METABOLIC ACTIVATION IN THE RAT, DOG, GUINEA PIG AND HUMAN OF THE SUSPECT CARCINOGEN 4,4'-METHYLENEBIS(2-CHLOROANILINE)

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

1988

ABSTRACT

METABOLIC ACTIVATION IN THE RAT, DOG, GUINEA PIG AND HUMAN OF THE SUSPECT CARCINOGEN 4,4'-METHYLENEBIS(2-CHLOROANILINE)

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4,4'-Methylenebis(2-chloroaniline) (MBOCA), similar to other arylamines, is carcinogenic in a variety of tissues of several species. The mechanism of MBOCA oxidation and the reactivity of metabolites was investigated. Metabolite structure was confirmed by synthesis, mass spectrometry and NMR. A microsomal enzyme system was used to study Phase I metabolism of MBOCA by dog, guinea pig, rat and human liver. N-, and o-Hydroxylations of MBOCA increased with incubation time, microsomal protein, substrate, and NADPH concentration and were inhibited by 2,4-dichloro-6-phenylphenoxyethylamine and carbon monoxide.

The direct mutagenicities of MBOCA and all oxidized derivatives except the hydroxylamine were negative in a Salmonella/mutagenicity assay employing the strains TA98 and TA100. This study is the first to show that N-OH-MBOCA is a potent mutagen. The effect of these oxidized metabolites on gap junctional communication of WB-F344 cells was also determined. The parent compound was the only one that inhibited communication, which may reflect tumor promotion potential.

The capacity of N-oxidized metabolites of MBOCA to form hemoglobin (Hb) adducts was determined in vitro, and the

formation of Hb adducts following *in vivo* administration of MBOCA and N-OH-MBOCA was assessed. Hb adduct formation was determined by acid hydrolysis and measurement of free MBOCA. Administration of 0.04 μmol/kg N-OH-MBOCA iv to rats resulted in measurable formation of MBOCA-Hb adducts (3.4 pmol/50 mg Hb). Administration of 1.9-190 μmol/kg MBOCA ip to rats and of 19-1,900 μmol/kg sc to rats and 15-380 μmol/kg sc to guinea pigs resulted in dose-related formation of Hb adducts. Following pretreatment of rats with phenobarbital (PB) or ß-naphthoflavone (ß-NF), MBOCA-Hb levels were increased in the ß-NF group. The *in vitro* metabolism of MBOCA to N-OH-MBOCA was also elevated by ß-NF treatment.

These studies provide evidence that several species, including man, are capable of oxidizing MBOCA to N-OH-MBOCA, a potential proximate carcinogen. In addition, it was found that MBOCA-Hb adducts may be useful for monitoring the production of the N-OH-MBOCA metabolite *in vivo*.

ACKNOWLEDGMENTS

This thesis would not have been possible without the support of many. I would first like to express my thanks to the members of my advisory committee, especially to my mentor Dr. Emmett Braselton for his guidance and encouragement.

I am most grateful to many who provided technical knowledge and assistance. Dr. James Trosko was very generous with his resources and encouragement with the gap junctional communication assay. Dr. J.E. Sinsheimer at the University of Michigan taught me the Salmonella mutagenicity assay. Dr. Klaus Halenga and Long Lee of the MSU NMR facility produced the nuclear magnetic resonance spectra and helped with the interpretation. John Spitsbergen, Ivy Su and Mark Shieh were all invaluable in carrying out many of the experiments.

Special thanks go to Dr. T-H. Chen who was always around to offer advice and support and was in fact responsible for the direction that much of the research was to take.

To Sunjay Gupta, who was so supportive during the time it was needed most.

Last, and most important, I want to express my greatest appreciation to my wife Molly for her love and understanding during these years that must have seemed so very uncertain.

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ACN	acetonitrile
2-AAF	2-acetylaminofluorene
2-AF	2-aminofluorene
4-AB	4-aminobiphenyl
ACS	aqueous counting solution
[B-^{14}C]MBOCA	4,4′-[^{14}C]methylenebis(2-chloroaniline) (bridge labeled)
BSA	bovine serum albumin
BZ	benzidine
CPBA	3-chloroperoxybenzoic acid
DCB	3,3′-dichlorobenzidine
DCNP	2,6-dichloro-4-nitrophenol
diNO-MBOCA	1,1′-methylenebis[3-chloro-4-nitrosobenzene]
DPEA	2,4-dichloro-6-phenylphenoxyethylamine
DPM	disintegrations per minute
EI-MS	electron-impact mass spectrometry
GJIC	gap junctional intercellular communication
GSH	glutathione
GSSG	reduced glutathione
Hb	hemoglobin
HFBA	heptafluorobutyric anhydride
ip	intraperitoneal
iv	intravenous
LDH	lactate dehydrogenase
LSS	liquid scintillation spectrometry
MBOCA	4,4′-methylenebis(2-chloroaniline)
MFO	mixed-function oxidase
ß-NF	ß-naphthoflavone
2-NA	2-naphthylamine
NMR	nuclear magnetic resonance
NO-MBOCA	2-chloro-4-[(3-chloro-4-nitrosophenyl) methyl]benzenamine
NH_4Ac	ammonium acetate
N-OH-MBOCA	4[(4-amino-3-chlorophenyl)methyl]-2-chloro-N-hydroxybenzenamine
Q-OH-MBOCA	2-amino-5-[(4-amino-3-chlorophenyl)methyl)]-3-chlorophenol
Q-OH-MBOCA -sulfate	5-hydroxy-3,3′-dichloro-4,4′-diamino-diphenylmethane-5-sulfate
PB	phenobarbital
RCM	radial compression module
sc	subcutaneous
TCA	trichloroacetic acid
[U-^{14}C]MBOCA	4,4′-methylenebis(2-chloro[U^{14}C]aniline) (uniformly ring labeled)

INTRODUCTION TO THESIS

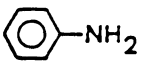
INTRODUCTION

Background

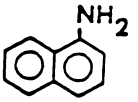
Environmental factors have been suggested to be the cause of a great portion of cancer in man (Searle, 1976; Miller, 1978). However, relatively few such factors have been identified conclusively as causative agents. The concept that environmental factors are involved in causing cancer is first attributed to Sir Percival Pott (1775) who associated occupational exposure to soot with the high incidence of scrotal cancer among chimney sweeps in London. Perhaps the first such realization was fourteen years earlier when John Hill reported the danger of using tobacco products (Redmond, 1970). He warned the public of the occurrence of cancer of the nasal passages in men who used snuff.

Aromatic amines are one class of compounds that are generally believed to be carcinogenic in humans. Exposure to aromatic amines has been associated with the development of urothelial cancer. The foundation for the study of aromatic amine induced carcinogenesis was established during the 1860's with the emergence of the commercial German synthetic dyestuff industry. A correlation between exposure to aromatic amines and human cancer was first reported by

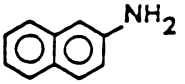
the German surgeon, Ludwig Rehn after noting that 4 of 45 industrial dye workers engaged in the manufacture of fuchsin from crude aromatic amines had contracted urinary bladder cancer (Rehn, 1895). Outbreaks of occupational bladder cancer were associated with high exposure and could readily be detected by clinical observation. The manufacture of these dyes was dependent largely on aniline, naphthylamine, benzidine and azobenzene. Rehn labeled these clinical observations, "aniline cancer". Leichtenstern (1898) later suggested that "naphthylamines" were a more likely cause of these outbreaks. Aniline in fact has been shown not to be carcinogenic (Case et al., 1954). By the late 1940's, a large body of epidemiological data, taken primarily from the european dye industry, established that long term exposure to certain aromatic amines constituted a serious bladder cancer hazard (Parkes and Evans, 1984). The compounds implicated at this time included 2-naphthylamine, 1-naphthylamine, and benzidine. The structures of these compounds are presented in Figure 1. The carcinogenicity of these amines was further established by the very comprehensive epidemiological studies of R.A.M. Case and colleagues (Case et al., 1954). By studying the records kept on over 4600 British factory workers, Case defined the risk of contracting bladder cancer as over 30 times as great for exposed chemical workers as for the general population. The relative potencies for suspected carcinogens was also



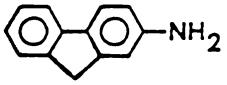
Aniline



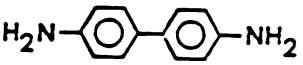
1-naphthylamine
(1-NA)



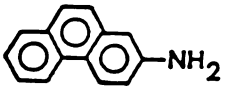
2-naphthylamine
(2-NA)



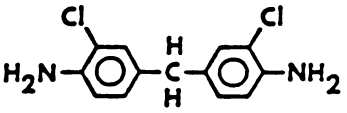
2-aminofluorene
(2-AF)



benzidine
(BZ)



2-aminophenanthrene



4,4'-methylenebis(2-chloroaniline)
(MBOCA)

Figure 1 Structures of selected primary aromatic amines.

established, with 2-naphthylamine representing the greatest risk, followed by benzidine and 1-naphthylamine. Aniline was found not to represent a significant hazard. The carcinogenicity ascribed to 1-naphthylamine was later attributed to its contamination by 3-6% 2-naphthylamine (Purchase et al., 1981). Epidemiological studies of other arylamines showed that, in two plants in the United States that manufactured 4-aminobiphenyl, 16.1% and 18.5% of the workforce developed bladder cancer (Melick et al., 1971). Goldwater et al. (1965) showed that 26.2% of a workforce of 366 male employees developed bladder cancer after exposure to 2-naphthylamine and benzidine in a coal tar dye factory. More recent estimates suggest that between 8 and 20% of bladder cancers can be attributed to occupational exposure (Cole, 1973; Cartwright, 1982; Vineis and Simonato, 1986).

Case et al. (1954) showed that the mean latent period for bladder tumors in these high risk industries was 18 years from first exposure. Parkes and Evans (1984) reported that the mean latent period for tumors in the rubber and chemical industry was 25 years. After such a long interval of time it becomes increasingly difficult to establish the nature of the exposures that did occur, as the factory may have been closed and records destroyed. Present epidemiological studies are made even more difficult as worker exposures may be controlled to very low levels, and new, less potent, carcinogenic arylamines are being used

(Wallace, 1988).

Epidemiological studies were given support by work on aromatic amine induced cancer in animals. The first tumors induced in animals were reported by Japanese workers (Yoshida, 1933; Sasaki and Yoshida, 1935; and Kinosita, 1936). They established the basis for experimental chemical carcinogenesis by demonstrating the hepatic carcinogenicity of 3,2'-dimethyl-4-aminoazobenzene administered subcutaneously to rats. Two years later, Hueper et al. (1938) reported that 2-naphthylamine (2-NA) induced bladder tumors in 13 of 16 dogs. This provided the first successful animal model for human bladder cancer and supported the explanation of excess bladder cancer in dye workers as first described by Rehn. Shortly thereafter, data were published on the induction of tumors in various rodent tissues by 2-acetylaminofluorene (2-AAF) (Wilson et al., 1941), 2-aminostilbene (Haddow et al., 1948), benzidine (BZ) (Spitz et al., 1956), and N,N-dimethyl-4-aminobiphenyl (Miller et al., 1949). Historically, fluorene derivatives have been among the most intensely studied animal carcinogens of the last forty years, and have served as models for investigations into the mechanisms of chemical carcinogenesis (Miller, 1978).

Arylamines, which are byproducts of the coal tar and gas industries, provide starting material for the manufacture of dyes used in leather, paper, textiles and

paints. They have been used as antioxidants and as polymerizing agents in the rubber and cable industries (Tola, 1980). Benzidine, for example, can be used to synthesize many different dyes. It is used in detection of blood, as a plastics and rubber hardener, and as a starting material in the chemical synthesis of many compounds (IARC, 1982). Aromatic amines have the additional toxicological property of causing methemoglobinemia which results from the oxidation of hemoglobin. If severe, it can result in central nervous system depression. Human absorption is usually through inhalation of the vapor or skin exposure.

Largely because of the work of Case, the British Cancer Substances Regulations of 1967 banned the manufacture of the principal aromatic amines, listing them as either prohibited or controlled. In 1974, 13 chemicals (9 aromatic amines) were identified and subsequently regulated in the U.S. (U.S. Dept. of Labor, 1974).

These regulations have not removed the danger of arylamine-induced bladder cancer from the population. Dozens of occupations (Cartwright, 1983) still involve exposure to hundreds of dye derivatives (Fishbein, 1981). Occupations where epidemiological studies show an increased risk of bladder cancer and where arylamines may play a role include chemical manufacture, leather work, printing and hairdressing. The process of recycling rubber for example, releases 2-naphthylamine exposing the workers involved.

Cerniglia and coworkers (1982) found that the dye C.I. Direct Black 38, used in the leather industry, was metabolically reduced by human intestinal bacteria to benzidine (U.S. Dept. Labor, 1974). The general population is also exposed directly to primary aromatic amines as environmental contaminants. Patriankos and Hoffmann (1979) have identified at least 14 amines, including 2-naphthylamine and 4-aminobiphenyl, in the mainstream and sidestream smoke of cigarettes. The importance of this exposure may be related to the increased risk of bladder cancer in smokers (Mommsen et al., 1983). Haugen et al. (1981) have isolated several primary aromatic amines from synthetic fuels, including 4-aminobiphenyl, aminonaphthylenes and aminofluorenes.

Based on the long latency of development, bladder cancers observed today are the result of exposures during the last 25 years. The result of present exposure to arylamines in the general population will in all likelihood take another 25 years to assess.

Metabolic Activation of Aromatic Amines

It was noted from animal studies that arylamines frequently produce tumors at sites distant from where administered, and that these tumors were species and organ specific. These observations led to the concept that aromatic amines require metabolic activation to exhibit their toxic effects (reviewed in Garner *et al.*, 1984). The concept of metabolic activation of chemical carcinogens can be traced to studies by the Millers and collaborators, who showed that the carcinogen *N*,*N*-dimethyl-4-aminoazobenzene formed covalent adducts with the protein of rat liver. It was suggested that the metabolism of this compound yielded a chemically reactive form which bound to protein (Miller and Miller, 1947). Other researchers suggested that metabolism to the *o*-hydroxylamine might be important in aromatic amine carcinogenesis (Clayson, 1953).

The degree of activation has been shown to vary for a compound and so the concept of "pro-", "proximate" and "ultimate" carcinogen was introduced. The term "pro-" refers to the parent or unmetabolized compound, "proximate" as a more reactive metabolite, and "ultimate" as the final reactive form that binds to nucleic acids and protein. The first demonstration of activation *in vivo* of an aromatic

amine to a "proximate" carcinogenic metabolite occurred when N-hydroxy-2-AAF was isolated from the urine of rats fed 2-AAF (Cramer et al., 1960). This was followed by the demonstration that this metabolite was more carcinogenic than the parent amine (Miller et al., 1961a; Miller et al., 1964). Subsequent studies with a variety of aromatic amines, including, 4-aminobiphenyl, benzidine, and 2-aminofluorene have demonstrated the importance of N-hydroxylation in the metabolic activation of these compounds (Kriek, 1974; Miller, 1978).

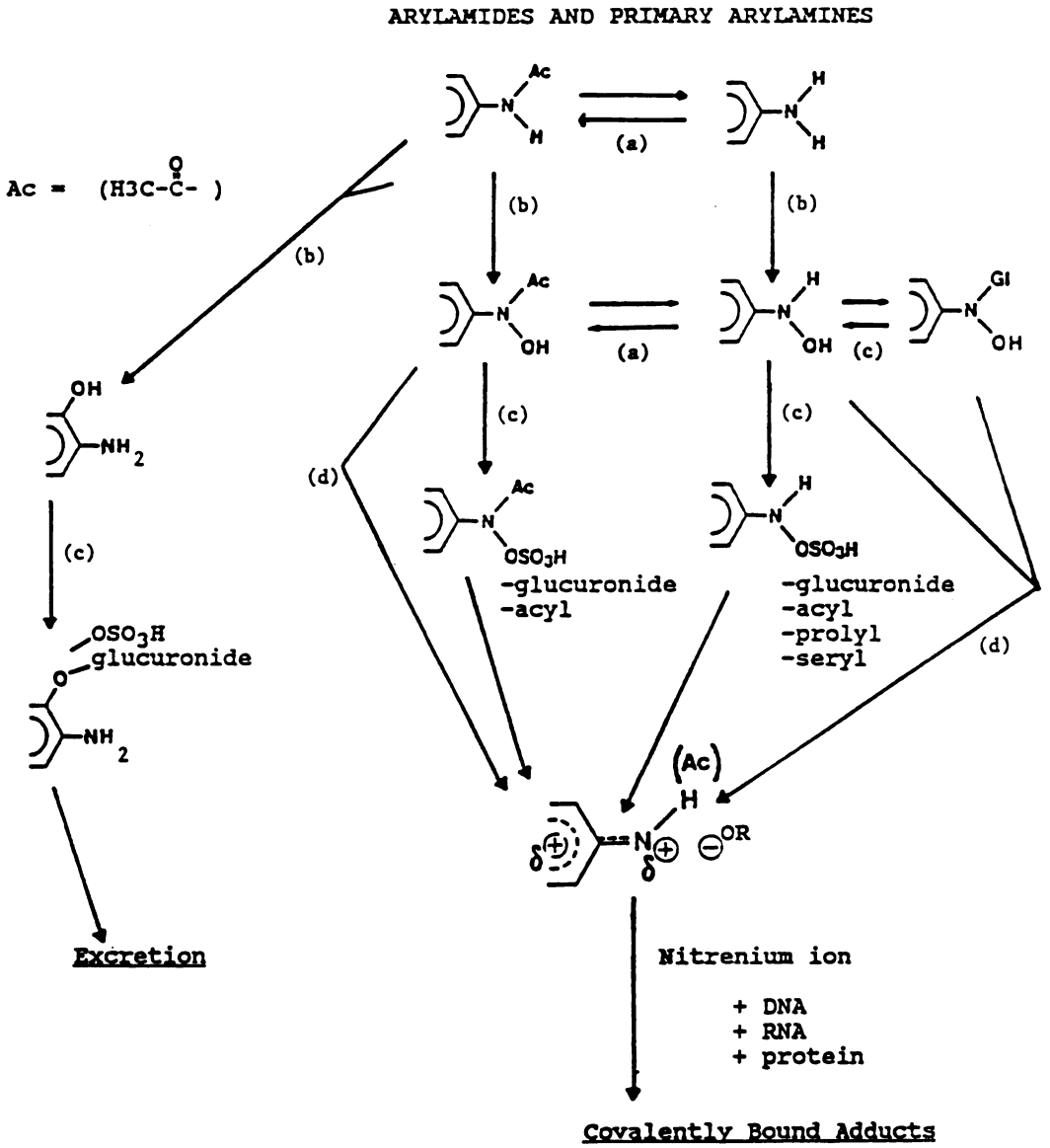
It was expected that the "ultimate" forms of chemical carcinogens must react with cellular macromolecules to give derivatized nucleic acids or proteins. The search for electrophilic derivatives of the N-hydroxy intermediates therefore ensued. Kriek (1965) first demonstrated that the acid-catalyzed protonation of N-hydroxy-2-AF (followed by hydrolysis to yield the nitrenium ion) led to reaction with the guanine residues of nucleic acids. Soon after, the benzoyloxy ester of N-hydroxymethylaminoazobenzene was shown to be an extremely potent carcinogen at the site of injection (Poirier et al., 1967). This derivative was further shown to react covalently with macromolecules in vitro, to yield the same derivatives found after in vivo administration of N-methylaminoazobenzene (Miller and Miller, 1969). These experiments provided the first indication that activation of aromatic amines to esters

could result in ultimate carcinogens. The discovery of other activation pathways soon followed.

By 1972, five enzymatic systems for the conversion of the model carcinogen N-hydroxy-2-AAF to electrophilic reactants had been discovered in the rat liver (Miller and Miller, 1983). The first of these is microsomal deacetylation to yield N-hydroxy-2-AF (Irving, 1966), which may then undergo protonation and hydrolysis as described above. Second, the glucuronide conjugate of N-hydroxy-2-AAF may lose the acetyl group with increasing pH, resulting in an N-glucuronide of N-hydroxy-2-AF, which has much greater electrophilic reactivity than the acetylated derivative (Irving and Russell, 1970). Third, cytosolic acyl transferases can transfer the acetyl group to the oxygen resulting in the N-acetoxyarylamine (Bartsch et al., 1972a; Bartsch et al., 1973). Fourth, a cytosolic sulfotransferase of the liver may utilize 3'-phosphoadenosine-5'-phosphosulfate to form N-sulfonoxy-2-AAF (King and Phillips, 1968; DeBaun et al., 1968), which is a much stronger electrophilic ester than N-acetoxy-2-AAF (DeBaun et al., 1970). These reactions along with others are summarized in Figure 2. Finally, the one-electron oxidation of N-hydroxy-2-AAF by purified peroxidases was shown to result in the formation of a nitroxide free radical, which dismutates to give N-acetoxy-2-AAF and 2-nitrosofluorene (Bartsch and Hecker, 1971; Bartsch et al., 1972b).

Figure 2 The metabolism of aromatic amines (amides) leading to activated and deactivated products.

a) N-acetyltransferases and deacetylases
b) C- or N-oxidation by cytochrome P-450 and flavin- containing monooxygenases
c) UDPGA-, PAPS-, seryl-, prolyl-, or acyl-dependent transferase
d) non-enzymatic acid catalysis


ARYLAMIDES AND PRIMARY ARYLAMINES
Ac = (H3C-C-)
(a)
(b)
(c)
(d)
-glucuronide
-acyl
-glucuronide
-acyl
-prolyl
-seryl
glucuronide
Excretion
Nitrenium ion
+ DNA
+ RNA
+ protein
Covalently Bound Adducts

The feature common to all of these activation pathways is that an electrophilic metabolite is produced. Activation of arylamines and arylamides results after oxidation to an electrophilic species that can form covalently bound adducts with DNA. The strength of this concept is based on the observation that the carcinogen-DNA adducts identified from the acid-catalyzed reaction of N-hydroxyarylamines (-arylamides), or from the reaction of the active esters at neutral pH, are the same as those which are recovered from DNA isolated from animals treated *in vivo* with the parent amine (amide) (reviewed in Kadlubar and Beland, 1985). Some examples include: 1-naphthylamine (Kadlubar *et al.*, 1978; Beland *et al.*, 1983), 2-naphthylamine (Kadlubar *et al.*, 1980a, 1981), 4-acetylaminobiphenyl (Kriek and Westra, 1979; Gupta and Dighe, 1984), 4-aminobiphenyl (Kadlubar 1982; Beland *et al.*, 1983), 2-acetylaminofluorene (Kriek, 1972; Westra *et al.*, 1976; Kriek and Westra, 1980; Poirier *et al.*, 1982), benzidine (Martin *et al.* 1982, 1983; Kennelly *et al.*, 1984), and N-methyl-4-aminoazobenzene (Lin *et al.*, 1975; Beland *et al.*, 1980, 1982; Delclos *et al.*, 1984).

The interaction of electrophilic aromatic amine metabolites with nucleic acids, and the structures of the resulting adducts have since been studied in great detail (reviewed in Schut and Castonguay, 1984). Despite these studies, the role of carcinogen-DNA adducts in the mechanism of chemical carcinogenesis is not completely resolved.

Based on model building studies and available physico-chemical data, Kadlubar (1980b) has proposed a mechanism whereby carcinogens bound to DNA through the N^2 atom of guanine could produce guanine to cytosine or thymidine transversions. Hypotheses have also been advanced whereby derivatization of O^6 of guanine or O^4 of thymidine by carcinogens in DNA sequences could result in guanine to adenine and thymidine to cytosine transitions (reviewed by Swenson and Kadlubar, 1981). The importance of these proposals is enhanced by the characterization in the past few years of several cellular transforming genes (oncogenes) which differ by only a single base transition or transversion from the normal gene (reviewed by Shih and Weeks, 1984; DeFeo-Jones et al., 1985). Regardless of the final mechanism by which carcinogen-DNA adducts initiate neoplasia, a knowledge of DNA adducts formed by carcinogens has been important to studies investigating the metabolic activation pathways of new and unique chemical carcinogens.

A logical extension of the DNA-adduct studies has centered on monitoring the extent of exposure to carcinogens. These studies may be useful in risk assessment, based on work suggesting that there is a relationship between DNA-adduct formation and carcinogen exposure (Brookes and Lawley, 1964). Initial studies used highly sensitive enzyme radioimmunoassays with specific antibodies to carcinogen-DNA adducts such as benzo(a)pyrene

modified DNA (Hsu, I.-C. et al., 1981; Poirier, M.C. et al., 1980). Perera et al. (1988) reported that in 35 foundry workers, estimated benzo(a)pyrene exposure was significantly related to DNA adduct levels as measured by benzo(a)pyrene antigenicity, after adjustment was made for cigarette smoking and time since vacation. The exposed group had significantly elevated adduct levels compared to 10 unexposed controls similar in age and sex distribution. Biophysical methods include ^{32}P labeling methods whereby nucleotides, including those modified with adducts, are labeled with ^{32}P, resolved by anion-exchange chromatography and detected using autoradiography. These recently introduced techniques show great promise because of their high sensitivity, with the ability to detect one modified base in 10^9. Since activation of arylamines results in protein binding as well as DNA binding, and since the extent of protein binding has been suggested to reflect DNA binding, the use of other macromolecules as dose monitors is being investigated. Ehrenberg and coworkers demonstrate a correlation between the amount of protein-adduct formation and DNA-adduct formation for direct alkylating agents as well as compounds requiring bioactivation (Ehrenberg et al., 1974; Ehrenberg, 1984). Hemoglobin for example has been used successfully to evaluate exposure to many compounds. The formation of hemoglobin-adducts from arylamines has been demonstrated for 4-aminobiphenyl, 2-naphthylamine and

benzidine (Farmer *et al.*, 1984; Skipper *et al.*, 1986; Stillwell *et al.*, 1987).

The biological consequence of covalent binding of electrophilic metabolites to DNA can be genotoxicity. One major area of research was developed from a study which showed that ultimate derivatives of two aromatic amide carcinogens were strongly mutagenic for *Bacillus subtilis* transforming DNA (Maher *et al.*, 1968). Subsequently, Ames and collaborators developed a now widely used mutagenicity assay for the detection of potential carcinogens (Ames *et al.*, 1973b; McCann *et al.*, 1975b; McCann *et al.*, 1976). This assay employs histidine deficient bacterial strains of *Salmonella typhimurium*. The mutated revertants, which are histidine independent, form visible colonies and are easily quantitated. A mammalian microsomal mixed function oxidase enzyme system or S9 is used to provide the metabolic activation required of the test compound. This assay is based on two important concepts: that mutagens (and carcinogens) generally require metabolic activation to an electrophilic species and that this species can bind covalently to DNA which results in mutation, if DNA repair mechanisms are inadequate. The validity of this *in vitro* assay as a predictor of animal carcinogenicity has recently been seriously questioned (Tennant *et al.*, 1987). Zeiger (1987) reports that only 54% of 149 compounds deemed as carcinogens or equivocal carcinogens were mutagens, and 58%

of the nonmutagens tested, were carcinogens. Despite these results, this researcher suggests that use of this assay as a preliminary screen for potential carcinogens may yet be useful, if factors such as chemical class, type of metabolism, and stability of metabolites are taken into consideration.

The present discussion has emphasized the activating pathways of arylamine metabolism. However, it must be remembered that not all enzymatic pathways are activating ones. The quantity of an electrophilic species formed depends not only on the rate of its formation, but rates of competing pathways that serve to inactivate the compound. The study of metabolism can be complex not only because of the many pathways involved, but also due to the relative importance of these pathways. These pathways may vary between species or even individuals of a given species.

Role of Monooxygenases in Activation

The specific enzyme systems responsible for aromatic amine oxidation in mammalian tissues have received much less attention than have the metabolic pathways and reactions of electrophilic products of these compounds. Monooxygenases are the enzymes of greatest interest since they are responsible for the initial activation of the majority of procarcinogens. It is now widely accepted that the bulk of aromatic amine oxidation takes place in the liver. Primary arylamines are oxidized chiefly by the cytochrome P-450 dependent monooxygenase system. The most common reaction carried out by this enzyme system is hydroxylation or monooxygenation of a hydrophobic substrate. The activation energy barrier is very high since a molecule of dioxygen must undergo heterolytic cleavage with incorporation of one atom into the carbon-hydrogen bond, and reduction of the other atom by two electrons to water. Electrons from NADPH are supplied to the enzyme via the membrane-bound flavoprotein, NADPH-cytochrome P-450 reductase. Enzyme activity is inhibited by carbon monoxide as well as antibodies against NADPH-cytochrome P450 reductase. In addition to arylamines, the system can metabolize a wide variety of foreign and endogenous compounds.

Metabolism studies with this enzyme system *in vitro* are often carried out using a liver microsomal preparation, supplemented with NADPH or an NADPH-generating system. The oxidation of aromatic amines using this preparation was first demonstrated by Kiese and Uehleke (1961). Several studies have since demonstrated that the microsomal cytochrome P-450 dependent monooxygenase *in vitro* is capable of N-hydroxylating 2-AAF (Thorgeirsson *et al.*, 1973), 2-AF (Frederick *et al.*, 1982; Razzouk and Roberfroid, 1982; Hammons *et al.*, 1985), and 2-NA (Poupko *et al.*, 1983; Hammons *et al.*, 1985). Evidence for the role of this enzyme system in aromatic amine N-oxidation *in vivo* has also been presented (von Jagow *et al.*, 1966; Uehleke, 1973; Razzouk *et al.*, 1980).

Many different chemicals have been described which can preferentially induce *de novo* synthesis of one or more forms of hepatic cytochrome P-450 (Conney, 1982). It is now known that hundreds of chemicals induce these metabolic enzymes. Examples include drugs such as phenobarbital and phenytoin, industrial solvents such as xylene (Toftgard *et al.*, 1983), and environmental contaminants such as chlordane, polycyclic hydrocarbons and 2,3,7,8-tetrachlorodibenzodioxin (Poland and Glover, 1973). Unlike the immune system, the cytochrome P-450 system gets its diversity from low substrate specificity rather than a great variety in highly substrate specific gene products.

