immediately perfused with 0.9% NaCl and then the liver and kidneys were removed separately into isocitric 250 mM sucrose-10 mM Tris-HCl buffer, pH 7.4. Liver and kidney homogenates were prepared as described below.

2.2.2 Preparation of microsomes and cytosols

All the following procedures were performed at 0-3°C according to the method described by Rahimtula et al. (1979). Liver and kidneys were separately blotted dry, weighed and transferred into beakers containing 4 volumes of 250 mM sucrose-10 mM Tris-HCl buffer, pH 7.4. Liver and kidneys were minced into small pieces and homogenized by a motor-driven Potter-Eljevhem homogenizer. The homogenates were centrifuged at 10,000 g for 15 minutes.

Table 2.1 Treatment regimen of drilling fluids to rats

	24 hours (1 dose)	72 hours (1 dose)	6 days (2 doses)	12 days (4 doses)
Day 1	administration	administration	administration	administration
Day 2	sacrifice			
Day 3				
Day 4		sacrifice	administration	administration
Day 5				
Day 6				
Day 7			sacrifice	administration
Day 8				
Day 9				
Day 10				administration
Day 11				
Day 12				
Day 13				sacrifice

1. Rats were administered intraperitoneally with either 1 ml of IPAR or NEODENE each time.

The supernatants from the 10,000 g spin were filtered through two layers of Kimwipes in order to get rid of fat and then recentrifuged at 105,000 g for one hour to yield cytosol. The pellets were resuspended in ice-cold 250 mM sucrose-10 mM Tris-HCl buffer, pH 7.4 and homogenized using 6 strokes of a tissue homogenizer. In the case of liver microsomes, the resuspended pellet was washed with above buffer by recentrifugation at 105,000 g for 1 hour.

Both microsome and cytosol samples of liver and kidney were stored at -70°C in aliquots for future enzymatic assays.

2.2.3 Determination of protein concentration

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.2.4 Measurement of microsomal cytochrome P450 levels

Microsomal cytochrome P450 levels were determined according to Omura and Sato (1964). Microsomal samples were diluted with 0.1 M Tris-HCl buffer, pH 7.4 containing 20% glycerol to a volume of 5 ml and containing approximately 2 mg protein per ml. A few grains of sodium dithionite were added and the contents mixed to reduce cytochrome P450. The reduced sample was divided equally between two matched cuvettes and a baseline was recorded between 400-

500 nm. Only the sample cuvette was gently bubbled with carbon monoxide for 30 seconds. The absorbance was re-scanned between 400-500 nm. P450 levels were determined using the molar extinction coefficient of $91cm^{-1}mM^{-1}$ for absorbance between 450-490 nm.

2.2.5 Enzymatic assays

2.2.5.1 Measurement of 7-ethoxyresorufin-O-deethylase (EROD) and 7-pentoxyresorufin-O-dependentylase (PROD) activities

EROD and PROD activities were measured respectively using ethoxyresorufin (ER) and pentoxyresorufin (PR) as substrates by a modification of the method described by Burke et al. (1985). The reaction mixture containing either 100 ug liver microsomal protein or 200 ug kidney microsomal protein, 5 uM ER or PR (12.5 ul of a 0.5 mM ER solution in DMSO or 6.5 ul of a 1 mM PR solution in DMSO), BSA 1 mg/ml, 0.1 M potassium phosphate buffer, pH 7.6 was equilibrated for 1 min at 37°C. The reaction was then started by the addition of 125 ul NADPH regenerating system. The NADPH regenerating system contains 50 ul of 1.0 M $MgCl_2$ solution, 15 mg DL-isocitrid acid, 4 mg NADP, 65 ul isocitric dehydrogenase and 885 ul of 0.1 M potassium phosphate buffer, pH 7.6 per ml. Blanks received 125 ul of buffer. The final reaction volume was 1.25 ml.

After 10 minutes incubation at 37°C, the reaction was stopped with 2 ml methanol. Precipitated protein was centrifuged and the fluorescence of the supernatant was measured at the excitation wavelength of 550 nm and the emission wavelength of 585 nm. Enzyme activity was determined by the increased fluorescence of the reaction mixture and standardized by constructing a standard curve with resorufin.

2.2.5.2 Measurement of lauric acid hydroxylase activity

Lauric acid hydroxylase activity was measured according to a modification of the method described by Viswalingam and Caldwell (1997). One hundred ug liver microsomal protein or 200 ug kidney microsomal protein, 210 ul of 1 mM lauric acid solution (approximately 1.4 $x10^6$ dpm/tube), and 190 ul of 0.1 M sodium phosphate buffer (pH 7.4) were pipetted into individual tubes and placed in a 37°C water bath for 5 min. The reaction was started by adding 50 ul NADPH regenerating system (composition mentioned above) to all tubes except the blank to which 50 ul buffer was added. The reaction was allowed to continue for 20 minutes at 37°C and then terminated with 400 ul of 3 M HCl.

Three ml ether was added to each tube and the mixture was vortexed and centrifuged at 8,000 rpm for 5 min. After allowing the phases to separate, the ether layer was pipetted out into a fresh tube and the aqueous phase was extracted again with 3 ml ether. The combined ether phases were evaporated to dryness by

nitrogen. The residue was taken up in 100 ul methanol and 25 ul was counted for ^{14}C in 7 ml Scintiverse II. The counting efficiency was assessed using an external standard. A further 25 ul was streaked on a TLC plate which was developed with hexane:ether:glacial acetic acid (49:49:1.5). Radioactive bands were located by autoradiography with Kodak scientific imaging film. Individual bands thus visualized were scraped into separate sintillation vials to which 7 ml Scintiverse II was added before counting. The ^{14}C radioactivity was determined by liquid scintillation counting. Lauric acid hydroxylase activity was expressed as nmol hydroxylated lauric acid formed/ min/ mg protein.

2.2.5.3 Measurement of palmitoyl-CoA oxidase activity

Palmitoyl CoA oxidase activity was determined by the fluorometric assay of Walusimbi-Kisitu and Harrison (1983). For this assay, fatty acyl-CoA dependent H_2O_2 production is coupled in a peroxidase-catalyzed reaction to the oxidation of scopoletin (6-methoxy-7-hydroxycoumarin), a highly fluorescent compound, to a nonfluorescent product.

The reaction mixture in a final volume of 1 ml contained 60 mM Tris-HCl , pH 8.3, 35 uM palmitoy-CoA, 50 uM FAD, 1 uM scopoletin, 10 ug (3 units) peroxidase, 0.6 mg BSA, 0.01% Triton X-100, and 100 ug liver microsomal protein or 200 ug kidney microsomal protein. The incubation was carried out

under subdued light at 37°C with shaking for 20 minutes. The reaction was terminated by adding 4 ml of 0.1 M borate buffer, pH 10. The fluorescence was measured at room temperature with emission wavelength at 470 nm and excitation wavelength at 395 nm. A control without enzyme was included in each assay. A standard curve was constructed at the same time by adding known amounts of H_2O_2 to the reaction mixture containing all components except the microsomal protein.

2.2.5.4 Measurement of carnitine acetyl transferase activity

Carnitine acetyl transferase activity was determined by the method of Böck et al. (1980). The assay was carried out at 25°C in a spectrophotometer set at a wavelength of 412 nm. The sample cuvette contained 0.5 mM DNBT-2.5 mM EDTA-0.2% Triton X-100 in 116 mM Tris-HCl buffer, pH 8.0, 0.1 mM acetyl-CoA, 5 mM carnitine, and 100 ug microsomal protein in a total volume of 1 ml. The reference cuvette contained the same mixture but without carnitine. The molar extinction coefficient of the reaction is $13.6 cm^{-1}$ mM^{-1}.

2.2.5.5 Measurement of glutathione S-transferase activities

Glutathione S-transferase activities were measured using 1-chloro-2,4-dinitrobenzene (CDNB), 1-nitro-2,4-dichlorobenzene (DCNB), ethacrynic acid,

and trans-4-phenyl-3-buten-2-one as substrates. All reactions were carried out at 30°C in 3 ml spectrophotometer cuvettes and were initiated by the addition of GSH. Individual conditions varied as follows:

2.2.5.5.1 CDNB

The reaction mixture contained 0.1 M potassium phosphate buffer, pH 6.5, 1 mM CDNB, 1 mM GSH and 50 ug cytosol protein. The reaction was followed by measuring the change in absorbance at 340 nm. The extinction coefficient of CDNB is 9.6 cm^{-1} mM^{-1}.

2.2.5.5.2 DCNB

The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.5, 1 mM DCNB, 5 mM GSH and 400 ug cytosol protein. The reaction was followed by measuring the change in absorbance at 345 nm. The extinction coefficient of DCNB is 8.5cm^{-1} mM^{-1}.

