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Canine In Vivo and In Vitro Metabolism of the Bladder Carcinogen 4,4'-Methylenebis(2-Chloroaniline)

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CANINE *IN VIVO* AND *IN VITRO* METABOLISM OF THE BLADDER CARCINOGEN 4,4'-METHYLENEBIS(2-CHLOROANILINE)

By

Melanie Otten Manis

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ABSTRACT

Canine *In Vivo* and *In Vitro* Metabolism of the Bladder Carcinogen 4,4'-Methylenebis(2-Chloroaniline)

By

Melanie Otten Manis

Metabolism of arylamines enhances excretion and results in both detoxified and activated products. Metabolism of known bladder carcinogens has been investigated in the liver, kidney medulla, and bladder but not in the renal cortex. 4,4'-Methylenebis(2-chloroaniline) (MBOCA), an arylamine bladder carcinogen in dogs, is a potential human carcinogen whose biotransformation has not been extensively investigated. The objectives of this investigation were 2-fold: (1) to determine the structure of the major canine urinary metabolite of MBOCA and (2) to test the hypothesis that liver and kidney play a role in arylamine metabolism, using MBOCA as a model.

Analysis of the major urinary metabolite of MBOCA demonstrated that it was 5-hydroxy-3,3'-dichloro-4,4'-diaminodiphenylmethane-5-sulphate, an ortho-hydroxy sulphate conjugate. Arylsulfatase but not β-glucuronidase or citric acid hydrolyzed the metabolite *in vitro*, indicating that it was a sulphate conjugate. Electron impact mass spectrometry following derivatization and transesterification indicated that the metabolite was ring hydroxylated and fast atom bombardment mass spectrometry confirmed the molecular weight as consistent with a sulfate

ester. Proton nuclear magnetic resonance studies indicated that the ring substitution was ortho to an amine. The major urinary metabolite of MBOCA corresponded to the same type of conjugate formed in dogs administered similar to known bladder carcinogens.

Tissue slice incubations from liver and kidney produced glucuronide, glucoside, and sulphate metabolites of MBOCA as determined by enzymatic, physical, and instrumental methods. Liver slices produced seven metabolites separable by high performance liquid chromatography and kidney cortex slices produced six. An N-glycoside and the ortho-hydroxy sulphate were identified in both organs. A hepatic metabolite was characterized as an o-glucuronide and both liver and kidney produced a metabolite with characteristics of an N-hydroxy-N-glucuronide.

^{14}C-MBOCA bound covalently to both liver and kidney slices in a substrate concentration and time-dependent manner. Binding was not altered in the presence of D(+)galactosamine or p-nitrophenyl sulphate. Binding decreased with a general inhibition of metabolism in the presence of 2,6-dichloro-4-nitrophenol, indicating that oxidation but not conjugation may be important.

This investigation provided the first demonstration of production of conjugated arylamine metabolites by the renal cortex. Thus, both liver and kidney may play a role in arylamine metabolism in the dog.

In memory of

Hans Hinnrich Mehrkens

ACKNOWLEDGEMENTS

My deepest thanks go to my husband Dr. Jack Jay Manis who makes this possible by his patience, love, wit, and stamina.

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LIST OF ABBREVIATIONS

ACN	acetonitrile
2AAF	2-acetylaminofluorene
2AF	2-aminofluorene
4AB	4-aminobiphenyl
ACS	aqueous counting solution
BZ	benzidine
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
DCB	3,3'-dichlorobenzidine
DCNP	2,6-dichloro-4-nitrophenol
DPM	disintegrations per minute
EI-MS	electron-impact mass spectrometry
FAB-MS	fast atom bombardment mass spectrometry
HPLC	high performance liquid chromatography
ip	intraperitoneal
iv	intravenous
LSS	liquid scintillation spectroscopy
MBOCA	4,4'-methylenebis(2-chloroaniline)
2NA	2-naphthylamine
NMR	nuclear magnetic resonance
PIC A	tetrabutylammonium phosphate; reagent for paired ion chromatography, acids
PNPS	p-nitrophenyl sulphate
RCM	radial compression module
RNA	ribonucleic acid
sc	subcutaneous
TCA	trichloroacetic acid

INTRODUCTION

A. Background

Bladder cancer is a significant cause of human death in industrialized nations. Bladder tumors represent about 2% of the tumors of the body and result in some 3% of the cancer deaths each year (Maltry, 1971). Urban populations have higher rates of bladder cancer than rural populations (Mommsen, 1983) and men have a three-fold greater incidence than women (King, 1982; Mommsen, 1983; Kern, 1984). This sex difference is probably related to occupation and personal habits, e.g. smoking (King, 1982). In some areas occupation-related bladder cancer accounts for 15-30% of the total (Lower, 1983). This indicates that bladder cancer may be, in part, a preventable disease.

Human bladder cancer is also a problem in less developed countries where Schistosoma haematobium is endemic in the water supply. Larvae penetrate the skin and migrate to the bladder where the organism causes a chronic infection (Boyland, 1963). Ova are passed in the urine. The disease, bilharziasis, is associated with an increased risk of bladder cancer (Boyland, 1963; King, 1982) which occurs equally in men and women. The cause is unknown but may be due to the chronic irritation or to the production of carcinogenic compounds (King, 1982). Improvement of sanitation may combat bladder disease in these areas.

The urinary bladder is susceptible to toxicity and carcinogenicity due to its role as a holding tank for concentrated metabolic wastes. It is a highly distensible organ, whose transitional cell epithelium is unique in its ability to change thickness and cell shape during bladder distension and contraction. The epithelium extends from the bladder, up the ureters and throughout the renal pelvis. Because of its exposure to urine, the transitional cell epithelium is constantly in contact with concentrated metabolic wastes. It is from this cell layer that bladder cancer arises.

Bladder tumors vary in their origin and severity. They range from benign papillomas to transitional cell carcinomas, squamous cell carcinomas, and adenocarcinomas. The latter two groups comprise less than 3% of the bladder cancers (Vidyarthi, 1971). Bladder cancers are rated in severity based on their invasive properties in the bladder, on lymph node involvement and on metastatic behavior. They were thought to originate mainly as papillomas (Vidyarthi, 1971). Recent surveys have demonstrated that non-papillary tumors are more invasive (Kern, 1984) and that nonpapillary lesions may be a more frequent precursor of bladder cancer (Brawn, 1984). Early detection remains essential for effective treatment.

Epidemiologic studies have elucidated the etiology of bladder cancer. Various aspects of lifestyle including smoking habits, coffee drinking, diet, saccharin consumption, and type of employment have been investigated to determine their relationship to bladder cancer. For some of these including smoking, the results are definitive.

Cigarette smoking increases the risk of bladder cancer. Relative risk for cigarette smokers increased to 1.89-5.8 in men and 2.0-4.5 in women (Cole et al., 1971; Simon et al., 1975; Howe et al., 1980). It increased in a dose-dependent manner in both males and females according to smoking frequency per day, duration of smoking in years and total life cigarette consumption (Howe et al., 1980).

Other tobacco products have also been surveyed. In contrast to the strong positive correlation with cigarette smoking, investigations into other types of tobacco use have had conflicting results. Cole et al. (1971) and Wynder et al. (1963) reported a weak or absent correlation between bladder cancer and cigar or pipe smoking. However, Howe et al. (1980) reported a significant increase in risk for pipe smoking by males and Mommsen et al. (1983) found increased risk due to cheroot smoking by females. No differences were noted for tobacco chewing or snuff use (Wynder et al., 1963).

Any apparent relationship between coffee drinking and bladder cancer may be noncausal (Simon et al., 1975). An increased risk was reported for men but not women (Bross and Tiding, 1973; Cartwright et al., 1981; Hartge et al., 1983; Mommsen et al., 1983). Conversely, an increased risk for women has also been reported (Simon et al., 1975; Howe et al., 1980; Morrison et al., 1982). This contradiction, the low positive relationship between bladder cancer and coffee drinking and the lack of dose response indicated that, if an association exists, it is weak (Simon et al., 1975).

Diet is not often included in epidemiologic studies of bladder cancer but may play a role in its appearance. Mettlin and Graham (1979) studied the diet of patients and controls using 29 edibles in addition

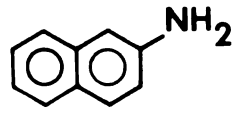
to coffee, tea, and milk. They reported that the risk of bladder cancer varied inversely with milk and carrot intake. Coffee drinkers, subdivided into groups by milk drinking habits, had a decreased risk of bladder cancer if they also drank milk. The differences observed were not significant, however. There was an inverse relationship associated with carrot consumption as well. Milk and carrots are high in vitamin A which has been associated with a protective role in lung cancer. The reduced risk of bladder cancer may have been due to vitamin A consumption (Mettlin and Graham, 1979).

Epidemiologic studies on saccharin usage had mixed results. Saccharin enhanced the risk of bladder cancer for men in a dose-dependent manner in one study (Howe et al., 1980), and enhanced risk only for male nonsmokers in another (Cartwright et al., 1981). In the latter investigation there was a nonsignificant increase in risk for female nonsmokers and no increase for smokers of either sex. Mommsen et al. (1983) reported an enhanced risk for nonsmoking women and Simon et al. (1975) found no increased risk for women using cyclamates or saccharin. This weak association has been difficult to characterize.

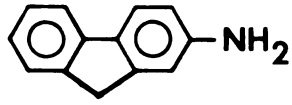
Other lifestyle factors have been studied. No increased risk was found for use of hair dyes (Howe et al., 1980; Hartge et al., 1982), use of estrogens (Mommsen et al., 1983), alcohol drinking habits (Cartwright et al., 1981) and tea consumption (Simon et al., 1975). Although most lifestyle factors are not associated with an increased risk of bladder cancer, certain occupations clearly stand out. Occupations with increased risk of bladder cancer include truck driving (perhaps due to exposure to diesel fumes) (Howe et al., 1980; Silverman et al., 1983),

food processing (Howe et al., 1980), and jobs in chemical and rubber industry, cable manufacture, textile works, leather works, coal tar and gas industry, and pigment and paint manufacture (Tola, 1980). Although the etiologic agent is unknown in most cases, exposure to arylamines is suspected in most of these industries. Arylamines provide the starting material for the manufacture of dyes used in leather, paper, textiles, and paints. They have been used as antioxidants and as polymeryzing agents in the rubber and cable industries and are a byproduct of the coal tar and gas industries (Tola, 1980). In a few well studied cases, arylamines in the workplace have been associated with an increased risk of bladder cancer.

