

METABOLISM AND POSSIBLE TOXICITY
OF 2,6 - DIMETHYLNAPHTHALENE,
A COMPONENT OF CRUDE OIL

CENTRE FOR NEWFOUNDLAND STUDIES

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ZUZZER A. SHAMSUDDIN



**METABOLISM AND POSSIBLE TOXICITY OF
2,6-DIMETHYLNAPHTHALENE, A COMPONENT
OF CRUDE OIL**

by

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

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In the name of Allah the Most Beneficent the Most Merciful

"It is your Lord, the Most Bounteous, who taught by the pen,
taught man that which he knew not...."

Al-Quran 96: 2-5

Dedicated to
Almighty Allah who blessed me with perception,
knowledge and skills to serve the fellowmen.

And to my parents whose love and affection augmented
aptitude and practice.

"And your Lord has commanded that you shall not serve
(any) but Him and goodness to your parents: If either or both of them
reach old age with you, say not to them (so much as) "ugh", nor chide
them, and speak to them a generous word.

And make yourself submissively gentle to them with compassion, and say:
O my Lord! have compassion on them, as they brought me up (when I was)
little.

Your Lord knows best what is in your minds; if you are good, then He
is surely Forgiving to those who turn (to Him) frequently.

Al-Quran 17: 23-24

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Chapter 1

Introduction

1.1. Alkylated Naphthalenes as Environmental Pollutants

Naphthalene and alkylated naphthalenes, especially the methylated naphthalenes, are the major constituents of the water soluble fraction of crude and fuel oils (Anderson et al., 1974) quantities of petroleum enter fresh waters from sources such as oil refineries, petrochemical waste waters, urban storm drainage and from disposal of used lubricants (Storrs et al., 1973; Boyd et al., 1976). After spills of crude oil on bodies of water, water-soluble components from the oil may remain even after cleanup, and continue to exert their hazardous effects on the aquatic environment. The rapid increase in the demand for and the utilization of petroleum and petrochemicals has resulted in steadily increasing levels of petroleum contamination of marine and estuarine waters. It has been estimated that the total annual influx of oil into the oceans is approximately 7 million tons (Anderson et al., 1974). Oil spills have caused the loss of large numbers of sea birds, marine and estuarine animals, and have become a serious threat to the environment (Clark, 1973). Low molecular weight polycyclic aromatic hydrocarbons such as naphthalene and methylated naphthalenes are known to be the major water-soluble constituents of crude oil, and are also major by-products of combustion processes (Schmeltz et al., 1974; 1976). Large numbers of aromatic hydrocarbons (chiefly alkylated benzenes and naphthalenes) in sea water extracts of crude oils and kerosene have been reported (Boylan and Tripp, 1971). Boyd et al. (1976) showed that in petroleum pollution incidents for 1974 in the U.S., 52% of the volume spilled was in inland waters (including marine waters).

The water solubility of dimethylnaphthalenes from various crude oils ranges from 0.06 ppm to .24 ppm (Anderson et al., 1974). Water-solubility of 2,6-DMN is 2mg/L (Mackay and Shiu, 1977).

In industry, methylated naphthalenes are used as dye carriers for polyester fibre. They are also constituents of cigarette smoke. An 85mm cigarette containing U.S. blended tobacco (without filter tips) would give out 220 ng. of dimethylnaphthalenes in smoke (Schmeltz et al., 1976). Methylated naphthalenes are also used as a mosquito and blackfly repellent. One commercial product available in the U.S. ("Mosquito Beater") is composed of 9% crude oil and 16% methylated naphthalenes (Witz et al., 1981).

1.2. Living Organisms and Alkylated Naphthalenes

Some tumor promoting and co-carcinogenic activity has been attributed to alkylated naphthalenes (Horton et al., 1957). Naphthalene and alkyl substituted naphthalenes are concentrated by marine animals, and metabolized with subsequent toxic effects (Anderson et al., 1974). 2-Methylnaphthalene (2-MN) is metabolized in fish and rats (Breger et al., 1981). 2,6-Dimethylnaphthalene (2,6-DMN) is toxic to polychaetes (Rossi and Neff, 1978). It accumulates in grass shrimp (Dillon, 1981), and decreases the resistance of estuarine shrimp to the combined challenge of hypoxia and reduced salinity (Dillon, 1981). 2,6-DMN also alters the electrophysiological responses of lateral line nerves in coho salmon (Falk et al., 1981). 2-MN is toxic to the Clara cells of lung and binds to tissue macromolecules in mice (Griffin et al., 1983). The LD₅₀ of naphthalene given intraperitoneally to male and female rats is 0.59 g and 0.60 g per kg body weight, respectively. 1-Methylnaphthalene and 2-MN are less toxic to the rat than naphthalene by a factor of about 3 (Rashestyuk, 1970). The acute toxicity of whole oil is often directly related to the concentration of naphthalenes in it (Anderson et al., 1974; Rossi and Neff, 1978; Tatem et al., 1978). 2,6-DMN is more toxic than naphthalene to many marine organisms (Anderson et al., 1974). Alkylation (methylation) probably substantially modifies the chemical and

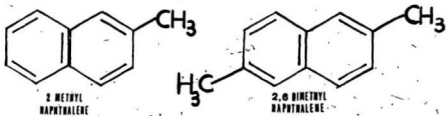
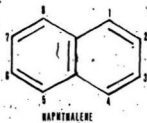
biological characteristics of an aromatic hydrocarbon (Rossi and Neff, 1978). The chemical structures of naphthalene and some alkylated naphthalenes are shown in Figure 1-1.

1.3. Hepatic Mixed Function Oxidases

In order to exert its biological effects, a xenobiotic compound in suitable concentrations must interact with receptors. The intensity and duration of action of a pharmacologically or toxicologically active substance is dependent on absorption, binding, metabolism, and excretion. Numerous foreign compounds including the polycyclic hydrocarbons are so hydrophobic that they would remain in the body indefinitely were it not for biotransformation. During phase I of metabolism, one or more polar groups (e.g. hydroxyl, carboxyl groups) are introduced into the parent molecule, thereby presenting the phase II conjugating enzymes (e.g. glucuronyl transferases and glutathione S-transferases) with a substrate. The conjugated products are sufficiently hydrophilic to be readily excreted through the kidney or in the bile (Williams, 1967). The biotransformation of xenobiotics can occur in several tissues, including liver, lung, intestine, skin, and kidney. The liver, however, is the major site of transformation of xenobiotics. The liver is particularly important for two main reasons: Firstly, it is well endowed with "drug-metabolizing enzymes" and secondly, by virtue of its blood supply, the liver tends to receive higher quantities of xenobiotics than do most other organs. Ingested, intravenously administered, or intraperitoneally-administered compounds are delivered most rapidly to the liver.

Phase I biotransformation is most often carried out by the cyt. P-450 mixed function oxidase system which resides primarily in the endoplasmic reticulum of cells. This enzyme system consists of a haemprotein termed cytochrome P-450 (cyt. P-450) and a flavoprotein called NADPH-cyt. P-450 reductase (also called NADPH-cyt. c reductase) and requires phospholipid for activity. There is also an absolute requirement for NADPH and molecular oxygen. Cyt. P-450 contains the substrate and oxygen-binding sites of the enzyme system, while the reductase

Figure 1-1:



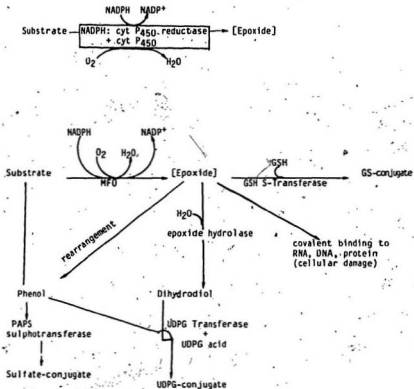
The chemical structures of naphthalene and some alkylated naphthalenes.

serves as an electron carrier shuttling electrons from NADPH to the cyt. P-450-substrate complex. Another haemprotein, cyt. b_5 , is also present in liver microsomes and may participate in the oxidation of xenobiotic and endogenous substrates (Cummings et al., 1984). The types of chemical transformations carried out by cyt. P-450 in hepatic microsomes include oxidative reactions in which an atom from molecular oxygen is inserted into an organic molecule, as in aliphatic and aromatic hydroxylations, N-oxidation, sulfoxidation, epoxidation, desulfuration, deamination, and N-, S-, and O-dealkylations. Cyt. P-450 also effects reductive reactions involving direct electron transfer (e.g. reduction of azo, nitro, N-oxide, and epoxide groups) as well as dehalogenation reactions (Gillfele, 1966; Coon, 1978; Guengerich et al., 1984). A general metabolic pathway for xenobiotic metabolism catalyzed by the microsomal mixed function oxidase enzyme system is shown in Figure 1-2. Hydrophobic substrates are readily hydroxylated either directly or through the formation of reactive epoxide intermediates. Epoxides may (a) chemically rearrange to phenols, (b) be hydrated to dihydrodiols in a reaction catalyzed by epoxide hydrolase, (c) and / or conjugate with cellular glutathione in a reaction catalyzed by the glutathione S-transferases. Phenols and dihydrodiols may readily be conjugated with glucuronic acid and sulfate, and excreted via the kidney or bile duct. The glutathione conjugate can be excreted in bile and, after conversion to mercapturic acids, be excreted by the kidney. In addition, being reactive electrophilic molecules, epoxides can bind to and modify cellular macromolecules such as DNA, RNA and proteins with possible resulting toxic and carcinogenic effects (Nebert, 1982).

The diversity of substrates for this enzyme system and the variety of transformations that it effects makes it a unique enzyme system among living organisms. It brings about chemical changes both in physiologically important substrates such as fatty acids, steroids, and prostaglandins, and also in a host of foreign substances such as petroleum products, drugs, pesticides, anaesthetics, and chemical carcinogens, as well as miscellaneous organic substances commonly found on the laboratory shelf (Coon and Persson, 1980).

Figure 1-2:

Scheme for the membrane-bound multicomponent monooxygenase system(s) and various possibly important pathways for substrates of cytochrome P-450 (simplified from Nebert, 1982).



The activity of hepatic mixed function oxidases can be enhanced or decreased by treating the animal with various chemicals or environmental agents (Coon and Persson, 1980). Different effects result. The rate of excretion of a chemical may be increased. It may be rendered more or less active pharmacologically or toxicologically. It is currently recognized that at least 10 sub-members of the cyt. P-450 family exist, and that different xenobiotics induce different cyt. P-450s. The different cyt. P-450 members are distinguished on the basis of their spectral characteristics, substrate specificities (in some cases), molecular weights and immunological reactivities (Bresnick et al., 1984). It has become increasingly apparent that different forms of cyt. P-450 may generate different ratios of metabolites from the same substrate. Two major submembers of the cyt. P-450 family are designated as cyt. P-450 and cyt. P-448. These are induced by barbiturates (e.g. phenobarbital, PB) and polycyclic aromatic hydrocarbons (e.g. 3-methylcholanthrene, 3-MC), respectively (Alvares et al., 1967; Bresnick et al., 1984). Various groups have shown that purified cyt. P-450 and cyt. P-448 hydroxylate substrates like biphenyl, testosterone, 2-acetylaminofluorene, bromobenzene, n-hexane, and benzo[a]pyrene, each at predominantly different positions thus producing different metabolic profiles (Jefcoate, 1983). Differences in the metabolite profile of a polycyclic hydrocarbon or other foreign chemical reflect presumed differences in the active-sites of various forms of cyt. P-450 and in the nature of the intermediates formed. Differences in the reactivity of these intermediates might therefore result in marked dissimilarities in the toxicity or carcinogenicity of a given compound (Nebert, 1982).

1.4. Enzymes of Phase I and Phase II Detoxication

Detoxication is frequently not the act of a single enzyme but the result of sequential action by several of them. Benzo[a]pyrene, for example, is sequentially subjected to oxidation by the cyt. P-450 oxidases to form an epoxide, hydration of the epoxide catalyzed by epoxide hydrolase to form a diol, and a second stage of oxidation to yield a diol-epoxide before the transformation into a powerful electrophile and carcinogen is complete. The subsequent detoxication of the

epoxide could be catalyzed by glutathione transferases to form the corresponding thioether conjugate with GSH, or by enzymic hydration of the epoxide ring catalyzed by epoxide hydrolase (Jakoby, 1980).

1.4.1. Microsomal Enzymes of Phase I Metabolism of Xenobiotics

1.4.1.1. NADPH-cyt.c reductase

NADPH-cyt.c reductase is a flavoprotein and is localized in the endoplasmic reticulum of the cell. This flavoprotein supplies electrons from NADPH for the oxidative reactions mediated by cyt. P-450 (Masters, 1980). The hydroxylation cycle mediated by cyt. P-450 requires two electrons to be introduced into the cyt. P-450 molecule during substrate binding, oxygen binding, and the concomitant intramolecular oxidation-reduction steps leading to the final products of the reaction. It is known that NADPH-cyt.c reductase is necessary for this process, but it is not known whether it is sufficient in the microsomal system for supplying both electrons. The oxidation-reduction states which NADPH-cyt.c reductase undergoes during catalytic turn-over have been the subject of much discussion (Masters, 1980).

1.4.1.2. 7-Ethoxyresorufin O-Deethylase

7-Ethoxyresorufin is preferentially O-deethylated by liver microsomes from 3-methylcholanthrene treated rats containing cyt. P-448, while untreated and phenobarbital treated rat liver microsomes exhibit negligible activity (Burke et al., 1977).

1.4.1.3. Benzo[a]pyrene Hydroxylase

Certain forms of cyt. P-450 have associated aromatic hydrocarbon hydroxylase (AHH) activity induced by polycyclic aromatic hydrocarbons. The assay for AHH activity with the use of benzo[a]pyrene (BP) as substrate and 3-hydroxy-BP as the product formed has remained a relative index of BP metabolism. Benzo[a]pyrene hydroxylase can both detoxify polycyclic hydrocarbons as well as activate them to more carcinogenic forms (Whitlock and Gelboin, 1982).

1.4.1.4. Epoxide Hydrolase

Epoxide hydrolase is linked functionally and perhaps structurally with mixed function oxygenases in liver endoplasmic reticulum. It hydrolyzes arene oxides, which are formed as reaction intermediates during several, perhaps all, aromatic hydroxylation reactions and produces aromatic dihydrodiols (Oesch, 1973).

1.4.2. Microsomal Enzymes of Phase II Metabolism of Xenobiotics

1.4.2.1. GSH S-Transferases

The GSH S-transferases are a family of detoxifying enzymes that catalyze the conjugation of the sulfhydryl group of reduced glutathione with a wide variety of electrophilic substances. The glutathione adduct formed in the first step is usually converted to a mercapturic acid derivative which is excreted via the kidney (Boylard and Chasseaud, 1969). In rat the major portion of the activity is in the cytosolic fraction of the hepatocytes, but GSH S-transferase activities have also been observed at a lower level in microsomes and mitochondria (Mannervik, et al, 1983).

1.4.2.2. UDP-Glucuronyl Transferases

UDP-Glucuronyl transferases are located in the endoplasmic reticulum of the cell and catalyze the conjugation of a variety of endogenous and exogenous compounds with UDP-glucuronic acid thereby rendering the products more water-soluble and more readily excretable via the kidney (Jefcoate, 1983). UDP-glucuronyl transferases function in close association with the microsomal mixed function oxidase system, since many substrates hydroxylated by the latter are then subjected to conjugation. The enzymes can be induced by 3-MC, PB, or stilbene oxide (Jefcoate, 1983).

1.5. Metabolism of Alkylated Naphthalenes

Few investigations concerning the biotransformation of low molecular weight aromatic hydrocarbons like naphthalene and alkylated naphthalenes have been conducted. This is probably due to their lower carcinogenic potential when compared to polycyclic hydrocarbons such as benzo[a]pyrene and 7,12-dimethyl benzantracene. Boyland (1950) proposed that aromatic hydrocarbons are metabolized via epoxides. The latter may give rise to dihydrodiol-epoxide intermediates (Daly et al., 1972; Wong et al., 1980). These reactions all involve the cyt. P-450-linked monooxygenase system (Coon and Persson, 1980).

The apparent K_m for 2,6-dimethylnaphthalene for coho salmon liver microsomes was found to be $15.3 \mu M$ and that for naphthalene to be $300 \mu M$ (Schnell et al., 1980). Salmon metabolize 2,6-DMN to naphthols and dihydrodiols, probably through reactive epoxide intermediates (Malins et al., 1979). Orally administered 2,6-DMN was readily taken up and metabolized by coho salmon, and both the hydrocarbon and its metabolites were found in liver, muscle, blood and brain of DMN-exposed fish (Collier et al., 1983). Among different species of fishes there is a marked difference in the primary metabolism of alkylated naphthalenes. Alkyl oxidation of 2,6-DMN predominates over aryl oxidation in starry flounder, whereas aryl oxidation is dominant in coho salmon (Collier et al., 1983). Sea urchins metabolize alkyl substituted aromatic hydrocarbons primarily through aromatic ring oxidation (Malins and Roubal, 1982). The bile of the non-induced rainbow trout exposed to aqueous [^{14}C]2-MN has a greater ratio of 2-hydroxymethylnaphthalene to dihydrodiols than that of the β -naphthoflavone induced trout (Melancon and Lech, 1984). β -Naphthoflavone pretreated trout have greater concentrations of total metabolites, glucuronide conjugates and dihydrodiol metabolites of 2-MN in the bile (Melancon and Lech, 1984).

Considerable work on the metabolism of methylated naphthalenes, (especially dimethylnaphthalenes), has been done on marine life, but less has been reported in mammals. In mice 2-MN causes pulmonary toxicity (Griffin et al., 1983). Grimes

and Young (1956) studied the metabolism of 2-MN in rabbit, rat, guinea pig and mouse. They reported the formation of naphthalene-2-carboxylic acid and the dihydrodiol of 2-MN. Kaubish et al. (1972) provided further information by synthesizing 2-methyl- and 1,2-dimethyl-naphthalene 1,2-oxides and their phenolic rearrangement products, and compared them to the phenols produced by rat hepatic microsomal metabolism. They concluded that the formation of arene oxides with alkyl substituents on the oxirane rings did not appear to be a major pathway for hepatic metabolism. Migration of methyl groups as well as apparent migration of oxygen occurred during rearrangement of methyl substituted arene oxides. The ratio and nature of isomerization products varied with reaction conditions, suggesting that multiple mechanistic pathways are operative in these rearrangements. Major products of 1-methylnaphthalene, 2-methylnaphthalene, and 1,2-dimethyl-naphthalene with guinea pig microsomes were 1-naphthoic acid, 2-naphthoic acid and 2-methyl-1-naphthoic acid, respectively. Breger et al. (1981) isolated three isomeric dihydrodiols from rat and rainbow trout microsomal incubations which were identical with those obtained from the urine of rats fed 2-MN. In *in vitro* studies the formation of metabolites was reduced by incubation in the presence of carbon monoxide, omission of NADPH, or use of heat denatured microsomes, implying the involvement of cyt. P-450 linked mixed function oxidase activity. Pretreatment with phenobarbital or β -naphthoflavone selectively altered the rate of formation of specific dihydrodiols by rat liver microsomes (Breger et al., 1981). Recently, 2-hydroxymethylnaphthalene and three isomeric dihydrodiols shown to be the 3,4-dihydrodiol, 5,6-dihydrodiol, and 7,8-dihydrodiol of 2-MN have been isolated (Breger et al., 1983). This suggests that the cyt. P-450 dependent mixed function oxidase system can form epoxides on an aromatic ring with an alkyl group attached to it.