Following induction, quantitative and qualitative changes take place. Changes in the metabolic profile may affect chemical carcinogenesis. First to demonstrate changes in carcinogenicity after enzyme induction was Brown *et al.*, 1954. Treatment of animals with 3-methylcholanthrene (3-MC) resulted in increased demethylation and increased hepatocarcinogenicity of 3-methyl-4-monomethylaminoazobenzene. Peraino *et al.* (1971) were able to inhibit 2-acetylaminofluorene carcinogenesis with prior feeding of phenobarbital, which induces different forms of cytochrome P-450 than does 3-MC. Induction of metabolic systems, therefore, may lead to an increase or decrease in the potency of a carcinogen.

In addition to the cytochrome P-450 system, a liver microsomal NADPH-dependent flavin containing monooxygenase is also capable of oxidation. This flavoprotein oxidase is free of cytochromes, iron and copper. It does not have cytochrome P-450 reductase activity, and has been shown to be immunologically different from cytochrome P-450 reductase which is also a microsomal flavoprotein (Masters and Ziegler, 1971). Although the *in vitro* N-oxidation of 2-NA and 2-AF has been demonstrated by this monooxygenase (Ziegler *et al.*, 1973; Frederick *et al.*, 1982; Hammons *et al.*, 1985), this enzyme appears to play a much more important role in the N-oxidation of alkylamines and tertiary amines (Ziegler *et al.*, 1973).

The question of how arylamines cause bladder cancer after N-hydroxylation in the liver has not yet been addressed. One hypothesis has been suggested from a series of metabolism studies. These studies began with the demonstration that the glucuronic acid conjugates of various N-hydroxylated arylamines were present in urine of animals exposed to the parent amine (Kiese et al., 1966; Radomski and Brill, 1970, Miller et al., 1961b). The glucuronic acid conjugate of N-hydroxy-4-aminobiphenyl was then isolated from the urine of dogs fed 4-aminobiphenyl and identified as the N-glucuronide (Radomski et al., 1973; Radomski et al., 1977). It was further demonstrated that hydrolysis of this conjugate occurred readily at acidic pH, liberating the free N-hydroxy compound. Subsequent acid-catalyzed hydrolysis of the N-hydroxylamine, in the presence of DNA, resulted in covalent binding to DNA. The hypothesis was therefore developed stating that the compounds are bioactivated to the hydroxylamine in the liver, inactivated and secreted as the glucuronide. The glucuronide remains inactive until it reaches the acidic medium of the urine, where acid hydrolysis and subsequent activation to a nitrenium ion lead to binding with the DNA of the bladder epithelium, initiating the carcinogenic process.

Alternatives to this hypothesis suggest that activation takes place in the target organ itself. The ability of isolated bladder to N-hydroxylate aromatic amines was first

demonstrated by Ueleke (1966). N-hydroxylation of 4-aminobiphenyl by bovine bladder microsomes (Poupko et al., 1981), and of 2-AAF by cultured bladder explants (Moore et al., 1982; Schut and Castonguay, 1984) has since been demonstrated. The N-hydroxylation of 2-aminofluorene by bladder mucosal microsomes was shown to be catalyzed by cytochrome P-450 (Vanderslice et al., 1985).

More recently, prostaglandin H synthase (PHS) mediated metabolism of some aromatic amines to reactive intermediates at neutral pH has been demonstrated (Kadlubar et al., 1982; Zenser et al., 1983, Moldeus et al., 1982; Boyd and Eling, 1981). This microsomal enzyme system is found in most mammalian tissues with high concentrations in the lung, skin, urinary tract, cardiovascular, and reproductive tissues (Hall and Behrman, 1981). The reaction is thought to be initiated by the direct oxidation of the primary aromatic amine to a free radical. The driving force of this oxidation may be the arachidonate hydroperoxide generated when PHS converts arachidonic acid to prostaglandin H_2 (reviewed in Eling et al., 1983) At least 3 mechanisms have been advanced to explain the subsequent binding of the amine to DNA (reviewed by Frederick et al., 1985). These mechanisms predict that the binding of aromatic amines to DNA or other cellular biomolecules occurs via an electron deficient free radical. The presence of PHS in renal medulla and bladder epithelium may contribute to the

generation of bladder cancer by arylamines activated by this system.

Initiation and Promotion of Cancer

The process of carcinogenesis can be separated into discrete initiation, promotion, and progression phases in numerous model systems and even these stages can be further subdivided (Foulds, 1975; Hecker et al., 1982; Slaga et al., 1978). In these systems, initiation consists of a low dose of a compound which results in a persistent, heritable lesion. This treatment is followed by a repetitive administration of an agent that may enhance the growth of initiated cells. Neither phase alone is sufficient for tumorigenesis, and initiation must occur first. Initiation is referred to as "persistent" because the promotion phase will still produce tumors even if delayed for months. Promotion, however, is not "persistent". Administration of the promoter must be repeated continuously or at relatively short intervals to be effective.

The promotion of tumors or cancers was originally described in the 1940's (Berenblum, 1941; Rous and Kidd, 1941). Later it was observed in a number of species and organ systems, including stomach, bladder, and liver (Slaga, 1983a). The model by which the process is divided into separate initiation and promotion stages is supported by several aspects of experimental carcinogenesis. It has been

offered as an explanation of such observations as the synergistic interaction of weak carcinogens in cancer causation and the long latency often observed between exposure to a carcinogen and the appearance of neoplasia.

Much experimental work with initiation and promotion strongly suggested that initiation results from somatic cell mutations. Slaga (1983a) reported that, in mouse skin initiation-promotion systems, the number of papillomas seen early in promotion can be correlated with the carcinogenic potency of the initiating compound and that tumor-initiating ability can be correlated with the extent of DNA binding. Likewise, in liver initiation-promotion systems, evidence also indicates a mutational basis for initiation. Tsuda et al. (1980) and Tatematsu et al. (1983) found a wide variety of carcinogens (although not necessarily liver carcinogens) to be active liver tumor initiators. The effect of the initiating compound can persist for long periods of time and has been considered essentially irreversible. Thus, Peraino et al. (1975) were able to demonstrate promotion of tumors following a lag of up to 4 months between initiation and promotion. The persistence of the initiating event is presumably due to permanent alterations in the genetic code of the affected cells, leading to mutations. The process begins with interaction of the electrophilic species with DNA resulting in DNA-adduct formation. During the process of replication, these adducts may cause deletions or base

pair substitutions leading to alterations in the primary structure of DNA and thereby to changes in genetic information. In this way, changes made to the genetic code become permanent or fixed in the affected cells.

Not all DNA binding, however, leads to initiation. Some adduct formation occurs at positions important for base pairing whereas others may have no affect on pairing. Likewise, enzymes that are responsible for the fidelity of the genetic information may repair damage before it is permanently fixed. Finally, not all changes in the genetic code affect the genes responsible for initiation. It is not surprising, therefore, that the relationship between biological effect and DNA binding, often times, cannot be correlated (Neumann H.-G., 1983).

Evidence suggests that initiation is an event that is not sufficient in producing tumors. Further progression of the process requires promotion. Promotion, in contrast to initiation, appears to be epigenetic, and to depend at least partly on proliferative stimulation and alterations in the regulation of gene expression (Shimada et al., 1981; Friedman and Steinberg, 1982; Williams, 1983). Examples of agents that act as promoters are phenobarbital and 12-O-tetradecanoylphorbol-13-acetate (TPA). Slaga et al. (1980a, 1980b) have shown that promotion can further be divided into at least two stages. This distinction was made with the observation that mezerein induced many of the cellular

events in a fashion similar to TPA, but was essentially a nonpromoting agent. It appeared then that TPA was responsible for additional cellular responses that were not elicited by mezerein. It was shown that only one application of a first-stage promoter such as TPA was enough for four to six weeks. Subsequent repeated treatment with mezerein, the second-stage promoter, was very effective in producing tumors (Slaga et al., 1982).

Carcinogenicity assays have been developed to distinguish between the promoting and initiating properties of a compound (Pitot and Sirica, 1980; Solt et al., 1977). These assays can also determine whether a compound is acting as a complete carcinogen, that is one having both initiating and promoting activity. Limited or short-term bioassays including the rat liver foci assay (Pereira, 1982) and the SENCAR mouse skin tumorigenesis assay (Slaga and Nesnow, 1985) have also been used to determine the tumor-initiating and promoting activities of a compound. In addition to assaying potential carcinogenic agents, these two assay systems can be used to study modifiers of carcinogenesis. Modifiers may have additive, synergistic, or a negative influence on tumor formation. Arylamines that tested positive as initiators or as complete carcinogens in the SENCAR mouse skin assay include, N-acetoxy-4-acetamidobiphenyl, N-acetoxy-2-acetamidofluorene, N-hydroxy-2-aminonaphthalene and N-acetoxy-2-acetamidostilbene

(reviewed in Slaga, 1983b). Examples of arylamines acting as pure promoters in these assays could not be found.

Many short term *in vitro* assays have been used to study the possible genotoxicity of new compounds. The assays can be divided into general classes that test for gene mutation, chromosomal damage, DNA damage and repair, and finally cellular transformation. The relevance of these assays to carcinogenicity testing has been based on the somatic theory of cancer that states that the initial damage is genetic in origin. This genetic damage would then correspond to the "initiation" stage. The primary limitation of these assays to predict a compound as being carcinogenic in man is that they cannot reflect the numerous biological processes that are evident in the human organism.

Short term *in vitro* assays that attempt to establish the promoting potential of compounds are far less numerous. One example is determination of ornithine decarboxylase (ODC) as a marker of carcinogenicity. Ornithine decarboxylase is elevated in target organs and cells in culture by complete carcinogens and tumor promoters. Boutwell *et al*. (1979) reported a close correlation between tumor-promoting potency and the induction of ODC. This correlation is likely due to the importance of ODC expression for nucleic acid synthesis, proliferation and differentiation. These processes are all altered by the action of carcinogens. One other *in vitro* assay determines

the effect that compounds have on gap-junctional intercellular communication. The assay is based on the fact that intercellular communication is an important mechanism involved in regulating cell growth (Loewenstein, 1979). Also, tumor promoting agents such as TPA have been shown to block gap-junctional intercellular communication (Yotti *et al.*, 1979; Murray and Fitzgerald, 1979; Chang *et al.*, 1985). A study of a compound's ability to block intercellular communication might therefore provide an indication of tumor promoting activity.

Despite their usefulness in assessing possible mechanisms of action, *in vitro* assays, however, can never replace the *in vivo* system in predicting the carcinogenicity of a compound. The *in vivo* system contains a much higher level of cellular organization not present in *in vitro* assays. Indeterminable influences on the toxicity of a compound due to this higher organization may never be successfully reproduced in the *in vitro* system.

MBOCA

An arylamine of current toxicological interest is 4,4'-methylenebis(2-chloroaniline), or MBOCA (Maltry, 1971; Wallace, 1988). Some of MBOCA's trade names include: MOCA, Curalin M, Curene 442 and Cyanaset. The compound was first developed and marketed by the DuPont Company in 1956 and has been widely used since as a crosslinking agent in the manufacture of diisocyanate based polymers and epoxy resins. It is used in the manufacture of polyurethane foams, industrial rubber products and rigid plastics such as plastic car moldings (IARC, 1974). Although domestic manufacture had stopped in 1979, it was being imported in amounts of 1-3.5 million pounds a year for the 200-400 plants that were using it (TSCA, 1983). In 1985 approval was granted for the installation of a facility to manufacture MBOCA in Michigan. NIOSH (1978) indicated that in the early 1970's approximately 55,000 U.S. workers were potentially exposed to this compound. NIOSH estimated that in 1977, 1,600 workers were directly exposed to MBOCA and that 16,000 employees in the same plants were at risk of exposure through indirect contamination.

MBOCA has been tested for carcinogenicity in rats, mice, and dogs. In 2 year studies, MBOCA caused liver,

lung, and Zymbal gland tumors in rats (Steinhoff and Grundmann, 1971; Russfield et al., 1975; Stula et al.,1975; Kommineni et al., 1978), although lung tumors from arylamines are not common in rats (Russfield et al., 1975). MBOCA caused hemangiomas and hemangiosarcomas in mice of both sexes and hepatomas in female mice (Russfield et al., 1975). Two years later, Stula et al. (1977) reported the results of a long-term feeding experiment in dogs. Five of 6 dogs developed transitional cell carcinomas of the bladder or urethra after 8 to 9 years feeding. Control animals had no tumors. These researchers suggest that MBOCA is a urothelial carcinogen of equal potency to benzidine. Taken together, these animal studies make it clear that MBOCA is an animal carcinogen.

MBOCA has been tested in vitro for mutagenicity and transformation. It is mutagenic in two assays, the Salmonella mutagenicity assay (procaryotic, reverse mutations) (McCann et al., 1975a; McCann et al., 1975b) and the mouse lymphoma assay (eukaryotic, forward mutation) (U.S. Dept. Health, 1983). MBOCA is interpreted as genotoxic to mouse and hamster hepatocytes (McQueen et al., 1981), and positive in the Balb C 3T3 mouse embryo cell transformation assay (U.S. Dept. Health, 1983). It inhibited DNA synthesis in cell culture (Aust et al., 1981). MBOCA was also shown to bind to DNA in explant cultures of human and dog bladder (Shivapurkar et al., 1987). Thus,

MBOCA should be considered a potential human health hazard based on its widespread industrial use, results from *in vitro* testing and its demonstrated carcinogenicity in animal models.

In addition to exhibiting the acute toxicity characteristics of aromatic amines, i.e. cyanosis and methemoglobinemia (Linch *et al.*, 1971), several reports indicate MBOCA may also be nephrotoxic. Humans and dogs exposed to MBOCA were seen to exhibit some kidney irritation (Mastromatteo, 1965). A worker exposed to liquid MBOCA in an industrial accident showed an immediate but transient decreased tubular reabsorption of low molecular weight proteins, and possible renal tubular damage and consequent inability to concentrate the urine (Hosein and Van Roosmalen, 1978).

MBOCA has been reported to be absorbed through the skin of workers exposed to it (Linch *et al.*, 1971). Chin *et al.* (1983) show that MBOCA is taken up rapidly from human neonate foreskin. They conclude that metabolism does not play a role in uptake and transport through the skin since little other than the parent compound was found after extraction and TLC analysis. Manis *et al.* (1984) report that in dogs, MBOCA was absorbed after dermal application with 1.9% excreted in urine and bile after 24 hrs., with lesser amounts present in liver, kidney, fat and muscle. Of the administered dose, 90% was still in skin at the site of

application. Metabolism and disposition of MBOCA have been studied in rats and dogs. The compound was extensively metabolized and rapidly excreted in both animals. In rats 69-73% of the administered dose was recovered in feces and 22-29% in urine in 48 hr (Farmer et al., 1981; Tobes et al., 1983). Although widely distributed, retained radioactivity was highest in the liver, followed closely by adrenals, small intestine and lung (Tobes et al., 1983). The parent compound represented just 1-2% of the excreted dose in the urine (Farmer et al., 1981; Tobes et al., 1983). In the dog, 46% of the administered iv dose was recovered in the urine by 24 hr, 0.54% of that as the parent compound (Manis et al., 1984). Thirty-two percent of the dose was excreted into the bile, none of it as the parent compound. The liver retained the highest tissue concentration followed by kidney, fat and lung (Manis et al., 1984). Extensive metabolism was demonstrated in both rat and dog by the low concentration of the parent compound in the urine and absence of parent compound in dog bile.

Few urinary metabolites of MBOCA have been identified to date. Farmer et al. (1981) hydrolyzed urine from MBOCA-dosed rats with sulfatase-glucuronidase. This treatment liberated two major metabolites from conjugation yet increased the yield of the parent compound from 1-2% to only 3-6% of the total dose. Others have identified the mono- and diacetates in human urine (Ducos et al., 1985). Manis

and Braselton (1984) identified the major canine urinary metabolite as the ortho-hydroxy sulfate. The ortho-hydroxy sulfate was also found to be the major canine urinary metabolite of benzidine, 4-aminobiphenyl and 2-naphthylamine (Wiley, 1938; Bradshaw and Clayson, 1955; Sciarini and Meigs, 1958).

Human exposure is currently monitored by determination of urinary levels of the parent compound. Linch et al. (1971) reported quantifiable urinary concentrations of occupationally exposed individuals. Preschool children residing in a two mile wide area surrounding a site of MBOCA manufacture in Adrian, Michigan had detectable urinary levels. MBOCA had also been detected in the urine of the workers' families (Williams, 1979). Thomas and Wilson (1984) measured the urinary levels in workers of a plastics manufacturing plant over a period of 5 years and showed at the start of the study that workers had levels of up to 250 nmol MBOCA/mmol creatinine in the urine. Levels fell to less than 10 nmol MBOCA/mmol creatinine after the introduction of protective measures for these workers.

Some epidemiological studies concerning MBOCA have been done or are reported to be under way. Linch et al. (1971) report that of 31 men exposed to MBOCA for 6 months to 16 years, no clinical or cytological evidence of bladder cancer was found. This study may have been inadequate due to the relatively short period and small number of subjects

involved. Ward et al. (1988) identified two noninvasive papillary tumors of the bladder in a screening of 540 workers who were exposed to MBOCA during its production at a Michigan chemical plant. Both tumors occurred in men under 30 years old who had never smoked. Though the incidence of grade 1-2 tumors for this age group is unknown, the normal incidence of clinically apparent tumors on males in the U.S. aged 25-29, is only 1 per 100,000 per year. Additional epidemiological studies are reported to be underway (Wallace, 1988; Ward, 1988).

Recently, Public Citizen Health Research Group and five industrial unions have petitioned OSHA for an emergency temporary standard (ETS) limiting worker exposure to MBOCA (Hanson, 1987). An ETS was issued for MBOCA in 1973, however the standard, along with 13 others, was abandoned on procedural grounds by a court order the following year. The present petition requests that exposure to MBOCA be limited to an eight hour time-weighted average of 3 mcg per cubic meter, with special provisions to minimize absorption from skin contact, the major route of MBOCA absorption. British regulations currently set an occupational exposure limit of 5 mcg per cubic meter with an action level of 30 nmol MBOCA/mmol creatinine in urine (Health and Safety Executive, 1987).

Animal studies to date have shown that MBOCA is extensively metabolized. Relatively few metabolites,

however, have been identified. Further elucidation of the structural identity of the oxidized metabolites of MBOCA is necessary. In addition, assessment of the potential mutagenicity and promotional activity of isolated oxidized metabolites is needed. Investigations into the metabolism of MBOCA, along with possible biological activity of identified metabolites, should help to elucidate mechanisms of MBOCA's established carcinogenicity.

Purpose

This thesis project involved the study of the commercially important arylamine MBOCA. MBOCA is a proven animal carcinogen whose metabolism has not been extensively characterized. These studies were undertaken to investigate the metabolic activation of this arylamine and demonstrate its potential as a human carcinogen. The objectives of the project were :

a) determine the structural identity of Phase I metabolites in several representative species including man;

b) to investigate the enzymatic nature of the formation of oxidized metabolites in various species;

c) to determine the relative activity of Phase I metabolites as potential mutagens and as compounds that are able to block gap-junctional intercellular communication;

d) to develop a means of measuring exposure to MBOCA; and

e) to determine the effect of cytochrome P-450 induction on the formation of these metabolites.

The hypothesis behind this work was that MBOCA Phase I metabolism leads to oxidized compounds that are more reactive than the parent, and thus may have the potential to act as proximate carcinogens. In addition, it was hypothesized that conversion to these reactive species can

be altered by induction of the metabolic enzymes involved in their formation.

CHAPTER 1

HYDROXYLATION OF MBOCA BY CANINE, GUINEA PIG, RAT AND HUMAN LIVER MICROSOMES

CHAPTER 1

INTRODUCTION

Most metabolic activation of procarcinogens begins with the mixed function oxidase system (Wiebel *et al.*, 1984), though in certain instances other enzymatic pathways such as the prostaglandin H synthase system may play a significant role (Zenser *et al.*, 1983). A study of Phase I metabolism is important in determining the relative involvement of initial activating and inactivating pathways. The technical advantage with this approach is that metabolic pathways involving conjugation are effectively eliminated removing much of the complexity in isolation and identification of products. Species comparisons of individual enzyme pathways would not otherwise be possible. The limitation of this simplification is that if one needs to study the "ultimate" electrophile important *in vivo*, all metabolic pathways need to be assessed, both activating and inactivating ones, both conjugating and those not involving conjugation.

A number of typical arylamine carcinogens have been found to be oxidized to *o*-hydroxy and N-hydroxy compounds. Although ring hydroxylation *ortho* to the amine may result in a reactive molecule (Bonser *et al.*, 1963), a large body of research suggests that N-hydroxylation is the obligatory

step in the activation of arylamine and arylamide carcinogens (Miller, 1978). Subsequent metabolism to form various esters is thought to be responsible for the ultimate electrophilic form of the toxic agent that binds to protein and DNA at the site of metabolism. As stated earlier, one hypothesis for the induction of bladder cancer as developed by Radomski *et al.* (1973), does not include formation of an ester. Bladder cancer in humans and dogs may result from N-hydroxylation of the amine, conjugation and then transport to the urinary bladder as the N-hydroxy-N-glucuronide. This acid labile form can release the activated N-hydroxy species through hydrolysis thereby generating the species responsible for interaction with bladder epithelial tissue. Regardless of the subsequent fate of the proximate species formed through Phase I metabolism, a study of the specific enzymes and kinetics involved in these initial reactions is important.

The mammalian metabolism of MBOCA has not been well characterized. *In vivo* studies in rats and dogs indicate that it is rapidly degraded and excreted in urine and bile. Rats eliminated 69-73% of the administered dose in feces and 16-29% in urine in 48 hr, only 0.2-2% of which was the parent compound (Farmer *et al.*, 1981; Groth *et al.*, 1984). Dogs excreted 46% of the dose in urine and 32% in bile in 24 hr, with only 0.54% of the urinary fraction as parent MBOCA (Manis *et al.*, 1984). The parent compound was not found in

bile from either the rat or dog (Farmer et al., 1981; Manis et al., 1984). Canine liver and kidney slice preparations have been utilized to investigate the potential for formation of Phase I and II metabolites by these tissues (Manis and Braselton, 1986). Liver slices formed seven metabolites of ^{14}C-MBOCA resolved by HPLC. The major metabolite, the o-OH-MBOCA sulfate, had previously been shown to be the major metabolite of canine urine (Manis et al., 1984). Other metabolites formed by liver slices included an MBOCA-glucoside, and O-glucuronide, and two additional glucuronide metabolites. Renal cortical slices produced six metabolites separable by HPLC. Three of these, the MBOCA-glucoside, a glucuronide, and the o-hydroxy sulfate were also formed by liver.

To further elucidate the structural identity of the oxidized metabolites of MBOCA, the more simplified in vitro hepatic microsomal mixed function oxidase enzyme system was used. Hepatic microsomal systems were used in these studies in order to assess the enzymatic nature of Phase I metabolism of MBOCA in a variety of species. Four species were utilized: the dog, because it is the animal model for human bladder carcinogenesis; the rat, because MBOCA is a proven carcinogen in this species; the guinea pig, because this species has been reported to form predominately the N-hydroxy species in the metabolism of other primary

arylamines; and finally human, to assess possible risk of MBOCA exposure to this species.

METHODS AND MATERIALS

Reagents and Chemicals

MBOCA was a gift from Dr. Daniel E. Williams (Michigan Department of Health). [B-^{14}C]MBOCA, 58 mCi/mmole, and [U-^{14}C]MBOCA, 10.9 mCi/mmole, were obtained from the Michigan Toxic Substances Control Commission. The compounds were purified by HPLC on a C_{18} μBondapak column (Waters Associates, Milford, MA) using ACN/H_2O (47:53 v/v). Radiochemical purity was judged to be greater than 99% by quantitative GC/MS and liquid scintillation spectrometry. DPEA was provided by Lilly Research Laboratories (Indianapolis, IN). All solvents were U.V. spectrometric grade. Alkylphenones were obtained as Alkylphenone Kit I and II (Pierce Chemical Company, Rockford, IL). Water was purified by passing distilled water through a 4-bowl Milli-Q water purification system (Millipore Corporation, Milford, MA) containing a 0.25 μm filter.

Animals

Dogs were healthy adult mongrel males, weighing 11-19 kg. They were anesthetized with pentobarbital on the day of the experiment, and kept under anesthesia for 2-4 hr for routine physiological laboratory experiments. Livers were taken at the end of the experiment prior to euthanasia with

an iv bolus of KCl. Rats were Sprague-Dawley adult males (Harlan, Indianapolis, IN), weighing 300-450 g. Guinea pigs were English short hair males (strain Mdh:(SR[A])) weighing 700-800 g, obtained from the Michigan State Health Laboratories (Lansing, MI). Rats and guinea pigs were sacrificed with light ether anesthesia followed by cervical dislocation. Human liver tissue was obtained at Ingham Medical Center and transferred to ice-cold saline for immediate transport to the laboratory at MSU. Three separate human liver samples were obtained. Liver biopsy samples were obtained from a 64 year old female patient diagnosed as having stomach cancer, a 56 year old male patient with a history of choleocystitis, and a 69 year old male with colon cancer that was metastatic to the liver. Liver specimens used for these assays were taken from seemingly non-cancerous tissue.

Microsomal Preparation

Livers were quickly removed and placed in ice-cold 0.01 M potassium phosphate buffer pH 7.4, containing 0.2% nicotinamide. The livers were minced and homogenized in a Potter-Elvehjem homogenizer in a sufficient volume of KCl solution to yield a homogenate containing the equivalent of 250 mg liver wet weight/ml. Microsomes were obtained by differential centrifugation of the homogenate through a 9000 x g supernatant and then precipitation at 105,000 x g. The 105,000 x g pellet was thoroughly washed by mixing with 0.1

M sodium pyrophosphate containing 0.3 M sucrose, pH 7.5, followed by a second centrifugation at 105,000 x g. The washed microsomes were suspended in 0.05 M Tris-HCl buffer, pH 7.4, to give a protein concentration of 10-20 mg/ml as determined by the Lowry procedure (Lowry et al., 1951). For routine use, this preparation was dispensed into 1 ml capped vials. After purging with argon, the vials were stored at -70°C for up to 4 months.

Incubation Conditions

Microsomes were incubated in a mixture consisting of 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM NADP, 0.5 mM NADH, 2.5 mM glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase/ml, 1-2 mg of microsomal protein/ml, 0.055 mM MBOCA (either ring or bridge labeled, with a specific activity of 0.5 μCi/μmole). The substrate was added in 20 μl MeOH/ml of incubation medium. Incubations were performed at 37°C for 15 to 60 min. In order to minimize loss of N-hydroxy metabolites, incubations and subsequent manipulations were carried out under yellow light.

Studies on the effect of atmosphere on the microsomal oxidations were carried out as above under conditions of 100% O_2, 90% CO: 10% O_2, or 100% N_2. One ml incubations with incubation mixture, but without added substrate were prepared. Samples were evacuated for 5 min under a vacuum of 0.01 mm Hg with swirling to evacuate dissolved gasses. The appropriate atmosphere was then allowed to fill the

incubation vials. Substrate was added (0.2 mM MBOCA), and incubations were carried out at 37°C for 30 min.

To terminate the incubations, sample vials were immersed in ice water, then immediately extracted two times with a double volume of ice-cold CH_2Cl_2 by vigorous swirling under Ar. The organic phase was separated by centrifugation and dried through anhydrous Na_2SO_4, then evaporated under a stream of N_2.

Thin-Layer Chromatography

Calibrated small volumes of CH_2Cl_2 were added to dissolve the metabolite residue, and samples were spotted on K5F or K5 silica gel TLC plates (Whatman). During spotting, the TLC plate was kept cold by placing on a 1-cm thick glass plate which had been prechilled in a -20°C freezer until just before spotting. Development was performed with toluene/ethanol (20:1) under vacuum in a desiccator or in a TLC development tank purged with Ar.

MBOCA and metabolites were visualized at a detection limit of approx. 0.1 μg by three different methods: 1) UV absorbance, as quenching of fluorescent TLC plates (K5F) viewed under short-wave UV light; 2) color development *in situ* by spraying with 4-dimethylamino-cinnamaldehyde, 0.2% in acidified ethanol, whereby MBOCA-derived compounds were shown as pink-purple; and 3) autoradiography using XAR-5 X-ray film (Kodak).

TLC spots were quantified by LSS. Spots were scraped

from the K5F plates, mixed with 0.2 ml of methanol and 10 ml ACS (Amersham) aqueous counting scintillant, and counted in a Packard Model 460C scintillation counter, using external quench correction. Counting efficiency for ^{14}C was 92%.

HPLC Analysis

TLC spots were further analyzed by HPLC and by mass spectrometry. Spots at the R_f of metabolites identified above were scraped from K5 plates run in parallel to the K5F plates used for visualization. Compounds were desorbed by acetone, and then the acetone was evaporated by N_2 without heating. The residue was dissolved in a small volume of ACN/H_2O (1:1) for analysis. The HPLC was carried out on a Waters Associates (Milford, MA) HPLC system with Waters 5μ C_{18} Nova-Pak (8 mm) radial compression column. The mobile phase was a 1-hr gradient from 10:90 to 75:25 ACN/0.01 M NH_4Ac, pH 6.4. Detection was by U.V. absorbance at 254 nm and 280 nm (Waters Model 440), and by radioactivity flow monitoring with a Radiomatic Instruments Flo-One HP (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL). Retention times were converted to normalized retention indices, RI_N, using the alkylphenone homologs acetophenone to hexanophenone and octanophenone (Hill et al., 1984).

Statistical Analysis

Statistical comparisons were made using analysis of variance, followed by the student's t test ($p < 0.05$).

