THE TOXICITY OF PRUDHOE BAY CRUDE OIL IN ERYTHROCYTES AND CHICK EMBRYOS

PAUL ALEXANDER JOSEPH WALTERS

THE TOXICITY OF PRUDHOE BAY CRUDE OIL IN ERYTHROCYTES AND CHICK EMBRYOS

by

© Paul Alexander Joseph Walters, B.Sc. (Honours)

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

Toxicology

Memorial University of Newfoundland
March 1987

St. John's Newfoundland

ISBN 0-315-36986-8

ABSTRACT

When it was incubated with herring gull or human erythrocytes Prudhoe Bay crude oil (PBCO) was found to induce methemoglobin formation, hemolysis and glutathione depletion. In the presence of a metabolic activation system such as rat liver microsomes plus NADPH, these effects were greatly enhanced.

Components of crude oil such as naphthalene and methylated naphthalenes induced methemoglobin formation *in vitro* in erythrocytes only when liver microsomes and NADPH were present in the incubation medium. However, naphthalene metabolites such as 1,2- and 1,4-naphthoquinone, 1,2-, and 1,4-dihydroxynaphthalene and 1-naphthol required no metabolic activation to produce toxic effects. In these studies naphthalene was used as a model to investigate the mechanism of PBCO toxicity in erythrocytes.

The aliphatic, aromatic and heterocyclic fractions of Prudhoe Bay crude oil were tested on the developing chick embryo for toxicity (in terms of mortality) and influence on cytochrome P-450 and aryl hydrocarbon hydroxylase induction. Induction of these enzymes by the fractions of crude oil was studied in the liver, kidney and lung. The aromatic fraction was found to be responsible for most of the embryo toxicity and enzyme inducing ability, based on its concentration in

PBCO. Although the heterocyclic fraction was less than 7% (w/v) of PBCO, on a weight equivalent basis, it was approximately as potent as the aromatic fraction in causing embryo toxicity and inducing increases in levels of cytochrome P-450 and aryl hydrocarbon hydroxylase. The aliphatic fraction had no toxic or inductive effects. These results suggest that embryo toxicity may be due to the metabolism of aromatic compounds to more toxic derivatives by aryl hydrocarbon hydroxylase.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my supervisor, Dr. A.D. Rahimtula for his advice, support and patience during the last couple of years. Also I wish to thank Marie Martin and Dr. S. Khan for technical assistance and helping to make bench work a more enjoyable experience.

The assistance of Dr. K. Keough and Dr. G. Herzberg in the preparation and the correction of this thesis is gratefully appreciated.

Special thanks is given to Dr. S. Macko of the Earth Science Department for devoting a considerable amount of time and effort towards the GC-Mass spectral studies performed on the PBCO and its fractions. The High Performance gas chromatograph and the mass selective detector were funded by NSERC equipment grants E5678 and E5779 respectively.

Acknowledgement is made to the Donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research.

Finally, I wish to express my gratitude for the financial assistance obtained from the Memorial University Bursary and to National Sciences and Engineering Research Council of Canada for the research project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AHH	aryl hydrocarbon hydroxylase
BPH	benzo[a]pyrene hydroxylase
DMSO	dimethyl sulfoxide
EROD	7-ethoxyresorufin-O-deethylase
GSH	glutathione (reduced)
GSSG	glutathione (oxidized)
Hb	hemoglobin
HbO_2	oxygenated hemoglobin
NADP$^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NMR	nuclear magnetic resonance
NOS	nitrogen, oxygen, sulfur
P450	cytochrome P-450
PAH	polycyclic aromatic hydrocarbon
PBCO	Prudhoe Bay crude oil
PBS	phosphate buffered saline
PCV	packed cell volume
RBC	red blood cell

CHAPTER 1

INTRODUCTION

1.1 Petroleum

1.1.1 The effect of oil on birds

1.1.1.1 Introduction

The death of sea birds from oil pollution receives a lot of publicity. In addition, because of its visual impact, oiling produces an emotional reaction stronger than does death through other pollutants. Because of this, much research has been performed on the impact of oil on individual birds, populations and ecosystems (Vermeer and Vermeer, 1975; Bourne, 1976; Holmes and Cronshaw, 1977). The following is a brief summary of some of the effects of oil on individual birds.

1.1.1.2 Biochemical and physiological effects

The direct effect of oil on a bird is to disrupt the feathers, which are responsible for maintaining water-repellance and heat insulation (Holmes and Cronshaw, 1977). The loss of this insulation increases metabolic activity to maintain body temperature (Hartung, 1967). Mortality can

result from rapid exhaustion of fat and muscular energy reserves.

Birds can also ingest oil by preening their oiled feathers (Hartung, 1963), or by eating contaminated food. Many physiological changes have been reported in studies involving ingested oil. Osmoregulator and hormone changes have been found (Holmes, 1975; Peakall et al., 1981), also impairment of weight gain of young birds (Miller et al., 1978), induction of hepatic enzymes (Gorsline et al., 1981), and other pathological effects (Holmes et al., 1978). The egg laying frequency and hatching success of eggs are known to be impaired by relatively small amounts of ingested oil (Ainley et al., 1981). Small quantities of oil or oil products, when applied to the surface of eggs, are known to kill the embryo at certain stages of development in the laboratory (see Introduction 4.1) and in the field (Birkhead et al., 1973).

1.1.2 Composition of crude oils

The composition of petroleum crude oil has been discussed in great detail by Tissot and Welt (1984) and Hunt (1979), and all information given in this section is based on these two sources unless stated otherwise.

The chemical composition of crude oil from different regions and even from a particular formation varies extensively. Hydrocarbons are the most abundant compounds in crude oils, accounting for 50-98% of the total composition (Clark and Brown, 1977). Most crude oils contain the higher relative amounts of hydrocarbons. The elemental composition consists mostly of carbon (80-87%) and hydrogen (10-15%). Sulfur (0-10%), nitrogen (0-1%) and oxygen (0-5%) are important minor elements and are present as elemental sulfur or as heterocyclic constituents and functional groups. The NOS compounds are compounds which contain the elements N, O and S. Trace metals such as V, Ni, Fe, Al, Na, Ca, Cu and U also exist in crude oil.

Table 1.1 presents examples of the composition of three crude oils (National Research Council, 1985). A discussion of the composition of crude oils will not be presented because they contain thousands of different chemical compounds due to "molecular scrambling" during formation. However, it is important to note that of the three crude oils presented in table 1.1, PBCO has the highest aromatic content. Naphthalene was chosen as a model to study the biochemical responses of red blood cells when exposed to PBCO because naphthalene and its derivatives comprise about 10% of the composition of PBCO (see table 1.1).

Table 1.1. The composition of three crude oils

Component	Crude Oil		
	Prudhoe Bay	South Louisiana	Kuwait
Sulfur (wt %)	0.94	0.25	2.44
Nitrogen (wt %)	0.23	0.69	0.14
Nickel (ppm)	10	2.2	7.7
Vanadium (ppm)	20	1.9	28
Naphtha fraction (wt %)	23.2	18.6	22.7
Paraffins	12.5	8.8	16.2
Naphthenes	7.4	7.7	4.1
Aromatics	3.2	2.1	2.4
Benzenes	0.3	0.2	0.1
Toluene	0.6	0.4	0.4
C_8 aromatics	0.5	0.7	0.8
C_9 aromatics	0.06	0.5	0.6
C_{10} aromatics	--	0.2	0.3
C_{11} aromatics	--	0.1	0.1
Indans	--	--	0.1
High-boiling fraction (wt %)	76.8	81.4	77.3
Saturates	14.4	56.3	34.0
n-paraffins	5.8	5.2	4.7
C_{11}	0.12	0.06	0.12
C_{12}	0.25	0.24	0.28
C_{13}	0.42	0.41	0.38
C_{14}	0.50	0.56	0.44
C_{15}	0.44	0.54	0.43
C_{16}	0.50	0.58	0.45
C_{17}	0.51	0.59	0.41
C_{18}	0.47	0.40	0.35
C_{19}	0.43	0.38	0.33
C_{20}	0.37	0.28	0.25
C_{21}	0.32	0.20	0.20
C_{22}	0.24	0.15	0.17
C_{23}	0.21	0.16	0.15
C_{24}	0.20	0.13	0.12
C_{25}	0.17	0.12	0.10
C_{26}	0.15	0.09	0.09
C_{27}	0.10	0.06	0.06
C_{28}	0.09	0.05	0.06
C_{29}	0.08	0.05	0.05
C_{30}	0.08	0.04	0.07
C_{31}	0.08	0.04	0.06
C_{32} plus	0.07	0	0.06
Isoparaffins	--	14.0	13.2
1-ring cycloparaffins	9.9	12.4	6.2

TABLE 1.1 (continued)

Component	Crude Oil		
	Prudhoe Bay	South Louisiana	Kuwait
2-ring cycloparaffins	7.7	9.4	4.5
3-ring cycloparaffins	5.5	6.8	3.3
4-ring cycloparaffins	5.4	4.8	1.8
5-ring cycloparaffins	--	3.2	0.4
6-ring cycloparaffins	--	1.1	--
Aromatics (wt %)	25.0	16.5	21.9
Benzenes	7.0	3.9	4.8
Indans and tetralins	--	2.4	2.2
Dinaphthenobenzenes	--	2.9	2.0
Naphthalenes	9.9	1.3	0.7
Acenaphthenes	--	1.4	0.9
Phenanthrenes	3.1	0.9	0.3
Acenaphthalenes	--	2.8	1.5
Pyrenes	1.5	--	--
Chrysenes	--	--	0.2
Benzothiophenes	1.7	0.5	5.4
Dibenzothiophenes	1.3	0.4	3.3
Indanothiophenes	--	--	0.6
Polar materials (wt %)	2.9	8.4	17.9
Insolubles	1.2	0.2	3.5

NOTE: These analyses represent values for one typical crude oil from each of the geographical regions; variations in composition can be expected for oils produced from different formations or fields within each region (National Research Council, 1985).

1.2 Naphthalene metabolism

Pathways of naphthalene metabolism have been extensively studied *in vivo* and *in vitro*. A general metabolic pathway is presented in Figure 1.1. Jerina et al. (1970) provided direct evidence for the formation of 1,2-naphthalene oxide as "the obligatory intermediate" in the formation of all *in vitro* naphthalene metabolites. Although the 1,2-naphthalene oxide has not been detected *in vivo*; evidence for its formation has been demonstrated in rats by using precursor-product relationships (Horning et al., 1980). The enzyme responsible for the above conversion is a cytochrome P-450 dependent monoxygenase. Once this highly reactive intermediate is formed, it can react with reduced glutathione to form the conjugate S-(1,2-dihydro-2-hydroxy-1-naphthyl) glutathione. 1,2-Naphthalene oxide can also be converted by microsomal epoxide hydrolase to trans-1,2-dihydro-1,2-dihydroxynaphthalene or it can rearrange nonenzymatically to 1-naphthol and 2-naphthol by the NIH shift. Experiments by Jerina et al (1970) demonstrated that rearrangement of naphthalene oxide to 1-naphthol is predominant over formation of 2-naphthol (88-98% versus 2-12% respectively).

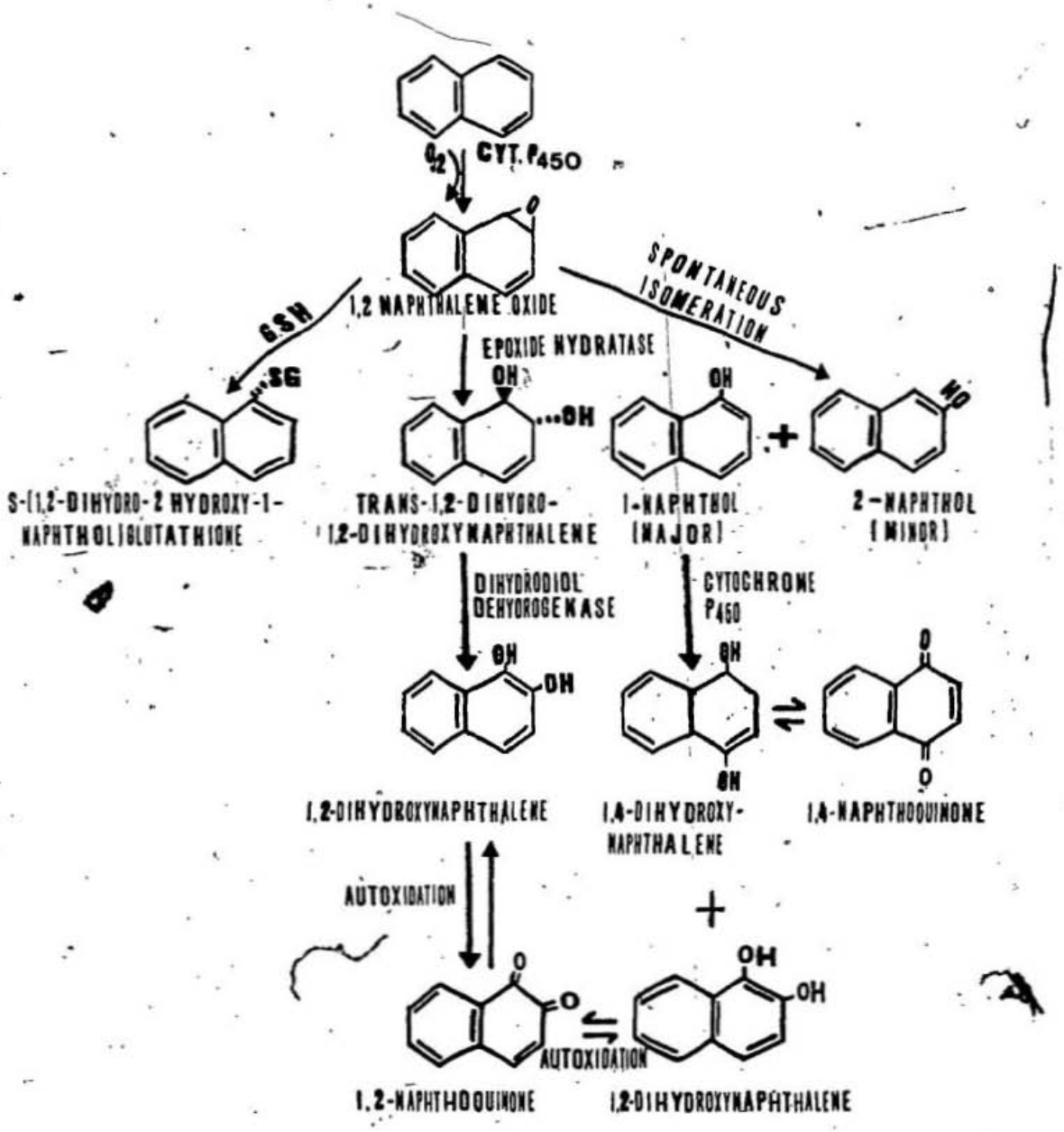


Figure 1.1: In vitro and in vivo metabolism of naphthalene

Although the primary metabolism of naphthalene involves the formation of trans-1,2-dihydro-1,2-dihydroxynaphthalene, 1-naphthol, and the glutathione conjugate of naphthalene, secondary metabolism of the first two compounds is responsible for producing toxic metabolites. Naphthalene dihydrodiol is converted to 1,2-dihydroxynaphthalene via oxidation by the cytosolic enzyme dihydrodiol dehydrogenase (Billings, 1985). This enzyme is present in liver homogenates and has been purified to apparent homogeneity by Vogel et al (1980). Billings (1985) reported that this may be the major route to 1,2 dihydroxynaphthalene. Autoxidation of 1,2-dihydroxynaphthalene to 1,2-naphthoquinone is quite rapid at physiological pH, and has been demonstrated by Van Heyningen and Pirie (1967).

1-Naphthol, another major product of naphthalene metabolism can be further metabolized to 1,2-naphthoquinone and 1,4-naphthoquinone (Doherty and Cohen, 1984) by microsomal systems or hepatocytes. It is believed (Doherty and Cohen, 1984) the quinones were formed by the autoxidation of 1,2-dihydroxynaphthalene and 1,4-dihydroxynaphthalene produced by the cytochrome P-450 dependent hydroxylation of 1-naphthol in the 2 or 4 position.

Although two pathways by which quinones can be generated via naphthalene metabolism has been discussed, it must be pointed out that over 30 metabolites have been detected

in rats. Horning et al (1980) demonstrated additional epoxides including dihydrodiol epoxides, diepoxides, variously substituted di-, tri-, and tetra-hydroxynaphthalenes and O-methylcatechols. Along with the other metabolites, glucuronide and sulfate conjugates of hydroxylated naphthalenes have been demonstrated in isolated hepatocytes and in liver microsomes (in the presence of uridine-5'-diphospho-α-D-glucuronic acid (UDPGA) and N-acetylglucosamine), (Bock et al., 1976).

1.3 Human erythrocyte toxicology

1.3.1 Pathophysiology

Erythrocytes are susceptible to peroxidation. The outer plasma membrane is rich in polyunsaturated fatty acids. The cells are continuously exposed to high oxygen tensions, and contain hemoglobin, one of the most powerful catalysts for the initiation of peroxidative reactions. Hemoglobin can undergo autoxidation in the presence of oxygen with the resultant generation of superoxide radicals. The mechanism as discussed by Carrell et al (1975) involves the polarization of an electron from the heme iron to the bound oxygen in oxyhemoglobin (Figure 1.2). Normally this shared electron is returned to the iron when oxygen is

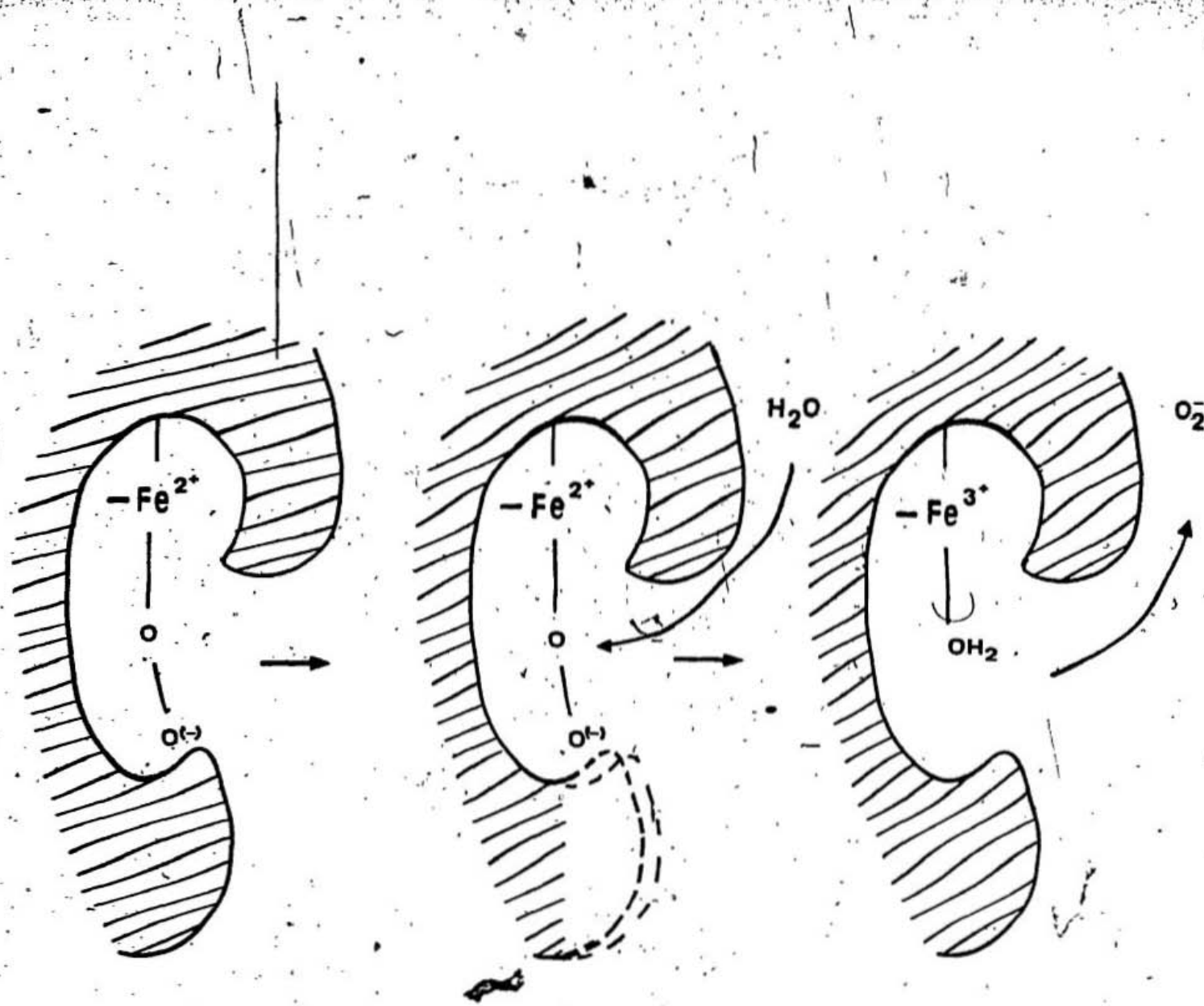
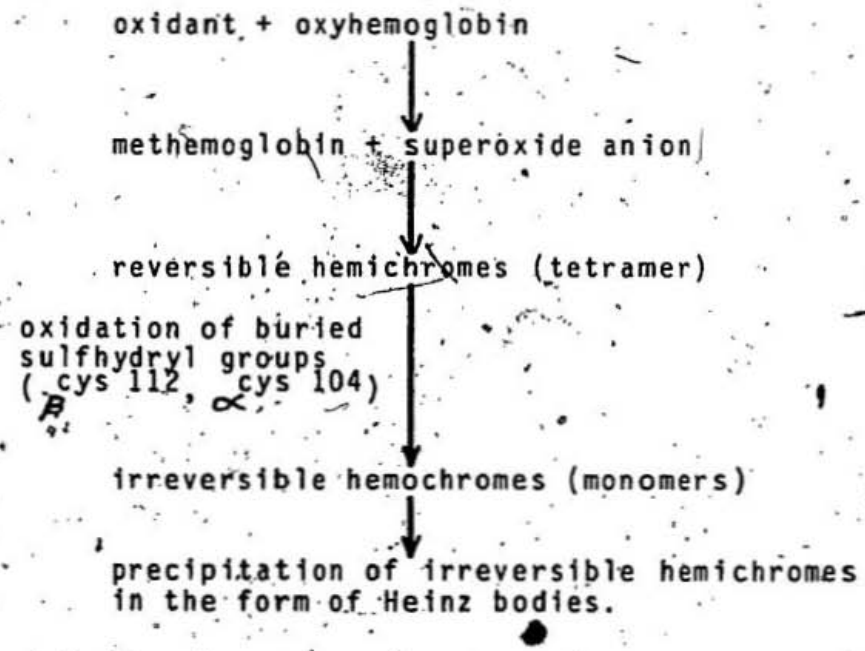


Figure 1.2: Autoxidation of oxygenated hemoglobin

released from hemoglobin, and the iron retains its ferrous (Fe^{2+}) state. The presence of displacing anions in the heme pocket, however, can interfere with this process. Although the heme pocket is hydrophobic, random fluctuations in the surrounding globin may allow the entry of water from time to time. The entry of water or other small anions will result in the displacement of oxygen with an extra electron, ie. superoxide. In the process, the heme iron loses an electron, resulting in the formation of ferric (Fe^{3+}) methemoglobin. This process is believed (Carrell et al., 1975) to explain the observation that 3% of the total body hemoglobin is converted to methemoglobin each day. This methemoglobin is rapidly reduced by methemoglobin reductase which is linked, through its requirement for NADH, to the glycolytic pathway. Glycolysis is the major pathway for NAD^+ reduction to NADH in the human erythrocyte. Oxidant drugs are also known to produce methemoglobin. Oxidant drugs are classified as chemicals which can oxidize hemoglobin *in vivo* and(or) *in vitro* (Bunn and Forget, 1986). Chemicals such as ferricyanide and hydrogen peroxide can oxidize ferrous hemoglobin directly because of their higher redox potential. In contrast, other agents such as nitrites and arylhydroxylamines can produce methemoglobin indirectly by reducing oxygen to superoxide and hydrogen peroxide.

Oxidative damage to erythrocytes has been studied mostly with respect to hemoglobin denaturation and membrane destruction. Oxidative damage to hemoglobin has been shown to cause changes in hemoglobin structure and function. These changes can result in hemoglobin denaturation and precipitation in red cells as Heinz bodies. Based on studies (in vitro) the proposed mechanism of Heinz body formation induced by oxidative stress can be represented as follows.



Studies (in vitro) with phenylhydrazine and dapsone have helped to clarify the mechanism for Heinz body formation by oxidative insult. Cohen and Hochstein (1964) demonstrated that H_2O_2 is generated when the oxidant drug interacts with hemoglobin. The reaction of H_2O_2 with hemoglobin leads to

the formation of methemoglobin and concomitant production of the superoxide radical (Weaver et al., 1973). The superoxide radical that is generated can be broken down (dismutated) to form O_2 and hydrogen peroxide by the action of superoxide dismutase. Some drugs (e.g. phenylhydrazine, dialuric acid) are capable of forming free radicals that can oxidize GSH with formation of the superoxide radical as an intermediate (Kosower et al., 1969).

The sulfhydryl groups in hemoglobin can react with glutathione (Garel et al., 1986). The oxidative attack of GSH on the $\beta^{cys\ 93}$ sulfhydryl group appears to precede attack on other sulfhydryl groups of hemoglobin. The results of Birchmeier et al (1973) indicate that this may lead to denaturation of hemoglobin. Rachmilewitz et al (1974) reported that the mixed disulfide causes instability in the hemoglobin molecule, producing conformational changes that expose interior sulfhydryl groups, enhancing the dissociation of the tetramer into monomers (Rachmilewitz et al., 1974). This observation may explain the formation of Heinz bodies (Rachmilewitz et al., 1974). Although Heinz bodies are formed within the red cell, they may coalesce and migrate towards the membrane where they become attached.

The relationship between hemolysis (in vivo) and Heinz bodies is that the latter may reduce the deformability of the cell, leading to early reticuloendothelial entrapment in

the spleen (Rifkind, 1965) or increased membrane permeability resulting in osmotic damage (Jacob et al., 1968).

It is well known that the lipids comprising the red cell plasma membrane are very susceptible to direct attack by oxidants. Jacob and Lux (1968) observed that phosphatidylethanolamine (PE) is lost before lysis. This is not surprising considering that PE contains a high concentration of polyunsaturated fatty acids, which are liable to autoxidation. Depletion of PE in the red cell membranes after peroxidation may result from fatty acid destruction. It was suggested by Jacob and Lux (1968) that injured cells had holes in the membranes of approximately 70 Å, resulting in hemolysis. It has also been demonstrated that lipid peroxidaton was followed by increased membrane rigidity (Dobretsov et al., 1977).

Proteins located in or on the red cell membrane are also targets for free radical attack. In particular, the membrane structural protein, spectrin is susceptible because it has exposed sulfhydryls. It was demonstrated by Haest et al (1977) that in intact human erythrocytes, SH-oxidizing agents cross-linked spectrin via disulfide bonds.

1.3.2 Protective systems against peroxidation

The following discussion has been outlined in Figure 1.3.

1.3.2.1 Superoxide dismutase - Superoxide dismutase (SOD) is the first line of defense against the superoxide radical. SOD catalyses the disproportion or dismutation of superoxide by the following equation.

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

It has been proposed by Lynch et al (1977) that a major function of this enzyme in the red cell is to prevent the formation of methemoglobin. The greatest danger of O_2^- in the red cell is probably its ability to form hydroxyl radicals (Thomas et al., 1978) which can attack the red cell membrane and cause hemolysis.

1.3.2.2 Glutathione peroxidase - Glutathione Peroxidase (GSH-PX) catalyses the breakdown of organic hydroperoxides (ROOH) and hydrogen peroxide by the following equation (Little and O'Brien, 1968).

$$ROOH + 2GSH \xrightarrow{GSH\text{-}PX} GSSH + ROH + H_2O$$

$$HOOH + 2GSH \xrightarrow{GSH\text{-}PX} GSSG + 2H_2O$$

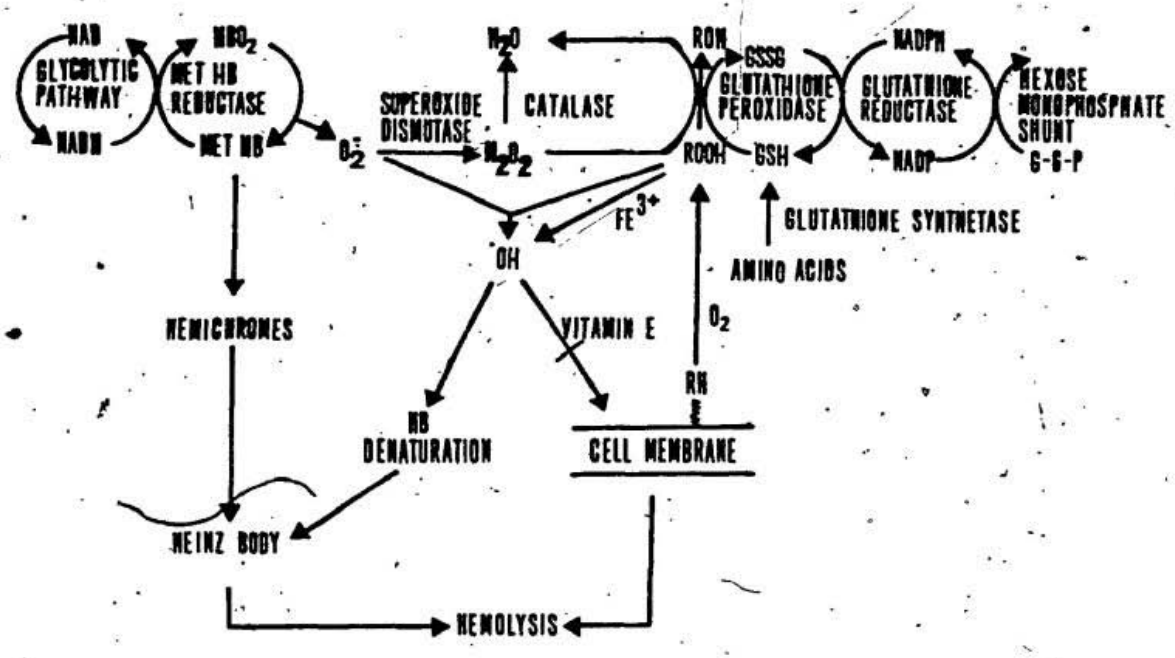


Figure 1.3: Protective systems against peroxidation

The catalytic site of human erythrocyte GSH-PX (Perona et al., 1978) is similar to rat liver GSH-PX in that it contains selenocysteine (Forstrom et al., 1978). This enzyme can protect HbO_2 from oxidative damage due to breakdown of H_2O_2 and RO_2H.

The action of GSH-PX on hydroperoxides is dependent on the level of GSH, which is maintained by de novo synthesis of GSH and NADPH conversion of GSSG to GSH by glutathione reductase. In the red cell, the only way to reduce $NADP^+$ is by oxidation of glucose via the hexose monophosphate shunt.

1.3.2.3 Catalase - The only known function of this enzyme is the removal of H_2O_2 from the cell via the following reaction.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Catalase has a low affinity for H_2O_2 (Cohen and Hochstein, 1963). At a high glutathione and relatively low peroxide concentration, glutathione peroxidase decomposes H_2O_2 faster than catalase. But at high H_2O_2 concentration, catalase removes H_2O_2 at a faster rate. Catalase has a much higher Km for H_2O_2 than GSH-peroxidase (Nicholls, 1972). Therefore, both catalase and GSH-PX play a role in protection of the red cell against H_2O_2.

Catalase is a conjugated protein with protohematin as its prosthetic group. However, catalase differs from all

known hematin derivatives in that it cannot be reduced even by such powerful reducing agents as sodium hyposulphite ($Na_2S_2O_4$). The breakdown of hydrogen peroxide occurs when the iron is in the ferric form. Sodium azide was used as an inhibitor of catalase because it forms an azide-catalase complex. The ferric iron of this complex can be reduced to the ferrous form by H_2O_2, rendering the catalase inactive (Keilin and Hartree, 1945).

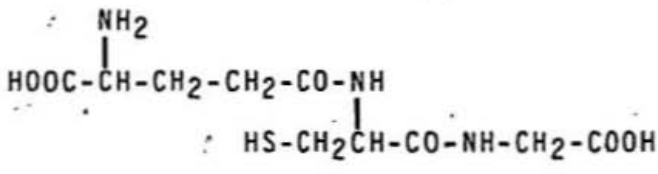
1.3.2.4 Vitamin E.- The major role of Vitamin E in human red cells is probably as a biological antioxidant protecting red cell membranes from peroxidative damage.

Oski and Barness (1967) demonstrated a relationship between vitamin E deficiency and hemolytic anemia in premature infants. Further studies by this group indicated that premature infants whose diet was supplemented with vitamin E had significantly higher hemoglobin concentrations, lower erythrocyte hydrogen peroxide-hemolysis values and lower reticulocyte counts than a corresponding group of non-vitamin E-supplemented infants.

The mechanism of action of vitamin E (α-Tocopherol) involves donation of its hydroxyl hydrogen atom to peroxy free radicals resulting in the formation of hydroperoxides. These hydroperoxides can be decomposed to the corresponding non-toxic hydroxy compounds by glutathione peroxidase.

There is evidence by Packer et al (1979) that the resulting vitamin E radical then reacts with ascorbic acid (vitamin C) to regenerate vitamin E. The vitamin C radical is in turn enzymatically reduced back to vitamin C by NADH-dependent systems. One question which remains to be answered, however; is how does the membrane bound vitamin E interact with Vitamin C which is located in the cytosol.

1.3.2.5 Glutathione - Although this is the last protective system to be discussed, it is extremely important in cellular protection. Glutathione is a tripeptide of glutamic acid, cysteine, and glycine with the following structure.



γ-glutamylcysteinylglycine.

It is readily oxidized by a variety of substances to give a disulfide. With molecular oxygen, iodine, $Fe(CN)_6^{3-}$, H_2O_2 or organoperoxides it forms the dimer GSSG. Reactions with other thiol compounds give rise to mixed disulfides. The reaction with molecular oxygen is slow in the absence of metal ions but greatly accelerated by the presence of copper or iron.

The de novo synthesis of GSH enables human red cells to maintain a steady state concentration of GSH of about 70mg per dl of cells or 2.2 mM. Neither GSH or GSSG can enter red cells from the extra cellular fluid (Srivastava and Beutler, 1969), and neither has been detected in normal plasma. Intracellular GSH cannot leave red cells but GSSG can, in an energy dependent reaction when the dimer is present at a high concentration (Srivastava and Beutler, 1967). GSH is in a dynamic state of turnover with a half-life of about three days (Dimant et al., 1955).

The roles of GSH in the red cell are varied. It is known to protect S-H groups in enzymes and act as a radical scavenger. GSH is also a co-factor in the glyoxalase reaction. Normal human red cells contain a high activity of glyoxalase but its purpose has not been determined yet. The enzyme acts in a two stage reaction to convert ketoaldehydes like methylglyoxal into hydroxyacids like lactic acid (Valentine and Tanaka, 1961). Methylglyoxal itself can be formed from dihydroxyacetone and glycerol and is considered to be toxic to the cell.