2.2.5.5.3 Ethacrynic acid

The reaction mixture contained 0.1 M potassium phosphate buffer, pH 6.5, 0.2 mM ethacrynic acid, 0.25 mM GSH and 1 mg cytosol protein. The reaction

was followed by measuring the change in absorbance at 270 nm. The extinction coefficient is 5.0 cm^{-1} mM^{-1}.

2.2.5.5.4 Trans-4-phenyl-3-buten-2-one

The reaction mixture contained 0.1 M potassium phosphate buffer, pH 6.5, 50 uM trans-4-phenyl-3-buten-2-one, 0.25 mM GSH and 1 mg cytosol protein. The reaction was followed by measuring the change in absorbance at 290 nm. The extinction coefficient is -24.8 cm^{-1} mM^{-1}.

2.2.6 Measurement of serum cholesterol and triglyceride concentration

Serum cholesterol was determined using Sigma diagnostics procedure No. 352 based on the method described by Allan et al. (1974). Serum triglycerides were measured by Sigma diagnostics procedure No. 336 based on the procedure of Bucolo and David (1973).

2.2.7 Western Blot analysis

Microsomal and cytosol proteins were boiled with an equal volume of 0.065 M Tris-HCl, pH 6.8, 4.5% sodium dodecyl sulphate (SDS-w/v), 13% glycerol (v/v), 0.3% sodium azide and 0.001% bromophenol blue (w/v) for 5 min. Aliquots of microsomal or cytosol protein along with corresponding standard

protein (Table 2.2) were loaded on 10% acryamide gels. Electrophoresis was performed at 180 V at the room temperature by the method of Laemmli (1970). Proteins were then transferred from the gel to a PVDF membrane in 10 mM CAPS-10% methanol buffer following the method of Towbin et al. (1979). The membrane was then soaked in 3% skim milk for one hour at room temperature. Blots were washed twice with a solution of 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl and 0.05% Tween 20 (Tris-Tween buffered saline, TTBS) for 5 min each. They were then incubated with primary antibody (Table 2.3) in TTBS buffer for one hour. After washing with TTBS buffer, the blots were incubated with alkaline phosphatase-conjuated secondary antibody (Table 2.3) for 30 min. Finally, the PVDF membrane was washed as above again and the blots were visualized by the addition of 0.165 mM NBT and 0.0825 mM BCIP in 20 ml of 100 mM Tris-HCl buffer, pH 9.7, containing 5 mM $MgCl_2$. Immunoreactive protein bands were quantified by Corel Draw Photo-Paint 8 software and the density was expressed in reversed gray level. The molecular weights of immunopositive bands were determined from simultaneously run pre-stained molecular weight markers.

2.2.8 Statistical analysis

Statistical comparisons between control and treated groups were carried out using student's t-test. A significant difference is expressed at $P < 0.05$ or $P < 0.01$. All values were expressed as mean ± SD.

Table 2.2 Proteins loaded on the 10% acryamide gel

	Microsomal protein	Cytosolic protein	Standand protein
CYP 1A1	10 ug		2 ug
CYP 2B1	10 ug		5 ug
GST Ya		10 ug	0.5 ug
GST Yb		10 ug	not available

Table 2.3 Primary and secondary polyclonal antibodies and working dilutions

	Microsomal protein		Cytosolic protein	
	CYP 1A1	**CYP 2B1**	**GST Ya**	**GST Yb**
Primary antibody	goat anti-rat CYP 1A1	goat anti-rat CYP2B1	goat anti-rat GST Ya	goat anti-rat GST Yb
Dilution	1/1000	1/500	1/1000	1/1000
Secondary antibody	dog anti-goat	dog anti-goat	dog anti-goat	dog anti-goat
Dilution	1/5000	1/5000	1/5000	1/5000

C H A P T E R 3

3. Results

3.1 Biological effects of IPAR and NEODENE administration

It was observed that in both IPAR and NEODENE treated groups, rats had an accumulation of slippery fluid in their abdominal cavities and cohesion between liver and intestine. The effects were more severe in the NEODENE group than in the IPAR group and also increased with the number of doses administered.

IPAR administration did not cause any significant weight loss in rats in any of the treated groups, nor did it alter the liver or kidney weights (Table 3.1).

Although NEODENE did not cause weight loss 24 hours after administration, it decreased body weight significantly in the other three administration groups: about 10% to 14% in the 72 hour (1 dose) group, 16% in the 6 day (2 doses) group, and 7% to 11% in the 12 day (4 doses) group (Table 3.2). Moreover, the decrease in weight was consistent in repeat experiments. Generally, liver weight and liver weight to body weight ratio remained unchanged after NEODENE administration. In contrast, kidney weight decreased significantly in the three treated groups. The decreases were about 13% in the 24 hour (1 dose) group, about 17% in the 72 hour (1 dose) group, and about 20% in

the 6 day (2 doses) group. In the 12 day (4 doses) group, kidney weight decreased slightly and was not significant as compared to the control group. However, the kidney weight to body weight ratio was unchanged.

3.2 Effect of IPAR and NEODENE administration on cytochrome P450 levels

IPAR had no impact on either hepatic or renal cytochrome P450 levels in any of the four treated groups (Table 3.3).

In contrast, NEODENE caused hepatic cytochrome P450 levels to decrease significantly in each of the four treated groups (Table 3.4). The extent of inhibition was 25%-33% in the 24 hour (1 dose) group, 28%-81% in the 72 hour (1 dose) group, 50% in the 6 day (2 doses) group and 36%-38% in the 12 day (4 doses) group. The inhibiting effect of NEODENE was consistent in replicate experiments, but did not change with the increasing doses. NEODENE did not alter renal cytochrome P450 levels after any treatment.

In one replicate experiment of hepatic P450 activity in the 72 hour (1 dose) NEODENE treated group, the P450 values were only 0.1 nmol/ mg $\pm$ 0.04. This P450 estimation was repeated on a different day with the same result. The reason for why the activity is so low is not obvious.

Table 3.1 Effect of IPAR administration to rats on body, liver, and kidney weights

		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR
Initial weight	(a)	208.0±2.53	207.0±4.65	208.3±3.93	207.3±1.51	194.0±5.73	191.5±2.35	206.3±6.09	203.0±3.46
(g)	(b)							210.8±9.60	208.7±11.96
Weight at death	(a)	189.3±6.28	188.5±2.95	210.2±4.02	205.3±3.78	224.0±14.81	217.0±5.19	318.5±7.50	290.5±20.33*
(g)	(b)							290.3±13.52	288.3±10.93
Liver weight	(a)	7.60±1.35	6.71±0.46	7.13±0.36	7.51±0.43	8.47±0.84	8.14±0.54	13.45±2.93	12.29±2.20
(g)	(b)							11.75±1.38	12.16±1.63
Liver weight to body	(a)	4.01±0.66	3.56±0.22	3.44±0.20	3.64±0.19	3.77±0.22	3.75±0.21	4.22±0.90	4.23±0.67
weight ratio (g/100g)	(b)							4.06±0.52	4.22±0.54
Kidney weight	(a)	2.07±0.13	1.91±0.14	2.14±0.13	2.11±0.02	2.43±0.39	2.22±0.11	2.84±0.29	2.62±0.21
(g)	(b)							2.55±0.31	2.71±0.20
Kidney weight to body	(a)	1.09±0.05	1.02±0.07	1.02±0.05	1.03±0.02	1.08±0.11	1.02±0.04	0.89±0.09	0.90±0.10
weight ratio (g/100g)	(b)							0.88±0.08	0.94±0.08

I. Values are expressed as mean±SD from 6 rats.

II. Significantly different from control at * $p < 0.05$ or ** $p < 0.01$.

III. Results of replicate experiments are expressed at (a) and (b) in corresponding columns.

Table 3.2: Effect of NEODENE administration to rats on body, liver, and kidney weights