Arylamines had been suspect bladder carcinogens for over 40 years before their carcinogenicity was demonstrated in an animal model. It was another 15 years before epidemiologic studies demonstrated the role of a particular arylamine, benzidine (BZ) (Figure 1), in human bladder cancer. In 1894 Rehn, an industrial surgeon, noted an increased incidence of bladder tumors in dye workers (Parkes, 1978). The dye industry in Germany was a scant 30 years old. He and others catalogued the cases in the early twentieth century as they developed parallel to the development of the dye industry. Then, in 1938, Heuper and Wolfe reported the development of bladder cancer in dogs treated with 2-napthylamine (2NA) (Figure 1) (Clayson and Garner, 1978). At that time it was the only animal tested which developed bladder cancer from 2NA (Clayson and Garner, 1978) and it has been used to test other industrial arylamines since then. It was not until the 1950's that Case and coworkers (1954) finished a controlled epidemiologic study and definitely demonstrated the role of 2NA and BZ in human bladder cancer. They demonstrated that



2 - naphthylamine



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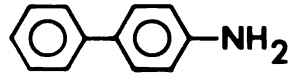
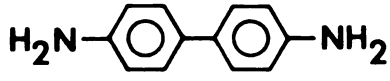
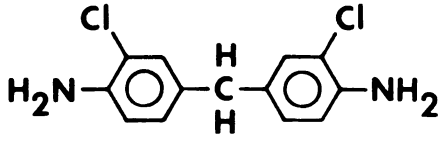


4 - aminobiphenyl



benzidine



4,4′ - methylenebis
(2 - chloroaniline)

Figure 1. Structures of Aromatic Amines

2NA and BZ, but neither 1-naphthylamine (1NA) nor aniline, were human bladder carcinogens.

Because of the efforts of Case and others the industrial use of demonstrated potent arylamine bladder carcinogens has been curtailed. 2NA and 4-aminobiphenyl (4AB) (Figure 1) are no longer used at all. BZ, although not used in many countries, remains a potential problem because of its use in the manufacture of imported dyes, which retain a residue (Boeniger et al., 1981). Exposure in the workplace is controlled more tightly than before and is monitored by testing for urinary excretion of the parent compound (Meal et al., 1981). Bladder cancer remains a concern in paint, pigment, dyestuff, chemical, rubber, and cable manufacture as well as in textile and leather works and the coal tar and gas industries.

The sensitivity of humans to arylamine-induced bladder cancer may, in part, be determined by acetylator phenotype. Acetylation is believed to be a detoxication step for arylamine bladder carcinogens. Liver N-acetyltransferase is a polymorphic enzyme in humans and in some recently developed animal models. These models have not yet been used to determine whether or not a different pattern of tumorigenesis will occur in rapid and slow acetylators but human epidemiologic studies suggest a direct relationship between slow acetylators and sensitivity to arylamine-induced bladder cancer. Cartwright et al. (1982) reported that although no relationship existed between acetylator phenotype and bladder cancer on a population-wide basis, a significant proportion of dye workers with bladder cancer were slow acetylators (96%). Evans et al. (1983), also in the United Kingdom, found that bladder cancer in

general but not industrial exposure specifically was associated with slow acetylators. Miller and Cosgriff (1983) (Rochester, NY) reported no difference from control in acetylator status of their bladder cancer population as a whole or when subgrouped according to industrial exposure. In these studies, the number of exposed workers was small (less than 25), the control groups were from different populations, and patients were not phenotyped at diagnosis. The differences in bladder cancer according to phenotype may have been due to a greater survival of slow acetylators (Evans, 1983). However, differences in the physiology of bladder cancer patients grouped by geographical region has been noted previously (Brown and Price, 1969) and may play a role in epidemiologic studies of this kind. Clearly more research is needed. Fast and slow acetylator animal models will be useful to study the problem.

The tumorigenesis of arylamines has been investigated in a number of animal species. Although arylamines differ in their potency and organ specificity, a general pattern of tumorigenesis is observed in each species. In rats, arylamines with two aromatic rings generally cause Zymbal and mammary gland tumors. Benzidine congeners including BZ, 3,3'dichlorobenzidine (DCB), dimethylbenzidine and dimethoxybenzidine cause Zymbal gland tumors (IARC, 1971; 1973; 1982b). BZ, DCB, 4AB, dimethylbenzidine and 4,4'-methylenebis(2-methylaniline) produce mammary tumors (IARC 1971; 1973; 1982b). Arylamines with one aromatic ring produce bladder cancer in rats. These include o-anisidine, m- and p-cresidine and 4-chloro-o-phenylenediamine (IARC, 1982a). Bladder cancer is also produced in rats by a few arylamines with two aromatic rings, including 2NA (Hicks et al., 1982) and tetrachlorobenzidine (IARC, 1982a).

Other animal species have not been as extensively used. Arylamines cause hepatomas in mice and can result in bladder cancer in rabbits and hamsters. Mice are sensitive to hepatomas from 4AB, BZ, 2NA, and DCB but not 1NA (IARC, 1973; 1982b). Although bladder cancer is observed in rabbits treated with 4AB, BZ and 2NA, the latter two are but weak carcinogens (IARC, 1971; 1982b). In hamsters 2NA and DCB produce bladder cancer, DCB and BZ produce hepatomas and dimethoxybenzidine produces forestomach carcinoma (IARC, 1973; 1982b).

Dogs have been used as a test animal for bladder carcinogens ever since Heuper and Wolfe demonstrated bladder cancer from 2NA in 1938 (Bonser, 1969). 4AB, BZ and DCB also produced bladder cancer in dogs (IARC, 1973; 1982b). In addition, the carcinogenic potency of these amines has been evaluated partly on the basis of the latency in dogs. Using this method, 4AB is the most potent bladder carcinogen; where administration of 30 g produced transitional cell carcinoma after 33 months (IARC, 1971). 2NA is also a potent carcinogen, it produces cancer in 30 months following administration of 100-200 g (IARC, 1973). Both BZ and DCB were weak carcinogens. They required 150-325 g and latent periods of 6-10 years (IARC, 1982b). Dogs have been a test animal for arylamine bladder carcinogens because they are sensitive to bladder tumors from arylamines, the tumors are of the same type and cell origin as observed in humans, and because carcinogenic potency can be evaluated using the latent period.

In spite of the reasons in favor of using dogs in arylamine bladder carcinogenicity testing their use in routine testing has been discouraged due to the long latent period and concomitant costs involved

(Bonser, 1969). Compounds that are bladder carcinogens in dogs are also carcinogens in rodents. Tests in rodents require significantly less time and resources. In addition, although spontaneous tumors are rare, they are observed in dogs after age 7, making results difficult to interpret (Bonser, 1969). Thus, the use of dogs in long-term testing might be inappropriate. They might be useful, however, in metabolism studies. Metabolism is often necessary for carcinogenesis and differences therein are reflected in differences in sensitivity and target organ specificity.

B. Arylamine Metabolism

Arylamines are lipophilic compounds which require metabolism for rapid excretion. Metabolism results primarily in detoxication, e.g. ring hydroxylation and conjugation (Clayson and Garner, 1978; Masson et al., 1983) resulting in metabolites that are more polar, more water soluble, and more rapidly eliminated than the parent compound. Some metabolites are common to several species. The major canine urinary metabolite of BZ, 2NA, 4AB, and 1NA, the ortho-hydroxy sulphate (Wiley, 1938; Clayson and Ashton, 1956; Sciarini and Meigs, 1958; Clayson et al., 1959) has been identified in rats (2NA, Booth et al., 1955; 1NA, Clayson and Ashton, 1956; BZ, Clayson et al., 1959) and rabbits (1NA, Clayson and Ashton, 1956). The ortho-hydroxy glucuronide is also formed in mice (BZ, Sciarini and Meigs, 1961), dogs (2NA, Booth et al., 1955; 1NA, Clayson and Ashton, 1956) and rabbits (1NA, Clayson and Ashton, 1956).

Arylamines can be detoxified by direct conjugation to the amine. The N-glucuronides of BZ (dog, rabbit, rat, Clayson et al., 1959; rat,

Lynn et al., 1983), 2NA (rat, rabbit, Boyland et al., 1957) and 1NA (rat, Clayson and Ashton, 1956) and sulphamates of 2NA and 1NA(rabbit, Boyland et al., 1957) and BZ (Clayson et al., 1959) are examples of the detoxifying reaction.

N-Acetylation, sometimes with added ring metabolism is observed in animal species except dogs. Rats, rabbits and mice produce N-acetyl- and N,N'-diacetylbenzidine (Sciarini and Meigs, 1961; Lynn et al., 1980, 1983). N-Acetylation with ring hydroxylation produces metabolites such as 4'-acetamido-4-amino-3-hydroxybenzidine sulphate (rat, mouse, rabbit, Clayson et al., 1959), 2-acetamido-6-naphthol (rat, rabbit, Booth et al., 1955) and monoacetyl-3-hydroxybenzidine sulphate or glucuronide (mouse, Sciarini and Meigs, 1961). Other metabolites include hydroxylation and conjugation at ring positions farther from the amine (Booth et al., 1955; Clayson and Ashton, 1956). Induction of ring hydroxylation also decreases tumorigenicity (Lotlikar et al., 1967). These metabolites are all considered detoxication products because of the decrease in tumorigencitiy, and due to their stability and rapid elimination.

Arylamine N-acetylation may play a role in the target organ specificity. In most species, except the dog, a rapid equilibrium exists bewteen the arylamine and the arylamide, favoring the arylamide (Irving, 1979; Kriek, 1979). As described above, these species, including rats, mice, and hamsters develop tumors of the liver, mammary gland, Zymbal gland and occasionally the bladder. This organ specificity may be due in part to arylamine metabolism in the individual organ. Hepatic carcinogenesis is believed to be due, in part, to sulfotransferase mediated

formation of a labile ester from hydroxamic acids (Weisburger et al., 1972; Beland et al., 1982; Poirer et al., 1982) and arylamines may require acetylation prior to initiating liver cancer. Zymbal gland tumors may be initiated by another route. The Zymbal gland is sensitive to arylamine and arylamide carcinogenesis and contains a high deacetylase activity in addition to P450 monooxygenase activity (Irving, 1979; Pohl and Fouts, 1983). Thus, arylamides may be deacetylated during or prior to the activation process. Bladder cancer from arylamines and arylamides in dogs may be due, in part, to their inability to acetylate arylamines (Poirer et al., 1963; Kriek and Westra, 1979; Weber et al., 1980) and their renal deacetylase activity (Lower and Bryan, 1976). The renal deacetylase activity towards 2-acetylaminofluorene (2AAF), 4-acetylaminobiphenyl, and 2-acetylaminonaphthalene was directly related to the susceptibility of the dog to bladder cancer from these arylamides (Lower and Bryan, 1976). Sensitivity to bladder cancer in humans may also be due, in part, to acetylator status (see Background). These observations, indicate that acetylation and deacetylation can alter the target organ specificity of carciongenesis.

The carcinogenicity of specific metabolites has been tested. The orthohydroxy metabolites of arylamines have not been consistently positive or negative in the mouse bladder implantation model. 3-Hydroxybenzidine, 3-hydroxybenizidine sulphate, 2-amino-1-naphthylsulphate, and 2-amino-1-fluorenol were negative (Allen et al., 1957; Bonser et al., 1963; IARC, 1982). However, 1-amino-2-naphthol, 2-amino-1-naphthol, 2-amino-1-naphthyl glucosiduronate, and 3-hydroxy-4-aminobiphenyl sulphate produced bladder tumors (Bonser et al., 1963). The mouse bladder model

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has been questioned because pellet implantation alone has enhanced tumor formation. Thus, the carcinogenicity of orthohydroxy metabolites remains unclear.