GSH is also known to detoxify foreign compounds by conjugating with them non-enzymatically and(or) enzymatically via GSH S-transferases. Acetylation of the conjugate occurs after removal of glycine and glutamate to yield mercapturic acids. Another function of GSH is as a firmly

bound prosthetic group on glyceraldehyde 3-phosphate dehydrogenase (Krimsky and Racker, 1952).

1.4 The effect of oil on the chick embryo

There are numerous studies which show that eggs of various species of birds that have been contaminated by crude oil or refined oils have a low hatchability. (Rittinghaus, 1956; Hartung, 1965; Kopishke, 1972; Hoffman, 1978; Hoffman, 1979a; Hoffman and Gay, 1981; Ellenton, 1982; Lee et al., 1986). The embryotoxicity of crude oils is directly correlated with the aromatic hydrocarbon content of the oil (Hoffman, 1979a; Hoffman and Gay, 1981; Ellenton, 1982). Crude oil was found to be most toxic when applied to the embryo during the earlier stages of development. Hoffman (1979b) demonstrated that when oil was applied on day 1 of incubation, maximum toxicity occurred 6 to 9 days later. Hamilton et al (1983) demonstrated that the embryo can exhibit differential response to chemicals by either an increase in basal enzymes levels or an increased inducibility of xenobiotic metabolizing enzymes. It has been previously shown that crude oils are very effective inducers of cutaneous, hepatic and renal mixed function oxidase in rodents (Rahimtula et al., 1982; Rahimtula et al., 1984) and

of hepatic mixed function oxidase in the developing chick embryo (Lee et al., 1986).

1.5 The monooxygenase system

1.5.1 Introduction

Animals and plants are constantly exposed to a vast group of chemicals that are foreign to their systems (xenobiotics). Xenobiotics can be of natural origin (eg. petroleum) or man-made. Many of these chemicals are lipophilic and can accumulate within the organism unless an effective means of disposal is present. Lipophilic compounds that are present in excretory fluids tend to diffuse through cellular membranes and are re[illegible]bed.

Fortunately, there are biochemical processes that convert these compounds into more hydrophilic metabolites. This process is termed biotransformation and is carried out by a variety of enzymes.

Most information concerning xenobiotic biotransformation has been derived from studies on the liver but it is known that this process occurs in most non-hepatic tissues.

Biotransformation is classified as occuring in two phas[illegible]s (Okey et al., 1986). Hydrophobic substrates are rendered more water-soluble by the introduction of polar

groups in Phase I reactions. Examples of some of these reactions are aromatic or aliphatic hydroxylation, N-hydroxylation, and N-or O-dealkylations. Phase II reactions involve the conjugation of polar groups with hydrophilic molecules such as glucuronic acid, glutathione and sulfate. In general, these conjugated products are inactive (non-toxic) and are readily excreted.

1.5.2 Cytochrome P-450

Phase I reactions are mostly carried out by microsomal enzymes, expecially various species of cytochrome P-450 which are components of the monooxygenase system. Monooxygenase activity requires cytochrome P-450, NADPH, NADPH-cytochrome P-450 reductase (a flavoprotein) and a suitable phospholipid matrix (Bentley and Oesch, 1982). Cytochrome b_5 and its reductase are also associated with the monooxygenase system. Cytochrome P-450 is so named because its reduced form binds with carbon monoxide, yielding a complex with an absorbance maximum at 450 nm. Cytochrome P-450-dependent oxidations have been termed mixed-function oxidases because in its reduced form, the hemoprotein catalyses the consumption of a molecule of oxygen, with one atom of oxygen appearing in the oxidized form of the substrate and the other atom being reduced to form water

(Mason et al., 1955). A general scheme for the cytochrome P-450 reduction-oxidation cycle is shown in Figure 1.4. The substrate combines with the oxidized form of cytochrome P-450 to form a ferric hemeprotein-substrate complex. The complex undergoes a one-electron reduction via the NADPH-dependent transport chain forming the ferrous-substrate complex. The latter reacts with molecular oxygen to form an oxygenated intermediate. This oxygenated-P-450 intermediate then undergoes a second electron reduction and through an internal rearrangement, one atom of oxygen is reduced to water, while the other atom of oxygen is introduced into the substrate molecule.

In general, the cytochromes P-450 are the rate-determining components in microsomal Phase I metabolism; the P-450 components also determine the substrate specificity. Although most species of P-450 metabolize a wide variety of compounds, those which act on physiologic substrates that are critical to survival, are much more substrate specific. These critical forms of P-450 are generally not induced by exposure to xenobiotic chemicals (Nebert and Gonzalez, 1985).

Although cytochromes P-450 collectively are capable of metabolizing thousands of different substrates, present evidence indicates that within any particular organism there are only a few dozen to a few hundred different forms of

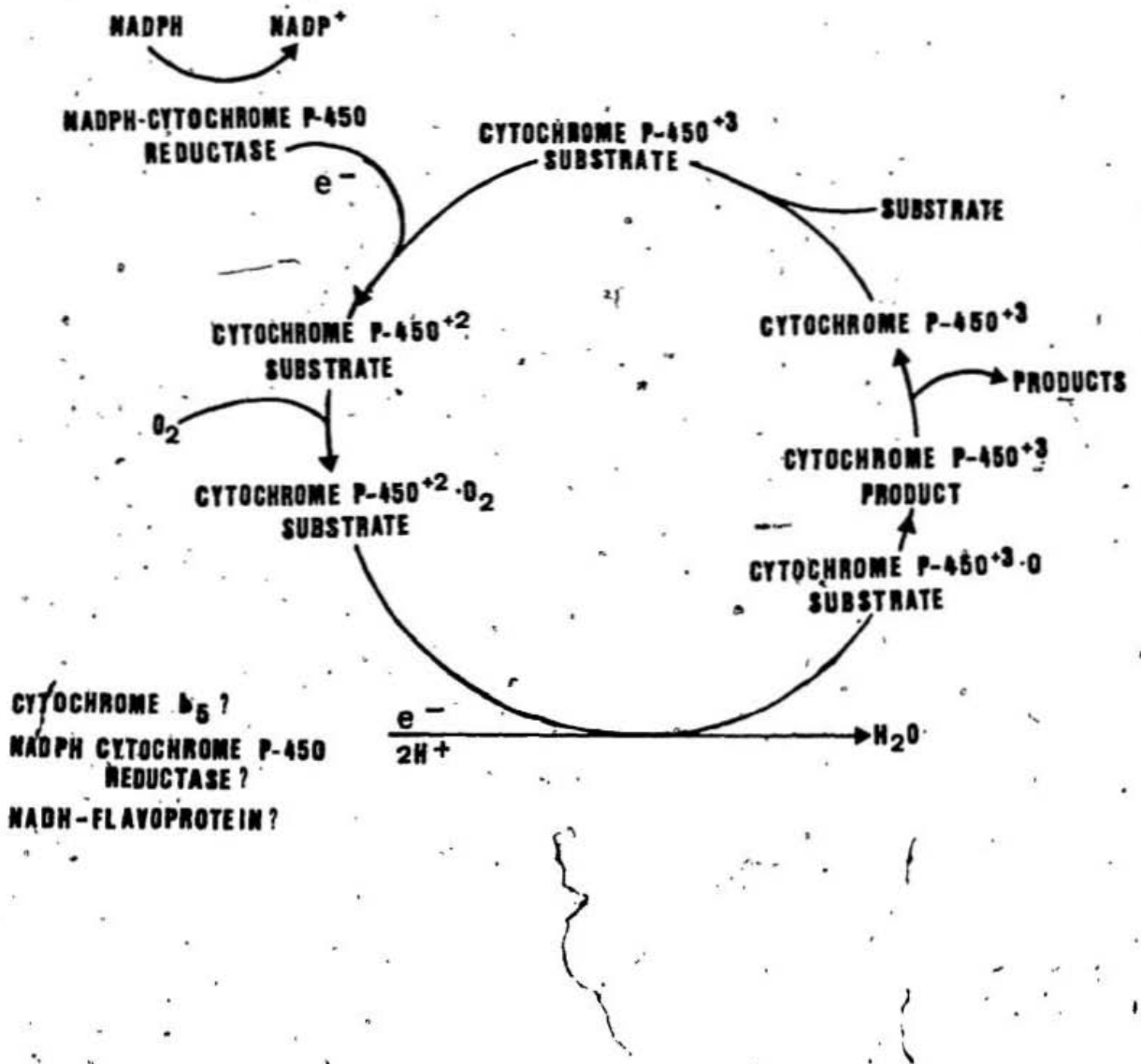


Figure 1.4: Mechanism of cytochrome P-450 enzyme system

cytochrome P-450 (Nebert and Gonzalez; 1985). Alterations in the levels of cytochrome P-450 can have profound effects on the response of the organism to xenobiotics.

The inducibility of cytochromes P-450 generally is higher than the inducibility of Phase II conjugating enzymes. This creates a potential imbalance between the rate at which chemically reactive intermediates are generated (by Phase I metabolism) and the rate at which these reactive metabolites can be inactivated and removed by conjugation. Those reactive metabolites that are not conjugated by Phase II reactions may covalently attack proteins, membrane components, or nucleic acids, thereby leading to cytotoxicity, mutations, and cancer. Reactive metabolites can also generate active oxygen species which are also known to be cytotoxic.

1.5.3 Induction of cytochrome P-450

For many years inducers of cytochrome P-450 were thought to fall into two major categories: 1) those that acted like 3-methylcholanthrene (3-MC) and 2) those that acted like phenobarbital (PB) (Conney, 1967). As techniques for characterizing P-450s improved it became clear that there were many more inducible species of P-450 and that

many inducers do not act like either 3-MC or PB (Nebert et al., 1981).

1,1,1-trichloro-2,2-bis (p-chlorophenyl)ethane (DDT) is another PB-like inducer. It enhances the metabolism of a large variety of substrates by these liver enzymes. In rats, this group of enzyme inducers has been shown to markedly increase liver P-450 content and associated enzyme activities, such as ethylmorphine N-demethylase and testosterone 16-hydroxylase. In control rats administered sedative doses of phenobarbitol, the barbituate caused an enhanced rate of plasma elimination of the test drug antipyrene. It was also revealed in PB induction studies that an enhanced rate of metabolism of drugs was associated with proliferation of smooth endoplasmic reticulum (ER) in the hepatocytes (Fouts and Rogers, 1965) and increased concentrations of the components of the monooxygenase enzyme system, cytochrome P-450, and NADPH-cytochrome P-450 reductase activity (Orrenius and Ernster, 1964).

In the 3-MC category, polycyclic aromatic hydrocarbons (PAH) such as benzo[a]pyrene induce the synthesis of cytochrome P-448, a hemoprotein that differs in spectral and catalytic properties from the cytochrome P-450 present in untreated rats or in rats pretreated with PB (Alvares et al., 1967; Sladek and Mannering, 1966). Cytochrome P-448 is also called P_1-450 or aryl hydrocarbon hydroxylase (AHH).

Several enzyme activities which are induced by 3-MC type inducers are 7-ethoxycoumarin-O-deethylase, 7-ethoxyresorufin-O-deethylase and benzo[a]pyrene hydroxylase. A receptor protein is involved in induction by 3-MC type inducers (Poland et al., 1976), but no such receptor protein has been detected for PB type inducers.

The first inducer of rat liver microsomal cytochrome P-450 to be distinguished from the classical inducers was pregnenalone 16α-carbonitrile (PCN) (Lu et al., 1972). Recent studies (Gorski et al., 1985) revealed that inducibility of benzo[a]pyrene hydroxylase activity decreased with age in male but not female rats indicating a sex and age dependence. PCN induced hepatic benzo[a]pyrene hydroxylase activity 5- to 8-fold in immature male, immature female and mature female rats. However, mature male rats only demonstrated a slight increase. Another cytochrome P-450 enzyme, 7-ethoxyresorufin-O-deethylase was not induced. This provided evidence that P-448 was not fully responsible for the benzo[a]pyrene hydroxylase induction. 7-Ethoxyresorufin can be dealkylated to a single product, resorufin by cytochrome P-448 (Burke et al., 1977).

1.6 Benzene metabolism and toxicity

1.6.1 Introduction

Benzene is used extensively in industry as a solvent or as starting material for chemical syntheses of paints and plastics. Today, because of its anti-knock properties, a mixture of benzene-enriched aromatics is being added to gasoline as a replacement for alkyl lead compounds. In the 1870's, benzene facilitated the rapid development of the rubber industry because of its ability to dissolve rubber latex and its ease of removal from formed rubber products. It also played a significant role in the high speed printing processes because it was a good solvent for ink and could be removed readily by evaporation following printing.

1.6.2 Toxicity of benzene

Benzene has a high vapor pressure at ambient temperatures and hazardous exposure may occur by inhalation. Acute exposure to benzene may kill by depressing the central nervous system, leading to unconsciousness and death or by producing cardiac arrhythmias (Snyder and Kocsis, 1975).

Benzene is well known for its ability to induce pancytopenia, a condition characterized by decreased number

of erythrocytes, leukocytes and thrombocytes (Snyder and Kocsis, 1975). Benzene is a known human carcinogen, being responsible for the production of acute myelogenous leukemia, an endpoint to the pancytopenia and aplastic anemia (Snyder et al., 1977).

1.6.3 Metabolism of benzene *in vivo*

Current evidence indicates that the toxicity of benzene is due to its metabolism. The metabolite(s) responsible for the hemopoietic toxicity of benzene is still uncertain, but an experiment by Park and Williams (1953), using ^{14}C-benzene administered to rabbits identified all the *in vivo* metabolites. Analysis of the urine revealed that phenol, hydroquinone (1,4-dihydroxybenzene), catechol (1,2-dihydroxybenzene) and trans-trans-muconic acid represented 23%, 4.8%, 2.2%, 0.3% and 1.3% respectively of the administered dose. In 1963, Sato et al identified trans-1,2-dihydro-1,2-dihydroxybenzene as an *in vivo* metabolite.

In our experiments we used benzene and its metabolites to study their effect and chick embryos, in order to determine if they can be used as a model for testing the effects of metabolism on toxicity.

1.7 Problem of Investigation

The major objective of the studies performed in this thesis was to determine the importance of metabolism in the toxicity of PBCO to erythrocytes and chick embryos.

Ingestion of PBCO by Herring Gulls (Larus argentatus) and Atlantic Puffins (Fratercula arctica) has been reported to induce hemolytic anemia (Leighton et al., 1983). Part of this study is to characterize some of the biochemical alterations in red blood cells when exposed to PBCO, under in vitro conditions.

Another purpose of this study was to attempt to correlate embryo toxicity with the induction of hepatic and renal cytochrome P-450 and arylhydrocarbon hydroxylase activities in the developing chick embryo. For this purpose, drilling fluids, blowout crude oil and diesel fuel, and the aliphatic, aromatic and NOS fractions of PBCO were tested.

The search for the answers to these questions were divided into several lines of investigation.

1) The polycyclic aromatic hydrocarbon extract of PBCO was metabolized in vitro to determine if it was more potent than the unmetabolized extract in terms of hemolysis,

glutathione depletion and metHb elevation in erythrocytes. The effects of naphthalene and some of its metabolic derivatives were investigated in order to determine the most toxic metabolites.

2) The second investigation involved determining the fraction of PBCO which is most toxic to the chick embryo in terms of mortality and induction of cytochrome P-450, 7-ethoxyresorufin-O-deethylase and benzo[a]pyrene hydroxylase. For these purposes, PBCO was fractionated into aliphatic, aromatic and NOS fractions.

3) Finally, to further study the importance of metabolism with respect to embryo toxicity, benzene and some of its known <u>in vivo</u> metabolites were applied to chick embryos in order to determine if any metabolites were more toxic than the parent compound.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Naphthalene, 1-naphthol, 2-naphthol, 1,2-naphthoquinone, 1,2-dihydroxynaphthalene, 1,3-dihydroxynaphthalene, 1,4-dihydroxynaphthalene, 2-methylnaphthalene, 2-methyl-1-naphthol, 2,6-dimethylnaphthalene, 1,4-benzoquinone, resorcinol, catechol, hexadecane, metaphosphoric acid and aluminium oxide (activated neutral 1) were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin.

Benzo(a)pyrene, 2,4-dinitrofluorobenzene (DNFB) sodium azide, dimethylsulfoxide, $NADP^+$, N-ethyl maleimide (NEM), GSH, glutathione reductase, DL-isocitrate, isocitric dehydrogenase, 5,5-dithiobis (2-Nitrobenzoic acid) (DTNB), catalase and superoxide dismutase were obtained from Sigma Chemical Co. St. Louis, Missouri.

Benzene, methanol, hexane, acetone and ethyl acetate were of HPLC grade and were obtained from Fisher Scientific, Fair Lawn, New Jersey.

Hydroquinone was obtained from J.T. Baker Chemical Co. Phillipsburg, New Jersey. Phenol was purchased from BDH

Chemicals, Toronto. Resorufin and 7-ethoxyresorufin were purchased from Pierce Chemical Co., Rockford, Illinois. Silica gel (60-200 mesh) was purchased from Matheson, Coleman and Bell, Norwood, Ohio.

The Fisher Diagnostics Cyanmethemoglobin Standard Set 251 was purchased from Fisher Scientific Co., Orangeburg, New York.

2.1.2 Gifts

Prudhoe Bay crude oil (PBCO) was kindly donated by Dr. David Peakall, Canadian Wildlife Service, Ottawa, Ontario.

Drilling fluids (Safver Oils #1, 3, 4, 5), Shell blowout and diesel fuel were provided by Dr. Jerry Payne, Dept. of Fisheries and Oceans, St. John's, Newfoundland.

Cis-cis muconic acid was donated by Dr. Eric Barnsley, Memorial University of Newfoundland, St. John's, Newfoundland.

Human whole blood (outdated) was provided by the local Red Cross.

2.2 Methods related to erythrocyte studies

2.2.1 Preparation of PBCO (PBCO-DMSO extracts, and fractionation)

2.2.1.1 Preparation of DMSO extracts of PBCO

DMSO was the solvent of choice for selectively extracting polycyclic organic compounds from PBCO. Natusch and Tomkins (1978) have demonstrated that DMSO is suitable for this purpose. PBCO (5 ml) and DMSO (5 ml) were mixed in a 17 x 100mm polypropylene vial and capped. The sample was shaken in a rotary mixer for 30 minutes, followed by centrifugation in a table top centrifuge (600 g, room temp.) for 5 minutes to separate the two layers. The DMSO extract (bottom layer) was collected by poking a pin hole in the bottom of the tube. Samples were stored as 5ml aliquots at -80 C.

2.2.1.2 Preparation of metabolized PBCO extract

Metabolized PBCO extract was prepared by incubation of the following components in 125ml of 0.1M potassium phosphate buffer (pH 7.5) at 37°C for 2 hours: 2.5ml of the DMSO extract of PBCO, 62 mg of microsomal protein (prepared from the livers of untreated rats) and 12.5ml of NADPH regenerating system. The regenerating system consisted of 625

μmoles of sodium isocitrate, 62.5 μmoles of $NADP^+$, 625 μmoles of $MgCl_2$ and 81.3 units of isocitric dehydrogenase in 12.5ml phosphate buffer. At the end of the incubation period, the mixture was extracted with ethyl acetate (2 x 100ml). The combined extracts were dried with anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in the original volume of DMSO (2.5ml). Microsomes were prepared from untreated Sprague Dawley rats as described in section 2.3.2.1.

2.2.1.3 Fractionation of PBCO

The fractionation of PBCO was carried out as described by Gearing et al (1976) with modifications. A (1cm x 20cm) column was set up containing silica (60-200 mesh) overlaid by alumina (activated neutral 1) at a ratio of 2:1 respectively. Both chemicals were heated at 200°C overnight to remove any water present, and stored in a dessicator before use. The PBCO was prepared by mixing a 0.5ml aliquot with 2.5ml pentane. By centrifuging this sample for 20 minutes in a table top centrifuge (600 g, room temperature), ashphaltenes were precipitated. The pentane mixture was gently applied to the hexane equilibrated column and allowed to flow until the meniscus reached the top of the alumina. The column was eluted successively with 50ml of hexane, 50ml of benzene and 50ml of methanol to yield respectively the

aliphatic, aromatic and NOS (nitrogen, oxygen and sulphur heterocyclic compounds) fractions. Each solvent and its dissolved components was collected separately in flasks. The number of columns depended on the volume of PBCO that had to be processed. All fractions were pooled correspondingly to solvent (hexane extract, etc.).

The solvent from each fraction was removed by the use of a rotary evaporator (Rotovapor RE 120). The percentage of fractions in PBCO were determined to be: aliphatic (38.4%; w/v), aromatic (38.5%; w/v), NOS (6.8%; w/v). The asphaltene content of the PBCO was determined to be 5.0%. After the volume, or the weights of the residues were recorded, each fraction was diluted in hexadecane to the original volume of PBCO that was processed. A sample of each fraction was submitted to Dr. Stephen Macko (Earth Science Dept., MUN) for GC-Mass spectral analysis to determine separating performance of the method as well as to identify some of the major components in each fraction. For results of GC-mass spectral analysis, see Appendix A.

2.2.2 Synthesis of trans-1,2-dihydroxy-1,2-dihydronaphthalene

Trans-1,2-dihydroxy-1,2-dihydronaphthalene was synthesized according to the method of Booth et al (1950). 200mg

of 1,2-Naphthoquinone, dried in vacuo over phosphorous pentoxide, was placed in a thimble of a Soxhlet apparatus. Diethyl ether (50ml; dried over sodium), was placed in the flask of the apparatus with 0.1g lithium aluminium hydride and refluxed until the naphthoquinone appeared to be extracted. After the mixture had cooled to room temperature, 35 mls of 1.7 M sulfuric acid was added slowly to decompose the exdess lithium aluminium hydride. The solution was centrifuged to separate the ether layer, which was then extracted with 2N sodium hydroxide and evaporated under reduced pressure. The residue was crystallized from benzene and then from cyclohexane. The crystallized product had a melting point range of 104-105°C. Reported melting point of trans-1,2-dihydroxy-1,2-dihydronaphthalene is 103-105°C. Yield = 26mg.

2.2.2.1 Nuclear magnetic resonance spectroscopy analysis

^{1}H Fourier Transform NMR for trans-1,2-dihydroxy-1,2-dihydronaphthalene was performed on a Bruker WP80 NMR spectrophotometer using a proton frequency of 80 MHz at ambient temperature.

The ^{1}H-FT NMR spectrum of trans-1,2-dihydroxy-1,2-dihydronaphthalene is shown in Figure 2.1.

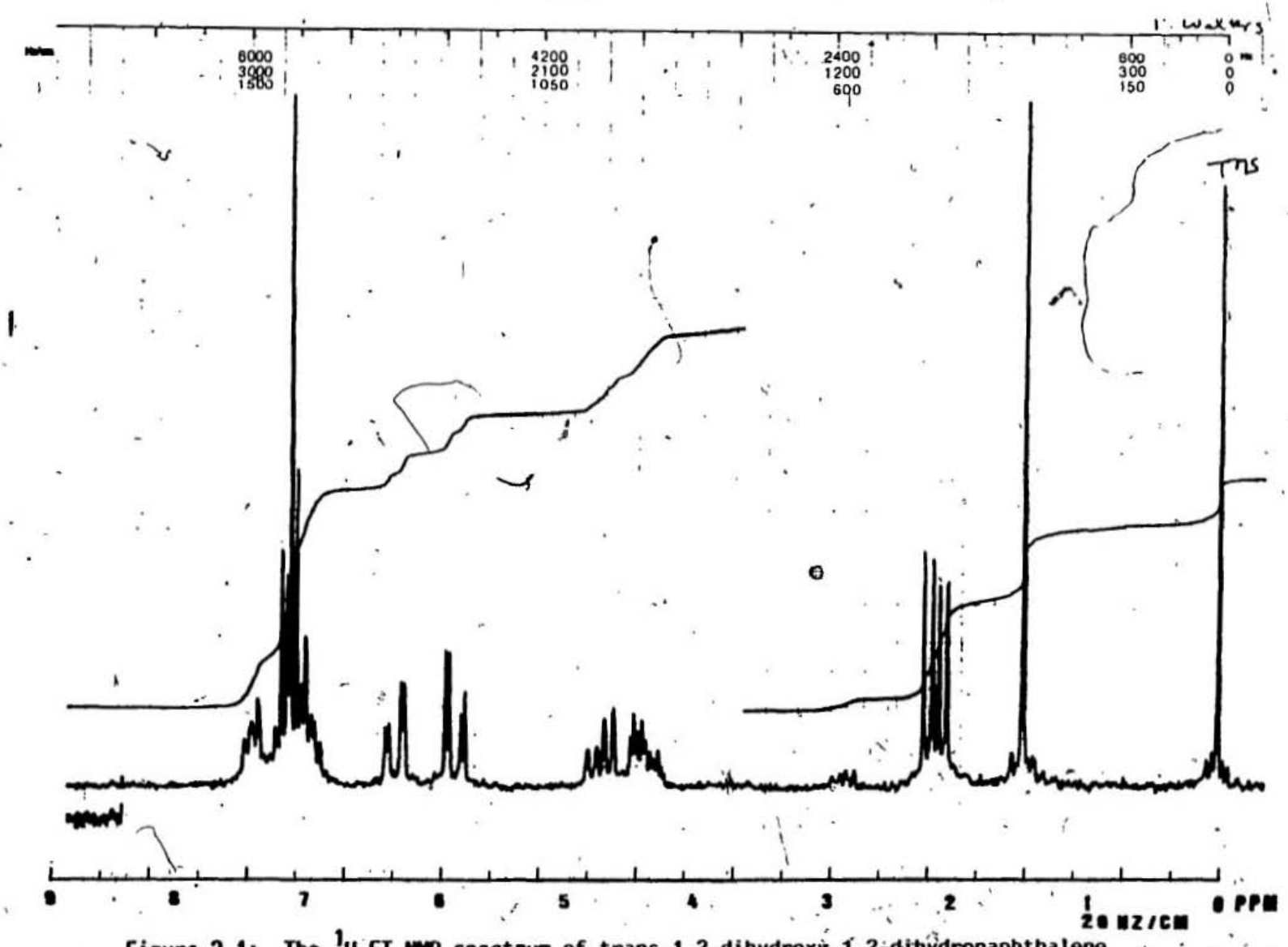


Figure 2.1: The ^{1}H FT NMR spectrum of trans-1,2-dihydroxy-1,2-dihydronaphthalene

NMR ($CDCl_3$): 1.5; H_2O - highfield when dilute in $CDCl_3$

2.0-2.25 - 1,2-dihydro

4.0-5.0 - hydroxyls (1-OH downfield of 2-OH)

5.5-6.5 - double bond hydrogens

6.5-8.0 - aromatic hydrogens

7.27 - $CHCl_2$

2.2.2.2 Mass spectroscopy analysis

Determination of the mass spectrum of trans-1,2-dihydroxy-1,2-dihydronaphthalene was carried out on a VG 7070 HS double focussing mass spectrometer equipped with a 2035 data system. A direct insertion probe, which was heated if necessary to obtain a spectrum, was used to introduce all samples, the ionization chamber temperature was 200°C and ions were generated by electron impact using 70 eV electrons. Mass spectral data from perfluorokerosene was input into the data system. This was then used to create a calibration file for the mass calibration of data from subsequent samples. High resolution data were obtained in the presence of perfluorokerosene calibration peaks using a resolving power of 8,000-10,000; low resolution data were obtained in the absence of perfluorokerosene and a resolving power of approximately 1,000. Whenever possible, a series of consecutive scans was averaged using the data system. Fragment ions were ignored if less than 2% intensity.

The mass spectrum of trans-1,2-dihydroxy-1,2-dihydronaphthalene is shown in Figure 2.2.

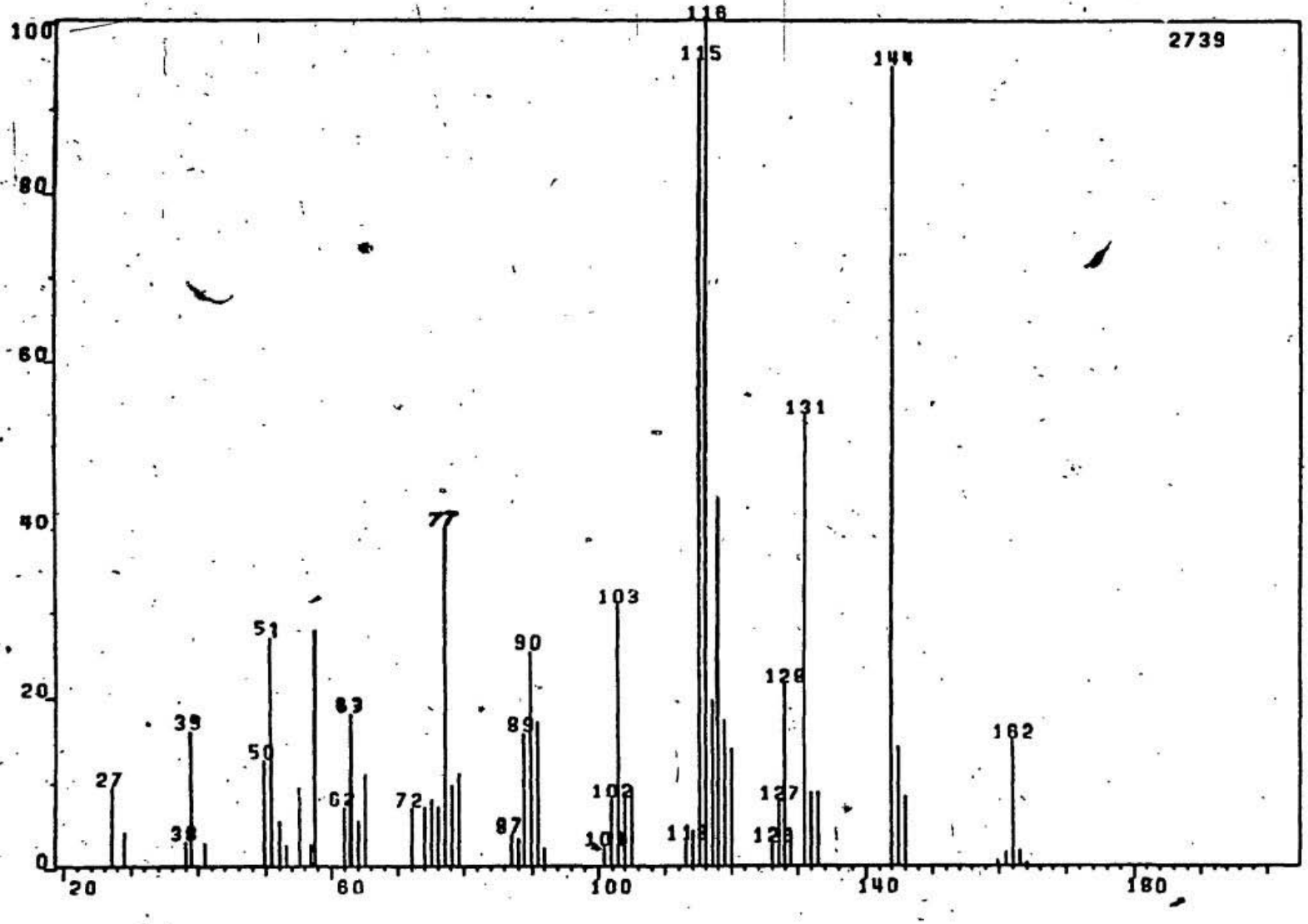


Figure 2.2: The mass spectrum of trans-1,2-dihydroxy-1,2-dihydronaphthalene

Mass spectrum = 162 (M^+) molecular ion

144 ($M^+ - H_2O$)

131 ($M^+ - CH_2OH$)

116 ($M^+ - H_2O$, - C = O), most abundant ion

115 (most abundant ion - H)

2.2.3 Collection and preparation of erythrocytes

Young Herring gulls (Larus argentatus) were collected on Great Island, 50 km south of St. John's, Newfoundland and held in pens at the University. They were fed unlimited amounts of capelin (Mallotus villosus) and seawater. Blood samples (5ml) were drawn from a wing vein into heparinized vacutainer tubes. At least 7 days were allowed to elapse between successive blood withdrawals from the same bird.

Gull or human erythrocytes were obtained from whole blood by centrifugation for 5 minutes in a table top centrifuge (600 g, room temperature), followed by four washings with 2 volumes of PBS (0.1M potassium phosphate buffer (pH 7.4), 0.9% (w/v) NaCl). The washed erythrocytes were finally suspended in an equal volume of PBS. Hematocrit values were determined and final results reported on the basis of % hematocrit (Brown, 1976).

2.2.4 Preparation of oxygenated hemoglobin (HbO_2).

Oxyhemoglobin was prepared according to the method of Geraci et al (1969). Approximately 5ml of washed human erythrocytes (see section 2.2.3) were centrifuged to pack the cells. After the supernatant was removed with a Pasteur pipette, 1ml of the packed cells was added to 5ml of

distilled water and allowed to stand at room temperature for 30 minutes in order to induce hemolysis. The membranes were removed by centrifugation in a table top centrifuge for 20 minutes (600 g, room temperature). A few crystals of sodium dithionite were added to the hemolysate to reduce ferric heme. The dithionite was removed by passing this solution through a Sephadex G-25 column (1.25cm x 30cm) equilibrated with 0.01M potassium phosphate buffer, pH 7.0. The hemolysate was collected and was bubbled with a mixture of 95% O_2 and 5% CO_2 for 2 minutes to generate HbO_2.

2.2.5 Determination of HbO_2 concentration

The concentration of HbO_2 was determined as described by Fairbanks (1976). A 0.02ml sample of HbO_2 solution (see section 2.2.4) was mixed with 6ml of Drabkin's reagent (0.20g $K_3Fe(CN)_6$, 0.05g KCN, 1.0g $NaHCO_3$ made up to 1L in H_2O). The absorbance (540mm) of the solution was recorded after 5 minutes in a spectrophotometer and designated as A_b.

The following calculation was used to determine the concentration of HbO_2 in the sample.

$$\text{Concentration of } HbO_2 \text{ (mg/100ml)} = \frac{\left(\frac{V + 0.02}{0.02}\right) \cdot \left(\frac{A_b \times C_s}{A_s}\right)}{1000}$$

V = volume of Drabkin's reagent used (usually 6ml).

A_s = Absorbance (540nm) of standard.

C_s = Concentration of standard (Fisher Diagnostics Cyanmethemoglobin Standard Set 251) - 80mg/100ml.

A_b = Absorbance (540nm) of sample.

2.2.6 Incubation conditions

All incubations involving erythrocytes and HbO_2 were carried out in phosphate buffered saline (PBS) (pH 7.4) at 37°C with gentle shaking, and contained either washed erythrocytes (20% or 25% packed cell volume (PCV) final concentration) or HbO_2 (50mg/ml). To achieve a final concentration of 20% or 25%, an appropriate aliquot of prewashed erythrocytes (see section 2.2.3) with known PCV was pipetted into a test tube, followed by the other reagents. A final PCV determination was performed as a check to determine if the desired PCV had been obtained.