		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE
Initial weight	**(a)**	202.7±4.46	203.8±4.49	213.0±12.95	214.0±5.71	228.2±2.95	223.4±4.78	206.3±6.09	211.0±3.66
(g)	**(b)**	207.0±6.89	211.5±5.07	203.0±4.63	199.0±6.26			210.8±9.60	209.0±5.38
	(c)			202.8±3.11	208.0±7.78				
Weight at	**(a)**	196.5±4.32	197.5±7.66	212.8±11.79	191.8±10.34*	255.0±3.08	213.2±8.79*	318.5±7.50	297±13.84**
death (g)	**(b)**	191.6±1.02	197.0±4.24	213.2±5.27	182.5±1.64**			290.3±13.52	257.8±26.1*
	(c)			212.8±4.49	192.0±1.49*				
Liver weight	**(a)**	7.19±0.24	6.86±0.32	8.39±1.08	7.90±0.36	9.27±0.39	9.43±1.34	13.45±2.93	12.41±2.21
(g)	**(b)**	6.64±0.92	7.23±0.52	7.78±0.24	6.81±0.80*			11.75±1.38	11.03±0.80
	(c)			7.72±0.27	7.50±0.45				
Liver weight	**(a)**	3.66±0.11	3.48±0.22	3.94±0.49	4.13±0.28	3.63±0.12	4.43±0.65*	4.22±0.90	4.16±0.58
to body weight	**(b)**	3.45±0.37	3.67±0.24	3.65±0.11	3.73±0.24			4.06±0.52	4.32±0.60
ratio (g/100g)	**(c)**			3.62±0.09	3.91±0.21*				
Kidney weight	**(a)**	1.88±0.13	1.62±0.10*	2.16±0.31	1.86±0.18	2.40±0.16	1.92±0.16*	2.84±0.29	2.56±0.24
(g)	**(b)**	1.83±0.10	1.82±0.14	2.09±0.13	1.73±0.24*			2.55±0.31	2.23±0.21
	(c)			2.21±0.11	1.95±0.13**				
Kidney weight	**(a)**	0.96±0.05	0.82±0.08*	1.02±0.14	0.97±.080	0.94±0.07	0.90±0.08	0.89±0.09	0.86±0.06
to body weight	**(b)**	0.96±0.04	0.93±0.07	0.98±0.05	0.95±0.07			0.88±0.08	0.88±0.01
ratio (g/100g)	**(c)**			1.04±0.05	1.01±0.03				

I. Values are expressed as mean±SD from 6 rats.

II. Significantly different from control at * $p< 0.05$ or ** $p< 0.01$.

III. Results of replicate experiments are expressed at (a), (b), and (c) in corresponding columns.

Table 3.3 Effect of IPAR administration on liver and kidney microsomal P450 activities

		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR
Liver P450	**(a)**	0.46±0.05	0.49±0.09	0.53±0.05	0.53±0.07	0.63±0.12	0.53±0.09	0.68±0.03	0.61±0.14
	(b)							0.58±0.10	0.76±0.04*
kidney P450	**(a)**	96.20±26.85	97.07±35.38	97.23±26.29	112.6±43.96	87.00±35.68	95.44±36.09	65.80±27.90	75.00±24.41
	(b)							54.60±30.00	105.91±50.2

I. Values are expressed as mean±SD from 6 rats.

II. Significantly different from control at * $p< 0.05$ or ** $p< 0.01$.

III. Results of replicate experiments are expressed at each row in corresponding columns.

IV. Liver and kidney P450 activities are expressed as nmol/mg protein and pmol/mg protein

Table 3.4 Effect of NEODENE administration on liver and kidney microsomal P450 activities

		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE
Liver P450	(a)	0.58±0.12	0.39±0.09*	0.62±0.19	0.59±.080	0.61±0.07	0.30±0.08**	0.68±0.03	0.42±0.08**
	(b)	0.69±0.06	0.52±0.05**	0.61±0.08	0.44±0.09**			0.58±0.10	0.37±0.10*
	(c)			0.53±0.07	0.10±0.04**				
Kidney P450	(a)	78.76±24.30	47.16±16.52*	97.94±20.46	93.12±23.19	95.58±24.08	88.95±47.53	65.80±27.90	60.20±21.22
	(b)	70.06±25.13	42.13±13.83	86.54±35.60	103.8±37.29			54.60±30.00	74.50±15.00
	(c)			75.81±18.47	87.36±33.33				

I. Values are expressed as mean±SD from 6 rats.

II. Significantly different from control at * $p < 0.05$ or ** $p < 0.01$.

III. Results of replicate experiments are expressed at (a), (b), and (c) in corresponding columns.

IV. Liver and kidney P450 activities are expressed as nmol/mg protein and pmol/mg protein

3.3 Effect of IPAR administration on EROD activity and CYP1A1 protein levels

EROD activity has been used as a marker for CYP1A (Kedderis et al., 1991). In liver, the EROD activity was significantly induced by 46% 24 hours after IPAR administration, but had returned to normal by 72 hours (Table 3.5). Further administration of IPAR caused the EROD activity to increase again. Following 2 doses of IPAR, the EROD activity was significantly higher by 27% as compared to control rats while, after 4 doses, there was still a nearly 30% increase, but no significant difference between the treated and control groups. This lack of significance is probably due to the higher standard deviation. Western blots of liver microsomes incubated with polyclonal anti-CYP1A1 were carried out using microsomes from every treated group and the results are described in Figure 3.1. CYP1A1 protein was visualized at an approximate molecular weight of 48 KDa, which was confirmed by using standard CYP1A1 protein. Western blots of replicate experiments were quite similar but result of only one such experiment was shown. Overall, the alteration of CYP1A1 level in the treated groups was correlated with that of EROD activity. The CYP1A1 protein was increased about 100% after 24 hours of IPAR administration. The extent of CYP1A1 protein induction exceeded the rate of EROD activity induction. However, there was no change in protein level after 72 hours of administration. Similar to the increase in

EROD activity, the CYP1A1 protein was increased again after further administration of IPAR. After 2 doses, CYP1A1 protein level was about 30% higher than that in control rats and increased by 39% after 4 doses.

The effect of IPAR administration on kidney microsomal EROD activity was similar to that on liver microsomal EROD activity (Table 3.5). IPAR also caused a significant 256% increase in renal EROD activity at 24 hours but the activity returned to the normal level after 72 hours. A second dose of IPAR again increased the activity but not as much (129%). However, after 4 doses, the EROD activity was unchanged. In the case of two treated groups (24 hours and 6 days) in which the EROD activities were induced, CYP1A1 protein was not detected by Western blot analysis even by increasing the protein load on the gel. Results of Western blot analysis were not shown.

Table 3.5 Effect of IPAR administration on liver and kidney microsomal EROD activities

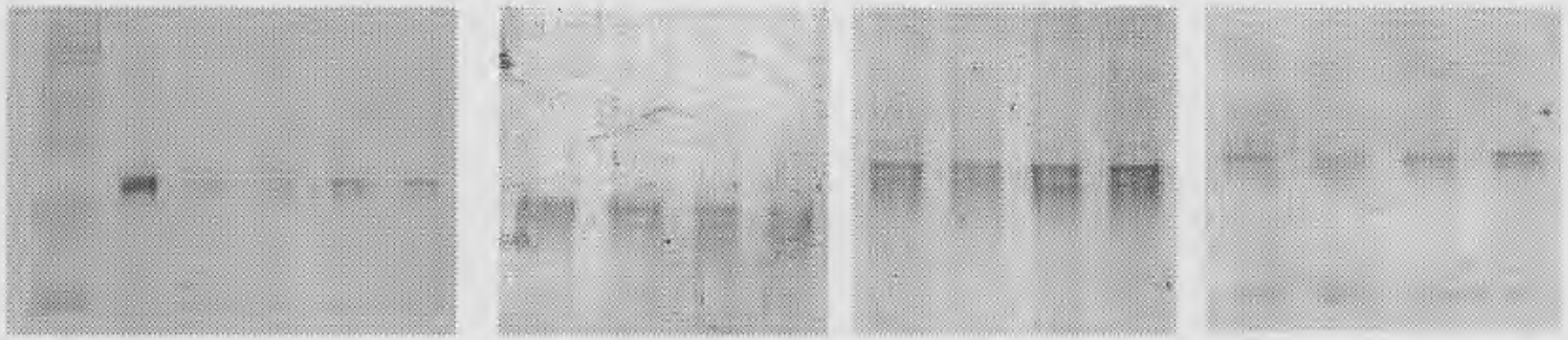
		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR
Liver EROD	(a)	55.85±9.95	83.56±30.59*	71.67±12.82	75.45±11.34	70.17±7.45	88.84±11.67*	139.79±24.0	186.39±55.0
	(b)							165.4±35.68	212.2±37.65
Kidney EROD	(a)	0.64±0.27	2.34±2.81*	0.56±0.33	0.55±0.17	0.55±0.12	1.26±0.51*	0.70±0.26	0.80±0.32
	(b)							0.42±0.24	0.39±0.17

I. Values are expressed as mean±SD from 6 rats.

II. Significantly different from control at * $p< 0.05$ or ** $p< 0.01$.

III. Results of replicate experiments are expressed at (a) and (b) in corresponding columns.

IV. EROD activity is expressed as pmol/min/mg protein



Lane:	I	II	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Grey level:			51	57	107	94	85	107	80	88	109	100	131	148	76	64	95	114
(reversed)		**24 hours (1 dose)**						**72 hours (1 dose)**				**6 days (2 doses)**				**12 days (4 doses)**		

*Lane I: molecular weight marker
*Lane II: standard CYP1A protein
*Lane 1, 2: control
*Lane 3, 4: treated

Figure 3.1: Western blots employing anti-CYP1A1 polyclonal antibodies on liver microsomes after IPAR administration

3.4 Effect of NEODENE administration on EROD activity and CYP1A1 protein levels

The effect of NEODENE administration on hepatic and renal microsomal EROD activity was quite different from that after IPAR administration (Table 3.6). The hepatic EROD activity was inhibited significantly about 34% 24 hours after 1 dose. But after 72 hours, the EROD activity data was more difficult to interpret. In three separate experiments, EROD activity increased significantly by 19% in only one experiment (Table 3.6) while, in the other two, there was no statistical difference between the control and treated groups. However, the inhibitory effect was significant after 4 doses with EROD activity being decreased by about 21% to 45% in duplicate experiments. Although EROD activity decreased approximately 20% in the 2 dose group, the difference was not statistically significant from the control group because of the relatively high standard deviation.