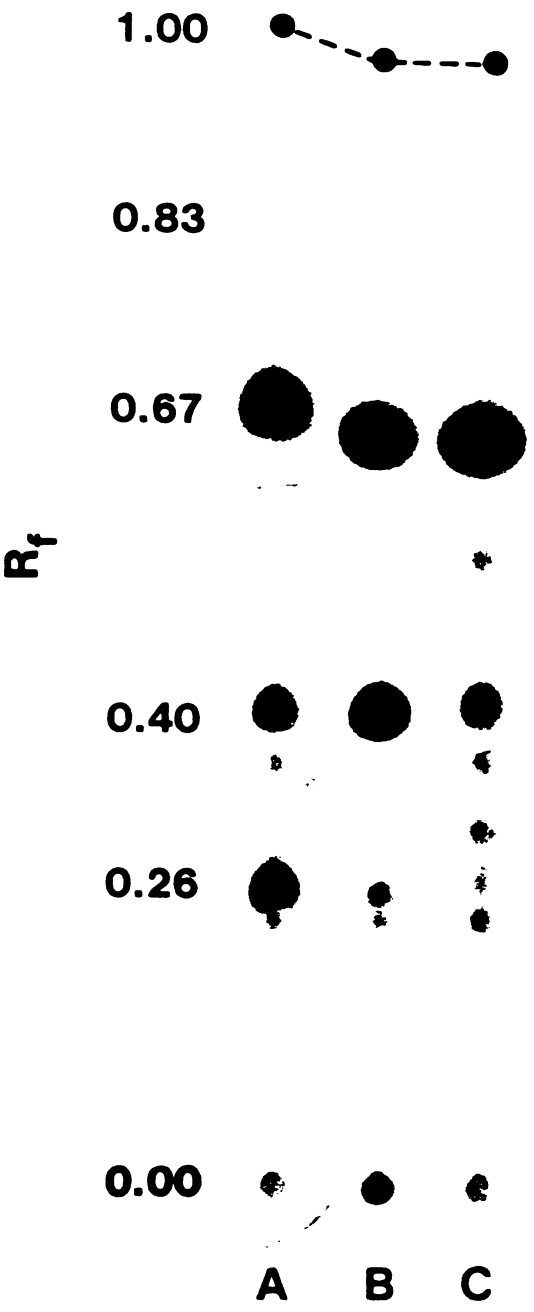
RESULTS

Identification of Hydroxylated Metabolites

Using highly purified MBOCA as substrate, and under stringently controlled conditions during purification (darkness, cold, oxygen-free atmosphere, dryness), two major metabolite spots, M1 at R_f 0.26 and M2 at R_f 0.40, were consistently found by TLC following incubations with canine, guinea pig and rat liver microsomes (Figure 3). An additional metabolite, M3, was often observed at R_f 0.83. Several minor polar metabolites were visible on the autoradiograms from all three species, but the profile from the rat microsomes was somewhat more complicated in this respect than the others. The metabolite profile visualized by autoradiography was identical when [B-^{14}C]MBOCA and [U-^{14}C]MBOCA were used as substrates, indicating that the Phase I metabolites seen in this study did not include products cleaved at the bridge carbon. Subsequently, all experiments were conducted with the [B-^{14}C]MBOCA, which had higher specific activity.

A metabolite, M3, was often observed at R_f 0.83 upon TLC of microsomal incubation extracts. The metabolite was also observed in samples of the N-hydroxy MBOCA that were

Figure 3 **An autoradiograph of the TLC separation of metabolites formed by the microsomal oxidation of [B-^{14}C]MBOCA.**


1.00
0.83
0.67
R_f
0.40
0.26
0.00
A
B
C

allowed to degrade, and could be readily generated from N-hydroxy MBOCA by oxidation with potassium ferricyanide.

Mass spectrometry results gave evidence that metabolite M1 was o-OH-MBOCA, M2 was N-OH-MBOCA, and M3 the N-O-MBOCA oxidized metabolite. Mass spectrometry results and further structural verification of these compounds and their synthesized standards are presented in chapter 2.

Manipulation of Microsomal Oxidation of MBOCA

In order to confirm that the hydroxylations of MBOCA observed in vitro were enzymatic, the rates of formation of the N-and o-hydroxy metabolites were measured under varied reaction conditions. The effect of increasing concentration of microsomal protein from dog and guinea pig liver on the formation of N- and o-hydroxy MBOCA is seen in Figure 4. The formation of N-OH-MBOCA by microsomes from both species increased with protein concentration to a maximum at 1 mg/ml under these conditions, while the formation of o-OH-MBOCA by dog liver microsomes increased linearly to a maximum at a protein concentration of 2 mg/ml. As was evident in the TLC autoradiograph (Figure 3), the guinea pig microsomes formed the N-hydroxy metabolite almost exclusively, with only trace amounts of the o-hydroxy metabolite present.

The time-dependent formation of N- and o-hydroxy metabolites of MBOCA by dog liver microsomes is shown in Figure 5. The reaction was linear with time from 0-15 min for both N- and o-hydroxylation. Evidence is presented in

Figure 4 Effect of microsomal protein concentration on formation of the N- and o-OH metabolites of [B-^{14}C]MBOCA by dog and guinea-pig liver microsomes. Following 30-min incubations with 2 mM NADPH, and 55 μM MBOCA, extracts were chromatographed by TLC, metabolites were located by autoradiography, scraped and quantified by LSS.

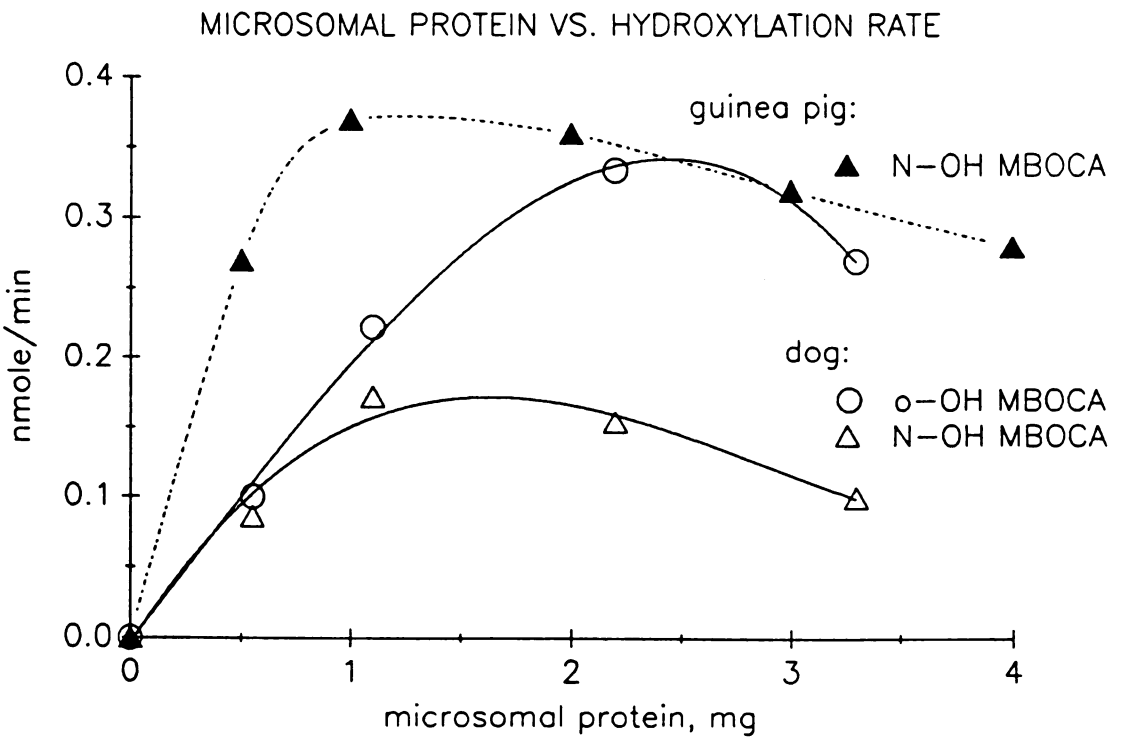

MICROSOMAL PROTEIN VS. HYDROXYLATION RATE
0.4
0.3
0.2
0.1
0.0
nmole/min
guinea pig:
N–OH MBOCA
dog:
o–OH MBOCA
N–OH MBOCA
0
1
2
3
4
microsomal protein, mg

Figure 5 Effect of incubation time on dog liver microsomal hydroxylation of 55 μM [B-^{14}C]MBOCA. Following 0-60 min incubations, metabolites were isolated and quantified as described in the legend to figure 4.

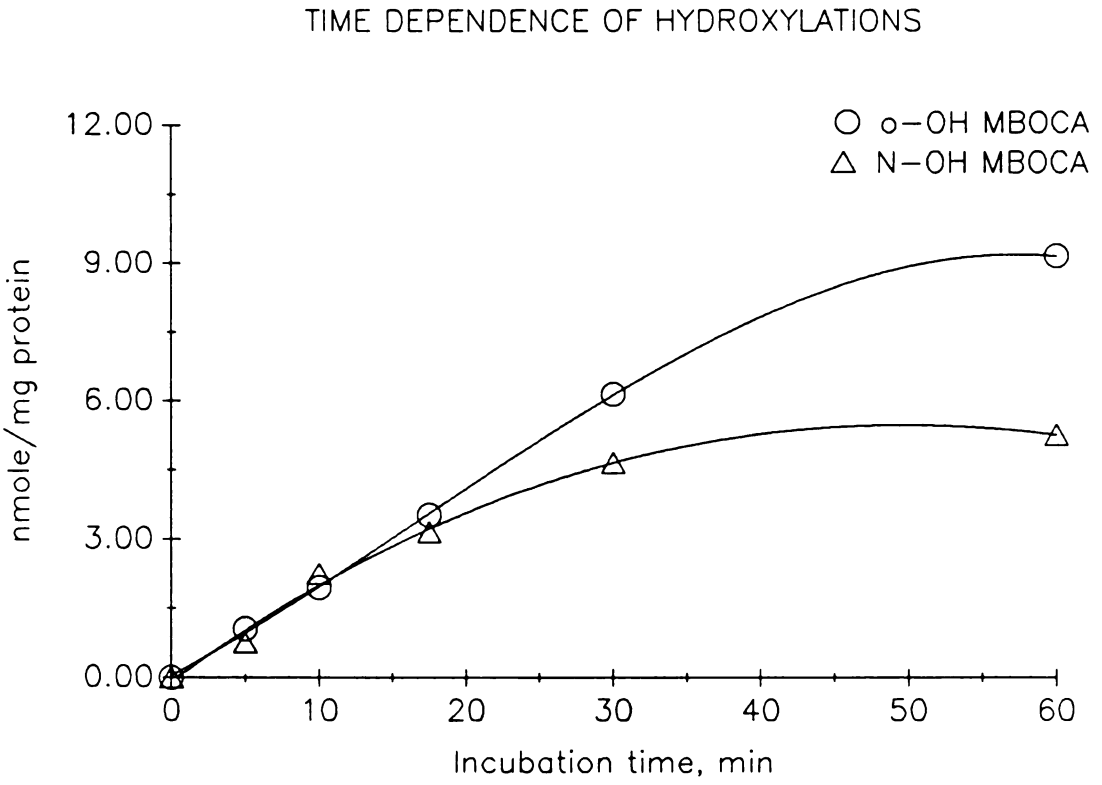

TIME DEPENDENCE OF HYDROXYLATIONS
o-OH MBOCA
N-OH MBOCA
12.00
9.00
6.00
3.00
0.00
nmole/mg protein
0
10
20
30
40
50
60
Incubation time, min

Figure 6 for the involvement of the cofactor NADPH in the enzyme catalyzed N- and o-hydroxylations by dog liver microsomes. The N-hydroxylation rate increased linearly with NADPH concentration until 0.125 mM NADPH, while o-hydroxylation was linear until 0.25 mM NADPH. Similar results were obtained with an NADPH-generating system.

A Lineweaver-Burk plot of the substrate concentration dependence on the velocity of N- and o-hydroxylation of MBOCA by dog liver microsomes is shown in Figure 7. The kinetic curves for the two hydroxylations showed similar apparent V_{max} (130 pmol/mg protein/min) but different apparent K_m values (0.038 mM for N-hydroxylation and 0.027 mM for o-hydroxylation).

The sensitivity of these enzymes to carbon monoxide and an inert atmosphere using dog liver microsomes is depicted in Table 1. Sensitivity to carbon monoxide of enzymatic oxidation of other arylamines has previously been shown (McMahon et al., 1980). In the present study, an atmosphere of carbon monoxide at a ratio of 9:1 to oxygen was effective as was a complete nitrogen atmosphere in inhibiting the enzymatic formation of these metabolites. The results in Table 1 show that an atmosphere consisting of 9:1 carbon monoxide to nitrogen was effective in reducing ortho-hydroxylation of MBOCA by 70%, whereas N-hydroxylation was reduced by 48%. In comparison, a 100% nitrogen atmosphere

Figure 6 **Effect of NADPH concentration on dog liver microsomal hydroxylation of 55 μM [B-^{14}C]MBOCA. Following 30-min incubations with 0 to 0.5 mM NADPH, metabolites were isolated and quantified as described in the legend to figure 4.**

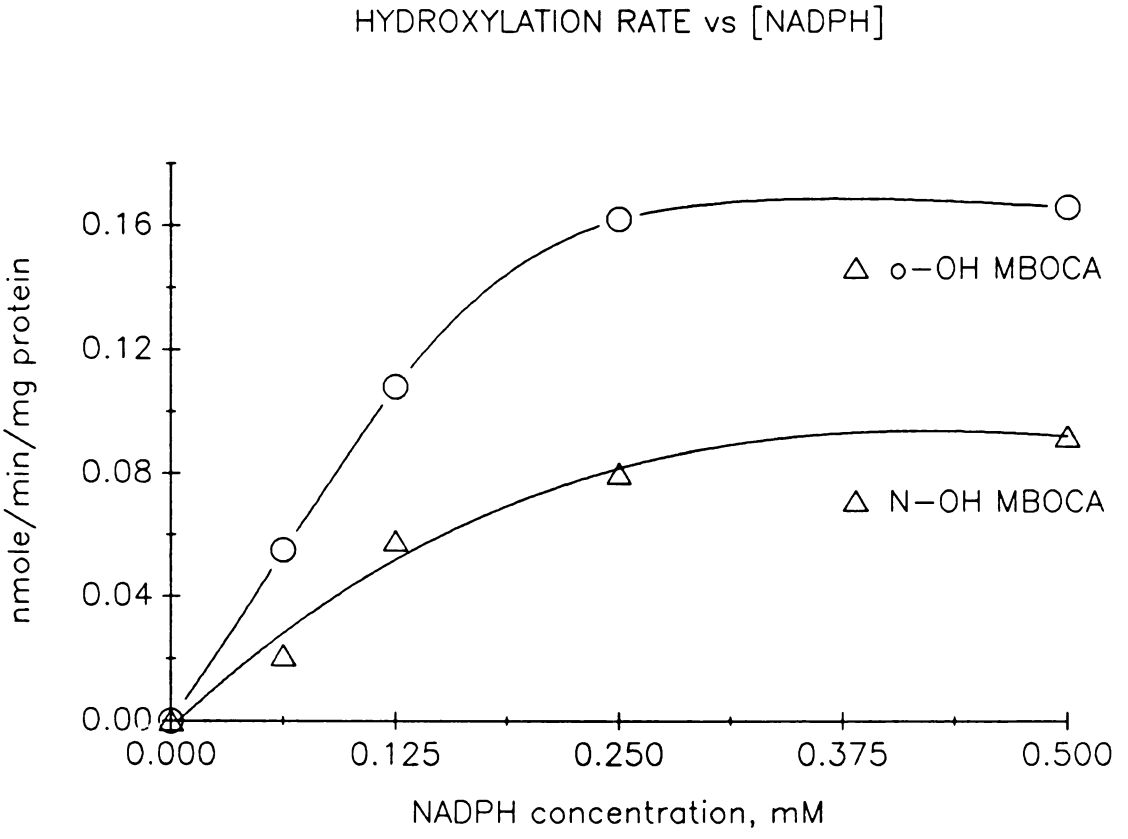

HYDROXYLATION RATE vs [NADPH]
nmole/min/mg protein
0.16
0.12
0.08
0.04
0.00
0.000
0.125
0.250
0.375
0.500
NADPH concentration, mM
o-OH MBOCA
N-OH MBOCA

Figure 7 Lineweaver-Burk plots of the hydroxylation of [B-^{14}C]MBOCA by dog liver microsomes and its inhibition by $3x10^{-5}$ mM DPEA. Following 15-min incubations, metabolites were isolated and quantified as described in the legend to figure 4.

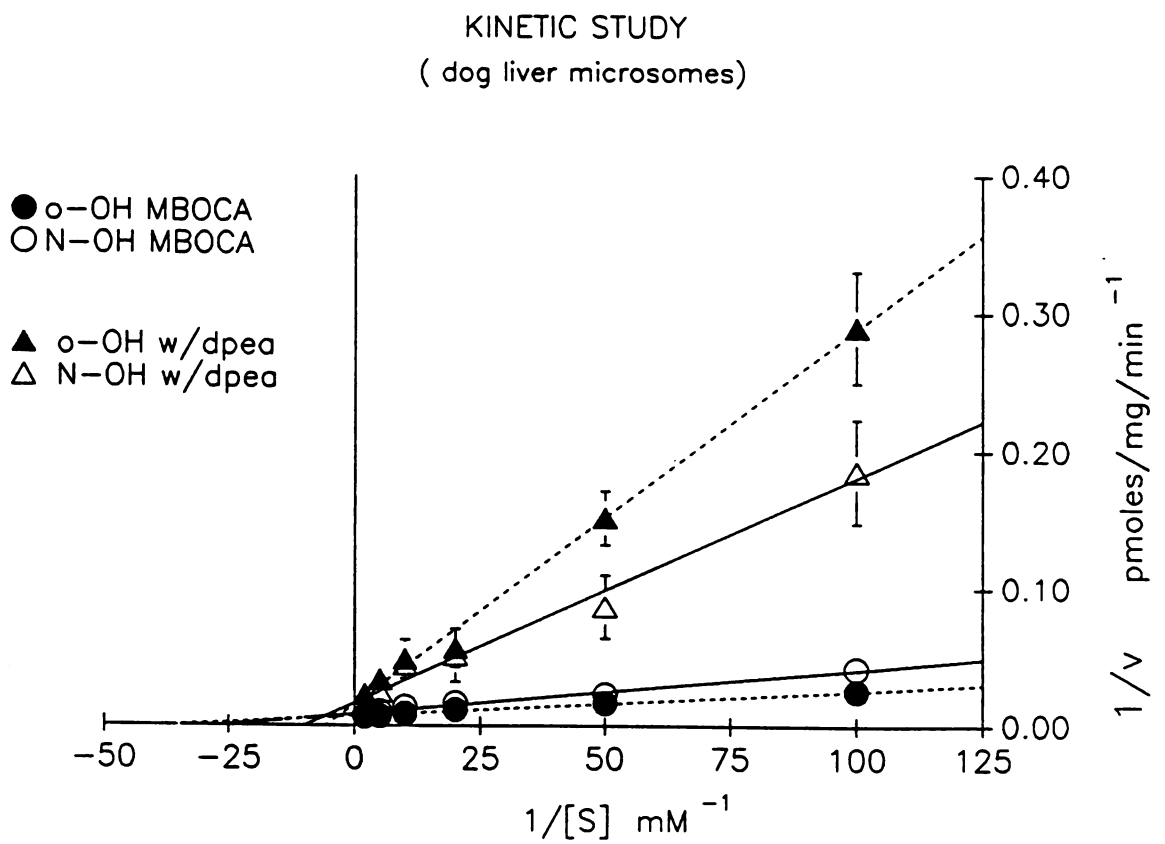

KINETIC STUDY
(dog liver microsomes)
● o–OH MBOCA
○ N–OH MBOCA
▲ o–OH w/dpea
△ N–OH w/dpea
0.40
0.30
0.20
0.10
0.00
1/v pmoles/mg/min $^{-1}$
−50
−25
0
25
50
75
100
125
1/[S] mM $^{-1}$

TABLE 1

Effect of atmosphere on formation of hydroxylated MBOCA derivatives

	N-OH-MBOCA	o-OH-MBOCA
100% O_2	8.4 ± 1.0[a]	16.8 ± 1.7
90% CO: 10% O_2	2.6 ± 0.9[b]	8.8 ± 3.3[b]
100% N_2	2.1 ± 0.9[b]	5.1 ± 1.7[b]

[a] nmol/mg
[b] different from respective control, $p < 0.05$ (Student's t test)

reduced ortho-hydroxylation by 75% and N-hydroxylation by 70%.

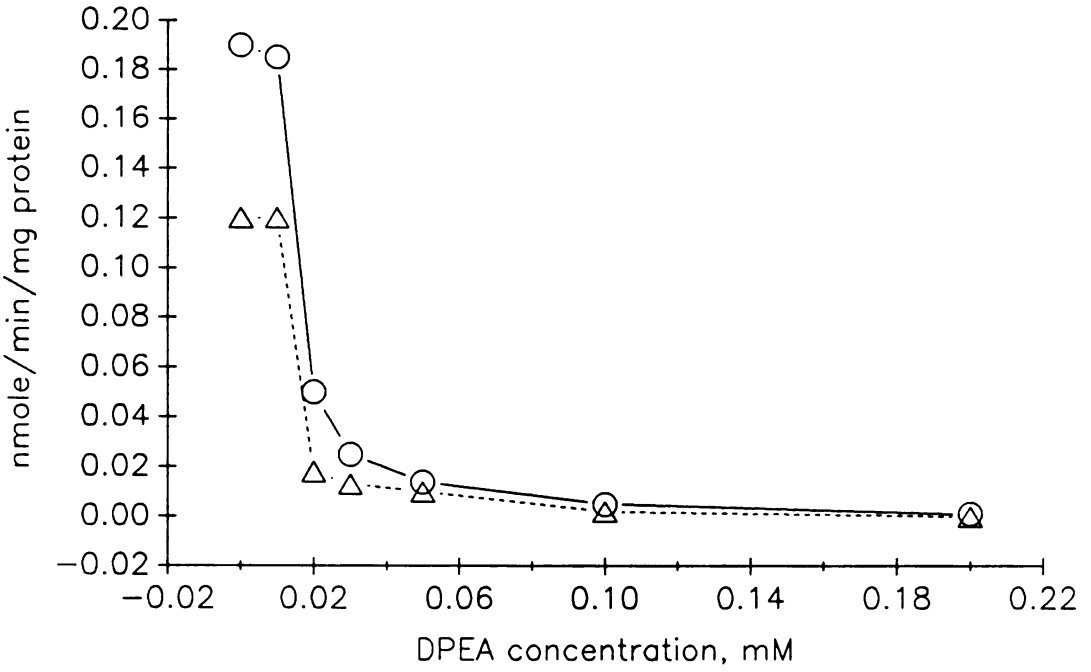
DPEA has been utilized by others as a specific inhibitor of the cytochrome P-450 enzyme system (McMahon et al., 1969; McMahon et al., 1980). In similar studies, DPEA effectively inhibited cytochrome P-450 N-hydroxylation of an imine (Parli et al., 1971), but was ineffective against the N-hydroxylation of methylaminoazobenzene, a reaction which is not cytochrome P-450 mediated (Kadlubar et al., 1976). Figure 8 indicates that DPEA was also a potent inhibitor of hydroxylation of MBOCA. MBOCA N- and o-hydroxylations were inhibited by 86% and 74%, respectively, at a concentration of approximately $2x10^{-5}$M DPEA, and by 98% at $1x10^{-4}$M DPEA. Thus, it was a more potent inhibitor of MBOCA hydroxylations than of 4-aminobiphenyl (McMahon et al., 1980) where 27-54% inhibition occurred at 10^{-4}M.

Lineweaver-Burk plots of the inhibition of N- and o-hydroxylation by DPEA are shown in Figure 7. The results indicate a competitive inhibition for both N-hydroxylation and o-hydroxylation with a similar V_{max} value of 31 pmol/mg protein/min, and apparent K_m values of 0.125 mM for o-hydroxylation, and 0.05 mM for N-hydroxylation.

Species Differences

Species differences in the formation of N- and o-hydroxyl metabolites of MBOCA between dog, rat and guinea pig are readily apparent in Figure 3. The transformation

Figure 8 Effect of DPEA concentration on dog liver microsomal hydroxylation of 55 μM [B-^{14}C]MBOCA. Following 0-30 min incubations, metabolites were isolated and quantified as described in the legend to figure 4.


0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
−0.02
nmole/min/mg protein
−0.02
0.02
0.06
0.10
0.14
0.18
0.22
DPEA concentration, mM

rates are compared in Table 2. Dog liver microsomes show a much greater capacity for ω-hydroxylation than rat and guinea pig liver microsomes, while the guinea pig microsomes formed the N-hydroxy metabolite in greatest proportion. Rat liver microsomes formed a greater proportion of additional unidentified polar metabolites.

In a separate experiment, a comparison between human, rat and dog in the formation of the N- and ω-hydroxyl metabolite was made (Figure 9). Human microsomes formed the reactive N-hydroxy metabolite at a rate greater than the rat and dog, while the ω-metabolite was formed to the least extent by human microsomes. The ω-OH metabolite was the major Phase I metabolite formed by the dog, bearing out earlier experiments. Unexplainably, the rate of formation of the N-OH metabolite for the dog was significantly less than it had been for previous experiments (compare with Figure 4). As with the other species, the formation of these two metabolites by human microsomes was dependent on NADPH (Figure 10A) and inhibited by DPEA (Figure 10B).

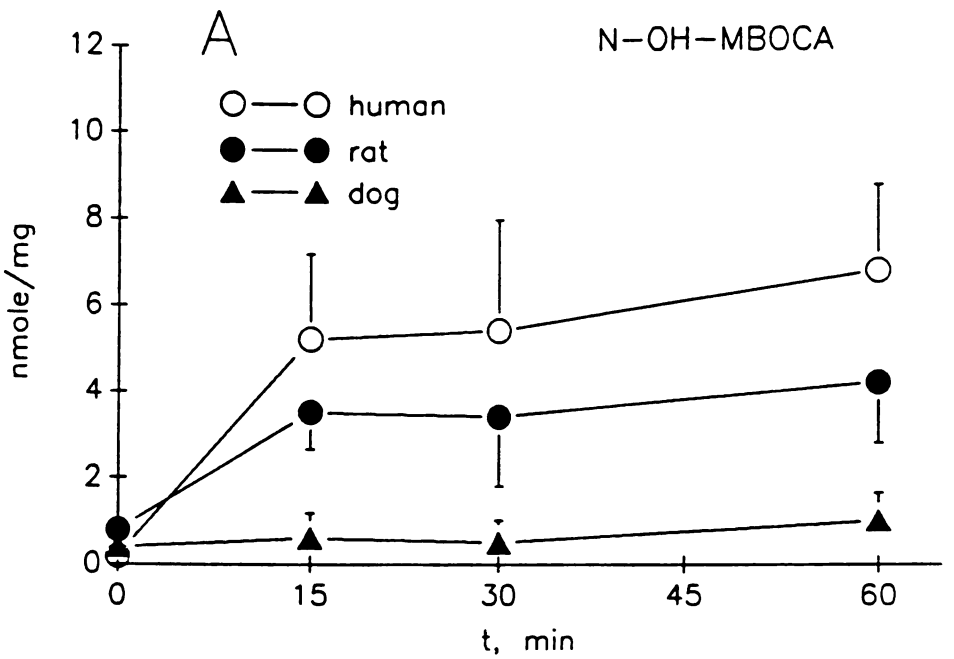
TABLE 2

Hydroxylation of MBOCA by dog, rat and guinea-pig liver microsomes

Experiments were conducted for 30 min at 37°C in 0.05 M Tris-HCl, pH 7.4 2 mM NADPH, 0.1 mM MBOCA, 5 mM $MgCl_2$ and 0.8 mg/ml microsomal protein.