2.2.6.1 Studies involving DMSO extracts of PBCO and metabolized PBCO.

Incubations contained per ml: washed RBC (25% PCV) or HbO_2 (50mg/ml), 5-25µl of DMSO extract of PBCO or metabolized PBCO. Whenever it was necessary to determine if the metabolism of PBCO was needed to produce toxicity, a NADPH

regenerating system (5.0 μmole of sodium isocitrate, 0.5 μmole of $NADP^+$, 5 μmole of $MgCl_2$, 0.65 units of isocitric dehydrogenase) in 100μl of PBS was added in the presence of 1mg of rat liver microsomes (control or pretreated) (see section 2.3.2.1) to give a final volume of 1ml of incubation mixture. Blanks were performed using erythrocytes and PBS in the absence or presence of DMSO. Time curves and concentration curves were also performed on these experiments. At suitable time intervals or at the end of 1 hour, aliquots of the reaction mixture involving erythrocytes were removed for the determination of hemolysis, metHb, reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione (GSH + GSSG). Only metHb levels were measured in incubations involving HbO_2. Percent hemolysis was determined as described by Draper and Sarri Csallany (1969) on 500μl of sample (see section 2.2.7). MetHb was determined as described by Fairbanks (1976) (see section 2.2.8) on 100μl of sample withdrawn from the incubation mixture. GSH was determined by the alloxan method (Patterson and Lazarow, 1955) on 200μl of reaction mix (see section 2.2.9.1). Total glutathione and GSSG were measured as described by Tietze (1969) on 10μl and 100μl of sample respectively (see section 2.2.9.2).

2.2.6.2 Studies involving naphthalene and some of its derivatives

Incubation conditions were the same as described in the previous section, with the exception that 0.05mM-1.0mM final concentrations of substrates (dissolved in a maximum of 10µl DMSO/ml reaction mixture) were added to the reaction mixture instead of PBCO mixtures. The following substrates were used: naphthalene, 1,2-naphthoquinone, 1,4-naphthoquinone, 1,2-dihydroxynaphthalene, 1,3-dihydroxynaphthalene, 1,4-dihydroxynaphthalene, 1-naphthol, 2-naphthol, trans-1,2-dihydroxy-1,2-dihydronaphthalene, 2,6-dimethylnaphthalene, 2-methyl-1-naphthol and 2-methylnaphthalene. MetHb was determined as previously discussed on erythrocyte or HbO_2 studies but GSH and GSSG were determined on 500µl of sample according to the HPLC method of Reed et al (1980) (see section 2.2.9.3).

2.2.7 Determination of hemolysis in erythrocytes

Hemolysis of erythrocytes was determined according to a modified method of Draper and Sarri Csallany (1969). A 500µl aliquot was withdrawn from the incubation medium. From this sample a 100µl aliquot was added to 3ml of distilled H_2O to hemolyse the erythrocytes completely. The remaining 400µl sample was centrifuged in a table top

centrifuge for 10 minutes (600 g, room temperature) to spin down unhemolysed erythrocytes and ghosts. A 100µl aliquot of the supernatant was transferred to a test tube containing 3ml of PBS. (0.1M potassium phosphate buffer (pH 7.4), 0.9% NaCl). After 30 minutes the absorbance of both tubes was measured at a wavelength of 415nm. The absorbances were measured against a PBS blank. Percent hemolysis was calculated by dividing the absorbance value for the tube containing the buffer by that of the completely hemolysed tube containing added water and multiplying by 100.

2.2.8 Determination of methemoglobin in erythrocytes and HbO_2

Methemoglobin was determined as described by Fairbanks (1976). 0.1ml of sample was removed from the incubation and mixed with 3.9ml of distilled H_2O in a 10ml test tube. After 10 minutes a 4.0ml solution of 0.15M potassium phosphate buffer pH 6.6 was added followed by thorough mixing. After centrifugation for 10 minutes in a table top centrifuge (600 g, room temp.), two 3.0ml aliquots of hemolysate were added to two tubes designated C_2 and C_3. A 100ul solution of 20% potassium ferricyanide was added to tube C_3 and the absorbance of both tubes were measured in a spectrophotometer at 630nm. The absorbance of contents of

tubes C_2 and C_3 were designated as A_{2a} and A_{3a} respectively. A 0.1ml solution of 5% KCN was then added to the contents of these tubes and the absorbance (630nm) was recorded again at least 5 minutes later. These absorbance values were designated as A_{2b} and A_{3b} respectively. The spectrophotometer was zeroed with a blank composed of 1.5ml buffer and 1.5ml of distilled water. Calculations were performed as follows:

$$\text{Methemoglobin (\% of total pigment)} = 100\left(\frac{A_{2a} - A_{2b}}{A_{3a} - A_{3b}}\right)$$

2.2.9 Determination of glutathione (GSH), oxidized glutathione (GSSG) and total glutathione in erythrocytes

2.2.9.1 Determination of GSH by the alloxan method

Determination of GSH was performed as described by Patterson and Lazarow (1955). A 0.2ml aliquot of reaction medium was mixed with 1.4ml of distilled water to hemolyse the red cells. A 0.4ml aliquot of 25% (w/v) metaphosphoric acid was added, mixed and then centrifuged for 5 minutes (600 g, room temp.) to precipitate the protein. For each sample, two tubes were labelled X and X_0 with both containing a 0.5ml aliquot of supernatant. A 0.5ml aliquot of 0.1M alloxan and H_2O were distributed to tubes X and X_0 respectively. X_0 corresponded to the sample blank.

Standards and blanks were set up as above but instead of 0.5ml supernatant, 0.5ml of buffer containing 50µg GSH or 0.5ml of 5.0% metaphosphoric acid was added, respectively. After all tubes were prepared, 0.5ml of 0.5 M phosphate buffer pH 7.5 was added, immediately followed by 0.5ml of equivalent NaOH solution. Equivalent NaOH was prepared by titrating a mixture of 20ml of 5% metaphosphoric acid and 20 ml of 0.1 M alloxan with 0.5 N NaOH to pH 7.5 with a pH meter. The amount of 0.5 N NaOH that was needed for the titration was diluted to 20ml. After six minutes, a 0.5ml aliquot of 1N NaOH was added. This stops the reaction and stabilizes the product absorbing at 305 nm for several hours. The concentration of GSH was determined by the following calculation.

$$\text{GSH (mg/100ml)} = \frac{A_x - A_b}{A_s - A_b} \times 50$$

A_x = Absorbance of sample minus sample blank (305nm).

A_s = Absorbance of standard minus standard blank (305nm).

Final calculation was recorded as µmoles GSH/ml packed RBCs (100% RBC).

2.2.9.2 Determination of total glutathione and GSSG

Determination of total glutathione (GSH and GSSG) and

oxidized glutathione (GSSG) was performed as described by Tietze (1969).

Total glutathione is defined as the sum of GSH and GSSG in GSH equivalents. The following protocol was used in its determination. 10µl of reaction mixture was hemolysed in 990µl of cold 0.01M phosphate/0.005M EDTA buffer, pH 7.5. For analysis, 50µl of the resulting hemolysate was added to a 1ml sample cuvette containing 0.6µmole of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 10µg of glutathione reductase, 0.2 µmoles of NADPH, and phosphate-EDTA buffer. The final volume was 1ml. The rate of reaction was expressed as change in absorbance per 6 minutes at 412nm wavelength, and was compared to a standard curve using 1-100 ng GSH. The contents of the standard cuvettes were the same as stated above, but the addition of hemolysate step was omitted. Final results were expressed as µmoles/1ml packed RBCs.

For determination of GSSG content, 100µl of sample was incubated with 0.02 M N-ethylmaleimide for 1 hr at 25°C as a 1:10 hemolyzate in EDTA-buffer. Following the precipitation of proteins by addition of 1.0ml 10% trichloroacetic acid (TCA) the suspension was centrifuged for 10 minutes in a table top centrifuge (600 g, room temp.). The supernatant solution was extracted 10 times with diethyl ether. Finally, 100µl of the extracted supernatant was added to a 1ml sample cuvette containing 0.6 µmoles of DTNB, 10 µg of

glutathione reductase, 0.2 of μmoles NADPH, and phosphate EDTA buffer. The final volume was 1ml. The rate of the reaction was expressed as the change in absorbance per 6 minutes at 412 nm wavelength, and was compared to a standard curve using 1-100 ng of GSSG. Results were converted to μmoles/ml packed RBCs and expressed as % of total glutathione.

For studies involving PBCO the above methods were found to be the most suitable for determination of GSH, GSSG, and total glutathione. Due to the complexity of components of crude oil, interference occurred in analysis using fluorimetric or HPLC methods.

2.2.9.3 GSH and GSSG determination (HPLC method).

Determination of reduced and oxidized glutathione was carried out by high-performance liquid chromatography as described by Reed et al (1980). 0.5ml of incubation mixture was mixed vigorously with a 0.5ml solution of 7.0% metaphosphoric acid (w/v). After centrifugation (5 min., 600 g, room temp.) to precipitate the protein, 0.4ml of supernatant was added to a 0.4ml solution of iodoacetic acid (7.5mg/1ml H_2O) and incubated for 60 minutes in the presence of approximately 20mg of sodium bicarbonate. A 0.5ml solution of 1-fluoro-2,4-dinitrobenzene (DNP) (1.5% v/v) in absolute ethanol was added and allowed to react for 4 hours at room

temperature in the dark to form N-DNP derivatives. N-DNP derivatives of standard GSH and GSSG were also formed in a similar way as described above.

Separation of DNP-derivatives were performed on a Waters - Bondapack amine liquid chromatography column. The 10µl samples were injected with a Perkin Elmer ISS-100 autosampler. The solvents were delivered with a Perkin-Elmer Series 4 Liquid Chromatograph Microprocessor-controlled solvent delivery system. The column was washed with methanol and then equilibrated for 5 minutes with a solvent system containing 640ml methanol, 160ml water and 200ml from a solution consisting of 272g sodium acetate trihydrate, 122ml water and 378ml glacial acetic acid. The elution of DNP derivatives of GSH and GSSG was carried out using the same solvent system as described for 15 minutes at a flow rate of 1.0ml per minute. The eluted compounds were detected at 365nm using a Perkin-Elmer model LC-85B dual beam spectrophotometer, controlled through a Perkin-Elmer L-C Autocontrol with variable wavelength. The signals from the detector were integrated on the Perkin-Elmer 3600 Data System through a Perkin-Elmer Chromatographics 2 (CIT2) software package. Data and graphics were recorded on a Perkin-Elmer 660 Graphics-Printer. After elution, the column was then washed for 5 minutes with methanol:water (4:1, v/v) and a further 5 minutes with methanol (100%)

before analysing the next sample. The concentrations of GSH and GSSG in the samples were calculated from respective standard curves eluted in the same manner. Figure 2.3 shows elution profile of GSH and GSSG. The concentrations of GSH and GSSG in the reaction mixture are expressed as μmoles GSH or GSSG/ml packed RBC. Percentage change in levels of GSH or GSSG from control values were sometimes used for consistency.

2.3 Methods related to chick embryo studies

2.3.1 Treatment of eggs

2.3.1.1 Handling of chick eggs

Fertile eggs (White Leghorn) were purchased from Cooks Chick Hatchery (Truro, Nova Scotia). Eggs were incubated at 37.5°C in commercial incubators. Before application of the test substance (day 7 or day 12 of incubation) eggs were selected for fertility and normal development by candling, and randomly divided into treatment groups of 30 eggs each.

2.3.1.2 Studies involving PBCO, PBCO fractions, drilling fluids, diesel fuel and blowout crude oil

Mortality studies involving PBCO, PBCO fractions (aliphatic, aromatic, NOS; see section 2.2.1.3), Drilling

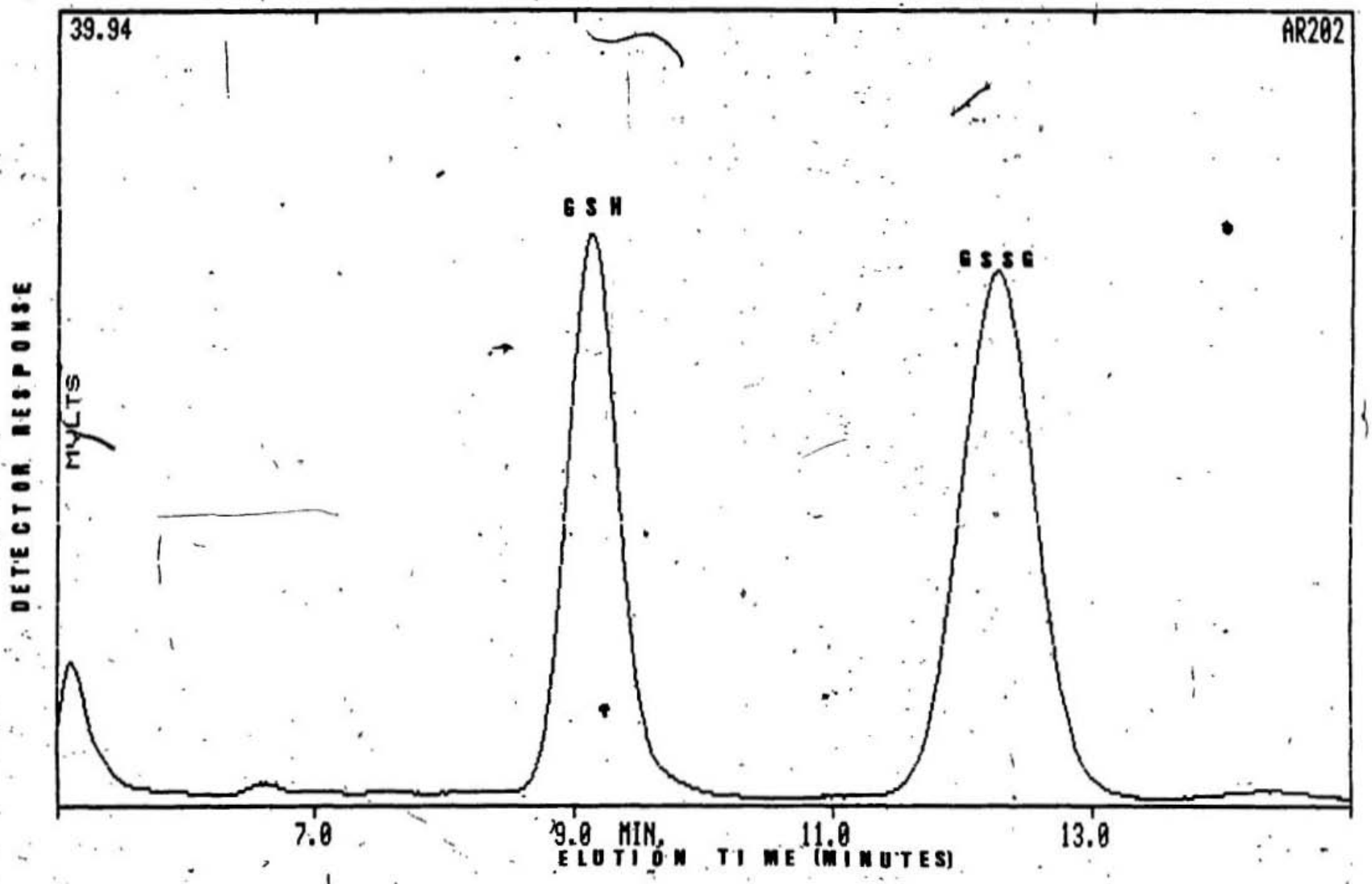


Figure 2.3: The HPLC profile of standard GSH and GSSG N-DNP derivatives

fluids (Safver oil 1, 3, 4, 5), Shell blowout and diesel fuels were carried out by application of samples on day 7 or 12 of incubation. 0-40µls of sample were applied by a microlitre syringe just below the airspace of upright eggs as described by Ablers (1977). Oil was spread around the surface of the egg with the tip of the syringe. Immediately after oil application, the eggs were returned to the incubator. Eggs were candled once every two days and were opened on day 15 for eggs treated on day 7 or on day 19 for eggs treated on day 12. Percent mortality was calculated by dividing the number of dead embryos by the sample number X100.

For enzyme induction studies, the above samples were applied to the surface of egg as described above on day 12 of incubation and the livers, kidneys and lungs excised 24 hours later. Kidney and lung studies were not peformed on drilling fluids, diesel fuel and blowout crude. Microsomes were prepared (see section 2.3.2.2) and assayed for cytochrome P-450 (section 2.3.3.1), 7-ethoxyresorufin O-deethylase (section 2.3.3.2) and benzo[a]pyrene hydroxylase activities (section 2.3.3.3).

2.3.1.3 Benzene and benzene metabolite studies

The injection of benzene or its metabolic derivatives was performed as described by Korhonen et al (1984).

Benzene, resorcinol, catechol, hydroquinone, 1,4-benzoquinone, phenol and cis-cis muconic acid were injected into eggs in amounts of 3.5-79.1 μmoles per egg. All compounds except benzene were injected in a total volume of 50μl of PBS. (0.1M phosphate buffer (pH 7.4), 0.9% NaCl). Benzene was delivered without previous dilution.

Each compound was injected into the airspace using a Hamilton syringe via a small hole made in the shell. After injection, the hole was sealed with critoseal. The treated eggs were then returned to the incubator and racks held in a horizontal position for 1 day, before resuming periodic rotation. All eggs were treated on day 7 of incubation and opened on day 15 to determine the mortality rate.

2.3.2 Preparation of microsomes

2.3.2.1 Preparation of liver microsomes from rats

Microsomes were prepared from untreated male Sprague Dawley rats (225-250g) or rats pretreated with sodium phenobarbitol (PB), (0.1% solution in drinking water for 4 days), or 3-Methylcholanthrene (3MC), (20mg/ml in corn oil administered i.p. as 2 daily injections of 40mg/kg body weight). Animals were fasted overnight after final treatment and killed the following day by cervical dislocation. All of the following procedures were performed at 0-4°C according

to the method described by Rahimtula et al (1979). Livers were removed and perfused through the portal vein with ice-cold 1.15% KCl to remove blood, blot-dried and weighed. Each liver was minced into fine pieces with scissors and homogenized with 3 volumes (w/v) of 0.1M potassium phosphate buffer (pH 7.5) by a motor-driven Potter-Eljevhem homogenizer. The homogenate was centrifuged at 10,000g for 10 minutes in a Sorval RC-2B centrifuge (using GSA rotor). The supernatant was decanted, filtered through cheese cloth and centrifuged at 105,000g for 75 minutes in a model L3-50 ultracentrifuge, equipped with a 50 Ti rotor. The microsomal pellets were resuspended in 0.1M phosphate buffer pH 7.5 by gentle homogenization with a hand homogenizer (4 strokes), and recentrifuged at 105,000g for 60 minutes. The supernatants were discarded and the pellets were resuspended by homogenization in phosphate buffer in a volume equal to the initial weight of the liver. The washed microsomal suspensions were frozen in suitable aliquots at -80°C. Protein determination was peformed as described by Lowry et al (1951), using bovine serum albumin as a standard.

2.3.2.2 Preparation of microsomes from chick embryos

Chick embryos were killed by decapitation. The lungs and kidneys were removed as quickly as possible and rinsed in ice cold 0.1M potassium phosphate buffer (pH 7.5). Each

organ was pooled separately with those of the same treatment group (20-30 embryos per group). Homogenizing and centrifugation of samples were carried out as described for rat microsomes with the exception that microsomal pellets were resuspended in buffer in a total volume of 600µl. Three 200µl aliquots were then frozen at -80°C until assayed (usually within 1 week). Protein determination was performed as described by Lowry et al (1951).

2.3.3 Enzyme assays

2.3.3.1 Measurement of cytochrome P-450 levels

Cytochrome P-450 levels were measured as described by Omura and Sato (1964). A 2ml sample containing 2mg microsomal protein, 0.25M potassium phosphate buffer (pH 7.5) was mixed gently with a few crystals of sodium dithionite. The solution was distributed equally in two cuvettes (1ml, 1cm pathlength) which were placed in the reference and sample cell compartments of a double beam spectrophotometer. After a baseline was recorded between 400 and 500nm, the sample cuvette contents were gassed with carbon monoxide by bubbling it for at least one minute at approximately 1 bubble per second. The spectrum was recorded again between 400nm and 500nm to determine the maximum absorbance peak at approximately 450nm. Calculations to determine cytochrome

P-450 content involved use of the extinction coefficient difference of 91mM^{-1} cm^{-1} between 450 and 490nm.

2.3.3.2 Determination of 7-ethoxyresorufin O-deethylase activity

7-Ethoxyresorufin O-deethylase O-dealkylase activity was determined according to the methods of Pohl and Fouts (1980). A total incubation volume of 1.25ml contained 0.125 mg of microsomal protein, 2.0mg of bovine serum albumin, 0.1ml of potassium phosphate buffer (pH 7.8), 125 µl of NADPH regenerating system (6.25 µmoles of sodium isocitrate, 0.63 µmoles of $NADP^+$, 6.25 µmoles of $MgCl_2$ and 0.81 units of isocitric dehydrogenase made up to 0.125µl with PBS) and 1.5µM 7-ethoxyresorufin. The sample was preincubated at 37°C for 2 minutes in the absence of the NADPH regenerating system before starting it by its addition. The reaction was stopped after 10 minutes by the addition of 2.5ml of methanol. After the precipitated protein had been removed by centrifugation (10 min., 600 g, room temp.), the fluorescence of the supernatant was measured using an excitation wavelength of 550nm and an emission wavelength of 585 nm. Determination of product quantity was calculated by use of a standard curve of a range of amounts of resorufin (0.0-10.0 nmoles). Due to light sensitivity of resorufin the precaution of using dim lighting was necessary. This was achieved

by the use of curtains which extended from the floor to the ceiling to exclude most of the ambient light from the working area.

2.3.3.3 The determination of benzo[a]pyrene hydroxylase activity

Benzo[a]pyrene hydroxylase activity was measured as described by Nebert and Gelboin (1968). The total incubation volume of 1ml contained 0.2mg of microsomal protein, 0.1M potassium phosphate buffer (pH 7.5), 80μM benzo[a]-pyrene (in 20μl acetone) and 100μl of an NADPH regenerating system (5.0 μmoles of sodium isocitrate, 0.5 μmoles of $NADP^+$, 5 μmoles of $MgCl_2$ and 0.65 units of isocitric dehydrogenase made up to 0.100ml with PBS). Samples were preincubated at 37°C for 2 minutes in a shaking water bath before starting the reaction by the addition of the NADPH regenerating system. After 10 minutes, the reaction was stopped by the addition of 4.25ml of acetone-hexane (1:3, V:V) and immediately vortexed vigorously for 30 seconds. The sample was then centrifuged for 2 minutes (600 g, room temp.) to produce a better separation of the upper and lower layers. 2.5ml of the upper organic layer was transferred to another test tube containing 2.5ml of 1N NaOH. After vortexing and centrifugation, the fluorescence in the lower aqueous phase was measured using an extinction wavelength of

398nm and an emission wavelength of 522nm. Determination of product quantity was calculated by use of a standard curve consisting of various amounts (0-10nmoles) of 3-hydroxy-benzo[a]pyrene in 1N NaOH.

CHAPTER 3

RESULTS

3.1 Erythrocyte Studies

3.1.1 The effect of Prudhoe Bay crude oil (PBCO) on herring gull erythrocytes

Data in Table 3.1 show that a DMSO extract of PBCO (15µl) induced metHb formation (3.0%, $P < .01$[1]) in herring gull erythrocytes. However, when an activation system consisting of microsomes and NADPH was present, the extent of metHb formation was increased significantly to 11.6% ($P < .005$). The activation system and the DMSO extract of PBCO also significantly increased metHb to 3.6% ($P < .01$) and 3.0% ($P < .01$) respectively.

Figure 3.1 shows the effect of incubating gull erythrocytes for 1 hour with varying amounts of a DMSO extract of PBCO or a DMSO solution of metabolized PBCO on metHb formation, hemolysis and GSH depletion. DMSO was run as a control with 50µl inducing metHb formation (2.5%; Figure 3.1A), hemolysis (2.7%; Figure 3.1B) and significant GSH depletion from 7.94 nmoles/ml packed RBC to 5.84 nmoles/

---

[1]All statistical calculations were performed by using the Student's t-test.

Table 3.1. The effect of a DMSO extract of PBCO on the levels of metHb in herring gull erythrocytes.

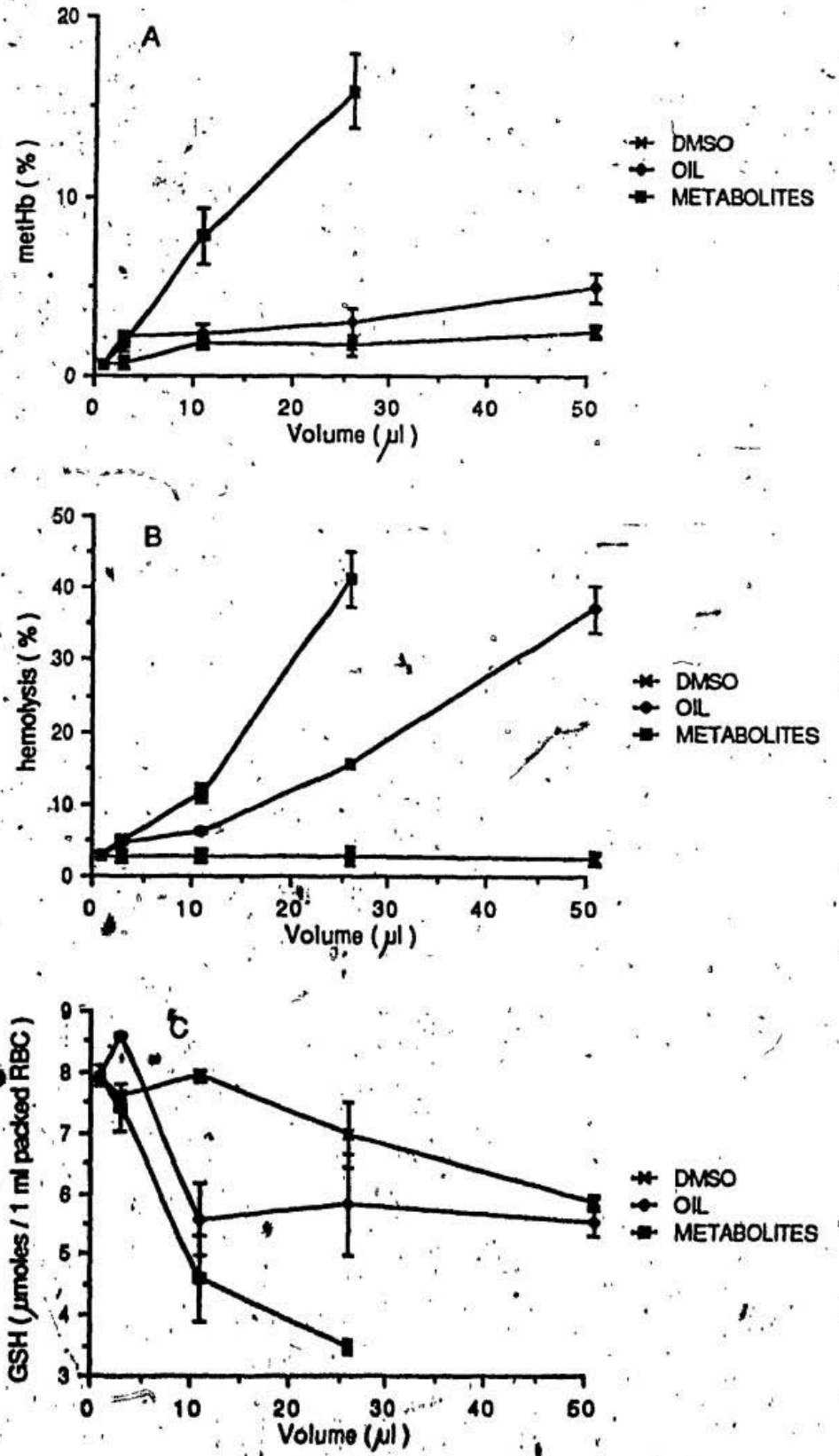
All incubations were carried out at 37°C for 60 minutes (rates were linear during this time period) and included in a total volume of 1 ml: PBS (pH 7.4), Herring gull erythrocytes (25% PCV). The activation system per 1 ml of reaction mixture consisted of: 1 mg of PB induced rat liver microsomes (prepared as described in Methods (2.3.2.1), 5 μmoles of sodium isocitrate, 0.4 μmoles of $NADP^+$, 5 μmoles of $MgCl_2$ and 0.65 units of isocitric dehydrogenase. Three separate experiments (3 separate batches of blood) were carried out with duplicate assays. Values are means ± standard deviations. All the following experiments that involved erythrocytes were performed using three different batches of blood.

SUBSTRATE	ACTIVATION SYSTEM	metHb(%)
Control (no PBCO)	-	1.8±0.4
Control (no PBCO)	+	3.6±0.7
PBCO (DMSO extract, 15µl)	-	3.0±0.3
PBCO (DMSO extract, 15µl)	+	11.6±0.6

Figure 3.1. The effect of the DMSO extract of PBCO or metabolized PBCO on metHb, hemolysis, and GSH levels in gull erythrocytes.

Blood was collected from two animals and pooled. Incubations were carried out at 37°C for 60 minutes and contained in a total volume of 1 ml: PBS, gull erythrocytes (25% PCV), and 2-50 µl of DMSO, DMSO extracts of PBCO or metabolized PBCO. Details of metHb (A), hemolysis (B) and GSH (C) measurements are outlined in Methods (2.2.8, 2.2.7, 2.2.9.1, respectively). Three separate experiments (3 separate batches of blood) were carried out. Values are means ± standard deviations.

Abbreviations: OIL; DMSO extract of PBCO
METABOLITES; metabolized DMSO extract of PBCO


A
metHb (%)
Volume (µl)
DMSO
OIL
METABOLITES
B
hemolysis (%)
Volume (µl)
DMSO
OIL
METABOLITES
C
GSH (µmoles / 1 ml packed RBC)
Volume (µl)
DMSO
OIL
METABOLITES

ml packed RBC ($P < .005$) (Figure 3.1C). Increasing concentrations of DMSO extract of PBCO (oil) induced a significant elevation in metHb formation (5.0% with 50µl; $P < .005$; Fig. 3.1A), a significant increase in the level of hemolysis (38% with 50µl; $P < .005$; Figure 3.1B) and a substantial decrease in GSH levels (5.53 nmole/ml packed RBCs with 50µl; $P < .005$; Figure 3.1C). There was no significant difference ($P > .05$) between 50µl of DMSO and the DMSO extract of PBCO in their ability to reduce GSH levels. Metabolized PBCO, however, induced metHb (15.7% with 25µl; Figure 3.1A) and hemolysis (42.7% with 25µl; Figure 3.1B). Addition of 25µl of metabolized PBCO significantly ($P < .005$) depleted GSH from the control value of 7.94 nmoles/ml packed RBCs to 3.47 nmoles/ml packed RBCs (Figure 3.1C).

3.1.2 The effect of PBCO on human erythrocytes

The results in Table 3.2 demonstrate that the levels of metHb and glutathione in human erythrocytes are also affected by PBCO. At the end of incubation the levels of metHb in the erythrocytes were 2.7%, 5.6% and 17.2% in the presence of 25µl/ml DMSO, DMSO extract of PBCO, and metabolized PBCO respectively.

Total glutathione decreased from the control value of 2.47 µmoles/ml packed RBC to 2.18 ($P < .01$), 1.92 and 1.53

Table 3.2. The effect of PBCO and metabolized PBCO on metHb, total glutathione and GSSG levels in human erythrocytes.

Incubations were carried out at 37°C for 60 minutes and contained in a total volume of 2 ml; PBS (pH 7.4), human erythrocytes (25% PCV) and 25 µl of DMSO, PBCO (DMSO extract) or metabolized PBCO. At the end of incuation, metHb, total glutathione (Tietze method) and GSSG (Tietze method) were measured. For further details, see Methods (2.2.8, 2.2.9.2 and 2.2.9.2) respectively. Three separate experiments were carried out. Values are means ± stardard deviations.

INCUBATION CONDITIONS	metHb %	Total Glutathione (μmoles/ml packed RBC)	GSSG % of total glutathione
No addition	1.7±0.6	2.47±0.10	4.2±0.2
25μl DMSO/ml	2.7±0.5	2.18±0.06	4.8±0.4
25μl PBCO (DMSO Extract)/ml	5.6±0.4	1.92±0.10	7.4±0.6
25μl metabolized PBCO/ml	17.2±0.5	1.53±0.06	11.2±0.5

in the presence of DMSO, DMSO extract of PBCO, and metabolized PBCO respectively.

The elevation in levels of oxidized glutathione (GSSG) from the control value of 4.2% (% of total glutathione) was observed. DMSO, DMSO extract of PBCO, and metabolized PBCO significantly elevated GSSG levels to 4.8% ($P < .05$), 7.4% ($P < .005$) and 11.2% ($P < .005$) respectively.

3.1.3 The effect of PBCO on human HbO_2

The effect of the amount of PBCO on human HbO_2 is summarized in figure 3.2. The DMSO extract of PBCO and metabolized PBCO induced a concentration dependent increase in metHb formation. Consistent with results obtained with erythrocytes, metabolized PBCO was found to be more effective in inducing metHb formation. There was a significant ($P < .05$) increase in metHb with 50µl of DMSO (5.43%) versus 50µl of the DMSO extract of PBCO (8.81%).

Figure 3.3 shows the time course of metHb formation from HbO_2 during incubation with a DMSO extract of PBCO or metabolized PBCO. There was a time dependent increase in metHb formation from PBCO (Figure 3.3A) to 1 hour of incubation but from metabolized PBCO, there was an increase in metHb formation up to 4 hours. Incubations containing

Figure 3.2. The effect of volume of the DMSO extract of PBCO or metabolized PBCO on metHb formation from human HbO_2.

Incubations were carried out at 37°C for 60 minutes, and contained in a total volume of 1 ml; PBS (pH 7.4), 50 mg of human HbO_2 (for detailed procedure of preparation of human HbO_2, see methods (2.2.4)), and 5, 10, 25 or 50 µl of DMSO extract of PBCO or metabolized PBCO. Samples were removed at the end of 60 minutes for metHb determination as outlined in methods (2.2.8). Three separate experiments were carried out. Values are means ± standard deviations.