Values between the three control EROD activities in the 72 hour (1 dose) group in liver were different. This difference may be due to the individual differences between rats because the difference persisted when these activities were assayed on the same day under identical conditions.

Liver microsomal CYP1A1 protein concentration was measured by Western blot in each of the four treated groups (Figure 3.2). Results of replicate

experiments were similar but results of only one such experiment were shown. Replicate experiments with different results were shown seperately. In general, CYP1A1 protein showed an increase while EROD activities decreased. Thus, there was a 24% to 32% induction of CYP1A1 protein content 24 hours after one dose. It is also interesting that CYP1A1 protein increased by 22% in the 6 day (2 doses) group and by about 26% in the 12 day (4 doses) group as opposed to the EROD activities observed in these groups. In the 72 hour (1dose) NEODENE treated group, CYP1A1 protein was either slightly increased or slightly decreased. Furthermore, unchanged CYP1A1 protein level was also observed. It is hard to establish a relationship between the alteration of EROD activity and that of CYP1A1 protein level in that treated group.

NEODENE did not have a significant impact on renal microsomal EROD activity as showed in Table 3.6.

Table 3.6 Effect of NEODENE administration on liver and kidney microsomal EROD activities

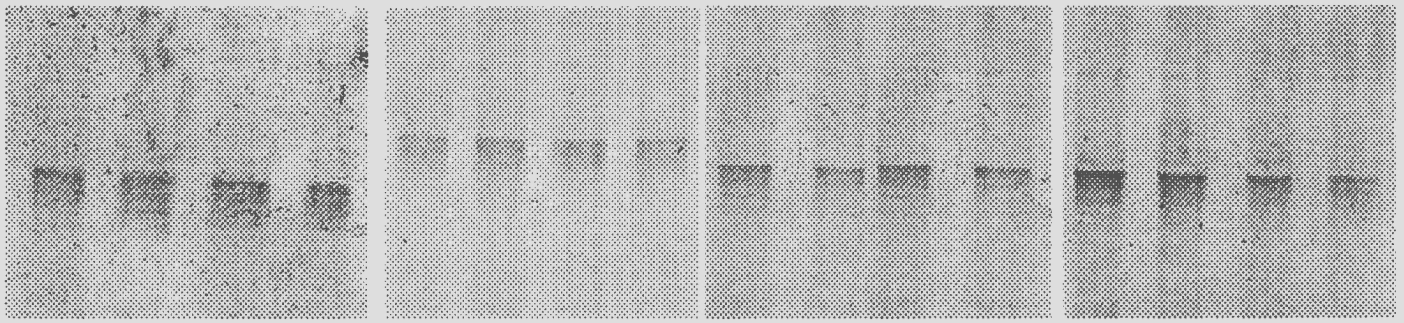
		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE
Liver EROD	**(a)**	115.50±16.8	70.81±9.91**	127.10±21.4	150.60±20.5	113.50±28.3	90.93±10.92	139.79±24.0	110.20±14.1
	(b)	114.60±9.72	79.17±14.3**	79.89±8.45	95.14±13.66*			165.40±35.6	90.19±36.4*
	(c)			155.30±32.0	99.94±32.61				
Kidney EROD	**(a)**	0.65±0.07	0.59±0.13	0.67±0.18	1.26±0.42*	0.61±0.10	0.87±0.56	0.70±0.26	0.81±0.28
	(b)	0.89±0.26	0.35±0.19*	0.50±0.17	0.65±0.26			0.42±0.24	0.86±0.40
	(c)			0.63±0.62	0.39±0.23				

I. Values are expressed as mean±SD from 6 rats.

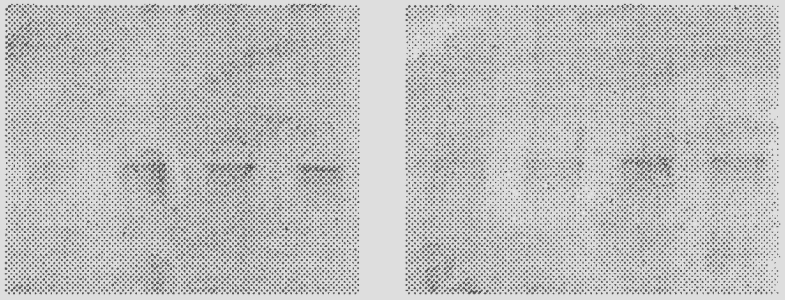
II. Significantly different from control at * $p< 0.05$ or ** $p< 0.01$.

III. Results of replicate experiments are expressed at (a), (b), and (c) in corresponding columns.

IV. EROD activity is expressed as pmol/min/mg protein



Lane:	1	2	3	4	1	2	3	4	1	3	2	4	1	2	3	4
Grey level:	104	99	123	144	65	73	74	60	61	71	173	106	209	189	158	119
(reversed)	**24 hours (1 dose)**				**72 hours (1 dose)**				**72 hours (1 dose)**				**72 hours (1 dose)**			



Lane:	1	2	3	4	1	2	3	4
Grey Level:	83	100	97	126	72	64	89	83
(reserved)	**6 days (2 doses)**				**12 days (4 doses)**			

*Lane 1, 2: control
*Lane 3, 4: treated

Figure 3.2: Western blot employing anti-CYP1A1 polyclonal antibodies on liver microsomes after NEODENE administration

3.5 Effect of IPAR administration on PROD activity and CYP2B1 protein levels

IPAR caused liver microsomal PROD activity to increase significantly in all treated groups as shown in Table 3.7. In particular, 24 hours after administration, PROD activity was increased nearly 9-fold (775%), even though the standard deviation was higher than that in other treated groups. In the other three treated groups, PROD activities were all induced about 3-fold. Therefore, the extent of induction did not correlate with the multiple dosing. The Western blot results indicated that induction of CYP2B1 protein correlated with induction of PROD activity (Figure 3.3). The degree of CYP2B1 protein induction was 67% in the 24 hour (1 dose) group, 53% in the 72 hour (1 dose) group, 18% in the 6 day (2 doses) group, and 141% in the 12 day (4 doses) group.

In kidney, IPAR administration inhibited PROD activity (Table 3.7). However, only in the 72 hour (1dose) group (47%) and the 12 day (4 dose) group (19%) were the decreases significant. In the other groups, there was a trend toward a decrease, but the results were not significant due to the high standard deviation.

3.6 Effect of NEODENE administration on PROD activity

NEODENE administration had no effect on PROD activity both in liver and kidney microsomes in any of the four treated groups (Table 3.8). The only

exception was a significant 103% increase in PROD activity in kidney microsomes after 2 doses. The difference in the values between the control PROD activities in the 72 hour (1 dose) group persisted when the activites were measured on the same day under identical conditions.

3.7 Effect of IPAR and NEODENE administration on microsomal lauric acid hydroxylase activity

Hydroxylation of lauric acid (ω-oxidation) representing the CYP 4A1 isoenzyme by liver and kidney microsomes was not altered in IPAR administered rats, except one significant increase (53%) was observed in kidney microsomes after 4 doses (Table 3.9). NEODENE caused hepatic lauric acid hydroxylase activity to decrease slightly (13%-29%) 24 hours after administration although this change was not significant (Table 3.10). By 72 hours, the enzyme activity was not different from that of the control. However, further administration of NEODENE again inhibited lauric acid hydroxylase activity. Significant drops were seen in the 6 day (2 doses) group (44%) and in one of two separate experiments (38 %) in the 12 day (4 doses) group. NEODENE did not cause any changes in renal enzyme activity.