	N-hydroxylation		o-hydroxylation	
Species	nmole/mg protein/min	% transformed in 30 min	nmole/mg protein/min	% transformed in 30 min
dog	0.29	9.2	0.55	18.7
rat	0.18	6.0	0.06	2.0
guinea pig	0.98	33	0.10	3.4

Figure 9 **Effect of incubation time on human, rat and dog liver microsomal A) N-hydroxylation and B) ortho-hydroxylation of 55 μM [B-^{14}C]MBOCA. Following 0-60 min incubations, metabolites were isolated and quantified as described in the legend to figure 4.**


A
N-OH-MBOCA
human
rat
dog
nmole/mg
12
10
8
6
4
2
0
0
15
30
45
60
t, min

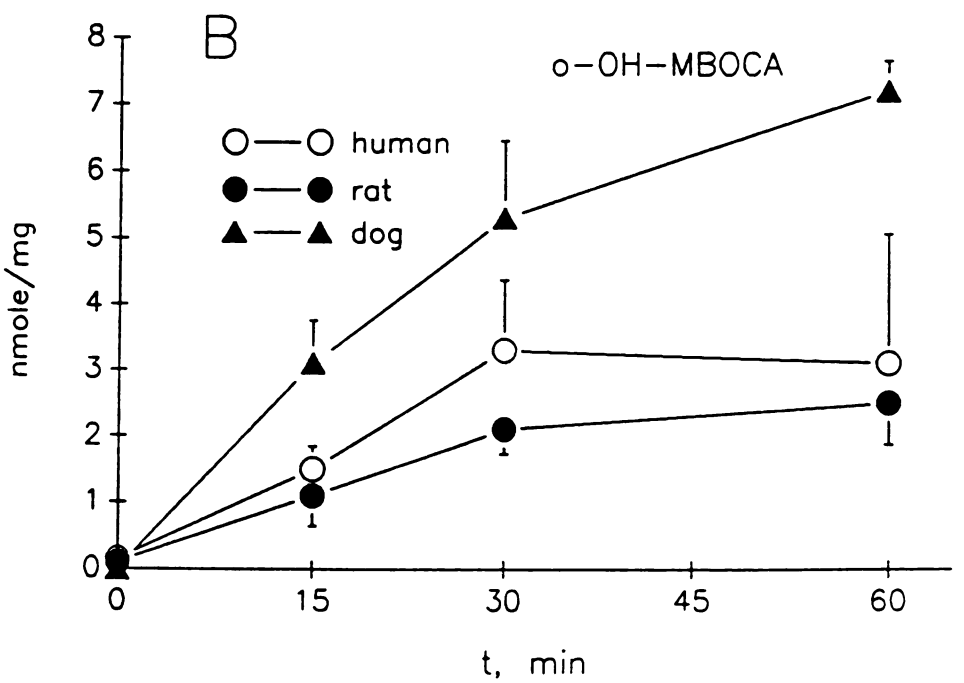
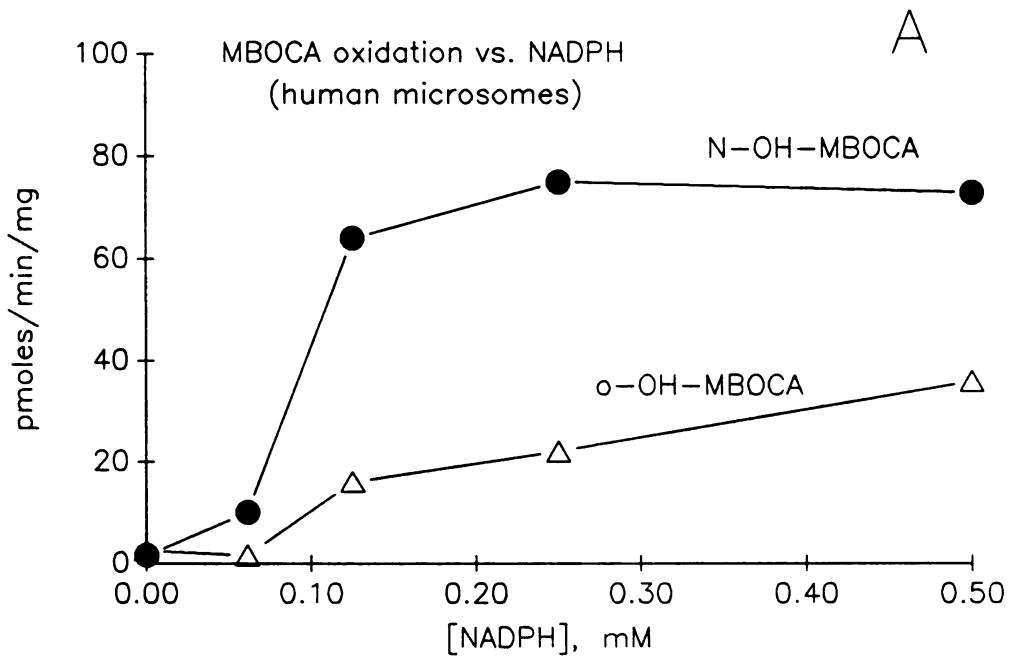
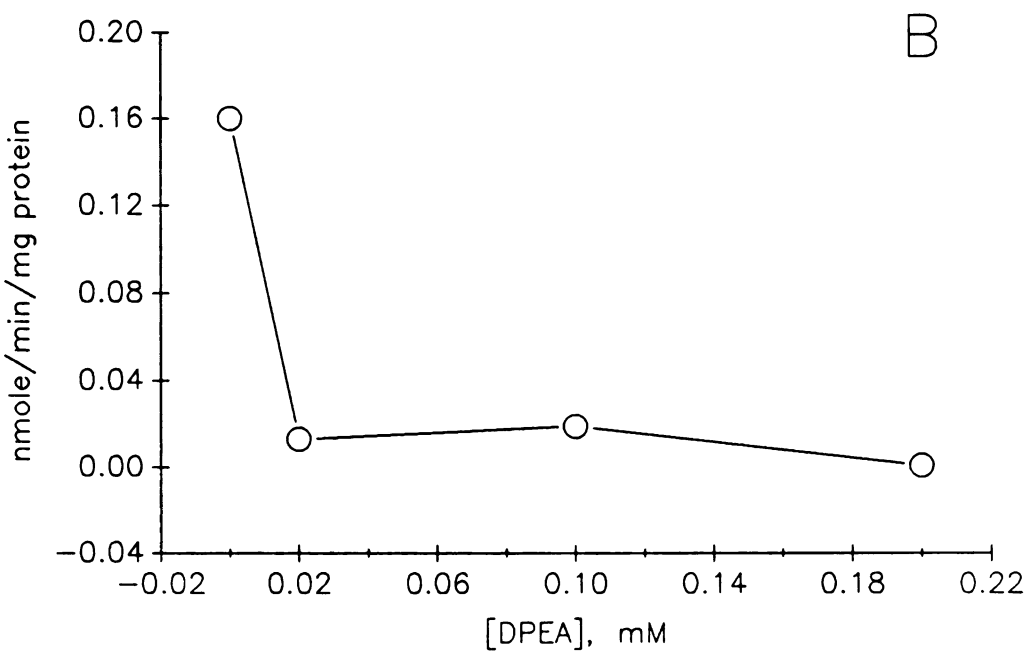

B
o-OH-MBOCA
human
rat
dog
nmole/mg
8
7
6
5
4
3
2
1
0
0
15
30
45
60
t, min

Figure 10A Effect of NADPH concentration on human liver microsomal metabolism of 55 μM [B-^{14}C]MBOCA. Following 30 min incubations with 0 to 0.5 mM NADPH, metabolites were isolated and quantified as described in the legend to figure 4.

10B Effect of DPEA concentration on human liver microsomal hydroxylation of 55 μM [B-^{14}C]MBOCA. Following 30 min incubations, metabolites were isolated and quantified as described in the legend to figure 4. Data points represent the sum of the N-OH and o-OH metabolites.


A
MBOCA oxidation vs. NADPH
(human microsomes)
N-OH-MBOCA
o-OH-MBOCA
pmoles/min/mg
100
80
60
40
20
0
0.00
0.10
0.20
0.30
0.40
0.50
[NADPH], mM


B
nmole/min/mg protein
0.20
0.16
0.12
0.08
0.04
0.00
-0.04
-0.02
0.02
0.06
0.10
0.14
0.18
0.22
[DPEA], mM

DISCUSSION

Liver microsomal preparations were used to study the formation of the major Phase I metabolites of the dog, rat, guinea pig and human *in vitro*. The compounds were characterized by their TLC and HPLC retention times. The major Phase I metabolite in the dog was shown to be 2-amino-5-[(4-amino-3-chlorophenyl)methyl]-3-chlorophenol, the aglycone of the major Phase II metabolite previously identified in canine urine (Manis and Braselton, 1984) and through *in vitro* incubations of liver and kidney slices (Manis and Braselton, 1986). While indirect evidence for the metabolic formation of some activated metabolites of MBOCA was obtained in dog liver and kidney slices (Manis and Braselton, 1986), the data presented here provide direct evidence for the enzymatic formation of the N-hydroxy metabolite. Since hepatic N-hydroxylation has been proposed as the first step in a series of essential metabolic transformations in arylamine-induced bladder carcinogenesis (Radomski and Brill, 1970; Clayson *et al.*, 1971; Poupko *et al.*, 1979; Kadlubar *et al.*, 1981), this evidence indicates the potential for MBOCA to act through a similar pathway in these species.

Kinetic data obtained with the microsomal incubations confirmed the enzymatic nature of both the N- and ring hydroxylations. The rate of formation of both metabolites was increased with incubation time, protein concentration, and substrate concentration.

The presence of NADPH and oxygen were obligatory requirements for enzymatic oxidation. In addition, the reactions were sensitive to inhibition by DPEA and carbon monoxide. DPEA and carbon monoxide are inhibitors of the cytochrome P450-dependent mixed function oxidases and are considered to be diagnostic for these enzymes distinguishing them from other amine oxidases such as flavoprotein dependent oxygenases (McMahon et al., 1980; Ziegler et al., 1973). The results, therefore, are consistent with the view that both N-and ortho- hydroxylation of MBOCA are catalyzed by the cytochrome P-450 dependent monooxygenase enzyme system.

The two major in vitro metabolites, N-OH and o-OH-MBOCA, were formed by microsomes of all four species studied, although there were significant differences in the ratio of these metabolites between species. Others have shown similar species differences in metabolism of arylamines (Radomski, 1979; McMahon et al., 1980; Gutmann and Ball, 1977). The finding here that the ring hydroxylation ortho to the amine is the major pathway in the dog confirms earlier studies with Phase II metabolites in

urine and in tissue slice preparations (Manis and Braselton, 1984; Manis and Braselton, 1986). The rat showed approximately equal formation of the N-hydroxy and o-hydroxy metabolites, with extensive formation of additional polar metabolites in this species. Although the N-OH- and o-OH-MBOCA metabolites were found in lesser amounts after incubation with rat liver microsomes, this may only reflect further oxidation of these metabolites to the more polar compounds seen on TLC. The results in the guinea pig, which indicated a predominance of N-hydroxylation, but little o-hydroxylation, were comparable to similar observations on the hydroxylation of 4-aminobiphenyl by guinea pig liver (McMahon et al., 1980).

The possibility of hydroxylation of the second amino group on MBOCA is hard to predict from these studies. Reports to date on metabolism of other diamines such as benzidine have failed to show evidence of a second N-hydroxylation.

Although species differences in the rate of formation of hydroxylated metabolites was noted in vitro, a comparison of the degree of MBOCA oxidation with the amount of tumor formation in the four species studied is not possible. First, carcinogenicity studies of MBOCA in the guinea pig, as certainly with the human, have not been done. Secondly, differences in the degree of Phase II metabolism, the rates of absorption and elimination of MBOCA, as well as in many

other variables between these species would preclude making a valid comparison.

The experiments presented here dealt with the enzymatic characterization of the microsomal metabolism of MBOCA. The results suggest that metabolism of MBOCA is similar to other arylamines such as 2-naphthylamine (Hammons et al., 1985) and 4-aminobiphenyl (McMahon et al., 1980), in that it involves the cytochrome P-450 system and that there are species differences in the relative importance in the sites of hydroxylation. These experiments also provide evidence that the hepatic microsomal enzyme systems of several species including man are capable of oxidizing MBOCA to the hydroxylamine, a highly reactive compound and potential proximate carcinogen.

CHAPTER 2

SYNTHESES AND STRUCTURAL ELUCIDATION OF OXIDIZED MBOCA METABOLITES

CHAPTER 2

INTRODUCTION

The microsomal enzyme studies presented in the previous chapter lacked unambiguous structural confirmation of the oxidized derivatives of MBOCA. Mass spectrometry was used as an initial confirmation. However, mass spectrometry could not be used to unambiguously determine the position of the oxygen atom in our proposed N-OH-MBOCA (M2) and NO-MBOCA (M3) metabolites. Further structural elucidation required the chemical synthesis of these N-oxidized compounds. The quantity of material that could only be obtained through chemical synthesis was needed in order to perform additional analyses that would yield the structural information required. Likewise, in order to successfully carry out proposed testing of the reactivities of individual Phase I metabolites, it would be necessary to have these compounds in quantities greater than could be obtained by microsomal oxidation alone. For these reasons, the chemical synthesis of these derivatives was undertaken. Additional o-OH-MBOCA could be obtained by scaled-up biosynthesis with dog liver microsomes.

The oxidation of arylamines may involve a number of pathways leading to the formation of phenylhydroxylamine,

nitroso, nitro, azo, azoxy, and benzoquinone derivatives. The relative yields of these products are determined by the nature of the oxidizing agent and the conditions of the reaction. The various modes of oxidation have been reviewed by Hedayatullah (1972). The reactions of primary arylamines with oxidizing agents proceed in two fundamentally different directions. The oxidizing agent can either donate oxygen to the arylamine molecule or it can remove hydrogen from the amino group. Reactions of the first type result in the formation of N-hydroxy-, nitroso-, or nitro-aromatics while the second type results in azobenzenes, azoxybenzenes, and quinones. When the reaction proceeds in the direction of the first type, the N-hydroxy- compound is the first that is detected. Further oxidation of this results in the nitroso- derivative, which can upon stronger oxidation yield the nitro- derivative.

Nitroso compounds can be obtained using suitable inorganic peroxy acids as the oxidizing agents. For example, oxidation of 2-nitroaniline with Caro's acid (peroxomonosulphuric acid) gives the corresponding nitroso compound (Page and Heasman, 1923). Further oxidation with nitric acid gives the nitro derivative. A solution of hydrogen peroxide in glacial acetic acid has been used successfully to generate the nitro derivative of p-toluidine (Holmes and Bayer, 1960). Oxidation to nitro or nitroso compounds can at times be accomplished using peroxy

carboxylic acids (D'Ans and Kneip, 1915). It was the peroxy carboxylic acid, m-chloroperbenzoic acid (CPBA), that proved to be a most useful oxidant of MBOCA in these studies.

METHODS AND MATERIALS

Reagents used were as described previously (chap 1). In addition, CPBA was from Aldrich Chemical Company, Inc. (Milwaukee, WI) and ascorbic acid was reagent grade from Fischer Scientific (Pittsburgh, PA).

NO- and diNO-MBOCA were synthesized by oxidation of MBOCA with CPBA based on modification of the procedures of Yost and Gutmann (1969) and Westra (1981). One gram (4 mmoles) of MBOCA (industrial purity) dissolved in 40 ml CH_2Cl_2 was cooled to 0°C and added to an ice-cold solution of 0.7 g CPBA (4 mmoles) in 40 ml CH_2Cl_2. The reaction mixture was agitated at 0°C for 5 min and the reaction then stopped by shaking with 40 ml of 0.5 N NaOH under Ar. The reaction mixture in CH_2Cl_2 was washed with 0.5 N NaOH and was further washed with N_2-purged H_2O under Ar. It was then extracted with 6 N HCl to separate the diNO-MBOCA (which remained in the CH_2Cl_2 phase) from the NO-MBOCA and MBOCA (in the aqueous phase). The aqueous phase was then neutralized using 2 N HCl and extracted with CH_2Cl_2 to isolate NO-MBOCA (in the CH_2Cl_2) from MBOCA (aqueous phase). After washing with O_2-free H_2O, the organic phases were dehydrated with anhydrous Na_2SO_4 and concentrated under vacuum to about 5 ml. Acetone (5 ml) was added and the

volume reduced under a stream of N_2. The residue in acetone was further concentrated to 1-2 ml by flushing carefully with N_2 and applied to a 2x40 cm column containing 50-60 g Kieselgel-60, 70-230 mesh. Chromatographic elution was accomplished by a mixture of hexane/acetone (10:2, v/v) with a flow rate of about 1 ml/min. Both CH_2Cl_2 phases, containing either the NO- or the diNO-MBOCA, were purified by the same chromatographic procedure. A sharp green zone containing the NO-or diNO-MBOCA was collected under Ar. Purity was assessed by TLC on K5F silica gel plates in a mobile phase of toluene/ethanol (20:1, v/v), with visualization by U.V. absorbance as fluorescence quenching. Following the Kieselgel column step above, compounds were > 90% pure.

The o-OH-MBOCA metabolite, or M1, was produced by incubation of MBOCA, dog liver microsomes, and a NADPH regenerating system scaling up the 1-2 ml incubation procedure described previously (chap 1) up to a volume no greater than 25 ml.

All derivatives were further purified by HPLC. Samples were transferred to ACN/H_2O (1:1, v/v) and applied to a Waters Associates HPLC system with a Waters 5μ Nova-pak (8mm) RCM column. The mobile phase was a 1-hr gradient from 10:90 to 75:25 ACN/0.01 M NH_4Ac, pH 6.4. Compounds were detected by U.V. absorbance at 254 and 280 nm and by radioactivity flow monitoring.

Attempts at crystallization proved largely unsuccessful, so that further chemical characterization was accomplished by mass spectrometry and proton NMR. Electron impact mass spectra were determined at 70 eV by direct insertion probe on a Finnigan 3200 mass spectrometer with Riber SADR data system. NMR spectra were obtained on a Bruker WM 250 spectrometer in the Fourier transform mode at a flip-angle of 90^{o} and a sweep width of 2500 Hz. Samples were prepared as 0.005 M solutions in $CDCl_3$ or CS_2 with 20% $CDCl_3$. Chemical shifts [δ] in parts per million were determined relative to $CDCl_3$ and converted into TMS scale using [$CDCl_3$]=7.24 ppm.

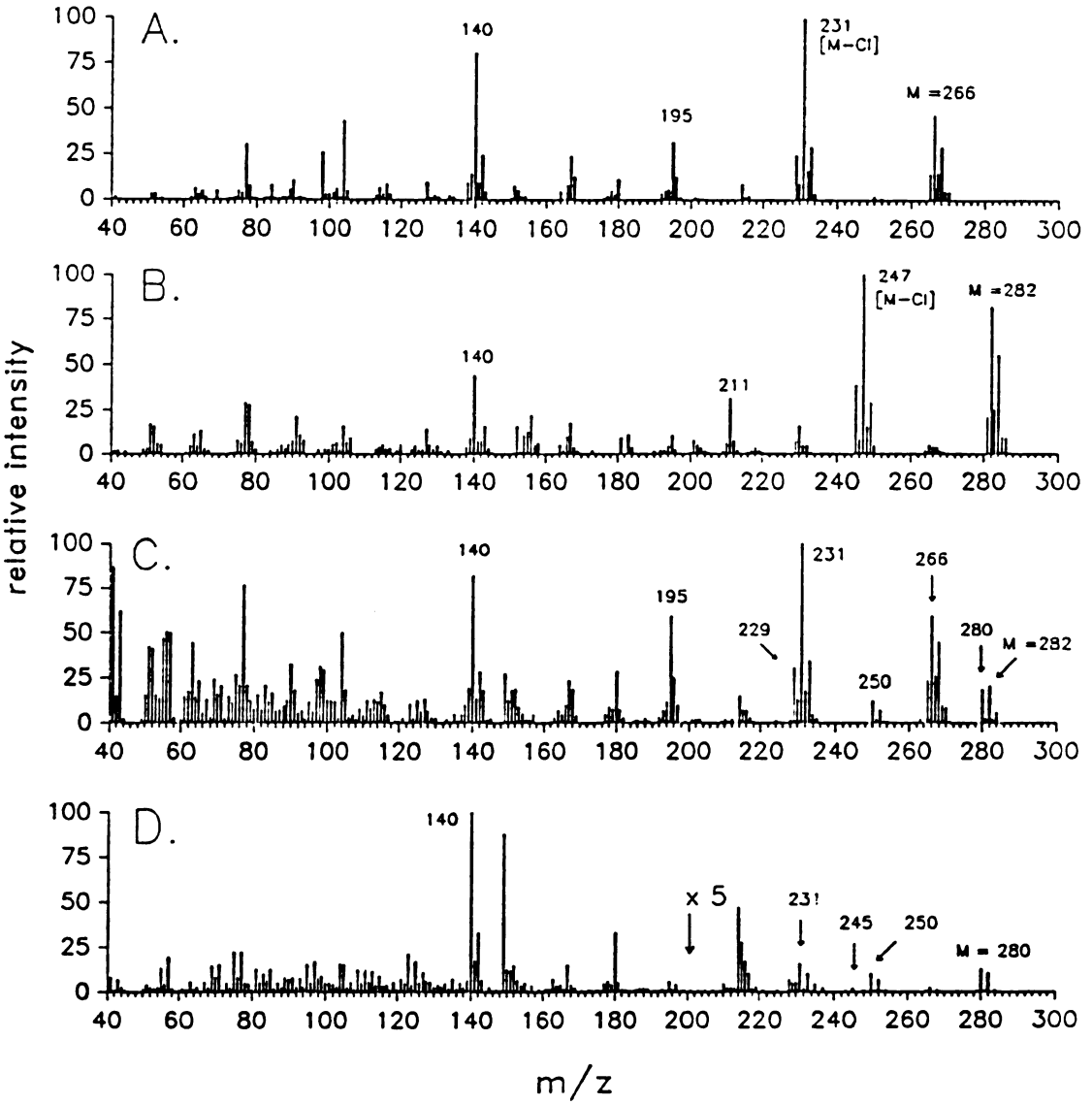
The labile NO-MBOCA was converted to N-OH-MBOCA by dissolving in an ascorbic acid saturated methanol medium. The N-OH-MBOCA was stable in this solution under Ar at -20^{o}C. NO-MBOCA could be regenerated quantitatively from the hydroxylamine by reaction with 2N HCl in methanol (1:1, v/v). CH_2Cl_2 was used to recover the compounds from the above added acids and the water miscible organic phase. Further purification by HPLC, and structure elucidation by mass spectrometry and NMR were as described above.

RESULTS

The nitroso- and dinitroso- derivatives were generated from the parent compound by reaction with the oxidant CPBA. The amount of MBOCA could be varied in scale from milligrams to several grams with a corresponding change in the amount of CPBA and in volume of CH_2Cl_2. As long as the molar ratio of MBOCA/CPBA was maintained at 1:1, the ratio of NO-MBOCA (approx. 50%) and diNO-MBOCA (approx. 30%) were constant, providing all procedures were conducted under yellow light (F40G0, gold, bug-away, North American Phillips Lighting Corp., Bloomfield, NJ) and Ar at 0°C. N-OH-MBOCA was formed by the rapid reduction of NO-MBOCA by ascorbic acid in a methanolic medium. Attempts at generating the o-OH-MBOCA derivative from N-OH-MBOCA by acid catalyzed rearrangement (Bamberger, 1921) proved unsuccessful. Scaled up incubations of MBOCA with dog liver microsomes and an NADPH regenerating system were used to produce the o-OH-MBOCA metabolite in yields of 20-40%.

The mass spectrum of the metabolite M1 is compared with the spectrum of parent MBOCA in Figure 11. The fragmentation is consistent with a compound hydroxylated on a ring carbon, with little loss of oxygen from the molecular

Figure 11 **Mass spectra of parent MBOCA and microsomal metabolites.**

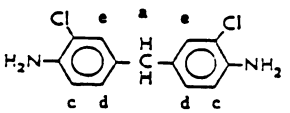
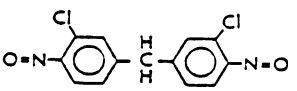
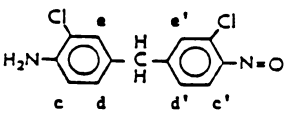
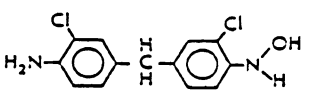
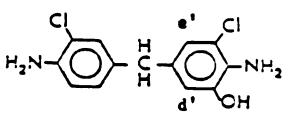

A.
140
231
[M-Cl]
195
M =266
B.
247
[M-Cl]
M =282
140
211
C.
140
231
266
195
229
280
M =282
250
D.
140
x 5
231
245
250
M = 280
relative intensity
m/z

ion (M^+=282). As in the parent MBOCA, the first major fragment occurred by loss of a chlorine from the molecular ion. The fragmentation of M1 proceeded in parallel with MBOCA, with notable ions at M-(35+36), m/z 211 and 195, respectively. A major fragment in both spectra occurred at m/z 140 (single chlorine isotope cluster), representing the chlorotoluidinium ion. Furthermore, the mass spectrum of the pentaacetyl derivative (not shown) was identical to that of the o-hydroxy-MBOCA pentaacetate (Manis et al., 1984) derived by transesterification of the o-hydroxysulfate. NMR analysis was used to confirm the position of the hydroxyl group. From Table 3, structure E, it can be seen that the methylene protons were not coupled to ring protons, and were in the aliphatic region of the spectrum, showing a singlet at 3.65 ppm consistent with the methylene bridge protons seen for all the metabolites shown. Referring to structure E from Table 3, it can be seen that protons c and d were split by 8.2 Hz, typical of benzylic coupling. Proton e' was downfield from proton d' being deshielded by the chlorine adjacent to e'. Both were split by 1.8 Hz, indicating that they were meta to each other. This second spin system is made up of only two protons, which confirmed that the hydroxyl constituent was ortho to the amine, and consistent with the proposed structure.

The mass spectrum of metabolite M2 is also shown in Figure 11. Characteristic fragments are the molecular ion,

Table 3

Proton NMR of MBOCA and oxidative metabolites

		chemical shifts (ppm)	coupling constants (Hz)
A) MBOCA		a 3.707 c 6.660 d 6.861 e 7.062	J(c,d) 8.1 J(d,e) 2.0
B) diNO-MBOCA		a 4.043 c 6.217 d 7.024 e 7.578	J(c,d) 8.3 J(d,e) 1.7
C) NO-MBOCA		a 3.855 c 6.725 d 6.858 e 7.048 c' 6.181 d' 6.981 e' 7.520	J(c,d) 8.1 J(d,e) 2.0 J(d',c') 8.3 J(e',d') 1.7
D) N-OH-MBOCA		a 3.748 c 6.656 d 6.827 e 7.005 c' 7.187 d' 7.025 e' 7.028	J(c,d) 8.1 J(d,e) 2.0 J(d',c') 8.6 J(e',d') 1.7
E) o-OH-MBOCA		a 3.650 c 6.669 d 6.831 e 7.013 d' 6.400 e' 6.693	J(c,d) 8.2 J(d,e) 2.0 J(e',d') 1.8

M^+=282, and the ions at m/z 280 ($M-H_2$), m/z 266 (M-16), and m/z 250 (M-32) which are distinctive of N-hydroxy arylamines (Coutts and Mukherjee, 1970; Iorio et al., 1985; Saito et al., 1985). The fragmentation then proceeds to give the same sequence of ions as the parent MBOCA, with characteristic fragments at m/z 231, 229, 195 and 140. The mass spectrum was identical to that of the synthesized N-OH-MBOCA. Likewise the TLC R_f and HPLC retention time indices (Table 4) were identical. This showed conclusively that M2 and the synthesized hydroxylamine were the same compound.

NMR was used to confirm the structure of the synthesized compound. The NMR spectrum showed a complex pattern such that computer simulation techniques were required to resolve the individual spectra of the two ring spin systems. Figure 12 shows the actual and the computer resolved spectra for this compound. Spectrum D shows the strongly coupled spin system of the 2-chloro-N-hydroxyaniline ring. Spectrum C is of the 2-chloroaniline ring, showing moderate second-order coupling effects. The simulated spectra C and D are added in spectrum B for direct comparison with the experimental spectrum A. In the first spin system (spectrum C), proton e is deshielded by the adjacent chlorine and is downfield from d, both split at 2.0 Hz (Table 3, structure B) consistent with benzylic meta coupling. Furthermore, protons d and c were split at 8.6 Hz indicating benzylic ortho coupling. In the strongly coupled

TABLE 4

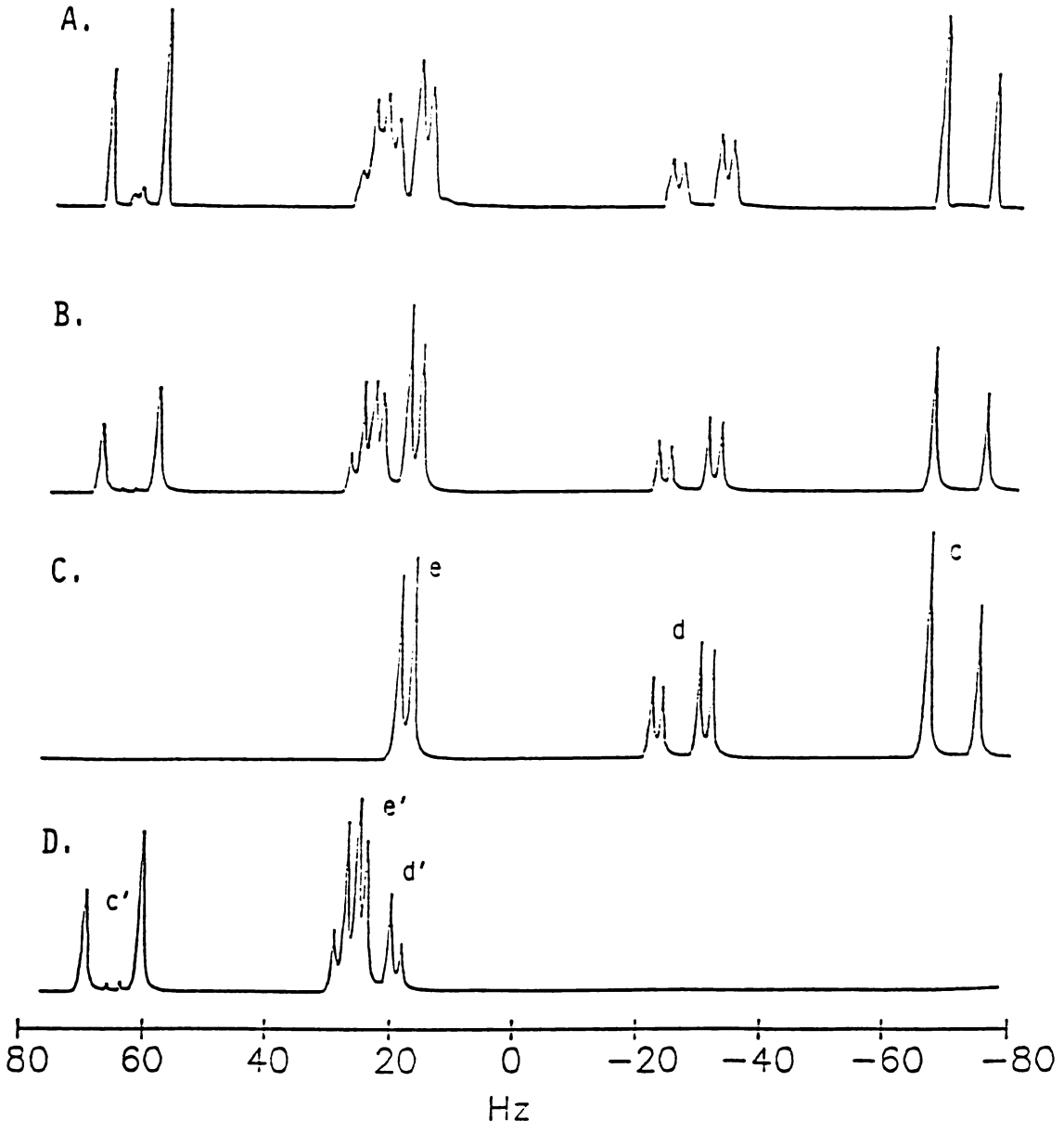
HPLC of MBOCA and oxidative metabolites

HPLC was carried out on a Waters 5μ C_{18} Nova-Pak (8 mm) radial compression column with a 1-hr gradient from 10:90 to 75:25 ACN/0.01 M NH_4Ac, pH 6.4. RI_N was calculated using alkylphenone homologs.

Compound	R_t	S.D.	RI_N	S.D.
MBOCA	42.1[a]	0.32	1043	1.3
M1	38.2[a]	0.54	977	12.8
N-OH-MBOCA	37.2[b]	0.34	961	0.52
M2	37.3[c]		961	
NO-MBOCA	50.2[b]	0.11	1176	1.15
M3	50.2[c]		1177	
diNO-MBOCA	56.0[b]	0.09	1262	0.58

[a]mean, N=4
[b]mean, N=3
[c]N=1

Figure 12 Comparison of experimental and simulated NMR spectra of newly synthesized N-OH-MBOCA. A) experimental spectrum of N-OH-MBOCA B) combined computer simulated spectra C and D C) computer simulated spectrum of the 2-chloroaniline ring D) computer simulated spectrum of the strongly coupled spin system of the 2-chloro-N-hydroxyaniline ring.


A.
B.
C.
e
d
c
D.
e'
d'
c'
80
60
40
20
0
−20
−40
−60
−80
Hz

spin system (spectrum D), computer simulation techniques indicate proton c' is highly deshielded by the N-hydroxy group on this system and is split 8.6 Hz along with proton d' indicating benzylic ortho coupling. Protons e' and d' were split at 1.7 Hz indicating benzylic meta coupling. NMR was therefore used to account for all the ring protons in this oxidized metabolite. The combination of mass spectrometry and NMR conclusively show that this compound is hydroxylated on the amine.