Other metabolites have also been tested for carcinogenicity. Benzidine metabolites N,N'-diacetylbenzidine and N-hydroxy-N,N'-diacetylbenzidine injected i.p. produced mammary and Zymbal gland tumors in rats (Morton et al., 1981), N-hydroxy-2-NA and N-hydroxy-1NA injected s.c. produced pulmonary and hepatic tumors in mice (IARC, 1973) and injected i.p. produced peritoneal cancer in rats (Belman et al., 1968). N-hydroxy-2NA also produced bladder cancer in mice by bladder pellet implantation (Bonser et al., 1963) and produced bladder cancer in dogs when instilled into the bladder in dimethylsulfoxide (IARC, 1973). These data clearly demonstrate the carcinogenicity of the hydroxylamine.

Arylamines require metabolic activation to exert their carcinogenic effect. N-Oxidation is the first step and a requirement for carcinogenic activity (Irving, 1979; Kriek, 1979). N-Hydroxylation is a minor reaction that takes place in the microsomal subcellular fraction in both the cytochrome P450 monooxygenase system and in the FAD containing monooxygenases (Frederick et al., 1982; Cummings and Prough, 1983). Formation of the hydroxylamine does not increase polarity and it must be further metabolized to be excreted. The relative degree of carcinogenicity of 4AB, 2NA, and 1NA is related to the concentration of their N-oxidation products in canine urine (Radomski and Brill, 1970, 1971). Phenobarbital induced N-hydroxylation of 4AB and 2NA (Uehleke and Brill, 1968) and reduced the latency of bladder cancer from 4AB in dogs (MacDonald et al., 1973). The hydroxylamine is not considered the ultimate carcinogen, however.

Hydroxylamines may be activated by conjugation with a sulphate or glucuronide. O-Esterification results in an intermediate which rapidly decomposes to a reactive electrophile, the arylnitrenium ion. This ion may be the ultimate carcinogenic species (Kriek, 1979). It has a positive charge on the nitrogen and interconverts at slightly acidic pH with the carbocation (Kadlubar et al., 1981), a resonance structure with the positive charge delocalized over the aromatic ring system. The carbocation reacts with nucleophiles at a position ortho to the amine (Morton et al., 1980) and the arylnitrenium ion reacts at the nitrogen atom. Since the majority of DNA binding occurs via the nitrogen atom (Martin and Ekers, 1980) and is thought to result in the heritable changes involved in initiation, the arylnitrenium ion is believed important in carcinogenesis.

Hartman and Schlegel (1979) concluded that the arylnitrenium ion singlet stability is related to carcinogenicity. Molecular orbital calculations indicated that carcinogenic arylamines formed arylnitrenium ions with singlet and triplet states of similar energy, the singlet being a stable species. Noncarcinogenic arylamines formed singlet states of higher energy and less stability than the corresponding triplet states. The carcinogenic or mutagenic potential of substituted arylamines was successfully predicted. This work indicated that arylnitrenium ions with enhanced opportunity to react as a singlet, i.e. longer singlet lifetime, would be more likely to act in initiation processes. In addition, calculation of nitrenium ion singlet stability might be useful as a predictive tool.

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Labile hydroxylamine conjugates can be formed from arylhydroxamic acids. Cytosolic N,O-acetyltransferase activity is capable of transferring the acetyl group of a hydroxamic acid from the nitrogen to the oxygen (King and Glowinski, 1983). This produces an extremely labile ester, the N-acyloxyarylamine. It has not been isolated but its existence has been inferred by C-8 guanine adducts formed on addition of DNA to *in vitro* incubations. They lack the acetyl group (King and Glowinski, 1983). Microsomal N,O-acetyltransferase activity exists as well but has not been as thoroughly characterized. In the rabbit but not the mouse, liver cytosolic N,O-acetyltransferase and N-acetyltransferase activities are on the same enzyme (Glowinski *et al.*, 1980), (N-acetyltransferase is the polymorphic enzyme responsible for acetylator phenotype). Dog tissues have neither N-acetyltransferase nor N,O-acetyltransferase activity (King and Glowinski, 1983). This species difference may be involved in differential sensitivity to arylamine carcinogenesis.

Formation of free radicals following oxidation may play a role in the carcinogenicity of arylamines. Free radicals may be formed on single electron transfer by NADH-cytochrome b5 reductase or NADPH-cytochrome c reductase and during peroxidation by horseradish peroxidase and prostaglandin synthase (Mason and Chignell, 1982; Mason, 1982). Formation of free radicals from arylamines has been studied in microsomal preparations by measuring the electron spin resonance signal. Free radical formation by N-hydroxy-N-methylaminoazobenzene and its congeners paralleled the carcinogenicity of the compounds (Nakayama *et al.*, 1980). Nakayama *et al.* (1982) found that 2NA readily formed

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products representing free radical forms of 2-naphthylhydroxylamine and 2-amino-1-naphthol but 1NA produced little free radical signal. Both cytochrome P450 enzymes and mixed function amine oxidase were involved. These free radicals can react with DNA *in vitro* and may be involved in arylamine carcinogenesis.

Labile ester or reactive free radical arylamine metabolites produced by the liver may not be involved in bladder carcinogenesis. These metabolites are not long lived enough to act at sites distant from their formation, such as the bladder. Other metabolites, detoxified by ring hydroxylation and conjugation are excreted in the urine, unreactive. The N-hydroxy-N-glucuronide conjugate, however, may be involved in bladder carcinogenesis. It is stable at physiological pH and releases the hydroxylamine at a slightly acidic pH such as is found in the urine of dogs and man (Kadlubar *et al.*, 1977, 1978; Poupko *et al.*, 1979).

Arylamine N-hydroxy-N-glucuronide conjugates are produced by the liver and excreted in the urine. Kadlubar *et al.* (1977) produced N-hydroxy-N-glucuronide conjugates from the hydroxylamine of 2NA, 1NA, and 4AB using uridine-5'-diphosphoglucuronic acid fortified canine hepatic microsomes. Poupko *et al.* (1979) repeated these results and isolated those conjugates of 2NA and 1NA from canine urine after administration of the parent compound. The N-hydroxy-N-glucuronide of 4AB was isolated from canine urine by Radomski *et al.* (1977). In addition, human and rat glucuronyltransferase-dependent metabolism of the three arylhydroxylamines was observed (Kadlubar *et al.*, 1977).

Support for a role of these glucuronide conjugates in the etiology of bladder cancer comes from several studies. The N-hydroxy-N-glucuronide conjugates are stable at neutral pH but release the hydroxylamine

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at slightly acidic pH or with β-glucuronidase (Kadlubar et al., 1977, 1978). Altering urinary pH of rats to 5.7 and 7.7 altered the amount of 2NA recovered as N-hydroxy-N-glucuronide in the expected manner (Kadlubar et al., 1981). The lipophilic hydroxylamines may react directly with the bladder epithelium as arylnitrenium ions produced in the acidic environment of the urine or may be absorbed by the bladder epithelium and further activated (Kadlubar et al., 1977). Oglesby et al. (1981) demonstrated that 2NA, N-hydroxy-2NA, and N-hydroxy-1NA were readily absorbed by rat bladder epithelium at pH 5 and 7 and distributed throughout the body. The N-hydroxy-N-glucuronide of 2NA was absorbed only at pH 5, under conditions of hydrolysis. Their study indicated that these compounds may be absorbed and recirculated and perhaps metabolized or re-excreted. These studies demonstrated that arylhydroxylamines may be delivered to the bladder from the liver in the form of an N-glucuronide. Metabolism by the bladder epithelium and other extrahepatic metabolism needs to be considered as well.

C. Extrahepatic Metabolism

Early studies indicated that the bladder played a minor role, if any, in metabolic activation of arylamines. N-Hydroxy-2NA but not 2NA was carcinogenic on direct instillation into dog bladder (Radomski and Brill, 1970). The same was true for 4AB in mice (Kadlubar et al., 1977). These early studies indicated that metabolic activation of arylamines was necessary prior to reaching the bladder. When a section of the bladder was protected from exposure to urine, it did not develop tumors. Exposed areas of the bladder in the same animal, treated with 2NA, developed tumors (Kadlubar et al., 1977). This demonstrated that

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the systemic circulation to the bladder did not contribute to carcinogenesis but that exposure to urine was necessary.

Recently, metabolism by the urothelium has been investigated more thoroughly. Poupko et al. (1983) compared the metabolism of 4AB by rat, bovine, and canine liver and bovine and canine bladder P450 enzymes. They demonstrated that N-hydroxylation occurred in both bladder microsomal preparations as well as in liver microsomes from dogs and rats. The relative rates of N-hydroxylation of 4AB, 2NA, and 1NA were correlated with their carcinogenic potency (4AB>2NA>1NA). Canine liver and bladder mucosal microsomes metabolized 4AB at equal rates when normalized per nmol P450 but the liver rate was ten times that of the bladder when normalized to microsomal protein. This study demonstrated that bladder mucosal cells may contribute to N-hydroxylation of arylamines.

Bladder organ and cell culture have been used to study arylamine metabolism. 2AAF is converted to ring hydroxylated detoxication products as well as to 2AF and N-hydroxy-2AAF in human and rat bladder culture (Moore et al., 1982). Glucuronides of the ring hydroxylated compounds were also formed. Bovine urothelial cells activated 2AF, 2AAF, 4AB, 2NA, and BZ to mutagens in S. typhimurium TA98 or TA100 and 2AF, 2AAF, and 4AB to mutagens in V79 cells (Hix et al., 1983; Oglesby et al., 1983). Thus, recent investigations have demonstrated that bladder cells are capable of oxidation, conjugation, and mutagenic activation of arylamines. Parent compound excreted in the urine or released by hydrolysis in the bladder may be metabolized by the urothelium.

Arylamine metabolism in the bladder may involve prostaglandin synthase. The transitional cell epithelium contains prostaglandin

synthase activity (Brown et al., 1980). Microsomal metabolism of 2-amino-4-(5-nitro-2-furyl)thiazole produced reactive metabolites which bound to added DNA and tRNA (Mattammal et al., 1981). Aspirin reduced prostaglandin production and lesions from N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide in rat bladder (Cohen et al., 1981). Thus, if an arylamine reaches the bladder unmetabolized, it may be metabolized there and contribute to carcinogenesis.

The kidney may also play a role in arylamine metabolism and activation. The kidney contains two distinct regions, cortex and medulla. There is a decreasing gradient of P450 enzymes from the cortex to the medulla and an increasing gradient of prostaglandin synthase activity in the same direction (Zenser et al., 1978; Armbrecht et al., 1979; Davis et al., 1981). This results in a difference in metabolic potential in the two regions. The proximity of the renal medulla to the bladder has sparked interest in the potential metabolism of arylamine bladder carcinogens by prostaglandin synthase.