Table 3.7 Effect of IPAR administration on liver and kidney microsomal PROD activities

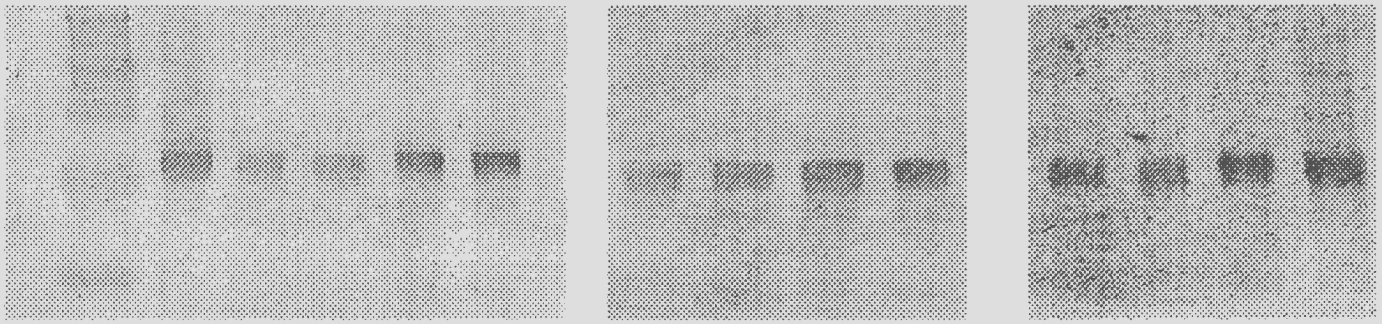
		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR
Liver PROD	(a)	12.24±2.74	107.1±91.62*	13.10±4.53	43.18±13.0**	12.61±4.27	38.03±24.69*	24.49±2.76	36.63±10.37*
	(b)							29.69±8.71	128.1±50.16*
Kidney PROD	(a)	0.83±0.51	0.48±0.42	0.78±0.16	0.41±0.34*	0.79±0.18	0.76±0.52	2.44±0.19	1.97±0.37*
	(b)							1.84±0.79	1.27±0.68

I. Values are expressed as mean±SD from 6 rats.

II. Significantly different from control at * $p< 0.05$ or ** $p< 0.01$.

III. Results of replicate experiments are expressed at (a) and (b) in corresponding columns.

IV. PROD activity is expressed as pmol/min/mg protein



Lane:	I	II	1	2	3	4	1	2	3	4	1	2	3	4
Grey Level:			69	62	100	119	73	88	116	131	107	92	137	145
(reversed)			**24 hours (1 dose)**				**72 hours (1 dose)**				**6 days (2 doses)**			

Lane:	1	3	2	4
Grey Level:	72	111	120	134
(reversed)	**12 days (4 doses)**			

*Lane 1, 2: control
*Lane 3, 4: treated

*Lane I : molecular weight marker
*Lane II : standard CYP2B1 protein

Figure 3.3: Western blots employing anti-CYP2B1 polyclonal antibodies on liver microsomes after IPAR administration

Table 3.8 Effect of NEODENE administration on liver and kidney microsomal PROD activities

		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE
Liver PROD	(a)	12.89±3.50	11.63±2.26	19.51±5.18	19.27±4.94	24.32±5.76	16.61±2.21	24.49±2.76	26.82±3.21
	(b)	14.52±2.07	11.99±2.19	9.83±6.12	12.70±3.88			29.69±8.71	19.15±6.93*
	(c)			29.04±8.94	17.52±9.76				
Kidney PROD	(a)	2.20±0.50	2.23±0.30	0.80±0.30	1.04±0.24	1.47±0.30	2.99±0.98*	2.44±0.19	2.18±0.81
	(b)	1.53±0.16	1.66±0.12	1.69±0.57	1.92±0.40			1.84±0.79	1.10±0.59
	(c)			1.79±0.16	1.99±0.21				

I. Values are expressed as mean±SD from 6 rats.

II. Significantly different from control at * $p < 0.05$ or ** $p < 0.01$.

III. Results of replicate experiments are expressed at (a), (b), and (c) in corresponding columns.

IV. PROD activity is expressed as pmol/min/mg protein

Table 3.9 Effect of IPAR administration on liver and kidney microsomal lauric acid hydroxylase activities

		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR
Liver lauric acid	(a)	0.29±0.12	0.37±0.09	0.26±0.05	0.22±0.07	0.26±0.10	0.34±0.03	0.53±0.25	0.68±0.26
hydroxylase	(b)							0.42±0.03	0.59±0.19
Kidney lauric	(a)	0.31±0.13	0.32±0.08	0.23±0.04	0.25±0.06	0.18±0.09	0.21±0.04	0.22±0.05	0.26±0.09
acid hydroxylase	(b)							0.38±0.10	0.58±0.15*

I. Lauric acid hydroxylase activity is expressed in nmol hydroxylated lauric acid formed/min/mg protein

II. Values are expressed as mean±SD from 6 rats.

III. Significantly different from control at * $p < 0.05$ or ** $p < 0.01$.

IV. Results of replicate experiments are expressed at (a) and (b) in corresponding columns.

Table 3.10: Effect of NEODENE administration on liver and kidney lauric acid hydroxylase activities

		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE
Liver lauric acid	(a)	0.75±0.19	0.65±0.16	0.88±0.17	1.01±0.37	0.87±0.30	0.49±0.19*	0.53±0.25	0.30±0.07
hydroxylase	(b)	1.01±0.24	0.72±0.18	0.37±0.13	0.40±0.15			0.42±0.03	0.26±0.14*
	(c)			0.38±0.06	0.44±0.08				
Kidney lauric	(a)	0.13±0.04	0.14±0.02	0.20±0.02	0.22±0.05	0.22±0.03	0.20±0.03	0.22±0.05	0.20±0.03
acid hydroxylase	(b)	0.20±0.03	0.19±0.02	0.17±0.04	0.19±0.06			0.38±0.10	0.41±0.15
	(c)			0.12±0.06	0.09±0.05				

I. Lauric acid hydroxylase activity is expressed in nmol hydroxylated laurid acid formed/min/mg protein

II. Values are expressed as mean±SD from 6 rats.

III. Significantly different from control at * $p < 0.05$ or ** $p < 0.01$.

IV. Results of replicate experiments are expressed at (a), (b), and (c) in corresponding columns.

3.8 Effect of IPAR and NEODENE administration on palmitoyl CoA oxidase and carnitine acetyl transferase activities

Both IPAR and NEODENE increased hepatic palmitoyl CoA oxidase activity significantly after 4 doses and this increase was consistent in repeated experiments (Tables 3.11 and 3.12). The degree of induction caused by NEODENE was much higher (92% and 327%) than that caused by IPAR (66% and 75%). However, neither liver nor kidney palmitoyl CoA oxidase activity was changed in the other treated groups.

As for carnitine acetyl transferase, IPAR or NEODENE administration did not displaly any major effect on either liver or kidney in any of the four treated groups. In some treated groups, such as after 4 doses of IPAR in the kidney or after a single dose of NEODENE in the liver and kidney, the difference was significant. However, these changes were not observed in replicate experiments.

Table 3.11: Effect of IPAR administration on liver and kidney peroxisomal palmitoyl CoA oxidase and carnitine acetyl transferase activities

			24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
			CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR
Liver	**Palmitoyl CoA oxidase**	**(a)**	0.89±0.04	1.01±0.40	0.55±0.17	0.52±0.27	0.63±0.26	0.59±0.26	0.62±0.33	1.03±0.15*
		(b)							0.40±0.16	0.70±0.16*
	Carnitine acetyl transferase	**(a)**	13.30±2.37	16.09±0.51*	10.48±0.60	10.23±1.64	13.88±1.90	15.59±2.46	16.95±4.7	25.49±9.0
		(b)							19.91±5.06	19.26±1.86
Kidney	**Palmitoyl CoA oxidase**	**(a)**	0.66±0.24	0.45±0.09	0.59±0.14	0.52±0.11	0.46±0.10	0.52±0.16	0.44±0.17	1.09±0.36*
		(b)							0.41±0.10	0.49±0.19
	Carnitine acetyl transferase	**(a)**	18.23±2.73	19.36±1.91	14.64±1.48	15.69±2.84	13.20±2.86	14.77±2.82	24.38±7.44	34.89±3.01*
		(b)							25.83±4.62	25.59±3.30

I. Values are expressed as mean±SD from 6 rats.

II. Significantly different from control at * $p< 0.05$ or ** $p< 0.01$.

III. Results of replicate experiments are expressed at (a) and (b) in corresponding columns.

IV. Palmitoyl CoA oxidase is expressed as nmol H_2O_2/min/mg protein and carnitine acetyl transferase is expressed as nmol/min/mg protein

Table 3.12: Effect of NEODENE administration on liver and kidney peroxisomal palmitoyl CoA oxidase and carnitine acetyl transferase activities