Degradation of N-OH-MBOCA also led to formation of a compound with TLC R_f 0.64, which was very close to that of the parent MBOCA (R_f 0.67). The compound was identified as azoxy-MBOCA by mass spectrometry, which revealed a molecular ion at M^+=544, with an isotope cluster indicative of four chlorines. Prominent fragments occurred at m/z 528 (M-16), 509 (M-35), 250, and 140.

A metabolite, M3, was often observed at R_f 0.83 upon TLC of microsomal incubation extracts. The metabolite was also observed in samples of the N-OH-MBOCA that were allowed to degrade, and could be readily generated from N-OH-MBOCA by oxidation with potassium ferricyanide. The TLC R_f and HPLC retention index (RI_N) (Table 4) were identical to those of authentic NO-MBOCA. The identity was confirmed by the mass spectrum (Figure 11), which was the same as that of the synthesized compound, with M^+=280, a characteristic loss of 30 a.m.u. (NO) at m/z 250, and fragment ions at m/z 245 (M-

Cl), 231, and 140 (Figure 11). NMR was also used to confirm the structure of the synthesized NO-MBOCA (Table 3) Protons c and d were split by 8.1 Hz, typical of benzylic ortho coupling. Protons d and e were split by 2.0 Hz consistent with benzylic meta coupling. In the same fashion, protons d' and e' could be seen to be meta coupled with a split of 1.7 Hz and protons d' and c' were split by 8.3 Hz, again indicating benzylic ortho coupling. These data are consistent with a compound containing two spin systems, with three distinct protons on each system, and in conjunction with the mass spectrum confirm the structure as the NO-MBOCA.

A side product of the synthesis of NO-MBOCA was a compound with an apparent molecular ion at M^+=294, suggesting the diNO-MBOCA derivative. Compatible with this was a loss of 30 a.m.u. at m/z 265, and the base peak (single chlorine isotope cluster) at m/z 199 (M-[2x30+35]). The structure was confirmed by NMR (Table 3). The data show that protons c and d are split by 8.3 Hz and protons d and e are split by 1.7 Hz, indicating benzylic ortho and meta coupling, respectively. These data are consistent with a symmetrical two ring spin system and taken together with the mass spectral data confirm the proposed structure for this compound. No evidence of the diNO-MBOCA metabolite was seen in microsomal incubation extracts by TLC or HPLC.

DISCUSSION

The mono- and di- nitroso derivatives of MBOCA were successfully synthesized by oxidation with CPBA. The N-OH-MBOCA derivative was formed by reduction of isolated NO-MBOCA with ascorbic acid. Saito et al. (1983a) were unable to oxidize arylamines derived from the pyrolysis of tryptophan and glutamic acid using H_2O_2 or CPBA and found it necessary to include the catalyst Na_2WO_4 in order to oxidize these compounds to nitro-derivatives. Partial oxidation of arylamines to nitroso- or hydroxylamines is not always easily achieved and a partial reduction of the nitro-derivative must be included to generate the desired nitroso-compounds. Partial reduction of aromatic nitro compounds to the corresponding hydroxylamines or nitroso compounds is usually difficult, producing low yields of the desired derivative. N-hydroxyfluorene was obtained by careful reduction of 2-nitrofluorene with alkaline hydrogen sulfide (Lotlikar et al., 1965). Westra et al. (1981) successfully achieved catalytic reduction to yield several arylhydroxamic acids using hydrazine hydrate and Pd/C at low temperatures. The successful partial oxidation of MBOCA then was indeed fortuitous in that it circumvented the need of a partial

reduction of a nitro- derivative.

Attempts at synthesis of the o-OH-MBOCA from N-OH-MBOCA through a rearrangement of the oxygen proved less successful. This type of intramolecular rearrangement of a hydroxylamine in dilute aqueous sulfuric acid was first described by Bamberger (1921). Heller et al. (1951) suggest that the preferred position of rearrangement is at the para position yielding the iminoquinol and the quinol of the original para substituted hydroxylamine. This may have been the preferred molecular rearrangement of MBOCA as well which would explain the failure in generating the o-OH-MBOCA derivative.

The structural analysis of the MBOCA derivatives, using NMR and mass spectrometry, conclusively proves that the structures for these compounds are as presented. Metabolites M2 and M3 isolated from microsomal incubations are identical to chemically synthesized N-OH-MBOCA and NO-MBOCA as shown by mass spectrometry, TLC and HPLC results.

CHAPTER 3

DIRECT MUTAGENICITIES OF MBOCA DERIVATIVES TOWARDS *SALMONELLA TYPHIMURIUM* TA98 AND TA100

CHAPTER 3

INTRODUCTION

The *Salmonella* mutagenicity assay developed by Ames and coworkers (Ames *et al.*, 1972; Ames *et al.*, 1975; and McCann *et al.*, 1976) has been used by many regulatory agencies and testing laboratories to screen, rapidly and inexpensively, large numbers of chemicals for potential carcinogens. This assay, as well as other short term mutagenicity tests, had been gaining widespread use due to the increase in supporting evidence of the somatic theory of carcinogenesis (Crawford, 1979; Straus, 1981). The ability of the *Salmonella* mutagenicity assay to predict whether or not an agent was likely to be an animal carcinogen was initially reported as quite good. This means that there were few reported false negatives (good sensitivity) and few false positives (good selectivity). The assay was reported to have a sensitivity of 90% and a selectivity of around 87%. (Ames *et al.*, 1973a; McCann *et al.*, 1975; Purchase *et al.*, 1976). Purchase *et al.* (1976) however, did report that a large number of false positives were possible and suggested the use of specific controls to monitor possible changes in assay conditions. It has been suggested that these early reported correlations were based on biased data (Zeiger,

1985). The investigators knew the identities of the chemicals and their "expected" mutagenicity, and so the test procedures could be modified until the desired results were obtained. A more recent evaluation of the Salmonella mutagenicity assay reported correlations of as low as 60% (Tennant et al., 1987). This study found that with a battery of four different short term mutagenicity tests, no complementarity could be achieved in predicting chemical carcinogenicity. These four assays included: the Salmonella mutagenicity assay, the assays of chromosome aberration and sister chromatid exchange induction in Chinese hamster ovary cells, and the mouse lymphoma L5178Y (MOLY) cell mutagenesis assay. Morevover, no improvement over the use of the Salmonella mutagenicity test alone could be seen. Recent thinking, therefore, has been to deemphasize short term mutagenicity testing in the risk assessment of new chemicals.

Many chemicals that are toxic to animals are not in themselves toxic, but must be metabolized to toxic forms. One disadvantage with working with a bacterial system is that it lacks mammalian metabolic enzymes. To overcome this shortcoming, the standard Salmonella mutagenicity assay employs a mammalian metabolic activation system (usually the 9,000 g supernatant fraction or S-9 of liver) from rats treated with Aroclor 1254. Modifications can be made to this metabolic system to study the enzymatic pathways

important in the activation of chemicals. Also, the test can be modified to study structural requirements of a chemical class necessary for activation. For example, Bartsch et al. (1977) found a lack of correlation between the comparative electrophilicity and mutagenicity of a series of highly lipophilic N- and O-acyl derivatives of N-hydroxy-aminofluorene. Nelson and Thorgeirsson (1976) studied the relationship between the structure of a series of acylaminofluorenes and their mutagenicity in the Salmonella system. Saito et al. (1983b) compared the mutagenic activities of synthesized hydroxyamino, nitroso and nitro derivatives of amino acid pyrolysate mutagens. Though the Salmonella mutagenicity assay has limited usefulness as a predictor of carcinogenicity, it can be used successfully for a better understanding of the molecular events critical to mutagenesis. A positive result in this assay confirms the capability of a compound or a bioactivated species to bind to DNA. The testing of the potential of oxidized metabolites to bind to DNA, without further metabolic activation, in something more than a direct in vitro DNA binding study was the intent of the present study. It must be remembered, however, that bacterial mutagenesis may not reflect the molecular mechanisms important in mammalian mutagenesis (Trosko, 1984; Trosko, 1988). Reasons may include availability of DNA for binding, and differences in DNA-adduct repair mechanisms

between the bacterial and the mammalian systems.

MBOCA has been interpreted as genotoxic to mouse and hamster hepatocytes (McQueen et al., 1981) and positive in the Balb C 3T3 mouse embryo cell transformation assay (U.S. Dept. Health, 1983). MBOCA binds to DNA in explant cultures of dog and human bladder (Shivapurkar et al., 1987). MBOCA has been shown to be mutagenic in two assays. It is positive in the mouse lymphoma assay (eukaryotic, forward mutation) (U.S. Dept. Health, 1983), and positive in the Salmonella mutagenicity test in a highly strain-dependent manner (McCann et al., 1975a). MBOCA or its activated metabolites appear to be capable of binding with DNA, although other explanations of genotoxicity have been suggested that do not include direct interaction with DNA (Trosko, 1984). The requirement of a mammalian liver enzyme activation mixture (S-9) for MBOCA to yield a positive result in this bacterial system, suggested studies directed toward the assessment of the mutagenic potential of individual MBOCA metabolites.

Some testing of the direct mutagenicity of metabolites of MBOCA has been done. Hesbert et al. (1985) chemically prepared the mono- and di-acetates of MBOCA and tested their activity in the Salmonella mutagenicity assay. These compounds were detected in the urine of occupationally exposed individuals (Ducos et al., 1984). The acetate derivatives were tested with strain TA100 with and without

metabolic activation. This study confirmed previous work which showed that MBOCA requires metabolic activation to give a positive result in this mutagenicity assay. In addition, it was found that, as with MBOCA itself, the acetate derivatives were not mutagenic without rat liver S-9. In the presence of S-9, reversion was highest with MBOCA, intermediate with N-acetyl-MBOCA, and lowest with N,N'-diacetyl-MBOCA. The o-hydroxy MBOCA sulfate metabolite, the major urinary metabolite found in the dog, did bind to protein and DNA *in vitro* upon hydrolysis with arylsulfatase (Manis, 1984), which suggests that the o-OH-MBOCA metabolite is a reactive compound which is detoxified after conjugation with sulfate. However, the o-hydroxy MBOCA sulfate was not mutagenic in strain *S. typhimurium* TA1538 under conditions that would hydrolyze the sulfate. These earlier studies therefore were unable to identify an activated mutagenic intermediate.

Synthesis of the oxidative metabolites of MBOCA (chap 2) made it possible to test for the direct mutagenicity of these compounds in the *Salmonella* assay. The hypothesis was that oxidized MBOCA metabolites are chemically more reactive than the parent compound, and that this reactivity will be reflected in their mutagenicity. A modified *Salmonella* mutagenicity assay without a metabolic activation system was used to test this hypothesis.

METHODS AND MATERIALS

Synthesis of N-oxidized metabolites

N-OH-MBOCA, diNO-MBOCA and NO-MBOCA were synthesized by oxidation of parent MBOCA with CPBA. The o-OH-MBOCA metabolite was extracted and purified from microsomal incubations as previously described (chap 2).

Bacterial Strains

On the basis of previous reports indicating that the mutagenic effectiveness of MBOCA and arylamines in general are fairly strain-dependent (Ames et al., 1973a,b; McCann et al., 1975a; Bos et al., 1982), the mutant strains of TA98 and TA100 were selected. These tester strains are S. typhimurium LT2 histidine-deficient auxotrophs which were supplied by Dr. B.N. Ames, University of California, Berkeley. The auxotrophs are converted to prototrophy by mutagenic agents that cause predominantly base-pair substitution (TA100) or frame-shift mutation (TA98) at G-C sites (Ames et al., 1975; Maron and Ames, 1983). These two of the four recommended standard strains carry the pKM101 plasmid which is absent in strains TA1535 and TA1537 (McCann et al., 1975b). The plasmid appears to promote cellular processing of DNA damage by an error-prone repair system,

thereby enhancing mutational events. One effect of this plasmid however is that TA100 is no longer specific for base-pair substitution mutagens but is reverted to some extent by frame-shift mutagens as well (Maron and Ames, 1983).

Mutagenicity tests

Oxidized derivatives of MBOCA were tested for their direct mutagenicity with the Salmonella mutagenicity assay according to the method of Maron and Ames (1983). Briefly, the test chemical in 0.1 ml of acetonitrile and 0.1 ml of an overnight culture of cells were preincubated at 37^{o}C with light shaking for 10 min before adding 2.0 ml of overlay agar. An aliquot of the overnight culture was diluted 10^{6} and 10^{7} fold with phosphate buffered saline and plated in triplicate on nutrient broth plates for a colony count. Overlay agar was allowed to solidify and plates were incubated for 48 hr at 37^{o}C. At the end of the incubation period, revertant colonies per plate were counted. Concurrent solvent controls were performed. To confirm the identity of the bacterial strain, sodium azide and 4-nitro-o-phenylenediamine were used as positive controls with no activating systems. Toxicity was indicated by a thinning or loss of the background lawn of his^{-} bacteria, and by a reduction in the number of his^{+} revertant colonies compared to the solvent control. Each chemical or control solvent was assayed with 3 to 4 plates at each dose level. The

results expressed as revertants/plate are means from 3-4 experiments ± S.E.M.

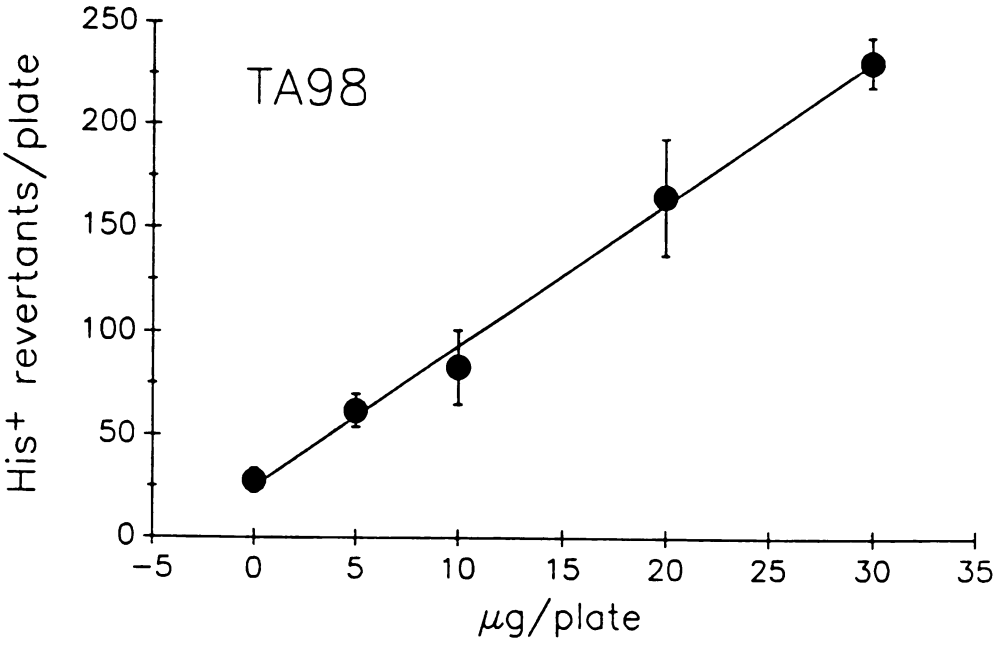
Statistical Analysis

Differences in the results were analyzed statistically by the Scheffe's test, taking $p < 0.05$ to be the level of significance. A linear regression equation was estimated by the method of least squares for mutagenic responses deemed to be linear.

RESULTS

The mutagenicities of four oxidized derivatives of MBOCA were assayed without added activating systems. In these studies, the frame-shift sensitive strain TA98 and the base-pair substitution sensitive strain TA100 were used. The increase in the formation of mutant colonies in both strains using N-OH-MBOCA as the test substance was regarded as approximately linear (Figure 13). A linear regression equation for each set of results was estimated by the method of least squares. The slopes of the dose response curves were 7.6 rev/μg (2 rev/nmol) for strain TA98 and 80 revertants/μg (21 rev/nmol) for strain TA100. The correlation coefficients for these curves were 0.974 and 0.963 for TA98 and TA100, respectively. The value of 2 rev/nmol for the mutagenicity of N-OH-MBOCA on strain TA98 compares with a value of 15 revertants/nmol for phenylhydroxylamine (Suzuki et al., 1987) and 60,000 revertants/nmol for the hydroxylamine derivative of the amino acid pyrolysate of tryptophan (Trp-P-2) (Saito et al., 1983b). The mutagenic activity of NO-MBOCA towards TA100 (Table 5) appeared to be masked by its toxicity towards this strain. At a level of 50 μg/plate (90 nmol/plate), the

Figure 13 Direct mutagenicity of N-OH-MBOCA with the two mutant strains of Salmonella, TA98 and TA100. The number of revertant colonies/plate were determined in the presence of 0-40 μg/plate of N-OH-MBOCA. Each point represents the mean value ± S.E.M. of 3 independent experiments with 3-4 plates each.


TA98
His+ revertants/plate
250
200
150
100
50
0
−5
0
5
10
15
20
25
30
35
μg/plate

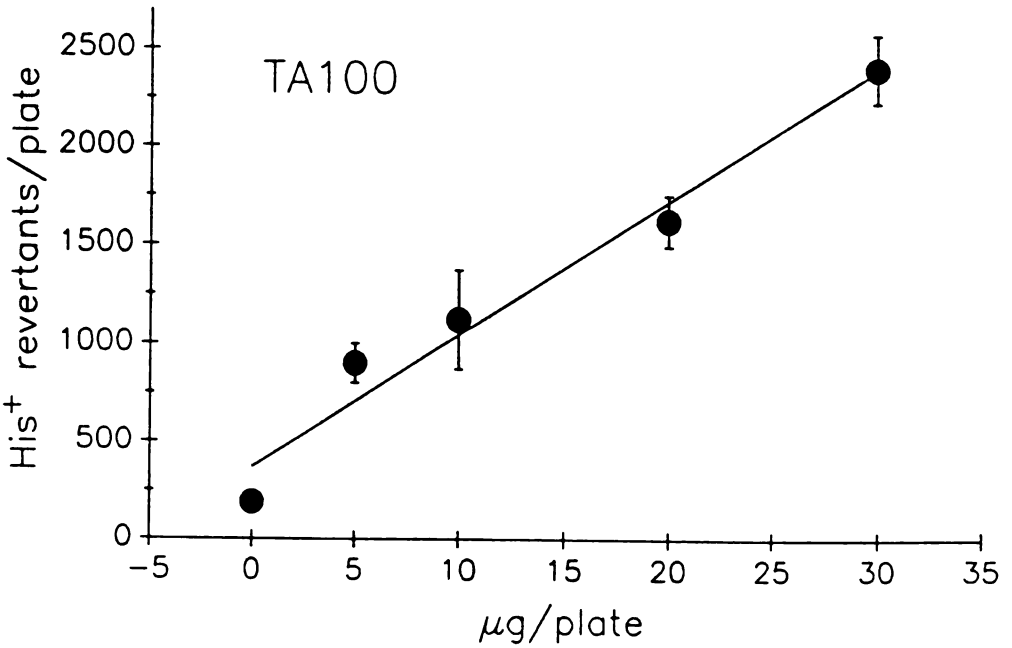

TA100
His+ revertants/plate
2500
2000
1500
1000
500
0
−5
0
5
10
15
20
25
30
35
μg/plate

TABLE 5

Compounds (μg/plate)		TA98: Number of revertants/plate	TA100: Number of revertants/plate
o-OH-MBOCA	0	32.0 ± 5.2	134.6 ± 15.7
	5	38.2 ± 8.3	135.0 ± 17.3
	10	39.6 ± 9.1	142.4 ± 13.5
	20	29.2 ± 3.1	152.6 ± 6.1
	50	32.0 ± 2.9	140.5 ± 10.6
diNO-MBOCA	0	34.9 ± 2.1	166.1 ± 12.7
	10	30.1 ± 2.9	172.3 ± 23.0
	50	26.2 ± 3.3	158.5 ± 13.5
	100	38.3 ± 2.8	148.0 ± 16.2
	500	25.4 ± 5.0	152.0 ± 7.5
NO-MBOCA	0	34.7 ± 5.8	124.3 ± 12.0
	1	37.3 ± 3.8	122.5 ± 4.6
	5	39.3 ± 7.6	116.1 ± 18.7
	10	40.0 ± 12.5	150.4 ± 12.3
	50	50.0 ± 11.1	206.7 ± 23.5[a]
	100	41.0 ± 5.2	20.0 ± 15.0[a]

[a]Curve differs form control at $p < 0.05$ level of significance

number of revertants was significantly increased over the number of revertants seen with the lesser doses or controls. However, at 100 μg/plate (180 nmol/plate), the number of revertants was significantly less then the number of spontaneous revertants seen in controls. In addition, the 100 μg/plate set had a noticeable thinning of the bacterial "lawn" which indicates a decrease in the number of his^- colonies. These two results indicate that the NO-MBOCA derivative was toxic at these levels. Interestingly, this derivative was neither mutagenic nor toxic to strain TA98 at the same concentrations. The results of the other oxidized derivatives of MBOCA can be found in Table 5. The o-OH-MBOCA derivative, which can be detected after microsomal incubation with MBOCA, and the diNO-MBOCA derivative, which is not formed by liver microsomes, were both negative for mutagenicity at the concentrations tested. The upper concentration of o-OH-MBOCA was limited by the availability of this derivative.

DISCUSSION

MBOCA has been shown by others to be positive in the Ames test only with metabolic activation (McCann *et al.*, 1975a; Hesbert *et al.*, 1985). This study is the first to show that the N-OH-MBOCA metabolite is a potent mutagen. This result is consistent with the findings others have obtained with the N-hydroxylated metabolites of other arylamines (Saito *et al.*, 1983b; Scribner *et al.*, 1979; Weeks *et al.*, 1978).

That the NO-MBOCA and diNO-MBOCA derivatives failed to show a clear positive response is surprising. NO-MBOCA is easily reduced by ascorbic acid to N-OH-MBOCA (chap 2). The nitroso derivatives of the amino acid pyrolysates tryptophan and glutamic acid are easily reduced to their respective hydroxylamines by ascorbic acid, NADPH, and reduced glutathione (Saito *et al.*, 1983a). Saito *et al.* (1983b) found that the positive mutagenic response with these nitroso-derivatives in *S. typhimurium* TA98 and the nitroreductase deficient strain TA98NR closely paralleled the response seen with the corresponding hydroxylamines. The mutagenic action of the nitroso- compounds was postulated to be indirect. Reduction to the hydroxylamine

by nonspecific materials in the bacteria was suggested to be responsible for the action of these derivatives. In contrast, the nitroso- derivatives of MBOCA did not give a clear response. The diNO-MBOCA derivative was clearly negative up to the highest dose tested (890 nmol/plate) in both strains. The NO-MBOCA metabolite was negative in strain TA98 up to a dose of 180 nmol/plate, while in TA100, the mutagenic response which began to appear at 90 nmol/plate was masked by a toxic response at 180 nmol/plate. It would appear therefore that either the nitroso-derivatives of MBOCA were not activated in the same manner as nitroso- derivatives of other compounds, or that they had failed to reach target DNA sites.

The o-OH-MBOCA metabolite was not positive at the concentrations tested in this study. These results are consistent with previous mutagenicity testing of the sulfate conjugate after arylsulfatase hydrolysis (Manis, 1984). This result is also consistent with reports that suggest that ortho-hydroxylation of arylamines is not responsible for mutagenic activity in the Salmonella mutagenicity assay (Scribner et al., 1979).

In summary, this study suggests that oxidation of MBOCA can lead to a reactive intermediate. The results are consistent with the responses seen with oxidized derivatives of other arylamines whereby N-hydroxylation leads to a potent mutagenic species and ring or C-oxidation of

arylamines is usually a detoxification pathway. Unlike the corresponding derivatives of other arylamines, the mono- and di-nitroso products of MBOCA failed to definitively exhibit a mutagenic response.

CHAPTER 4

EFFECT OF TREATMENT OF MBOCA DERIVATIVES ON GAP-JUNCTIONAL COMMUNICATION IN RAT LIVER EPITHELIAL, WB, CELLS.

CHAPTER 4

INTRODUCTION

Studies in animals and in humans indicate that most tumors evolve through a multi-step process that can take a considerable part of the lifespan of the species. In some instances the process can be divided into at three stages, initiation, promotion, and progression. Even these stages are probably divisible into substages (Foulds, 1975; Hecker, et al., 1982; Slaga et al., 1978). These steps are also apparent during the transformation of cells in culture, whether the process is induced by chemical carcinogens or certain oncogenic viruses (Barrett and Ts'o, 1978; Fisher et al., 1979; Morris, 1981).

Tumor promoters can be defined as compounds which have very weak or no carcinogenic activity when tested alone, but markedly enhance tumor yield when applied repeatedly following a low or suboptimal dose of an initiator (Hecker et al., 1982). Most of our information on the mechanism of action of this class of agents derives from studies on the potent skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), and related phorbol esters. TPA in its chemical structure contains a portion that is very similar to diacylglycerol. Diacylglycerol is transiently produced

from inositol phospholipid metabolism in response to extracellular signals. Diacylglycerol activates protein kinase C. The physiological substrates of protein kinase C are still largely unknown. It is known that protein kinase C is important in signal transduction, and has been implicated in the TPA-inhibition of gap junctional intercellular communication (GJIC). Tumor promoting agents have been shown to block GJIC in various cell lines. It has been suggested that the blockage of the exchange of important messenger ions or molecules between normal communicating cells could lead to abnormal cell proliferation. The precise biochemical mechanisms involved in the regulation of the gap junction, however, are not well understood. Inhibition of this form of intercellular communication by various chemicals has been postulated to be a factor in the tumor promotion phase of carcinogenesis (Murray and Fitzgerald, 1979; Trosko et al., 1983).

In addition to causing DNA damage, at least one arylamine has been shown to be a very effective promoter. 2-Acetyl-aminofluorene is generally believed to act as a selective growth inhibitor when given in sub-carcinogenic doses (Solt and Farber, 1976; Solt et al., 1977). However, some recent reports suggest that 2-AAF may be acting as a promoter of liver carcinogenesis by a non-cytotoxic mechanism (Saeter et al., 1988). This compound or its metabolites have not been reported to have been tested in

short term assays to determine if they have an effect on GJIC.

Although MBOCA has not been tested by any protocol to determine whether it might be acting as an initiator or a complete carcinogen, available animal studies do suggest that it may be acting as a complete carcinogen (Russfield et al., 1975; Stula et al., 1975). One might therefore postulate that MBOCA or products of its metabolism may be acting to enhance proliferation of initiated cells through selective growth inhibition or alternatively, as promoters through non-cytotoxic mechanisms.

Metabolic activation leading to DNA damage and possible mutagenesis has been extensively studied for many arylamine carcinogens. That some of these metabolites might be important in other stages of carcinogenesis such as proliferation or tumor progression, has not been as well characterized.

The hypothesis tested in the work presented in this chapter was that products of Phase I metabolism of MBOCA or MBOCA itself may be acting as inhibitors of GJIC at non-cytotoxic levels.

METHODS

Chemicals

N-OH-MBOCA, diNO-MBOCA and NO-MBOCA were synthesized by oxidation of parent MBOCA with CPBA, while the o-OH-MBOCA metabolite was extracted and purified from microsomal incubations as previously described (chap 2). Compounds were purified by HPLC or TLC just prior to use. Compounds were dissolved in methanol. The final concentration of this solvent in culture medium was 0.1%. Lucifer yellow and tetramethyl rhodamine dextran, M.W. 10,000, were purchased from Molecular Probes Inc., Eugene, OR.

Cell culture

WB-F344 rat liver epithelial cells, passages 10-25, were used. The cells were cultured in D medium, a modified Eagle's medium containing Earle's balanced salt solution with a 50% increase of vitamins and essential amino acids except glutamine, a 100% increase of non-essential amino acids (Gibco Laboratories, Grand Island, NY) and 1 mM sodium pyruvate, 5.5 mM glucose, 14.3 mM NaCl, 11.9 mM $NaHCO_3$, pH 7.3. The medium was supplemented with 5% fetal bovine serum and 50 μg/ml gentamicin. Cells were incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2. For all

experiments, WB cells were seeded at 20% and grown to confluence. Treatments were made on cells 1-2 days post confluence. The culture medium was replaced with serum-free D medium prior to addition of test chemicals and then incubated with the test chemical for 30 min.

Measurement of Gap-Junctional communication

The method of scrape loading/dye transfer described by El-Fouly et al. (1987) was used with a slight modification. Confluent cultures in 35 mm plates (1.2-1.6 x 10^6 cells) were rinsed three times with PBS and drained after treatment with the test chemicals. Two milliliters of 0.05% Lucifer yellow in PBS was added to the plates and three scrape lines were made in the center of the monolayer with a surgical blade with three additional scrape lines made at right angles to the initial set of lines. After 3 min to allow dye uptake and transfer at room temperature, the cells were rinsed several times with PBS to remove excess dye and immediately examined under a Nikon epifluorescence phase microscope. Rhodamine dextran at 0.04% was occasionally added to the dye to verify that Lucifer yellow transfer was through the gap junctions.

Cells were fixed in 4% phosphate-buffered formalin and air-dried. The fixed cells were examined on the ACAS 470 fluorescence workstation (Meridian Instruments, Okemos, MI) with a laser beam (Schindler et al., 1987). Using an appropriate computer program, a scan of the scrape-loaded

cell image was generated and an integrated value of fluorescence intensity over a boxed area (78 mm x 100 mm) of the scrape line was obtained as a measure of the extent of dye transfer. Dieldrin at 7 μg/ml (25 nmol/ml) was used as a positive control.

Measurement of cytotoxicity

Cytotoxicity was determined for the same plates that gap-junctional communication determinations were made. After treatment with the chemical, the serum-free D medium was collected for the enzyme determinations. The kit for lactate dehydrogenase (LDH) determinations from Sigma St. Louis, MO was used. The sample (100 μl) was added to a solution containing NAD and lactate. The rate of formation of NADH was determined at 340 nm on a Beckman model 42 spectrophotometer.