Prostaglandin synthase has a combination of two enzymatic activities, fatty acid cyclooxygenase and prostaglandin hydroperoxidase (Mattammal et al., 1981; Marnett, 1981). Arylamines may be oxidized during the second step, prostaglandin G_2 reduction. Thus, in the presence of cyclooxygenase inhibitors such as aspirin and indomethacin, other peroxides e.g. cumene peroxide, can substitute for prostaglandin G_2. These tools have been used to investigate prostaglandin synthase-mediated arylamine cooxidation.

Prostaglandin synthase was capable of cooxidation of arylamines in microsomal preparations from ram seminal vesicle and rabbit renal

medulla. BZ, 2AF, 4AB, and 2-amino-4-(5-nitro-2-furyl)thiazole were cooxidized during arachadonic acid metabolism (Zenser et al., 1979a,b, 1980; Mattammal et al., 1981; Morton et al., 1983). Metabolism resulted in reactive products as determined by DNA binding (Zenser et al., 1980; Mattammal et al., 1981), RNA binding (Zenser et al., 1980; Mattammal et al., 1981; Morton et al., 1983), macromolecular binding (Zenser et al., 1979b) and mutagenesis (Robertson et al., 1983). Binding decreased in the presence of indomethacin or aspirin (Zenser et al., 1979a,b, 1980; Mattammal et al., 1981). Binding increased in the presence of the substrates arachadonic acid (Zenser et al., 1979b, 1980) and cumene hydroperoxide (Mattammal et al., 1981).

Metabolism of arylamines in renal medullary slices has also been demonstrated. BZ metabolism, measured by macromolecular binding, increased with addition of arachadonic acid and was inhibited by aspirin, indomethacin, and meclofenamate (Rapp et al., 1980). Mixed function oxidase inhibitors metyrapone and SKF-525A did not inhibit binding, demontrating that P450 enzymes were not involved.

Renal cortical arylamine metabolism has been observed, using mutagenicity in the Ames test as an index. Both S9 and microsomal fractions from mouse kidney activated 2AAF (Reddy et al., 1980). S-9 fractions also activated AF to mutagens (Aune and Dybing, 1979; Robertson and Birnbaum, 1982; Sutter et al., 1982). Renal mutagenic activation of 2AAF but not 2AF was inducible by 3-methylcholanthrene and 2,3,7,8-tetrachloro-p-dibenzodioxin (Aune and Dybing, 1979; Reddy et al., 1980). As in the liver, renal activation decreased with increased age (Robertson and Birnbaum, 1982; Sutter et al., 1982).

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Production of arylamine metabolites in the renal cortex has not been extensively studied. The cortex contains mixed function oxidase activities (Rush et al., 1983) and can form glucuronide (Dutton, 1980), sulphate (Mulder, 1981) and glutathione conjugates (Jakoby and Habig, 1980) with xenobiotics other than arylamines. 2AAF oxidation was observed in mouse renal S9. Renal hydroxylation of 2AAF was induced by 2,3,7,8-tetrachloro-p-dibenzodioxin, and N-hydroxylation was decreased (diGiovanni, 1979). Booth et al. (1955) noted that rat kidney slices did not metabolize 2NA. The slices were capable of hydrolyzing and acetylating 2-amino-6-naphthol sulphate and deacetylating several metabolites. Zenser et al. (1979) investigated BZ metabolism in rabbit hepatic and renal microsomes. They observed no metabolism with either fraction using NADPH or arachadonic acid as cosubstrates. Thus, attempts to demonstrate metabolism in the renal cortex have had limited success and the production of conjugated metabolites has not been demonstrated.

The renal cortex may be well suited for metabolism of bladder carcinogens for several reasons. First, it has a direct route of excretion of metabolites in the urine. Second, the cortex receives a high blood flow. The kidney represents 0.5% of the body weight but receives up to 20% of the cardiac output (de Wardener, 1973; Cohen and Kamm, 1976). The cortex represents 70% of the renal weight and receives 94% of the renal blood flow (de Wardener, 1973). Third, the renal cortex contains the enzymes required for oxidation. P450 monooxygenase activity toward AF and AAF was mentioned above. Other substrates include phenetidine, aniline, and 4AB (Jones et al., 1980). Finally, the potential for conjugation with glutathione, sulphate, and glucuronide

moieties exists, as outlined above and by Aitio and Marniemi, Powell and Roy, and Chasseaud (1980). Thus, the kidney has the potential to play a role in arylamine metabolism.

D. 4,4'-Methylenebis(2-chloroaniline) (MBOCA)

MBOCA (Figure 1), an arylamine, is the main component of an industrial curing agent. It is used in the manufacture of polyurethane foams, industrial rubber products, e.g. gaskets, belts, gears, solid tires, and consumer products, e.g. heels and soles of shoes, skate wheels. Although MBOCA is no longer manufactured in the USA, it is imported in amounts of 1-3.5 million pounds a year for the 200-400 plants that use it (TSCA, 1983). Exposure to MBOCA presents a health hazard due to its potential human carcinogenicity.

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). 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). Dogs developed only transitional cell carcinoma of the bladder when MBOCA was administered over 8-9 years (control animals had no tumors) (Stula et al., 1977). MBOCA is thus clearly a carcinogen in animals.

MBOCA has been tested in vitro for mutagenicity, genotoxicity, and transformation. It is mutagenic in two assays, the Ames test (procaryotic, reverse mutations) (McCann et al., 1975) and the mouse lymphoma assay (eukaryotic, forward mutation) (U.S. Dept. Health, 1983).

MBOCA is 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 also inhibited DNA synthesis in cell culture (Aust et al., 1981). Thus, MBOCA may be considered a potential human health hazard based on its widespread industrial use, its demonstrated carcinogenicity in animal models and its in vitro mutagenicity.

Human exposure has occurred during manufacture and use. Workers have had quantifiable urinary concentrations (Linch et al., 1971) and have experienced acute toxicity due to an industrial accident (Hosein and van Roosmalen, 1978). A two-mile wide area surrounding the site of its previous manufacture in Adrian, Michigan was contaminated and pre-school children residing in the area had detectable urinary MBOCA concentrations. MBOCA has also been detected in the urine of workers' families (Williams, 1979). Because metabolism is required for rapid excretion and for carcinogenic activation, these observations point to the need for in-depth studies of MBOCA metabolism.

Metabolism and disposition have been studied in rats and dogs. MBOCA 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. 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 dose was recovered in the urine by 24 hours, 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 (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. Excretion in the feces represents an opportunity for reabsorption, enterohepatic circulation, and continued exposure.

Few metabolites of MBOCA have been identified. Farmer et al. (1981) hydrolyzed urine from MBOCA-dosed rats with sulphatase-glucuronidase, increasing the yield of the parent compound from 1-2% to 3-6%. Two deconjugated metabolites were characterized by their HPLC retention time. Manis and Braselton (1984) identified the major canine urinary metabolite as the ortho-hydroxy sulphate, 5-hydroxy-3,3'-dichloro-4,4'-diaminodiphenylmethane-5-sulphate. This was similar to the major canine urinary metabolite of BZ, 4AB and 2NA (Wiley, 1938; Bradshaw and Clayson, 1955; Sciarini and Meigs, 1958). Further investigation of MBOCA metabolism will aid elucidating the mechanism of its carcinogenicity.

E. Purpose

The objective of this thesis project was to test the hypothesis that the liver and kidney both play a role in the metabolism of arylamines and that both can produce reactive metabolites. MBOCA was used as a model arylamine and the dog was used as a test animal due to its sensitivity to arylamine induced bladder carcinogenesis. The initial phase of the investigation concerned elucidation of the structure, binding characteristics and mutagenicity of the major MBOCA metabolite

identified in canine urine. Determination of the complete structure indicated that MBOCA may be metabolized in the dog similar to other arylamines and would be a good model for metabolism studies. Binding studies demonstrated the reactivity of the metabolite with DNA and protein in vitro. Methods developed in this phase of the investigation were then used to elucidate structure and reactivity of metabolites including the major urinary metabolite, produced in liver and kidney in vitro. Metabolism and reactivity in vitro were measured in terms of metabolite production, detected by high performance liquid chromatography (HPLC) and macromolecular binding in tissue slices. The results were used as a measure of the potential to produce metabolites and reactive species in vivo. The structures of metabolites were elucidated using chemical, physical, and enzymatic methods both during and after incubation. The studies included an investigation of the contribution of glucuronidation and sulphation to binding in hepatic and renal cortical slices.

Induction of bladder cancer by arylamines may involve metabolism at a number of sites in the organism. The potential for metabolism in liver, kidney and bladder have been demonstrated using other arylamines. Metabolism in the liver can produce an array of detoxication products, one of which, the N-hydroxy-N-glucuronide, is stable at physiological pH and labile at the acidic pH of the urine (Kadlubar et al., 1977, 1978). It can release a reactive hydroxylamine in the bladder. The renal medulla is physiologically proximal to the bladder and contains prostaglandin synthase. It is capable of metabolizing arylamines to reactive products that could be excreted in the urine and react with the bladder epithelium (Zenser et al., 1979a,b, 1980). The renal cortex contains

mixed function oxidase activity and the capacity for conjugation. Arylamine oxidation in the renal cortex has been observed in vitro (diGiovanni, 1979).

The specific objectives of this investigation are to determine:

1. The structure, protein and DNA binding, and mutagenicity of the major metabolite of MBOCA detected in liver slices and in canine urine.
2. The metabolism of MBOCA in canine liver and kidney slices in terms of
   a. rate of metabolism.
   b. types of conjugates produced.
   c. degree of macromolecular covalent binding.
   d. the role of specific types of conjugates in covalent binding.

METHODS

A. Animals

Healthy adult male mongrel dogs weighing 11-19 kg were used for slice incubation studies. They had free access to food and water prior to the experiments and were used within 3 days of receipt. Dogs of less than about 1.5 years did not produce the same metabolic profile in *in vitro* incubation.

Urine for isolating the major metabolite was obtained in a previous investigation (Manis *et al.*, 1984) where adult, male, conditioned, beagle-type mongrel dogs of 11-17 kg were used.

B. Chemicals

Methane sulfonic acid, gold label deuterium oxide, gold label m-cresol, glucuronic acid and diethyl maleate were obtained from Aldrich Chemical Co. (Milwaukee, WI), aqueous counting solution (ACS) from Amersham Corporation (Arlington Heights, IL), 2,6-dichloro-4-nitrophenol (DCNP) from Alpha (Danvers, MA), tetrabutylammonium phosphate (PIC A) from Waters Associates (Milford, MA), D-glucose [U-^{14}C] 13 mCi/mmol, $Na_2{}^{35}SO_4$ 43 Ci/mg, 220 mCi/ml from ICN Pharmaceuticals (Irvine, CA), ^{14}C-toluene (4×10^5 DPM/ml) from New England Nuclear (Boston, MA), anhydrous sodium sulphate from Mallinckrodt (Paris, KY), β-glucuronidase free-arylsulfatase, β-glucuronidase, Type I DNA, D(+)galactosamine,

p-nitrophenyl sulphate, 5,5'-dithiobis(2-nitrobenzoic acid), methionine-DL-sulfoximine and deoxycholic acid, sodium salt from Sigma Chemical Corporation (St. Louis, MO) and Soluene 350 and Demilume from United Technologies Packard (Downers Grove, IL). Solvents were HPLC, UV, spectrometric grade and were purchased from Burdick and Jackson (Muskegon, MI). Water was purified by passing distilled water through a four-bowl Milli-Q (Millipore Corporation) water purification system containing a 0.25μ filter.