			24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
			CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE
Liver	**Palmitoyl CoA oxidase**	(a)	0.70±0.27	0.92±0.30	0.32±0.14	0.41±0.10	0.42±0.14	0.46±0.11	0.62±0.33	1.19±0.87*
		(b)	1.11±0.27	0.89±0.37	0.37±0.22	0.96±0.19*			0.40±0.16	1.71±0.23**
		(c)			0.83±0.18	0.97±0.12				
	Carnitine acetyl transferase	(a)	12.33±2.98	10.65±1.82	13.58±2.82	22.62±2.82*	17.52±1.65	17.05±4.79	16.95±4.70	23.10±3.66*
		(b)	11.63±0.83	9.48±0.95*	11.39±1.90	11.15±1.79			19.91±5.06	15.46±2.67
		(c)			14.03±3.40	14.24±1.70				
Kidney	**Palmitoyl CoA oxidase**	(a)	0.47±0.14	0.29±0.21	0.23±0.10	0.24±0.09	0.18±0.05	0.37±0.14	0.44±0.17	0.62±0.11
		(b)	0.44±0.12	0.55±0.07	0.53±0.13	0.37±0.20			0.41±0.10	0.81±0.17*
		(c)			0.60±0.11	0.80±0.17				
	Carnitine acetyl transferase	(a)	11.59±0.99	12.47±1.47	20.70±3.40	24.10±4.70	26.38±3.56	32.50±2.53*	24.38±7.44	23.65±5.15
		(b)	12.27±0.65	13.66±0.53	12.96±1.67	15.12±1.52*			25.83±4.62	20.32±4.15
		(c)			14.45±0.93	15.17±2.30				

I. Values are expressed as mean±SD from 6 rats.

II. Significantly different from control at * $p< 0.05$ or ** $p< 0.01$.

III. Results of replicate experiments are expressed at (a), (b), and (c) in corresponding columns.

IV. Palmitoyl CoA oxidase is expressed as nmol H_2O_2/min/mg protein and carnitine acetyl transferase is expressed as nmol/min/mg protein

3.9 Effect of IPAR and NEODENE administration on glutathione S-transferase (GST) activity, GST Ya and GST Yb protein levels

IPAR administration did not significantly alter hepatic glutathione S-transferase activity with the four test substrates in any of the four treatment groups (Table 3.13).

In contrast, NEODENE administration had varying effects on the liver activity with the four test substrates in the four treated groups (Table 3.14). NEODENE had no significant effect on glutathione S-transferase activity in any of the four treated groups when ethacrynic acid was used as substrate. With CDNB as substrate, a small but significant decrease in hepatic glutathione S-transferase activity was observed in the 1 dose (24 hours) group (21%), the 1dose (72 hours) group (19%) and the 2 dose group (20%). However, this inhibitory effect was not consistent and was seen in only one of two and one of three repeat experiments in the 1 dose (24 hours) and the 1 dose (72 hours) groups respectively. The 2 dose experiment was not repeated. With trans-4-phenyl-3-buten-2-one as substrate, a significant decrease (21% to 42%) was seen only in the 4 dose treated group in both replicate experiments. Finally, with DCNB as substrate, a more consistent decrease was seen in all treated groups except the 1 dose (24 hours) group. The glutathione S-transferase activity was decreased by 13% 24 hours after 1 dose and further decreased by 26% after 72 hours. Following 2 doses, the glutathione S-

transferase activity reached its maximal inhibition (40%). By the end of 4 doses, a 17% to 30% drop in activity was observed.

Control values in replicate experiments were different in the 24 hour (1 dose) and the 12 day (4 doses) groups using ethacrynic acid as substrate and in the 72 hour (1 dose) group using trans-4-phenyl-3-buten-2-one as sustrate. However, similar results were obtained when these activities were measured at the same day under identical condition.

Immunoblotting was performed simultaneously to examine the effect of NEODENE on the levels of Ya and Yb subunits of glutathione S-transferase, which represent the alpha and mu classes of glutathione S-transferase, respectively. As shown in figure 3.4, the level of Ya was unaltered except after 4 doses where about a 24% decrease was observed. NEODENE did not cause any effect on Yb protein in any of the treated groups as shown in figure 3.5. Thus, immunoblots did not reveal a positive correlation between the activity of glutathione S-transferase and levels of Ya and Yb subunits in liver.

In general, neither IPAR nor NEODENE caused any major changes in glutathione S-transferase activities with CDNB and ethacrynic acid as substrates in kidney (Tables 3.15 and 3.16). With the other two substrates: DCNB and trans-4-phenyl-3-buten-2-one, the glutathione S-transferase activity was not detectable in the kidney cytosols.

Table 3.13: Effect of IPAR administration on liver cytosol glutathione S-transferase activities

		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR
CDNB[I]	(a	1.24±0.09	1.19±0.09	1.26±0.09	1.53±0.14*	1.53±0.14	1.48±0.22	1.08±0.05	1.02±0.14
	(b)							1.61±0.35	1.66±0.27
DCNB[II]	(a	59.31±7.43	52.65±2.49	56.85±5.88	62.72±12.90	64.64±11.46	65.57±6.63	60.88±6.35	65.30±3.54
	(b)							60.82±10.92	49.19±4.47
Ethacrynic acid[II]	(a	35.60±7.20	38.0±4.52	53.00±7.01	44.0±4.90*	52.50±13.81	44.75±11.16	31.9±3.34	35.16±3.28
	(b)							65.6±7.47	63.80±4.66
Trans-4-phenyl-3-buten-2-one[II]	(a	13.66±1.94	14.0±2.66	16.73±2.76	17.64±2.11	13.10±2.13	12.55±2.20	12.42±1.47	12.04±3.43
	(b)							23.33±4.82	15.08±2.90*

I. umol/min/mg protein

II. nmol/min/mg protein

III. Values are expressed as mean±SD from 6 rats.

IV. Significantly different from control at * $p < 0.05$ or ** $p < 0.01$.

V. Results of replicate experiments are expressed at (a) and (b) in corresponding columns.

Table 3.14: Effect of NEODENE administration on liver cytosol glutathione S-transferase activities

		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	NEODENE	CONTROL	NEODENE	CONTRO	NEODENE	CONTROL	NEODENE
CDNB[I]	(a)	1.75±0.18	1.39±0.23*	1.22±0.14	1.20±0.09	1.18±0.13	0.94±0.16*	1.08±0.05	0.97±0.23
	(b)	1.23±0.17	1.23±0.17	1.73±0.19	1.40±0.21*			1.61±0.35	1.29±0.19
	(c)			1.25±0.12	1.13±0.25				
DCNB[II]	(a)	70.94±7.01	61.38±4.93*	88.59±14.31	65.85±7.95*	69.06±7.23	41.33±16.2*	60.88±6.35	50.3±4.44*
	(b)	72.34±4.78	78.54±1.16	89.25±10.28	66.05±11.10*			60.82±10.92	42.40±7.86*
	(c)			77.19±10.10	70.88±9.35				
Ethacrynic acid[II]	(a)	53.79±4.02	51.17±2.95	41.15±3.66	45.87±2.70*	42.69±4.6	47.37±4.09	31.9±3.34	54.1±3.09**
	(b)	39.60±2.72	40.50±3.0	45.90±5.50	38.70±5.79			65.60±7.47	65.3±3.54
	(c)			43.44±5.53	46.80±6.57				
Trans-4-phenyl-3-buten-2-one[II]	(a)	14.49±3.34	16.32±2.39	23.09±2.89	18.82±3.69	16.71±4.78	12.76±3.13	12.42±1.47	9.82±2.09*
	(b)	11.61±1.52	12.64±3.12	13.47±2.01	12.92±1.76			23.33±4.82	13.53±3.51*
	(c)			9.37±2.76	7.02±2.33				