Abbreviations: OIL; DMSO extract of PBCO
METABOLITES; metabolized DMSO extract of PBCO

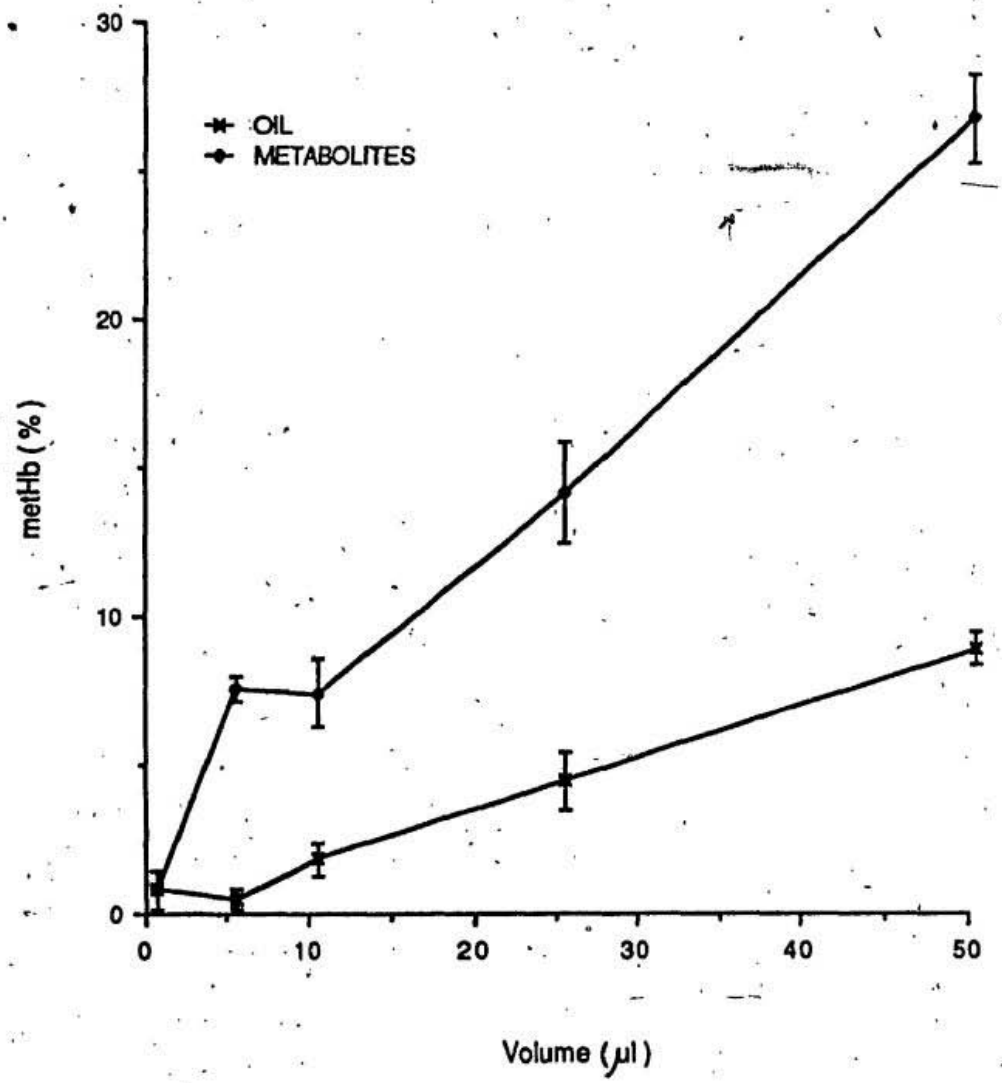
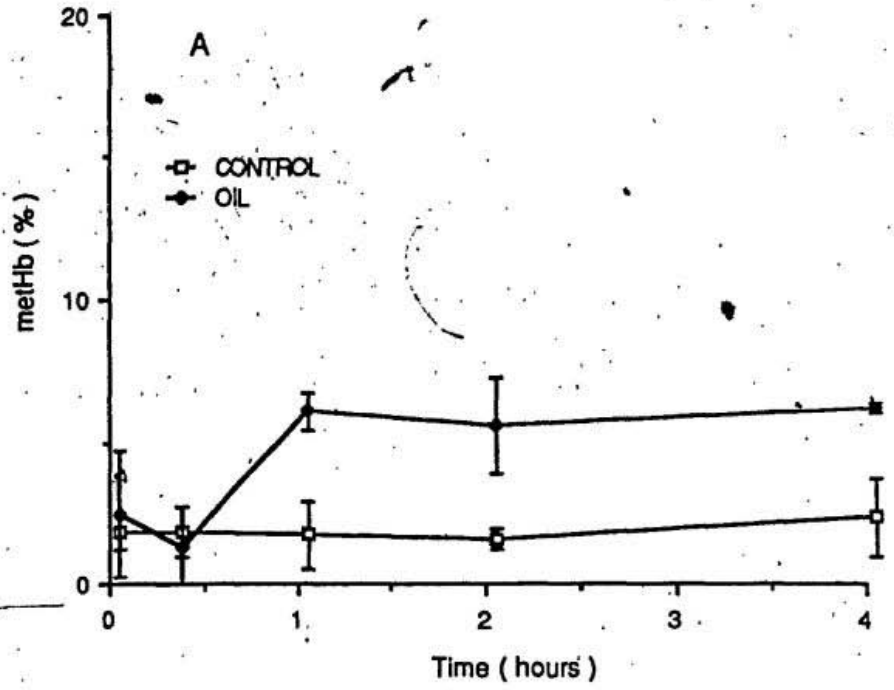
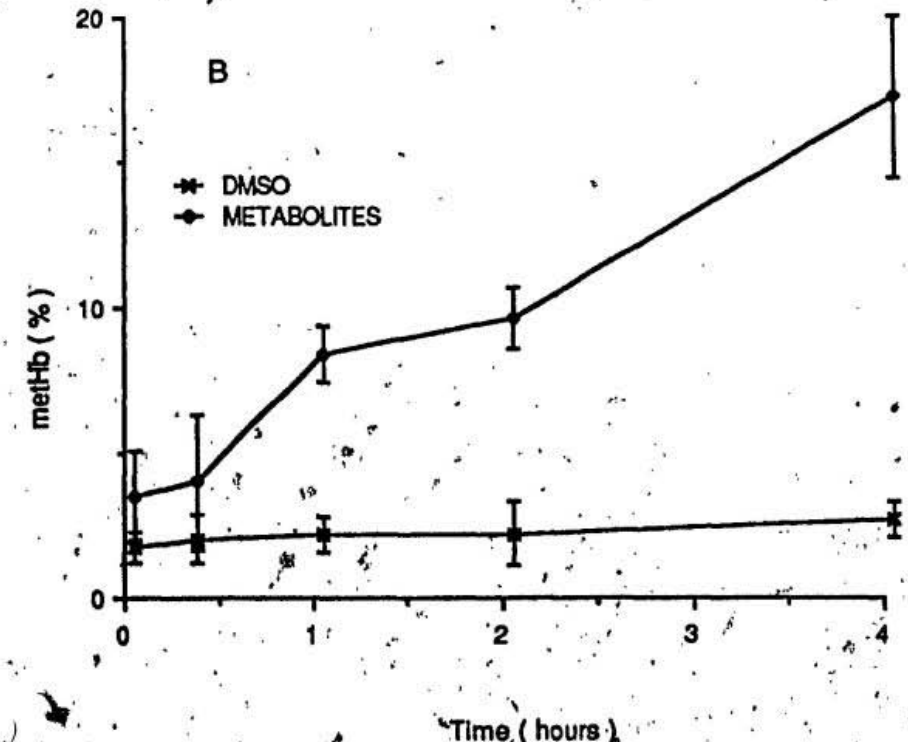

OIL
METABOLITES
metHb (%)
Volume (µl)
0
10
20
30
40
50

Figure 3.3. Time course on the effect of the DMSO extract of PBCO and metabolized PBCO on metHb formation in human HbO_2.

Incubations were carried out at 37°C for 4 hours and contained in a total volume of 1 ml; PBS (pH 7.4), 50 mg HbO_2, 10 µl of DMSO or DMSO extracts of PBCO. Samples were removed at 0 mins, 20 mins, 1 hr, 2 hrs and 4 hrs for metHb determination as outlined in Methods (2.2.8). Three separate experiments were carried out. Values are means ± standard deviations.

Abbreviations: OIL; DMSO extract of PBCO
METABOLITES; metabolized DMSO extract of PBCO


A
CONTROL
OIL
metHb (%)
20
10
0
0
1
2
3
4
Time (hours)


B
DMSO
METABOLITES
metHb (%)
20
10
0
0
1
2
3
4
Time (hours)

DMSO (Figure 3.3B) or no substrate (Figure 3.3A) did not demonstrate any increase in MetHb formation.

3.1.4 The effect of naphthalene and its derivatives on herring gull erythrocytes

Aromatic hydrocarbons like naphthalene and methylated naphthalenes such as 2-methylnaphthalene and 2,6-dimethylnaphthalene induce metHb (6.9%, 6.2% and 4.3% respectively) in gull erythrocytes as indicated in Table 3.3. These values are slightly higher than the 1.5% metHb observed in erythrocytes alone. But as observed with PBCO, (Table 3.1) inclusion of microsomes and NADPH significantly elevated metHb formation to 34.0%, 48.3%, and 18.2% respectively after 60 minutes of incubation. Incubation of hydroxylated naphthalenes such as 1-naphthol, 1,2- or 1,4-dihydroxynaphthalene or 2-methyl-1-naphthol gave rise to extensive metHb formation (26.3%, 78.0%, 79.0%, and 96.6% respectively) without any microsomal activation. 1,2 naphthoquinone also induced a substantial increase of MetHb levels (67.8%).

Table 3.3. The effect of naphthalene and some of its derivatives on the levels of metHb in herring gull erythrocytes.

All incubations were carried out at 37°C for 60 minutes and in a total volume of 1 ml: PBS (pH 7.4), Herring gull erythrocytes (25% PCV), and naphthalene, 2-methylnaphthalene, 2,6-dimethylnaphthalene, 1-naphthol, 1,2-dihydroxynaphthalene, 1,4-dihydroxynaphthalene, 2-methyl-1-naphthol or 1,2-naphthoquinone. Each substrate was dissolved in 5µl of DMSO at an amount to give a final concentration to give 0.5 mM. The activation system per 1 ml of reaction mixture consisted of 1 mg of PB induced rat liver microsomes (preparation as described in Methods (2.3.2.1), 5 µmoles of sodium isocitrate, 0.4 µmoles of $NADP^+$, 5 µmoles of $MgCl_2$ and 0.65 units of isocitric dehydrogenase. Three separate experiments were carried out. Values are means ± standard deviations.

SUBSTRATE	ACTIVATION SYSTEM	MetHb(%)
No addition	-	1.5±0.3
No addition	+	4.5±0.3
Naphthalene	-	6.9±0.5
Naphthalene	+	34.0±2.2
2-Methylnaphthalene	-	6.4±0.3
2-Methylnaphthalene	+	48.3±2.4
2,6-Dimethylnaphthalene	-	4.3±0.4
2,6-Dimethylnaphthalene	+	18.2±1.8
1-Naphthol	-	26.3±1.5
1,2-Dihydroxynaphthalene	-	78.0±2.9
1,4-Dihydroxynaphthalene	-	79.0±5.3
2-Methyl-1-naphthol	-	96.8±3.0
1,2-Naphthoquinone	-	67.8±4.2

3.1.5 The effect of naphthalene and its derivatives on metHb formation in human HbO_2.

Table 3.4 shows that naphthalene but not 1-naphthol, 1,2-naphthoquinone, 1,4-naphthoquinone, 1,2-dihydroxynaphthalene require metabolic activation to induce metHb formation from HbO_2. This is consistent with data obtained using herring gull erythrocytes (Table 3.3). 2,6-Dimethylnaphthalene gave a slight but significant ($P < .05$) increase in metHb (6.1% at 60 minutes). Although 1-naphthol by itself gave a substantial increase in metHb (38.4% at 60 minutes), the addition of microsomes and NADPH increased the metHb level to 47.2%.

2-Naphthol and 1,3-dihydroxynaphthalene induced a slight but significant increase in metHb from HbO_2 (5.5%; $P < .005$), 5.2%; $p < .05$ respectively) (Table 3.4). In the presence of the activation system there was a decrease in metHb formation for 1,2 naphthoquinone, 1,4-naphthoquinone, 1,2-dihydroxynaphthalene and 1,4-dihydroxynaphthalene.

1,3-Dihydroxynaphthalene (Figure 3.5A) did not significantly ($P > .05$) elevate metHb levels in human erythrocytes after a 60 minute incubation period.

Table 3.4. the effect of naphthalene and its derivatives on metHb formation from human HbO_2.

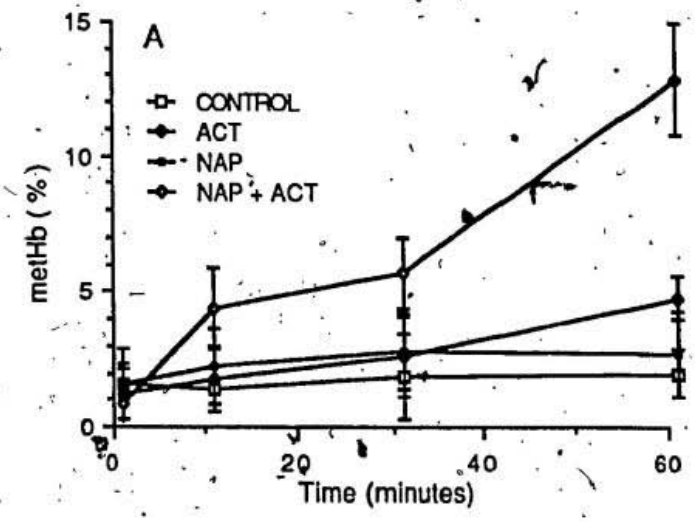
All incubations were carried out at 37°C for 60 minutes and contained in a total volume of 1 ml: PBS (pH 7.4), human HbO_2 (50 mg), and naphthalene, 2,6-dimethylnaphthalene, 1-naphthol, 2-naphthol, 1,2-naphthoquinone, 1,4-naphthoquinone, 1,2-dihydroxynaphthalene, 1,3-dihydroxynaphthalene or 1,4-dihydroxynaphthalene. All compounds were dissolved in 5µl of DMSO to give a final concentration of 0.5 mM. The activation system consisted of 1 mg of PB induced rat liver microsomes (preparation as discussed in Methods (2.3.2.1), 5 µmoles of sodium isocitrate, 0.4 µmoles of $NADP^+$, 5 µmoles of $MgCl_2$ and 0.65 units of isocitric dehydrogenase. Three separate experiments were carried out. Values are means ± standard deviations.

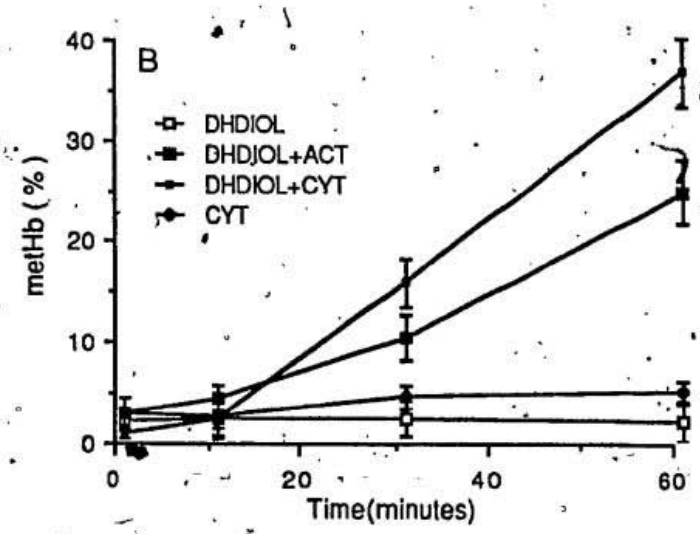
SUBSTRATE (0.5 mM)	ACTIVATION SYSTEM	MetHB (%) 5 MIN	60 MIN
No addition	-	2.0±1.4	1.00±0.0
No addition	+	2.1±1.5	4.0±1.0
Naphthalene	-	0.9±1.2	0.0±0.0
Naphthalene	+	2.3±0.5	18.5±0.9
2,6-Dimethylnaphthalene	-	1.5±0.7	6.1±3.9
2,6-Dimethylnaphthalene	+	4.2±0.6	16.7±5.4
1-Naphthol	-	7.5±2.2	38.4±4.1
1-Naphthol	+	9.1±0.6	47.2±1.3
2-Naphthol	-	2.8±0.1	5.5±1.7
2-Naphthol	+	4.6±1.2	30.7±0.4
1,2-Naphthoquinone	-	42.4±6.8	73.6±2.8
1,2-Naphthoquinone	+	23.6±1.2	50.1±2.3
1,4-Naphthoquinone	-	3.6±0.6	42.2±2.5
1,4-Naphthoquinone	+	4.2±3.0	23.4±0.6
1,2-Dihydroxynaphthalene	-	36.6±2.4	73.4±3.1
1,2-Dihydroxynaphthalene	+	21.7±3.0	41.3±1.8
1,3-Dihydroxynaphthalene	-	2.1±0.1	5.2±3.1
1,3-Dihydroxynaphthalene	+	4.3±0.3	9.0±0.5
1,4-Dihydroxynaphthalene	-	3.3±1.7	33.2±8.1
1,4-Dihydroxynaphthalene	+	5.4±0.3	20.3±4.4

Figure 3.4. The effect of naphthalene and naphthalene derivatives on metHb formation in human erythrocytes.

Incubations were carried out at 37°C for 1 hour and contained in a total volume of 1 ml; PBS (pH 7.4), human erythrocytes (25% PCV), 0.5 mM naphthalene (Figure 3.4A), trans 1,2-dihydroxy-1,2 dihydronaphthalene (Figure 3.4B), 1-naphthol or 2-naphthol (Figure 3.4C). The activation system (ACT), consisted of 1 mg of PB induced rat liver microsomes (prepared as described in Methods 2.3.2.1), 5 μmoles of sodium isocitrate, 0.4 μmoles of $NADP^+$, 5 μmoles of $MgCl_2$ and 0.65 units of isocitric dehydrogenase. Trans-1,2-dihydroxy-1,2- dihydronaphthalene was also incubated in the presence of rat liver cytosol (CYT), nontreated; equivalent to 1 mg of protein, and 2.3 mM $NADP^+$. Samples were removed at 0, 10, 30 and 60 minutes for metHb determination as described in Methods (2.2.8). Three separate experiments were carried out. Values are means ± standard deviations.

Abbreviations: NAP; naphthalene
DHDIOL; trans-1,2-dihydroxy-1,2-dihydronaphthalene
1-NAPOL; 1-naphthol
2-NAPOL; 2-naphthol
CYT; rat liver cytosol
ACT; activation system


A
CONTROL
ACT
NAP
NAP + ACT
metHb (%)
Time (minutes)


B
DHDIOL
DHDJOL+ACT
DHDIOL+CYT
CYT
metHb (%)
Time(minutes)

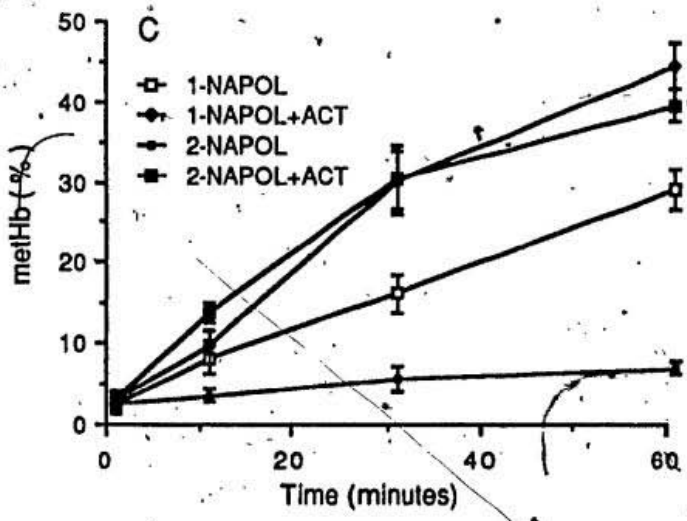
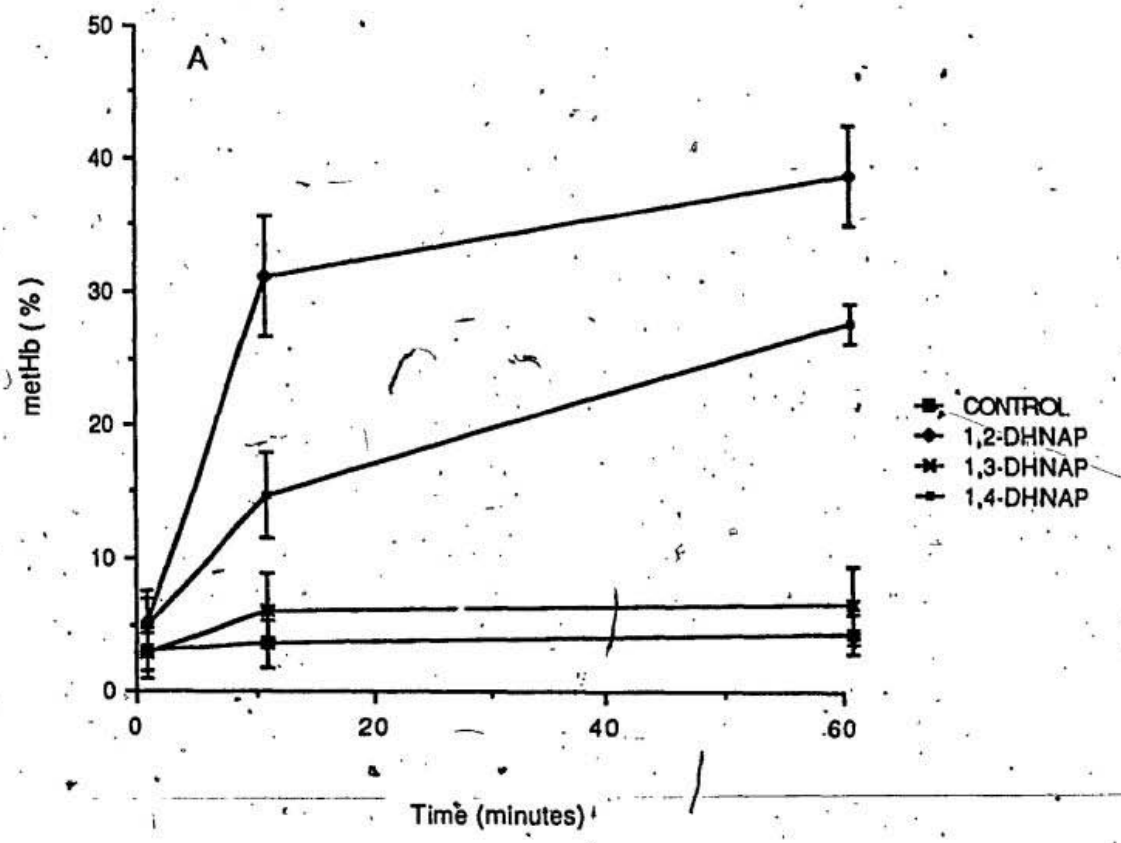
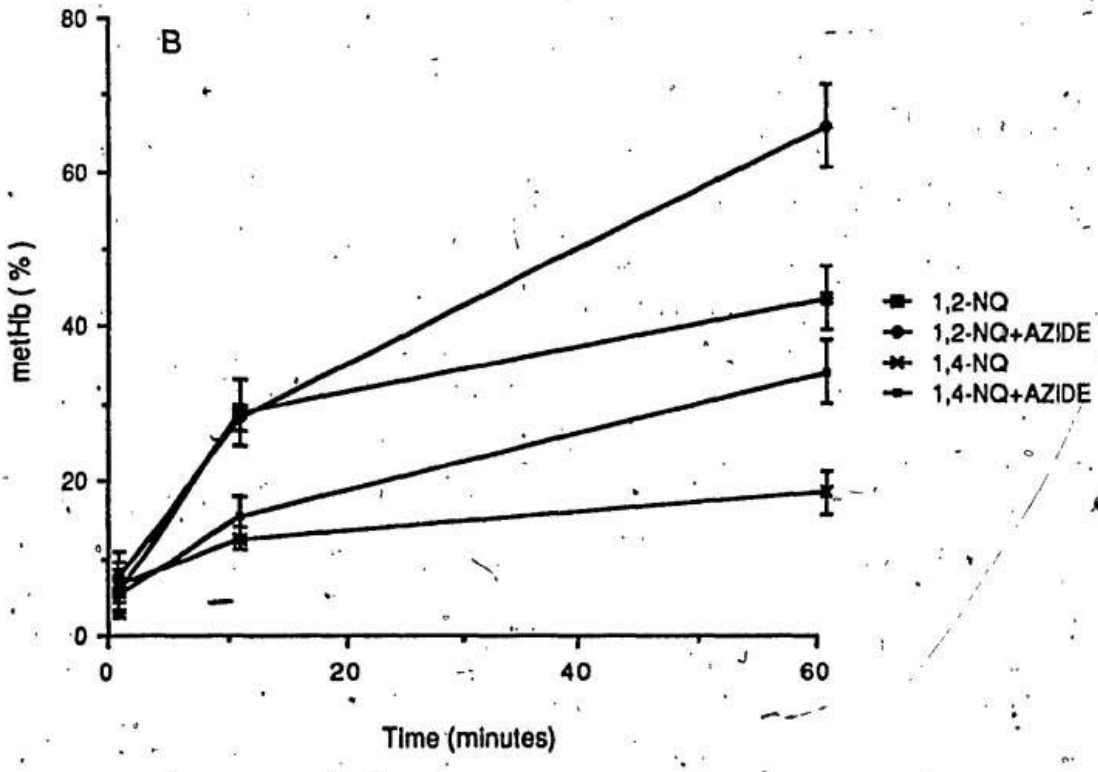

C
1-NAPOL
1-NAPOL+ACT
2-NAPOL
2-NAPOL+ACT
metHb (%)
Time (minutes)

Figure 3.5. Time course of metHb formation from human erythrocytes by naphthoquinones and dihydroxynaphthalenes.

Incubations were carried out at 37°C and contained in a total volume of 1 ml; PBS (pH 7.5), human erythrocytes (25% PCV), 50 μM 1,2-naphthoquinone (1,2-NQ), 1,4-naphthoquinone (Figure 3.5B), 1,2-dihydroxynaphthalene (1,2-DHNAP), 1,3-dihydroxynaphthalene, or 1,4-dihydroxynaphthalene (Figure 3.5A). 1,3-Dihydroxynaphthalene had a concentration of 0.5 mM. The control contained no substrate. The naphthoquinones were also incubated in the presence of 1.0 mM sodium azide (NaN_3). All substrates were dissolved in DMSO (5μl per 1 ml incubation). Samples were removed at 0, 5 and 60 minutes for determination of metHb as described in Methods (2.2.8). Three separate experiments were carried out. Values were means ± standard deviations.

Abbreviations: DHNAP; dihydroxynaphthalene
NQ; naphthoquinone


A
metHb (%)
50
40
30
20
10
0
0
20
40
60
Time (minutes)
CONTROL
1,2-DHNAP
1,3-DHNAP
1,4-DHNAP


B
metHb (%)
80
60
40
20
0
0
20
40
60
Time (minutes)
1,2-NQ
1,2-NQ+AZIDE
1,4-NQ
1,4-NQ+AZIDE

3.1.6 The effect of naphthalene and its derivatives on the level of metHb in human erythrocytes.

In human erythrocytes, naphthalene (figure 3.4A) and its derivative such as trans-1,2-dihydroxy-1,2-dihydronaphthalene (figure 3.4B) did not significantly elevate metHb levels after 60 minutes of incubation (2.63%; $P > .05$, 2.13%; $P > .05$ respectively). 2-Naphthol produced a slight but significant ($P < .005$) increase in metHb from 1.97% to 7.04%. However, when an activation system was present, naphthalene, trans-1,2-dihydroxy-1,2-dihydronaphthalene, and 2-naphthol elevated metHb levels to 12.7%, 24.9% and 39.6% respectively at the end of the 60 minute incubation period. Microsomes and NADPH alone significantly elevated metHb levels to 4.7% ($P < .05$).

When trans-1,2-dihydroxy-1,2-dihydronaphthalene was incubated in the presence of rat liver cytosol and $NADP^+$, metHb was elevated to 36.0% after 60 minutes of incubation (Figure 3.4B).

Also consistent with metHb formation from HbO_2 (Table 3.4) is the significant increase in metHb in human erythrocytes by compounds such as 1,2-dihydroxynaphthalene (Figure 3.5A), 1,4-dihydroxynaphthalene (Figure 3.5A), 1,2-naphthoquinone (Figure 3.5B) and 1,4-naphthoquinone (Figure 3.5B). With these compounds, metHb levels were elevated to

38.1%, 27.5%, 42.3% and 18.3% respectively at the end of the 60 minute incubation period. These compounds were very potent considering that the concentration in the incubations were 0.05 mM, while the other substrates tested were 0.50mM.

In the presence of 1.0 mM sodium azide (Figure 3.5B), 1,2- and 1,4-naphthoquinone significantly ($P < .005$) elevated metHb levels above those obtained with 1,2- and 1,4-naphthoquinone alone. Sodium azide is an inhibitor (reversible) of catalase.

3.1.7 The effect of naphthalene and its derivatives on the level of reduced glutathione (GSH) in human erythrocytes

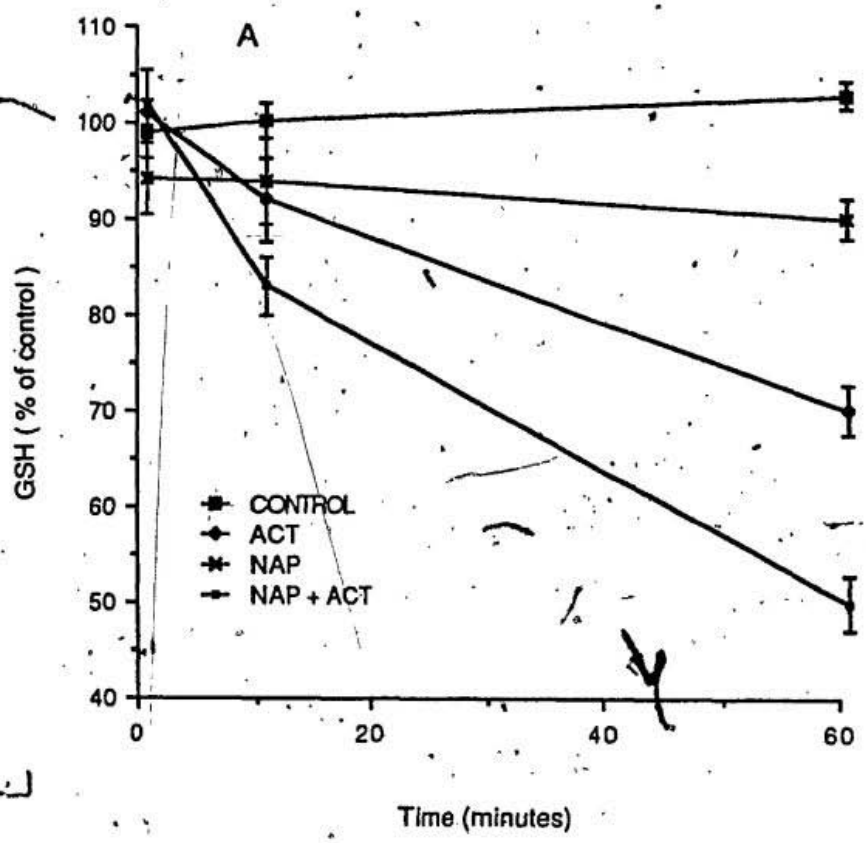
Depletion of GSH was similar to changes in metHb levels. Figure 3.6A shows that in the presence of 0.5mM naphthalene, the GSH level was approximately 93% of the control (no substrate) throughout the 60 minute incubation period. However, in the presence of NADPH and microsomes, a final value of 50.0% of control was observed. Similarly, with 0.5mM 2-naphthol (Figure 3.6B) activation was needed to cause depletion of GSH to 51.8% of control values.

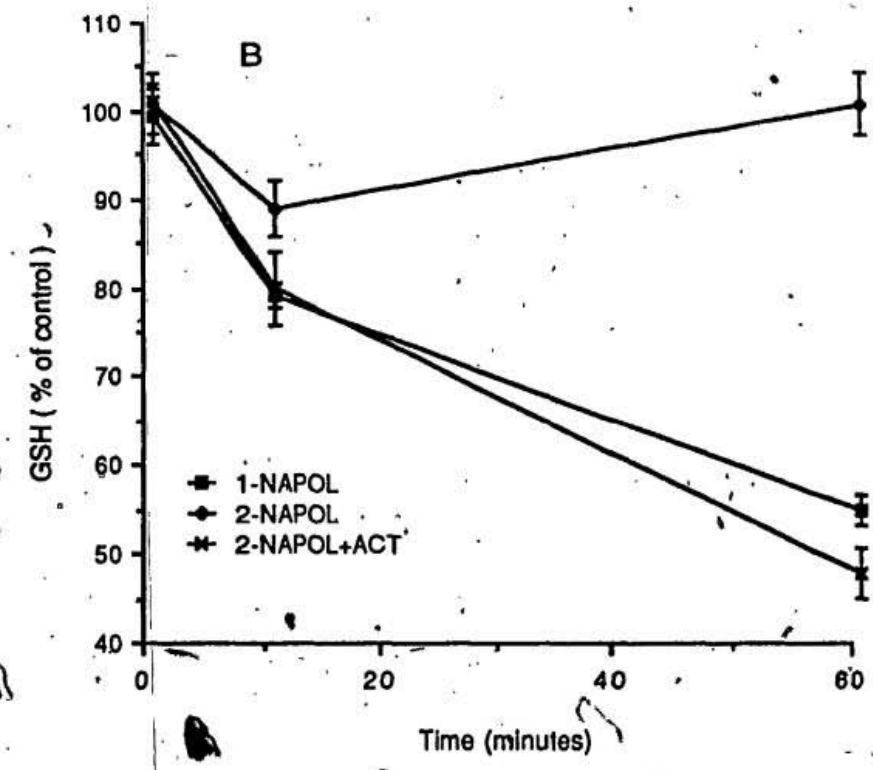
Of special interest was the observation that microsomes and NADPH depleted GSH to about 69.6% of the control value (Figure 3,6). However, evidence indicates the microsomal

Figure 3.6. The effect of naphthalene, 1-naphthol and 2-naphthol on GSH depletion in human erythrocytes.

Intubations were carried out at 37°C for 60 minutes and contained in a total volume of 2 ml; PBS (pH 7.5), human erythrocytes (25% PCV), 0.5 mM naphthalene (NAP), (Figure 3.6A), 1-naphthol or 2-naphthol (1-NAPOL, 2-NAPOL), (Figure 3.6B). Compounds were added in 5 µl of DMSO per 1ml of reaction mixture. The activation system consisted of; 1 mg PB induced rat liver microsomes (preparation as described in Methods (2.3.2.1), 5 µmoles of sodium isocitrate, 0.4 µmoles of $NADP^+$, 5 µmoles of $MgCl_2$ and 0.65 units of isocitrate dehydrogenase. Samples were removed at 0, 10, and 60 minutes for GSH determination as described in Methods (2.2.9.3). The value of GSH in tube containing no substrate was 1.93 ± 0.14 µmoles/ml packed RBC's. Three separate experiments were carried out. Values are means ± standard deviations.

Abbreviations: NAP; naphthalene
NAPOL; naphthol
ACT; activation system


A
GSH (% of control)
110
100
90
80
70
60
50
40
0
20
40
60
Time (minutes)
CONTROL
ACT
NAP
NAP + ACT


B
GSH (% of control)
110
100
90
80
70
60
50
40
0
20
40
60
Time (minutes)
1-NAPOL
2-NAPOL
2-NAPOL+ACT

oxidation of NADPH may produce superoxide radicals (Fridovich and Handler, 1961) and hydrogen peroxide (Gillette et al., 1957).