In rat, the major urinary metabolite of 2-MN was the glycine conjugate of 2-naphthoic acid suggesting extensive metabolism of the alkyl group (Melancon et al., 1982). Minor products were dihydrodiols, indicating some ring oxidation as well (Melancon et al., 1982).

In guinea pig, the major urinary metabolites were oxidative products of the methyl group of 2-MN, namely 2-naphthoic acid and its glycine and glucuronic acid conjugates (Teshima et al., 1983). These studies suggest that oxidation of the methyl group rather than ring oxidation is the major reaction which facilitates the urinary excretion of metabolites. S-(7-Methyl-1-naphthyl) cysteine, and glucuronic acid and sulfate conjugates of 7-methyl-1-naphthol, were also identified as minor urinary metabolites (Teshima et al., 1983).

A summary of these transformations is shown in Figure I-3.

Pulmonary and hepatic microsomes from DBA/2J mice metabolized 2-MN to three dihydrodiols and 2-(hydroxymethyl)naphthalene whereas renal microsomes produced 2-(hydroxymethyl)naphthalene, but no detectable dihydrodiols (Griffin et al., 1983).

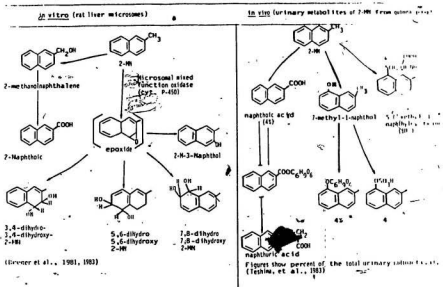
1.6. Binding to Cellular Macromolecules

The incubation of naphthalene or 1-naphthol with rat liver microsomes in the presence of a NADPH-regenerating system leads to the formation of reactive metabolites which bind irreversibly to protein. It was suggested that either quinone or semiquinone metabolites were involved (Hesse and Mezger, 1979). A recent study with isolated hepatocytes supports the suggestion that the toxicity of 1-naphthol may be mediated by the formation of 1,2-naphthoquinone and/or 1,4-naphthoquinone, which may then undergo one electron reduction to naphthosemiquinone radicals (Doherty et al., 1984a). These, in turn, may covalently bind to important cellular macromolecules or enter a redox cycle with molecular oxygen thereby generating active oxygen species (Doherty et al., 1984a). It is noteworthy, however, that in mice the Clara cell is a target for the cytotoxic actions of 2-MN, which binds irreversibly to the cell microsomal proteins (Griffin et al., 1982, 1983). Lung tissue has a low capacity for metabolizing xenobiotics, and glutathione concentrations *in vivo* are not much depressed. *In vivo* compounds which depress glutathione concentrations in liver may, after a large

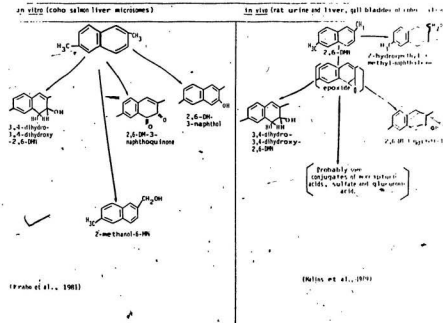
Figure 1-3:

In Vitro and In vivo Metabolism of Methylated Naphthalenes

a) Metabolism of 2-Methylnaphthalene (2-MN)



b) Metabolism of 2,6-Dimethylnaphthalene (2,6-DMN)



dose, cause effects in the lungs, including a decrease in glutathione. This suggests that active metabolites are transported from the liver (Buckpitt and Warren, 1983).

1.7. Reduced Glutathione (GSH) and Detoxication

Boyland (1950) suggested that polycyclic aromatic hydrocarbon metabolism proceeds via epoxide (arene oxide) formation. This is mediated by microsomal mixed function oxidases (Jerina and Daly, 1974). Epoxides can react with GSH in a reaction catalyzed by a series of enzymes known as GSH S-transferases. Booth et al. (1961) showed that conjugation of GSH with naphthalene required not only the presence of liver microsomes and NADPH but also the presence of rat liver cytosol which contains the GSH S-transferases. The formation of epoxides which can react with GSH, has also been demonstrated for other polycyclic hydrocarbons (Grover et al., 1972). Most epoxides are reactive and therefore potentially toxic to biological systems. The intrinsic stability of the epoxide with respect to spontaneous isomerization to the phenol or hydration to form the dihydrodiol, and the relative affinities of the different enzyme systems for a particular epoxide in different species may decide which route of metabolism is dominant, and may regulate the potential toxicity of the epoxide to biological systems (Grover et al., 1972; Jerina et al., 1974). The formation of epoxides and diol-epoxides is thought to be of great importance in carcinogenesis by polycyclic aromatic hydrocarbons, and diol epoxides are regarded as ultimate carcinogens in most cases (Sims et al., 1974).

The liver is an important organ of biotransformation and, appropriately, GSH levels are relatively high (about 170 mg/100g) in rat liver (Chasseaud, 1976). Measurement of hepatic GSH depletion, or even extrahepatic GSH depletion, can provide a useful indication of the protective role of GSH against potentially toxic compounds (Chasseaud, 1976). Hence, GSH may be regarded as an endogenous protective agent with which drugs, pesticides, and other reactive compounds (that have the capability to covalently bind to biological macromolecules) react to form conjugates which are readily excreted.

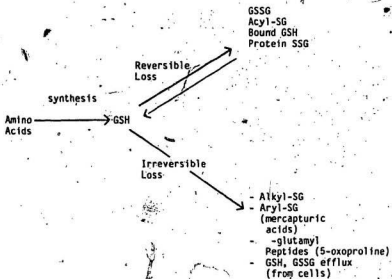
Nearly all of the glutathione in tissues is present as reduced glutathione (GSH) with less than 5% of the total being present as glutathione disulfide (GSSG). This thiol redox status is maintained by intracellular GSSG reductase and NADPH (Reed and Fariss, 1984). Depletion of GSH by rapid conjugation can cause GSH synthesis at rates upto 2 to 3 $\mu\text{moles/h/g}$ of wet liver tissue (White, 1976). The liver GSH content in rat undergoes diurnal or circadian variations, and is altered by starvation and chemical treatments. The diurnal variation in hepatic GSH content results in the highest GSH levels in the night and early morning and lowest levels in the late afternoon with maximum variation of as much as 25% to 30% (Reed and Fariss, 1984).

A general pathway for GSH turnover is shown in figure 1-4. Experiments carried out using mice indicated that administration of GSH or cysteine before and after a dose of 2-MN greatly diminished the toxicity of 2-MN, while the administration of diethyl maleate (which depletes GSH) before 2-MN enhanced the toxicity of 2-MN (Teshima et al., 1983). 2-MN has been shown to conjugate with GSH in the presence of guinea pig liver 9,000g supernatant and, when 2-MN was given to guinea pigs orally at a dose of 500mg/kg body wt., the acid soluble sulphhydryl fraction in the liver decreased by 40% (Teshima et al., 1983). It is possible that some of the ring oxidation metabolites of 2-MN are responsible for its toxic effects, and that these are detoxified by conjugation with GSH and cysteine. As mentioned earlier, GSH conjugation is one of the main pathways of detoxification of xenobiotics in mammalian species.

1.8. Methaemoglobin

The ability of haemoglobin (Hb) to bind reversibly with oxygen is called "oxygenation". Under certain conditions the iron of the haem moiety binds oxygen so closely that it is unable to dissociate and is therefore not available for respiration. Oxidation of Hb, in which the iron of the haem moiety is bivalent ferrous(II), to the trivalent ferric (III) form results in its transformation into a brown pigment called methaemoglobin (metHb), which can accumulate in red

Figure 1-4: A general pathway for GSH turnover



Representation of the reversible and irreversible processes which effect the GSH-GSSG status of cells. (Kosower and Kosower, 1976).

cells. It is critical to the physiological function of Hb that the ferrous (II) should not be oxidized to ferric(III), as metHb is not able to bind oxygen. In normal physiological conditions metHb is continuously being formed within erythrocytes but is reduced back to ferrous(II) enzymatically (Jaffe, 1964). This is done within the cell by a specific NADH-metHb reductase, which maintains a physiological metHb concentration at about 1% of the total Hb present (Jaffe, 1964). Usually high amounts of metHb of pathological importance can result (i) when the rate of formation and the amount of metHb overwhelm the physiological reducing enzyme system; (ii) when there is a genetic defect in the intraerythrocytic enzyme system that normally reduces metHb or (iii) from the presence of an abnormal amino-substitution (M-Type Hb and Hb Freiburg) (Bauer, 1982).

Methaemoglobinaemia can also result from exposure to certain toxic agents or drugs such as phenols, arylamines, hydrazines, nitrites, copper, sulphonamides, acetanilid, nitroglycerin, and phenacetin. All these accelerate the oxidation of haemoglobin (Beutler, 1985). Toxic methaemoglobinaemia is characterized by elevated levels of the globin component of Hb without any NADH-metHb reductase deficiency. Newborn infants and those up to about 3 months of age have a tendency to form metHb and are thus particularly sensitive to these oxidizing substances (Bauer, 1982). Normally, after prolonged standing, oxyhaemoglobin is converted to metHb which is brown. MetHb formation also accompanies some forms of haemolytic anaemias, so that metHb is found in the serum and urine, both of which turn brown.

1.9. Purpose of Study

The metabolism of naphthalene in biological systems has been periodically investigated in mammals and fish (Melancon and Lech, 1979; Buckpitt and Warren, 1983). The *in vitro* and *in vivo* metabolism of 2-MN has been reported in rats, mice, guinea pigs and in marine species (Breger, et al., 1981; Griffin et al., 1982; Teshima et al., 1983; Melancon et al., 1982; Malins et al., 1979). A comparative study of the fate of naphthalene and 2-MN in mammals and fish indicates that inducers of hepatic monooxygenase activity such as β naphthoflavone or phenobarbital, selectively altered the rate of formation of specific dihydrodiols by hepatic microsomes (Breger et al., 1981; Melancon et al., 1984; Griffin et al., 1983). Similar studies on the metabolism of 2,6-DMN, either *in vitro* or *in vivo*, in mammalian systems (especially in rat) have not been carried out. It was considered both important and interesting to extend these studies to the dimethylnaphthalenes which are one of the most abundant components of crude oil. In addition, dimethylnaphthalenes have been shown to be toxic to some marine species (Anderson et al., 1974).

In the present study, 2,6-DMN was selected as a representative dimethylnaphthalene because (i) hardly any work has been done on its metabolism in mammalian system. (ii) the 2,6-position of the methyl groups gives a symmetrical molecule which might limit the number of metabolites. (iii) 2,6-DMN is available as a radioactive compound. 2,6-DMN is only one of the several possible isomers. It may be viewed as a model but alone cannot tell the whole story.

The metabolism and toxicity of 2,6-DMN was investigated using primarily rats or liver microsomes obtained therefrom. The metabolism of 2,6-DMN was examined in liver microsomes isolated from untreated rats as well as rats that had been pretreated with a variety of agents known to alter the hepatic mixed function oxidase activity. This was done in the hope of identifying the possible pathways by which the different metabolites are formed. Binding to

macromolecules, depletion of GSH, and methb formation are generally indicators of toxicity of a compound. Hence, such phenomena were examined to demonstrate the potential of 2,6-DMN as a toxic compound.

Chapter 2

Materials and Methods

2.1. Materials

The following chemicals were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin: 2,6-dimethylnaphthalene, potassium nitrosodisulfonate, lithium aluminium hydride, acetyl chloride, pyridine and o-phenylenediamine.

Sodium phenobarbital was obtained from J.T. Baker Chemical Co., Phillipsburg, N.J.(U.S.A.). 3-Methylcholanthrene, NADP⁺, isocitrate dehydrogenase (type N), DL-isocitrate (type 1), reduced glutathione and oxidized glutathione, were obtained from Sigma Chemical Co., St. Louis, Mo. Cobalt protoporphyrin IX was purchased from Porphyrin Products, Logan, Utah (USA). All other chemicals were of the highest grade commercially available.

2,6-Dimethyl[8-¹⁴C]naphthalene (specific activity 2.1 mCi/mmole) was purchased from California Bionuclear Corporation, Sun Valley, Ca. The radiochemical purity of this compound was shown to be greater than 98% by High Performance Liquid Chromatography (HPLC) on a C₁₈ μ Bondapak (4.6 x33 mm) reverse phase column using acetonitrile:water (20:80) which was changed in a stepwise gradient to acetonitrile:methanol:water (60:10:30).

2.2. Methods

2.3. Synthesis of standard metabolites of 2,6-DMN

The metabolites of 2,6-DMN were prepared according to established procedures.

2.3.1. 2,6-DM-3-Naphthol

2,6-DM-3-naphthol was synthesized by the method of Fieser and Seligman (1934) and Vogel (1966).

First, 2,6-dimethylnaphthalene-3-sulphonate was prepared (Feiser and Seligman, 1934) by melting 55g of 2,6-DMN in a 250ml flask with constant stirring at 135-140°C (oil-bath). Concentrated H_2SO_4 (38ml), preheated to 100°C, was added slowly. After four hours, 10ml more of the acid was added and the stirring and heating were continued for one-half hour longer. The mixture was poured into 160ml of water and the 3-sulphonic acid which separated on cooling was collected by filtration (the 4-sulphonic acid passes into the mother liquor). The solid was taken up in hot water, the solution was filtered from a little unchanged 2,6-DMN and slowly neutralized while hot with 2M potassium hydroxide. The potassium salt of 2,6-DM-3-sulphonic acid separated as large, colourless plates. The preparation of the naphthol was done by the method of Vogel (1966). 30g of KOH and 1.25ml of water were heated to melt in a 100ml nickel crucible. When the temperature reached to 250°C, the flame of the Bunsen burner was removed. Immediately 14g of powdered potassium 2,6-DMN-sulphonate was added. The flame was replaced, and the stiff pasty mass was stirred till the temperature reached to 300°C in 5 to 10 min. After frothing, the mass became clear. The temperature was raised to 310°C for 5 min. The flame was removed and the material was pushed down from the sides into the crucible and was heated again for 2 min at 310°C. The mass was allowed to cool to become pasty and was added in small portions to 250ml of water containing crushed ice. The residual material in the crucible was extracted with water and added to the contents of the beaker. The naphthol was precipitated by slowly adding concentrated HCl with stirring.

The solution was warmed until the finely divided precipitate of the naphthol coagulated. It was cooled on ice and then filtered. The precipitate was washed into a beaker containing cold water. Enough 5% NaOH was added to dissolve the precipitate in the presence of 0.28g of sodium hyposulphite (to prevent any oxidation). Any insoluble traces were removed by filtration and the filtrate was acidified with acetic acid and warmed to precipitate the naphthol. It was cooled on ice and filtered. The precipitate was dried by pressing the filter paper and then finally under vacuum. The melting point was found to be 172-174°C (reported melting point 170-174°C). Yield 40%.

The structure of 2,6-DM-3-naphthol is shown in figure 2-1.

2.3.2. 2,6-DM-3,4-Naphthoquinone

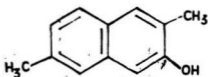
2,6-DM-3,4-naphthoquinone was synthesized by the method of Cassebaum (1957). 6.6g of 2,6-DM-3-naphthol was dissolved in 760 ml of acetone (solution A). Solution B contained 23.0g of Fremy's salt (potassium nitrosodisulphonate) dissolved in 1520ml of water and 380ml of 0.15M potassium phosphate (monobasic). Solution B was added to A while stirring on a magnetic stirrer. The 2,6-DM-3-naphthol precipitated out and then redissolved, followed immediately with the precipitation of orange crystals of 2,6-DM-3,4-naphthoquinone. The naphthoquinone was washed with ethanol and then recrystallized from ethanol. The melting point was found to be 150-152°C. (reported melting point 151.0-151.4°C). Yield was 5.2g (81%).

The structure of 2,6-DM-3,4-naphthoquinone is shown in figure 2-1.

2.3.3. 2,6-DM-3,4-dihydro-3,4-dihydroxynaphthalene

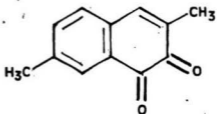
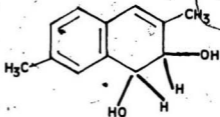
2,6-DM-3,4-dihydro-3,4-dihydroxynaphthalene was prepared by reducing 2,6-DM-3,4-naphthoquinone with lithium aluminium hydride by the method of Booth et al. (1950).

Figure 2-1: The chemical structures of synthetic metabolites of 2,6-DMN



2,6-DIMETHYL-3-NAPHTHOL

TRANS-3,4-DIHYDRO-3,4-DIHYDROXY
-2,6-DIMETHYLNAPHTHALENE



2,6-DIMETHYL-3,4-NAPHTHO-QUINONE

2,6-DM-3,4-naphthoquinone was dried under vacuum and 4.0g were placed in the thimble of a Soxhlet apparatus. Anhydrous ether (200ml) was placed in the flask of the apparatus with 1.0g of lithium aluminium hydride and refluxed until all the naphthoquinone appeared to be extracted. The ethereal solution was cooled and 5ml water and 30ml of 2N sulphuric acid were added slowly to decompose the excess of lithium aluminium hydride. The ethereal layer was separated by centrifugation, and the residue extracted with ether. The combined ethereal extracts were washed with 2N NaOH and evaporated under nitrogen. The residue was first crystallized from benzene and then from cyclohexane. The melting point was found to be 107-108°C (reported melting point 107-108°C). Yield 1.2g (30%).

The structure of 3,4-dihydro-3,4-dihydroxy-2,6-DMN is shown in figure 2-1.

2.4. Confirmation of Structures of Synthetic Metabolites

2.4.1. Ultraviolet Spectroscopy

Ultraviolet absorbance for each synthetic standard was obtained on a Hitachi dual wavelength spectrophotometer. The compound was dissolved in ethanol and wavelength scan was performed from 200 nm to 400 nm. Matched cuvettes with 1cm pathlength were used.