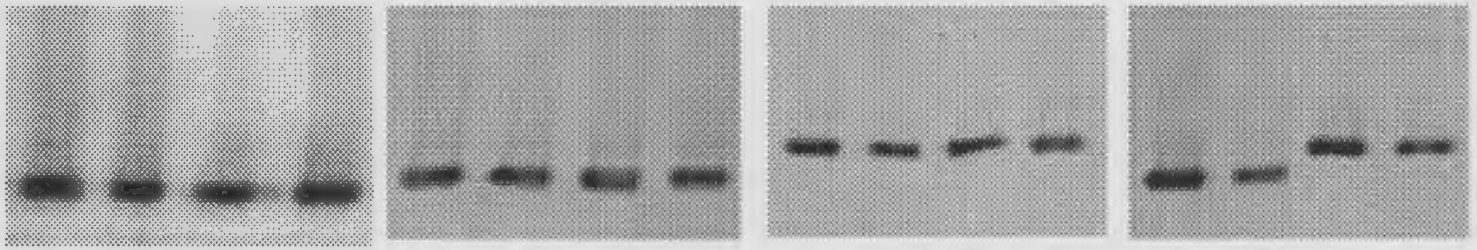
I. umol/min/mg protein

II. nmol/min/mg protein

III. Values are expressed as mean±SD from 6 rats.

IV. Significantly different from control at * $p < 0.05$ or ** $p < 0.01$.

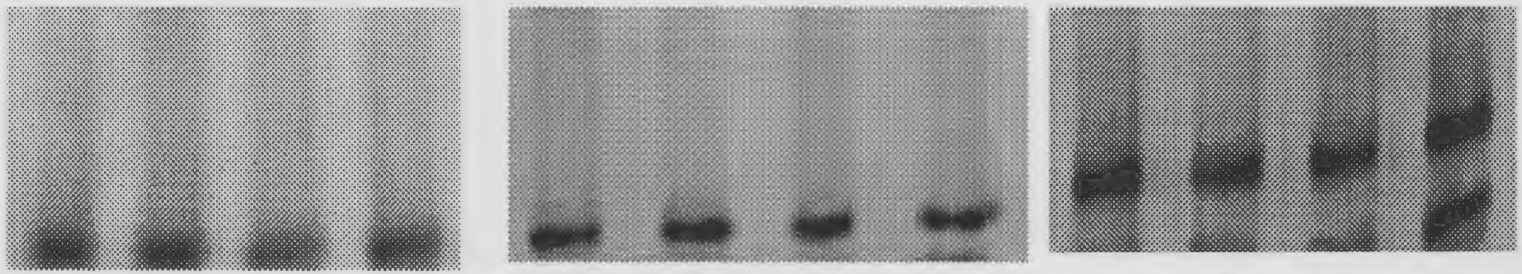
V. Results of replicate experiments are expressed at (a), (b), and (c) in corresponding columns.



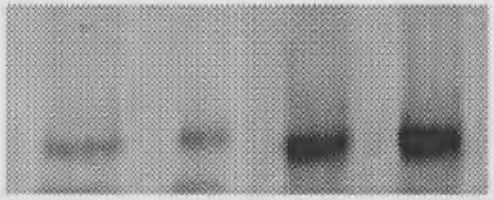
Lane:	1	2	3	4	1	2	3	4	1	2	3	4	1	3	2	4
Grey Level: (reversed)	222	211	216	195	219	220	195	229	248	212	242	196	255	203	255	180
	24 hours (1 dose)				**72 hours (1 dose)**				**6 days (2 doses)**				**12 days (4 doses)**			

*Lane 1, 2: control
*Lane 3, 4: treated

Figure3. 4: Western blots employing anti-GSTYa polyclonal antibodies on liver cytosols after NEODENE administration



Lane:	1	2	3	4	1	2	3	4	1	2	3	4
Grey Level:	203	186	192	255	255	255	255	255	238	209	192	244
(reversed)		**24 hours (1 dose)**				**72 hours (1 dose)**				**6 days (2 doses)**		



Lane:	1	3	2	4
Grey Level:	137	150	255	255
(reversed)		**12 days (4 doses)**		

*Lane 1, 2: control
*Lane 3, 4: treated

Figure 3.5: Western blots employing anti-GSTYb polyclonal antibodies on liver cytosols after NEODENE administration

Table 3.15: Effect of IPAR administration on kidney cytosolic glutathione S-transferase activities

		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR	CONTROL	IPAR
CDNB[I]	(a)	0.34±0.04	0.36±0.03	0.39±0.02	0.44±0.02**	0.35±0.06	0.37±0.08	0.30±0.08	0.29±0.07
	(b)							0.29±0.03	0.33±0.04
Ethacrynic acid[II]	(a)	27.01±7.59	28.08±5.0	29.50±5.00	32.00±5.82	30.0±3.15	28.5±3.15	85.62±8.89	57.38±8.03**
	(b)							44.94±8.78	54.56±7.19

I. umol/min/mg protein

II. nmol/min/mg protein

III. Values are expressed as mean±SD from 6 rats.

IV. Significantly different from control at * $p< 0.05$ or ** $p< 0.01$.

V. Results of replicate experiments are expressed at (a) and (b) in corresponding columns.

Table 3.16: Effect of NEODENE administration on kidney cytosolic glutathione S-transferase activities

		24 hours (1 dose)		72 hours (1 dose)		6 days (2 doses)		12 days (4 doses)	
		CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE	CONTROL	NEODENE
CDNB[I]	(a)	0.27±0.03	0.29±0.03	0.34±0.04	0.38±0.03*	0.34±0.04	0.33±0.05	0.30±0.08	0.27±0.05
	(b)	0.39±0.08	0.31±0.02	0.24±0.03	0.29±0.04*			0.29±0.03	0.32±0.05
	(c)			0.43±0.05	0.46±0.03				
Ethacrynic acid[II]	(a)	29.0±2.45	28.51±3.14	36.77±3.16	42.76±3.98*	36.7±3.64	41.78±4.80	85.62±8.89	79.94±1.88
	(b)	36.98±6.59	33.73±3.81	28.5±2.48	27.01±1.88			44.94±8.78	49.31±4.21
	(c)			35.96±3.53	37.41±2.39				

I. umol/min/mg protein

II. nmol/min/mg protein

III. Values are expressed as mean±SD from 6 rats.

IV. Significantly different from control at * $p< 0.05$ or ** $p< 0.01$.

V. Results of replicate experiments are expressed at (a), (b), and (c) in corresponding columns.

3.10 Effect of IPAR and NEODENE administration on serum cholesterol and triglyceride concentrations

Serum cholesterol and triglyceride concentrations were only determined in the 4 doses administration group. Neither IPAR nor NEODENE had any effect on serum cholesterol or triglyceride levels (Table 3.17).

Table 3.17 Concentrations of serum cholesterol and triglycerides after administration of 4 doses of IPAR or NEODENE

		CONTROL	IPAR	NEODENE
Cholesterol (mg /dL)	**(a)**	123.98±29.58	137.83±32.62	139.87±45.87
	(b)	102.6±14.21	106.1±28.21	87.3±15.44
Triglyceride (mg/dL)	**(a)**	187.02±75.0	166.08±78.97	234.2±45.66
	(b)	97.58±23.10	86.52±27.75	86.08±11.55

I. Values are expressed as mean±SD from 6 rats.
II. Significantly different from control at * $p< 0.05$ or ** $p< 0.01$.
III. Results of replicate experiments are expressed at (a) and (b) in corresponding columns.

C H A P T E R 4

4. Discussion

IPAR and NEODENE caused different effects on biotransforming enzymes in rats. Overall, IPAR had no impact on total cytochrome P450 activity, nor on glutathione S-transferase activity in both liver and kidney samples. However, IPAR specifically induced two isoforms of cytochrome P450: CYP1A1 and CYP 2B1 in liver. This induction is significant and consistent as evidenced by EROD and PROD activities as well as by Western blot analysis of CYP1A1 and CYP 2B1 proteins. Induction of kidney EROD activity was also observed 24 hours after administration of one dose and after two doses. In contrast, NEODENE exerted an inhibiting effect on rat hepatic biotransforming enzymes, but had no effect on renal enzymes. NEODENE not only significantly inhibited total cytochrome P450 levels (30%-50%), but also significantly inhibited the specific cytochrome P450 isoform: CYP1A1 as expressed by EROD activity (20%-45%). Furthermore, glutathione S-transferase as measured by one substrate: DCNB was significantly inhibited (13%-30%) in all four treated groups. Lauric acid hydroxylase, catalyzed by another isoform of cytochrome P450: CYP 4A1, also had a trend to decrease as a result of NEODENE administration though a significant difference was only seen in the 6 day (2 doses) group and one of the 12

day (4 doses) groups. The difference in response to IPAR and NEODENE is most likely due to their different chemical composition. These results thus provide a clue to identify the specific chemicals present in the drilling fluids which are responsible for either induction or inhibition. Since synthetic-based muds are typically based on ester, ether, olefin and paraffin, these chemicals can be either hydrolysed or oxidized by xenobiotic biotransforming enzymes. Therefore, the metabolites associated with different metabolic pathways may also affect the enzyme activity and exert toxic effects. One possible type of metabolite could be olefin epoxides because olefin epoxidation is a widespread reaction catalyzed by cytochrome P450 (Vaz et al., 1998; Wang et al., 1998). Propylene oxide, a aliphatic oxide was found to inactivate human erythrocyte glutathione S-transferase (Ansari et al., 1987). Others such as glycidol and 2-methyl-2,3-epoxybutane are carcinogenic and mutagenic to rats (Irwin et al., 1986; Gollapudi et al., 1995).