4,4'-[^{14}C]Methylenebis(2-chloroaniline) (MBOCA*) specific activity 58 mCi/mmole and 4,4'-methylenebis(2-chloro[U-^{14}C]aniline) (^{14}C-MBOCA) specific activity 10.9 mCi/mmole (Amersham Corporation) were provided by the Michigan Toxic Substances Control Commission through the Office of Radiation, Chemical, and Biological Safety, Michigan State University. They were purified by HPLC on a C_{18} reverse-phase column in acetonitrile:water (47/53, v/v) prior to use. 4,4'-Methylenebis(2-chloroaniline) (MBOCA) was a gift from Daniel E. Williams (Division of Environmental Epidemiology, Michigan Department of Public Health) and was purified before use in a similar manner.

C. Isolation and Characterization of the Major Metabolite in Canine Urine and Hepatic Tissue Slices

1. HPLC characterization

Dog urine was collected in the course of a study on the absorption, disposition, and excretion of 4,4-[^{14}C]methylenebis(2-chloroaniline) 4.3 mCi/mmol (Manis et al., 1984). Aliquots of dog urine representing 5% of the urine at each timepoint 1/2-6 hours following intravenous administration of MBOCA* were pooled, brought to 20% acetonitrile

(ACN) and 0.001 M PIC A and filtered for analysis by HPLC. A 7.8 mm x 30 cm C_{18} μBondapak column (Waters Associates) was used. Fractions were collected every 30 sec for 30 min using a 25 min linear gradient with a 4 min lag of from 20-75% ACN:H_2O, 0.001 M PIC A, at at 4.5 ml/min. The radioactivity of each fraction was determined by liquid scintillation spectroscopy (LSS). Recovery of radioactivity from the column was 100%.

2. Isolation of the major metabolite

Dog urine was extracted 3 times with methylene chloride and stored at 4°C until use. The major metabolite was partially purified and concentrated using C_{18} reverse phase Sep Paks (Waters Associates) which had been activated with ACN and rinsed with water. Extracted urine, in 10 ml aliquots was applied to a Sep Pak. The metabolite was eluted with 5 ml 30% ACN:H_2O. The ACN was evaporated under N_2, two such aliquots combined and this procedure repeated. The second eluant was adjusted to 20% ACN, 0.001 M PIC A and filtered for HPLC. This method concentrated 95 ml of urine to about 25 ml.

Two HPLC purification steps were used to isolate the metabolite. First, the metabolite was eluted at 16.5 min on the 25 min linear gradient described above with a flow rate of 2.5 ml/min. The ACN was evaporated under N_2, the PIC A removed using Sep Paks, and the eluant adjusted to 10% ACN. Second, the metabolite was eluted at 14 min on a 10 min linear gradient of 10-40% ACN, 0.010 M ammonium acetate, pH 6.4, with a flow rate of 2.3 ml/min. The HPLC system was as described above, but used different 7.8 mm x 30 cm C_{18} μBondapak columns for each isolation step so that the second column was not exposed to PIC A. Following the second HPLC purification the ammonium acetate was removed using Sep

Paks and the metabolite was stored in water at 4°C until use. It was extremely stable under these conditions.

3. Hydrolysis

Hydrolysis of the metabolite was attempted using 3 different methods: citric acid, β-glucuronidase, and arylsulfatase. First, purified metabolite was evaporated to dryness under N_2, resuspended in 0.033 M citric acid, pH 3.9, and incubated at 37°C for 1 hour. The reaction mixture was extracted 3 times with dichloromethane, and the extracts pooled, dried over sodium sulfate, evaporated under N_2, resuspended in 40% ACN:H_2O (v/v) and filtered for HPLC. The metabolite was isolated and collected at 10 min on a 15 min 10-30% linear gradient of ACN:H_2O at 1.8 ml/min using a 3.8 mm x 30 cm μBondapak C_{18} column. The amount of metabolite present was quantified by LSS, and control and experimental values compared.

Hydrolysis with β-glucuronidase was performed in 0.075 M phosphate buffer at pH 6.0 using 312 units of enzyme and about 3 μg purified metabolite in a 1.5 ml assay. This was incubated for 1 hr at 37°C before extraction. The HPLC analysis was performed as described above. Phenophthalein glucuronide was used to assess enzymatic activity.

Arylsulfatase hydrolysis was performed using 0.005, 0.05, or 0.5 units of enzyme in 0.2 M Tris buffer, pH 7.1 and 2 μg of the metabolite in a 3 ml reaction mixture. The reaction was terminated by the addition of 0.76 ml 0.005 M PIC A and 3 ml dichloromethane. The reaction mixture was extracted three times and the organic phase prepared for HPLC by drying, evaporating, resuspending and filtering as described

for citric acid hydrolysis. The metabolite was collected from a 3.8 mm x 30 cm μBondapak column using a 15 min linear gradient from 4 to 75% ACN (v/v), 0.001 M PIC A at 1.8 ml/min and was quantified by LSS. The enzyme was inactivated for control experiments by placing it in a boiling water bath for 10 min followed by a quick cooling on ice.

4. Mass spectrometry

a. EI-MS. EI-MS was done by direct probe on a Finnegan 3200 at 70 eV. Because sulfate conjugates are not volatile and do not give good mass spectra the metabolite was acetylated using a method which simultaneously transesterified the sulfate conjugate (Paulson and Portnoy, 1970). Two hundred μl of 40:1 acetic anhydride:methane sulfonic acid were added to 2 μg of the metabolite and incubated at 100°C for 60 min. The reaction was stopped by placing the sample on ice and adding a chip of ice to the vial. After 5 min, the sample was extracted 3 times with 0.5 ml benzene and the extracts pooled and dried under N_2. The sample was resuspended in 20 μl dichloromethane for transfer to the probe capillary. An MBOCA control was derivatized and analyzed in the same manner.

b. FAB-MS. FAB-MS was done on a Varian CH5 double focusing instrument at 6.5 KeV using an argon atom beam. A KCl saturated glycerol matrix was used. The sample of 10-20 μg was applied to the probe tip and dried in a gentle stream of air.

5. Nuclear magnetic resonance

The metabolite was prepared for proton nuclear magnetic resonance (NMR) by hydrogen-deuterium exchange on the amine. About 500 μg was added to an activated, D_2O rinsed Sep Pak. The Sep Pak was rinsed twice with 5 ml D_2O, allowing 10 min between and after rinses for

exchange. The metabolite was eluted with 50:50 v/v ACN:D_2O, evaporated to dryness, resuspended in 0.325 ml D_2O, and passed through an 0.2μ filter before use. NMR was carried out on a Bruker WM 250 with fourier transform using peak suppression by selective inversion.

6. Covalent binding in vitro to DNA and protein

a. DNA binding. DNA binding was determined under conditions of arylsulfatase hydrolysis using 0.05 or 0.5 units of enzyme and 1 mg/ml DNA in a 2 ml reaction volume. The reaction was terminated and the DNA isolated as described by Mattammal et al. (1981) using 2% potassium acetate in ethanol to precipitate the DNA. Following extractions with ethanol and ether the DNA was resuspended overnight at 2°C in 1 ml 0.015 M NaCl-0.0015 M sodium citrate pH 7.0. The sample was divided for LSS and DNA determinations. DNA was hydrolyzed by addition of 2 volumes 10% TCA and incubation at 100°C 20 min prior to determination of DNA concentration by the Ceriotti method (Ceriotti, 1952). DNA recoveries were about 50%.

b. Protein binding. Protein binding was determined under conditions of arylsulfatase hydrolysis using 0.5 or 0.05 units of enzyme. The reaction was stopped by the addition of carrier BSA, PIC A to 0.001 M and 3 ml dichloromethane. Following 3 dichloromethane extractions protein was precipitated by adjusting the mixture to 10% TCA and placing at 0°C for 5 min. Protein was pelleted and the precipitate extracted with 10% TCA twice, followed by acetone and ethylacetate 3 times each. This was sufficient to reach background radioactivity in the extracts. The pellet was solubilized overnight at 37°C in 1 ml 1 N NaOH and divided for LSS and protein determination. Binding was

quantified by LSS following addition of an equal volume of 1 M acetic acid and protein was determined by the Lowry method (Lowry et al., 1951).

7. Mutagenicity

Salmonella typhimurium strains TA100 and TA1538, containing respectively base pair (McCann et al., 1975b) and frameshift (Ames et al., 1973b) mutation in the histidine operon, where chosen for the assay due to their susceptibility to mutation by arylamines and their widespread use with compounds similar to MBOCA (Ames et al., 1973a,b; McCann et al., 1975a; Bos et al., 1982). However, TA100 was not viable under the preincubation conditions of the assay and only TA1538 was used.

Bacteria for the assay were grown overnight in nutrient broth and were washed and resuspended with 0.2 M Tris, pH 7.1. Tris buffer was used due to the inhibition of the arylsulfatase by phosphate (Rammler et al., 1964) and resulted in a 30-40% loss of viability. The bacteria were preincubated for 0 or 1 hr with one unit arylsulfatase and 0.4, 4, 40, 80, or 200 μg of the metabolite of MBOCA in a 2 ml volume. Following the incubation 0.5 ml was plated in triplicate on Vogel Bonner E medium (Vogel and Bonner, 1956) using top agar supplemented with 0.05 M histidine and 0.05 M biotin. An aliquot was diluted 10^6 and 10^7 fold with phosphate buffered saline and plated in triplicate on nutrient broth plates for a colony count. All plates were incubated at 37°C. Colonies were scored at 36-48 hr.

D. Assessment of Metabolism In Vitro

1. Incubation procedure

Dogs were killed by lethal injection of pentobarbital (80 mg/kg). Kidneys were quickly excised, divided into 4 pieces by transverse cuts, rinsed by agitation in cold saline (0.85% NaCl) and placed in ice-cold phosphate buffered medium (slice buffer) containing 96.7 mM NaCl, 7.4 mM sodium phosphate buffer, 40 mM KCl and 0.74 mM $CaCl_2$ at pH 7.4 (Cross and Taggart, 1950). Liver samples were excised, cut into approximately one inch pieces, rinsed in cold saline and placed in a separate container of ice-cold slice buffer. Renal medullary tissue was carefully dissected away from cortico-medullary tissue and placed on ice in slice buffer. All phases of the experiments were initiated within 3 hours.