2.4.2. Infrared Spectroscopy

2.4.2.1. 2,6-DM-3,4-naphthol

The infrared spectrum of 2,6-DM-3,4-naphthol is shown in figure 2-2.

a) Two peaks are clearly observed at 3595 and 3323 cm^{-1} and attributed to an OH group. The first peak is due to a free OH group and the low frequency peak is attributed to a H-bonded OH group.

b) Peaks appearing around 3000 cm^{-1} are due to $\nu(\text{CH})$ of the aromatic rings, and absorptions observed around 2900 cm^{-1} are attributed to the stretching mode of $\nu(\text{CH})$ of CH_3 .

c) A peak is observed at 1642 cm^{-1} and probably is attributed to the $\nu(\text{C}=\text{O})$ of the carbonyl group as a result of auto-oxidation of the OH group.

d) Group of peaks are observed in the range $1615\text{--}1447\text{ cm}^{-1}$ are attributed to $\nu(\text{C}=\text{C})$ of the aromatic rings.

2.4.2.2. 2,6-DM-3,4-naphthoquinone

The infrared spectrum of 2,6-DM-3,4-naphthoquinone is shown in figure 2-3.

a) Asymmetric stretching mode $\nu(\text{CH})$ of phenyl rings appear in the vicinity of 3000 cm^{-1} .

b) $\nu(\text{CH})$ of CH_3 groups are observed around 2900 cm^{-1} .

c) Two characteristic peaks are observed at 1696 and 1665 cm^{-1} are attributed to the carbonyl groups $\nu(\text{C}=\text{O})$.

d) Pairs of absorptions appear in the region of $1450\text{--}1603\text{ cm}^{-1}$ and are attributed to ring stretching absorption $\nu(\text{C}=\text{C})$ of the phenyl rings.

2.4.2.3. 3,4-Dihydro-3,4-dihydroxy-2,6-DMN

The infrared spectrum of 3,4-dihydro-3,4-dihydroxy-2,6-DM is shown in figure 2-4.

a) Two peaks are observed at 3580 and 3455 cm^{-1} and these are attributed to OH groups. The high frequency peak is due to free OH groups, and the low frequency one is attributed to H-bonded OH groups.

b) $\nu(\text{CH})$ of the aromatic rings appear around 3000 cm^{-1} .

c) $\nu(\text{CH})$ of aliphatic protons are observed at 2941 and 2864 cm^{-1} .

d) A strong peak is clearly observed at 1703 cm^{-1} and is attributed to $\nu(\text{C}=\text{O})$

Figure 2-3: Infrared spectrum of synthetic 2,6-DM-3,4-naphthoquinone

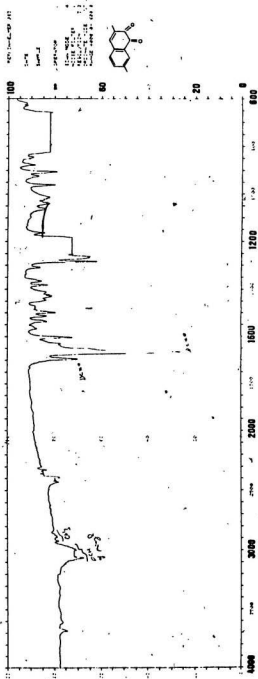
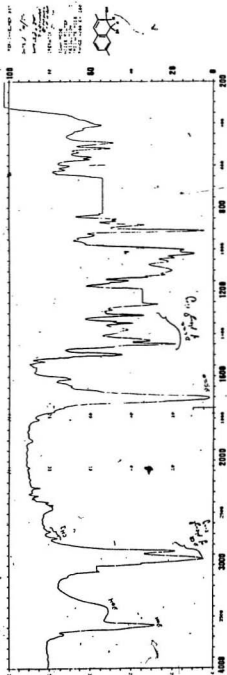


Figure 2-4:
Infrared spectrum of synthetic 3,4-dihydro-3,4-dihydroxy-2,6-DNN



of a carbonyl group. The carbonyl group most probably arose from auto-oxidation of an OH group.

e) Absorption patterns appear in the range $1612-1448\text{ cm}^{-1}$ and are attributed to $\nu(\text{C}=\text{C})$ of the aromatic rings.

2.4.3. Nuclear Magnetic Resonance Spectroscopy

^1H Fourier Transform NMR for synthetic metabolites were performed on a Bruker WP80 NMR spectrophotometer using a proton frequency of 80 MHz at ambient temperature.

The ^1H FT NMR spectrum of 2,6-DM-3-naphthol is shown in figure 2-5.

N.M.R. (CDCl_3): δ 2.37 (s, 3H), 2.44 (s, 3H)
 3.95 (s, 1H), 6.96 (s, 1H)
 7.11 (dd, 2H), 7.38 (s, 1H),
 7.52 (s, 1H).

The ^1H FT NMR spectrum of 2,6-DM-3-naphthoquinone is shown in figure 2-6.

N.M.R. (CDCl_3): δ 2.0 (s, 3H), 2.38 (s, 3H)
 7.38 (dd, 2H), 7.82 (s, 1H).

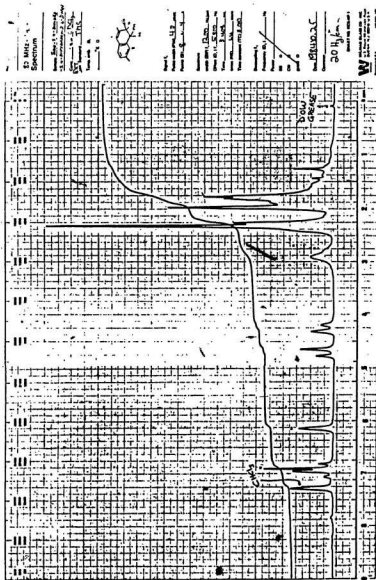
The ^1H FT NMR spectrum of 3,4-dihydro-3,4-dihydroxy-2,6-DMN is shown in figure 2-7.

N.M.R. (CDCl_3): δ 1.95 (s, 3H), 2.31 (s, 3H),
 2.89 (s, 2H), 4.48 (dd, 2H),
 6.15 (s, 1H), 6.80 (s, 1H),
 7.13 (dd, 2H).

2.4.4. Mass Spectroscopy

Determination of mass spectra was carried out on a VG 7070HS 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

Figure 2-7: ^1H FT NMR spectrum of 3,4-dihydro-3,4-dihydroxy-2,6-DMH



generated by electron impact using 70 eV electrons. Mass spectral data from perfluorokerosene was acquired 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 1000. Whenever possible a series of consecutive scans was averaged using the data system. Fragment ions were ignored if less than 2% intensity.

2.4.4.1. 2,6-DM-3-naphthol

The mass spectrum of 2,6-DM-3-naphthol is shown in figure 2-8.

Mass spectrum= 172 (M^+), molecular ion (most abundant ion),
 171 ($M^+ - H$), H of the OH group.
 157 ($M^+ - CH_3$),
 141 ($M^+ - 2CH_3; - H$).
 128, 115.

2.4.4.2. 2,6-DM-3,4-naphthoquinone:

The mass spectrum of 2,6-DM-3,4-naphthoquinone is shown in figure 2-9.

Mass spectrum= 186 (M^+), molecular ion,
 158 ($M^+ - C=O$) most abundant ion,
 143 ($M^+ - C=O, - CH_3$),
 115 ($M^+ - 2xC=O, - CH_3$),
 129.

2.4.4.3. 3,4-dihydro-3,4-dihydroxy-2,6-DM

The mass spectrum of 3,4-dihydro-3,4-dihydroxy-2,6-DM is shown in figure 2-10.

Mass spectrum= 190 (M^+) molecular ion,
 172 ($M^+ - H_2O$),
 144 ($M^+ - H_2O, - C=O$) most abundant ion.
 129 (most abundant ion - CH_3),
 115 (most abundant ion - $CH_3 - CH_3$);
 159, 89.

Figure 2-8: Mass spectrum of 2,6-DI-2-naphthol

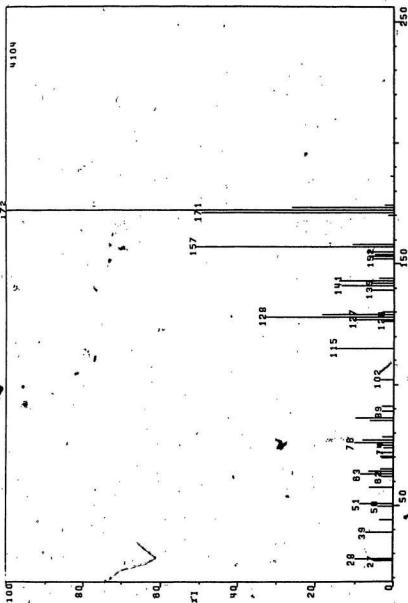


Figure 2-9: Mass spectrum of 2,6-DH-3,4-naphthoquinone.

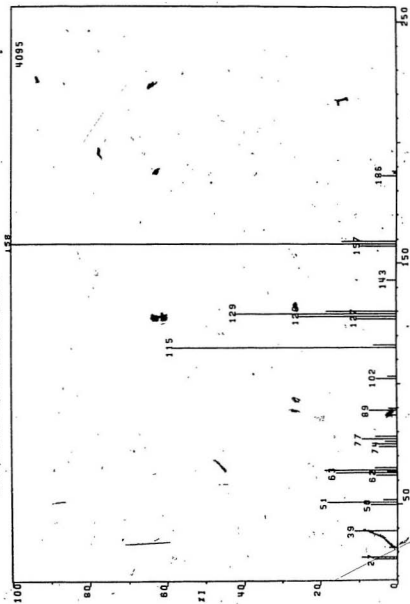
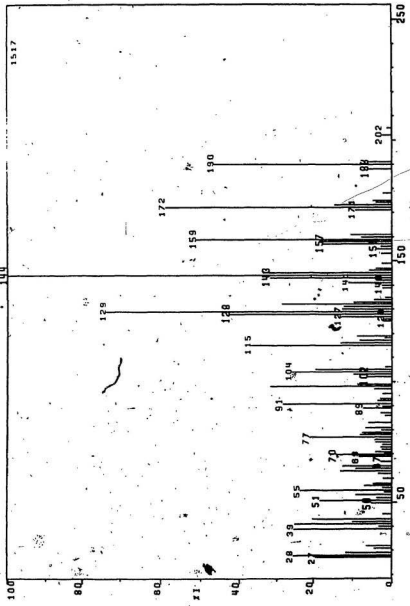


Figure 2-10:
Mass spectrum of 3,4-dihydro-3,4-dihydroxy-2,6-DNH.



2.4.5. Chemical Tests to Correlate Synthetic Standards with in vitro Formed Metabolites of 2,6-DMN

2.4.5.1. 2,6-DM-3-naphthol

A HPLC profile of synthetic 2,6-DM-3-naphthol is shown in figure 2-11.

To confirm that 2,6-DM-3-naphthol is a phenolic derivative of the 2,6-DMN, it was converted to 2,6-DM-3-naphthyl acetate in the following manner. 15mg of 2,6-DM-3-naphthol was dissolved in 0.5ml pyridine and then 0.5ml of acetyl chloride was added. The reaction was immediate. Excess pyridine was reacted with dilute HCl. The precipitated acetate derivative was filtered and washed with several volumes of water. The naphthyl acetate was dissolved in chloroform. Aliquots of 2,6-DM-3-naphthyl acetate and 2,6-DM-3-naphthol were chromatographed on thin layer (silica gel 250 μ m, 20x20cm) containing a UV indicator. The plate was developed with benzene:acetone (2:1). R_f values for the naphthol and the acetate were 0.78 and 0.87, respectively.

The test was repeated with synthetic 2,6-DM-3-naphthol mixed with the radioactive compound obtained after separation on HPLC by injecting an aliquot from an ethyl acetate extract of a microsomal incubation of 2,6-DM-[8- 14 C]N. Maximum radioactivity (51% of total radioactivity of the metabolite) was obtained at an R_f value corresponding to that of 2,6-DM-3-naphthyl acetate. 22% of the original radioactivity was associated with the 2,6-DM-3-naphthol. There was no other radioactive band on the plate. The remaining 27% of the radioactivity was presumably lost during workup.

2.4.5.2. 2,6-DM-3,4-naphthoquinone

A HPLC profile of synthetic 2,6-DM-3-naphthoquinone is shown in figure 2-12.

It is known that the o-quinones react with o-phenylenediamine to yield quinoxalines (Vogel, 1966). Therefore, to demonstrate that the quinone formed in vitro incubations of 2,6-DMN is an o-quinone (3,4-quinone) the following method was used.

Figure 2-11:
HPLC profile of synthetic 2,6-DH-3,4-naphthol.

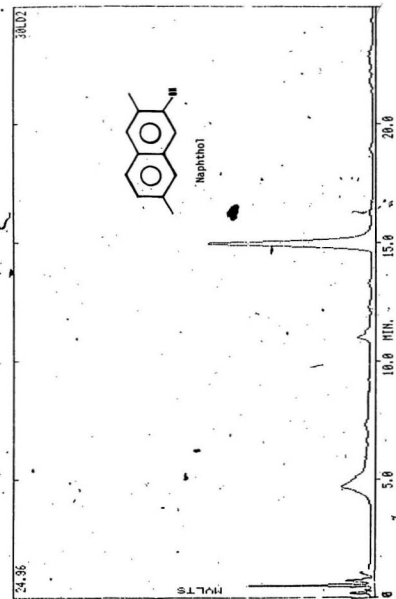
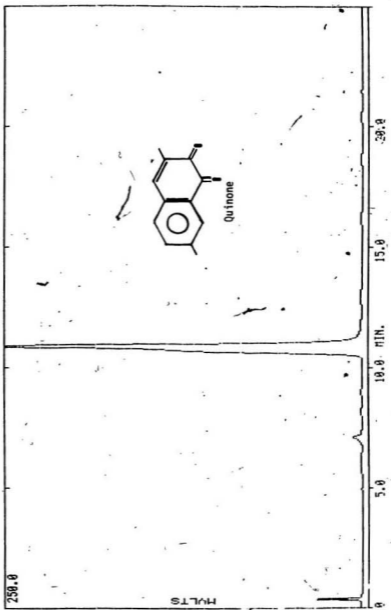


Figure 2-12;
HPLC profile of synthetic 2,6-DM-3,4-naphthoquinone.



20mg of 2,6-DM-3,4-naphthoquinone was dissolved in 0.25ml of glacial acetic acid. An equivalent amount of o-phenylenediamine (in 0.1ml methanol) was added. The reaction mixture was kept at 35°C for 15 min then cooled to room temperature and diluted with 2ml of water to preprecipitate the quinoxaline derivative. The solution was filtered and the quinoxaline derivative was recrystallized from dilute ethanol. The crystals were dried under vacuum. An aliquot of the quinoxaline derivative was dissolved in methanol and chromatographed on thin layer (silica gel 250 μ m, 20x20cm) with UV indicator, along with the aliquots of the quinone and the quinoxaline. The plate was developed with benzene:dioxane:acetic acid (45:5:1). The R_f values for the quinone, o-phenylenediamine, and the quinoxaline were found to be 0.52, 0.72, and 0.92, respectively.

Radiolabelled 2,6-DM-3,4-naphthoquinone was obtained from HPLC, after injection of an aliquot of an ethyl acetate extract of a microsomal incubation of 2,6-DM[8-¹⁴C]N. It was mixed with the cold quinone and was treated as described above. Maximum radioactivity (46% of the total radioactivity of the metabolite) cochromatographed with the quinoxaline derivative. 28% of the original radioactivity was associated with the 2,6-DM-3,4-naphthoquinone. There was no other radioactive band on the plate. The remaining 28% of the radioactivity was presumably lost during workup.

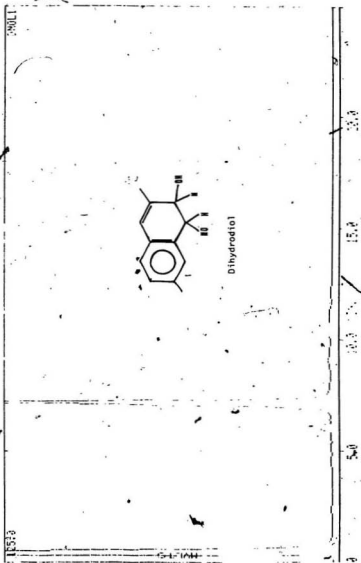
2.1.6.3. 3,4-Dihydro-3,4-dihydroxy-2,6-DMN

A HPLC profile of synthetic 3,4-dihydro-3,4-dihydroxy-2,6-DMN is shown in figure 2-13.

The dihydrodiol was converted into the 3-naphthol by using the method of Booth and Boyland (1958). The synthetic compound (20mg) was dissolved in 0.1ml methanol and 3ml of 2N HCl were added. Two 1.5ml portions were removed into separate test tubes and one of the tubes was placed in a boiling water-bath for 8 min to convert the dihydrodiol into the naphthol. The tube was cooled to room temperature and both tubes were extensively extracted with 2ml

Figure 2-13:

HPLC profile of synthetic 3,4-dihydro-3,4-dihydroxy-2,6-DMA.



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portions of ethyl acetate. The ethyl acetate extracts from the respective tubes were pooled and reduced to 0.1ml. An aliquot of each was chromatographed on a thin-layer plates with⁴ UV indicator. The plate was developed with benzene:dioxane:acetic acid (45:5:1). The dihydrodiol ($R_f = 0.21$) in the acidified-heated reaction mixture was converted into the 3-naphthol ($R_f = 0.86$).

Radioactive 3,4-dihydro-3,4-dihydroxy-2,6-DMN obtained on HPLC at the retention time corresponding to synthetic dihydrodiol was subjected to the above experiment in the presence of the unlabelled compound. Most of the radioactivity (44%) was transferred to the R_f value of the naphthol. 17% of the original radioactivity was associated with the 3,4-dihydro-3,4-dihydroxy-2,6-DMN. There was no other radioactive band on the plate. The remaining 39% of the radioactivity was presumably lost during the acid and heating treatment.

2.5. Pretreatment of Animals

Male Sprague Dawley rats (225-250g) and male CD-1(20-30g) mice were purchased from Charles River Canada. Animals were housed (3/cage) on hardwood bedding with food and water available *ad libitum*. Rats in groups of three were pretreated with a variety of xenobiotics. Sodium phenobarbital (PB) was administered as a 0.1% solution in drinking water for 4 days. 3-Methylcholanthrene (3MC) was dissolved in corn oil (20mg/ml) and administered i.p. as 2 daily injections (40mg/kg body weight). 2,6-DMN was dissolved in corn oil (82.5mg/ml) and administered i.p. for 3 consecutive days (200mg/kg body weight). Prudhoe Bay Crude Oil (PBCO) was orally intubated for 3 consecutive days (5ml/kg body weight). Control animals were given equal amounts of dissolving vehicle. Animals were fasted overnight after the final treatment and killed on the following day.