As a separate measure of cytotoxicity, the effect of MBOCA concentration was tested on the colony-forming ability of this same WB-F344 cell line. Initially, 200 cells were plated in 60-mm dishes. MBOCA was added after about 4 hr when cells attached on the bottom of the dishes. The cell cultures were incubated for 3 days with MBOCA. The medium was replaced at Days 4 and 7 with fresh medium. Growth was continued for a total of 10 days until colonies had grown to sufficient sized to score. Colonies were rinsed with 0.85% saline, fixed, and stained with crystal violet. The resulting colonies were scored visually. Negative control

plates were treated with solvent and no MBOCA.

Statistical Analysis

Differences in the results were analyzed statistically using analysis of variance followed by the Scheffe's test, taking $p < 0.05$ to be the level of significance.

RESULTS

The concentrations of oxidized MBOCA metabolites at which cytotoxicity was observed as determined by LDH release are presented in Table 6. N-OH-MBOCA exhibited the greatest cytotoxicity towards WB cells (0.2 nmol/ml), NO-MBOCA was cytotoxic at 18 nmol/ml, while o-OH-MBOCA had no apparent cytotoxic effect even at the highest level tested (180 nmol/ml). The amount of the o-OH-MBOCA metabolite obtainable precluded testing at higher levels. Figure 14A is a presentation of the effect of MBOCA treatment of WB cells on fluorescence intensity as a measure of gap-junctional communication along with LDH release from these cells as a measure of overt cytotoxicity. Beginning at a concentration of MBOCA of 7.5 nmol/ml a decrease in fluorescence preceded the increase in LDH release. This decrease continued at 11.3 nmol/ml, a concentration at which LDH release was still not significantly different from its control. LDH release became significantly increased at a level of 15 nmol/ml. Figure 14B is a presentation of parallel studies done to determine cytotoxicity through plating efficiencies after treatment with MBOCA. Although the incubation time with the test chemical was 3 days

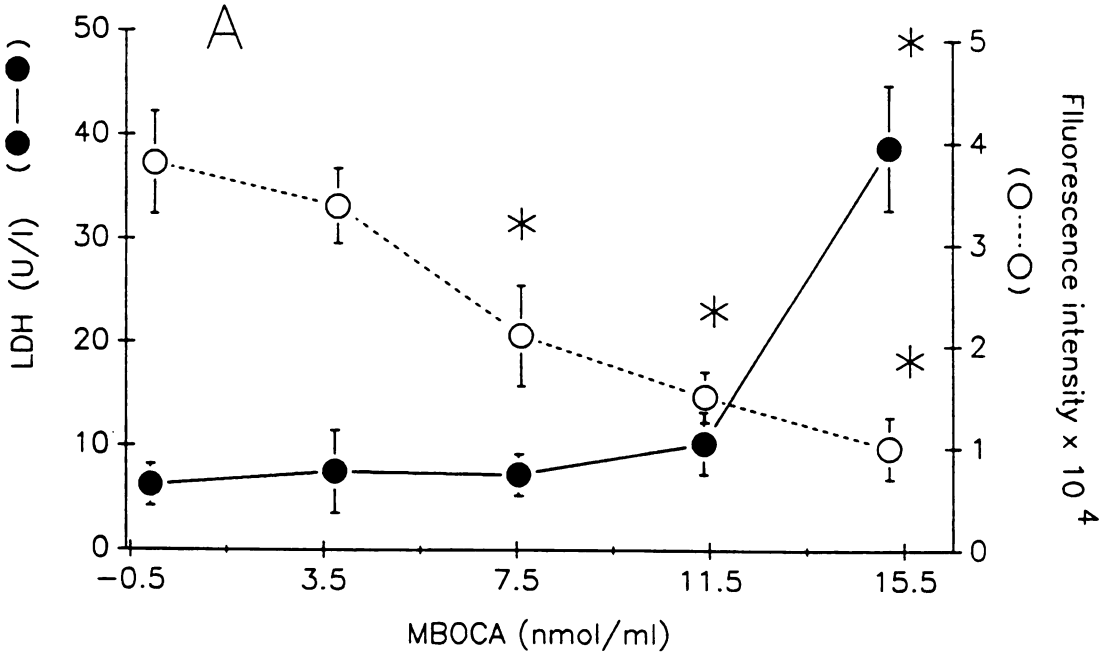
Table 6

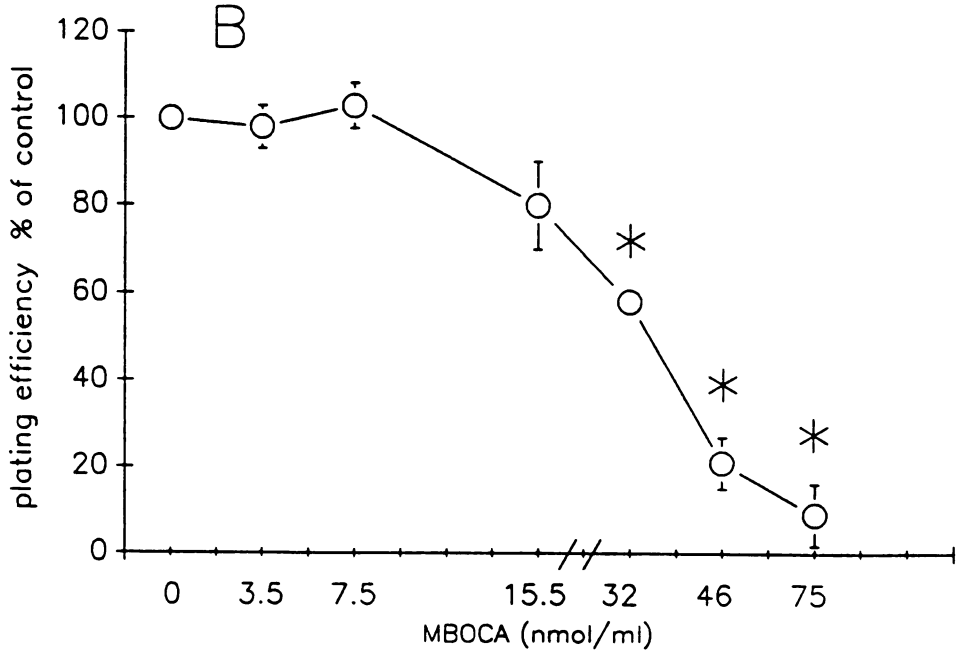
Effect of treatment of oxidized derivatives of MBOCA on LDH release and fluorescence transfer in WB cells.

	concentration (nmol/ml)	(n)	LDH (U/l)	Fluorescence Intensity ($x10^4$)
N-OH-MBOCA	4.0	2	35	0.8
	0.4	5	40 ± 10[a]	1.2 ± 0.6[b]
	0.2	8	25 ± 6[a]	2.1 ± 0.5[b]
	0.1	8	10 ± 5	3.5 ± 1.2
NO-MBOCA	72	2	30	2
	18	6	12 ± 2[a]	2.3 ± 0.7[b]
	12	6	7 ± 2	3.9 ± 0.7
	4	8	7 ± 3	4.2 ± 1.0
o-OH-MBOCA	180	3	4 ± 2	3.0 ± 0.1
	18	6	5 ± 2	5.1 ± 1
	4	6	5 ± 3	3.5 ± 2
dieldrin	25 (7 µg/ml)	10	5 ± 4	0.5 ± 0.2[b]

[a]Significant difference ($p<0.05$), Control values were 6 ± 2 U/l
[b]Significant difference ($p<0.05$), Control values were 4 ± 0.5

Figure 14 **Effect of treatment of MBOCA on A) gap-junctional communication and LDH release; B) plating efficiency using WB cells.**


A
LDH (U/l) (●—●)
Fluorescence intensity x 10 4
(O····O)
50
40
30
20
10
0
5
4
3
2
1
0
−0.5
3.5
7.5
11.5
15.5
MBOCA (nmol/ml)


B
plating efficiency % of control
120
100
80
60
40
20
0
0
3.5
7.5
15.5
32
46
75
MBOCA (nmol/ml)

instead of 30 min, the plating efficiency studies appear to confirm the LDH results in that cytotoxicity of these cells towards MBOCA begins to appear at 15 nmol/ml although its not significant until 30 nmol/ml.

There were no concentrations of N-OH-MBOCA, NO-MBOCA, or o-OH-MBOCA tested that were non-cytotoxic at a point where a decrease in fluorescence of the dye could be measured (Table 6).

DISCUSSION

There is much to suggest that carcinogenesis is a multi-step process, and that metabolic activation of some chemical carcinogens is important in the initiation stage. The concept that the parent compound or activated metabolites might also be important in the promotional phase or play an epigenetic role in cancer causation has been much less studied. Furthermore, short-term *in vitro* assays for promotional activity have not been well worked out. Since GJIC is inhibited by known tumor promoters, such as TPA and PB, this test may provide some insight into the potential activity of MBOCA and its metabolites to act as tumor promoters.

The results of this study show that MBOCA itself has a role in blocking cell-cell communication. Results also suggest that Phase I metabolites of MBOCA do not have an effect on GJIC at levels that are non-cytotoxic. The N-oxidized metabolites are seen to be relatively cytotoxic, and may be acting as promoters *in vivo* by acting to enhance cellular proliferation by a cytotoxic effect. Since inhibition of GJIC has been suggested to be a reflection of tumor promoter potential, MBOCA may be acting as a promoter

to the initiating effects of mutagenic MBOCA metabolites. This suggestion that the MBOCA - N-OH-MBOCA metabolite mixture contains both initiation and promotion potential is intriguing, and warrants further testing of the MBOCA molecule in standard in vivo assays for promotion activity.

The finding that the N-OH-MBOCA metabolite is the most cytotoxic compound of all tested here is not surprising since highly reactive species can bind to protein and cause cell death. The relative cytotoxicity of the NO-MBOCA species is surprising. It is two orders of magnitude less cytotoxic than the N-OH-MBOCA compound. This suggests that NO-MBOCA is not being reduced to the N-OH-MBOCA species in the target cells or the surrounding media, or possibly that NO-MBOCA is being diverted into another pathway before it can be chemically reduced.

The work presented in this chapter attempts to dissect the effect that individual products of arylamine metabolism have on gap-junctional communication. It is known that even the process of tumor promotion may be divided into various stages, and the effect on GJIC may only represent one such stage. The different metabolites of MBOCA could act at separate stages not represented by the short term assay used here. The final result in vivo therefore may be an additive, antagonistic, or a synergistic promotional effect. These separate properties may not be determinable through short-term assays.

CHAPTER 5

DEVELOPMENT OF MBOCA-HEMOGLOBIN BINDING ASSAY

CHAPTER 5

INTRODUCTION

Currently, exposure to MBOCA is monitored by determination of urinary concentrations of the parent compound and acetyl metabolites (Gristwood et al., 1984; Thomas and Wilson, 1984; Ducos et al., 1984). These measurements may only indicate a relatively recent exposure, and provide little information on the production of reactive metabolites. The use of macromolecules as dose monitors for exposure to carcinogens has recently become possible for many compounds. The prime prerequisite for the use of any macromolecule as a dose monitor is to demonstrate a linear dose-dependence of binding to the amount of chemical administered. The formation of carcinogen-protein adducts as a result of carcinogen administration to experimental animals has now been evaluated for more than 30 chemicals including such typical arylamine carcinogens as 4-aminobiphenyl, 2-naphthylamine, and benzidine (Farmer et al., 1984; Skipper et al., 1986; Stillwell et al., 1987). Determination of the carcinogen-protein binding of hemoglobin (Hb) has been proposed for several reasons. Hemoglobin is more readily obtainable and in much larger quantities than DNA. It exists in the erythrocyte at

concentrations of 1-2 mM or 10-15% by weight. Green et al. (1984) showed that 2.5 sulfhydryl groups per hemoglobin tetramer were susceptible to binding by the metabolically activated form of 4-aminobiphenyl. The potential binding capacity for this arylamine might therefore represent a concentration of 2-5 mM. The stability of hemoglobin which is dependent on the red blood cell survival time is on the order of months. Depending on the stability of the hemoglobin adducts of a chemical, monitoring of an exposure could be made long after it had ceased. Likewise, accumulation of repeated exposures could be reflected in higher levels of binding.

The measurement of Hb-adduct formation may be a measure of the formation of reactive metabolites. The mechanism of Hb-adduct formation proposed for arylamines begins with the formation of the reactive N-hydroxyl metabolite of the arylamine. A nitroso derivative is believed to be generated from the hydroxylamine with hemoglobin being cooxidized to methemoglobin. The nitroso derivative binds directly to the sulfhydryl groups of proteins forming a sulfinamide bound adduct. The arylamine adducts bound to the cysteine residues of hemoglobin are believed to be sulfinamides (Green et al., 1984; Neumann, 1984; Bryant et al., 1987). These are readily hydrolyzed by mild acid to release the parent amine which can be analyzed appropriately. The work of Ehrenberg and associates indicates that the determination

of protein adducts is a relevant and quantifiable measure of DNA adduct formation by direct alkylating agents as well as ones requiring bioactivation (Ehrenberg et al., 1974; Ehrenberg, 1984). The measurement of Hb-adduct formation therefore may be useful in estimating an effective dose after in vivo activation of the arylamine carcinogen to the proximate N-oxidized forms, and in addition, may reflect DNA binding.

In this phase of the study, the capacity of N-oxidized metabolites of MBOCA to form hemoglobin adducts was determined in vitro and the formation of Hb adducts following in vivo administration of MBOCA was assessed. The specific objectives of this study were to develop analytical procedures to measure very low levels of parent MBOCA expected to be hydrolyzed from hemoglobin. Reaction conditions needed to be determined that would release the covalently bound adduct as the parent amine. In vitro binding capability of N-OH-MBOCA, NO-MBOCA and MBOCA itself needed to be assessed and determined if binding was dose-dependent. In the same way, it was necessary to determine in vivo binding, and demonstrate that it too was dose-dependent. After these assessments, animal experiments were planned to determine the time course of binding after dosing.

METHODS

Chemicals

MBOCA (97%) was obtained from Pfalz & Bauer, Inc. (Waterbury, CT), [^{14}C]MBOCA, 58 mCi/mmole, was obtained from the Michigan Toxic Substances Control Commission, and was purified by HPLC on a C_{18} μBondapak column (Waters Associates, Milford, MA) using ACN/H_2O (47:53 v/v). NO-MBOCA was synthesized by oxidation of MBOCA with CPBA and N-OH-MBOCA by reduction of NO-MBOCA in an ascorbic acid saturated methanol medium (chap 2). The compounds were stored in ACN and ascorbic acid saturated ACN respectively, under Ar and at -70^{o}C. Immediately before use the compounds were purified by TLC on K5 silica gel plates (Whatman, Inc., Clifton, NJ). The plate was chilled during spotting, and development was performed with toluene/ethanol (20:1) under Ar as described previously (chap 2). Heptafluorobutyric anhydride (HFBA) was purchased from Regis Chemical Co., Morton Grove, IL. Human and rat hemoglobin (2x crystallized, dialyzed and lyophilized) were from Sigma Chemical Co, St. Louis, MO, and solvents were U.V. spectrometric grade from American Burdick and Jackson, Muskegon, MI. All other chemicals were reagent grade.

Trimethylamine in hexane was prepared by adding 1 g reagent grade trimethylamine hydrochloride to 2 ml H_2O, neutralizing with NaOH, and extracting into 5 ml hexane. Water was purified by passing distilled water through a 4-bowl Milli-Q water purification system and 0.25 μm filter (Millipore Corporation, Milford, MA).

Animals

Male Sprague Dawley rats with an average weight of 190 g were obtained from Harlan Sprague Dawley, Indianapolis, In. Guinea pigs were English short hair males (strain Mdh:(SR[A])) with an average weight of 245 g obtained from the Michigan State Health Laboratories, Lansing, MI. The animals had free access to food and water throughout the experiment. For intraperitoneal and subcutaneous administration, MBOCA was dissolved in a mixed vehicle consisting of propylene glycol/DMSO/0.9% NaCl (4:4:2 v/v), and injected in a volume of 2.0 ml/300 g body weight. N-OH-MBOCA was administered iv through the penile vein in a mixture of MeOH/0.9% NaCl (2:1, v/v), at 1 ml/kg body weight. Blood was obtained from rats by docking the tip of the tail while the animals were under ether anesthesia. Guinea pig blood was obtained from an incision in the lateral marginal vein of the hind limb while the animals were anesthetized with a mixture of ketamine-HCl, 35 mg/kg, and acepromazine, 5 mg/kg. Animals were sacrificed with ether anesthesia followed by exsanguination.

In vitro Hb binding

Ten ml of rat blood was obtained by sacrificing several control rats. Human blood was taken by venipuncture. All samples were anticoagulated with heparin or EDTA before further processing. Samples were mixed with an equal volume of heparinized saline and blood cells collected by centrifugation. Red cells were washed 3 times by suspending in 10 volumes of 0.9% NaCl followed by centrifugation, lysed by mixing with 3-4 volumes of 10^{-4}M EDTA solution, pH 7.5, and allowed to stand for 30 min. The lysed blood was further diluted with four volumes of CO_2 saturated distilled water. After standing for two hr, the membranes and other cellular debris were removed by centrifugation at 25,000xg for 20 min. The supernate, which contained about 10 mg Hb protein/ml as determined by the Lowry procedure (Lowry et al., 1951), was pooled and stored at 0-4^{o}C after purging with N_2.

To determine dose-dependent binding, serial amounts of MBOCA, N-OH-MBOCA and NO-MBOCA were added to screw-capped centrifuge tubes containing 5 mg Hb (rat, human or guinea pig) in 0.5 ml phosphate buffer (or lysed when whole blood was used), inhibitors (if being tested), and 1.0 ml phosphate buffer, pH 7.0. Reactions were carried out under Ar at room temperature with shaking for two hr. Samples were chilled on ice and added dropwise to 12 ml of ice cold acetone containing 0.2% HCl while vortexing vigorously. The

Hb precipitate was centrifuged (3000xg for 5 min) and washed with: 1) 12 ml acidified acetone containing 0.2% HCl (once); 2) 12 ml acetone (3 times); and 10 ml ether (twice). The final ether wash was retained and analyzed for MBOCA. Washing was continued if necessary until no additional MBOCA residue (as determined by GLC) could be extracted from the Hb.

To the washed Hb powder, 4 ml of 0.5% SDS and 0.4 ml of 1 N HCl were carefully added with vortexing. Hydrolysis was carried out for 2 hr or longer. The solution was then adjusted to pH 9-10 and extracted twice with equal volumes of hexane. The hexane extract was evaporated to dryness under a stream of N_2 prior to derivatization and GLC analysis (vide infra).

In vivo Hb binding

Blood samples, 0.5-2 ml, were collected into 2 ml heparinized saline. Red cells were washed 3 times by suspending in 10 volumes of 10^{-4}M EDTA solution, pH 7.5, and allowed to stand for 30 min. Ice cold hemolysate was added dropwise to ice cold acetone containing 0.2% HCl while vortexing vigorously. Globin precipitate was obtained by centrifugation (3000xg, 5 min) and washed consecutively with 15 ml of acidified acetone (0.2% HCl), 15 ml acetone (twice) and 15 ml ether (twice) by vortexing and centrifugation. The globin pellet was allowed to dry overnight at room temperature or in a 50^{o}C water bath for 30 min.

A 25 mg aliquot of the dried red cell globin powder was weighed and carefully dispersed into 8 ml of 0.5% SDS. Hydrolysis and recovery of MBOCA was then carried out as described above, with reagents scaled to the amount of Hb used.

Red cell globin positive and negative controls were included in each individual set of assays. The positive control was prepared by *in vitro* reaction of N-OH-MBOCA with rat red cell hemolysate on an enlarged scale. The average adduct level was obtained through replicated determinations. The negative control was prepared with pooled blood samples from control animals.

The Hb content of four *in vitro* preparations and the *in vivo* red cell powder was quantified by determination of the iron content (Riggs, 1981) using inductively coupled argon plasma (ICAP) spectrometry to measure iron (Braselton *et al.*, 1981). Samples (10-12 mg dry weight) were combined with 1 ml of conc HNO_3 and 1 ml of conc H_2SO_4 in a test tube (13 x 100 mm) and heated over a flame for 5 min. Samples were cooled, then quantitatively transferred to 10 ml volumetric flasks. Yttrium was added as an internal reference standard. Samples were diluted to the final volume with H_2O and analyzed on a Jarrell-Ash Model 955 Atomcomp spectrometer. The Hb content was expressed as mg Hb/mg protein (*in vitro*) or mg Hb/mg red cell powder (*in vivo*). Reagent grade Hb was taken as standard for iron

content/mg. Protein was determined by the procedure of Lowry (Lowry et al., 1951).

Analytical procedures

Preparation of the MBOCA-HFB derivative was conducted immediately after evaporation of hexane from the final extract. To each screw-capped tube was added 0.5 ml isooctane, 2-3 µl TMA (in hexane) and 3 µl HFBA. After vortexing, sealed tubes were allowed to stand for 10 min at room temperature and then 0.5 ml of 5% NH_4OH were added, mixed thoroughly by vortexing, and the phases allowed to separate. One to 4 µl of the hexane phase were injected onto the GLC. A Varian series 2100 gas chromatograph equipped with a Ni^{63}ECD and 1.6 m x 2 mm glass column packed with 1% SP2100 on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA) was used. Chromatography was conducted with N_2 flow at 45 ml/min and column temperature at 240°C. The minimum detectable amount of MBOCA-HFB was 0.5 pg (4 x noise), and the ECD signal was linear from 0.5 pg to 80 pg. All samples with greater responses were diluted to this range.

To confirm the identity of the isolated MBOCA, the isooctane fraction from above was concentrated under a stream of N_2 to about 100 ng MBOCA/10 µl, and analyzed on a Hewlett-Packard 5890 GLC and 5970 MSD. The column used was a 15 m x 0.25 mm fused silica capillary DB-5 bonded phase.

RESULTS

Hydrolysis of the MBOCA-Hb adduct released free MBOCA, which was determined as the MBOCA-*bis* HFB derivative. Figure 15 compares a chromatogram of reference MBOCA with chromatograms of derivatized extracts of blood from a control rat, a rat treated *in vivo* with MBOCA, and Hb reacted with N-OH-MBOCA *in vitro* to form the Hb adduct. The latter sample was used routinely as a positive control for the total procedure (hydrolysis, extraction, derivatization and EC-GLC).

The identity of the MBOCA-*bis* HFB peak was confirmed by gas chromatography-mass spectrometry (70eV) (Figure 16A). The spectrum revealed a molecular ion at M^+=658, with ions at m/z 623 (M-35), m/z 336 (corresponding to the derivatized chlorotoluidinium ion), and m/z 300. The ion at m/z 300, formed by loss of HCl from m/z 336, was the base peak in the spectrum. Figure 16B is the GC/MS with selected ion monitoring of a reference standard of MBOCA-*bis* HFB. Figure 16A is the GC/MS with selected ion monitoring of Hb samples pooled from MBOCA treated rats. The retention time of 8.36 min is the same for both standard and sample. Likewise, the relative heights of the peaks at the ions being monitored

Figure 15 Gas chromatography of the bis-heptafluorobutyryl derivative of A) reference MBOCA (MBOCA-HFB); B) an extract of hydrolyzed Hb from a control rat; C) an extract of hydrolyzed Hb pooled from MBOCA treated rats D) and an extract of hydrolyzed Hb following in vitro reaction with N-OH-MBOCA under Ar.

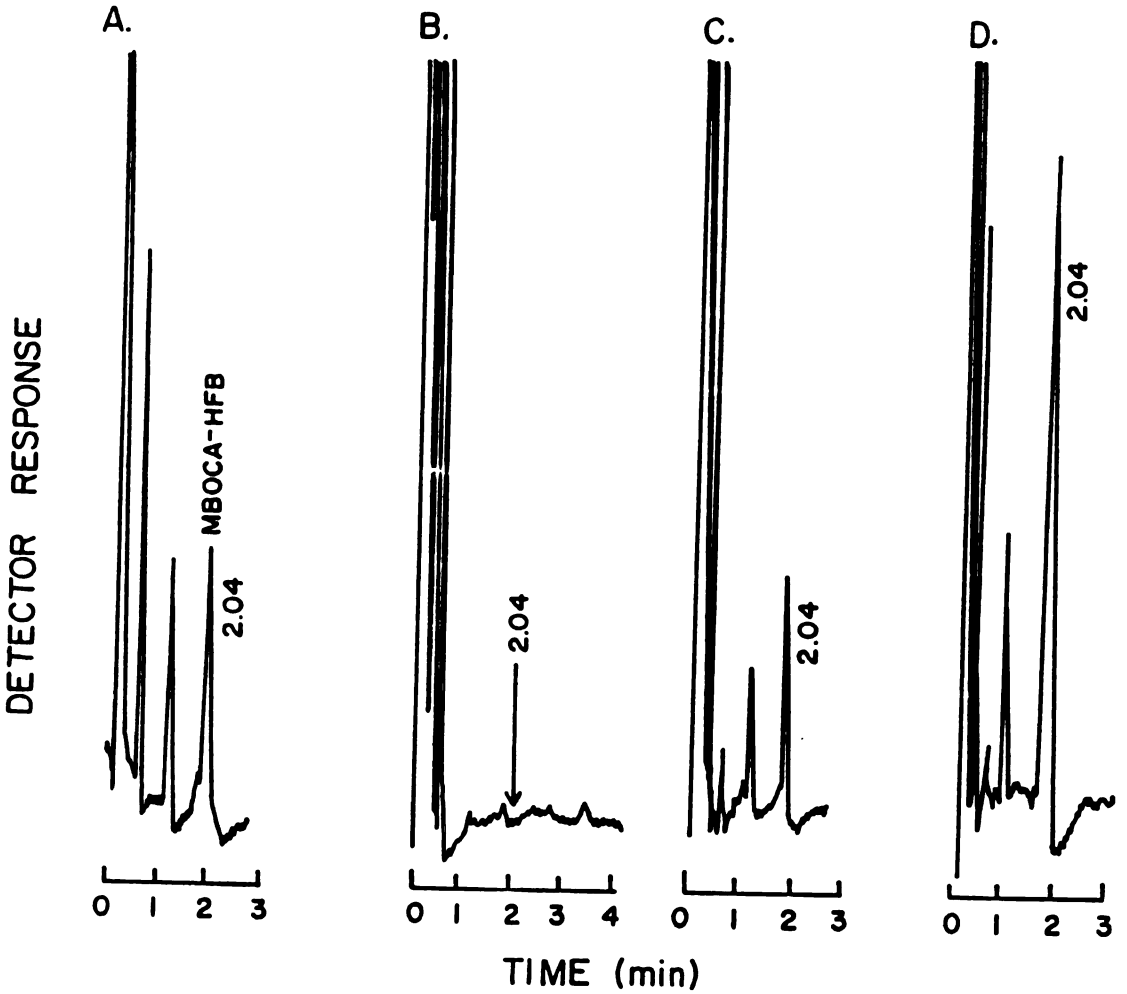
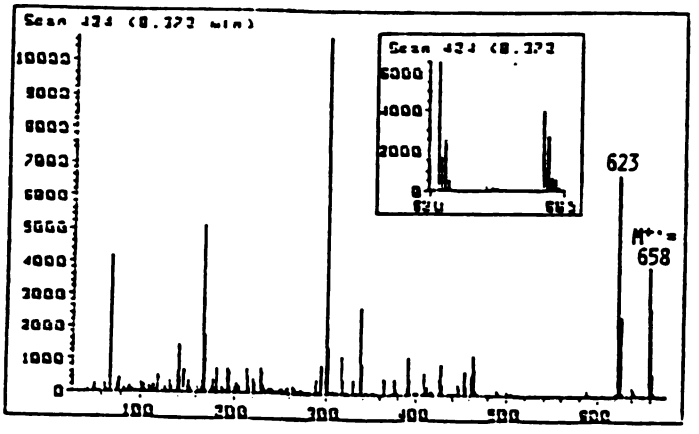
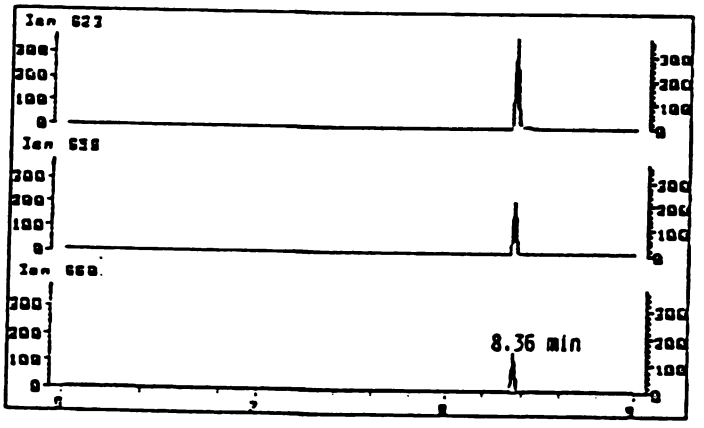

A.
B.
C.
D.
DETECTOR RESPONSE
MBOCA-HFB
2.04
2.04
2.04
2.04
0 1 2 3
0 1 2 3 4
0 1 2 3
0 1 2 3
TIME (min)

Figure 16 **Mass spectrum (70 eV) of A) reference MBOCA-bisHFB; GC/MS with selected ion monitoring of B) 20 ng reference MBOCA-bisHFB; C) derivatized extract of hydrolyzed Hb from MBOCA-treated rats.**

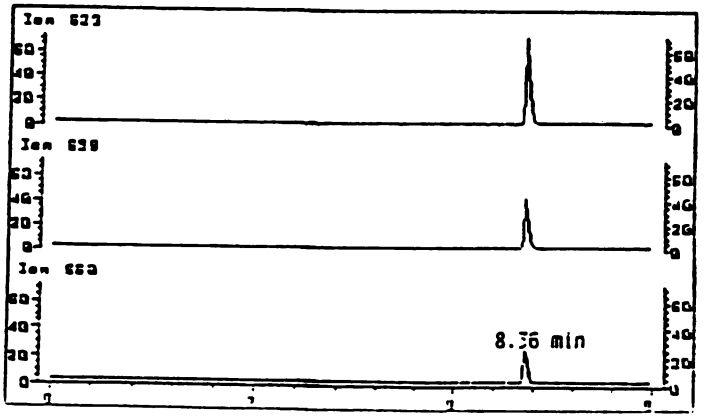
A.



B.



C.



are also the same. These two characteristics are very strong evidence that these two peaks are in fact the same compound.