Thin slices were prepared from the renal cortex (400 mg $\pm$ 5%) renal medulla (400 mg $\pm$ 5%), or liver (200 mg $\pm$ 5%) by hand using a slicing block and razor blade. The slices were thin enough to curl when they were picked up with forceps. They were incubated in 4 ml of slice buffer. ^{14}C-MBOCA, 100 nmol/ml (9.09×10^{-4} μCi/nmol) was added in 20 μl dimethyl sulfoxide (DMSO). Incubations were performed in a Dubnoff shaker at 90 cycles/min at 37°C under 100% O_2 for 60 min with liver slices and 90 min with kidney slices. Changes in the above protocol including slice weight, time, preincubation additions, and substitutions are noted where they occur. Incubations were stopped by addition of an equal volume of ice-cold ACN and were placed on ice for 15 min.

2. Metabolite separation and quantitation

Incubated samples were prepared for HPLC through a multi-step procedure. Slices were homogenized in the incubation media with a Wheaton teflon-glass homogenizer and 1 ml 0.05 M PIC A and 3 ml dichloromethane were added. The homogenate was extracted 3 times with 3 ml dichloromethane. The extracts were dried over anhydrous sodium sulphate, evaporated under N_2, resuspended in 150 µl 1:1 ACN:H_2O, and filtered through 0.2µ nitrocellulose filters (BAS, from Anspec, Ann Arbor, MI). Glass fiber prefilters (Millipore Corporation) were sometimes used. Samples were kept at -20°C until use. Total recovery of radioactivity was 90-100% in the extract and 75-85% in the filtrate. Calculations were based on DPM in the incubation.

An HPLC method for separation of the metabolites was developed based on their anionic nature as glucuronide or sulphate conjugates. HPLC was performed using M6000 and M45 pumps, a model 720 system controller, U6K injector, and model 440 detector (Waters Associates, Milford, MA). Absorbance at 254 nm was monitored and recorded on a Linear chart recorder (Linear Instruments, Corporation, Irving, CA). Separation of metabolites was achieved using a 5µ C_{18} Novapak radial compression column (Waters Associates) with a 35 min linear gradient of 23-42.5% ACN, 0.0025 M PIC A at 3 ml/min. A second set of conditions was also used, including a 25 min linear gradient of 23-33% ACN, 0.0025 M PIC A followed by a 20 min linear gradient of from 33-42.5% ACN, 0.0025 M PIC A at 2.3 ml/min. During methods development, metabolite peaks were identified by collecting one minute or peak fractions through 60 min and LSS. For metabolite quantitation during the experiments, radioactive peaks only were collected and quantified by LSS using a Packard

B4600 refrigerated scintillation counter encoded with a predetermined quench curve. Most samples contained radioactivity at 10 times or more background although none less than 3 times background were used for quantification.

3. Assessment of macromolecular covalent binding

Macromolecular binding was determined on the extracted homogenate. The homogenate was brought to 10% trichloroacetic acid and macromolecules were allowed to precipitate on ice for 15 min. Following centrifugation the supernatant was discarded. The precipitate was extracted 6 times with 3:1 methanol:ether and 4 times with ethylacetate. Each extraction was a minimum of 15 min on ice and one of the first 6 was overnight at 4°C. Using this protocol background DPM were reached with 4 extractions of 3:1 methanol:ether and 3 extractions of ethylacetate. Following the last extraction the precipitates were allowed to dry in a fume hood. They were moistened with 0.3 ml (200 mg) or 0.6 ml (400 mg) 5% deoxycholic acid overnight and solubilized with 2-3 ml Soluene 350 at 50°C. Fifteen ml demilume was added and binding was quantified by LSS using the internal standard method with ^{14}C-toluene. Binding was expressed as pmol/mg wet weight of tissue.

E. Individual Experiments

1. Preliminary investigation of metabolism

a. Enzyme concentration dependence. In a preliminary experiment renal cortical slices of 100, 200, and 400 mg and liver slices of 50, 100 and 200 mg were incubated with 0.05 and 0.1 μmol/ml ^{14}C-MBOCA (0.05 μmol/ml was 1.8×10^{-3} μCi/nmol). Hepatic slice incubations were 90 min and renal cortical slice incubations were 120 min.

Metabolism was evaluated by HPLC as described in section C.2. and by macromolecular binding as described in section C.3.

b. Concentration dependence of MBOCA binding to tissue slices. The effect of MBOCA concentration on binding was determined using 0, 0.005 ($1.8x10^{-2}$ μCi/nmol - MBOCA*), 0.01 ($9x10^{-3}$ μCi/nmol - ^{14}C-MBOCA), 0.025 ($3.6x10^{-3}$ μCi/nmol), 0.05 ($1.8x10^{-3}$ μCi/nmol), 0.100, and 0.200 ($4.5x10^{-4}$ μCi/nmol) μmol/ml with 200 mg liver incubated 60 min and 400 mg renal cortex incubated 90 min. Macromolecular binding was determined after dichloromethane extraction as described in section C.3. Controls were inactivated with ACN prior to adding ^{14}C-MBOCA.

c. Time dependence of metabolism. Appearance of metabolites was studied in liver slices 100 mg ± 5%, 0.05 μmol/ml ^{14}C-MBOCA ($1.8x10^{-3}$ μCi/nmol), 0-90 min and in 400 mg kidney, 0.1 μmol/ml ^{14}C-MBOCA, 0-120 min. Time dependence of macromolecular binding was determined in 200 mg liver or renal cortical slices over a 0-90 min time period for liver, and 0-120 min for kidney using 0.1 μmol/ml ^{14}C-MBOCA.

2. Elucidation of metabolites - sulfation

a. Incubation with $Na_2{}^{35}SO_4$. Renal cortical and hepatic slices were incubated as described in section C.1 except 100 nmol/ml cold MBOCA and 2 mM $Na_2{}^{35}SO_4$ ($8x10^6$ DPM and $7.4x10^8$ DPM) were used. Incubations were prepared for HPLC as described in section C.2. One-minute or peak fractions were collected across the gradient (36 min-kidney; 32 min-liver) and DPM were quantified by LSS using the quench curve for ^{14}C. As a control, CPM were measured 0-167 KeV and found to be no different than the 0-156 KeV used for ^{14}C.

b. Incubation with p-nitrophenyl sulphate (PNPS). Renal cortical and hepatic slices were preincubated 10 min in 0, 0.1, 0.5, and 2.0 mM PNPS under incubation conditions described in D.1. prior to the addition of ^{14}C-MBOCA. Metabolism was evaluated as described in sections D.2-D.3.

c. Incubation with 2,6-dichloro-4-nitrophenol (DCNP). Renal cortical and hepatic slices were preincubated 10 min in slice buffer containing 2 mM Na_2SO_4 and 0, 0.1, 1.0, 10.0, and 100.0 μM DCNP under incubation conditions described in D.1. prior to adding ^{14}C-MBOCA. Metabolism was evaluated as described in sections D.2.-D.3.

3. Elucidation of metabolism - glucuronides

a. Incubation with ^{14}C-glucose. Hepatic liver and renal cortical slices were incubated as described in C.1. except that 100 nmol/ml cold MBOCA and ^{14}C-glucose (3.7×10^7 DPM, 1.29 μmol) were used. In another experiment 1.85×10^8 DPM (6.47 μmol) ^{14}C-glucose were used with hepatic slices. Incubations were prepared for HPLC as described in section D.2. Fractions were collected across the gradient and DPM were quantified by LSS.

b. Incubation with D(+)galactosamine. Renal cortical and hepatic slices were preincubated 10 min in 0, 0.6, 3.0, or 15.0 mM D(+)galactosamine under incubation conditions described in D.1. prior to adding ^{14}C-MBOCA. Metabolism was evaluated as described in sections D.1.-D.3.

4. Elucidation of metabolism - glutathione

Preliminary studies were done to inhibit glutathione conjugate formation by depleting available glutathione or inhibiting its formation. The decrease in nonprotein sulfhydryl groups due to the treatment

was determined on a second set of slices preincubated 10 min with the inhibitor. Nonprotein sulfhydryl groups were determined by the method of Ellman (1959). Briefly, tissues were homogenized, then precipiated with 20 vol 6% TCA and centrifuged to pellet the precipitate. For the colorimetric reaction 0.5 ml of the supernatant was used with 2.0 ml 0.3 M Na_2PO_4 pH 8.2 and 0.5 ml 0.04% 5,5'-dithio-bis(2-nitrobenzoic acid) in 10% Na citrate. Optical density at 412 nm was determined immediately and compared to a standard curve of 0-50 μg/ml reduced glutathione.

a. Incubation with diethyl maleate. Renal cortical or hepatic slices were preincubated 10 min with 0, 0.2, 1.0, or 5.0 mM diethyl maleate under incubation conditions before adding ^{14}C-MBOCA. Metabolism was evaluated as described in sections C.1.-C.3.

b. Incubation with methionine sulfoximine. Renal cortical and hepatic slices were preincubated 10 min with 0, 0.2, 2.0, or 10.0 mM methionine sulfoximine before adding ^{14}C-MBOCA. Metabolism was evaluated as described in sections C.1.-C.3.

5. Post-incubation analyses

For post-incubation analysis the dichloromethane extracts for each tissue described in section C.2. were pooled and stored at -20°C until use. Extracts were divided, evaporated under N_2 and resuspended in the appropriate medium for each test.

a. Citric acid hydrolysis. Dried extracts were resuspended in 2 ml 0.075 M phosphate buffer pH 7.0. Samples received 50 or 100 μl 1 M citric acid (final pH 3.60-4.70). All tubes were incubated 60 min at 37°C in a shaking water bath. Samples were neutralized with 4 drops

0.1 N NaOH. Tubes were extracted and analyzed as described in section C.2. following the addition of 0.2 ml 0.05 M PIC A.

b. β-glucuronidase hydrolysis. Dried extracts were resuspended in 2 ml 0.075 M phosphate buffer pH 7.0. Samples received 500-100 units glucuronidase and all tubes were incubated 4 hours at 37°C in a shaking water bath. Following addition of 0.2 ml 0.05 M PIC A the tubes were extracted with dichloromethane, prepared for HPLC, and analyzed as described in section C.2.

c. Arylsulfatase hydrolysis. Dried extracts were resuspended in 2 ml 0.2 M Tris-HCl buffer at pH 7.1. Samples received 4-8 units of arylsulfatase and all tubes were incubated at 37°C, 4 hours in a shaking water bath. Samples and controls were extracted, prepared for HPLC and analyzed as described in section C.2. following addition of 0.2 ml 0.05 M PIC A.

d. Thermal hydrolysis. Dried samples were resuspended in 2 ml 0.075 M phosphate buffer pH 7.0. Controls were placed in the refrigerator and samples were placed at 37°C in a shaking waterbath for 24 hr. After adding 0.2 ml 0.05 M PIC A samples and controls were extracted, prepared for HPLC, and analyzed as described in section C.2.