2.6. Preparation of Microsomes

Rats were killed by cervical dislocation. All of the following procedures were performed at 0-4°C. Livers were removed and perfused through the portal vein with ice-cold 1.15% KCl to remove blood. Livers were 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) in a hand homogenizer (4 to 5 strokes), followed by homogenization with a motor-driven Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000g for 10 min in a Sorval RC-2B centrifuge (using GSA rotor). The supernatant was decanted, filtered through cheese cloth and centrifuged at 105,000g for 75 min. 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 min. The supernatants were discarded and the pellets were resuspended by homogenization in 0.1M 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 (Rahimtula et al., 1970). Protein was estimated by the method of Lowry et al. (1951).

2.7. Microsomal Incubation Conditions

Unless otherwise specified, incubation mixtures consisted of 0.1M phosphate buffer pH 7.4, microsomal protein (1mg/ml), 0.2mM of 2,6-DM[8-¹⁴C]naphthalene and a 0.1ml NADPH-regenerating system consisting of 50mM MgCl₂, 50mM DL isocitrate, 5mM NADP⁺, and 6.5 units of isocitrate dehydrogenase. Corresponding blanks were without the regenerating system. Incubations were carried out for 20 min at 37°C in a total volume of 2ml. Reactions were stopped with 4ml of ice-cold ethyl acetate. The mixtures were shaken and the ethyl acetate layer separated by centrifugation. The aqueous layer was extracted repeatedly with ethyl acetate (5 to 10 times) until no more radioactivity was detected in the ethyl acetate extracts. The ethyl acetate extracts were combined, concentrated under nitrogen and chromatographed by thin layer and high-performance liquid chromatography.

2.8. Determination of K_m

The K_m for 2,6-DMN was determined using microsomes from four groups of pretreated rats (PB, 3MC, and 2,6-DMN as well as control microsomes). Incubations were carried out for 10 min at 37°C with shaking and contained in a total volume of 2ml; 2mg microsomal protein, 50 μ M to 250 μ M 2,6-DM[8-¹⁴C]naphthalene 10 μ l of acetone and 0.2ml of a NADPH-regenerating system. Incubations were terminated by the addition of 4ml of ice cold ethyl acetate followed by shaking. The ethyl acetate extracts were concentrated and chromatographed on thin layer (silica gel 250 μ m 20x20cm; developed in hexane) to separate 2,6-DMN from its metabolites. The solvent system separated unmetabolized 2,6-DM[8-¹⁴C]naphthalene (R_f 0.90) from all metabolites which remained at the origin. The rate of production of metabolites was linear for at least 10 min. When reactions were carried out for different concentrations of 2,6-DMN, the results gave a linear Lineweaver-Burke plot.

2.9. In Vitro Study of 2,6-DMN Metabolism

Incubation mixtures consisted of 0.1M phosphate buffer pH 7.4, microsomal protein (1mg/ml), 0.2mM of 2,6-DM[8-¹⁴C]naphthalene, and a NADPH-regenerating system. Corresponding blanks were without the regenerating system. Incubations were carried out for 20 min at 37°C in a total volume of 2ml. Reactions were stopped with 4ml of ice-cold ethyl acetate. The mixtures were shaken and centrifuged to separate the ethyl acetate layers. The aqueous layer was extracted repeatedly with ethyl acetate (5 to 10 times) until no more radioactivity was detected in the ethyl acetate extracts. The combined ethyl acetate extracts were concentrated under nitrogen and chromatographed on thin layer or high-performance liquid chromatography.

2.10. High Performance Liquid Chromatography

Separation of 2,6-DMN metabolites was performed on a P.E.-3x3 (4.6x33mm) C₁₈- μ Bondapak reversed-phase analytical column with no guard column. All samples were filtered through a 0.45 μ m Millipore filter (Millipore, Bedford, MA, U.S.A.) prior to their injection into the column. The samples were injected with a Rheodyne injection valve (Model 7120). The solvents were delivered with a Perkin-Elmer Series 4 Liquid Chromatograph Microprocessor-controlled solvent delivery system. Analyses were carried out at room temperature utilizing a mobile phase of acetonitrile:water (3:97) which was changed stepwise (in stages) to acetonitrile:methanol:water (60:10:30). Separations were effected using different gradients over a 25 min time period in the following manner: acetonitrile:water (3:97) at a flow rate of 2.5ml/min for 1 min, acetonitrile:water (20:80) at a flow rate of 2.0ml/min for 1 min, acetonitrile:methanol:water (60:2:38) at a flow rate of 1.2ml/min for 20 min, and finally acetonitrile:methanol:water (60:10:30) for 3 min. The column was equilibrated with acetonitrile:water (1:99) at a flow rate of 2ml/min for 2 min prior to injection of the sample. The pressure never exceeded 16 MPa during any analysis. The eluted compounds were detected at 254nm using a Perkin-Elmer model LC-85 B 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. The eluted radioactive fractions (30 sec each) were collected using a LKB fraction collector. A LS-85 Beckman Scintillation counter was used to measure radioactivity of the eluted fractions after addition of 10ml of "Scintiverse" scintillation cocktail.

2.11. Determination of Phase I and Phase II Microsomal Enzyme Activities

Liver microsomes from untreated and treated rats were used and were obtained as mentioned in section 2.5. All enzyme activities were measured in quadruplicate.

2.11.1. Measurement of Cyt. P-450 Levels

Cyt. P-450 levels were measured as described by Omura and Sato (1964). A total volume of 5ml contained 0.25mM phosphate buffer pH 7.5, 5mg microsomal protein, and a few mg of sodium dithionite. The contents of the tube were gently mixed and distributed equally in two cuvettes (1-cm pathlength) which were placed in the sample and reference cell compartments of a dual-wavelength spectrophotometer. A baseline was recorded between 400nm and 500nm. The sample cuvette was then saturated with 30-40 bubbles of carbon monoxide. The spectrum was recorded again between 400nm and 500nm to determine the maximum absorbance peak at 450nm (for PB type) or 448nm (for 3-MC type). The cyt. P-450 content was determined by using the extinction coefficient of $91\text{mM}^{-1}\text{cm}^{-1}$.

2.11.2. Benzo[a]pyrene Hydroxylase

Benzo[a]pyrene hydroxylase activity was measured by the method of Nebert and Gelboin (1968). Liver microsomes (0.1mg protein/ml) were placed in a test tube along with 100mM phosphate buffer (pH 7.5), 80 μ M benzo[a]pyrene in 20 μ l acetone, and water to make the final volume to 0.9ml. Samples were preincubated at 37°C for 2 min in a shaking water bath. The reaction was started by adding 100 μ l of a NADPH-regenerating. After 10 min, the reaction was stopped by the addition of 4.25ml of acetone-hexane (1:3), and immediately mixed by vortexing. After centrifugation, 2.5ml of the upper layer was transferred to a clean tube and 2.5ml of 1N NaOH was added to it. After vortexing and centrifugation, the lower aqueous phase was used to read the fluorescence with excitation at 398nm and emission at 522nm. Samples were measured against a standard curve consisting of various concentrations of 3-hydroxybenzo[a]pyrene in 1N NaOH.

2.11.3. 7-Ethoxyresorufin O-deethylase

7-Ethoxyresorufin O-deethylase activity was measured by the method of Pohl and Fouts (1980). A total incubation volume of 1.25ml contained liver microsomes (0.1mg protein), 0.32mg bovine serum albumin, and 1.5 μ M 7-ethoxyresorufin. The sample was preincubated at 37°C for 2 min, before initiating the reaction by addition of 150 μ l NADPH-regenerating system. The reaction was stopped after 10 min by adding 2.5ml of methanol. Precipitated protein was centrifuged down, and the fluorescence of the supernatant was measured with excitation at 550nm and emission at 585nm. The amount of product produced was calculated against a standard curve of various concentrations of resorufin.

2.11.4. NADPH-Cyt. P-450 Reductase

NADPH-cyt.P-450 reductase was assayed using cyt.c as an external electron acceptor as described by Phillips and Langdon (1962). The reaction was carried out in a 1ml cuvette containing in a total volume of 1ml: 0.1ml potassium phosphate buffer (pH 7.7), 0.1mg rat liver microsomal protein and 40 μ M cyt.c. The contents were mixed and preincubated at 30°C in a cuvette in a recording spectrophotometer and a baseline recorded at 550nm. The reaction was started by adding 0.1ml of a NADPH-regenerating system, and the increase in absorbance at 550nm was recorded for at least 4 to 5 min. The rate of cyt.c reduction was determined by using an extinction coefficient of 21mM⁻¹ cm⁻¹.

2.11.5. Epoxide Hydrolase

The activity of epoxide hydrolase was measured fluorometrically by using benzo[a]pyrene-4,5-oxide as a substrate (Densette et al., 1979). The reaction was carried out in a fluorimeter cuvette in a volume of 2.5ml which contained 0.15M Tris-HCl (pH 8.7) and liver microsomes (0.125mg protein). The reaction was initiated by the addition of 10 μ M of the substrate. Relative fluorescence (with excitation wavelength of 310nm and emission wavelength of 385nm) was recorded as a function of time. The activity in the sample was calculated by measuring the

fluorescence of, known concentrations of the product, benzo[a]pyrene-4,5-dihydrodiol.

2.11.6. GSH S-Transferases

GSH S-transferase activity was determined according to the method of Habig et al. (1974), using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The reaction was carried out in a 3ml spectrophotometric cuvette and contained: 0.1M phosphate buffer (pH 6.5), 1mM CDNB, and 0.3mg liver microsomal protein. A baseline was recorded at 340nm before initiating the reaction by adding 1mM of GSH. The increase in absorbance was recorded and activity was calculated by using an extinction coefficient of $9.8\text{mM}^{-1}\text{cm}^{-1}$ for CDNB.

2.11.7. UDP-Glucuronyltransferases

UDP-Glucuronyltransferase activity was assayed spectrofluorometrically by the method of Bock and White (1974). A total volume of 1ml contained 0.1M Tris-HCl buffer (pH 7.5), 5mM MgCl_2 , 0.5mM 1-naphthol dissolved in 0.24% dimethyl sulfoxide, and 0.3mM UDP-glucuronic acid. After preincubation for 2 min at 37°C , the reaction was started by the addition of 0.1mg of liver microsomal protein. After 4 min of incubation, the reaction was stopped with 1ml of 0.6M glycine-0.4M trichloroacetic acid buffer (pH 2.2) followed by centrifugation. To 1ml of the aqueous phase was added 2ml of 0.45N NaOH to give a final pH between 10.0-10.5. The fluorescence of 1-naphthyl glucuronide was determined using an excitation wavelength of 290nm and emission wavelength of 330nm. Activity was determined by using a standard curve constructed from various concentrations of 1-naphthyl glucuronide.

2.12. Depletion of GSH in Tissues of Rats and Mice

Rats and mice (3/group) were orally intubated with 2,6-DMN (500mg/kg body weight; dissolved in 10ml corn oil/kg body wt.) at zero time and at intervals of 4, 6 and 8 hours (also 12 hours for rats only). Control animals received equal amounts of corn oil at corresponding time intervals. In this and the following experiments (unless otherwise mentioned), the animals were fasted overnight prior to 2,6-DMN administration. Animals were sacrificed by cervical dislocation between 4 p.m. and 5 p.m. throughout this and subsequent experiments in order to avoid diurnal variations of the hepatic glutathione levels. Individual tissues were immediately freeze-clamped and stored at -80°C . Tissue GSH was determined essentially by the method as described by Akerboom et al. (1982). The frozen tissue was pulverized and an aliquot (0.2-0.5g) of powdered tissue was treated with four volumes of 1M perchloric acid containing 2mM EDTA. The contents were rapidly mixed and homogenized by polytron for 20 sec at maximum speed, keeping the temperature of the sample at 4°C . The acid extracts were centrifuged at 5000g for 5 min to remove protein. The supernatants contained the soluble components (GSH, GSSG, and acid-soluble mixed sulphides) whereas the pellet contained acid-insoluble mixed disulphides. An aliquot of the deproteinized extract was neutralized with a solution of 2M KOH containing 0.3M N-morpholinopropanesulphonic acid (MOPS) and assayed immediately. The final assay mixture was 1ml and contained an aliquot of the neutralized sample, $50\mu\text{l}$ of o-phthaldialdehyde (OPT; 1mg/ml), and 0.1M phosphate buffer (pH 8.0) containing 5mM EDTA. After mixing and incubating at 25°C for 15 min, the fluorescence of the OPT-GSH adduct was determined at 420nm with excitation at 350nm. Values were calculated against a standard GSH curve (ranging from 0.1 nmoles to 5 nmoles).

2.12.1. Effect of Various Doses of 2,6-DMN on GSH Levels in Rat Liver

Groups of rats were orally intubated with 2,6-DMN dissolved in corn oil (50mg/ml). The doses varied from 20mg/kg body weight to 500mg/kg body weight. Controls received an equal amount of corn oil. Rats were sacrificed after 8 hours and GSH levels were measured in freeze-clamped livers as described earlier in section 2.12.

2.12.2. Effect of 2,6-DMN on the Levels of Hepatic GSH in Pretreated Rats

Groups of 3 rats were pretreated with PB, 3MC, or 2,6-DMN as mentioned in section 2.5. 2,6-DMN was dissolved in corn oil, and was administered by oral intubation (500mg/kg). Controls were given only corn oil. Rats were sacrificed at 4, 6 and 8 hours after 2,6-DMN dosing. GSH levels were measured as mentioned in section 2.12.

2.12.3. Effect of Fasting

Rats were divided into two groups of 3 animals each. One group was fasted overnight (as usual) and the other was given free access to food. Both groups were given 2,6-DMN (500mg/kg body weight) in corn oil. Controls of both groups were given corn oil only. Animals were sacrificed after 8 hours and hepatic GSH levels were measured as described in section 2.12.

2.12.4. Effect of Cobalt-Protoporphyrin IX

A group of rats (n=3) was injected subcutaneously with a single dose of cobalt-protoporphyrin IX (125 μ mol/kg body weight) as described by Drummond and Kappas, (1982). This dose of cobalt-protoporphyrin IX is known to decrease the level of hepatic cyt.P-450 to 20% of controls by within 48 hours of administration (Drummond and Kappas, 1982). A solution of cobalt-protoporphyrin IX was prepared by dissolving the compound in a small volume of 0.1M sodium hydroxide, and the pH was adjusted to 7.4 with 1.0M hydrochloric acid. The final

volume was made up, with 0.9% NaCl. The cobalt-protoporphyrin IX solution was administered (4ml/kg body weight) within 10 min of preparation. Control animals received an equivalent volume of saline. After 48 hours of cobalt-protoporphyrin IX or saline treatment, the rats were fasted overnight and subdivided into two groups. One subgroup from control and cobalt-protoporphyrin IX treated rats was intubated orally with 2,6-DMN (500mg/kg body weight) while the other received as equivalent amount of corn oil. Animals were sacrificed after 8 hours and hepatic GSH levels were determined as described before in section 2.12.

2.13. In Vitro Binding of 2,6-DMN to Hepatic Microsomal Protein

Microsomal incubations were carried out as described earlier in section 2.7, and contained in a final volume of 2ml of 0.1M phosphate buffer pH 7.4: hepatic microsomal protein (1mg/ml), 0.2mM 2,6-DM[8-¹⁴C]N, and a NADPH-regenerating system. Corresponding blanks lacked the NADPH-regenerating system. All the analyses were done in quadruplets. Test tubes were capped to prevent the evaporation of 2,6-DMN and were incubated for 20 min at 37°C in a shaking waterbath. Reactions were stopped by the addition of 4ml of ice-cold ethyl acetate followed by vortexing. The ethyl acetate phase was removed. Protein in the aqueous phase was precipitated with an equal volume of 10% TCA followed by centrifugation. The supernatant was removed, and the precipitate was washed repeatedly by mixing, with 5ml of methanol:ether (3:1) to remove non-covalently bound substrate and metabolites. After each washing, the mixture was centrifuged and the solvent was removed by aspiration. The washing procedure was continued until the organic wash contained background levels of radioactivity (usually a matter of 5 to 10 washes). The pellet which consisted mainly of protein, was dissolved in 1ml of 1.0N sodium hydroxide by overnight incubation at 60°C followed by neutralization with 5N HCl. An aliquot of the clear solution was counted in scintillation fluid for radioactivity determination. Protein was determined with another aliquot of the dissolved precipitate by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.13.1. In Vitro Protein Binding by 2,6-DMN in the Presence of GSH or both GSH and Hepatic Cytosol

The procedure was the same as above (2.13) except that the incubation mixtures contained various concentrations of GSH (ranging from 50 μ M to 5mM) and liver cytosolic protein (when included). Liver cytosol was dialyzed to remove any endogenous GSH present and was used in the incubation mixture at a concentration of 0.3mg/ml.

2.14. Determination of GSH and GSSG in Human Red Blood Cells

Human red blood cells were obtained from the blood bank unit of the General Hospital, St. John's, NF. The erythrocytes were obtained by centrifugation and extensively washed with isotonic saline prior to use, and were diluted in a ratio of 1:1 (v/v) with isotonic 0.1M phosphate buffer, pH 7.4. Haematocrit values were determined and final results reported on the basis of % haematocrit (Brown, 1976).

2.14.1. Incubation Conditions

The incubation mixture contained liver microsomal protein (1mg/ml) from 3MC pretreated or control rats, 0.2mM 2,6-DMN or any one of its three metabolites (namely 2,6-DM-3-naphthol, trans-3,4-dihydro-3,4-dihydroxy-2,6-DMN, and 2,6-DM-3,4-naphthoquinone). It also contained 0.4ml of red blood cells, a NADPH regenerating system and isotonic 0.1M phosphate buffer (pH 7.4) to make the final volume to 1ml. Blanks were run simultaneously and lacked the NADPH regenerating system. Other "controls" were also run by excluding certain components. All incubations were done in quadruplicate. Reactions were stopped by taking a aliquot of the incubation mixture and placing it immediately in (a) 2.0M perchloric acid containing 4mM EDTA for GSH or GSSG determination, or (b) 3.9 ml distilled water for determination of methaemoglobin.