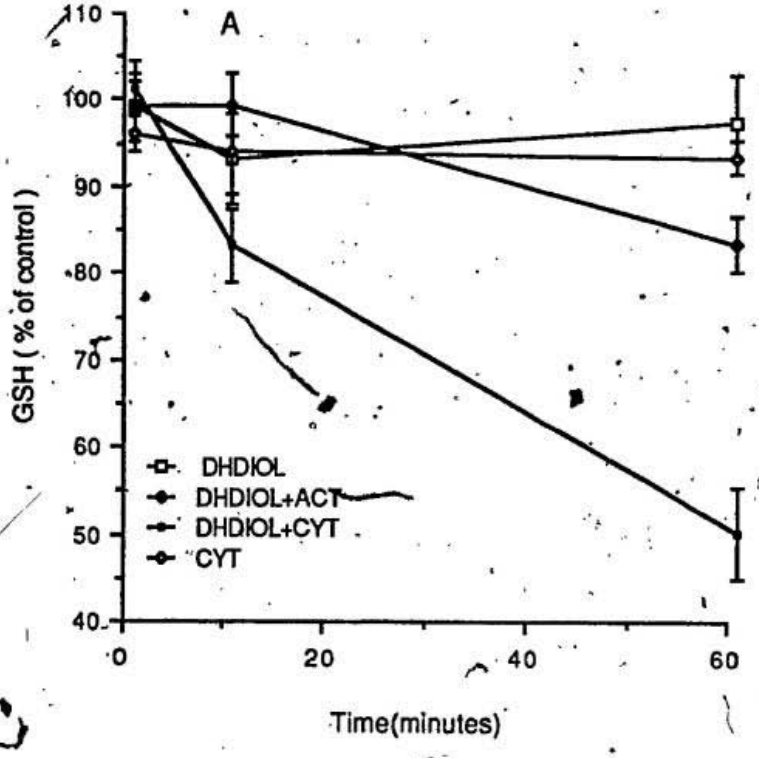
Consistent with the results of metHb studies, 0.5mM trans 1,2-dihydroxy-1,2-dihydronaphthalene by itself did not significantly ($P > .05$) change the GSH level at 60 minutes (Figure 3.7A) but in the presence of NADPH and microsomes or rat liver cytosol and $NADP^+$, GSH decreased from the control value by 17.8% and 50.4% respectively after 60 minutes of incubation. The other naphthalene derivatives such as 1-naphthol (Figure 3.6B), 1,2-dihydroxynaphthalene and 1,4-dihydroxynaphthalene decreased GSH by 44.9%, 90.3% and 60.5% respectively as compared to the control.

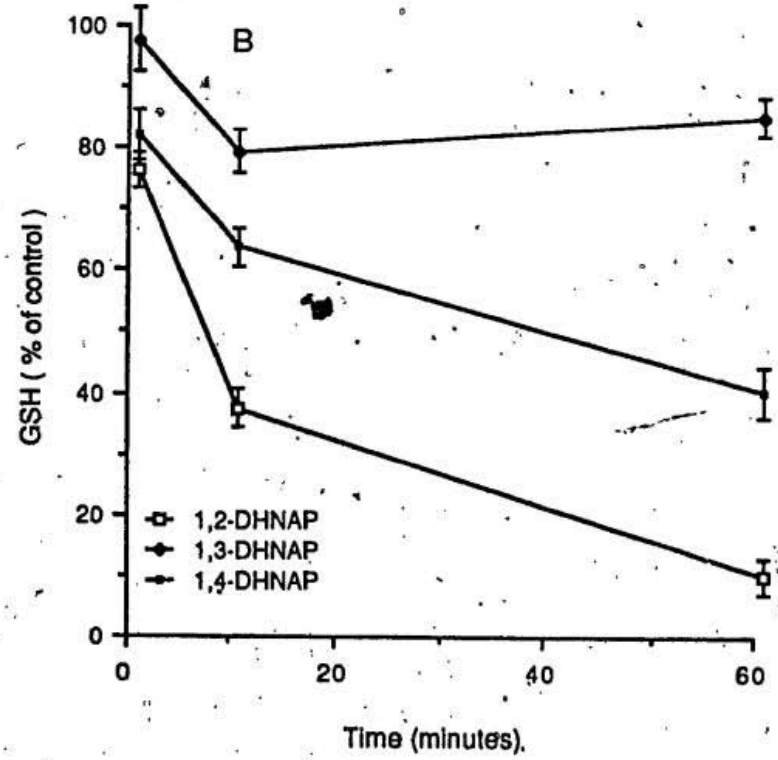
1,2-Dihydroxynaphthalene (Figure 3.7B) and 1,4-dihydroxynaphthalene (Figure 3.7B) gave substantial depletion of GSH at concentrations 10 fold lower (0.05mM) than the other compounds while 0.5mM 1,3-dihydroxynaphthalene significantly ($P < .05$) depleted GSH to 15.2% of control after 60 minutes of incubation.

Figure 3.7. The effect of trans-1,2-dihydroxy-1,2-dihydronaphthalene; 1,2-dihydroxynaphthalene, 1,3-dihydroxynaphthalene and 1,4-dihydroxynaphthalene on GSH depletion in human erythrocytes.

Incubations were carried out at 37°C for 60 minutes and contained in a total volume of 2 ml; PBS (pH 7.5), human erythrocytes (25% PCV), 0.5 mM trans 1,2-dihydroxy-1,2-dihydronaphthalene (DHDIOL) (Figure 3.7A), 1,2-dihydroxynaphthalene (1,2-DHNAP), 1,3-dihydroxynaphthalene or 1,4-dihydroxynaphthalene. Compounds were added in 5 µl DMSO/ml of reaction mixture. The activation system (ACT) per ml of reaction mixture consisted of: 1 mg PB induced rat liver microsome (preparation as described in Methods (2.3.2.1), 5 µmoles of sodium isocitrate, 0.4 µmoles of $NADP^+$, 5 µmoles of $MgCl_2$ and 0.65 units of isocitric dehydrogenase. Trans-1,2-dihydroxy-1,2-dihydronaphthalene was also incubated in the presence of rat liver cytosol (CYT), (nontreated; equivalent to 1 mg of protein) and 2.3 mM $NADP^+$. Samples were removed at 0, 10 and 60 minutes for GSH determination as described in Methods (2.2.9.3). The value of GSH in tubes containing no substrate was 1.97 ± 0.22 µmoles/ml packed RBC's. Three separate experiments were carried out. Values are means ± standard deviations.

Abbreviations: DHDIOL; trans-1,2-dihydroxy-1,2-dihydronaphthalene
DHNAP; dihydroxynaphthalene.
ACT; activation system
CYT; rat liver cytosol


A
GSH (% of control)
110
100
90
80
70
60
50
40
0
20
40
60
Time(minutes)
DHDIOL
DHDIOL+ACT
DHDIOL+CYT
CYT


B
GSH (% of control)
100
80
60
40
20
0
0
20
40
60
Time (minutes).
1,2-DHNAP
1,3-DHNAP
1,4-DHNAP

3.1.8 The effect of 1,2-naphthoquinone and 1,4-naphthoquinone on the levels of GSH and oxidized glutathione (GSSG) in human erythrocytes

One of the functions of GSH is to prevent the oxidation of physiologically important compounds in the body in which role it is oxidized to GSSG or conjugated with reactive metabolites. Using 0.05mM 1,2-naphthoquinone and 0.05mM 1,4-naphthoquinone as substrates, it is shown in Figure 3.8A and 3.8C that GSH levels were decreased in both cases. 1,2-naphthoquinone was more potent than 1,4-naphthoquinone, which resulted in decreases (after 60 minutes of incubation) by 65% and 40% of control values respectively. Also the levels of GSSG increased but the magnitude was much higher for 1,2-naphthoquinone (Figure 3.8B) than 1,4-naphthoquinone (Figure 3.8D).

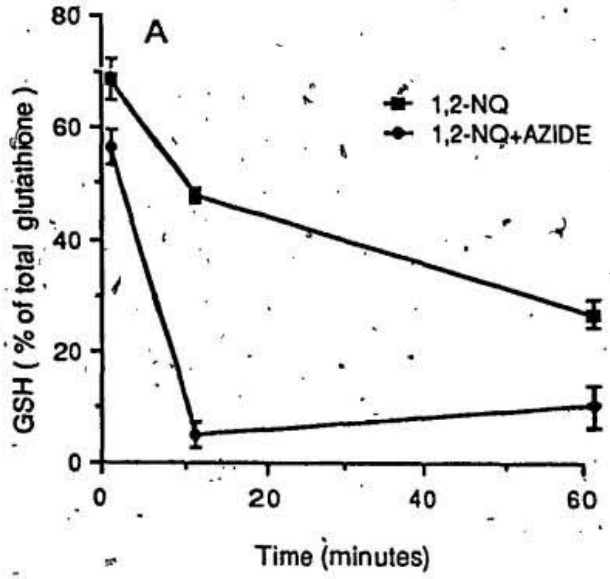
In the presence of sodium azide, 1,2-naphthoquinone and 1,4-naphthoquinone deplete GSH even further, and simultaneously GSSG increased (Figure 3.8B and Figure 3.8D respectively).

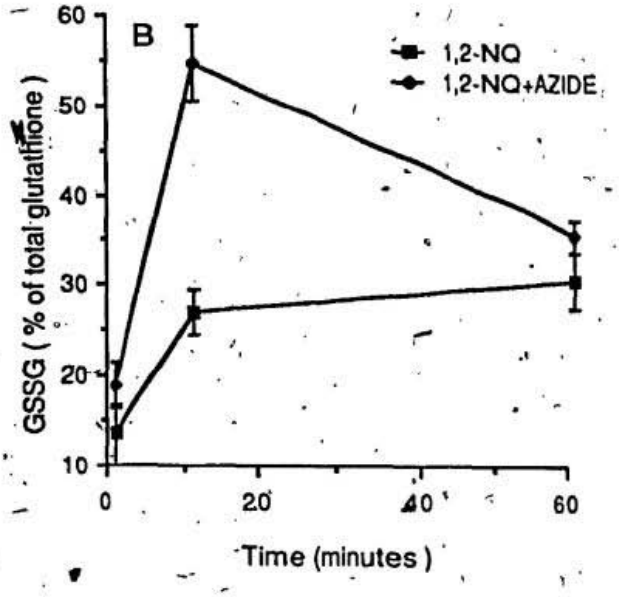
Also from these results, it is evident that the quinones produce drastic changes in GSH and GSSG within 10 minutes of incubation. The only exception to this trend is the change of GSH with 1,4-naphthoquinone.

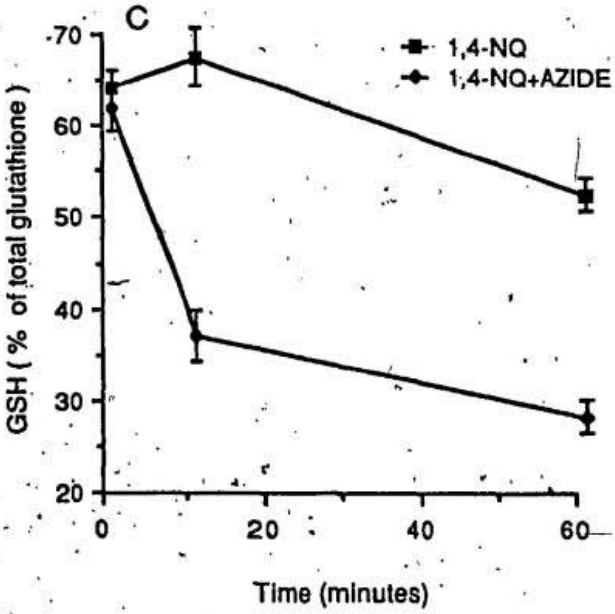
Figure 3.8. The effect of 1,2-naphthoquinone and 1,4-naphthoquinone on GSH and GSSG in human erythrocytes.

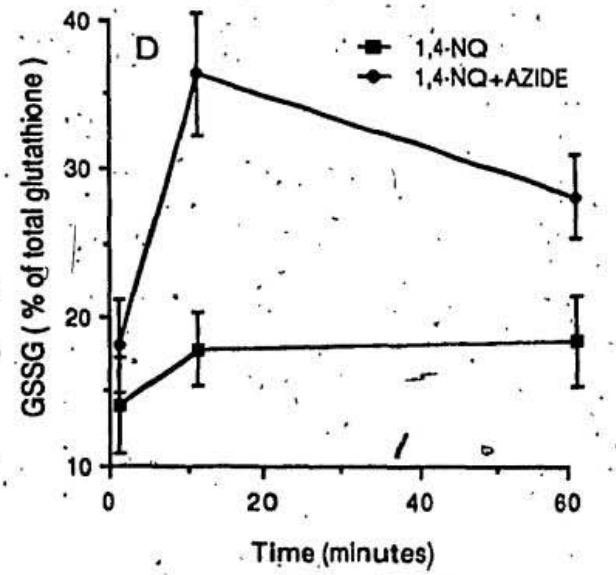
Incubations were carried out at 37°C for 60 minutes and contained in a total volume of 2 ml; PBS (pH 7.5), human erythrocytes (25% PCV), 50 μM 1,2-naphthoquinone (1,2-NQ) (Figure 3.8A and B) or 1,4-naphthoquinone (1,4-NQ) (Figures C and D) and when included, 1 mM sodium azide. Compounds were dissolved in 5 μl DMSO per ml reaction mixture. Samples were removed at 0, 10 and 60 minutes for the determination of GSH (Figure 3.8A and C) and GSSG (Figure 3.8B and D) as described in Methods (2.3.2.1). GSH is expressed as percentage of total glutathione (in GSH equivalents) in control tube. GSSG is expressed as percentage of total glutathione (in GSH equivalents). Total glutathione in erythrocytes was 2.13 ± 0.25 μmoles/ml packed RBCs. Three separate experiments were carried out. Values are means ± standard deviations.

Abbreviations: NQ; naphthoquinone
AZIDE; sodium azide


A
1,2-NQ
1,2-NQ+AZIDE
GSH (% of total glutathione)
Time (minutes)


B
1,2-NQ
1,2-NQ+AZIDE
GSSG (% of total glutathione)
Time (minutes)


C
1,4-NQ
1,4-NQ+AZIDE
GSH (% of total glutathione)
Time (minutes)


D
1,4-NQ
1,4-NQ+AZIDE
GSSG (% of total glutathione)
Time (minutes)

In all cases of these experiments, the decrease of GSH was not equivalent to the increase of GSSG. An interpretation of this result is given in the Discussion.

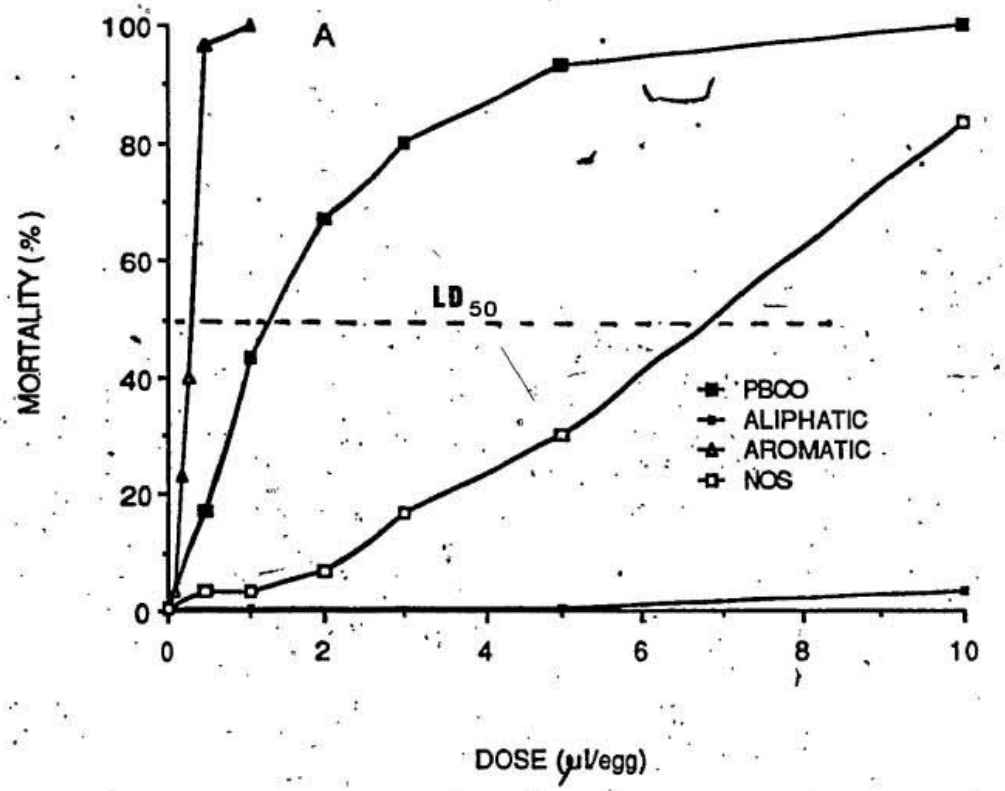
3.2 The toxicity of PBCO and its fractions on chick embryos

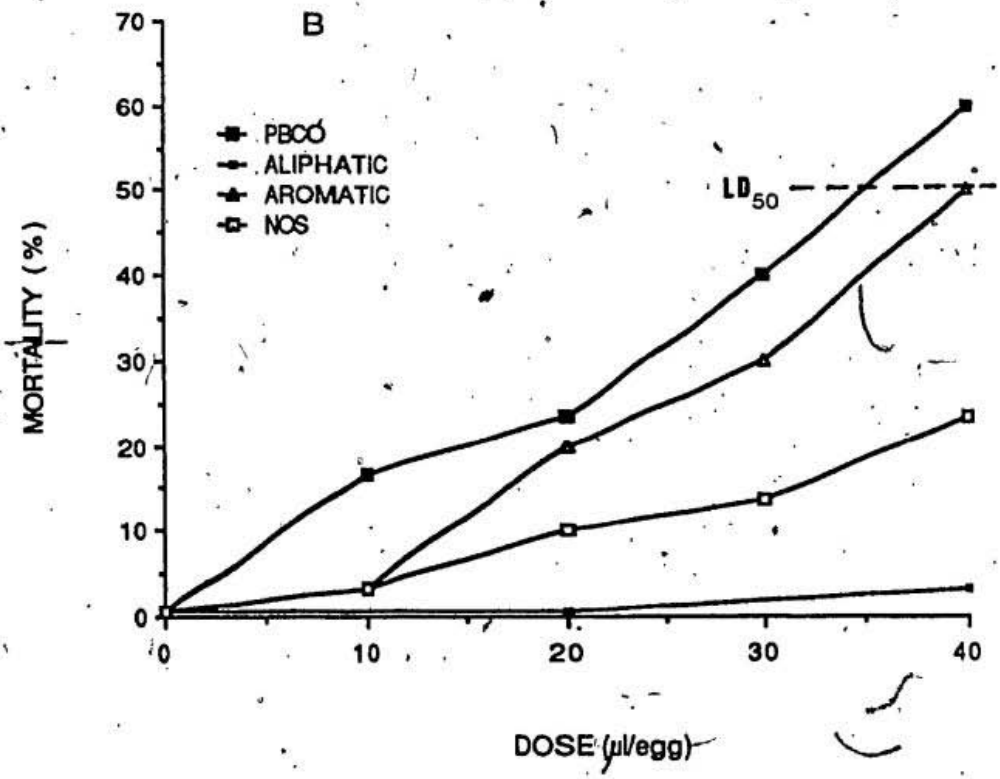
3.2.1 The effect of PBCO on mortality rates in 7 and 12 day old chick embryos

Figure 3.9A shows the effect of application of various amounts of PBCO or its fractions on chick embryo mortality. In general, there was increasing embryo mortality with increasing dosage. The 7 day old embryo was very sensitive to the effects of PBCO. The LD50s (day 7) for PBCO and its aliphatic, aromatic and NOS fractions were found to be 1.4µl, >>10µl, 0.4µl and 6.8µl respectively (Figure 3.9A). In contrast, the 12-day-old embryo was much less sensitive. In those embryos the LD50s for PBCO and its aliphatic, aromatic and NOS fractions were found to be 35µl, >>40µl, 40µl and >40µl respectively (Figure 3.9B).

Figure 3.9. Toxicity of PBCO and its aliphatic, aromatic and NOS fractions on the chick embryo.

Indicated volumes of PBCO or its aliphatic, aromatic, and NOS fractions were applied just below the airsac of fertile chicken eggs on day 7 (A) or 12 (B) of incubation as described in Methods (2.3.1.2). 30 eggs were used per group. Eggs were opened up on day 15 and 19 respectively, and dead embryos were counted.


A
MORTALITY (%)
100
80
60
40
20
0
LD50
PBCO
ALIPHATIC
AROMATIC
NOS
0
2
4
6
8
10
DOSE (µl/egg)

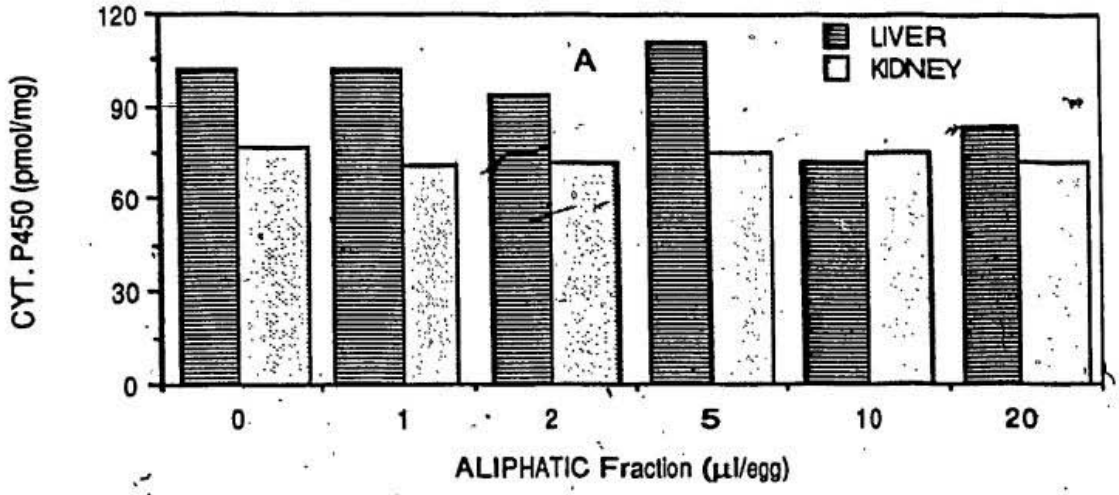

B
MORTALITY (%)
70
60
50
40
30
20
10
0
PBCO
ALIPHATIC
AROMATIC
NOS
LD50
0
10
20
30
40
DOSE (µl/egg)

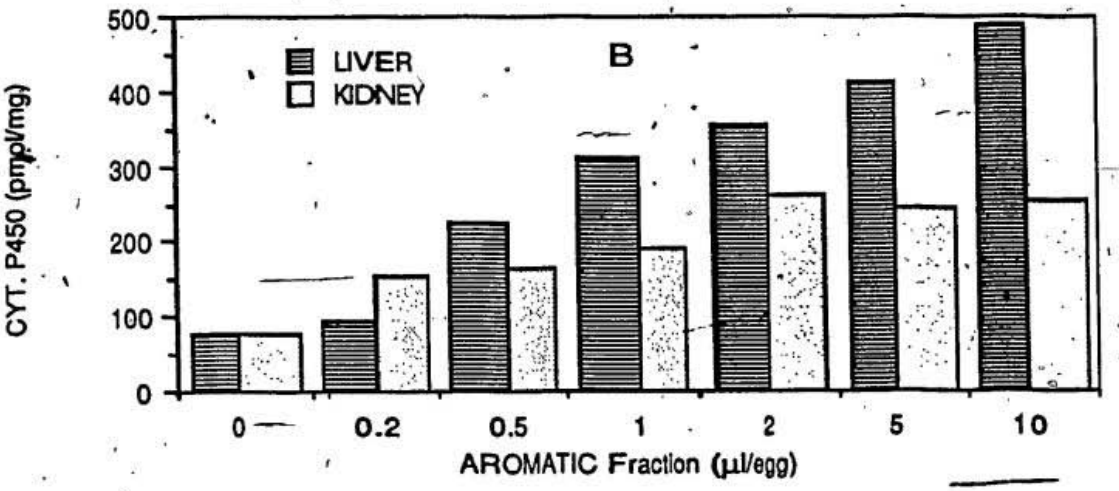
3.2.2 The effect of PBCO fractions on hepatic and renal cytochrome-P450 (P450) levels in 12 day old chick embryos

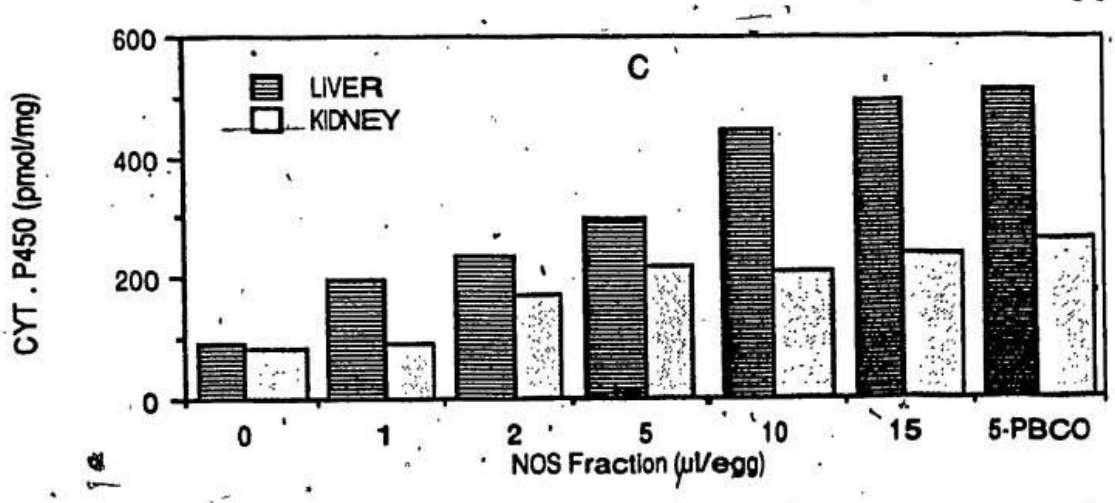
In figure 3.10, the effect of application of the aliphatic, aromatic or NOS fraction to the 12-day-old egg on embryo hepatic and renal P450 levels is shown. As seen in figure 3.10A, the aliphatic fraction up to 20µl failed to substantially elevate either the hepatic or renal P450 levels. The aromatic fraction (Figure 3.10B) showed a concentration-dependent increase in both hepatic and renal P450 levels. A 3-fold increase in hepatic P450 levels was seen with 0.5µl of the aromatic fraction while 10µl showed a maximum elevation of 5-fold. A doubling of renal P450 levels was seen with as little as 0.2µl of the aromatic fraction while application of 2µl gave a maximum elevation of 3.4-fold (Figure 3.10B). The NOS fraction (Figure 3.10C) also elevated both hepatic and renal P450 levels to approximately those seen with the aromatic fraction but required higher concentrations. For comparison, the elevation of hepatic and renal P450 levels with 5µl PBCO is also shown (Figure 3.10C). Previously it was shown that 5µl of PBCO was sufficient to cause maximal elevations of hepatic P450 levels as well as BPH and EROD activities. (Lee et al., 1986).

Figure 3.10. Inducibility of chicken embryo hepatic and renal cytochrome P-450 by aliphatic, aromatic, or NOS fractions of PBCO.

Indicated volumes of aliphatic (A), aromatic (B), and NOS fractions (C) were applied just below the airsac of fertile chicken eggs on day 12 of incubation as described in Methods (2.3.1.2). 30 eggs were used per group. Twenty-four hours later, livers and kidneys from each treatment group of embryos were pooled separately, and used immediately to prepare microsomes (Methods, 2.3.2.2). Cytochrome P-450 levels were determined as described in Methods (2.3.3.1). Results are means of duplicate assays.


A
LIVER
KIDNEY
CYT. P450 (pmol/mg)
120
90
60
30
0
0
1
2
5
10
20
ALIPHATIC Fraction (µl/egg)


B
LIVER
KIDNEY
CYT. P450 (pmol/mg)
500
400
300
200
100
0
0
0.2
0.5
1
2
5
10
AROMATIC Fraction (µl/egg)


C
LIVER
KIDNEY
CYT. P450 (pmol/mg)
600
400
200
0
0
1
2
5
10
15
5-PBCO
NOS Fraction (µl/egg)

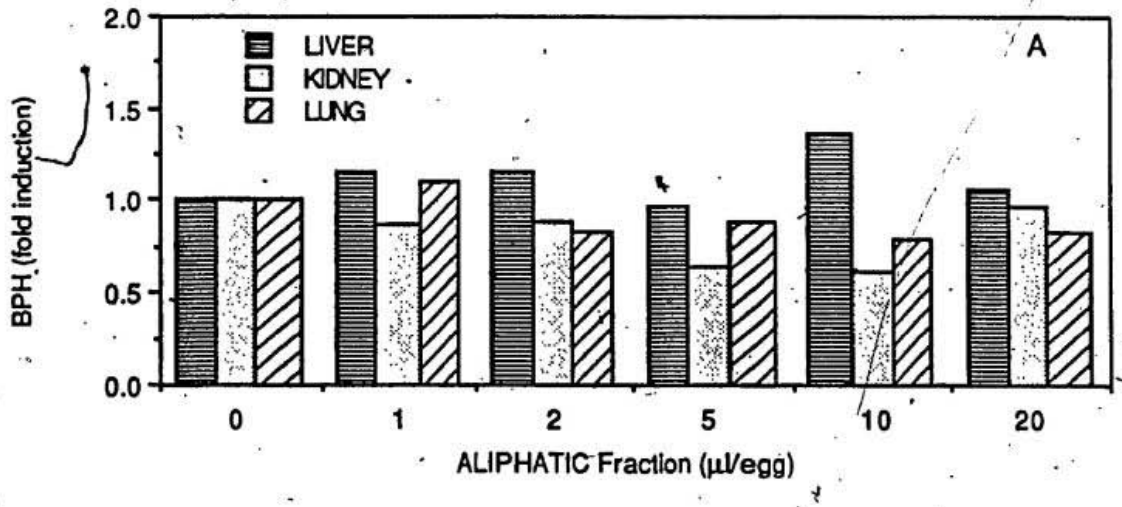
3.2.3 The effect of PBCO fractions on hepatic, renal and pulmonary benzo[a]pyrene hydroxylase (BPH) levels in 12 day old chick embryos

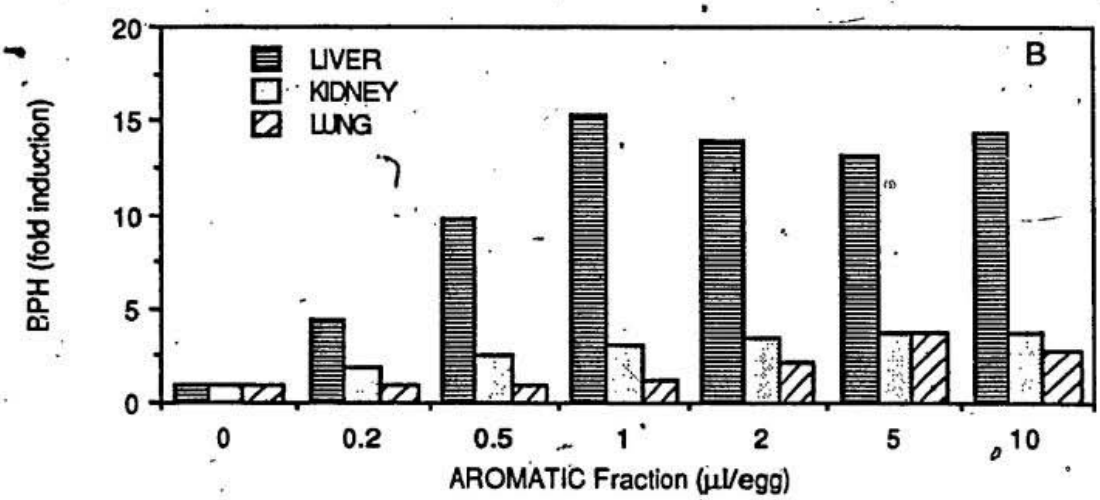
Changes in activities of hepatic, renal and pulmonary BPH levels following application of the aliphatic, aromatic or NOS fractions to 12-day-old eggs are shown in Figure 3.11.

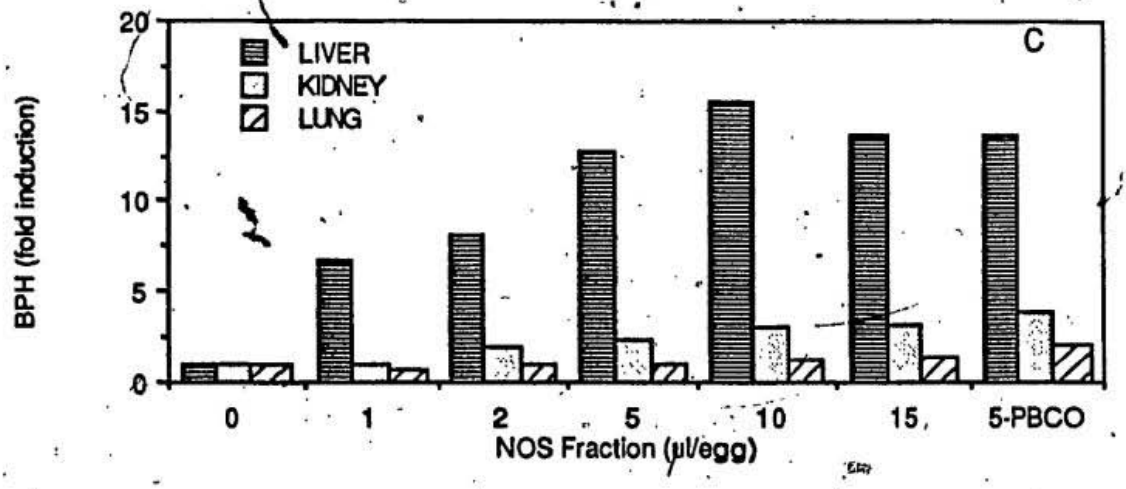
The aliphatic fraction up to 20µl/egg showed no substantial induction of BPH (Figure 13.11A). The aromatic fraction (Figure 3.11B) was very potent and caused a maximum induction of over 15-fold of hepatic BPH on application of only 1µl. Even 0.2µl induced hepatic BPH over 4-fold. Renal BPH was also elevated in a dose dependent manner by the aromatic fraction. 0.2µl doubled renal BPH activity while 5µl showed a 4-fold maximum induction. Low doses of the aromatic fraction (<1µl) did not significantly alter pulmonary BPH levels but 5µl elevated this activity to a maximum of 4-fold. The NOS fraction (Figure 3.11C) also elevated BPH activity but only in the liver and kidney. Application of 10µl of the NOS fraction resulted in a maximum induction of 15.5-fold and 3-fold of the hepatic and renal activity respectively. For comparison, the changes in

Figure 3.11. Inducibility of chick embryo hepatic, renal and pulmonary benzo[a]pyrene hydroxylase activities by aliphatic, aromatic, or NOS fractions of PBCO.

Indicated volumes of aliphatic (A), aromatic (B) and NOS (C) fractions were applied just below the air sac of ferile chicken eggs on day 12 of incubation as described in Methods (2.3.1.2). 30 eggs were used per group. Twenty-four hours later, livers, kidneys, and lungs from each treatment group of embryos were pooled separately and immediately followed by microsomal preparation as described by Methods (2.3.2.2). Benzo(a)pyrene hydroxylase levels were determined as described in Methods (2.3.3.2). Results are means of duplicate assays.