6. Mass spectrometry

a. Isolation of peaks from in vitro incubations. Metabolites eluting at 9.5 min (liver) and 15.5 min (kidney) were isolated from in vitro incubations for mass spectrometry. Incubations were homogenized and extracted as described in section C.2. The dichloromethane extracts from 3 incubations were pooled, dried and resuspended in 5 ml H_2O. This was passed over a C_{18} Sep Pak (Waters Associates)

previously activated with 20 ml ACN and equilibrated with H_2O. The Sep Pak was rinsed with 10 ml water and the metabolites eluted with 1:1 ACN:H_2O. The ACN was evaporated under N_2 and the sample filtered for HPLC. A 5μ C_{18} radial compression module (RCM) column (Waters Associates) and a 40 min hyperbolic (#8) gradient was used to isolate the metabolites. The column eluant was evaporated to dryness and resuspended in ACN for storage.

b. Fast atom bombardment mass spectrometry (FAB-MS). FAB-MS was done on a Varian CH5 double focusing instrument at 6.5 KeV using a xenon atom beam. A KCl saturated glycerol matrix was used. The samples of 1-5 μg were applied to the probe tip and dried in a gentle stream of air. The FAB-MS was done at the MSU Mass Spectrometry Facility which is supported by NIH grant RR00480-13.

c. Electron-impact mass spectrometry (EI-MS). The structure of the peak at 9.5 min was investigated by EI-MS. Earlier observations indicated that it was N-conjugated, possibly with a sugar molecule. The conjugate was too unstable to derivatize and run directly but the sugar molecule, released on hydrolysis was suitable for derivatization and mass spectrometry. Thus, 75 nmol of the isolated conjugate was dissolved in water maintained at room temperature for 48 hours. This resulted in complete disappearance of the peak at 9.5 min and concomitant appearance of MBOCA as determined by HPLC and LSS as described in section C.3.

For EI-MS 60 nmol were derivatized with 1:1:3 trimethylsilyl imidazole: N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA): pyridine for 20 min at 100°C. This produced a penta-TMS (trimethylsilyl) derivative. The reagents were evaporated under N_2 and the sample

was resuspended in dichloromethane. The derivative was analyzed by gas chromatography-mass spectrometry (GC-MS) on a Finnigan 3200 GC/MS with Riber SADR data system. Chromatography was carried out on 2 separate columns, 1% OV17 and 1% OV1 both on gas chrom Q using 30 ml/min helium and a temperature program of 140-260°C at 12°C/min beginning 0.5 min after injection. This method was adequate for detection of 3 nmol of the derivatized sugar. Mass spectra were obtained at 70 eV.

7. Renal medullary metabolism

Renal medullary metabolism was evaluated through binding studies done on 400 mg tissue incubated 90 min. These studies included the substrate concentration dependence of binding and binding in the presence of 0.2 or 1 mM arachadonic acid, 0.28 mM indomethacin or 1.0 mM aspirin. Incubations with added compounds were done under yellow lights due to the light sensitivity of arachadonic acid. Arachadonic acid was diluted in ethanol and stored in the dark, under N_2, in the freezer. Its purity was tested prior to use. TLC on LHP-K plates was done using 99:1 ethylacetate:acetic acid and visualizing in iodine vapors. Arachadonic acid had an R_f of 0.67 under these conditions.

F. Statistical Methods

Differences from control values were determined at $p \leq 0.05$ by one-way or two-way analysis of variance. Metabolites from renal cortical and hepatic slice incubations with added inhibitors or enhancers of conjugation and associated macromolecular binding studies were analyzed. The least significant differences test was used for individual comparisons where the analysis of variance indicated that differences existed.

Renal medullary metabolism was evaluated by one-way analysis of variance in arachadonic acid studies and by a t-test when indomethacin and aspirin were used.

RESULTS

A. Isolation and Characterization of the Major Metabolite Detected in Canine Urine and Hepatic Tissue Slices

1. HPLC characterization

The major metabolite of MBOCA in canine urine and hepatic tissue slices was initially characterized in urine by HPLC and LSS. Figure 2 illustrates a profile of radioactivity which eluted from the column under gradient conditions and indicated a major peak at 16.5 min. This peak contained an average of 75% of the eluted radioactivity in the urine of the 2 dogs tested. Under these conditions MBOCA eluted at 22.5 min.

2. Hydrolysis

Both chemical and enzymatic hydrolysis of the major metabolite were used to elucidate the structure of the isolated compound. Mild acid conditions using citric acid and enzymatic hydrolysis with β-glucuronidase have been used to hydrolyze N-hydroxy-N-glucuronides and O-glucuronides of arylamines, respectively (Kadlubar et al., 1978). Neither of these treatments altered retention time, peak height, or the amount of the major urinary metabolite of MBOCA recovered from the reaction mixture (data not shown).

Arylsulfatase hydrolysis, however, resulted in a time and enzyme concentration-dependent decrease in the amount of the metabolite

Figure 2. HPLC characterization of the major urinary metabolite of MBOCA. A representative plot of the LSS analysis of 30 sec fractions collected from a 25 min linear gradient with a 4 min lag, 23-75% acetonitrile:water, 0.001 M PIC A at 4.5 ml/min. A 7.8 mm x 30 cm 10μ C_{18} μBondapak column (Waters Associates) was used.

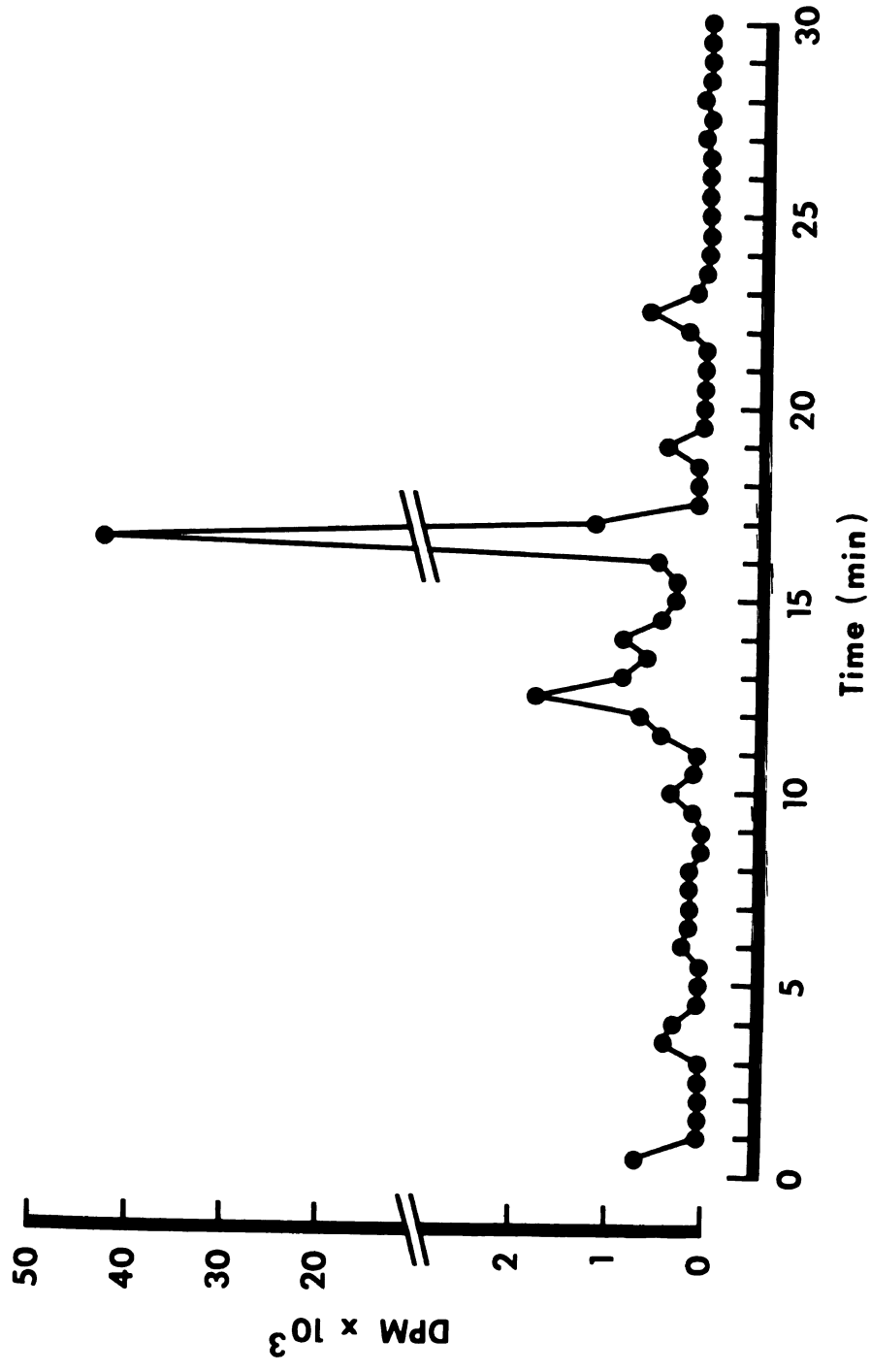

Figure 2

in the reaction mixture. Figure 3 is representative of the time course of the hydrolysis, demonstrating that the reaction was log-linear for 60 min at both 0.05 and 0.5 units of enzyme and indicating that the metabolite was a sulfate conjugate.

3. Mass spectrometry

EI-MS provided initial evidence that the metabolite was a product of ring metabolism of MBOCA. The derivatized metabolite had a molecular ion M^+=492, which represented a hydroxylated MBOCA metabolite with 5 acetyl substituents, and fragment ions at m/z 450 (M-42), m/z 390 (M-2x42-18), m/z 282 (M-5x42), and m/z 156. The derivatized MBOCA had a similar fragmentation pattern with a molecular ion at M^+=434 and fragment ions at m/z 392 (M-42), m/z 332 (M-2x42-18), m/z 266 (M-4x42) and m/z 140.

The FAB mass spectrum (Figure 4) yielded a molecular ion [M(K salt)+K]$^+$ at m/z 439. This corresponded to the K^+ salt of the ortho-hydroxy sulfate metabolite with a K^+ adduct. Formation of positive ion adducts in FAB is not unusual and has been observed for other conjugated metabolites (Liehr et al., 1982) under similar mass spectrometric conditions. The FAB data for the metabolite of MBOCA is consistent with a ring hydroxylated sulfate conjugate structure.

4. Nuclear magnetic resonance (NMR)

The NMR analysis provided additional data in support of the proposed structure for the major urinary metabolite of MBOCA. There were 5 ring protons and 2 methylene protons as determined by integration (Table 1). The methylene protons (A) (Figure 5) were in the substituted aliphatic region of the spectrum and not coupled to ring protons. Ring

Figure 3. Arylsulfatase hydrolysis of the major urinary metabolite of MBOCA. Hydrolysis was performed in 0.2 M Tris buffer, pH 7.1, using 2 μg of the metabolite. ▲ 0.005, ● 0.05, ■ 0.5 units of enzyme. N=2. Range bars are shown or are within the symbol.