The substrate-dependent *in vitro* Hb binding of the metabolites N-OH-MBOCA and NO-MBOCA are compared to that of the parent MBOCA in Figure 17. The binding levels were nearly identical for the NO- and N-OH-MBOCA metabolites, and were linear for 2-3 orders of magnitude, suggesting that the two metabolites were nearly equivalent in their capacity to form adducts. No difference could be seen between binding to Hb isolated from rats or humans, or to reagent grade human Hb from a commercial source. Binding of MBOCA to Hb was linear for the range that was tested. Its capacity to bind to Hb however was far less than N-OH-MBOCA. MBOCA under these conditions required approximately 100 ng to produce the same amount of binding as 1 ng of N-OH-MBOCA.

The ability of ascorbic acid and thiols such as glutathione and cysteine to inhibit *in vitro* binding is shown in Table 7. N-OH-MBOCA or NO-MBOCA binding to Hb was suppressed by cysteine and glutathione but not oxidized glutathione or methionine. Ascorbic acid also inhibited binding to Hb. The binding of NO-MBOCA to Hb was similar at pH 5 and pH 7, but binding of N-OH-MBOCA was more than doubled when the pH was lowered from 7 to 5.

To demonstrate that the MBOCA-Hb adduct could be formed *in vivo* and determined in blood samples, rats were treated

Figure 17 The *in vitro* binding of MBOCA, N-OH-MBOCA, and NO-MBOCA to lysed whole blood and to Hb from the rat, human (isolated from whole blood), and human (commercial source).

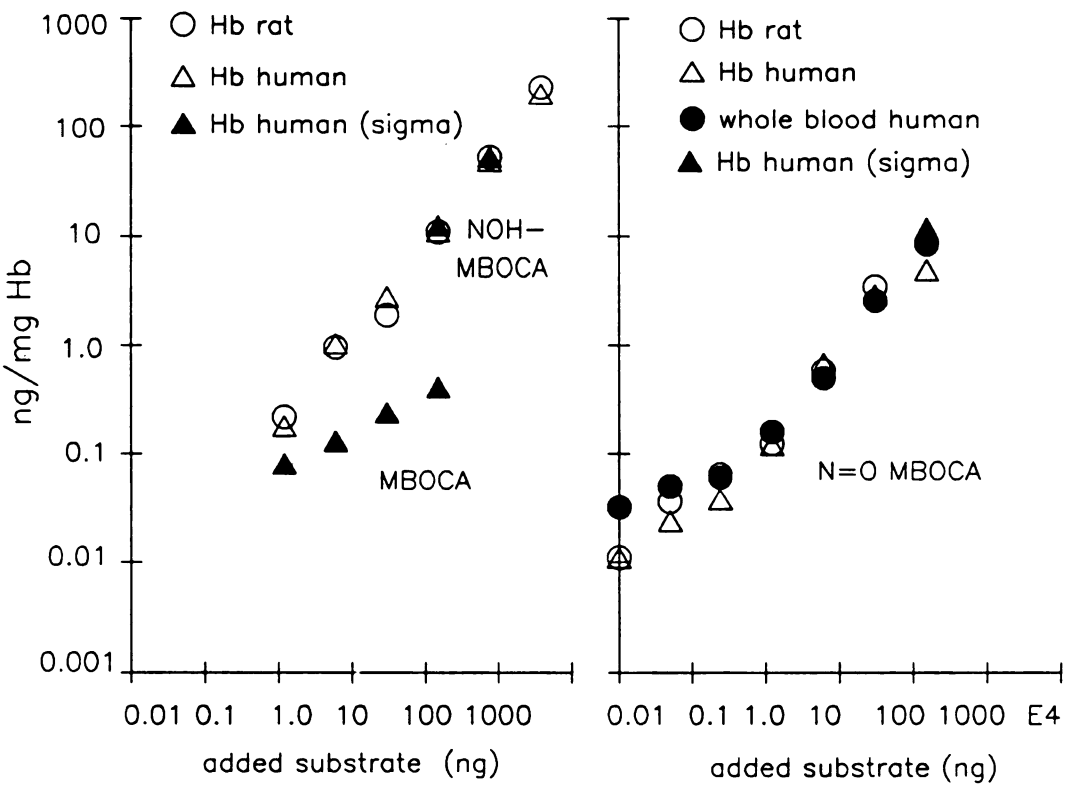

1000
100
10
1.0
0.1
0.01
0.001
ng/mg Hb
Hb rat
Hb human
Hb human (sigma)
NOH–
MBOCA
MBOCA
0.01 0.1 1.0 10 100 1000
added substrate (ng)
Hb rat
Hb human
whole blood human
Hb human (sigma)
N=O MBOCA
0.01 0.1 1.0 10 100 1000 E4
added substrate (ng)

Table 7

The effect of thiols and other factors on the *in vitro* binding between MBOCA metabolites and partially purified Hb from rats

	Macromolecular binding levels, nmole/mg Hb			
added MBOCA mtb.	NO-MBOCA (3 μM)		N-OH MBOCA (3 μM)	
conc. of chemical	5 mM	10 mM	5 mM	10 mM
cysteine	48.1 ± 8.0	3.9 ± 0.9	9.8 ± 1.6	4.3 ± 1.2
GSH	23.4 ± 2.9	16.0 ± 3.9	4.0 ± 1.8	4.5 ± 0.9
GSSG	61.6 ± 5.5	68.2 ± 2.7	86.9 ± 15.4	71.3
Methionine	75.8 ± 2.1	58.6 ± 14.2	78.0 ± 17.0	74.7 ± 22.1
Ascorbic acid	52.0 ± 13.3	37.9 ± 7.6	65.3 ± 19.0	39.0 ± 3.0
pH=5	70.2 ± 5.6		163.7 ± 5.7	
pH=7	70.2 ± 5.9		69.7 ± 5.4	

NO-, and N-OH-MBOCA were added to a final concentration of 3 μM in 10 μl of ACN. The reaction medium contained 5 mg rat Hb in 0.04 M phosphate buffer, pH 7.0. pH 5 was adjusted by 0.5 M citric acid. Reactions were conducted under Ar at room temperature for 4 hr. Mean ± S.D. (of triplicates)

iv with N-OH-MBOCA. The dose-related levels of adduct at 1 day and 1 week after exposure are shown in Table 8.

Figures 18A and 18B demonstrate that MBOCA administered to rats in ip or sc doses is activated to electrophilic intermediates which form Hb adducts in a dose-related manner. As might be expected, ip dosing led to greater levels of MBOCA-Hb adduct.

Figure 19 shows the formation and disappearance with time of the MBOCA-Hb adduct after a single sc administration of various doses of MBOCA to guinea pigs. The MBOCA-Hb adduct could still be determined 10 weeks after exposure to as little as 4 mg/kg MBOCA. Figure 20 is a representation of the data in Figure 19 redrawn for each dose level on a linear scale. The regression fits for these plots were better than those obtained from data plotted on semi-logarithmic plots. The regression coefficients for the 100, 20 and 4 mg/kg dose data were 0.96, 0.92 and 0.81 respectively when graphed on a linear scale, and 0.93, 0.85 and 0.77 on a semi-logarithmic scale. The elimination of Hb bound MBOCA, therefore, appears to more closely follow zero order kinetics. This is as would be expected if elimination were dependent on the life span of the red blood cells. Likewise, the elimination of Hb bound MBOCA (approximately 60-80 days) followed that of the red blood cell of the guinea pig which is reported to be 60-80 days (Sisk, 1976).

Table 8

Hb bound MBOCA following iv administration of N-OH MBOCA to rats

TIME (postdosing)	Dose (μmole/kg body wt)		
	0.04	0.2	1.0
		ng MBOCA/50 mg Hb	
0 hr	N.D.[a]	N.D.	N.D.
24 hr	0.9	1.7	13.3
		2.7	11.3
1 wk	0.8	1.7	5.0
		2.5	4.6

[a]N.D. = not detectable

Figure 18 **A) Formation of MBOCA-Hb adducts in rats treated ip with 0, 0.5, 5 and 50 mg/kg MBOCA.
B) Formation of MBOCA-Hb adducts in rats treated subcutaneously with 0, 4, 20, 100 and 500 mg/kg MBOCA.**

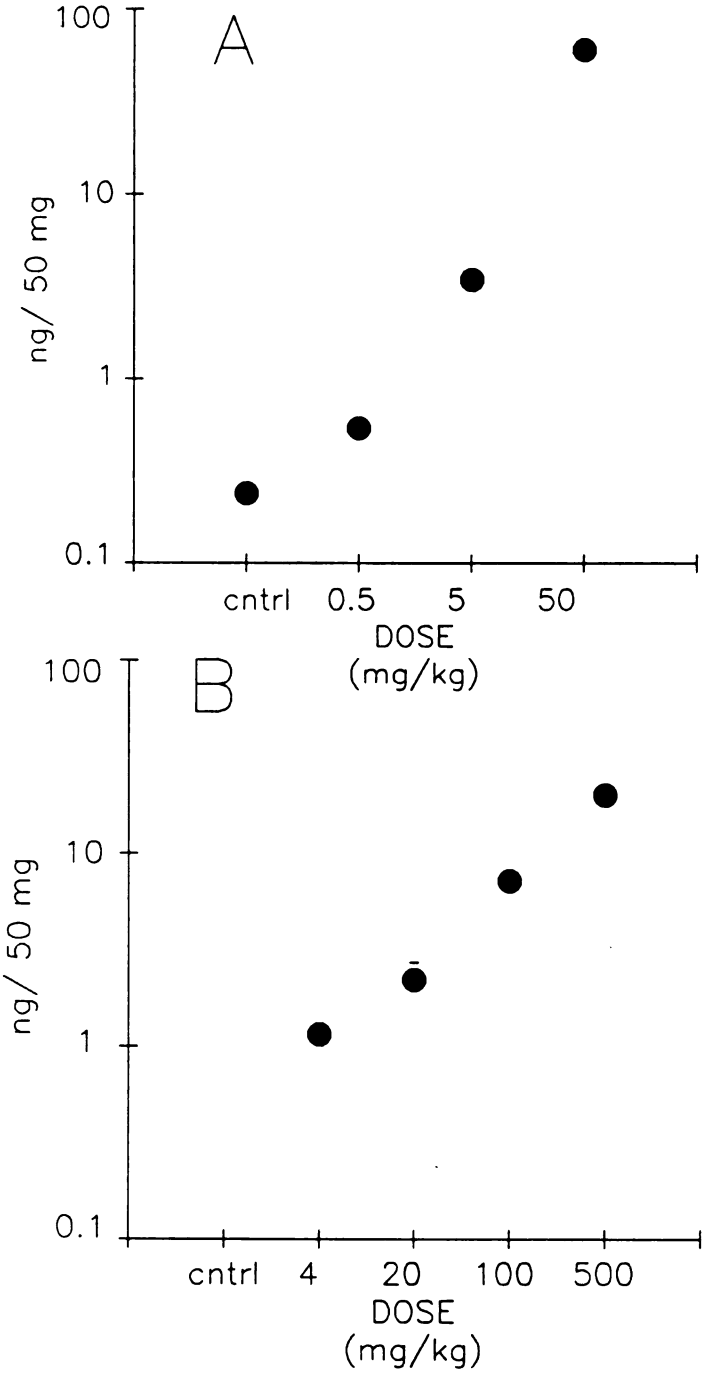

A
100
10
1
0.1
ng/ 50 mg
cntrl
0.5
5
50
DOSE
(mg/kg)
B
100
10
1
0.1
ng/ 50 mg
cntrl
4
20
100
500
DOSE
(mg/kg)

Figure 19 **Formation of MBOCA-Hb adducts in guinea pigs treated with a single subcutaneous dose of 0, 4, 20, or 100 mg/kg MBOCA.**

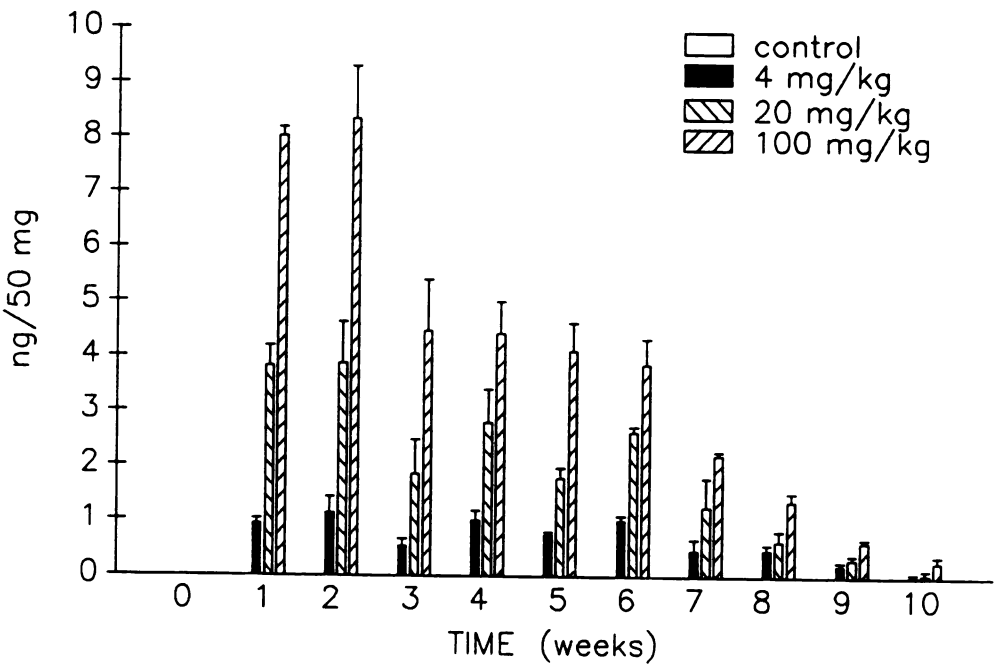
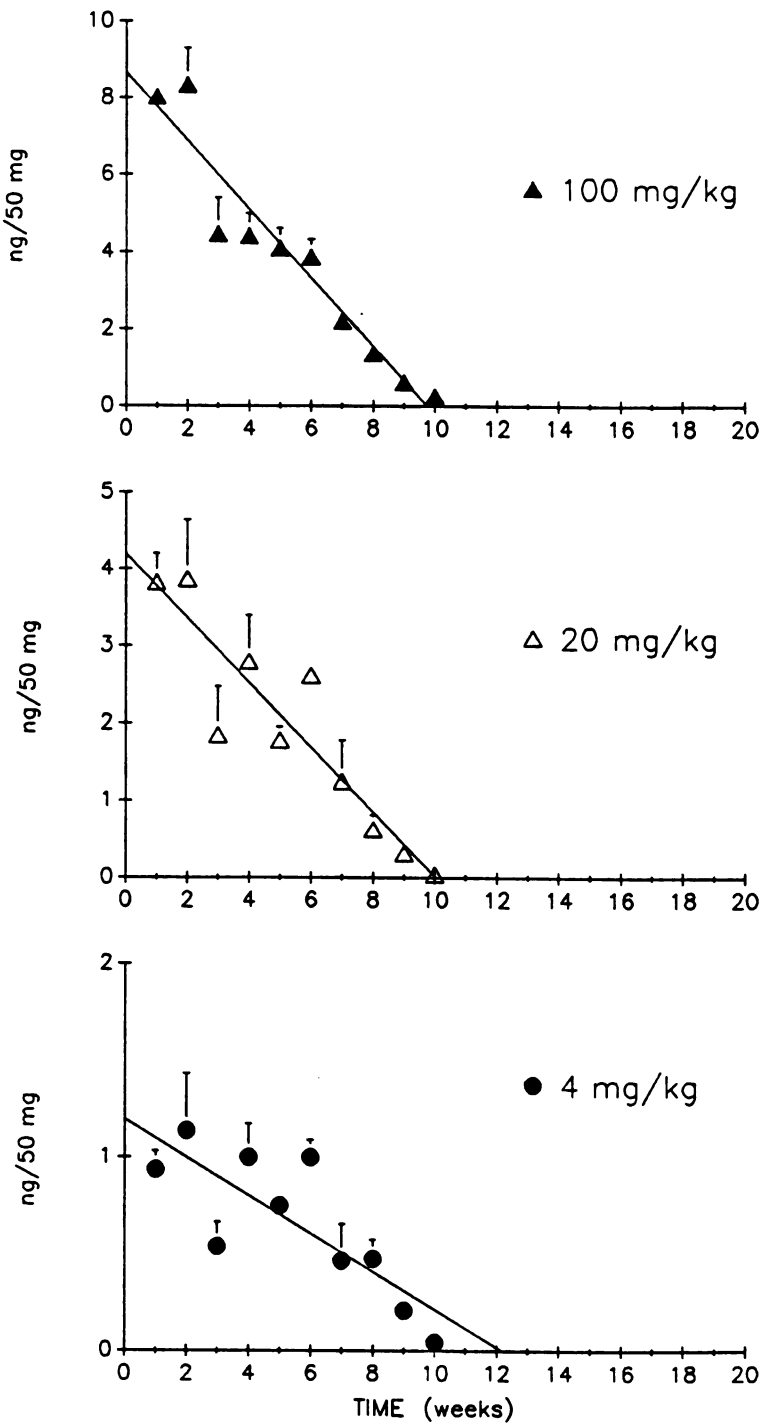

control
4 mg/kg
20 mg/kg
100 mg/kg
ng/50 mg
0
1
2
3
4
5
6
7
8
9
10
0
1
2
3
4
5
6
7
8
9
10
TIME (weeks)

Figure 20 **Elimination of MBOCA-Hb with time in guinea pigs treated with a single subcutaneaous dose of 4, 20 or 100 mg/kg MBOCA.**


ng/50 mg
▲ 100 mg/kg
ng/50 mg
△ 20 mg/kg
ng/50 mg
● 4 mg/kg
TIME (weeks)

DISCUSSION

The capacity of N-oxidized metabolites of MBOCA to form hemoglobin adducts was determined *in vitro*, and the formation of Hb adducts following *in vivo* administration of MBOCA was assessed. Hb adduct formation was determined by electron-capture GLC of MBOCA as the heptafluorobutyryl derivative following mild acid hydrolysis of protein bound MBOCA. The method was confirmed by gas chromatography-mass spectrometry with selected ion monitoring. N-hydroxy-, mononitroso-MBOCA, and MBOCA itself formed adducts to Hb *in vitro* in a dose-related manner. Binding was inhibited by cysteine and glutathione but not oxidized glutathione or methionine. Taken together, these results suggest that binding to Hb follows a similar mechanism as proposed for other arylamines (Eyer and Liebermann, 1980; Green *et al.*, 1984; Neumann, 1984; Bryant, 1987). The proposed mechanism states that the parent amine requires activation to the hydroxylamine predominantly by the cytochrome P-450 system in the liver. The hydroxylamine undergoes cooxidation resulting in formation of the nitroso derivative and methemoglobin. The nitroso derivative reacts with the sulfhydryl group of available cysteine residues resulting in

a covalently bound sulfinamide adduct. This adduct can be hydrolyzed under mild acidic conditions and the extent of binding determined by measurement of the parent amine.

MBOCA itself was observed to bind to a small degree to Hb *in vitro*. Hemoglobin has been shown to have some monooxygenase activity (Starke *et al.*, 1984; Golly and Hlavica, 1983), a peroxidase activity (Sok *et al.*, 1983; Eckert and Eyer, 1983; Yamaguchi *et al.*, 1984; Ha *et al.*, 1988), and biological Fenton reagent behavior (Sadrzadeh *et al.*, 1984; Puppo and Halliwell, 1988). Hemoglobin therefore may itself be contributing to the activation of MBOCA resulting in binding to its own sulfhydryl groups.

Intravenous administration of as little as 0.04 μmol/kg N-OH-MBOCA to rats resulted in measurable formation of MBOCA-Hb adducts (0.9 ng/50 mg). Intraperitoneal administration of 0.5-50 mg/kg MBOCA to rats, and subcutaneous administration of 5 to 500 mg/kg MBOCA to rats and 4 to 100 mg/kg to guinea pigs resulted in dose-related formation of Hb adducts. MBOCA-Hb was detectable in blood for approximately 10 weeks following a single subcutaneous dose in guinea pigs. Elimination of MBOCA-Hb was zero order with an estimated life span that did not appear to go beyond 10 weeks (70 days). This is in comparison with the elimination of the benzidine-Hb adduct which displayed first order kinetics, with a half-life of 11.5 days (Albrecht and Neumann, 1984). Since the bound MBOCA measured in these

experiments is associated with hemoglobin, it would be expected that elimination should follow the life span of the red blood cell and therefore, zero order kinetics. The life span of the guinea pig red blood cell is approximately 70 days (Sisk, 1976). The persistence of benzidine adducts may indicate that a deeper compartment exists which is responsible for the release of reactive metabolites. It would be of interest to determine if indeed there is a difference in elimination kinetics between the Hb adducts of these two arylamines and explore possibilities of why benzidine should display a greater persistence in its binding to Hb.

To relate the degree of Hb binding of a compound to the amount that was administered to an animal, a binding index has been suggested (Neumann, 1984). The index is defined as:

Binding index = Binding (mmol/mol Hb)/ Dose (mmol/kg). At a dose of 4 mg/kg, the binding index in rats treated iv with MBOCA was 0.33. This compares with a binding index of 147 $\pm$ 9.6 for trans-4-dimethylaminostilbene, 21 $\pm$ 3.7 for 2-acetylaminofluorene, and 0.4 $\pm$ 0.04 for acetaminophen (Neumann, 1984). The binding index of the N-OH-MBOCA treated rats at 0.04 μmol/kg was 110. The binding index of N-OH-MBOCA is almost three orders of magnitude higher than that of MBOCA. This supports the suggested mechanism of binding of MBOCA to Hb, in that it involves the activated N-

OH-MBOCA species. The binding index comparisons made above suggest that MBOCA binding is on the same order as that of acetaminophen. The consequence of this is that it may be more difficult to analyze the MBOCA-Hb adduct at a given dose than it would be to analyze the trans-4-dimethylaminostilbene adduct. Hemoglobin-binding indices may not be used for direct comparisons of the toxicity of different chemicals, the relationship between macromolecular binding and risk has yet to be elucidated. No such simple correlation to allow comparisons of different chemical toxicities should be anticipated.

These *in vivo* studies suggest that MBOCA is activated to an electrophilic intermediate capable of binding to Hb, that this binding is dose-dependent, and that elimination of Hb bound MBOCA adducts appear to be dependent on the life span of the red blood cell. The technical development of a highly sensitive GC-ECD method combined with GC/MS identification in the quantification of the Hb adduct should meet the demands for an *in vivo* surveillance system for the suspect carcinogen MBOCA.

CHAPTER 6

EFFECT OF β-NAPHTHOFLAVONE AND PHENOBARBITAL TREATMENT ON HEMOGLOBIN BINDING OF MBOCA IN RATS

CHAPTER 6

INTRODUCTION

Presently, there is a need to understand better the mechanism behind individual and species variations in the toxicological response to drugs and susceptibilities to chemical carcinogens. In many cases, the varied responses to drugs and chemicals can be related to differences in metabolism (Thorgeirsson and Nebert, 1977). The enzyme system that plays a key role in the metabolism of most foreign compounds is the cytochrome P-450 mono-oxygenase system. This system is composed of numerous isozymes that exhibit substrate selectivity for model compounds such as 2-AAF (Johnson et al., 1980; McManus et al., 1984), benzo(a)pyrene (Gelboin, 1980) and testosterone (Wood et al., 1983). Because of this selectivity, the relative activities of various cytochrome P-450 isozymes might be a factor in determining individual susceptibility to adverse drug reactions and chemical carcinogenesis.

An important property of these mono-oxygenases is the differential inducibility of the isozymes of cytochrome P-450. Early work in the rat suggested that there were two major forms of cytochrome P-450 induced by the prototypic compounds phenobarbital (PB) and 3-methylcholanthrene (3-

MC). The latter compound induces the enzyme accompanied by an absorption shift to 448 nm and is frequently referred to as cytochrome P-448. Subsequent work has shown that there are many additional isozymes in rat liver though the naming of them has not been uniform. In this discussion, the terminology of Ryan et al. (1982) will be used. By this method, isozymes are simply designated by a letter suffix in alphabetical order of first isolation. The number of isozymes by this system now extends to cytochrome P-450p (Siest et al., 1988). Phenobarbital induces primarily cytochromes P-450b and e (Ryan et al., 1982), while 3-methylcholanthrene and β-NF induce cytochrome P-450c, d and perhaps a third form (Seidel and Shires, 1986; Siest et al., 1988). Cytochromes P-450 a,f,g,h, and i are significantly expressed in untreated rats but their levels may rise or fall depending on the inducer (Ryan et al., 1985). Cytochrome P-450h is specific to male rats while cytochrome P-450i appears to be specific to female rats. The discovery of inducers proceeded largely because they produce significant changes in the total specific content of cytochrome P-450. However, certain inducers, such as isoniazid which induces cytochrome P-450j (Ryan et al., 1985), increase synthesis of one isozyme without significantly altering the total specific content of cytochrome P450.

The isozymic composition of cytochrome P-450 in a

microsomal sample can be differentiated by immunochemical, enzymatic or spectrophotometric methods. The latter method is least specific because of small spectral differences between isozymes. It is still useful in combination with other methods. Enzymatic characterization is frequently and successfully used to determine isozyme composition of a microsomal sample. Although there are hundreds of substrates for cytochrome P-450, some substrates are fairly restricted to a particular isozyme. In this regard the following substrates are of note. Benzphetamine, aminopyrine, and pentoxyresorufin are typical diagnostic substrates for dealkylase activity of cytochromes P-450 b and e induced by phenobarbital. Arylhydrocarbon hydroxylase activity of cytochrome P-450c is very specifically measured with benzo(a)pyrene or ethoxyresorufin. Immunochemical methods are useful in comparing and quantitating isozymes and provide the most direct and potentially most specific probe. The problem is that some isozymes exhibit so much sequence homology that polyclonal antibodies show significant cross reactivity. Therefore, enzymatic characterization of cytochrome P-450 was used for the work presented in this chapter.

Complicating the question of induction, cytochrome P-450 enzymes are not always the only xenobiotic detoxifying enzymes induced. Butylated hydroxyanisole, as an example, is an anti-oxidant that will induce aniline hydroxylase,

epoxide hydrolase and glucuronyl transferase activities in the liver microsomal fraction (Cha and Bueding, 1979). This compound will also increase the level of glutathione-S-transferase, glucose-6-phosphate dehydrogenase, and UDP-glucose dehydrogenase in the liver cytosolic fraction as well as the concentration of free sulfhydryl groups in tissue (Benson et al., 1978; Tsuda et al., 1988). Free sulfhydryl groups have been postulated to protect organisms from formed electrophilic metabolites of carcinogens by binding and thus inactivating them.

Pretreatment with inducers can result in an increase or a decrease in the carcinogenicity of a compound. Ethanol pretreatment enhanced the carcinogenicity of N-nitrosopyrrolidine in the liver (McCoy et al., 1981), while polycyclic hydrocarbons inhibited 2-acetylaminofluorene dependent tumor formation in the liver (Miller et al., 1958). The effect that an inducer has on the carcinogenicity of a compound, may be organ dependent. Pretreatment with butylated hydroxytoluene, for example, inhibited liver carcinogenicity, while enhancing bladder carcinogenicity of N-acetylaminofluorene (Williams et al., 1983). These effects are presumably due to modification of enzyme systems involved in the biotransformation of the carcinogen. However, a complicating feature with many inducers is that the inducing compound may be acting as a tumor promotor. Phenobarbital, a-hexachlorocyclohexane and

polychlorinated biphenyls, all enzyme inducers, also act as promoting agents for the development of preneoplastic foci when given after administration of initiators (Peraino et al., 1984; Pitot and Sirica, 1980). In contrast, treatment with other inducers including butylated hydroxyanisole and acetaminophen inhibit the formation of neoplastic and preneoplastic lesions following initiation (Tsuda et al., 1984). It is therefore very difficult to predict how the induction of metabolic pathways might affect the carcinogenicity of a chemical.

Changes in the toxicity of arylamines in general, or MBOCA specifically, following enzyme induction might be reflected in the amount of covalent binding to macromolecules. Pereira et al., 1981 were able to show that 2-AAF products bound to DNA and Hb to a much greater degree in rats than mice, presumably due to the greater bioactivation of 2-AAF in the rat, a species susceptible to the carcinogenicity of 2-AAF. In order to monitor the extent of bioactivation of MBOCA to the N-OH-MBOCA species, a probable proximate carcinogen, MBOCA-Hb adduct levels were measured (chap 5). The underlying hypotheses for this phase of the study were that a) the metabolism of MBOCA leads to activated and inactivated intermediates; b) enzyme induction will affect both inactivation and activation of MBOCA; and c) the overall change in the rate of formation of the N-OH-

MBOCA species, an activation product, will be reflected in Hb-adduct concentrations.

MATERIALS AND METHODS

Animals and Treatment

Male Sprague-Dawley rats weighing 180-210 g were obtained from Harlan Sprague Dawley, Indianapolis, IN. Food and water were available *ad lib*.. Animals were randomly distributed into one of three main groups. Each group of animals was injected with either sodium phenobarbital (PB), 80 mg/kg; β-naphthoflavone (β-NF), 80 mg/kg; or vehicle 1ml/kg. Phenobarbital was dissolved in 0.9% w/v NaCl solution, while β-NF was dissolved in corn oil. Control rats were treated with corn oil. After three days animals from each group were sacrificed for the enzyme studies. The remaining animals were further distributed into subsets that would be treated subcutaneously with either 0, 20, 100, or 500 mg/kg MBOCA. MBOCA treatment time was designated as day 0. MBOCA was dissolved in a mixed vehicle of 40:40:20 propylene glycol:DMSO:0.9% NaCl and injected in a volume of 2.0 ml/300 g body weight. Blood samples were obtained on day 0 just prior to dosing, and on days 3, 11, 18, 25, and 32 after MBOCA treatment. Blood was collected in EDTA tubes after docking the tip of the tail while the animal was under ether anesthesia. Two small hematocrit tubes were also

drawn for measurement of the hematocrit (Hct). The total volume of blood taken from each rat was between 0.5 ml and 1.0 ml.

Enzyme and Hb-binding assays

Microsomes were prepared from livers as previously described (chap 1). All protein concentrations were determined by the method of Lowry (Lowry et al., 1951) using bovine serum albumin as protein standard.