LIVER
KIDNEY
LUNG
A
BPH (fold induction)
2.0
1.5
1.0
0.5
0.0
0
1
2
5
10
20
ALIPHATIC Fraction (µl/egg)


LIVER
KIDNEY
LUNG
B
BPH (fold induction)
20
15
10
5
0
0
0.2
0.5
1
2
5
10
AROMATIC Fraction (µl/egg)


LIVER
KIDNEY
LUNG
C
BPH (fold induction)
20
15
10
5
0
0
1
2
5
10
15
5-PBCO
NOS Fraction (µl/egg)

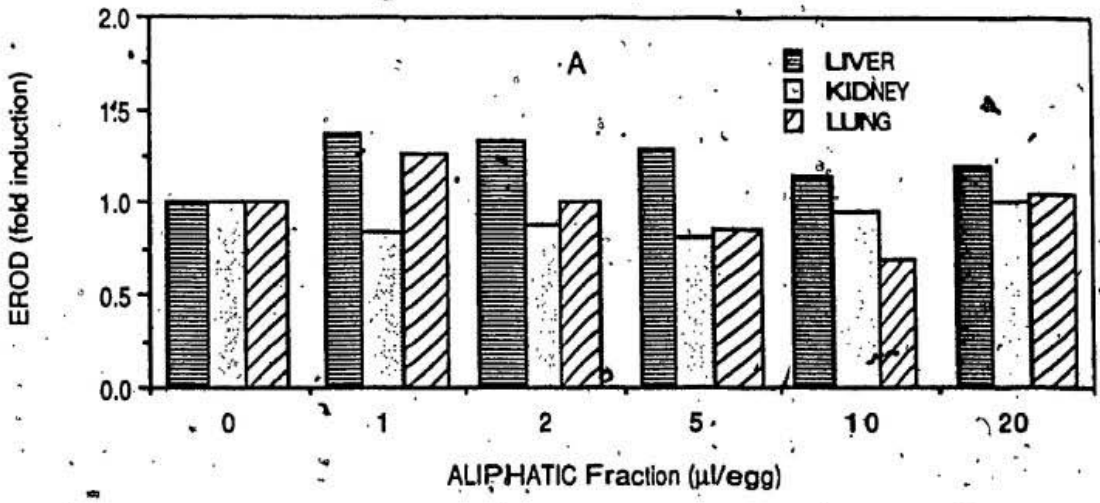
BPH activities with 5µl of PBCO are also shown (Figure 3.11C). In an earlier report, it was shown that 5µl of PBCO elevated hepatic BPH activity 14-fold (Lee et al., 1986).

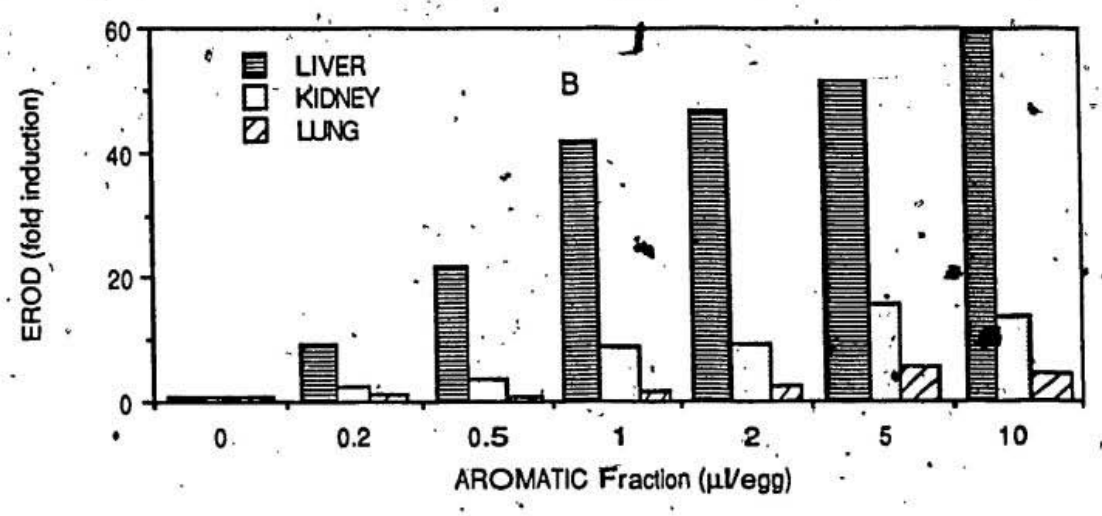
3.2.4 The effect of PBCO fractions on hepatic, renal and pulmonary 7-ethoxyresorufin-O-deethylase (EROD) levels in 12 day old chick embryos

In keeping with other results, the aliphatic fraction failed to substantially induce EROD (Figure 3.12A). In contrast, both the aromatic (Figure 3.12B) and NOS (Figure 3.12C) fractions induced hepatic and renal EROD. However, the aromatic fraction was more effective with 1µl inducing the hepatic activity over 40-fold and the renal activity about 9-fold (Figure 3.12B). In contrast, 1µl of the NOS fraction caused only a 12-fold increase in the hepatic activity and a 4-fold increase in the renal activity. A maximum elevation of about 60-fold of the hepatic activity was seen with higher concentrations of both aromatic or NOS fractions. In comparison, 5µl of PBCO caused a 65-fold increase in hepatic EROD activity and a 37-fold increase in the renal EROD activity (Figure 3.12C). It was previously shown that 5µl of PBCO elevated hepatic EROD by over 70-fold (Lee et al., 1986).

Figure 3.12. Inducibility of chick embryo hepatic, renal and pulmonary 7-ethoxyresorufin-O-deethylase activities by aliphatic, aromatic, ~~or NOS fractions~~ of PBCO.

Indicated volumes of aliphatic (A), aromatic (B) and NOS (C) fractions were applied just below the air sac of fertile chicken eggs on day 12 of incubation as described in Methods (2.3.1.2). 30 eggs were used per group. Twenty hours later, livers and kidneys from each treatment group of embryos were pooled separately, and used immediately to prepare microsomes (Methods, 2.3.2.2). 7-Ethoxyresorufin-O-deethylase levels were determined as described in Methods (2.3.3.3). Results are means of duplicate assays.


A
LIVER
KIDNEY
LUNG
EROD (fold induction)
2.0
1.5
1.0
0.5
0.0
0
1
2
5
10
20
ALIPHATIC Fraction (µl/egg)


B
LIVER
KIDNEY
LUNG
EROD (fold induction)
60
40
20
0
0
0.2
0.5
1
2
5
10
AROMATIC Fraction (µl/egg)

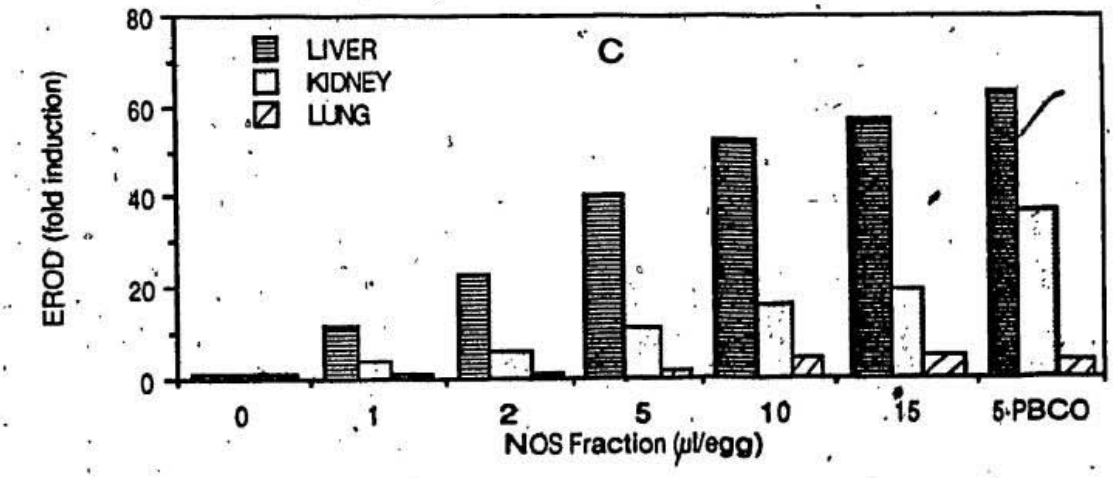

C
LIVER
KIDNEY
LUNG
EROD (fold induction)
80
60
40
20
0
0
1
2
5
10
15
5-PBCO
NOS Fraction (µl/egg)

Figure 3.13 provides a direct comparison of the results for the liver and shows the effective dose of the aromatic and NOS fractions that are required to cause half maximal induction (ED50) of P450 levels as well as of BPH and EROD activities. In terms of volume, approximately 3 times the amount of the NOS fraction (1.9µl as compared to the aromatic fraction (0.6µl), was required for half maximal induction of hepatic P450. Also, 4 times the amount of the NOS fraction was required for half maximal elevation of BPH (1.5µl vs. 0.4µl) and EROD (2.7µl vs. 0.7µl) activities.

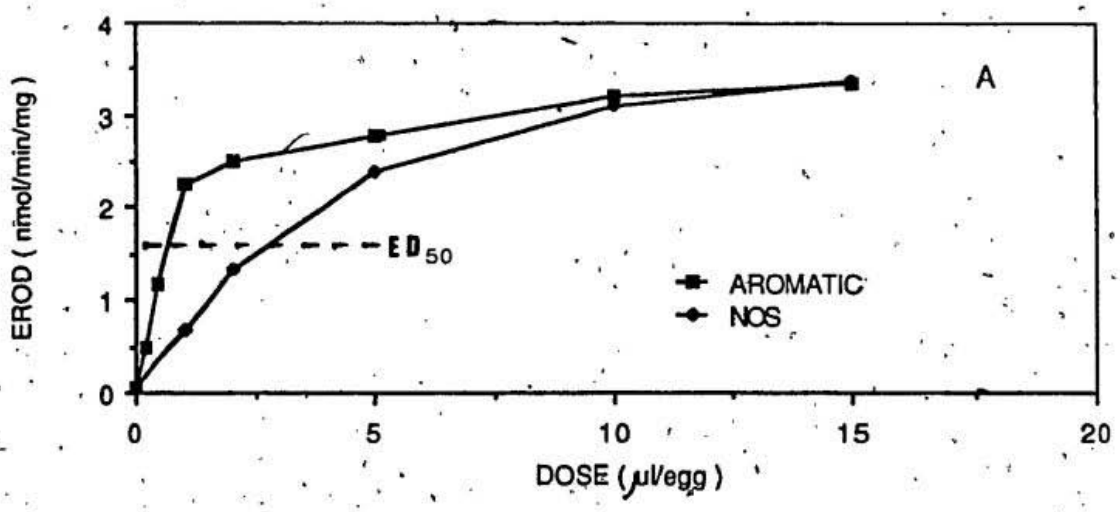
3.3 The toxicity of blowout crude oil, diesel oil and drilling muds on chick embryos

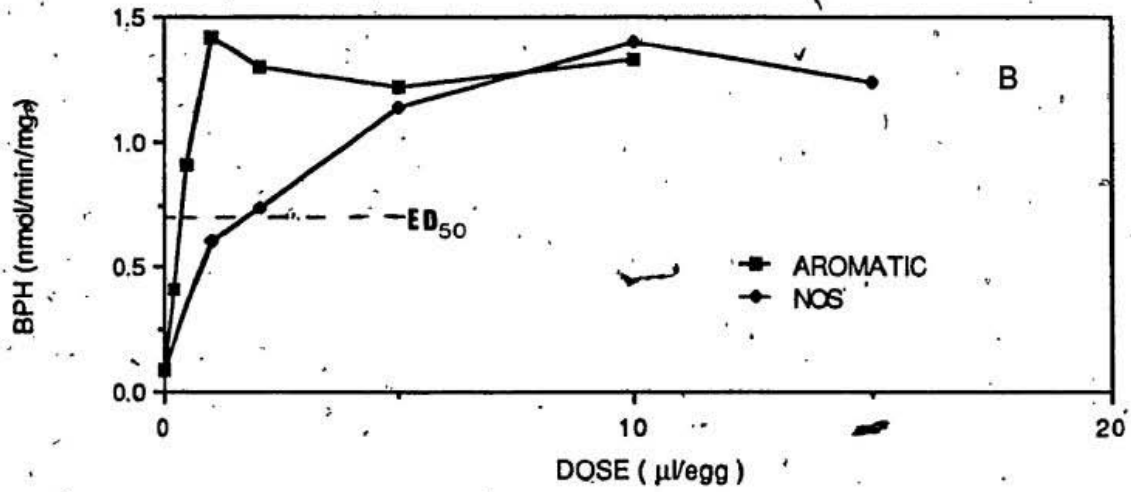
3.3.1 The effect on mortality rates in chick embryos

Both blowout crude oil and diesel oil cause significant increases in mortality when applied to the outside surface of eggs containing 7 day old chick embryos. As shown in Figure 3.14, the LD-50 for blowout or diesel fuel was 2.6µl or 3.6µl respectively. These results are in contrast to data obtained for drilling fluids. As indicated in Figure 3.15, none of the Safver oils (trade name of drilling fluids that were tested) induced a significant increase in mortality. Safver oils #1, 4, and 5 induced a mortality

Figure 3.13. The Effect of application of varying doses of aromatic or NOS fractions on day 12 chick embryo liver 7-ethoxyresorufin-O-deethylase, benzo[a]pyrene hydroxylase and cytochrome P-450 levels.

The various data points were obtained from figure 3.10-3.12. The doses (in μl) of the aromatic and NOS fractions required for half maximal induction (ED_{50}) of the EROD, BPH and P450 were obtained as shown from the graphs.


A
EROD (nmol/min/mg)
4
3
2
1
0
0
5
10
15
20
DOSE (µl/egg)
ED50
AROMATIC
NOS


B
BPH (nmol/min/mg)
1.5
1.0
0.5
0.0
0
10
20
DOSE (µl/egg)
ED50
AROMATIC
NOS

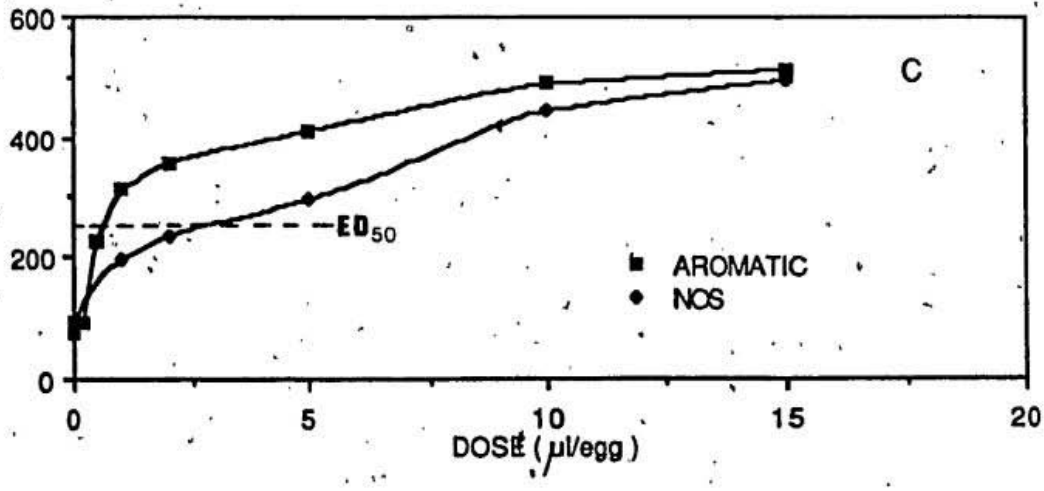

C
CYT. P450 (pmol/mg)
600
400
200
0
0
5
10
15
20
DOSE (µl/egg)
ED50
AROMATIC
NOS

Figure 3.14. The effect of dose of blowout crude and diesel oil on chick embryo mortality.

Results show mortality rates of chicken embryos on day 7 application of 0.3-20 µl volumes of blowout crude or diesel fuel. Samples were applied to the outside of the egg, just below the air sac as described in Methods (2.3.1.2). Eggs were opened on day 15 of incubation and dead embryos were counted. Mortality rate for 30 untreated eggs was 3.3%. Sample size for each dose was 30 eggs.

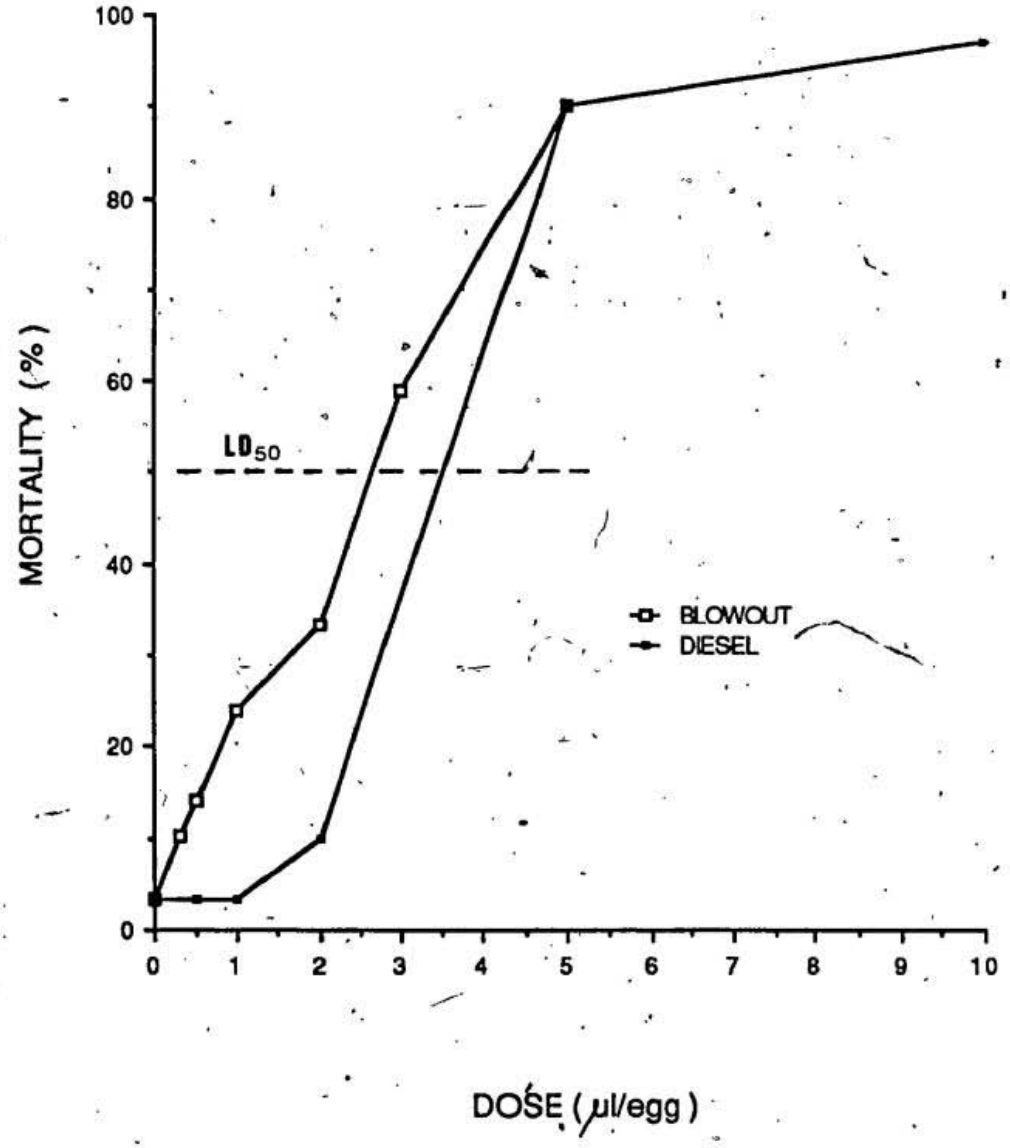
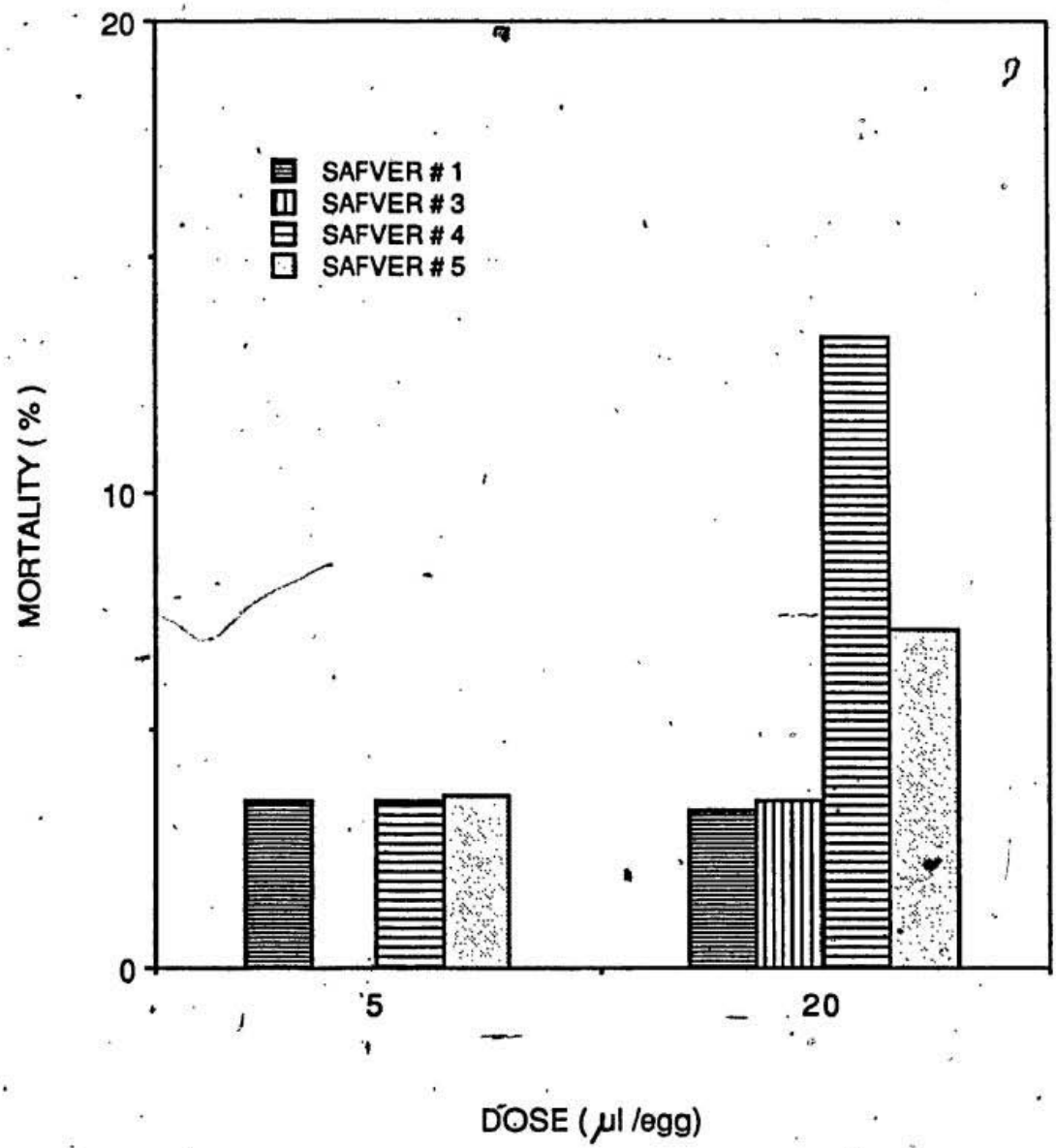

100
80
60
40
20
0
MORTALITY (%)
LD50
BLOWOUT
DIESEL
0
1
2
3
4
5
6
7
8
9
10
DOSE (µl/egg)

Figure 3.15. The effect of application of Safver drilling fluids on chick embryo mortality.

Results show mortality rates of chicken embryos on day 7 of application of 5 µl or 20 µl volumes of various Safver drilling fluids. Samples were applied to the outside of the egg, just below the air sac as described in Methods (2.3.1.2). Eggs were opened on day 15 of incubation and dead embryos were counted. Sample size for each dose was 30 eggs.


20
10
0
MORTALITY (%)
SAFVER # 1
SAFVER # 3
SAFVER # 4
SAFVER # 5
5
20
DOSE (µl /egg)

rate of 3.3% at a dose of 5µl. This result was not significant considering that out of a sample group of 30 eggs, only 1 embryo died. The mortality rate for nontreated eggs was 3.3%. However at a dose of 20µl, Safver oil #4 and #5 produced a mortality rate of 13.2% and 6.6% respectively.

3.3.2 The effect on the levels of 7-ethoxyresorufin-O-deethylase and benzo(a)pyrene hydroxylase in the 12 day old chick embryo liver.

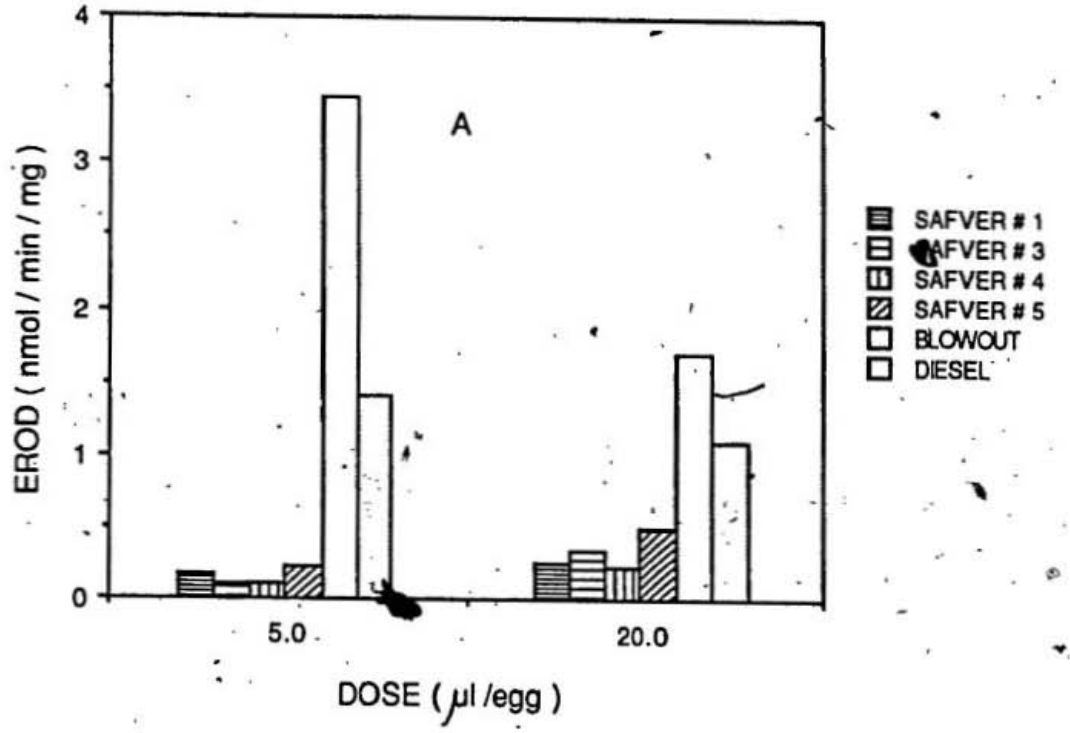
Consistent with the results of mortality studies, blowout crude and diesel were significantly more potent at inducing EROD and BPH levels than drilling fluids (Figure 3.16).

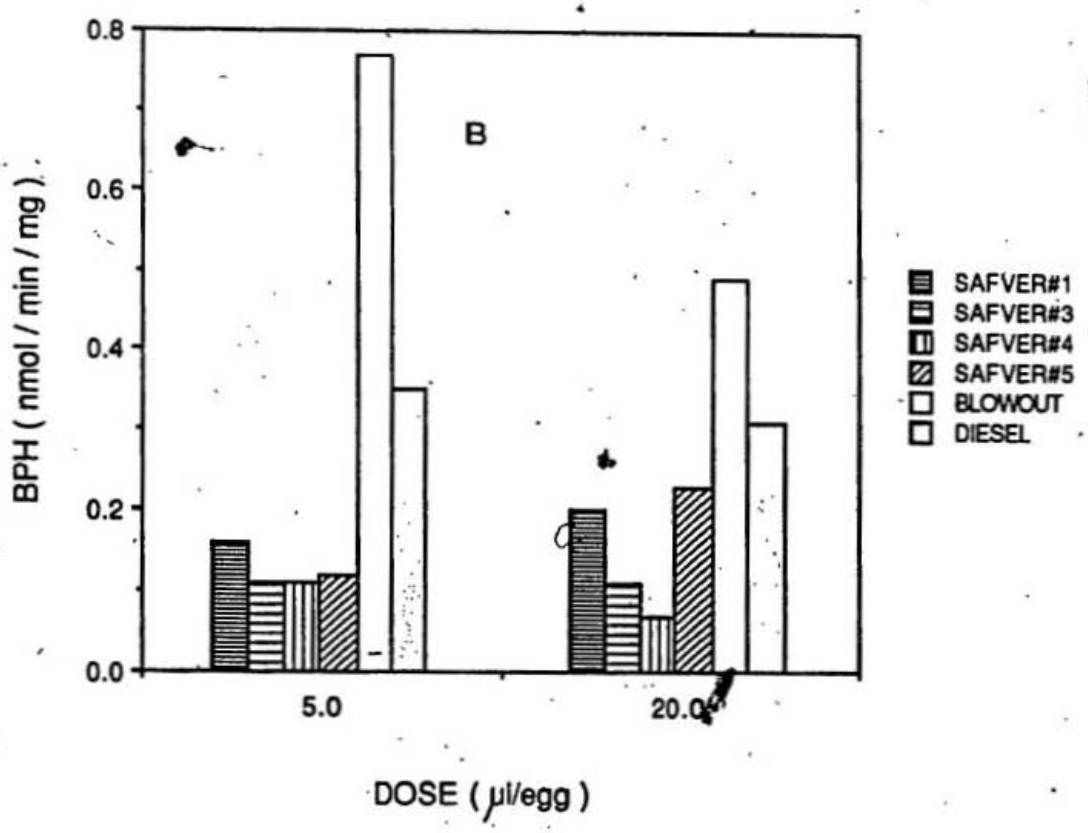
Although the EROD level (Figure 3.16A) in day 13 non-treated embryos was determined to be 0.25 nmoles/min/mg protein, a 5µl dose of blowout crude or diesel (24 hour pretreatment) induced enzyme activities to 14 fold and 6 fold respectively. However, when a 20µl dose was tested, the enzyme levels were elevated to 7 and 5 fold above control, so that the activity in 20µl treated embryos was less than that in 5µl treated embryos.

In contrast, drilling fluids were not very effective for inducing EROD levels, with the exception of 20µl of Safver oil #5 (2 fold induction).

Figure 3.16. Inducibility of chick embryo hepatic levels of 7-ethoxyresorufin O-deethylase and benzo[a]pyrene hydroxylase by drilling fluids, blowout crude and diesel fuel.

Five and 20 µl samples of Safver drilling fluids, blowout crude and diesel oil were applied just below the air sac on day 12 of incubation as described in Methods (2.3.1.2). Twenty-four hours later, the chicken embryos were killed and livers were excised, followed immediately by microsomal preparation as described in Methods (2.3.2.2). 7-Ethoxyresorufin O-deethylase (A) and benzo[a]pyrene hydroxylase (B) were assayed as described in Methods (2.3.3.3 and 2.3.3.2 respectively).


A
EROD (nmol / min / mg)
4
3
2
1
0
5.0
20.0
DOSE (µl /egg)
SAFVER # 1
SAFVER # 3
SAFVER # 4
SAFVER # 5
BLOWOUT
DIESEL


B
BPH (nmol / min / mg)
0.8
0.6
0.4
0.2
0.0
5.0
20.0
DOSE (µl/egg)
SAFVER#1
SAFVER#3
SAFVER#4
SAFVER#5
BLOWOUT
DIESEL

The induction of BPH (Figure 3.16B) was consistent with results obtained with EROD (Figure 3.16A). Again, blowout crude and diesel were effective at inducing BPH activities from 0.13 nmoles/min/mg, protein (nontreated embryos) to activities 6 and 2.7 fold higher respectively, at a dose of 5ul. The use of 20µl dose did not increase activities as well as the lower dose.

The drilling fluids did not induce BPH with the exception of Safver oil #5 (1.8 fold induction).

3.4 The toxicity of benzene and its metabolites on chick embryos

3.4.1 Mortality studies with seven day old chick embryos

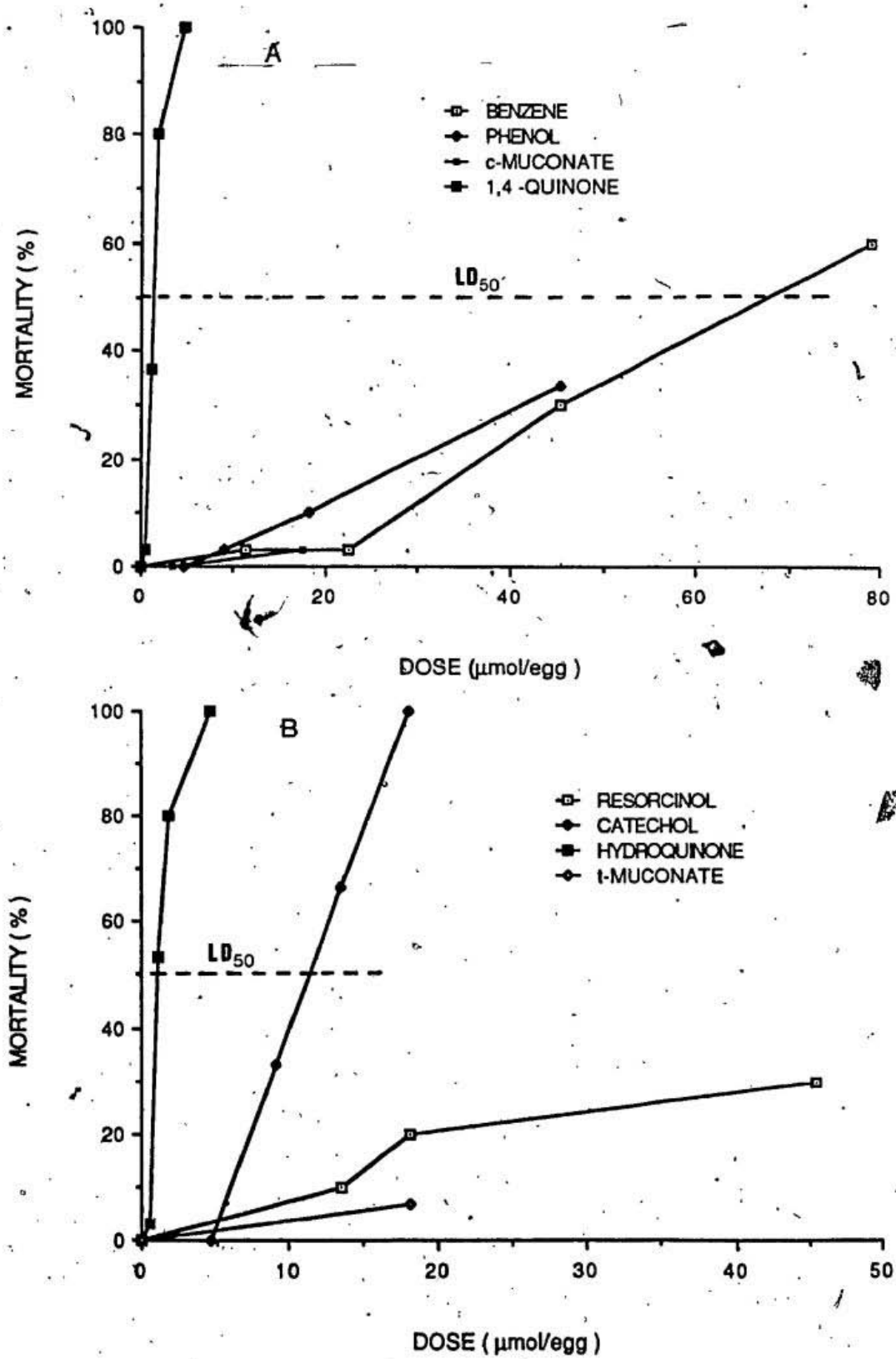
As shown in Figure 3.17, benzene and some of its metabolic derivatives can cause death when injected into the airsac of a seven day old embryo.