Moreover, this different pattern of changes in biotransforming enzymes by IPAR and NEODENE may play a role in their potential toxicity. In the present study, body weight as well as liver and kidney weights were monitored as indices of toxic effects. No weight loss was observed in any of the four treatments after IPAR exposure. Although the inducing responses of IPAR were similar to those obtained after exposure of rats to crude oil and other drilling fluids (Rahimtula et

al., 1985; Khan et al., 1987), this induction was not accompanied with liver enlargement which may be related to hepatotoxicity as observed in many cases (Knasmulle et al., 1997; Khan et al. 1989). Thus, from current data available, it is suggested that IPAR caused no significant sublethal effects in rats either after one dose or after exposure to multiple doses. The inducing property of IPAR could be considered as a protective mechanism which enhances detoxification of lipophilic compounds present in this drilling fluid.

In contrast, a substantial 10% weight loss was observed in three NEODENE treated groups except in the single dose group 24 hours after NEODENE exposure. With increasing time and dose, the weight loss effect due to NEODENE was apparent and persistent. The absolute kidney weight had a trend to decrease after all four treatments. However, because body weight also decreased, kidney weight to body weight ratio remained unchanged. Therefore, NEODENE may cause sublethal effects to rats after exposure for a period of time. This toxic effect is not immediate, but manifests itself in a few days. Since NEODENE inhibited both phase I and phase II metabolic enzyme activities, this inhibition could delay its clearance from the rats which could add to its toxic effects. In the present study, only body weight was used as an indicator of toxic effects; more work is needed to determine if there are other toxic effects.

IPAR had a preference to induce the CYP 2B isoform because the induction of PROD activity was much higher than the induction of EROD activity. This induction was similar to the effect caused by phenobarbital which was found to cause a marked (>20-fold) induction of CYP 2B and a 2-4-fold increase in the levels of CYP1A (Parkinson, 1996). The CYP1A1 protein induction is well correlated to its associated catalytic activity, EROD. It is suggested that the increased EROD activity is possibly due to the increased synthesis of CYP 1A protein. However, the increase in CYP2B protein was not as much as the induction of its associated PROD activity. Therefore, the induction of PROD activity may not be solely due to increased CYP2B protein synthesis, other mechanisms may also exist. One possibility is that cytochrome P450 isoforms other than CYP2B are also induced by IPAR which may be involved in catalyzing PROD activity. In a study of PCB contamination in polar bears (Letcher et al., 1996), similar results were obtained, i.e., induction of EROD activity correlated strongly with CYP 1A protein induction, while the induction of PROD activity did not correspond to the induction of CYP2B protein. The authors further pointed out that PROD activity correlated well with CYP 1A protein. With additional immunoinhibition assays, PROD activity was decreased about 60% employing anti CYP 1A1 IgG. They concluded that CYP 1A also played a role in the induction of PROD activity. It can be extrapolated from this study that

overlapping substrate specificity of CYP 2B may exist. However, in the case of my study, this possibility needs to be confirmed by immunoinhition studies of CYP2B and other cytochrome P450 enzyme assays.

It is interesting that NEODENE caused EROD activity to decrease significantly, while the CYP 1A1 protein content was only slightly increased or unaltered as shown by Western blot analysis. This lack of correlation between enzyme activity and enzyme content may be due to the mechanism of inhibition. As opposed to the well known mechanism of CYP 1A induction, which involves transcriptional activation of the CYP1A gene resulting in an increase in the levels of mRNA and newly synthesized CYP1A protein, the inhibition of CYP 1A can occur at any step of the catalytic cycle of cytochrome P450, such as competition at the substrate binding site (Paul and Maria, 1989) and interaction with the haem moiety of the cytochrome to prevent oxygenation of the substrates (Rodrigues et al., 1987). Therefore, CYP1A protein synthesis may not be affected even though the CYP1A enzyme activity is inhibited. A slight increase of CYP1A protein content may be considered as an adjustable response of the organism for the compensation of CYP1A enzyme activity loss.

Subunits Ya and Yb of glutathione S-transferase were analyzed by Western blot analysis as well as glutathione S-transferase activity. Although glutathione S-transferase activity was decreased with several substrates, Ya and

Yb protein content was not decreased except in the 12 day (4 doses) group in which Ya protein was decreased by about 30%. The decrease in Ya may be responsible for the significant inhibitory response seen with the two substrates: DCNB and trans-4-phenyl-3-buten-2-one. Since glutathione S-transferases exhibit broad, overlapping substrate specificities, neither Ya nor Yb could alone be responsible for the inhibition of glutathione S-transferase activity as measured by the different substrates.

The 72 hour time point after administration seems to be critical for both IPAR and NEODENE. In the case of IPAR, EROD activity increased at 24 hours and returned to normal by 72 hours. The EROD activity also increased with additional doses. The induction of CYP1A protein strongly correlated with the changes observed in EROD activity. Although the increased PROD activity did not return to normal by 72 hours, the increased level of PROD activity declined to 230% after it reached a maximal induction of 775% at 24 hours after administration. The alteration in EROD activity was also observed in the 72 hour NEODENE treated group. Although EROD activity was significantly decreased after 24 hours, by 72 hours after administration, EROD activity was either increased, in some cases substantially, or decreased. Moreover, CYP1A1 protein content was either elevated or decreased in keeping with the changes in EROD activity. Brady et al. (1991) also indicated a similar result. The induction of

EROD enzyme activity reached to a maximal level at about 18-24 hours and returned to normal by 72 hours. After administration, components of IPAR and NEODENE can be expected to interact with cells in the liver, a major metabolic site, and trigger perturbations in cell function such as induction or inhibition of cytochrome P450 enzyme activities as were seen in this study. The early alteration in enzyme activity thus could trigger homeostatic mechanisms that may account for the complicated patterns of enzyme activity by 72 hours. However, after additional exposure, the perturbations continue and enzyme activities do not return to normal.

Khan et al. (1989) found that crude induced peroxisome proliferation which was thought to play a role in its carcinogenic / tumorgenic potential. Therefore, the possibility of peroxisome proliferation induced by IPAR and NEODNE was also investigated in this study. Peroxisome proliferation has been observed in mammalian liver by many chemicals. It is characterized by increased activity of several peroxisomal enzymes which are involved in the β oxidation of fatty acids and in an increased activity of lauric acid ω-hydroxylase, which is catalyzed by cytochrome P450 4A1 and plays a role in the metabolism of long-chain fatty acids (Sharma et al., 1987; Sabzevari et al., 1994; Bentley et al., 1993). Furthermore, peroxisome proliferation is also associated with a decrease in serum cholesterol and/or triglycerides since peroxisomes are involved in lipid

metabolism (Moody et al., 1983). Therefore, two peroxisomal enzymes: palmitoyl CoA oxidase and carnitine transferase, which are biomarkers of peroxisome proliferation (Demoz et al., 1994; Khan et al., 1989), as well as microsomal lauric acid hydroxylase and serum cholesterol and tryglycerides were all measured in my study. Both palmitoyl CoA oxidase and carnitine transferase were only induced about 50% in liver microsomes after 4 doses of IPAR or NEODENE. However, neither lauric acid hydroxylase nor serum cholesterol and triglyceride levels changed in this 4 dose treated group. No changes in these enzyme activities were observed in either the one dose group or in the two dose group. Thus, the peroxisome proliferating property of either IPAR or NEODENE is not clear. Since peroxisomal enzyme activities are induced after four doses of treatment, it could be implied from these data that both IPAR and NEODENE may exert their effects on fatty acid metabolism because lipophilic chemicals present in them become more abundant in the body at that time point. The peroxisomal enzyme activity is much more induced by NEODENE than it is by IPAR. This may be due to the delay in the elimination of lipophilic chemicals because of the inhibiting property of NEODENE on the biotransforming enzymes. Alternately or additionally, NEODENE could contain chemicals that selectively induce peroxisomal enzyme activity.

C H A P T E R 5

5. Conclusion

1. IPAR administration did not cause weight loss while NEODENE administration caused a significant weight loss in rats in every treatment group.
2. Significant changes in hepatic enzyme activities but not in renal enzyme activities suggest that liver is the major effected site by both IPAR and NEODENE.
3. Cytochrome P450 enzyme activities as well as their associated protein levels were either increased or decreased by IPAR or NEODENE. However, the rate of alteration was not dose-dependent. Therefore, these endpoints may not be used as biomarkers of drilling fluid exposure.
4. NEODENE is a potential inhibitor of cytochrome P450 1A enzymes and glutathione S transferases which may in part account for its toxic effects.
5. Following IPAR or NEODENE administration, peroxisomal enzyme activities, lauric acid hydroxylase activity and serum lipid level were not changed coordinately. Thus, neither IPAR nor NEODENE is likely to cause peroxisomal proliferation.
6. Overall, it appears that both IPAR and NEODENE exhibit low toxicity.

C H A P T E R 6

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