Activity of ethoxyresorufin-O-deethylase (EROD) was determined (in vitro) by the method of Johnson et al. (1979). Each incubation tube contained 0.5 to 2.0 mg of microsomal protein per milliliter as determined by the method of Lowry (Lowry et al., 1951). Each tube contained an NADPH regenerating system (4.5 mM glucose-6-phosphate, 0.3mM NADH, 0.1mM NADPH, and 1U glucose-6-phosphate dehydrogenase) in a total volume of 1.0 ml. The reaction was started by the addition of 10 µl of 0.125 mM ethoxyresorufin (Pierce Chemical, Rockford, IL). The reaction mixture was incubated for 5 minutes at 37^{o} C, and the reaction was stopped by the addition of cold acetone. The resorufin present was quantified by spectrofluorimetry; excitation = 510 nm, emission = 586 nm.

Benzphetamine-N-demethylase (BND) activity was determined by the method of Prough and Ziegler (1977). Hepatic microsomes were incubated with an NADPH regenerating system as described above, and the reaction was started by

the addition of 1.5 μmole benzphetamine (donated by the UpJohn Corp., Kalamazoo, MI). After incubation for 10 minutes at 37°C, the reaction was stopped by the addition of 1.0 ml of 10% TCA. The formaldehyde produced by benzphetamine demethylation was measured using the acetate/acetoacetone method of Nash (1953), and the chromophore was measured by U.V. absorption at 415 nm.

Benzo(a)pyrene monooxygenase (aryl hydrocarbon hydroxylase-AHH) was determined by the radiometric assay of Nesnow et al. (1977). Hepatic microsomes were incubated with an NADPH regenerating system as described above, and the reaction was started by the addition of 60 nmol of [^{3}H]benzo(a)pyrene (BP) (1μCi) (Amersham, England). After a 15 min incubation at 37°C the reaction was terminated by addition of 1.0 ml of 0.5 N NaOH in 80% aqueous ethanol. Each sample was vortexed, after which 3.0 ml of spectrograde hexane was added, then vortexed again for 2.5 min. After centrifugation, 10 μl of trifluoroacetic acid was added to the hexane phase, and after 2 min, the assay mixture was vortexed and recentrifuged. An aliquot (300 μl) of the lower phase was removed and neutralized with 0.5 N HCl, and the radioactivity measured.

MBOCA hydroxylation studies were carried out with microsomes incubated in a mixture consisting of 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM NADP, 0.5 mM NADH, 2.5 mM glucose-6-phosphate, 2 units of glucose-6-phosphate

dehydrogenase/ml, 1-2 mg of microsomal protein/ml and 0.100 mM MBOCA (bridge labeled, with a specific activity of 0.5 mCi/mmole). The substrate was added in 20 μl MeOH/ml of incubation medium. Incubations were performed at 37°C for 30 min. Precautions were taken to minimize loss of N-OH-MBOCA, incubations and subsequent manipulations being carried out under yellow light.

To terminate the incubations, sample vials were immersed in ice water, then extracted two times with a double volume of ice-cold CH_2Cl_2 by vigorous swirling under Ar. The organic phase was separated by centrifugation and dried through anhydrous Na_2SO_4, then evaporated under a stream of N_2.

Calibrated small volumes of CH_2Cl_2 were added to dissolve the metabolite residue, and samples were spotted on K5F silica gel TLC plates (Whatman, Hillsboro, OR). Development was performed with toluene/ethanol (20:1) in a TLC development tank purged with Ar. Quantitation of the metabolites was made by liquid scintillation counting of the scraped TLC plate, the R_f being determined from authentic standards.

Hemoglobin binding was determined as previously described (chap 5). Dried red cell globin powder was stored in scintillation vials at 4°C until enough samples were collected to conveniently continue with Hb-binding determinations. Red cell globin positive and negative

controls were included in each set of assays.

Statistical Analysis

Statistical testing was done using the Student's t-test ($p<0.05$). Multiple comparisons were made using analysis of variance followed by Scheffe's Test ($p<0.05$).

RESULTS

Enzymatic characterization of cytochrome P-450 activity after PB or β-NF treatment is summarized in Table 9. AHH and EROD activities both specific for cytochrome P-450c were significantly increased over control values in the β-NF treated group. Enzymatic activity had increased for these two approximately 10 and 5 times respectively. BND activity which is specific for cytochrome P-450b was significantly increased in the PB treated group, the activity being approximately 8 times that of the control activity. The increase in enzymatic activity thus confirmed that the cytochrome P-450 enzymes were indeed induced.

Table 9 also shows the effect of enzyme induction on MBOCA hydroxylation. Phenobarbital treatment did not significantly alter the amount of N-OH-MBOCA or o-OH-MBOCA formed. Rats treated with β-NF showed a significant increase of 7.5 fold in the formation of N-OH-MBOCA not observed with PB treatment. Ortho- hydroxylation, though apparently increased, was not statistically significant with any of the treatments.

Animals treated with MBOCA, even at the high 500 mg/kg dose, did not show any overt signs of toxicity.

Table 9

Effect of administration of PB or ß-NF on hepatic oxidase activity in male Sprague-Dawley rats[a]

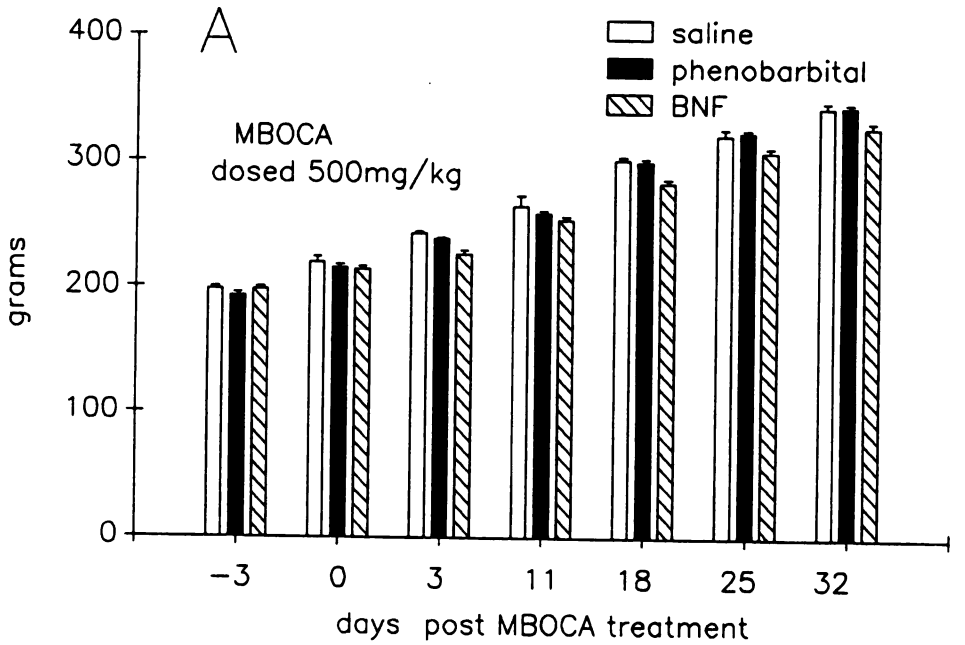
	VEHICLE (n=4)	PB (n=3)	ß-NF (n=3)
AHH	0.2 ± 0.1	0.5 ± 0.1	1.8 ± 0.2[b]
EROD	0.44 ± 0.2	0.31 ± 0.1	2.1 ± 0.3[b]
BND	1.2 ± 0.3	10.1 ± 0.5[b]	1.5 ± 0.1
N-OH-MBOCA	0.2 ± 0.1	0.6 ± 0.2	1.5 ± 0.2[b]
o-OH-MBOCA	0.1 +_ 0.05	0.3 ± 0.1	0.3 ± 0.1

[a]activities in nmol/mg/min
[b]significantly different from control
all measures are mean ± S.E.M.

No differences were observed in weight or hematocrit (Figure 21).

Figure 22 shows the formation and disappearance with time of the MBOCA-Hb adduct after a single sc administration of various doses of MBOCA to rats treated with vehicle, PB, or β-NF. MBOCA-Hb could still be detected in all groups after 32 days. The data in Figure 22 are redrawn in Figure 23 for days 3, 11, and 18 to show the dependence of binding on dose. Differences in the amount of Hb-adduct formation were observed for the β-NF treated group. Significantly higher MBOCA-Hb adduct levels were observed for the 500 and 100 mg/kg dose regimens but a difference was not evident for the low 20 mg/kg dose. On day 3, levels of MBOCA-Hb adduct were approximately 3 times higher in β-NF treated animals than the vehicle or PB treated animals. By day 32 this difference was no longer evident.

Figure 21 **Effect of treatment protocol on A) rat weights and B) hematocrits for the 500 mg/kg group.**


A
saline
phenobarbital
BNF
MBOCA
dosed 500mg/kg
grams
400
300
200
100
0
−3
0
3
11
18
25
32
days post MBOCA treatment

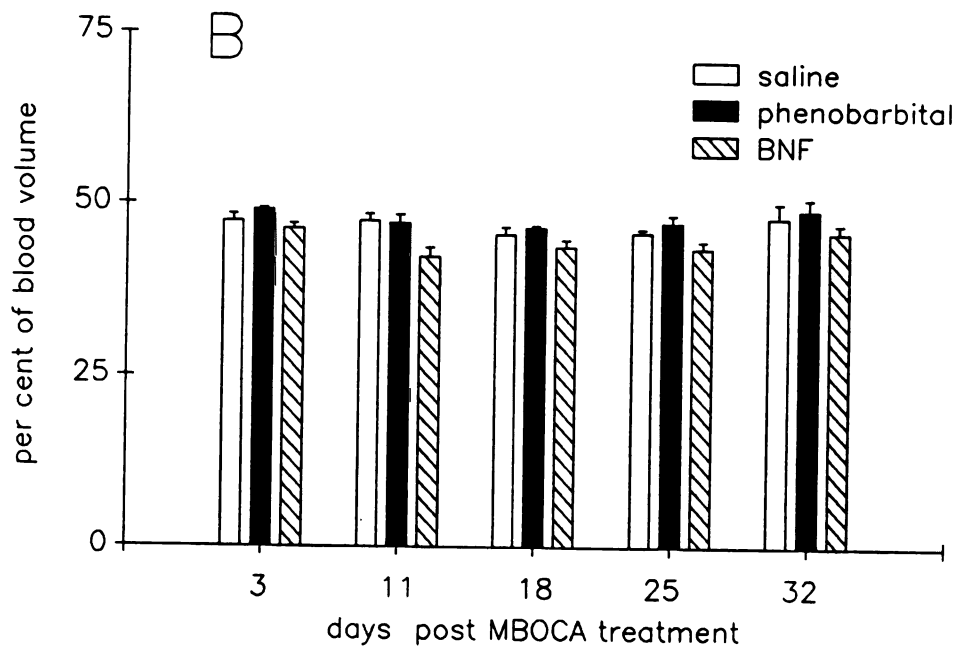

B
saline
phenobarbital
BNF
per cent of blood volume
75
50
25
0
3
11
18
25
32
days post MBOCA treatment

Figure 22 **Time course of MBOCA-Hb adducts in rats treated with vehicle, PB or β-NF followed by a single sc dose of MBOCA of either 20, 100 or 500 mg/kg. * Statistically different from the respective saline treated animals by Scheffe's test ($p<0.05$).**

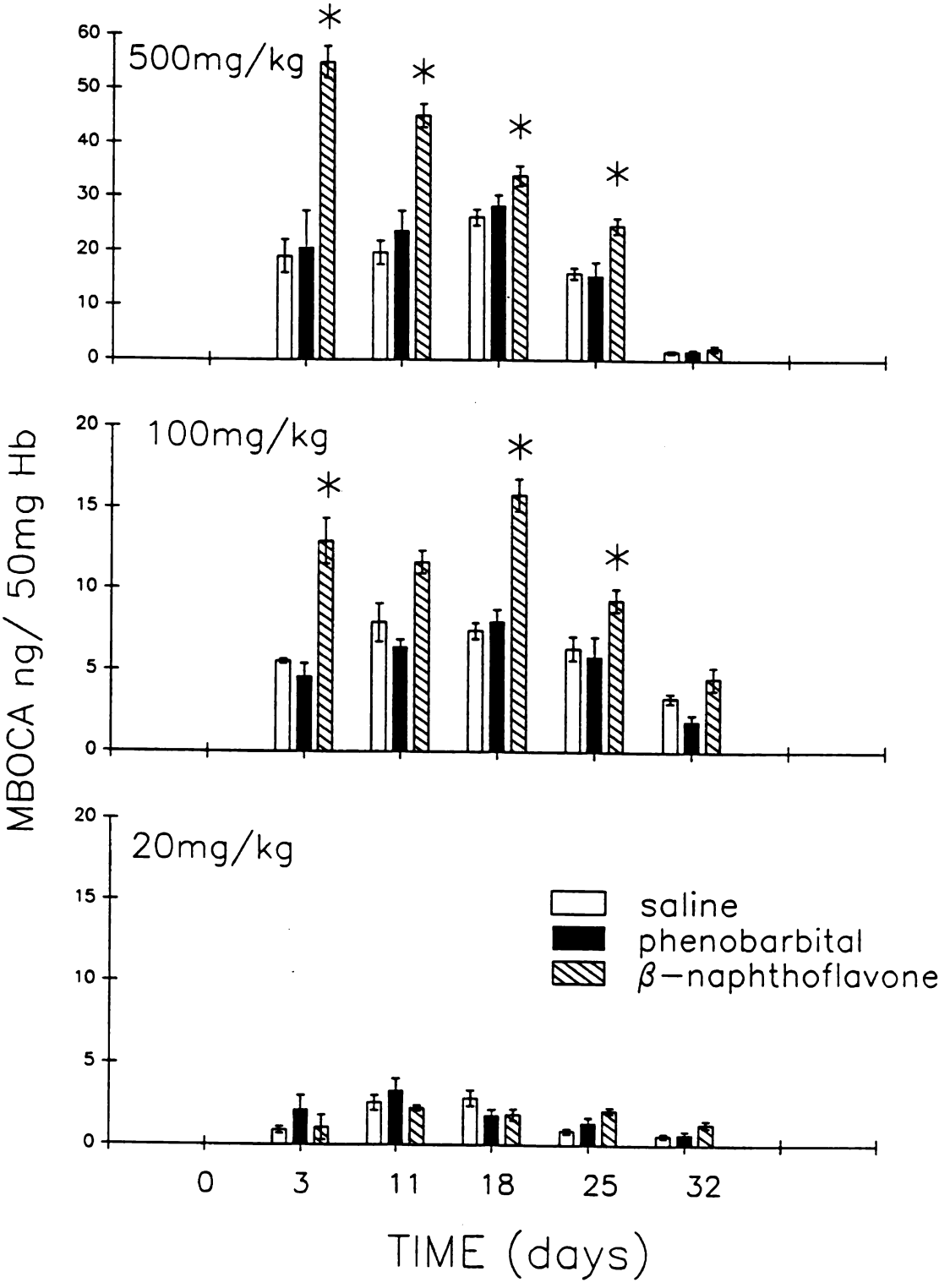
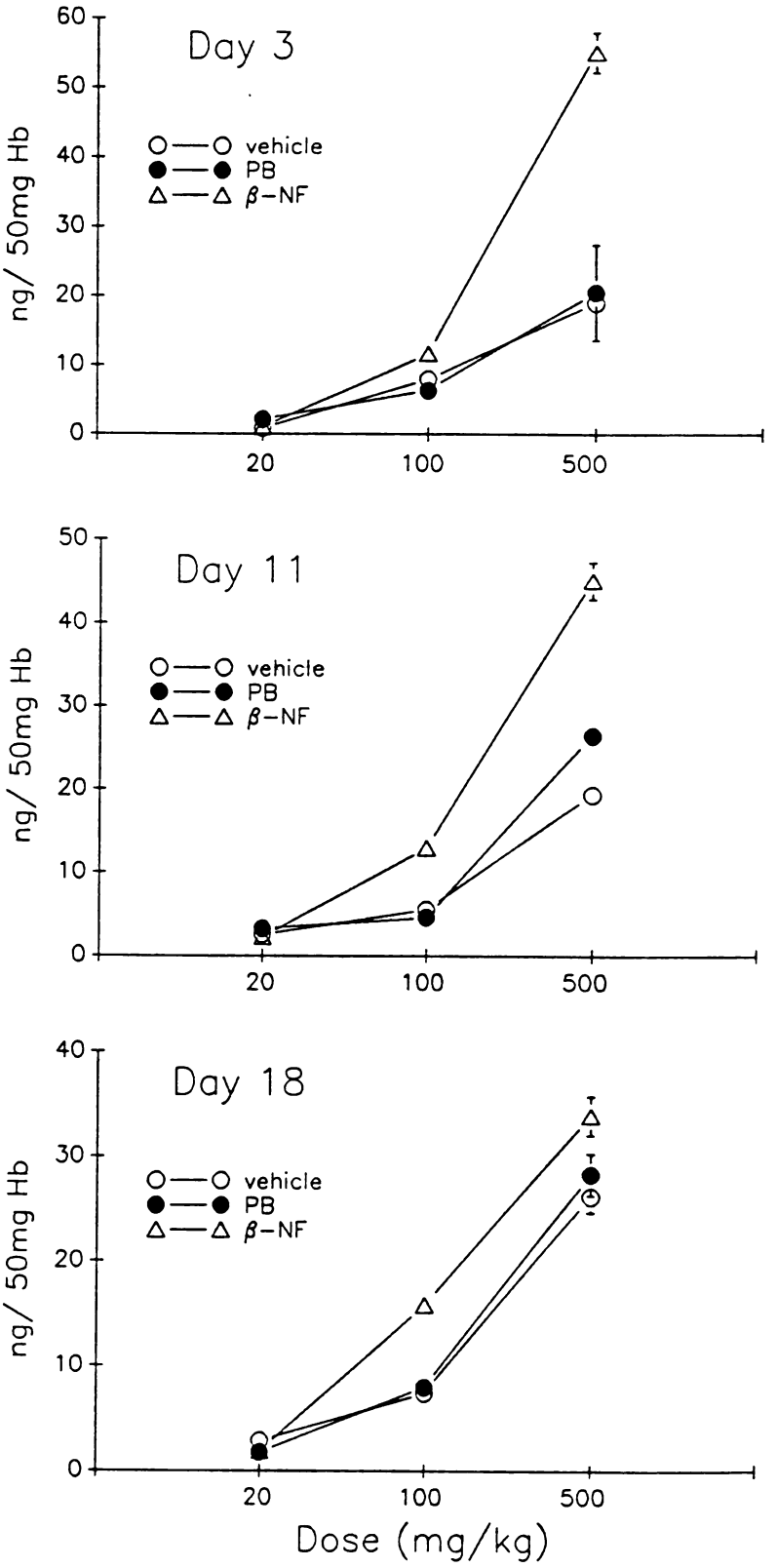

500mg/kg
100mg/kg
20mg/kg
MBOCA ng/ 50mg Hb
saline
phenobarbital
β−naphthoflavone
0
3
11
18
25
32
TIME (days)

Figure 23 **The effect of treatment of rats with vehicle, PB, or β-NF on Hb binding of MBOCA after subsequent treatment with 20, 100 or 500 mg/kg MBOCA. Data presented are for days 3, 11 and 18.**


Day 3
vehicle
PB
β-NF
ng/ 50mg Hb
60
50
40
30
20
10
0
20
100
500
Day 11
vehicle
PB
β-NF
ng/ 50mg Hb
50
40
30
20
10
0
20
100
500
Day 18
vehicle
PB
β-NF
ng/ 50mg Hb
40
30
20
10
0
20
100
500
Dose (mg/kg)

DISCUSSION

In the present study, a very sensitive assay for the determination of MBOCA-Hb binding was used to monitor changes in the formation of reactive metabolites *in vivo* after induction with representative cytochrome P-450 inducers. Induction by PB and β-NF of cytochrome P450-b and P450-c respectively was established. Two pathways of oxidative metabolism of MBOCA were studied; N-hydroxylation and *ortho*- hydroxylation. Induction by either PB or β-NF appeared to change the metabolic profile of Phase I metabolism of MBOCA. A significant difference was noted however for only the formation of N-OH-MBOCA after β-NF induction. Literature reports on the induction of arylamine N-hydroxylase have been varied. For example N-hydroxylation of some arylamines has been reported to be induced by 3-MC in rats only (Uehleke, 1967; McMahon *et al.*, 1980) while PB is reported to induce the same enzyme in rabbits and dogs (Uehleke and Nestel, 1967; Smith and Gorrod, 1978).

Levels of MBOCA-Hb adduct were significantly higher for β-NF treated animals. Whether or not this is a reflection of the increased formation of N-OH-MBOCA observed in the microsomal incubations is not certain. Differences in

activation and thus MBOCA-Hb binding almost certainly depend on activating pathways as well as inactivating ones. PB and β-NF have been shown to differentially induce different classes of UDP-glucuronosyltransferases (UDPGT), an enzyme that is generally considered as detoxifying (Boutin et al., 1985). One might argue that the increase in Hb-binding of MBOCA is not the result of an increase in N-OH-MBOCA formation as it is a relative decrease in the activity of detoxifying pathways.

The more rapid decrease of MBOCA-Hb levels in animals, treated with the highest dose of MBOCA and which initially showed high levels of binding, may be the result of an increase in the rate of red blood cell processing by the spleen due to red blood cell injury. This increase in the elimination of red blood cells may have been slow enough so as not to be reflected in changes in the hematocrit.

Studies have shown a difference in Hb-adduct formation due to differences in metabolic pathways. Lewalter and Korallus (1985) demonstrated that in humans, the fast acetylator phenotype consistently had lower levels of aniline-Hb binding. Pereira et al. (1981) showed that species with increased rates of N-hydroxylation had increased levels of 2-AAF-Hb binding. Prior to the currrent investigation, changes in Hb-adduct formation following induction had not been studied. This is the first report to show that enzyme induction will have an effect on Hb-

adduct formation.

Though the increase in Hb-MBOCA adduct formation due to induction of cytochrome P-450 isozymes by β-NF probably reflects an increase in activating pathways over inactivating ones, the biological significance of the increase in binding is not entirely clear. The animals in this study did not show any signs of overt toxicity, and the study was not designed to determine any changes in carcinogenicity due to this treatment protocol. The increase in binding may represent an increase in risk to the animal. This, however, has not yet been established. One other possibility is that there are no short or long term biological effects due to the induction of MBOCA metabolism. It has been suggested that binding of reactive species to nucleophiles, particularly those containing sulfhydryl groups may inhibit the carcinogenicity of a compound (Miller and Miller, 1972). Glutathione binds to the reactive intermediate of aflatoxin B_1 and has been shown to inhibit the carcinogenicity of this compound (Novi, 1981). It is possible therefore, that though there may be an increase in the formation of the reactive intermediate of MBOCA through induction of metabolism, cellular nucleophiles including Hb may bind to these before they reach critical cellular sites. Changes, therefore, in the levels of glutathione or available binding sites on Hb may affect the toxicity of MBOCA.

Future studies are needed to establish whether increased MBOCA-Hb binding is a reflection of increased risk. Studies are also needed to establish whether MBOCA-Hb binding is proportional to DNA-adduct formation, a relationship that has now been established for trans-4-dimethylaminostilbene and 4-aminobiphenyl (Neumann, 1984; Bryant et al., 1987).

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

A number of arylamines and arylamides are carcinogenic in a variety of tissues of several species. These compounds are metabolized to ultimate carcinogens through enzymatic and non-enzymatic pathways. An arylamine of current toxicological interest is 4,4'-methylenebis(2-chloroaniline) (MBOCA). MBOCA is a proven animal carcinogen whose metabolism has not been extensively characterized. The purpose of this theses project was to investigate the mechanism of MBOCA activation and the relative chemical reactivity of the oxidation products. It was hypothesized that MBOCA metabolism leads to oxidized compounds that are more toxic than the parent. In addition, it was hypothesized that conversion to these reactive intermediates can be altered by induction of the metabolic enzymes involved in their formation.

Similar to other arylamines, it has been proposed that the animal carcinogen MBOCA is metabolically activated to an electrophilic species, forming an N-hydroxy intermediate. Microsomal incubations with MBOCA did indeed result in the formation of the N-hydroxy as well as ortho-hydroxy and nitroso metabolites. These derivatives were formed in

varying degrees in rat, dog, guinea pig and human microsomes. The structures of these metabolites were confirmed by chemical synthesis, NMR, and/or EI-MS. DPEA as well as CO, both potent cytochrome P-450 inhibitors substantially reduced the formation of these oxidized MBOCA metabolites. The results suggest that metabolism of MBOCA is similar to other arylamines such as 2-naphthylamine and 4-aminobiphenyl (Hammons et al., 1985; McMahon et al., 1980), in that it involves the cytochrome P-450 system and that there are species differences in the relative importance in the sites of hydroxylation. The results of these experiments also provide evidence that the hepatic microsomal enzyme systems of several species including man are capable of oxidizing MBOCA to the hydroxylamine, a highly reactive intermediate and potential proximate carcinogen.

MBOCA has been shown by others to be positive in the Salmonella/mutagenicity assay only with the use of a metabolic activation system or S-9 (McCann et al., 1975a; Hesbert et al., 1985). The direct mutagenicities of the oxidized products of MBOCA were studied using a modification of this mutagenicity assay with no metabolic activation system. Of the oxidized derivatives of MBOCA, the N-hydroxy metabolite was the only one that was clearly mutagenic.

The effect of these oxidized metabolites on gap-junctional intercellular communication (GJIC) was also

assessed. An effect at non-cytotoxic levels on gap-junctional communication was not observed for any of the oxidized derivatives tested. In contrast, the parent compound did show an effect on dye-transfer in WB cells using the scrape-loading method for determination of chemical effects on GJIC. As it has been postulated that inhibition of GJIC is a reflection of tumor promoter potential, MBOCA may be acting as a tumor promoter.

The capacity of N-oxidized metabolites of MBOCA to form hemoglobin adducts was determined *in vitro*, and the formation of Hb adducts following *in vivo* administration of MBOCA was assessed. Hb adduct formation was determined by electron-capture GLC of MBOCA as the heptafluorobutyryl derivative following mild acid hydrolysis of protein bound MBOCA. The method was confirmed by gas chromatography-mass spectrometry with selected ion monitoring. N-hydroxy-, NO-MBOCA and MBOCA itself formed adducts to Hb *in vitro* in a dose-related manner. Binding was inhibited by cysteine and glutathione but not oxidized glutathione or methionine. Taken together, these results suggest that binding to Hb follows a similar mechanism as proposed for other arylamines in that it involves the activated N-hydroxylated derivative of the arylamine (Eyer and Liebermann, 1980; Green *et al.*, 1984; Neumann, 1984; Bryant, 1987). Intravenous administration of as little as 0.04 μmol/kg N-OH-MBOCA to rats resulted in measurable formation of MBOCA-Hb adducts

(0.9 ng/50 mg). Intraperitoneal administration of 0.5-50 mg/kg MBOCA to rats, and subcutaneous administration of 5 to 500 mg/kg MBOCA to rats and 4 to 100 mg/kg to guinea pigs resulted in dose-related formation of Hb adducts. MBOCA-Hb remained elevated in blood for greater than 10 weeks following a single subcutaneous dose in guinea pigs. These *in vivo* studies suggest that MBOCA is activated to an electrophilic intermediate capable of binding to Hb, that this binding is dose-dependent, and that it can be monitored using sensitive analytical methodologies.

The MBOCA-hemoglobin binding assay was used for the determination of MBOCA-Hb binding to monitor changes in the formation of reactive metabolites *in vivo* after induction with representative cytochrome P-450 inducers. Induction by PB and β-NF of cytochrome P450-b and P450-c respectively was established. Two pathways of oxidative metabolism of MBOCA were studied; N-hydroxylation and *ortho*- hydroxylation. Induction by either PB or β-NF appeared to change the metabolic profile of Phase I metabolism of MBOCA. A significant difference was noted however for only the formation of N-OH-MBOCA after β-NF induction. Literature reports on the induction of arylamine N-hydroxylase have been varied. For example N-hydroxylation of some arylamines has been reported to be induced by 3-MC in rats only (Uehleke, 1967; McMahon *et al.*, 1980) while PB is reported to induce the same enzyme in rabbits and dogs (Uehleke and

Nestel, 1967; Smith and Gorrod, 1978).

Levels of MBOCA-Hb adduct were significantly higher for β-NF treated animals. Whether or not this is a reflection of the increased formation of N-OH-MBOCA observed in the microsomal incubations is not certain. Differences in activation and thus MBOCA-Hb binding almost certainly depend on activating pathways as well as inactivating ones. Though the increase in Hb-MBOCA adduct formation due to induction of cytochrome P-450 isozymes by β-NF probably reflects an increase in activating pathways over inactivating ones, the biological significance of the increase in binding is not entirely clear.

In summary, the enzymatic formation of reactive intermediates of MBOCA in microsomal preparations in four species including man was established. The formation of a reactive hydroxlyamine has been shown to be characteristic of a number of arylmines (Uehleke, 1973). Evidence for the formation of a reactive species was also seen in the determination of MBOCA binding to hemoglobin in vivo. The amount of hemoglobin binding by MBOCA could be altered by induction of the metabolizing enzymes presumed to be involved in the initial hydroxylation of MBOCA. The oxidized metabolites of MBOCA were tested for direct mutagenic potential. The strong mutagenicity of NOH-MBOCA is further evidence for the reactivity of this species. The results from studies of GJIC effects of MBOCA and its

oxidized metabolites indicate that the parent compound is the only one that has a measurable effect on GJIC at non-cytotoxic levels. Since inhibition of GJIC has been suggested to be a reflection of tumor promoter potential, MBOCA may be acting as a promoter to the initiating effects of the activated metabolites of MBOCA. The SENCAR mouse skin tumor assay could be used to test this possibility. The MBOCA parent compound could be tested for promotion potential, and the N-OH metabolite could be tested to see if it is acting as an initiator.

The formation of reactive oxidative metabolites in microsomal preparations has not been correlated to risk. Likewise, risk has not been correlated to the amount of MBOCA-Hb adduct formation, degree of mutagenicity of oxidized metabolites, or GJIC inhibition effects by these compounds. Future work must establish the mechanism of DNA binding *in vitro* and *in vivo*, and correlate Hb binding with DNA binding for more accurate risk assessment. MBOCA is, however, a proven animal carcinogen. The results presented here show that it is capable of forming reactive intermediates both *in vitro* and *in vitro* in a manner similar to arylamines known to be human carcinogens (Case, 1954). For these reasons, MBOCA should continue to be considered a suspect human carcinogen.

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