The LD-50 of benzene (Figure 3.17A) was experimentally determined as approximately 67 µmoles per egg. However, 1,4-benzoquinone, 1,4-dihydroxybenzene (hydroquinone) and 1,2-dihydroxybenzene (catechol) had LD-50s of 0.07, 0.07 and 11.5 µmoles/egg respectively. Because high enough concentrations were not tested the LD_{50} of resorcinol and phenol were not clearly established. However, it appears that at

Figure 3.17. The effect of benzene and its metabolites on chick embryo mortality.

Eggs on day 7 of incubation were injected with 0.55-79.1 μmoles of benzene, phenol, muconate, 1,4-benzoquinone (Figure 3.17A), resorcinol, 1,2-dihydroxybenzene (catechol), 1,4- dihydroxybenzene (hydroquinone or muconate (Figure 3.17B). Samples were dissolved in 50 μl of PBS and injected into the air sacs of upright eggs as described in Methods (2.3.1.3). Eggs were opened on day 15 of incubation and dead embryos were counted. Sample size for each dose was 30 eggs.

Abbreviations: C-MUCONATE; cis-muconate
T-MUCONATE; trans-muconate
1,4-QUINONE; 1,4-benzoquinone


A
BENZENE
PHENOL
c-MUCONATE
1,4 -QUINONE
LD50
MORTALITY (%)
DOSE (µmol/egg)
B
RESORCINOL
CATECHOL
HYDROQUINONE
t-MUCONATE
LD50
MORTALITY (%)
DOSE (µmol/egg)

doses less than 45 μmoles/egg their potencies are similar to benzene.

Cis-cis muconate and trans-trans muconate did not cause a significant mortality at the doses tested.

CHAPTER 4

DISCUSSION

4.1 PBCO toxicity in erythrocytes

4.1.1 A possible explanation for the toxicity of PBCO in herring gull and human erythrocytes

From our studies it is evident that a DMSO extract of PBCO is capable of inducing metHb formation, GSH depletion and hemolysis in erythrocytes, especially after activation by liver microsomes and NADPH.

Liver microsomes contain mixed function oxidase enzymes which are capable of metabolizing a wide variety of substrates. An earlier study by Leighton et al (1983) has demonstrated that Heinz-body hemolytic anemia is a primary toxic response in seagulls upon ingestion of crude oil. Accompanying Heinz-body anemia was an increase in reticulocyte count, which is a further indication of hemolytic anemia (Leighton, 1985). The red cell lesions observed were similar to those that were observed when phenylhydrazine was injected into seagulls.

Crude oils are known to contain significant amounts of aromatic hydrocarbons like naphthalene, phenanthrene, and benzo[a]pyrene, and their alkylated derivatives (Peakall et

al., 1982; Lee et al., 1985; National Research Council, 1985).

Metabolism of aromatic hydrocarbons by mixed function oxidase is known to produce phenols, diols, and quinones as well as reactive intermediates that bind to cellular macromolecules (Hesse and Mezger, 1979; Prough et al., 1979; Pelkonen and Nebert, 1982).

It is also known that administration of naphthalene to rodents results in oxidative damage to erythrocytes (Smith, 1980). There are documented cases of infants poisoned by ingestion (Gidron and Leurer, 1956) or inhalation (Valaes et al., 1963) of naphthalene, leading to hemolytic crisis. In the latter case, Heinz bodies were detected in the erythrocytes. It was, therefore, likely that at least a portion of the oxidative damage to erythrocytes induced by PBCO is due to the aromatic hydrocarbons present in it.

The rational for the use of naphthalene derivatives such as 2-methylnaphthalene and 2,6-dimethylnaphthalene (Table 3.3) in these experiments is based on the observation that methylated naphthalenes are present in crude oil. Like naphthalene, they were also able to induce metHb formation in seagull erythrocytes when incubated in the presence of microsomes and NADPH. Because of the commercial availability of naphthalene metabolites, compared to methylated derivatives, we used the former compounds as model substrates to

study the effects of naphthalene metabolism which in turn served as a model for PBCO toxicity studies.

Our results indicate that 1-naphthol, 1,2-dihydroxynaphthalene, 1,4-dihydroxynaphthalene 1,2-naphthoquinone and 1,4-naphthoquinone are the most toxic of all naphthalene metabolites tested on the red cell or HbO_2.

The metabolism of naphthalene *in vivo* and *in vitro* has been outlined previously (see Introduction). A variety of naphthalenes and their hydroxylated derivatives were able to induce metHb formation in erythrocytes. As expected, the naphthalenes but not the phenols (with the exception of 2-naphthol) required metabolic activation to toxic metabolites.

The results in Table 3.2 show that there is a time dependent increase in metHb formation, a decrease in total glutathione level and an increase of GSSG level when a DMSO extract of PBCO is incubated with human erythrocytes. These effects were significantly enhanced when a metabolized extract of PBCO (see methods 2.2.1.2) was used instead. Payne and May (1978), have previously shown that incubation of a DMSO extract of crude oils with fish liver homogenates results in the production of a variety of fluorescent products. Lee et al (1985) and Rahimtula et al (1984) have observed the same with rodent liver and gull liver micro-

somes respectively, indicating that transformation of crude oil components does occur in these tissues.

A variety of chemicals including phenylhydrazine, phenacetin, aromatic amines, aminophenols and quinones are known to induce metHb formation (Jandl et al., 1960; Gault et al., 1974; Eyer et al., 1974; Goldberg and Stern, 1976). Phenylhydrazine is the classical oxidant hemolytic drug and Jandl and coworkers (1960) originally postulated that phenylhydrazine-induced hemolysis was caused by free radicals produced when the drug was oxidized within the red cell. The results of a later study (Babior, 1981) confirmed this and indicated that phenylhydrazine hemolysis and metHb formation required the oxidation of phenylhydrazine to phenyldiazene by oxidizing systems formed ultimately from hemoglobin and oxygen. Phenyldiazene itself or its further one-electron oxidation product, the phenyl radical, is believed to be the ultimate hemolytic species.

The fact that metabolized PBCO, 1-naphthol, 1,2-naphthoquinone, 1,4-naphthoquinone, 1,2-dihydroxynaphthalene, and 1,4-dihydroxynaphthalene are directly able to oxidize HbO_2 to metHb (Figure 3.2, Figure 3.3, Table 3.4) indicates that other components of the erythrocyte (such as the plasma membrane) are not required. Naphthalene, 2-naphthol and 2,6-dimethylnaphthalene were also able to convert HbO_2 to metHb but only after microsomal metabolism (Table 3.4).

4.1.2 A possible mechanism of 1-naphthol toxicity in erythrocytes

The ability of phenols to directly interact with HbO_2 and convert it into metHb is well established. Wallace and Caughey (1975), have shown that phenols can transfer an electron to the bound oxygen of HbO_2. Then the combination of an electron transfer from an external donor (a phenol) and an electron from Fe^{2+} of the protein allows the thermodynamically favoured two electron reduction of bound dioxygen to H_2O_2 to occur and opens the possibility of a very rapid reduction reaction. The rate of metHb formation would depend upon the electron donating capacity of the phenol (Wallace and Caughey, 1975).

Eyer and coworkers (1974) have shown that 4-dimethylaminophenol rapidly converts HbO_2 to metHb and that the role of H_2O_2 in metHb formation from 4-dimethylaminophenol was of little importance. Based on these observations it is likely that the conversion of HbO_2 to metHb by 1-naphthol or other phenols present in metabolized PBCO occurs via the following mechanism: the initial reaction is the one electron reduction of HbO_2 by a phenol resulting in the formation of metHb, H_2O_2 and a phenoxy radical:

$$HbO_2 + R\text{-}O^- + 2H^+ \longrightarrow \text{metHb } (Fe^{3+}) + H_2O_2 + RO\cdot$$

The metHb produced in this reaction then acts as a peroxidase, catalyzing the one-electron oxidation of additional phenol molecules by H_2O_2.

$$H_2O_2 + 2ROH \xrightarrow{metHb} 2H_2O + 2RO\cdot$$

The phenoxy radicals may be further oxidized by O_2 or HbO_2 to quinones, dimers or other products.

$$R\text{-}O\cdot + HbO_2 \text{ or } O_2 \longrightarrow \text{quinones, dimers etc.} + metHb$$

$$R\text{-}O\cdot + O_2 \longrightarrow \text{quinones, dimers etc.} + O_2^{-}$$

The phenoxy radicals may also be reduced back to the phenol by cellular GSH.

$$2\ R\text{-}O\cdot + 2\ GSH \longrightarrow 2\ ROH + GSSG$$

DMSO alone as well as PBCO decreased seagull erythrocyte GSH levels significantly but metabolized PBCO was much more effective (Figure 3.1). There is some increase in GSSG formation with the PBCO extracts but total glutathione levels decreased as well by 22-43% (Table 3.2) indicating that some of the components of PBCO must bind covalently to GSH.

It is well established that a variety of phenols can be activated *in vitro* and *in vivo* to give glutathione adducts (Moldeus and Jernstrom, 1983; Tunek et al., 1980). 4-Dimethylaminophenol is also known to reduce GSH levels, increase GSSG levels and give rise to a glutathione adduct in erythrocytes both *in vitro* (Eyer and Kiese, 1976) and *in vivo* (Eyer and Gaber, 1978). GSH is the primary intra-

cellular protective agent against oxidative damage and its depletion below a certain critical concentration usually precedes oxidative damage to erythrocytes and other cells (Babier, 1981; Allen and Jandl, 1961).

4.1.3 A possible mechanism of dihydroxynaphthalene and naphthoquinone toxicity in erythrocytes

Further experiments with other known metabolites of naphthalene on human red cells indicate that quinones may play a major role in toxicity by elevating metHb levels and depleting GSH levels (Figure 3.5B and 3.8) at a low centration of 50μM.

In view of the fact that these compounds can autoxidize to the corresponding quinones (Doherty and Cohen, 1984), as expected, 1,2- and 1,4-dihydroxynaphthalenes demonstrated the same trend (Figure 3.5A and 3.7B).

1,3-Dihydroxynaphthalene did not effect metHb or GSH levels (Figure 3.5A and 3.7B) probably due to its inability to form a quinone.

Trans-1,2-dihydroxy-1,2-dihydronaphthalene also had no effect on metHb or GSH levels (Figure 3.4B and 3.7A) unless microsomes and NADPH or liver cytosol and $NADP^+$ were included in the incubation. As previously stated, dihydrodiol dehydrogenase present in the rat liver cytosol probably

converted the dihydrodiol to the catechol (1,2-dihydroxy-naphthalene).

These studies indicate that quinones derived from the metabolism of naphthalene may play a major role in the toxicity of naphthalene to red cells. The conversion of HbO_2 to metHb by quinones most likely occurs by the following pathway (Winterbourn, 1985).

$$\text{quinone} + HbO_2 \longrightarrow \text{metHb } (Fe^{3+}) + O_2 + \text{semiquinone}^{\cdot -}$$

The semiquinone free radical intermediate reduces O_2 to the superoxide radical.

$$\text{semiquinone}^{\cdot -} + O_2 \longrightarrow \text{quinone} + O_2^{\cdot -}$$

The superoxide radical produced is converted to H_2O_2 by superoxide dismutase (SOD).

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

The hydrogen peroxide generated can be broken down by catalase and glutathione peroxidase. Involvement of glutathione peroxidase would explain why GSSG is elevated with these compounds.

It should be noted that at zero time, the level of GSH in the incubations containing 1,2- and 1,4-naphthoquinone (Figure 3.8A and 3.8C) were considerably lower than expected. This may be due to the possibility that the quinones (or reactive intermediates) were still reacting with GSH in the presence of metaphosphoric acid (see methods 2.2.9.3). Corresponding to initial low levels of GSH is the

relatively high amount of GSSG. It is therefore a possibility that the zero time values recorded are not accurate and may represent a reaction time of a few minutes.

Furthermore, the sum of GSH and GSSG (in GSH equivalents) does not add up to the total GSH in the control incubation (erythrocytes and buffer). It is possible that GSH may be lost due to forming conjugates with the quinones. Miller et al (1986) demonstrated that GSH non-enzymaticly reacts with 1,2- and 1,4-naphthoquinone to form one of more conjugates. Nickerson et al (1963) have demonstrated that quinones react with thiol groups by a nucleophilic substitution reaction.

Recycling of the quinone may be the reason why 1,2- and 1,4-naphthoquinones are very effective in elevating metHb levels at very low concentrations. The metabolism of quinones has been studied in isolated cell systems (Thor et al., 1982) and in subcellular fractions (Powis et al., 1981) and it has been demonstrated that they may undergo either one- or two-electron reduction. The one-electron reduction of a quinone forms a semiquinone radical; this process can be catalyzed by a variety of flavoenzymes, including NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase and NADH-ubiquinone oxidoreductase (Thor et al., 1982; Powis et al., 1981; Iyanagi and Yamazaki, 1970). In the presence of oxygen, the semiquinone radical can be reoxidized to the

parent quinone with the concomitant formation of superoxide anion $O_2^{\cdot-}$ (Thor et al., 1982; Lind et al., 1982). The enzymatic or spontaneous dismutation of $O_2^{\cdot-}$ yields O_2 and H_2O_2 (Kappus, 1986). The two-electron reduction of certain quinones (eg. menadione) to the corresponding hydroquinones is catalysed by the flavoenzyme NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorase) (Ernster, 1967) without formation of semiquinone free radical intermediates (Thor et al., 1982). The significant increase in GSSG levels in the presence of the naphthoquinones (Figure 3.8) is evidence that H_2O_2 is generated. The H_2O_2 can be detoxified by glutathione peroxidase with concomitant formation of oxidized glutathione.

Catalase, another enzyme present in red cells probably plays a role in protection against naphthoquinone toxicity. The addition of 1mM sodium azide (an inhibitor of catalase) to the incubations enhanced the quinone mediated GSH depletion and GSSG elevation (Figure 3.8). A recent study by Miller et al. (1986) demonstrates a similar mechanism of toxicity of naphthoquinones in hepatocytes.

4.2 The toxicity of PBCO, diesel oil, blowout oil and drilling fluids on chick embryos

The results of the present study indicate that the order of potency of the fractions of PBCO in (i) causing embryo mortality and (ii) inducing P-450 levels, benzo[a]pyrene hydroxylase activities, and 7-ethoxyresorufin-O-deethylase activities is aromatic > NOS >> aliphatic. Previously, Ellenton (1982) had shown that the aliphatic fraction of PBCO, when applied to chick eggs on day 3 of incubation, was ineffective in inducing embryo abnormalities or mortality.

The mechanism by which crude oils exert their toxicity on the avian embryo is not known. Components of crude oil could be directly toxic to the developing embryo or metabolic activation may be required. It is well established that many xenobiotics including the aromatic hydrocarbons, require metabolic activation before they can exert their toxic or carcinogenic effects (Miller and Miller, 1981). Benzo[a]pyrene has been shown to cause embryo resorption, fetal death and malformations when administered to pregnant rats (Rigdon and Rennels, 1964). 7,12-Dimethylbenzanthracene is also known to cause a high incidence of incomplete neural tube closures and other defects (Currie et al., 1970). Hoffman and Gay (1981) found that the temporal pattern of embryonic death following the administration of

the polycyclic hydrocarbons benzo[a]pyrene, chrysene and 7,12-dimethylbenzanthracene, was similar to that after exposure to crude oil, with additional mortality occurring after the outgrowth of the chorioallantois.

Lee et al (1986) have previously shown that chick embryo liver, at least from day 10 onwards, contains cytochrome P-450 and is capable of metabolizing hydrocarbons like naphthalene and benzo[a]pyrene. The chick embryo at earliest stages of development from day 3 onwards has also been shown to have AHH activity (Hamilton et al., 1983). This is supported by observations that disulfiram, a drug known to lower cytochrome P-450 levels in rodents, substantially reduced the embryotoxic effects of PBCO applied on day 7 of incubation (Lee et al., 1986). This suggests that aromatic hydrocarbons, and presumably other compounds present in crude oil, are metabolized by the embryo liver.

4.2.1 Mortality studies

4.2.1.1 The seven day old embryo

4.2.1.1.1 The effects of PBCO

In the 7 day old embryo, the LD50s of PBCO, aromatic fraction and NOS fraction were found to be 1.3 µl, 0.4 µl and 6.8 µl respectively (Figure 3.9A). Since each fraction was reconstituted in hexadecane up to the original volume of

PBCO, it follows that, on a volume equivalent basis, the aromatic fraction was 17 times more potent than the NOS fraction. This is in agreement with the results of Ellenton (1982) and Hoffman and Gay (1981) that the aromatic fraction is primarily responsible for the toxicity of crude oils like PBCO. However, the aromatic fraction comprised 38.5% (w/v) of PBCO while the NOS fraction accounted for only 6.8%. On a weight equivalent basis, therefore, the aromatic fraction was only 3 times more toxic to the embryos than the NOS fraction. To our knowlege, the "embryotoxic" potential of the NOS fraction has not been previously recognized. Hoffman (1979a) found that the application of heterocyclics like benzothiophene, dibenzothiophene and 2,3,3-trimethyl-indolenine, known to be present in South Louisiana crude, to mallard eggs did not result in any embryo mortality. This would suggest that the heterocyclic compounds responsible for the toxic effects of the NOS fraction of PBCO are different from those tested by Hoffman (1979a).

The fact that the aromatic fraction had a lower LD50 (0.4µl) than PBCO (1.3µl) suggests that some of the inert components of PBCO might be inhibiting the toxic effects of the aromatic components on the embryos. This would be particularly relevant if metabolic activation of the protoxic or promutagenic aromatic hydrocarbons (presumably by the cytochrome P-450 dependent mixed function oxidases)

was being competitively inhibited by other nontoxic components of PBCO which could also act as substrates. In support of this, it has previously shown that the liver microsomal metabolism of benzo[a]pyrene as well as the mutagenicity in the Ames assay was substantially inhibited in the presence of a dimethylsulfoxide extract of fuel oil No. 2 or of Kuwait crude (Rahimtula et al., 1984).

4.2.1.1.2 The effects of diesel oil, blowout crude, and drilling fluids

To test the hypothesis that aromatic content plays a role in toxicity, diesel, blowout crude and drilling fluids were applied to 7 day old chick embryos in order to determine if aromatic content plays a role in toxicity. All of the above samples, with the exception of blowout crude and Safver 3 were analysed for aromatic content (Table A.3) and the results were reported by Payne et al (1985). Their results indicated that the levels of monocyclic, bicyclic and tricyclic aromatic compounds were much higher in the diesel fuel than in the drilling fluids.

Consistent with the levels of aromatic compounds as determined by Payne et al (1985), diesel oil had an LD-50 of 2.6µl while Safver 1, 4, and 5 produced mortality rates of 3.3, 13.2 and 6.6% at a dose of 20µl respectively (Figure 3.14).

Because data on the composition of blowout crude and Safver 3 was not obtained, the aromatic content could be

predicted only. Blowout crude had an LD-50 of 3.6µl while Safver 3 produced 3.4% mortality at a dose of 20µl.

4.2.1.2 The twelve day old embryo

PBCO and its aromatic and NOS fractions were found to be considerably less toxic to the 12 day old embryo (Figure 3.9B). This may be due to the fact that the critical stage(s) of embryo development affected by PBCO occur(s) during an earlier growth phase. Hoffman (1978) reported that in chick embryos, a major increase in mortality occurred on the 7th and 8th days of incubation after crude oil had been applied to the eggshell surface on the 2nd day of development. The major period of lethality in embryos occurred during the time of rapid outgrowth of the chorio-allantoic membrane over the surface of the inner shell membrane, suggesting potential for rapid uptake of the xenobiotics by this membrane (Hoffman, 1978; Hoffman and Gay, 1981). Alternatively, the larger size of the 12 day old embryo may make it more difficult for the PBCO components to be absorbed and transported to the critical target sites. However, in the 12 day old embryo as well, the aromatic fraction was found to be more toxic than the NOS fraction on a volume equivalent basis (Figure 3.9B). In contrast to the 7 day old embryo, the 12 day old embryo was somewhat more sensitive to PBCO than its aromatic fraction.

Also, the NOS fraction was closer in toxicity to the aromatic fraction on day 12 than on day 7 (Figure 3.9A). The greater potency of PBCO on day 12 can be accounted for on the basis of the additive contributions of the aromatic and NOS fractions. It is not quite clear why the NOS fraction has increased toxic potency relative to the aromatic fraction on day 12. It is possible that the increased metabolic capacity due to induction of different cytochrome P-450s on day 12 (relative to day 7) is better able to activate compounds present in the NOS fraction. It is also possible that the NOS fraction affects embryo development at a later stage relative to the aromatic fraction.

4.2.2 Enzyme induction studies

4.2.2.1 The effects of PBCO

Both the aromatic and NOS fractions elevated embryo hepatic cytochrome P-450 levels a maximum of 5-fold and shifted the carbon monoxide binding spectrum of the reduced hemoprotein from 450 nm to 448 nm suggesting that these fractions are similar to benzo[a]pyrene and 3-methylcholanthrene in inducing cytochrome P-448. In support of this, benzo[a]pyrene hydroxylase (BPH) and ethoxyresorufin-O-deethylase (EROD) activities were elevated roughly 15-fold

and 60-fold respectively. Both benzo[a]pyrene and 7-ethoxyresorufin are excellent substrates for the cytochrome P-448 induced by benzopyrene or 3-methylcholanthrene (Conney, 1967; Lu and West, 1980). The larger induction seen with EROD as opposed to BPH is due to the fact that the constitutive forms of cytochrome P-450 present in untreated liver microsomes are better able to metabolize BP than ER (Conney, 1967).

In addition to the liver, both kidney and lung from the developing embryo are able to metabolize xenobiotics. In both these tissues as well, BPH and EROD are inducible (Figure 3.11 and Figure 3.12) but not to the same extent as the liver. Hamilton and Bloom (1983) previously showed that pulmonary BPH was not induced by 3,4,3',4'-tetrachlorobiphenyl in embryos of Cornell K-strain eggs from day 14 up to day 19. The lack of pulmonary BPH induction observed by them cannot be compared with the induction observed by us since our measurements were made a day earlier with a different strain of eggs and with different inducing agents.

The aromatic fraction was found to be more potent than the NOS fraction in inducing cytochrome P-450 levels and arylhydrocarbon hydroxylase activities (Figures 3.10-3.12).

Figure 3.13 provides a direct comparison of the results for the liver and shows the concentrations of the aromatic and NOS fractions that are required to cause half maximal

induction of cytochrome P-450 levels as well as of BPH and EROD activities. On a volume equivalent basis, roughly 3 times the amount of the NOS fraction (1.9µl) as compared to the aromatic fraction (0.6µl), was required for half maximal induction of hepatic cytochrome P-450, and 4 times the amount of the NOS fraction was required for half maximal elevation of BPH (1.5µl vs 0.4µl) and EROD (2.7µl vs 0.7µl) activities. This would indicate that the aromatic fraction, and presumably the polyaromatic components present in it, are primarily responsible for the elevation of hepatic cytochrome P-450 levels and related mixed function oxidase activities seen previously with PBCO (Lee et al., 1986).

Since the concentration of the NOS fraction is less than one fifth of the aromatic fraction, the results indicate that on a weight equivalent basis, the NOS fraction was at least as effective as the aromatic fraction in inducing hepatic cytochrome P-450 levels and mixed function oxidase activities. Similar conclusions could also be drawn for the kidney. The fact that the NOS fraction contains potent inducers of chick embryo mixed function oxidase activities has not been previously reported.

It is not known with certainty which components of crude oil are primarily responsible for its toxicity to embryos. Ellenton (1982) subfractionated the aromatic fraction of PBCO and fuel oil No. 2 into 2-3 ring aromatics and 4-5 ring

aromatics. Her results indicated that the teratogenic and toxic activities of both oils resided in the 2-3 ring aromatic fraction. This was due primarily to the much greater abundance of the 2-3 ring aromatics in these oils. Hoffman (1979a) tested the toxic effects on mallard eggs of a mixture of aromatic hydrocarbons similar in class composition to South Louisiana Crude, an American Petroleum Institute reference oil. He found that when individual classes of aromatic compounds were tested, only the tetracyclic like pyrene and chrysene caused significant embryonic death. However, the entire mixture of aromatic hydrocarbons was found to be far more toxic than the individual classes. One possible explanation for this observation could be that the polycyclic components of the mixture would induce the cytochrome P-450 levels in embryos. This would enable the more abundant 2-3 ring aromatic to be more efficiently metabolized and possibly activated to toxic species. In support of this, it has previously been shown that PBCO induces naphthalene hydroxylase 6-fold in the 12 day old chick embryo (Lee et al, 1986). In the present study, we did not subfractionate the aromatic fraction into various classes of compounds.

4.2.2.2 The effects of diesel oil, blowout crude and drilling fluids

Consistent with mortality studies involving day 7 chick embryos (see discussion 4.2.1.1.2), diesel oil and blowout crude were more effective than drilling fluids for inducing EROD and BPH (see figure 3.16). There appears to be a positive correlation between the aromatic content of these compounds (Payne et al., 1985) and these induction studies. This study is also supported by the 3-fold induction of EROD in liver and kidney of fish (Payne et al., 1985). Their studies also indicated that drilling fluids induced EROD to a lesser degree.

4.3 Benzene toxicity in chick embryos

In humans, one of the long term effects of repeated exposure to benzene is the increased risk of leukemia and chromosome abnormalities (Snyder and Kocsis, 1975). This is supported by Gill and Ahmed (1981) who reported that ^{14}C from benzene and its metabolites binds covalently to nucleic acids in the hematopoietic cells of mice.

Morimoto et al (1983) demonstrated that the induction of sister-chromatid exchanges (SCE) in human lymphocytes by microsomal activation of benzene metabolites was highest in the presence of catechol (1,2-dihydroxybenzene) in comparison to hydroquinone, phenol, and benzene. Their results

also indicated that the order of decreasing potency was hydroquinone (1,4-dihydroxybenzene), phenol and benzene. Benzene did not induce SCE, unless a microsomal activation system was present. The data of Morimoto et al (1983) demonstrated that catechol and hydroquinone can be metabolized to produce reactive species such as benzo(semi)-quinones under conditions of lower metabolic activity.

Phenol is a major metabolite of benzene and a rearrangement product of the putative reactive intermediate benzene oxide (Tunek et al., 1978). Depending on the animal species, when phenol was administered *in vivo*, up to 7% of the phenol was further oxygenated to form hydroquinone (Capel et al., 1972). The majority of phenol was directly conjugated and excreted. Although catechol was formed in trace amounts (Park and Williams, 1953), hydroquinone was the main further oxygenated metabolite.

Tunek et al (1980) have provided evidence that rat liver microsomes activate benzene via phenol and hydroquinone to p-benzosemiquinone and p-benzoquinone as quantitatively important reactive metabolites. Benzene is known to block liver regeneration in partially hepatectomized rats (Sammet et al., 1979), and ovarian hypertrophy in the hyperspayed rat (Souza et al., 1979). Gill and Ahmed (1981) reported that mitochondria are irreversibly labeled by ^{14}C from

benzene and its metabolites which correlate with previously described morphological and functional abnormalities of this organelle (Kaminski et al., 1978). These effects may impair respiration, especially in rapidly growing tissues (Gill and Ahmed, 1981).

The effect of benzene metabolites on mitochondria and possibly respiration may be responsible for the mortality rates observed on application of the above in the 7 day chick embryo (see Results 3.4.1). 1,4-Benzoquinone and 1,4-dihydroxybenzene had an LD-50 of 0.07 μmole/egg, indicating that these metabolites were the most toxic of the metabolites tested. In comparison, catechol which had an LD-50 of 11.5 μmoles/egg was still more potent than resorcinol, phenol, benzene, trans-muconate, and cis-muconate. Benzene and phenol were able to induce mortality, presumably because they were metabolized by cytochrome P-450 and other enzymes known to be present in the 7 day old chick embryo (Hamilton et al., 1983). Resorcinol (1,3-dihydroxybenzene) cannot serve as a precursor for quinone formation, therefore possibly explaining why it was not as effective as hydroquinone or catechol for inducing mortality. Cis-muconate is not a known metabolite of benzene, but it was tested with trans-muconate to determine if conformation may have an effect on mortality. However, both compounds produced the lowest toxicity over the dose tested in this experiment.

CHAPTER 5

CONCLUSIONS

1) Metabolized PBCO was more toxic than unmetabolized PBCO as determined *in vitro* by GSH depletion and metHb elevation in erythrocytes.

2) The metabolism of naphthalene was essential in order to produce toxicity in erythrocytes as determined by *in vitro* experiments. Naphthoquinones or compounds capable of autoxidizing to naphthoquinones were the most toxic in terms of GSH depletion and metHb elevation.

3) The aromatic fraction of PBCO was the most potent fraction in terms of mortality in 7 day and 12 day old chick embryos. The induction of several enzymes in the 12 day embryo was stimulated mostly by the aromatic fraction. For the above parameters the NOS fraction was more toxic than expected. Other oils tested which are known to have a high level of PAH's (diesel and blowout crude) produced mortality and enzyme induction in a similar fashion as PBCO. Drilling muds (synthetic oils), which have a low PAH content had low levels of toxicity.

4) The benzene metabolites which were most toxic in terms of mortality in the day 7 chick embryo were hydroquinone, catechol, and 1,4-benzoquinone. This study helps to support previous observations that quinones and compounds capable of autoxidizing to quinones produce greater toxicities than the other compounds tested.

REFERENCES

Ablers, P.H. Effects of external applications of fuel oil on hatchability of mallard eggs. In Fate and effects of petroleum hydrocarbons in marine ecosystems, Wolfe, D.A., (ed.), pp. 158-163. Pergamon Press, New York, 1977.

Ainley, D.G., Grau, C.R., Roudybush, T.E., Morrell, S.H., and Utts, J.M. Petroleum injestion reduces reproduction in Cassin's Auklets. Mar. Pollut. Bull., 1981, 12, 314-317.

Allen, D.W., and Jandl, J.H. Oxidative hemolysis and precipitation of hemoglobin. II. Role of thiols in oxidant drug action. J. Clin. Invest., 1961, 40, 454-475.

Alvares, A.P., Schilling, G., Levin, W., and Kuntzman, R. Studies on the induction of CO-binding pigments in liver microsomes by phenobarbital and 3-methylcholanthrene. Biochem. Biophys. Res. Commun., 1967, 29, 521-526.

Babior, B.M. Oxidizing radicals and red cell destruction. In The function of red blood cells: Erythrocyte pathobiology, pp. 173-195, Alan R. Liss Inc., New York, 1981.

Bentley, P., and Oesch, F. Foreign compound metabolism in the liver. In Progress in Liver Diseases, Vol. 7, Popper H., and Schaffner F. (eds.), pp. 157-178, Grune and Stratton, New York, 1982.

Billings, R.E. Mechanisms of catechol formation from aromatic compounds in isolated rat hepatocytes. Drug Metab. Disposition, 1985, 13, 287-290.

Birchmeier, W., Tuchschmid, P.E., and Winterhalter, K.H. Comparison of human hemoglobin A carrying glutathione as a mixed disulfide with naturally occurring human hemoglobin A_3. Biochemistry, 1973, 12, 3667-3672.

Birkhead, T.R., Lloyd, C. and Corkhill, P. Oiled seabirds successfully cleaning their plumage. Br. Birds, 1973, 66, 535-537.

Bock, K.W., Van Ackeren, G., Lorch, F., and Birke, F.W. Metabolism of naphthalene to naphthalene dihydrodiol glucuronide in isolated hepatocytes and in liver microsomes. Biochem. Pharmac., 1976, 25, 2351-2356.

Booth, J., Boyland, E., and Turner, E.E. The reduction of O-quinones with lithium aluminium hydride. J. Chem. Soc. (London), 1950, 1188-1190.

Bourne, W.P. Seabirds and Pollution. In Marine pollution, Johnson, R., (ed.), pp. 403-502, Academic Press, New York, 1976.

Brown, B.A. In Hematology: Principles and procedures. pp. 74-75, Lea and Febirger, Philadelphia, 1976.

Bunn, H.F., and Forget, B.G. Hemoglobin oxidation: methemoglobin, methemoglobinemia, and sulfhemoglobinemia. In Hemoglobin: molecular, genetic and clinical aspects, Dyson, J., (ed.), pp. 634-662, W.B. Saunders Co., Philadelphia, 1986.

Burke, M.D., Prough, R.A., and Mayer, R.T. Characteristics of a microsomal cytochrome P-448-mediated reaction. Ethoxyresorufin O-de-ethylation. Drug Metab. Dispos., 1977, 5, 1-8.

Capel, I.D., French, M.R., Millburn, P., Smith, R.L. and Williams, R.T. The fate of [^{14}C]phenol in various species. Xenobiotica, 1972, 2, 25-34.

Carrell, R.W., Winterbourn, C.C., and Rachmilewitz, E.A. Activated oxygen and haemolysis. Brit. J. Haematol., 1975, 30, 259-264.

Clark, R.C., and Brown, D.W. Petroleum: properties and analyses in biotic and abiotic systems. In Effects of petroleum on arctic and subarctic marine environments and organisms. Vol. 1, Nature and fate of petroleum Malins, D.C., (ed.), pp. 1-89, Academic Press, New York, 1977.

Cohen, G., and Hochstein, P. Glutathione peroxidase: The primary agent for the elimination of hydrogen peroxide in erythrocytes. Biochemistry, 1963, 2, 1420-1428.

Cohen, G., and Hochstein, P. Generation of hydrogen peroxide in erythrocytes by hemolytic agents. Biochemistry, 1964, 3, 895-900.

Conney, A.H. Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev., 1967, 19, 317-366.

Currie, A.R., Bird, C.C., Crawford, A.M., and Sims, P. Embryopathic effects of 7,12-dimethylbenz(a)anthracene and its hydroxymethyl derivatives in the Sprague-Dawley rat. Nature (London), 1970, 226, 911-914.

Dimant, E., Landsberg, E., and London, I.M. The metabolic behavior of reduced glutathione in human and avian erythrocytes. J. Biol. Chem., 1955, 213, 769-776.

Dobretsov, G.E., Borschevskaya, T.A., Petrov, V.A., and Vladimirov, Y.A. The increase of phospholipid bilayer rigidity after peroxidation. Febs Lett., 1977, 84, 125-128.

Doherty, M., and Cohen, G.M. Metabolic activation of 1-naphthol by rat liver microsomes to 1,4-naphthoquinone and covalent binding species. Biochem. Pharmac., 1984, 33, 3201-3208.

Draper, H.H., and Saari Csallany, A. A simplified hemolysis test for vitamin E deficiency. J. Nutrition, 1969, 98, 390-394.

Ellenton, J.A. Teratogenic activity of aliphatic and aromatic fractions of Prudhoe Bay crude and fuel oil No. 2 in the chicken embryo. Toxicol. Appl. Pharmacol., 1982, 63, 209-215.

Ernster, L. DT Diaphorase. In Methods in enzymology, Vol. X, Oxidation and phosphorylation, Estabrook, R.W., and Pullman, M.E. (eds.), pp. 309-317, Academic Press, New York, 1967.

Eyer, P., and Gaber, H. Biotransformation of 4-dimethylaminophenol in the dog. Biochem. Pharmac., 1978, 27, 2215-2221.