2.14.2. Determination of GSH in Microsome-Erythrocyte Incubation Mixtures

An aliquot (0.1-0.5ml) of the incubation mixture was added to an equal volume of 2.0M perchloric acid containing 4.0mM EDTA. The precipitated protein was removed by centrifugation. The acid extract was treated in the same manner as for the determination of GSH in tissues by the o-phthalaldehyde method as described earlier (section 2.12)

2.14.3. Determination of GSSG in Microsome-Erythrocyte Incubation Mixtures

Autoxidation of GSH in the neutralized extracts was circumvented by trapping the GSH with N-ethylmaleimide (NEM) as described by Akerboom and Sies, (1982). NEM was added to an aliquot of the acid extract of the incubation mixture to a final concentration of 50mM. The mixture was carefully neutralized to pH 6 with a solution containing 2M KOH and 0.3M MOPS. Excess NEM was removed by extraction with 4 volumes of diethyl ether. The extraction was repeated 5-8 times. Traces of diethyl ether were removed by bubbling nitrogen through the sample. An appropriate aliquot of this was used to determine the concentration of GSSG by the method as described by Akerboom and Sies (1982). An aliquot of the NEM-free sample made up to 1ml with 0.1M phosphate buffer pH 7.0 was placed in a cuvette. 10 μ l of 100mM EDTA and 10 μ l NADPH (dissolved in 0.5% NaHCO₃) were also added with mixing. The baseline level of NADPH absorbance at 340nm was measured. After equilibration of a temperature to 25°C, the reaction was started by the addition of 5 μ l of glutathione reductase (the commercial enzyme was diluted to give 20 units/ml in 0.1M phosphate buffer pH 7.0, containing 10 μ M NADPH). The rate of decrease of NADPH in the sample was determined. For the measurement of standard GSSG, buffer containing 5nmole of GSSG/ml replaced the sample.

2.15. Determination of Methaemoglobin (metHb) Formation

The spectrophotometric one-wavelength (630nm) method was used to determine erythrocyte metHb levels (Salvati et al., 1981). Incubations were carried out as described in section 2.14.1. An aliquot of the incubation mixture was immediately transferred to a test tube containing 3.9ml of water which both stopped the reaction and haemolyzed the red blood cells. After 5 min, 4.0ml of 0.15M phosphate buffer (pH 6.6) was added. Two 3ml aliquots were transferred to two test tubes, named C₂ and C₃. 20% potassium ferricyanide (0.1ml) was added to tube C₃ while mixing. After 2 min the absorbance of both the tubes was measured at 630nm, using phosphate buffer and water as blank. These absorbances were designated as A_{2a} and A_{3a}. Then 0.1ml of 5% potassium cyanide was added to both the tubes and mixed. Absorbances were again recorded at 630nm for C₂ and C₃, and the absorbance were recorded as A_{2b} and A_{3b}, respectively. The concentration of metHb was calculated as follows:

Methaemoglobin (percent of total pigment)

$$= 100 (A_{2a} - A_{2b}) / (A_{3a} - A_{3b})$$

2.16. Statistical Analysis

The data for all experiments were analysed by group t-test in which each treated group was compared against the corresponding control. When treatments were more than one the analysis of variance was performed and was followed by group t-test between the control and treated groups.

Chapter 3

Results

3.1. Percent of Total Metabolites Formed

Table 3-1 shows the extent of metabolism of 2,6-DMN by rat liver microsomes *in vitro*. The results give the percentage of the total metabolites that are formed with each microsome type. Metabolites were separated by TLC on silica gel plates which were developed in hexane. This solvent system separates 2,6-DMN from all its metabolites, which remain at the origin.

The results show that highest metabolism of 2,6-DMN *in vitro* occurred with liver microsomes obtained from PB pretreated rats (30%). The percent metabolism of 2,6-DMN by liver microsomes obtained from pretreatment with PBCO, 3-MC, and 2,6-DMN were 24%, 20%, and 14%, respectively. These values are significantly higher than those obtained from liver microsomes of control (6%) or corn-oil (i.p.) pretreated rats (7%).

3.1.1. Distribution of Microsomal Metabolites of 2,6-DMN

Table 3-2 summarizes the results for 5 major metabolites produced in the metabolism of 2,6-DMN by rat liver microsomes from control and variously pretreated rats *in vitro*. The results give the percentage of individual metabolites on the basis of the total metabolites (as 100%) that were recovered on HPLC. The metabolites were separated on a 3x3 C₁₈ reverse-phase column as described in Methods (2.10).

The identity of the metabolites was determined by comparison with synthetic

Table 3-1: Percent of total metabolites formed by liver microsomes from untreated and pretreated rats.

Treatments	metabolites formed nmol/min/mg protein (\pm standard deviation)	% metabolism
Control	0.6 \pm 0.05	6.0 \pm 0.50
Control (corn-oil)	0.71 \pm 0.07	7.1 \pm 0.70
PB	3.0 \pm 0.20	30 \pm 2.0
3-MC	2.0 \pm 0.21	20 \pm 2.1
2,6-DMN	1.4 \pm 0.16	14 \pm 1.6
PBCO	2.4 \pm 0.20	24 \pm 2.0

Incubations were performed in quadruplicate for 20 min and contained per ml: 200 μ M 2,6-DM[8- 14 C]N, 1 mg microsomal protein from control or variously pretreated rats and a NADPH-regenerating system. After incubation, ethyl acetate extracts of the reaction mix were spotted on TLC plates which were developed in hexane. Details are described in Methods (2.10). Percentage metabolism was calculated as the fraction of radioactivity present in the reaction mix that remained at the origin of the TLC plate \times 100.

Table 3-2: Effect of various pretreatments on the percent of microsomal metabolites of 2,6-dimethylnaphthalene separated by HPLC*.

Metabolites	Retention Time (In Mins) HPLC	Control	Corn Oil (8 ml/kg b.wt.)	Phenobarbital (0.1% in Drinking Water)	3-Methyl-Cholanthrene (1.p. 40 mg/kg b.wt.)	2,6-Dimethyl Naphthalene (1.p. 200 mg/kg b.wt.)	Purdue Bay Crude Oil (5 ml/kg b.wt.)
1) 3,4-dihydroxy-3,4-dihydro-2,6-dimethyl Naphthalene	7.18	7.8	9.2	24.0	8.4	10.1	17.2
2) 2,6-dimethyl-3,4-naphthoquinone	11.18	42.0	39.4	33.7	35.6	45.0	31.4
3) III (unidentified)	13.23	2.4	2.7	5.6	6.8	9.1	7.6
4) 2,6-dimethyl-3-naphthol	15.19	10.6	10.0	7.6	19.9	11.4	18.2
5) V (unidentified)	17.64	19.0	19.8	12.8	8.8	9.4	10.7

Incubations were carried out at 37°C for 60 min and contained in a final volume of 2 ml 200 nmol 2,6-¹⁴C₁₂H.

2 mg microsomal protein from control or variously pretreated rats and a NADPH regenerating system. Details of the work-up procedure and HPLC separation as described in Methods (2.9;2.10).

*Results give the percentage of individual metabolites on the basis of the total metabolites (I-IV):

standards on HPLC. The metabolites as well as the synthetic standards were clearly separable by HPLC using the column and solvent system described in Methods (2.10). The eluant system used also separated 1- and 2-naphthols (see figure 3-1) suggesting that if isomers of 2,6-DMnaphthols were formed in the incubation mixture they might also be separated.

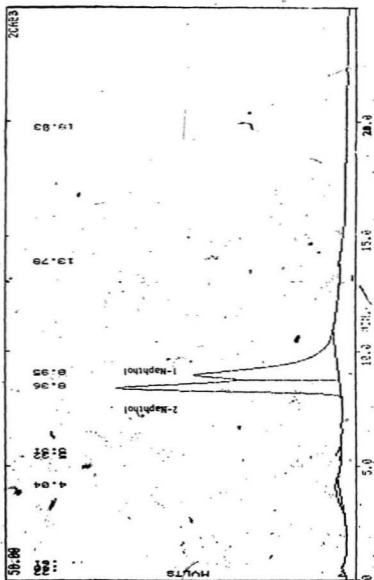
There was no change in the number of metabolites formed when microsomes from control animals (Figure 3-2) were substituted with those from animals treated with PB (Figure 3-3), 3-MC (Figure 3-4), 2,6-DMN (Figure 3-5) or PBCO (Figure 3-6). The quantity, however, of the individual metabolites changed with each microsome type. 2,6-DM-3,4-naphthoquinone, 2,6-DM-3-naphthol and 3,4-dihydro-3,4-dihydroxy-2,6-DMN were the major metabolites identified. They constituted about 80% of the total metabolites that were recovered on HPLC. Liver microsomes from PB or PBCO pretreated rats increased the formation of 3,4-dihydro-3,4-dihydroxy-2,6-DMN significantly (to 24% and 17.2% respectively of total metabolites) as compared to controls (7.8%). Similarly, 2,6-DM-3-naphthol formation was increased significantly with liver microsomes from 3-MC (19.9%) or PBCO (18.2%) pretreated rats, as compared to control microsomes (10.6%). The unidentified metabolites III (retention time 13.23 min) and V (retention time 17.64 min), respectively increased and decreased with pretreatments when compared to controls.

3.2. Determination of Apparent Michaelis Constant, K_m

The apparent K_m values for 2,6-DMN with hepatic microsomes obtained after different pretreatment regimens were determined (Methods 3.8). The results were determined by the Lineweaver-Burk method (Fig. 3-7). The best straight lines were determined by the method of Johansen and Lumry (1961), using an Apple II computer programme (Enzyme Kinetics I). The K_m for 2,6-DMN using control microsomes was found to be $110\mu\text{M}$, and the V_{max} was 18.7 nmol/min/mg protein. Pretreatment with 3-MC led to a lower K_m of $70\mu\text{M}$ and a lower V_{max} . Pretreatment with PB or 2,6-DMN led to a higher V_{max} but with a higher K_m of

Figure 3-1:

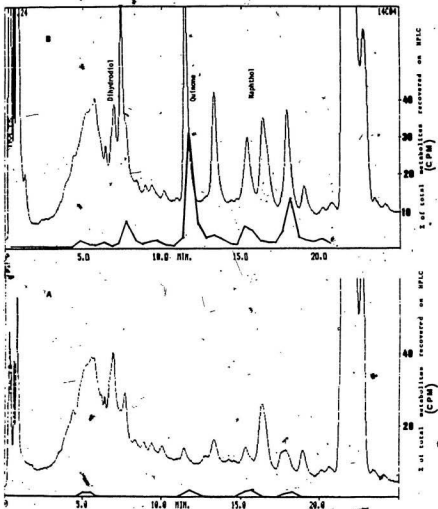
Separation of 1-naphthol and 2-naphthol on HPLC



Conditions were the same as for the separation of 2,6-DNH and its metabolites and are described in Methods (2-10).

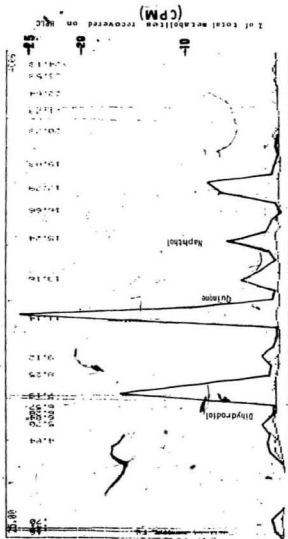
Figure 3-B:

HPLC profile of metabolites formed on incubation of 2,6-DH[6-¹⁴C] with liver microsomes from control rats.



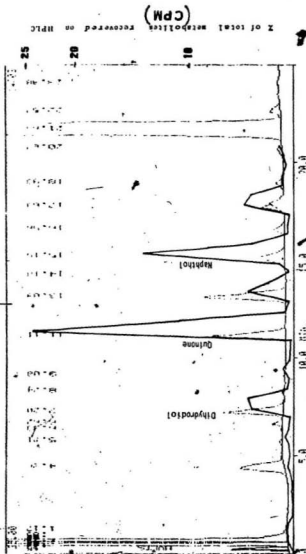
Incubations were carried out as described in footnote of Table 1. HPLC separation was carried out on a μ Bondapak C₁₈ reversed phase column using acetonitrile:water:methanol as described in Methods (2.20).
 (A) With ¹⁴C-DPN-regenerating system.
 (B) With ¹⁴C-regenerating system.
 (.....) Absorbance at 254 nm.
 (—) Radioactivity.

Figure 2: Profile of metabolites formed on incubation of 2,6-DI(9-¹⁴C)H with 1 liter microsomes from PB pretreated rats.



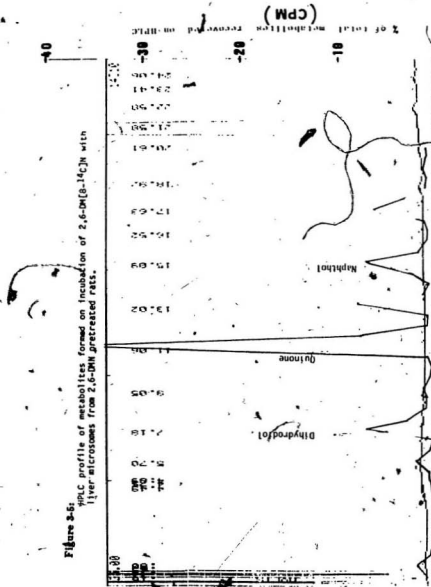
Incubations were carried out as described in footnote of Table 1. HPLC separation was carried out on a 3 x 3 C18 reversed phase column using acetonitrile-water-methanol as described in Methods (4,10). With this system, 100% radioactivity was recovered at 254 m.

Figure 3-4:
HPLC profile of metabolites formed on incubation of 2,6-DHAP- ^{14}C in with
liver microsomes from 3-HC-pretreated rats.



Incubations were carried out as described in footnote of Table 1
in the presence of the same column
using acetonitrile as eluent as described in Method (p. 10).
With NaOH-regenerating system (.....) Absorbance at 254 nm.
— Radioactivity.

Figure 3-5: HPLC profile of metabolites formed on incubation of 2,6-DN[8-¹⁴C]N with liver microsomes from 2,6-DN pretreated rats.



Incubations were carried out as described in footnote of Table 3. HPLC separation was carried out on a 3 x 3 C18 reversed phase 4.6 x 25 cm Acetonitrile-water-methanol as described in footnote of Table 3. ¹⁴C radioactivity was measured in a liquid scintillation counter using a 100% efficient system. Absorbance at 254 nm was measured in a spectrophotometer.

340 μ M and 240 μ M, respectively. The best adapted system, which maximizes V_{max}/K_m was that induced by PB.

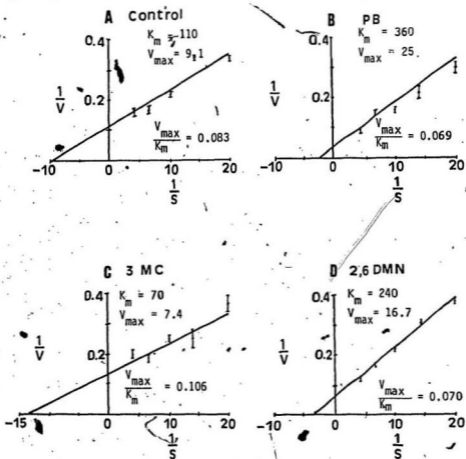
3.3. Effects of Pretreatment on Hepatic Microsomal Phase I and Phase II Enzymes of Rat

The effects of PB, 3-MC, 2,6-DMN or PBCO pretreatments on hepatic cyt. P-450 levels and some phase I and phase II activities are shown in Table 3-3. Levels of hepatic cyt. P-450 were increased with pretreatment of animals with PB (4-fold), PBCO (2-fold) and with 3-MC (36%). There was no significant increase in the levels of cyt. P-450 when animals were pretreated with 2,6-DMN.

Pretreatment of animals with different inducers caused a significant induction of some mixed function oxidase activities. 7-Ethoxyresorufin O-deethylase activity showed maximum induction after pretreatment with PBCO (12-fold), followed by an increase of 11-fold after 3-MC, 2-fold after PB and 70% after 2,6-DMN pretreatments. Activity of benzo[a]pyrene hydroxylase was also significantly increased by pretreatment of animals as compared to controls. There was an 11-fold increase in the case of PBCO, 7.5-fold for 3-MC, 4-fold for 2,6-DMN and 3-fold for PB. Epoxide hydrolase activity also increased 2-fold by pretreatment of rats with PBCO and 3-MC, and by 50% with PB. There was no significant increase in the activity with 2,6-DMN pretreatment. All the pretreatments significantly increased the levels of the liver microsomal flavoprotein NADPH-cyt.c reductase activity as compared to controls (Table 3-3).

The levels of two groups of the phase II detoxication enzymes from microsomes, namely, GSH S-transferases and UDP-glucuronyl transferases were also elevated with various pretreatments. There was a 100% increase in GSH S-transferase activity with 3-MC and PB pretreatments, and a 36% increase with 2,6-DMN and PBCO pretreatments. Similarly, UDP-glucuronyltransferase activity was also elevated. A 3.5-fold increase was observed with 3-MC pretreated microsomes, while PB, PBCO and 2,6-DMN pretreatments increased this activity by (2-fold), 34% and 34%, respectively.

Figure 3-7: Lineweaver-Burk plots of 2,6-DMN metabolized by hepatic microsomes from control and pretreated rats.



- Experiments were performed as described in 2.9, 2.10.
- The units of V and V_{max} are $\text{nmols of metabolites formed} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.
- The units of K_m and S are μM and mM respectively.
- The bars represent \pm SD.

Table 3-3: Effects of pretreatments on some hepatic microsomal enzymes of rat.

Pretreatments	Cyt. P450	NADPH-Cyt. C Reductase	Benzo(a)pyrene Hydroxylase	7-Ethoxycoumarin O-deethylase	Epoxide Hydrolase	GSH Transferase	³ H- Glucuronyl Transferase
Control	0.58±0.04	169±10	0.50±0.03	0.70±0.02	55±1.41	178±6.0	13.2±0.69
PB*	2.53±0.16	324±17	1.58±0.07	1.61±0.09	82.7±6.15	333±6.5	22.6±1.17
3-MC*	0.79±0.07	236±9.0	3.80±0.08	7.70±0.11	109±2.94	356±4.3	46.3±1.37
2,6-DMN*	0.66±0.04	227±7.0	2.06±0.05	1.19±0.06	54.3±0.87	244±12	16.4±0.71
PRCO*	1.21±0.05	265±11	5.70±0.38	8.52±0.12	118±5.7	226±0.85	16.4±0.71

* All analyses were performed as described in methods (2.11).

* Cyt. P450 levels are in nmol/mg protein.

* Enzyme activities are expressed as nmol min⁻¹mg⁻¹ microsomal protein.

* Results are mean ± S.E. for quadruplicate assays.

* *Significantly different from control (t-test for grouped data, P < 0.05).

* *Not significantly different from control (t-test for grouped data, P < 0.05).

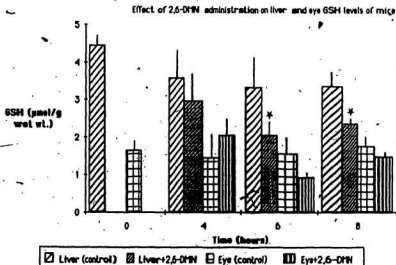
3.4. Depletion of GSH in Mice

Buckpitt and Warren (1982) showed that the administration of naphthalene (400mg/kg body wt.) to mice depleted GSH in liver, kidney and lungs. Griffin et al. (1981,1983) showed that a pulmonary toxic dose of 2-MN (400mg/kg body wt., i.p.) administered to mice significantly depleted GSH in the liver and lung and, to a lesser extent, in the kidney. To observe the effects of 2,6-DMN on the levels of GSH in different tissues, male CD-1 mice were administered an oral dose of 2,6-DMN (500mg/kg body wt.) by intubation. Animals were sacrificed at time intervals of 0, 4, 6, and 8 hours after 2,6-DMN administration. Levels of GSH were measured in different frozen-clamped tissues as described in Methods (2.12). Figures 3-8 and 3-9 show the results. A marked decrease in hepatic levels of GSH was observed. This depletion was maximum 6 hours after 2,6-DMN dosing, and reached 2.05 ± 0.35 $\mu\text{mols/g}$ wet wt. of liver from a control value of 3.34 ± 0.80 .