Eyer, P. and Kiese, M. Biotransformation of 4-dimethylaminophenol: Reaction with glutathione, and some properties of the reaction products. Chem.-Biol. Interact., 1976, 14, 165-178.

Eyer, P., Kiese, M, Lipowsky, G., and Weger, N. Reactions of 4-dimethylaminophenol with hemoglobin, and autoxidation of 4-dimethylaminophenol. Chem.-Biol. Interact., 1974, 8, 41-59.

Fairbanks, V.F. Hemoglobin, hemoglobin derivatives and myoglobin. In Fundamentals of clinical chemistry, Tietz, N.W., (ed.), pp. 401-450, W.B. Saunders Co., Philadelphia, 1976.

Forstrom, J.W., Zakowski, J.J., and Tappel, A.L. Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. Biochemistry, 1978, 17, 2639-2644.

Fouts, J.R., and Rogers, L.A. Morphological changes in the liver accompanying stimulation of microsomal drug metabolizing enzyme activity by phenobarbital, chlordane, benzpyrene or methylcholanthrene in rats. J. Pharmacol. Exp. Ther., 1965, 147, 112-119.

Fridovich, I., and Handler, P. Detection of free radicals generated during enzymic oxidations by the initiation of sulfite oxidation. J. Biol. Chem., 1961, 236, 1836-1840.

Garel, M.C., Domenget, C., Martin, J.C., Prehu, C., Galacteros, F., and Beuzard, Y. Covalent binding of glutathione to hemoglobin. 1. Inhibition of hemoglobin S polymerization. J. Biol. Chem., 1986, 261, 14704-14709.

Gault, M.H., Shahidi, N.T., and Barber, V.E. Methemoglobin formation in analgesic nephropathy. Clin. Pharmacol. Therap., 1974, 15, 521-527.

Gearing, P., Gearing, J.N., Lytle, T.F., and Lytle, J.S. Hydrocarbons in 60 northeast Gulf of Mexico shelf sediments: a preliminary survey. Geochim. Cosmochim. Acta, 1976, 40, 1005-1017.

Geraci, G., Parkhurst, L.J., and Gibson, Q.H. Preparation and properties of α- and β-chains from human hemoglobin. J. Biol. Chem., 1969, 244, 4664-4667.

Gidron, E. and Leurer, J. Naphthalene poisoning. Lancet, 1956, 270, 228-231.

Gill, D.P., and Ahmed, A.E. Covalent binding of [^{14}C] benzene to cellular organelles and bone marrow nucleic acids. Biochem. Pharmacol., 1981, 30, 1127-1131.

Gillette, J.R., Brodie, B.B., and La Du, B.N. The oxidation of drugs by liver microsomes: on the role of TPNH and oxygen. J. Pharmac. Exp. Ther., 1957, 119, 532-540.

Goldberg, B., and Stern. A. Production of superoxide anion during the oxidation of hemoglobin by menadione. Biochim. Biophys. Acta, 1976, 437, 628-632.

Gorski, J.R., Arlotto, M.P., Klaassen, C.D., and Parkinson, A. Age- and sex-dependent induction of liver microsomal benzo[a]pyrene hydroxylase activity in rats treated with pregnenolone-16α-carbonitrile (PCN). Carcinogenesis, 1985, 6, 617-624.

Gorsline, J., Holmes, W.N., and Cronshaw, J. The effects of injested petroleum on naphthalene metabolizing properties of liver tissue in seawater-adapted mallard ducks (Anas platyrhynchos). Environ. Res., 1981, 24, 377-390.

Haest, C.W.M., Kamp, D., Plasa, G., and Deuticke, B. Intra- and intermolecular cross-linking of membrane proteins in intact erythrocytes and ghosts by SH-oxidizing agents. Biochim. Biophys. Acta, 1977, 469, 226-230.

Hamilton, J.W., Denison, M.S., and Bloom, S.E. Development of basal and induced aryl hydrocarbon (benzo(a)pyrene) hydroxylase activity in the chicken embryo in ovo. Proc. Natl. Acad. Sci. USA, 1983, 80, 3372-3376.

Hamilton, J.W., and Bloom, S.E. Developmental differences in basal and aryl induced hydrocarbon (benzo(a)pyrene) hydroxylase activity in chick embryo liver and lung in ovo. Biochem. Pharmacol., 1983, 32, 2986-2988.

Hartung, R. Injestion of oil by water fowl. Papers Mich. Acad. Sci. Arts Lett., 1963, 48, 49-55.

Hartung, R. Some effects of oiling on reproduction of ducks. J. Wildl. Manage., 1965, 29, 872-874.

Hartung, R. Energy metabolism in oil-covered ducks. J. Wildlife Manage., 1967, 31, 798-804.

Hesse, S., and Mezger, M. Involvement of phenolic metabolites in the irreversible protein-binding of aromatic hydrocarbons: Reactive metabolites of [^{14}C] naphthalene and [^{14}C] 1-naphthol formed by rat liver microsomes. Mol. Pharmacol., 1979, 16, 667-675.

Hoffman, D.J. Embryotoxic effects of crude oil in mallard ducks and chicks. Toxicol. Appl. Pharmacol., 1978, 46, 183-190.

Hoffman, D.J. Embryotoxic and teratogenic effects of petroleum hydrocarbons in mallards (Anas platyrhynchos). J. Toxicol. Environ. Health., 1979a, 5, 835-844.

Hoffman, D.J. Embryotoxic and teratogenic effects of crude oil on mallard embryos on day one of development. Bull. Environ. Contam. Toxicol., 1979b, 22, 632-637.

Hoffman, D.J., and Gay, M.L. Embryotoxic effects of benzo(a)pyrene, chrysene, and 7,12-dimethylbenz(a)anthracene in petroleum hydrocarbon mixtures in mallard ducks. J. Toxicol. Environ. Health, 1981, 7, 775-787.

Holmes, W.N. Hormones and osmoregulation in marine birds. Gen. Comp. Endocrinol., 1975, 25, 249-258.

Holmes, W.N., and Cronshaw, J. Biological effects of petroleum on marine birds. In Effects of petroleum on arctic and subarctic marine environments and organisms. Vol. 2, Biological effects, Malins, D.C., (ed.), pp. 359-398, Academic Press, New York, 1977.

Holmes, W.N., Cronshaw, W.J. and Gorsline, J. Some effects of injested petroleum on seawater-adapted ducks (Anas platyrhynchos). Environ. Res., 1978, 17, 177-190.

Horning, M.G., Stillwell, W.G., Griffin, G.W., and Tsang, W.-S. Epoxide intermediates in the metabolism of naphthalene by the rat. Drug Metab. Disposition, 1980, 8, 404-414.

Hunt, J.M. Petroleum geochemistry and geology, W.H. Freeman and Co., San Francisco, 1979.

Iyanagi, T., and Yamazaki, I. One-electron-transfer reactions in biochemical systems. V. Difference in the mechanism of quinone reduction by the NADH dehydrogenase and the NAD(P)H dehydrogenase (DT-diaphorase). Biochim. Biophys. Acta, 1970, 216, 282-294.

Jacob, H.S. and Lux, S.E. Degradation of membrane phospholipids and thiols in peroxide hemolysis: Studies in vitamin E deficiency. Blood, 1968, 32, 549-568.

Jacob, H.S., Brain, M.C., and Dacie, J.V. Altered sulfhydryl reactivity of hemoglobins and red blood cell membranes in congenital Heinz body hemolytic anemia. J. Clin. Invest., 1968, 47, 2664-2677.

Jandl, J.H., Engle, L.K. and Allen, D.W. Oxidative hemolysis and precipitation of hemoglobin. I. Heinz body anemias as an acceleration of red cell aging. J. Clin. Invest., 1960, 39, 1818-1836.

Jerina, D.M., Daly, J.W., Witkop, B., Zaltzman-Nirenberg, P., and Udenfriend, S. 1,2-Naphthalene oxide as an intermediate in the microsomal hydroxylation of naphthalene. Biochemistry, 1970, 9, 147-155.

Kaminski, M., Jonek, J.J., Konecki, J, Kaminska, O., Gruszeczka, B. and Koehler, B. Histochemical and histoenzymatic changes in mouse-liver in sub-acute benzene intoxication. Acta Histochem., 1978, 61, 1-19.

Kappus, H. Overview of enzyme systems involved in bioreduction of drugs and in redox cycling. Biochem. Pharmacol., 1986, 35, 1-6.

Keilin, D., and Hartree, E.F. Properties of azide-catalase. Biochem. J., 1945, 39, 148-157.

Kopishke, E.D. The effect of 2,4-D and diesel fuel on egg hatchability. J. Wildl. Manage., 1972, 36, 1353-1356.

Korhonen, A., Hemminki, K., and Vainio, H. Embryotoxic effects of eight organic peroxides and hydrogen peroxide on three-day chicken embryos. Environ. Res., 1984, 33, 54-61.

Kosower, N.S., Song, K., Kosower, E.M., and Correa, W. Glutathione II. Chemical aspects of azoester procedure for oxidation to disulfide. Biochim. Biophys. Acta., 1969, 192, 8-14.

Krimsky, I. and Racker, E. Glutathione, a prosthetic group of glyceraldehyde-3-phosphate dehydrogenase. J. Biol. Chem., 1952, 198, 721-729.

Lee, Y.-Z., Leighton, F.A., Peakall, D.B., Norstrom, R.J., O'Brien, P.J., Payne, J.F., and Rahimtula, A.D. Effects of injestion of Hibernia and Prudhoe Bay crude oils on hepatic and renal mixed function oxidase in nestling Herring gulls (Larus argentatus). Environ. Res., 1985, 36, 248-255.

Lee, Y.-Z., O'Brien, P.J., Payne, J.F., and Rahimtula, A.D. Toxicity of petroleum crude oils and their effect on xenobiotic metabolizing enzyme activities in the chicken embryo in ovo. Environ. Res., 1986, 39, 153-163.

Leighton, F.A., Peakall, D.B., and Butler, R.G. Heinz-body hemolytic anemia from the injestion of crude oil: A primary toxic effect in marine birds. Science, 1983, 220, 871-873.

Leighton, F.A. Morphological lesions in red blood cells from herring gulls and atlantic puffins injesting Prudhoe Bay crude oil. Vet. Pathol., 1985, 22, 393-402.

Lind, C., Hochstein, P., and Ernster, L. DT-Diaphorase as a quinone reductase: A cellular control device against semiquinone and superoxide radical formation. Arch. Biochem. Biophys., 1982, 216, 178-185.

Little, C., and O'Brien, P.J. An intracellular GSH-peroxidase with a lipid peroxide substrate. Biochem. Biophys. Res. Commun., 1968, 31, 145-150.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. Protein measurement with the folin phenol reagent. J. Biol. Chem., 1951, 193, 265-275.

Lu, A.Y.H., Somogyi, A., West, S., Kuntzman, R., and Conney, A.H. Pegnenolone-16α-carbonnitrile: A new type of inducer of drug-metabolizing enzymes. Arch. Biochem. Biophys., 1972, 152, 457-462.

Lu, A.Y.H. and West, S.B. Multiplicity of mammalian microsomal cytochromes P-450. Pharmacol. Rev., 1980, 31, 277-295.

Lynch, R.E., Thomas, J.E., and Lee, G.R. Inhibition of methemoglobin formation from purified oxyhemoglobin by superoxide dismutase. Biochemistry, 1977, 16, 4563-4567.

Mason, H.S., Fowlks, W.L., and Peterson, E. Oxygen transfer and electron transport by the phenolase complex. J. Am. Chem. Soc., 1955, 77, 2914-2915.

Miller, D.S., Peakall, D.B. and Kinter, W.B. Injestion of crude oil: sublethal effects in Herring Gull chicks. Science, 1978, 199, 315-317.

Miller, E.C., and Miller, J.A. Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. Cancer, 1981, 47, 2327-2345.

Miller, M.G., Rodgers, A. and Cohen, G.M. Mechanisms of toxicity of naphthoquinones to isolated hepatocytes. Biochem. Pharmac., 1986, 35, 1177-1184.

Moldeus, P., and Jernstrom, B. Interaction of glutathione with reactive intermediates. In Functions of glutathione: Biochemical, physiological, toxicological and clinical aspects, Orrenius, S., Holmgren, A., and Mannervik, B., (eds.), pp. 99-108. Raven Press, New York, 1983.

Morimoto, K., Wolff, S., and Koizumi, A. Induction of sister-chromatid exchanges in human lymphocytes by microsomal activation of benzene metabolites. Mutation Res., 1983, 119, 355-360.

National Research Council. Chemical composition of petroleum hydrocarbon sources. In Oil in the Sea, Inputs fates and effects, pp. 17-42. National Academy Press, Washington, D.C., 1985.

Natusch, D.F.S., and Tomkins, B.A. Isolation of polycyclic organic compounds by solvent extraction with dimethyl sulfoxide. Anal. Chem., 1978, 50, 1429-1434.

Nebert, D.W., Eisen, H.J., Negishi, M., Lang, M.A., and Hjelmeland, L.M., and Okey, A.B. Genetic mechanisms controlling the induction of polysubstrate monooxygenase (P-450) activities. Ann. Rev. Pharmacol. Toxicol., 1981, 21, 431-462.

Nebert, D.W., and Gelboin, H.V. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of the induced enzyme. J. Biol. Chem., 1968, 243, 6242-6249.

Nebert, D.W., and Gonzalez, F.J. Cytochrome P-450 gene expression and regulation. Trends Pharmacol. Sci., 1985, 6, 160-164.

Nicholls, P. Contributions of catalase and glutathione peroxidase to red cell peroxide removal. Biochim. Biophys. Acta, 1972, 279, 306-309.

Nickerson, W.J., Falcone, G., and Strauss, G. Studies on quinone-thioethers. I. Mechanism of formation and

properties of thiodione. Biochemistry, 1963, 2, 537-543.

Okey, A.B., Roberts, E.A., Harper, P.A., and Denison, M.S. Induction of drug-metabolizing enzymes: Mechanisms and consequences. Clin. Chem., 1986, 19, 132-141.

Omura, T., and Sato, R. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem., 1964, 239, 2370-2378.

Orrenius, S., and Ernster, L. Phenobarbital-induced synthesis of the oxidative demethylating enzymes of rat liver microsomes. Biochem. Biophys. Res. Commun., 1964, 16, 60-65.

Oski, F.A., and Barness, L.A. Vitamin E deficiency: A previously unrecognized cause of hemolytic anemia in the premature infant. J. Pediatr., 1967, 70, 211-220.

Packer, J.E., Slater, T.F., and Willson, R.L. Direct observation of a free radical interaction between vitamin E and vitamin C. Nature, 1979, 278, 737-738.

Park, D.V., and Williams, R.T. Studies in detoxication 49. The metabolism of benzene containing [$^{14}C_{10}$]benzene. Biochem. J., 1953, 54, 231-238.

Patterson, J.W., and Lazarow, A. Determination of glutathione. In The methods of biochemical analysis. Vol. 2, Glick, D., (ed.), pp. 259-278, Interscience, New York, 1955.

Payne, J.F., Fancey, L., Kiceniuk, J., Williams, U., Osborne, J., and Rahimtula, A. Mixed-function oxygenases as biological monitors around petroleum hydrocarbon development sites: Potential for induction by diesel and other drilling mud base oils containing reduced levels of polycyclic aromatic hydrocarbons. Marine Environ. Res., 1985, 17, 328-332.

Payne, J.F., and May, N. Oxidative transformation of complex mixtures of pollutant aromatic hydrocarbons by fish. Chemosphere, 1978, 7, 815-819.

Peakall, D.B., Hallett, D.J., Bend, J.R., Foureman, G.L., and Miller, D.S. Toxicity of Prudhoe Bay crude oil and its aromatic fractions to nestling herring gulls. Environ. Res., 1982, 27, 206-215.

Peakall, D.B., Tremblay, J., Kinter, W.B., and Miller, D.S. Endocrine dysfunction in seabirds caused by injested oil. Environ. Res., 1981, 24, 6-14.

Pelkonen, O., and Nebert, D.W. Metabolism of polycyclic aromatic hydrocarbons: Etiologic role in carcinogenesis. Pharmacol. Rev., 1982, 34, 189-222.

Perona, G., Guidi, G.C., Piga, A., Cellerino, R., Menna, R., and Zatti, M. In vivo and in vitro variations of human erythrocyte glutatione peroxidase activity as a result of cells aging, selenium availability and peroxide activation. Br. J. Haematol., 1978, 39, 399-408.

Pohl, R.J., and Fouts, J.R. A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. Anal. Biochem., 1980, 107, 150-155.

Poland, A., Glover, E., and Kende, A.S. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. Evidence that the binding species is the receptor for the induction of aryl hydrocarbon hydroxylase. J. Biol. Chem., 1976, 251, 4936-4946.

Powis, G., Svingen, B.A., and Appel, P. Quinone-stimulated superoxide formation by subcellular fractions, isolated hepatocytes, and other cells. Mol. Pharmacol., 1981, 20, 387-394.

Prough, R.A., Patrizi, V.W., Okita, R.T., Masters, B.S., and Jakobsson, S.W. Characteristics of benzo(a)pyrene metabolism by kidney, liver, and lung microsomal fractions from rodents and humans. Cancer Res., 1979, 39, 1199-1206.

Rachmilewitz, E.A., Harari, E., and Winterhalter, K.H. Separation of α- and β-chains of hemoglobin A by acetylphenylhydrazine. Biochim. Biophys. Acta, 1974, 371, 402-407.

Rahimtula, A.D., O'Brien, P.J., and Payne, J.F. Induction of xenobiotic metabolism in rats on exposure to hydrocarbon-based oils. In Applied toxicology of petroleum hydrocarbons. Vol. V, MacFarland, H.N., Holdsworth, C.E., MacGregor, J.A., Call, R.A., and Kane, M.L. (eds.), pp. 71-79. Princeton Scientific Publ., Princeton., 1984.

Rahimtula, A.D., Zachariah, P.K., and O'Brien, P.J. Differential effects of antioxidants, steroids and other compounds on benzo(a)pyrene 3-hydroxylase activity in various tissues of rat. Brit. J. Cancer, 1979, 40, 105-112.

Rahimtula, A.D., Payne, J.F., and Martins, I. Hydrocarbon-based oils as inducers of cutaneous aryl hydrocarbon hydroxylase. Toxicol. Lett., 1982, 10, 213-217.

Reed, D.J., Babson, J.R., Beatty, P.W., Brodie, A.E., Ellis, W.W., and Potter, D.W. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide and related thiols and disulfides. Anal. Biochem., 1980, 106, 55-62.

Rifkind, R.A. Heinz body anemia: An ultrastructural study. II. Red cell sequestration and destruction. Blood, 1965, 26, 433-448.

Rigdon, R.H., and Rennels, E.G. Effect of feeding benzpyrene on reproduction in the rat. Experientia, 1964, 20, 224-226.

Rittinghaus, H. On the indirect spread of oil pollution in a seabird sanctuary. Ornithol. Mitt, 1956, 8, 43-46.

Sammett, D., Lee, E.W., Kocsis, J.J., and Snyder, R. Effect of partial hepatectomy on benzene metabolism and toxicity. Toxic. Appl. Pharmac., 1979, 48, A39 (Abst. #78).

Sato, T., Fukuyama, T., Suzuki, T., and Yoshikawa, H. 1,2-Dihydro-1,2-dihydroxybenzene and several other substances in the metabolism of benzene. J. Biochem., 1963, 53, 23-27.

Sladek, N.E., and Mannering, G.J. Evidence for a new P-450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. Biochem. Biophys. Res. Commun., 1966, 24, 668-674.

Smith, R.P. Toxic responses of the blood. In Toxicology, the basic science of poisons, 2nd edition, Doull, J., Klaassen, C.D., and Amdur, M.O. Macmillan Publishing Co. Inc., New York, 1980.

Snyder, R. and Kocsis, J.J. Current concepts of chronic benzene toxicity. CRC Crit. Rev. Toxicol., 1975, 3, 265-288.

Souza, M.D., Snyder, R. and Kocsis, J.J. Benzene inhibits ovarian hypertrophy in the hyperspayed rat. Toxic. Appl. Pharmac., 1979, 48, A40 (Abst. #79).

Srivastava, S.K., and Beutler, E. Permeability of normal and glucose-6-phosphate dehydrogenase deficient erythrocytes to glutathione. Biochem. Biophys. Res. Commun., 1967, 28, 659-664.

Srivastava, S.K., and Beutler, E. The transport of oxidized glutathione from human erythrocytes. J. Biol. Chem., 1969, 244, 9-16.

Thomas, M.J., Mehl, K.S., and Pryor, W.A. The role of the superoxide anion in the xanthine oxidase-induced autoxidation of linoleic acid. Biochem. Biophys. Res. Commun., 1978, 83, 927-932.

Thor, H., Smith, M.T., Hartzell, P., Bellomo, G., Jewell, S.A., and Orrenius, S. The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. J. Biol. Chem., 1982, 257, 12419-12425.

Tietze, F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. Anal. Biochem., 1969, 27, 502-522.

Tissot, B.P., and Welte, D.H. Petroleum formation and occurrence, 2nd edition, Springer-Verlag, New York, 1984.

Tunek, A., Platt, K.L., Bentley, P. and Oesch, F. Microsomal metabolism of benzene to species irreversibly binding to microsomal protein and effects of modifications of this metabolism. Mol. Pharmacol., 1978, 14, 920-929.

Tunek, A., Platt, K.L., Przybylski, M., and Oesch, F. Multi-step metabolic activity of benzene. Effect of superoxide dismutase on covalent binding to microsomal macromolecules, and identification of glutathione conjugates using high pressure liquid chromatography and field desorption mass spectrometry. Chem.-Biol. Interact., 1980, 33, 1-17.

Valaes, T., Doxiadis, S.A., and Fessas, P. Acute hemolysis due to naphthalene inhalation. J. Pediat., 1963, 63, 904-915.

Valentine, W.N., and Tanaka, K.R. The glyoxalase content of human erythrocytes and leukocytes. Acta Haematol., 1961, 26, 303-316.

Van Heyningen, R., and Pirie, A. The metabolism of naphthalene and its toxic effect on the eye. Biochem. J., 1967, 102, 842-852.

Vermeer, K. and Vermer, R. Oil threat to birds on the Canadian West Coast. Can. Field-Nat., 1975, 89, 278-298.

Vogel, K., Bentley, P., Platt, K.L., and Oesch, F. Rat liver cytoplasmic dihydrodiol dehydrogenase. J. Biol. Chem., 1980, 255, 9621-9625.

Wallace, W.J., and Caughey, W.S. Mechanism for the autoxidation of hemoglobin by phenols, nitrite and "oxidant" drugs. Peroxide formation by one electron donation to bound dioxygen. Biochem. Biophys. Res. Commun., 1975, 62, 561-567.

Wever, R., Oudega, B., and Van Gelder, B.F. Generation of superoxide radicals during the autoxidation of mammalian oxyhemoglobin. Biochim. Biophys. Acta, 1973, 302, 475-478.

Winterbourn, C.C. Free-radical production and oxidative reactions of hemoglobin. Environ. Health Perspect., 1985, 64, 321-330.

APPENDIX A

For GC-mass spectral analysis the sample was injected into an oven of a 5792A Hewlett Packard Gas Chromatograph and equilibrated at 70°C for 3 minutes. A temperature rise of 4°C per minute was sustained until a final temperature of 270°C was reached. The sample was then injected into a 5970A Hewlett Packard Mass Selective Detector equilibrated at 280°C. The data was analysed by a 9133 Hewlett Packard Data System.

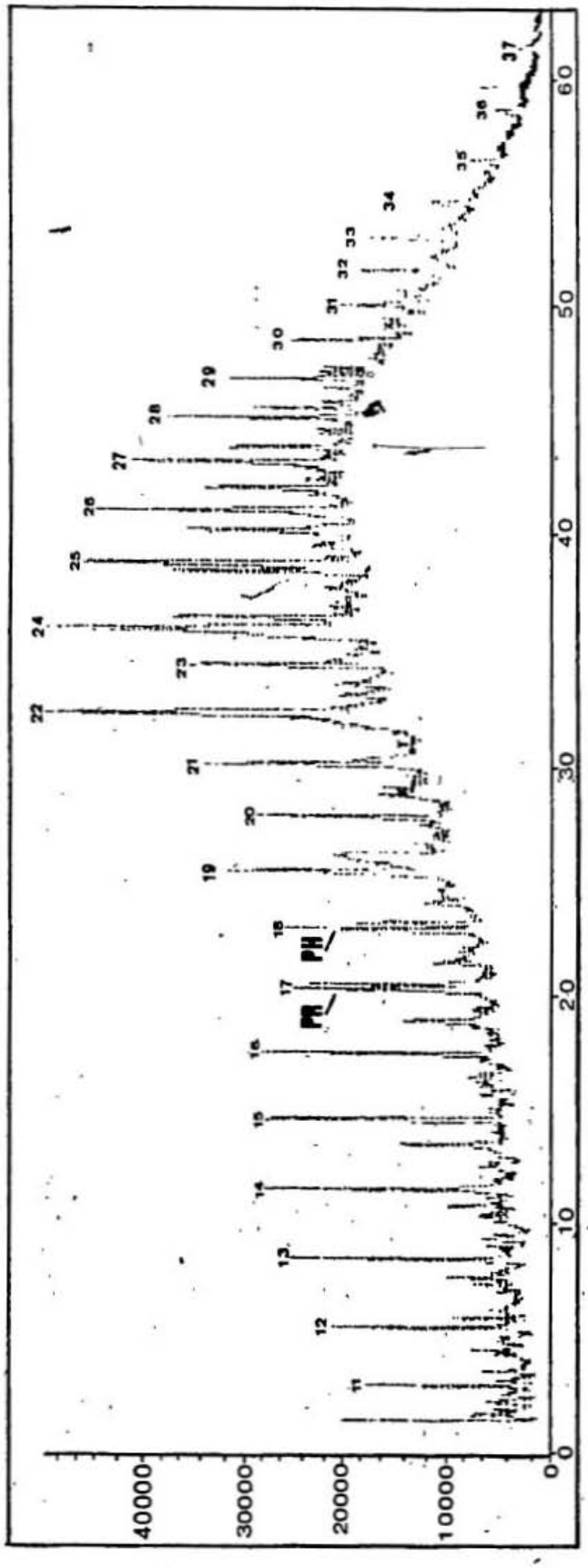


Figure A.1: GC-mass spectrum of the aliphatic fraction of PBCO

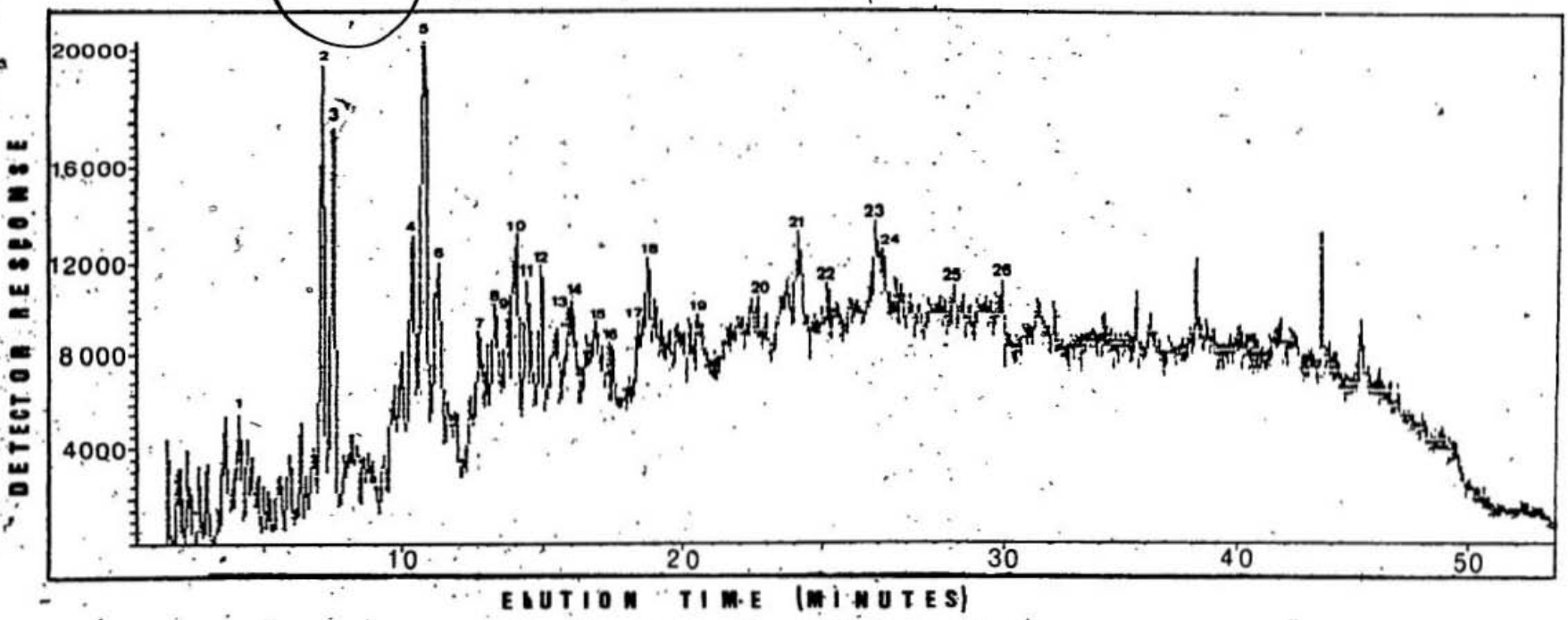


Figure A.2: GC-mass spectrum of the aromatic fraction of PBCO

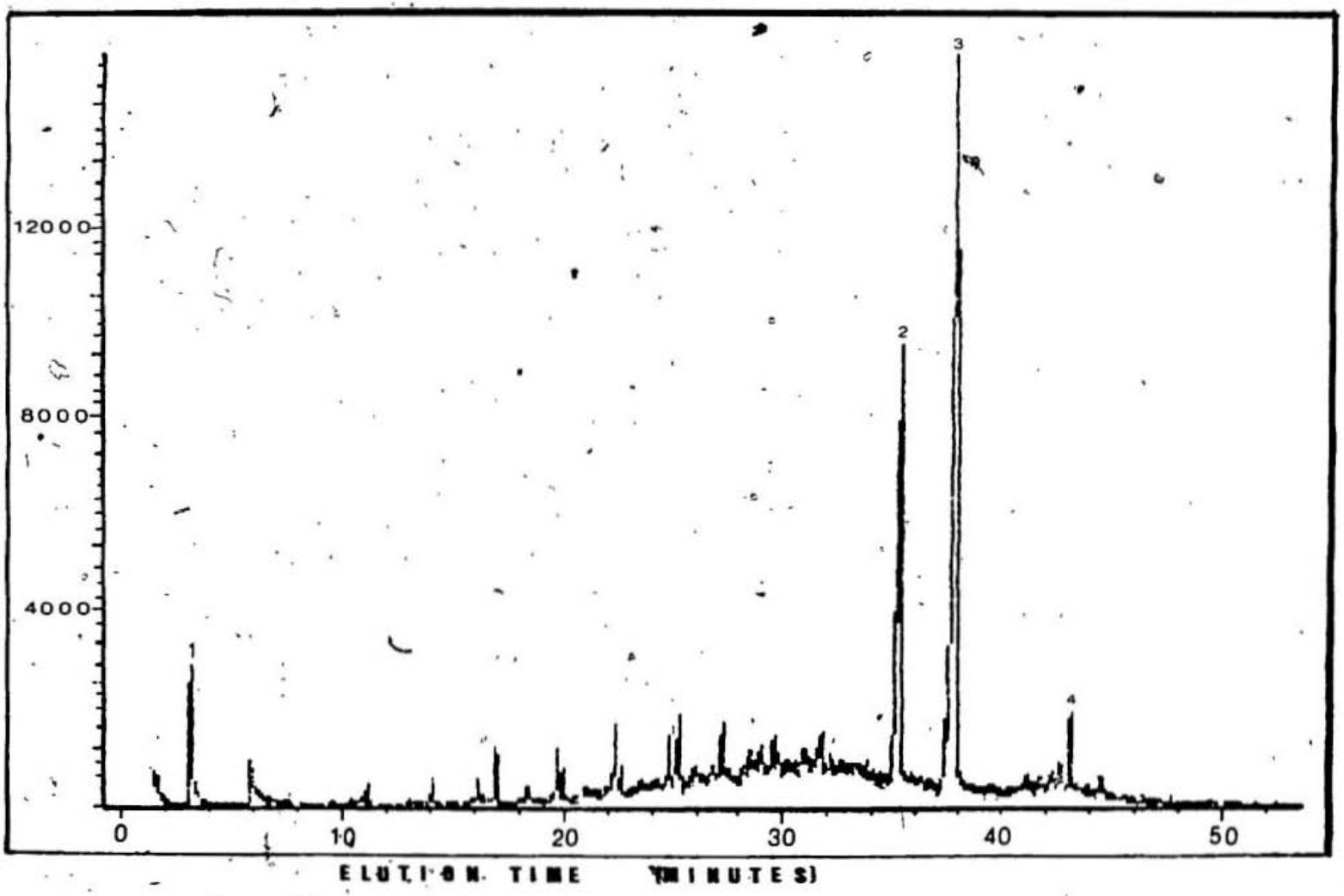


Figure A.3: GC-mass spectrum of the NOS fraction of PBCO

Table A.1: Identification of selected peaks in GC-mass spectrum of the aliphatic and NOS fractions of PBCO.

aliphatic fraction

Peak number	Compound(s)
11-37	C_{11} - C_{37} normal alkane series
PR	Pristane
PH	Phytane

NOS fraction

1	benzoquinoline
2	hexanedioic acid
3	1,2-benzenedicarboxylic acid
4	carbazole

The aliphatic and NOS fractions of PBCO were prepared as described in Methods.

Table A.2: Identification of selected peaks in GC-mass spectrum of the aromatic fraction of PBCO

Peak number	Compound
1	naphthalene
2	2-methylnaphthalene
3	1-methylnaphthalene
4	1,3-dimethylnaphthalene
5	C_2-methylnaphthalene
6	1,2-dimethylnaphthalene
7	C_1-biphenyl
8	C_3-trimethylnaphthalene
9	C_3-trimethylnaphthalene
10	1,4,5-trimethylnaphthalene
11	1,4,6-trimethylnaphthalene
12	C_3-trimethylnaphthalene
13	C_2-biphenyl
14	C_2-biphenyl
15	C_4-naphthalene
16	C_4-naphthalene
17	C_3-triphenyl
18	C_1-fluorene
19	phenanthrene
20	C_4-biphenyl
21	1-methylphenanthrene
22	C_1-phenanthrene
23	2,5-dimethylphenanthrene
24	2,3-dimethylphenanthrene
25	C_3-phenanthrene
26	2,3,5-trimethylphenanthrene

The aromatic fraction of PBCO was prepared as described in Methods. $C_\#$ represents side chain aliphatic group(s). eg. C_2 represents either 2 methyl groups or 1 ethyl group.

Table A.3: Analysis of drilling mud base oils

drilling fluid	concentration of aromatic compounds (ppm)			
	1-ring	2-rings	3-rings	4-rings
Safver 1	35,000	15,000	700	ND
Safver 4	10,000	500	ND	ND
Safver 5	145,000	6,000	ND	ND

ND - none detected
ppm - parts per million

Analysis was performed by Payne et al (1985). One, 2, 3 and 4-ring aromatic compounds were calculated as benzene, naphthalene, phenanthrene and pyrene equivalents.