Four hours after 2,6-DMN administration, an elevation of GSH was observed in the eye (2.04 ± 0.42 $\mu\text{mol/g}$ wet wt.), but by 6 hours the level of GSH came down to 0.92 ± 0.05 $\mu\text{mols/g}$ wet wt. and then rose to 1.47 ± 0.06 $\mu\text{mols/g}$ wet wt. 8 hours after dosing. Nakashima (1934) also observed a depletion of GSH in the liver and eye lens of rabbits treated with naphthalene, but our results were not statistically significant for eyes.

In kidney no significant GSH depletion was observed. In fact, 8 hours after 2,6-DMN administration there was an increase in the level of GSH from 0.15 ± 0.05 (control at 8 hours) to 0.24 ± 0.08 $\mu\text{mol/g}$ wet wt. Similarly, an increase in the level of GSH in the lung was observed after 4 hours of dosing with 2,6-DMN. The results for lung and kidney (Fig. 3-9) were not statistically significantly different from the control.

Figure 3-8:

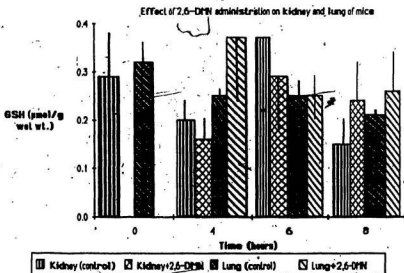


Groups of 3 mice were sacrificed 4, 6 and 8 hr after oral dosing with 2,6-DMN (500 mg/kg body wt. in corn oil) or vehicle alone. GSH levels were measured in duplicate in the liver and eyes of each mouse as described in Methods (2.12).

Results are mean values \pm S.D.

*Indicates significant difference in values from control mice (t-test for group data, $P < 0.05$).

Figure 3-9:



Groups of 3 mice were sacrificed 4, 6 and 8 hours after oral dosing with 2,6-DNH (500 mg/kg body wt in corn oil) or vehicle alone. GSH levels were measured in duplicate in the kidneys and lungs of each mouse as described in Methods (2.12).

Results are mean values \pm S.D.

3.5. Depletion of GSH in Rat

Figures 3-10 and 3-11 show the levels of GSH in tissues of rat treated with an oral dose of 500mg/kg body wt. of 2,6-DMN by intubation. A marked decrease in hepatic GSH levels to 25% of control values was observed within 8 hours after 2,6-DMN administration. There were no significant changes in the GSH levels of lung, kidney or eye, when compared to controls. There was an elevation in GSH levels of eye after 6 hours of 2,6-DMN dosing as compared to its corresponding control. However, the 6 hours control rats group had GSH levels lower than the controls of 0, 4, or 8 hours. The discrepancy in this case is not clear.

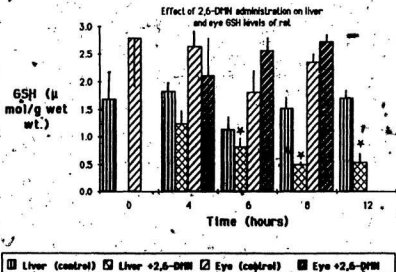
3.5.1. Effect of Various Doses of 2,6-DMN

The results in Figure 3-12 show the effect of various doses of 2,6-DMN on hepatic GSH levels of rats. Control rats received corn-oil only. Animals were sacrificed 8 hours after administration of the various doses of 2,6-DMN, since maximum depletion of hepatic GSH was observed at this time period (Figure 3-8). Results are mean \pm S.D. for three rats/group. Generally, doses above 120mg/kg body wt. caused maximal depletion, but least standard deviation was observed with a dose of 500mg/kg body weight.

3.5.2. Effect of Various Pretreatments

Figure 3-13 shows the depletion of GSH in liver by 2,6-DMN after pretreatment of rats with various inducers of microsomal mixed function oxidases. Control rats were given corn-oil and others the inducers dissolved either in corn-oil (3-MC and 2,6-DMN) or in water (PB). The inducers in the doses given to rats did not lower the level of GSH in the liver. 2,6-DMN was given orally (500mg/kg body wt.) by intubation, and rats were sacrificed at time intervals of 4, 6, and 8 hours. Results are shown as % of GSH in the corn-oil control livers. There was no depletion at 4 hours when PB or 2,6-DMN were used as inducers. However, significant loss of GSH was observed 8 hours after administration of an oral dose of 2,6-DMN. Only in the case of 3-MC pretreated rats was there a very substantial reduction in GSH.

Figure 3-10:

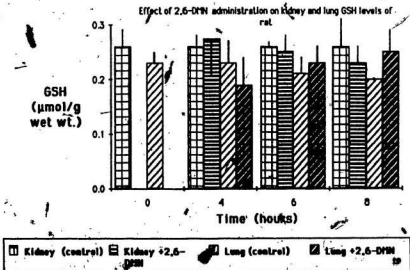


Groups of 3 rats were sacrificed 4, 6 and 8 hours after oral dosing with 2,6-DPM (500 mg/kg body wt in corn oil) or vehicle alone. GSH levels were measured in duplicate in the liver and eye of each rat as described in Methods (2.12).

Results are mean values \pm S.D.

*Indicates a significant difference from values in control rats (t-test, for group, $P < 0.05$).

Figure 3-11:

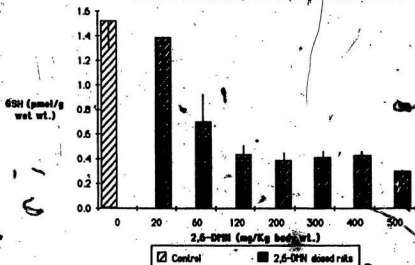


Groups of 3 rats were sacrificed 4, 6 and 8 hours after oral dosing with 2,6-DMN (500 mg/kg body wt in corn oil) or vehicle alone. GSH levels were measured in duplicate in the kidney and lung of each rat as described in Methods (2.12).

•Results are mean values \pm S.D.

Figure 3-12:

Effect of various doses of 2,6-DNN on liver glutathione levels of rat



Groups of 3 rats were sacrificed 8 hours after oral incubation with varying doses of 2,6-DNN in corn oil or corn oil alone. The GSH level in the liver of each individual rat was measured in duplicate as described in Methods (2.12.1).

Results are means \pm S.D. for 3 rats.

levels (nearly 60%) by 4 hours and this GSH depletion continued till levels reached to 15% of controls by 8 hours. It seems from the results that metabolites of 2,6-DMN produced by 3-MC pretreatment might effect the *de novo* synthesis of GSH because significant depletion (as early as 4 hours after the dose) is observed.

3.5.3. Effect of Fasting

Fasting enhances the hepatotoxicity of several chemicals and such increased hepatotoxicity has been extended to chemicals that are capable of depleting GSH (Reed and Fariss, 1984). There is critical level of GSH below which toxic effects became manifest (Mitchell et al., 1973). Fasting is known to decrease the liver-GSH content in rats (Jaeger et al., 1974) and since all the experiments were performed on overnight fasted rats, it was essential to demonstrate that the loss of hepatic GSH was solely due to 2,6-DMN administration and not to any other factor(s) caused by fasting.

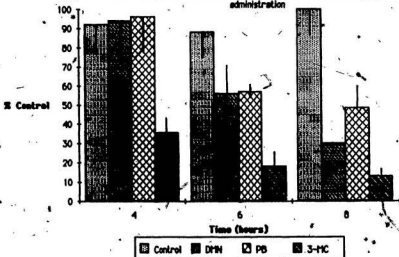
Figure 3-14 shows the results. After 2,6-DMN administration, GSH levels in the liver of rats which were not fasted overnight decreased from 2.35 to 1.2 $\mu\text{mols/g}$ wet wt., a loss of 49%. In rats which were fasted overnight before dosing with 2,6-DMN, the level of GSH went down from 1.45 to 0.63 $\mu\text{mols/g}$ wet wt., a loss of 45%. The total content of hepatic GSH in the fed animal was twice that in the fasted animal but the pattern of depletion was the same in both groups. Thus, the results confirm the conclusions that depletion of GSH is truly due to the administration of 2,6-DMN. The results also indicate that fasting does not aggravate the effects of 2,6-DMN in depleting hepatic GSH as the proportion of loss in fed and fasted animals were not significantly different.

3.5.4. Effect of Cobalt Protoporphyrin IX

A subcutaneous dose of cobalt protoporphyrin IX to rats is known to decrease the level of cyt.P-450 to 20% of controls within 48 hours of administration (Drummond and Kappas, 1982). An experiment was performed to show that *in vivo* metabolism of 2,6-DMN by liver cyt. P-450 was essential for hepatic GSH

Figure 13:

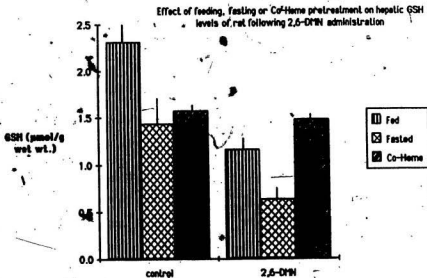
Liver GSH levels in control and variously pretreated rats following 2,6-DPN administration



Rats were intubated with 2,6-DPN (500 mg/kg body wt in corn oil) or vehicle alone. Groups of 3 rats from each treatment regimen were sacrificed 4 hr, 6 hr and 8 hr after dosing and liver GSH levels were measured in duplicate in individual livers as described in Methods (2.12.2).

Results are mean values + S.D. and are expressed as a percentage of corn oil treated rats. 100% GSH = $152 \pm 0.18 \mu\text{mol/g wet wt.}$

Figure 3-14:



Groups of 3 untreated-fed, untreated-overnight fasted and Co-Heme-overnight fasted rats were intubated with 2,6-DMN (500 mg/kg body wt in corn oil) or vehicle alone. Rats were sacrificed 8 hr after treatment and GSH levels in individual livers were determined in duplicate as described in Methods (2.12.3 and 2.12.4).

Results are mean values \pm S.D.

depletion. The results are shown in Figure 3-14. 2,6-DMN administration to cobalt protoporphyrin IX pretreated rats resulted in no GSH depletion by 8 hours whereas control rats suffered a 50% depletion in hepatic GSH levels within this period.

3.6. Binding of 2,6-DM[8-¹⁴C]N to Microsomal Protein

The ability of liver microsomes from control as well as PB, 3-MC, 2,6-DMN or PBCO pretreated rats to catalyze the irreversible binding of 2,6-DMN metabolites to microsomal proteins was determined. 2,6-DM[8-¹⁴C]N (0.2mM) was incubated under the conditions given in Methods (2.13), in the presence or absence of a NADPH-regenerating system. Negligible binding occurred if the incubation mixture lacked the NADPH-regenerating system (Table 3-4). There was a significant increase in protein binding of 2,6-DM[8-¹⁴C]N metabolites when microsomes from pretreated animals were used in place of controls. Maximum binding was 4.73nmol/min/mg of protein after pretreatment with PB, followed by 3.93 after PBCO, 3.35 after 3-MC and 1.55 after 2,6-DMN pretreatments. In comparison, control microsomes showed a binding of 0.56nmol/min/mg protein.

3.6.1. Protein Binding in the Presence of GSH

Several reactive metabolites, including many epoxides, react with glutathione to form glutathione-metabolite adducts (Mitchell and Jollow, 1975), and it is known that liver is capable of metabolizing naphthalene to reactive metabolites which can conjugate with GSH to form at least three adducts (Smart and Buckpitt, 1983). Using incubation conditions as described in Methods (2.13.1.), various concentrations of GSH ranging from 50 μ M to 5mM were added to the incubation mixture containing microsomes from rats pretreated with 3-MC. Microsomes from 3-MC pretreated rats were chosen because 2,6-DMN caused a dramatic drop in hepatic GSH levels of 3-MC pretreated rats (Figure 3-15) and also because 3-MC pretreated microsomes gave significant levels of 2,6-DM[8-¹⁴C]N binding to protein (Table 3-4). In the presence of GSH, a marked decrease in the binding of

Table 3-4: Effect of various pretreatments on the binding of 2,6-DN[8-¹⁴C] naphthalene to liver microsomes in vitro¹

Microsome Type	NADPH regenerating system	Binding to micr. protein ² , (nmols/min/mg)
Control	-	0.22±0.07
	+	0.56±0.08
PB	-	0.23±0.01
	+	4.73±0.57
3-MC	-	0.35±0.08
	+	3.35±0.39
DMN	-	0.27±0.05
	+	1.55±0.03
PBCO	-	0.31±0.01
	+	3.93±0.61

¹Incubations were carried out at 37°C for 20 min and contained in a total volume of 2 ml: 0.1 M potassium phosphate buffer (pH 7.5), 400 nmol 2,6-DN[8-¹⁴C]N, 2 mg microsomal protein and 200 µl NADPH-regenerating system. Details of protein binding are described in Methods (2.13).

²Results are mean values ± S.D. for quadruplicate assays.

2,6-DM[8-¹⁴C]N to protein was observed. This decrease in binding was proportional to an increase in the concentration of GSH (Figure 3-15). In the absence of GSH, 3.8 nmols of 2,6-DM[8-¹⁴C]N were bound per mg of microsomal protein. This value decreased to 0.87 ± 0.11 nmol/mg of protein at a GSH concentration of 5mM. Thus, 5mM GSH inhibited 2,6-DMN protein binding by more than 75%.

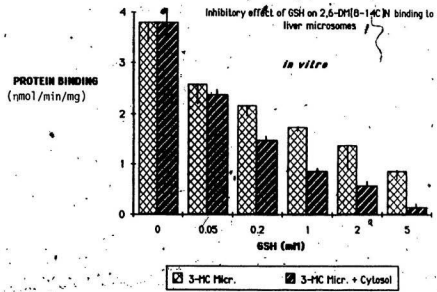
3.6.2. Protein Binding in the Presence of Cytosol and GSH

It has been shown by Booth et al. (1961) that addition of the soluble fraction of rat liver to microsomal incubations containing naphthalene and GSH increased the formation of the GSH derivative of naphthalene, namely, S-(1,2-dihydro-2-hydroxy-1-naphthyl) glutathione. Therefore, the effect of cytosol (0.3mg/ml incubation volume; dialyzed to remove endogenous GSH) on the binding of metabolites of 2,6-DM[8-¹⁴C]N to microsomal protein in the presence of various concentrations of GSH was examined. The results are shown in Figure 3-15. Inclusion of the cytosol in the incubation mix led to a further decrease in the binding of metabolites of 2,6-DM[8-¹⁴C]N to protein. This decrease was observed at all GSH concentrations but was more significant at high GSH concentrations. Thus, the addition of cytosol to the incubation mix containing 5mM GSH resulted in greater than 95% inhibition of protein binding, as opposed to about 75% in the absence of cytosol. The observations are in accord with Booth et al. (1961).

3.7. Methaemoglobin Formation in Human Erythrocytes

Certain chemicals and drugs have the capacity to greatly increase the rate at which haemoglobin is converted to methb, and this may overwhelm the capacity of the erythrocyte to reduce methb to haemoglobin (Beutler, 1985). It was of interest to study whether 2,6-DMN or any of its metabolites could cause methb formation in human erythrocytes. Figure 3-16 shows the results. When erythrocytes were incubated with 0.2mM 2,6-DMN (in 10 μ l acetone), control-microsomes and a NADPH-regenerating system for 20 min, 0.7 ± 0.2 % of

Figure 3-16:



¹Incubations were carried out at 37°C for 20 min and contained in a total volume of 2 ml; 0.1M potassium phosphate buffer (pH 7.5), 400 nmol 2,6-DM[8-¹⁴C]N, 2 mg microsomal protein from livers of rats pretreated with 3-MC, various concentration of GSH, 200 μ l NADPH-regenerating system and (when added) 0.6 mg cytosolic protein from livers of 3-MC pretreated rats. Details of protein binding are described in Methods (2.13).

²Results are mean values \pm S.D. for quadruplicate assays.

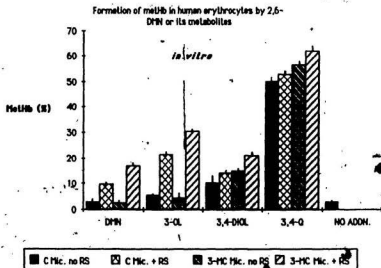
oxyhaemoglobin was converted to methHb. This amounted to a rise in methHb of about 5% as compared to methHb found in erythrocytes alone. The metabolism of 2,6-DMN by microsomes obtained from the liver of rats pretreated with 3-MC increased methHb formation in erythrocytes to 17.1% (Figure 3-16). This increase in methHb formation indicates either an increase in the rate of 2,6-DMN metabolism and/or formation of reactive metabolite(s).

It is known that naphthols formed from the microsomal metabolism of naphthalene can be further metabolized by microsomal enzymes to bind irreversibly to protein (Hesse and Mezger, 1979). It was of interest to know whether this was also true of 2,6-DM-3-naphthol. The results (Figure 3-16) indicate that 2,6-DM-3-naphthol is metabolized in the presence of microsomes and a NADPH-regenerating system. When erythrocytes were included, the methHb formed was found to be 21% and 30% by control and 3-MC-microsomes respectively. The corresponding blanks showed no significant methHb formation (Figure 3-16).

Metabolism of dihydrodiols is slow in rat liver (Bock et al., 1976). Though dihydrodiol dehydrogenase is absent from microsomes (Ayenger et al., 1959), if a product diol is derived from a large hydrophobic molecule, it may again serve as a substrate for the monooxygenases resulting in the formation of diol epoxides (Sims et al., 1974). When 3,4-dihydro-3,4-dihydroxy-2,6-DMN was incubated with erythrocytes, a NADPH-regenerating system and control or 3-MC microsomes, the methHb produced was 14% and 21% respectively. The corresponding blanks, (no NADPH-regenerating system) also produced methHb (10% and 15% for control and 3-MC-microsomes respectively). This increase in methHb levels in blanks (and possibly in test) may also be due to formation of an autooxidized product(s) of dihydrodiol, as no metabolism was expected in the blanks which lacked the NADPH-regenerating system.

2,6-DM-3,4-naphthoquinone is one of the major metabolites in the *in vitro* microsomal metabolism of 2,6-DMN. When this quinone was incubated with

Figure 3-16:



Incubations were carried out at 37°C for 20 min and contained in a final volume of 1 ml: 0.1M potassium phosphate buffer (pH 7.4), 200 nmol 2,6-DHN (DHN) or 2,6-DN-3-naphthol (3-OL) or 3,4-dihydro-3,4-dihydroxy-2,6-DHN (3,4-DIOL) or 2,6-DN-3,4-naphthoquinone (3,4-Q), 0.4 ml erythrocytes in isotonic saline, 1 mg microsomal protein (MIC) from either control (C), or 3-MC pretreated rats and 0.1 ml of NADPH-regenerating system (RS). Corresponding blanks lacked the NADPH regenerating system. Details of metHb measurement are described in Methods (2:15).

Results are mean \pm S.D. for quadruplicate assays.

erythrocytes, either in the presence of control or 3-MC microsomes, with or without a NADPH-regenerating system, a significant amount of methHb was formed ranging from 50% to 82% (Figure 3-16).

3.7.1. Depletion of GSH in Human Erythrocytes

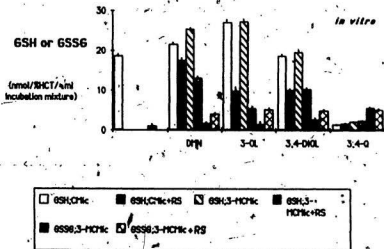
When human erythrocytes were incubated with 2,6-DMN, control-microsomes, and a NADPH-regenerating system the level of GSH in the cell was lowered from 21.6 ± 3.3 nmoles to 17.5 ± 3.3 nmoles/%Haematocrit/ml incubation mixture (Figure 3-17). When 2,6-DM-3-naphthol was used as a substrate in the microsome-erythrocyte incubation mixture, the GSH content of the erythrocytes decreased from 28.9 to 9.7 nmoles/%Haematocrit/ml. This depletion, which is greater than that caused by the parent molecule, seems appropriate in context with methHb formation, which was higher with 2,6-DM-3-naphthol (Figure 3-16).

3,4-Dihydroxy-3,4-dihydro-2,6-DMN also decreased the GSH levels of erythrocytes incubated with control-microsomes and a NADPH-regenerating system. Though the blanks were low (18.6 ± 1.4 nmol/%Haematocrit/ml) as compared to blanks (without NADPH-regenerating system) of 2,6-DMN (21.6 ± 3.3 nmol/%Haematocrit/ml) or 2,6-DM-3-naphthol (28.9 ± 2.8 nmol/%Haematocrit/ml), the inclusion of a NADPH-regenerating system further reduced the levels of GSH to 10.1 ± 1.1 nmol/%Haematocrit/ml). The pattern is similar to that of methHb formation, where the blanks were high.

In the case of 2,6-DM-3,4-naphthoquinone, the absence or presence of a NADPH-regenerating system in the microsome-erythrocyte incubation mixture did not affect the rapid loss of GSH from the cell. The data are similar to that of the experimental results of methHb formation with quinone as the substrate.

Figure 3-17:

Effect of 2,6-DPM or its metabolites on GSH or GSSG levels in human RBC



Incubations were carried out at 37°C for 20 min and contained in a total volume of 1 ml: 0.1M phosphate buffer (pH 7.4), 200 nmol 2,6-DPM or any of its metabolites, 0.4 ml erythrocytes in isotonic saline, 1 mg of microsomal protein (MIC) either from control (C) or 3-MC pretreated rats, 0.1 ml NADPH-regenerating system (RS). GSSG levels were determined only in incubation mixtures containing 3-MC microsomes. Blanks were run simultaneously and lacked the NADPH-regenerating system.

Results are mean values \pm S.D. for quadruplicate assays.

3.7.2. Formation of Oxidised-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. It was therefore of interest to examine the release of GSSG with loss of GSH in the microsome-erythrocyte incubation mixture. Using the 3-MC microsomes-erythrocyte incubation mixture, the level of GSSG was measured in the presence of 2,6-DMN or one of its metabolites. The results (Fig. 3-17) showed an increase in GSSG formation but this was not high enough to compensate for the large decrease in GSH levels. The value for GSSG in the incubation mixture without any additions was 0.96 ± 0.28 nmol/%Haematocrit/ml. When 2,6-DMN was added, the value of GSSG increased from 1.73 ± 0.28 nmol/%Haematocrit/ml (without a NADPH-regenerating system) to $4.03 \pm$ nmol/%Haematocrit/ml with the addition of a NADPH-regenerating system. In the presence of 2,6-DM-3-naphthol, a NADPH-regenerating system and 3-MC microsomes, a GSSG value of 5.02 nmol/%Haematocrit/ml was obtained. The blanks (lacking a NADPH-regenerating system) for 3,4-dihydro-3,4-dihydroxy-2,6-DMN and 2,6-DM-3,4-naphthoquinone were respectively 2.89 ± 0.55 nmol/%Haematocrit/ml and 5.69 ± 0.65 nmol/%Haematocrit/ml, which were higher than the blanks for 2,6-DMN or 2,6-DM-3-naphthol. Both of them had similar patterns of increase in methHb formation and the depletion of GSH (Figures 3-16 and 3-17). When a NADPH-regenerating system was added to the erythrocytes-microsomal incubation mixture containing 3,4-dihydro-3,4-dihydroxy-2,6-DMN, there was an increase in GSSG formation (4.83 nmol/%Haematocrit/ml), whereas, addition of a NADPH-regenerating system to 2,6-DM-3-naphthoquinone did not elevate the levels of GSSG.

Chapter 4

Discussion

4.1: In Vitro Microsomal Metabolism of 2,6-DMN

Results of the present investigation show that 2,6-DMN is converted by rat liver cyt. P-450s to a variety of metabolites including chemically reactive metabolite(s) that covalently bind to hepatic microsomal proteins. In keeping with all monooxygenase catalyzed reactions, there is an absolute requirement for molecular oxygen and NADPH.

There was no change in the number or types of metabolites formed when control microsomes were replaced with PB, 3-MC, 2,6-DMN, or PBCO microsomes. However, the individual quantities of the various metabolites changed with each microsome type. There were five major metabolites separated on HPLC. Three of these were identified as 2,6-DM-3-naphthol, 3,4-dihydro-3,4-dihydroxy-2,6-DMN and 2,6-DM-3,4-naphthoquinone on the basis of retention times as compared to synthetic standards. These three metabolites comprised 80% of the metabolites formed.

The chemically reactive metabolite(s) that appears upon oxidation of 2,6-DMN by cyt. P-450 is probably an epoxide. Though many phenols were believed to be formed via direct oxygen insertion, many have been demonstrated to arise by way of epoxide formation (Yang et al., 1977). The rationale for this is based on experiments by Jerina et al. (1974) and Kaubish et al. (1972) who demonstrated that arene oxides of naphthalene and 2-MN served as obligatory intermediates for the further formation of dihydrodiols and phenols. Nevertheless, the possibility

remains that a direct oxygen insertion occurs in some circumstances (Tomaszewski et al., 1975).

Microsomal epoxide hydrolase catalyzes the transformation of many chemically reactive and highly toxic epoxides to dihydrodiols which, in many instances, are devoid of these properties, but which in other instances serve as precursors of even more reactive dihydrodiol epoxides (Sims et al., 1974). The mechanism of the hydrolase reaction appears to be a nucleophilic attack by water or OH⁻ from the side of the molecule opposite to the epoxide ring (DuBois et al., 1978). Consequently, the resulting diols usually have a trans configuration (Oesch, 1973).

Doherty and Cohen (1984) have shown that 1-naphthol can be metabolized to 1,2- and 1,4-naphthoquinones by rat liver microsomes. It is known that dihydroxynaphthalenes may be readily oxidized to their respective quinones, i.e. 1,2- and 1,4-naphthoquinones (van Hegningen and Pirie, 1967). In the present investigation a significant amount of 2,6-DM-3,4-naphthoquinone was formed, which probably would arise either by further oxidation of 2,6-DM-3-naphthol or from autooxidation of 3,4-dihydro-3,4-dihydroxy-2,6-DMN.

As mentioned in Methods (2.4), all three synthetic metabolites of 2,6-DMN were identified by ultraviolet, infrared, nuclear magnetic resonance and mass spectroscopy. The metabolites isolated from the microsomal incubation of 2,6-DMN were further analyzed by chemical tests (Methods 2.4.5) to verify the identification. Therefore, on the basis of the metabolites obtained one can suggest the probable pathway(s) for their formation *in vitro*. The scheme is given in figure 4-1. These metabolites are the ring oxidized products of 2,6-DMN, which are similar to compounds obtained from metabolism of 2,6-DMN in sea urchin and rainbow trout. This indicates that rat liver microsomes also metabolized 2,6-DMN preferentially on the aromatic ring moiety of 2,6-DMN.

Formation of these metabolites in microsomes were affected by various pretreatments of rats. Formation of 3,4-dihydro-3,4-dihydroxy-2,6-DMN was

significant in microsomes obtained from livers of PB and PBCO pretreated rats. Breger et al. (1981) also observed an increase in the formation of 3,4-dihydro-3,4-dihydroxy-2-MN in microsomes obtained from livers of rats pretreated with PB, though Jerina et al. (1971) reported that the metabolism of 2-MN by rat liver microsomes leads to the production of naphthoic acids as major products and dihydrodiols as minor products. The pretreatment of rats with 3-MC or PBCO increased the formation of 2,6-DM-3-naphthol as compared to control-microsomes. Both pretreatments also increased liver microsomal aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity (Table 3-3), indicating increased attack on the aromatic rings. Induction of hepatic xenobiotic metabolism systems in mammals by agents such as PB and 3-MC has been shown to change the metabolic pathways for several compounds and apparently different forms of cyt. P-450 may generate different ratios of metabolites from the same substrate (Synder and Remmer, 1982). Purified cyt. P-448 (3-MC-type) and cyt. P-450 (PB-type) from the rat liver microsomes are both capable of metabolizing benzo[a]pyrene, but cyt. P-448 is much more active than cyt. P-450 (Yang et al., 1975). Yang et al. (1975) have also shown that the induction of cyt. P-450 usually does not result in an equal increase among the metabolites formed; the ratios of metabolites formed with 3-MC induced microsomes to those formed by control microsomes varied by a factor of ten.

Pretreatment of rats with various inducers of cyt. P-450 resulted in increased activity of microsomal enzymes of phase I and phase II metabolism (Table 3-3). There is an increase in the cyt. P-450 protein content of PB-microsomes as compared to control or other pretreated microsomes. However, this increase in microsomal enzyme activity is usually substrate specific, and can occur in the absence of an increase or decrease in the cyt. P-450 content of microsomal protein. This is due to the occurrence of several types of cyt. P-450s and to selective increases in a single isozyme which may not show a detectable increase in the total CO-binding haem-protein measurement. Under some circumstances there may be a better correlation between increase in metabolism and changes in

absorbances of binding spectra than to total cyt. P-450 levels (Synder and Remmer, 1982). It is possible that the effects of the inducers (PB, 3-MC, 2,6-DMN or PBCO) seen in the present study could have resulted from an overall increase in induction of microsomal phase I and phase II enzymes system.

A possible pathway for metabolism of 2,6-DMN by rat liver microsomes *in vitro* is shown in Figure 4-1.

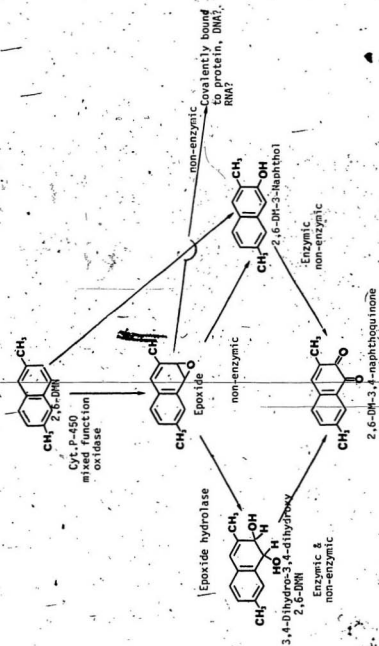
4.1.1. Michaelis Constant, K_m

The kinetic properties of membrane-bound enzymes are not well understood, and those of cyt. P-450-mediated drug metabolism are particularly difficult to interpret because of their complexity. The hepatic cyt. P-450 system is heterogeneous and the overall process leading to the formation of oxidized products involves several steps. Reaction rates for all these steps have not been determined and also it is not clearly known which step is rate limiting. The degree of substrate activation of cyt. P-450 varies with substrate and may vary with the species of cyt. P-450. Apart from all these, various other factors affect the kinetic data for cyt. P-450 mixed function oxidases (Gander and Mannering, 1982). Even under these circumstances, in most cases when the velocity of the monooxygenase reaction is plotted as a function of substrate concentration, the curve satisfies the criteria of Michaelis-Menten kinetics (Gander and Mannering, 1982).

We have measured the rate of 2,6-DMN metabolism by control or variously pretreated microsomes as a function of substrate concentration. As compared to controls, pretreatment with 2,6-DMN or PB increased both the K_m and V_{max} , where as 3-MC pretreatment led to a lower K_m and lower V_{max} . The best adapted system which maximizes V_{max}/K_m is that induced by PB. One important point to remember regarding cyt. P-450 dependent drug metabolism is that the observed V_{max} obtained with saturating concentrations of NADPH and drug substrate will be much less than the theoretical V_{max} . The number of nmol of product formed per min per nmol of cyt. P-450 should therefore not be referred to

Figure 4-1:

Metabolism of 2,6-DNH by rat liver microsomes *in vitro*



as the turnover number even when "saturating" concentrations of NADPH and substrate are used because the turnover number for an enzyme requires that the enzyme be saturated with substrate, a condition which cannot be met in a microsomal system (Gander and Mannering, 1982).

The apparent K_m for naphthalene and 2,6-DMN for liver microsomal preparations of coho salmon were reported to be $200\mu\text{M}$ and $15.3\mu\text{M}$, respectively (Schnell et al., 1980). The apparent K_m for naphthalene with rat liver microsomes was found to be $70\mu\text{M}$ (Nilsson, et al., 1976). The K_m for naphthalene is lower with liver microsomes from fish as compared to liver microsomes from rat but the maximum activity is only one-third of that of rat liver microsomes. Our data show that rat liver microsomal preparations (with or without pretreatments) were many fold more active than fish liver microsomes as assayed by Schnell et al. (1980). This may be due to a species difference for the metabolism of 2,6-DMN for hepatic mixed function oxidases.

4.2. Depletion of GSH

The present investigations have shown that 2,6-DMN decreases the concentration of GSH in livers of rats and mice. The decrease occurred as early as 6 hours and was extensive by 8 hours, suggesting a glutathione-depleting mechanism. Naphthalene (Buckpitt and Warren, 1983; Buckpitt et al., 1985) and 2-MN (Griffin et al., 1983) have also been shown to deplete hepatic GSH levels. It is generally accepted that the dose-dependent formation of reactive metabolites *in vivo* results in tissue GSH depletion by conjugation (Mitchell et al., 1976). It has been argued that a reduction in hepatic GSH concentrations below a critical value is an essential prerequisite for reactive metabolites to reach "target" molecules in sufficient concentrations to initiate cell damage. The depletion of hepatic GSH by 2,6-DMN was prevented by pretreatment of rats with cobalt-protoporphyrin IX, suggesting that GSH was depleted by cyt. P-450-generated reactive metabolites. Several reactive metabolites are produced by hepatic microsomal mixed function oxidases, including many epoxides which may react with GSH to form

glutathione-metabolite adducts (Mitchell and Jollow, 1975). The liver forms reactive metabolites of naphthalene which can conjugate with GSH to form three glutathione adducts (Smart and Buckpitt, 1983). Phenanthrene gave two GSH conjugates, and also yielded *trans*-1,2-dihydro-1,2-dihydroxy-*trans*-9,10-dihydro-9,10-dihydroxy-phenanthrenes, which are known urinary metabolites in the rat (Boylard and Wolf, 1950). The formation of epoxides which can react with GSH has also been demonstrated for other polycyclic hydrocarbons (Grover et al., 1971). The GSH conjugates are converted in separate stages to the N-acetylcysteine conjugates (mercapturic acids) which are excreted in the urine. Many xenobiotics are metabolized by the mercapturic acid pathway after conjugation with GSH. This conjugation is assumed to effect the detoxification of the xenobiotic upon excretion of the GSH-conjugate from the cell in which it was formed (Meister, 1983).

Apart from conjugation, a variety of compounds can undergo one electron reduction at the expense of cellular reducing equivalents to yield radicals. These free radicals rapidly autoxidize to generate the original compound in a process called redox cycling. Redox cycling leads to the formation of superoxide anion radicals, and its further degradation products such as H_2O_2 , hydroxyl radicals or singlet molecular oxygen which may be responsible for toxic and / or therapeutic effects (Kappus and Sies, 1981). Likely organic precursors for radical formation include quinones, nitroaromatic compounds, carbon tetrachloride etc. (Bridges et al., 1983).

We have not investigated the formation of the identified three metabolites of 2,6-DMN, *in vivo*. Extrapolating from the information obtained from *in vitro* metabolic studies and *in vivo* depletion of GSH, we speculate on the formation of GSH conjugates or a redox cycle reaction, perhaps involving quinones and hydroxy metabolites. When various doses of 2,6-DMN was intubated orally to rats the levels of GSH decreased rapidly in a dose dependent manner. About 70% of hepatic GSH was depleted by a dose of 120mg/kg body wt. At a dose of

500mg/kg body wt. GSH was further depleted to nearly 20% of control levels. It is well known that hepatic GSH levels are decreased by the administration of acetaminophen and there exists a good correlation between covalent binding and hepatic necrosis on the one hand and GSH depletion on the other (Mitchell and Jollow, 1975).

Fasting causes the liver GSH content to decrease to about 50% of fed levels in rats (Jaeger et al., 1974). Fasting also enhances the hepatotoxicity of several chemicals particularly those that are capable of depleting GSH (Reed and Fariss, 1984). Our experiments on *in vivo* depletion of GSH were performed on overnight fasted animals. In agreement with the results of others, we also found that hepatic GSH levels of these animals were about half of those in the fed animals. On administration of 2,6-DMN (by oral intubation) to both groups of rats, the percentage of GSH depletion was the same (Figure 3-14), indicating that fasting had not aggravated the effects of 2,6-DMN with respect to hepatic GSH levels. Short term depletion of GSH appears to be less harmful as GSH can be rapidly replenished (Thor et al., 1979) either by *de novo* synthesis from amino acids or by reduction of GSSG by glutathione reductase (Kosower and Kosower, 1976). The latter has been shown in the case of the anticancer drug adriamycin which is known to lower GSH levels. Protection against toxicity is observed even at low GSH levels (25% of controls) provided that glutathione reductase activity was not impaired (Bridges et al., 1983).

Oral administration of 2,6-DMN (500mg/kg body wt.) to rats pretreated with PB or 2,6-DMN did not significantly alter the time course of hepatic GSH levels. However, in the case of 3-MC pretreated rats there was a substantial reduction in GSH levels (nearly 80% of control) by 4 hours and the depletion continued till levels reached 15% of control by 8 hours. Since cyt. P-450-dependent metabolism is necessary for the expression of these biological effects, it is likely that the cyt. P-448 induced by 3-MC pretreatment results in the generation of a larger amount of the reactive metabolite(s) which can conjugate with GSH resulting in its

depletion. 3-MC also renders hamsters more susceptible to acetaminophen-induced liver cell injury than PB (Potter et al., 1974). Moreover, pretreatment of hamsters with 3-MC increased the depletion of hepatic GSH, the covalent binding, and severity of necrosis (Potter et al., 1974). In case of bromobenzene, cyt. P-448 (3-MC type) produces predominantly the 2,3-epoxide, as shown by the formation of 2-bromophenol, while cyt. P-450 (PB-type) leads primarily to an increase in the formation of 3,4-epoxide. Pretreatment with 3-MC did not sensitize rats to bromobenzene-induced hepatic necrosis. This is because formation of bromobenzene 3,4-epoxide leads to tissue necrosis while formation of 2,3-epoxide does not (Farber and Gerson, 1984). Therefore, it is also possible that metabolites of 2,6-DMN produced with 3-MC pretreatment effect the turnover of GSH. This might also contribute towards a more significant depletion of GSH. The lag observed before depletion when PB and 2,6-DMN have been used as inducers, suggests that other systems (e.g. glucuronidation) may initially be more important in preventing the depletion of GSH.

As mentioned earlier, the cyt. P-450 containing mixed function oxidases are required for the metabolism of 2,6-DMN to various ring-oxidized products *in vitro*. As a result, the reactive metabolites produced will covalently bind to cellular macromolecules. Much of the evidence implicating the covalent binding of reactive metabolites to subsequent cell death is based on studies of bromobenzene and acetaminophen-induced liver necrosis in rodents (Farber and Gerson, 1984). The extent of covalent binding of [^{14}C]bromobenzene to the total cellular proteins in primary cultures of hepatocytes was proportional to the number of dead cells after 18 to 20 hours, but the time course of the accumulation of covalently bound metabolites did not correlate with the course of the cell death (Casini et al., 1982). Also, bromobenzene-induced liver necrosis is predominantly centrilobular as is the covalent binding of [^{14}C]bromobenzene (Farber and Gerson, 1984). Griffin et al. (1983) administered the radio labelled pulmonary toxin, 2-MN to mice and examined the extent of covalent binding to proteins in a variety of tissues. They did not find a correlation between covalent binding and pulmonary toxicity.

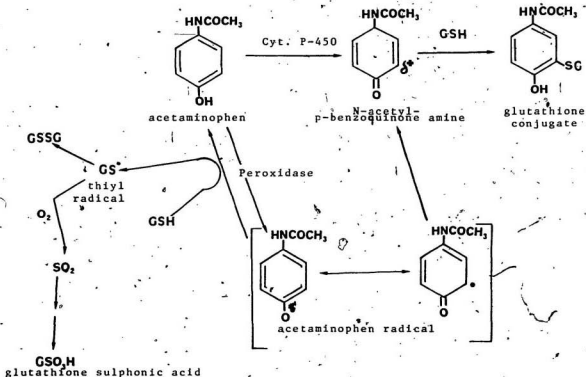
However, covalent binding is frequently used as an endpoint in studies on the metabolism of hepatotoxins to either explain the mechanism of action of a known hepatotoxin or to predict the biological activity of a suspected one. Hence, the covalent binding hypothesis remains a dominant organizing principle in molecular toxicology (Farber and Gerson, 1984).

When liver microsomes from various pretreated rats were incubated with 2,6-DMN, significant binding of 2,6-DMN metabolites to microsomal protein was observed as compared to control microsomes. These observations suggest that the chemically reactive metabolites of 2,6-DMN may either react with GSH or may covalently bind to microsomal proteins. If this is the case, then the protective effect of GSH should decrease the amount of metabolites of 2,6-DMN available for binding to protein. To test this hypothesis, liver microsomes from 3-MC pretreated rats were chosen because (i) significant covalent binding was observed with this microsome type and (ii) 2,6-DMN caused a dramatic drop in hepatic levels of GSH of 3-MC pretreated rats. The addition of various concentrations of GSH (ranging from $50\mu\text{M}$ - 5mM) to the incubation mixture markedly decreased the covalent binding to microsomal protein in a dose dependent manner (Figure 3-15). The binding of metabolites to GSH can occur nonenzymatically as well as enzymatically in the presence of GSH-S-transferases (Chasseaud, 1976). Since the major portion of activity of GSH-S-transferases resides in the cytosolic fraction of rat liver (Mannervik et al., 1983). We added dialyzed liver cytosol to the incubation mixture to see if it would further decrease the covalent binding of metabolites to proteins. This was found to be the case. This decrease was observed at all GSH concentrations but was more significant at high GSH concentrations. In the presence of low concentration of GSH, a lesser fraction of the formed metabolites reacted with GSH, whereas a greater fraction covalently bound to protein. It is suggested that the reactive metabolites produced by the microsomal mixed function oxidases reacted with GSH either directly or via a reaction catalyzed by GSH S-transferases to yield glutathione conjugates.

These observations for GSH binding *in vitro* implicate that a similar protective mechanism could be operating *in vivo*. The rapid depletion of liver GSH levels in rats indicates that conjugation of reactive metabolites of 2,6-DMN with GSH probably predominates. For acetaminophen, a large body of data exists that supports the hypothesis that acetaminophen initiates hepatocellular necrosis in laboratory animals and in man by its conversion into a highly electrophilic metabolite, N-acetyl-p-benzoquinoneimine, which arylates liver macromolecules. Glutathione is thought to protect susceptible cyt. P-450-containing target cells by preferentially adding to or reducing the quinoneimine metabolite (Smith et al., 1983). Figure 4-2 shows the interactions of GSH with reactive intermediates formed from acetaminophen as summarized by Moldeus and Jernstrom (1984). At least two types of metabolites, epoxides and quinones, can react with GSH accounting for its depletion from hepatic tissues. Glutathione S-transferases are capable of protecting against potential adverse effects of epoxides. Several substrates of GSH S-transferases have been identified which include naphthalene-1,2-oxide, 1-methylnaphthalene-1,2-oxide, 2-methylnaphthalene-1,2-oxide, and also various epoxides of phenanthrene, dimethylbenzanthracene and benzo[a]pyrene (Jerina, 1976). Naphthalene-1,2-oxide was found to be the best substrate for GSH S-transferases among the polycyclic aromatic hydrocarbons, but substitution of a methyl group at either the 1- or 2- carbon atom of the oxirane in the naphthalene oxide led to decreased activity (Jerina, 1976). Naphthalene is metabolized to not only the 1,2-oxide but to diepoxide and diolepoxide metabolites (Stillwell, et al., 1982) as well, and these metabolites are likely to conjugate with GSH also. Studies have shown that *in vivo* GSH depletion and covalent binding of reactive naphthalene metabolites are interrelated events (Warren et al., 1982) and that GSH conjugates are formed from naphthalene (Jeffrey, et al., 1975; Jerina, 1976). Smart and Buckpitt (1983) have shown that at least three GSH adducts are formed during the metabolism of naphthalene by mouse liver microsomes in the presence of GSH and cytosolic GSH S-transferases, demonstrating that nucleophilic attack by GSH can occur on the electrophilic carbons of naphthalenes oxides.

Figure 4-2:

Glutathione in Detoxification



Interactions of glutathione (GSH) with reactive intermediates formed from acetaminophen (Moldeus and Jernstrom, 1984).

As shown in the present study, the administration of 2,6-DMN to rats and mice significantly decreased hepatic levels of GSH. *In vitro* covalent binding of 2,6-DMN to liver microsomal protein was also prevented by GSH. These observations are consistent with previous studies. Naphthalene is three times as toxic as 1- or 2-methylnaphthalene (Rashestyuk, 1970), indicating that substitution of methyl groups for hydrogens on naphthalene may lead to a decrease in toxicity. However, our studies suggest that this may not be the case, at least not for 2,6-DMN.

Our *in vitro* studies have shown that 2,6-DM-3,4-naphthoquinone is produced in significant amounts by hepatic microsomes from control as well as from pretreated rats. If this is the case *in vivo* as well then the 2,6-DM-3,4-naphthoquinone produced could deplete the GSH as quinones are very reactive towards GSH. However, mercapturic acid excretion has not been detected *in vivo* after administration of quinones to animals (Bray and Garret, 1981). The latter finding does not preclude the possibility that conjugation with GSH occurs as a means of detoxifying and eliminating reactive quinones in the bile as GSH conjugates (Chasseaud, 1976). In a study of the metabolism of [¹⁴C]menadione (vitamin K₃) in perfused rat liver it was shown that about one-third of the biliary radioactivity was associated with ninhydrin-positive compounds, most probably resulting from glutathione conjugates. The major biliary product was the β -glucuronide, amounting to 57%. As the UDP-glucuronyl transferase requires a phenolic group, it is clear that the reduction step is obligatory and precedes the glucuronidation step (Losito et al., 1967). In the cell, quinones are more likely to (i) undergo redox cycling to give GSSG and (ii) be reduced to hydroquinones which could either be conjugated with UDP-glucuronic acid or GSH. Hence, a useful biological function of conjugate formation is, of course, the ease of elimination of the quinone moiety from the hepatocyte. Demonstration of redox cycling by the menadione-GSH conjugate, thiodione, revealed that quinone thioether formation as such does not necessarily mean a detoxification reaction, but only a facilitation of elimination (Wefers and Sies, 1983). In general, GSH-conjugation is assumed to effect the detoxification, usually via the mercapturic

acid pathway. Some recent evidence suggests that in certain cases this process provides intermediate metabolites that are substrates for mutagen / toxin-forming reactions in particular, reactive thiols (Bakke and Gustafssen, 1984). These thiols are formed upon cleavage of the cysteine conjugates by cysteine conjugate 7-lyase (Bakke and Gustafssen, 1984).

4.3. Formation of MetHb, GSSG, and Depletion of GSH in Human Erythrocytes

Naphthalene has been known to induce methaemoglobin formation both in experimental animals and in man (Zuelzer and Apt, 1949). We examined the ability of 2,6-DMN to induce methHb formation in human erythrocytes *in vitro*. Under normal physiological conditions, any methHb formed in erythrocytes due to normal oxidative processes is reduced back to Hb by NADH-methHb reductase. If the rate at which Hb is converted to methHb sharply increases, this may overwhelm the capacity of red cells to reduce methHb. Toxic methaemoglobinaemia results from the action of oxidizing chemicals and drugs, which accelerate the oxidation of Hb (Bauer, 1982).

Our investigations have shown that 2,6-DMN in the presence of liver microsomes produces methHb in human erythrocytes. MethHb formation was further elevated when liver microsomes from 3-MC pretreated rats were used. The data indicate that induction of hepatic microsomal mixed function oxidases by 3-MC produces considerably more toxic metabolite(s). As shown in the *in vitro* metabolism of 2,6-DMN (Table 3-2), 3-MC microsomes formed significantly higher levels of 2,6-DM-3-naphthol as compared to control-microsomes. The increase in methHb formation was accompanied by a depletion of GSH (Figure 3-17) in erythrocytes incubated in the presence of fortified rat liver microsomes (control or 3-MC). Probably GSH is essential for maintaining protein sulphhydryl groups in the reduced state, thereby protecting the haemoglobin. Oxidation or depletion of erythrocyte GSH by metabolites of 2,6-DMN, such as epoxides, naphthol or quinone, could result in the modification of cellular macromolecules.

Since metabolic activation is necessary for metHb formation, we examined the ability of the three metabolites 2,6-DM-3-naphthol, 3,4-dihydro-3,4-dihydroxy-2,6-DMN, and 2,6-DM-3,4-naphthoquinone to induce metHb formation in erythrocytes.

4.3.1. Studies with 2,6-DM-3-naphthol

When 2,6-DM-3-naphthol was incubated in the presence of a NADPH-regenerating system, microsomes (control or 3-MC) and erythrocytes, metHb formation was further increased by 75% to that caused by the parent molecule 2,6-DMN. GSH depletion was also increased. It is known that naphthols can be further metabolized by microsomal enzymes to bind irreversibly to protein (Hesse and Mezger, 1979). According to Doherty and Cohen (1984) 1-naphthol is metabolized by liver microsomal mixed function oxidases to 1,4-naphthoquinone, and an unknown product which may be the glutathione conjugate of naphthoquinone. In addition, covalent binding of 1-naphthol to microsomal protein was observed. According to Doherty and Cohen, (1984) the covalently bound species is derived primarily from 1,4-naphthoquinone, most likely via 1,4-naphthosemiquinone, and not from 1,2-naphthoquinone. Previously, Doherty et al. (1984) have shown that the depletion of GSH preceded the onset of cytotoxicity to freshly isolated hepatocytes obtained from PB pretreated rats. Contrary to these observations, Buckpitt et al. (1985) showed in *in vivo* studies that i.p. administration of 1-naphthol to mice did not significantly change hepatic or renal GSH levels, nor did it cause detectable tissue injury. According to them the apparent discrepancy in the *in vivo* vs *in vitro* response to 1-naphthol may be related to a) the different animal species used b) the fact that hepatocytes were isolated from PB-induced rats c) the fact that hepatocytes may not always mimic the *in vivo* situation. Our observations clearly demonstrate that 2,6-DM-3-naphthol is metabolized by a cyt. P-450-dependent reaction to reactive metabolites that can oxidize Hb. Exclusion of the NADPH-regenerating system from the incubation mixture resulted in no metHb formation in human erythrocytes. The data support the findings of Doherty and Cohen, (1984) that

naphthol is further metabolized either from untreated or 3-MC pretreated animals. Fluck et al. (1984) have further confirmed the findings of Doherty and Cohen, (1984) by reporting that 1-naphthol is converted to cytotoxic naphthoquinone metabolites by a mechanism(s) dependent upon the generation of free radicals in rat liver microsomes.

4.3.2. Studies with 3,4-dihydro-3,4-dihydroxy-2,6-DMN

Metabolism of dihydrodiol is slow in fat liver due to lack of microsomal dihydrodiol dehydrogenase (Ayenger et al., 1959). When 3,4-dihydro-3,4-dihydroxy-2,6-DMN was incubated with erythrocytes, a NADPH-regenerating system and control or 3-MC microsomes, the formation of methHb and depletion of GSH was less than that produced by 2,6-DM-3-naphthol but comparable to that produced by the parent molecule, 2,6-DMN. However, the corresponding blanks (without NADPH-regenerating system) had similar effects, suggesting that auto-oxidation of the dihydrodiol, possibly to the quinone, may be responsible for these effects since no metabolism is expected in the absence of a NADPH-regenerating system. However, the presence of a NADPH-regenerating system showed only slightly more toxic effects than the corresponding blanks, particularly in case of 3-MC microsomes. It is known that diols may again serve as substrates for the monooxygenases resulting in the formation of diol epoxides (Sims et al. 1974). One can assume that this may be the case, as epoxides would cause further depletion of GSH and methHb formation.

4.3.3. Studies with 2,6-DM-3,4-naphthoquinone

2,6-DM-3,4-naphthoquinone is one of the major metabolites in the *in vitro* microsomal metabolism of 2,6-DMN. When this quinone was incubated with erythrocytes either in the presence of control- or 3-MC- microsomes, with or without a NADPH-regenerating system, there was a large increase in methHb formation and a substantial decrease in GSH levels. As discussed earlier, quinones are known to be potent depleters of GSH and to bind covalently to

macromolecules. It is also known that menadione in the presence of oxyhaemoglobin will accelerate the formation of methHb while forming menadione semiquinone and this results in the generation of superoxide anions. The superoxide anion appears to be the source of hydrogen peroxide which accounts for most of the observed methHb formed (Goldberg and Stern, 1976). According to Lilienblum et al. (1985) the toxicity of quinones is thought to be mediated by redox cycling between quinones and hydroquinones with the intermediate formation of semiquinone radicals.

Considering these findings one would expect an increase in GSSG formation concomitant with GSH depletion. Our investigations showed that there was a significant increase in the formation of GSSG, as well as depletion of GSH and methHb formation in erythrocytes. However, the increase in GSSG formation was not sufficient to account for the large loss of GSH in erythrocytes. The case is similar to that of acetaminophen where no GSSG or lipid peroxides are observed to be formed during acetaminophen activation in liver. This does not necessarily exclude radical formation since most of the GSSG formed via a radical process would be reduced back to GSH via the very active GSSG reductase. In our studies the rapid depletion of GSH may be partly due to the fact that quinones could form conjugates with GSH. Nickerson et al. (1963) proposed an *in vitro* mechanism according to which formation of menadione-GSH is accompanied by reduction of menadione to menadiol. The latter can oxidize and give rise to excited oxygen species. This was further confirmed by Wefer and Sies (1983) who concluded that the conjugation of quinones with GSH may not in itself be protective since it does not abolish semiquinone formation. Therefore, it seems probable that GSH depletion may be largely due to conjugation followed by redox cycling which explains the small increase in GSSG formation. There are more Hb molecules (5mM) than glutathione molecules (3mM) and GSSG is roughly 1% of the concentration of GSH (Kosower and Kosower, 1976). Under normal physiological conditions, small amounts of GSSG can be rapidly reduced by glutathione reductase. However, if the rate of GSSG formation substantially

increases then reduction may not be able to keep pace due to the rate limiting step of NADPH regeneration (Kosower and Kosower, 1976). In experiments when only the NADPH-regenerating system, microsomes and erythrocytes were present, there was a 100% increase in GSH levels as compared to the GSH levels in erythrocyte-microsome incubation mixtures without the NADPH-regenerating system. It is possible that the presence of a NADPH-regenerating system in the incubation mixture would assist in the conversion of some GSSG back to GSH, which may in turn either be conjugated or be reoxidized to GSSG. Since we have neither investigated the formation of free radicals nor isolated the glutathione conjugate, it cannot be said with certainty which of the two are the major routes of GSH depletion.

Chapter 5

Conclusions

1. 2,6-DMN is metabolized *in vitro* by rat liver microsomal mixed function oxygenases to a variety of metabolites, including 2,6-DM-3-naphthol, 2,6-DM-3,4-naphthoquinone, and 3,4-dihydro-3,4-dihydroxy-2,6-DMN.

2. Formation of ring oxidized metabolites of 2,6-DMN suggests the production of a reactive metabolite(s), probably an epoxide.

3. Depletion of hepatic GSH in rodents and in human erythrocytes, covalent binding to liver microsomal proteins, and metHb formation in erythrocytes indicate the possible toxicity of 2,6-DMN.

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