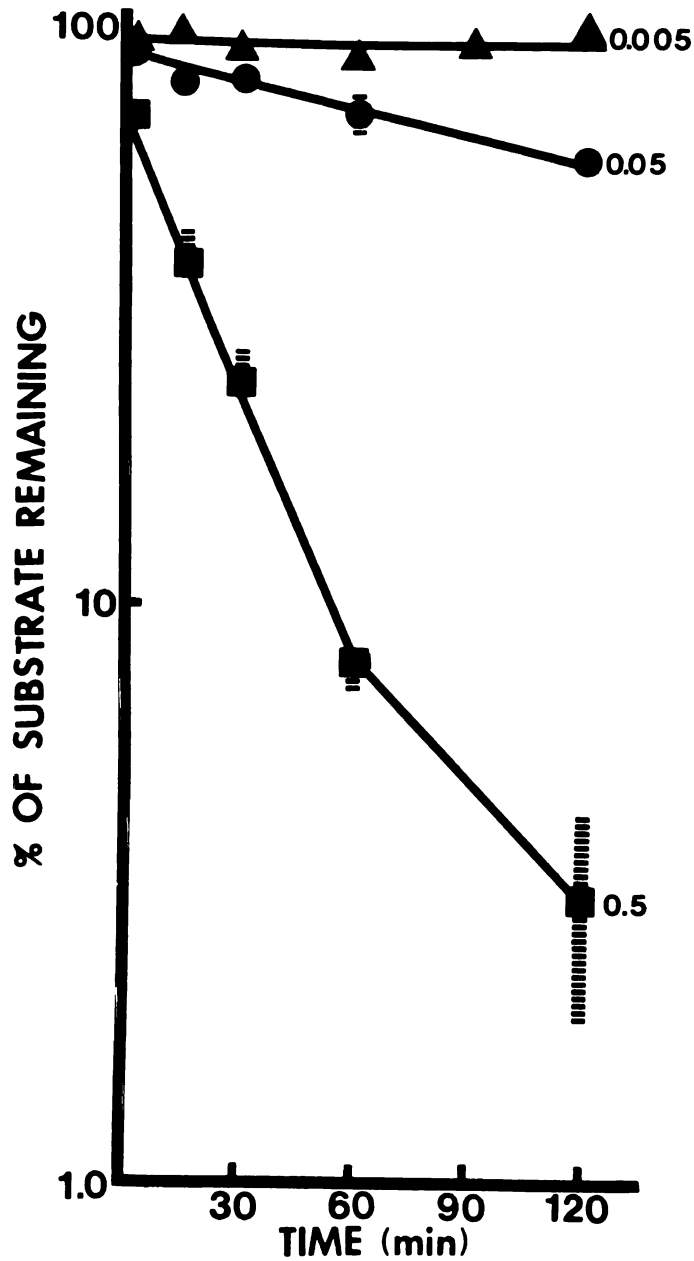
100
10
1.0
% OF SUBSTRATE REMAINING
0.005
0.05
0.5
30
60
90
120
TIME (min)

Figure 3

Figure 4. FAB-MS of the major urinary metabolite of MBOCA in a saturated KCl-glycerol matrix at 6.5 KeV.

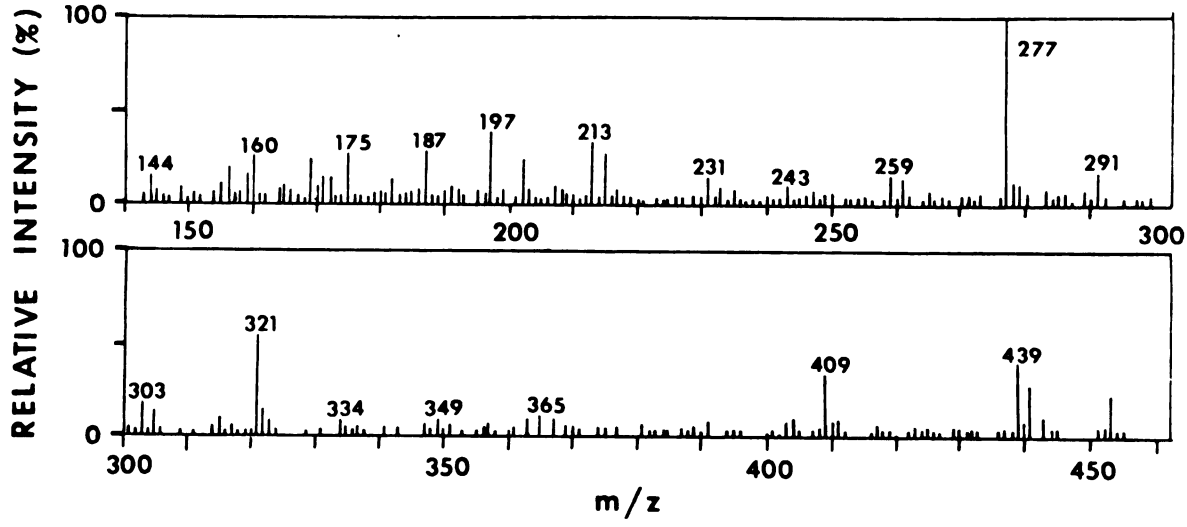
RELATIVE INTENSITY (%)
100
0
144
160
175
187
197
213
231
243
259
277
291
150
200
250
300
100
0
303
321
334
349
365
409
439
300
350
400
450
m/z

Figure 4

TABLE 1

250 MHz NMR Spectral Data of the Major Canine Urinary Metabolite of 4,4'-Methylenebis(2-Chloroaniline)

ppm	Integration (arbitrary units)	Assignment[a]
3.73	2	A
7.17	1.0	B
6.98	1.3	C
6.84	1.0	D
7.07 7.06	1.8	E-F

[a]Letters refer to proton position assignment. See diagram on Figure 5.

Figure 5. 250 MHz NMR of the major urinary metabolite of MBOCA. Amine hydrogens were exchanged with D_2O. Letters A-F refer to ring or bridge proton positions.

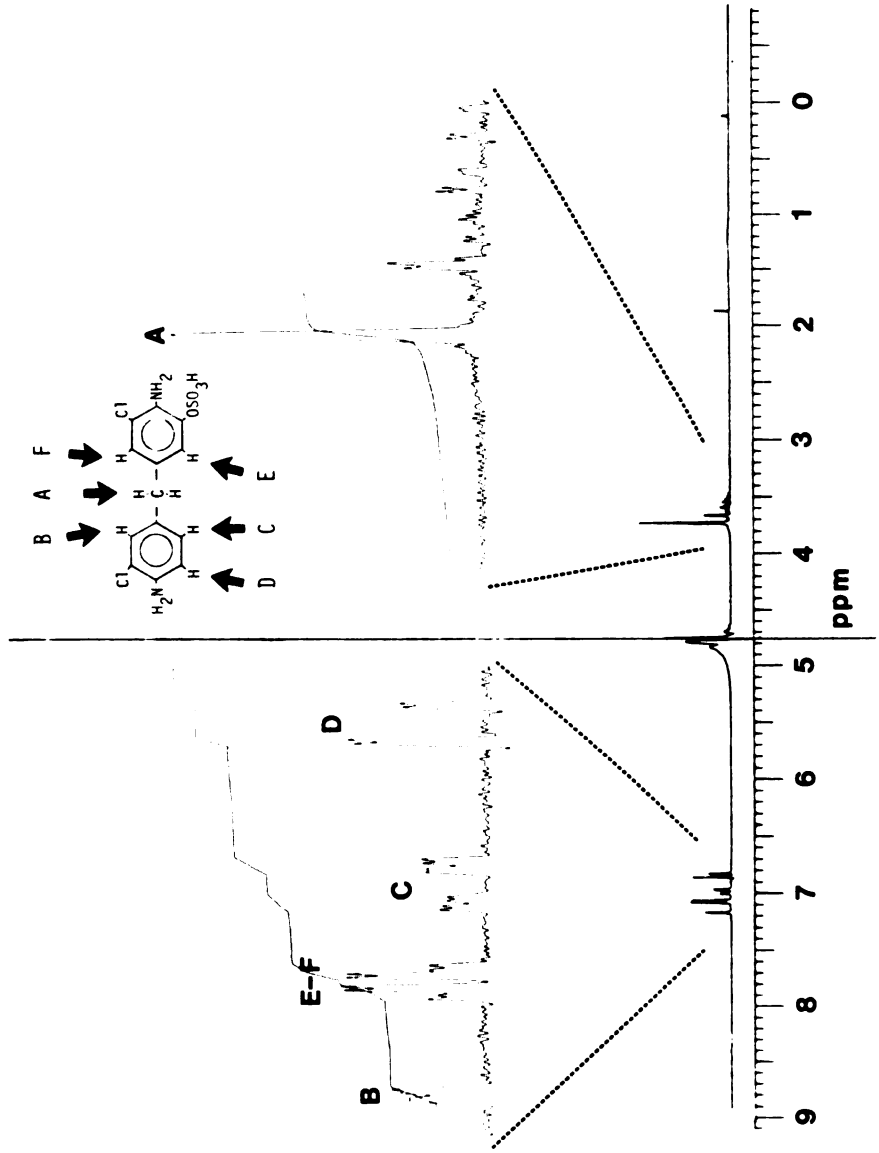

Figure 5

position assignments were proposed as follows. Protons C and D were split by 8.2-8.3 Hz, typical of benzylic ortho coupling. In addition, C showed splitting of 2.69 Hz typical of benzylic meta coupling. Proton B, which was downfield and deshielded by the adjacent chlorine, was also split by 2.55 Hz and therefore coupled to C. Although unambiguous assignment between E and F was not possible due to the complexity of the splitting pattern, the major coupling was 2.32 Hz, indicating that they were meta to one another. This determined that the ring conjugation was ortho to an amine and was consistent with the proposed structure.

5. Binding in vitro to protein and DNA

Reactivity of the major metabolite was investigated via binding and mutagenicity. Protein binding occurred during enzymatic hydrolysis in a time and enzyme concentration-dependent manner (Figure 6). With the 2 enzyme concentrations used, binding increased to 3.6% or 9.8% of the 2 μg of the major metabolite over 24 hours. Background activity, determined without enzyme present or in the presence of inactivated enzyme, did not exceed 0.3% of the reaction mixture.

DNA binding increased with time but not with enzyme concentration over the 24 hour period (Figure 7). This may have been due to binding to the enzyme or decrease in hydrolysis in the presence of DNA. It reached 1.1% and 1.3% of the reaction mixture which corresponded to 43 and 52 pmol/mg DNA. Binding was not detected without enzyme or in the presence of inactivated enzyme.

6. Mutagenicity

The major metabolite of MBOCA was not mutagenic in TA1538 under the conditions used. With 0.1, 1.0, 10, or 20 μg/plate no excess of colonies were observed over control values (Table 2). At 50 μg/plate

Figure 6. Protein binding of the major urinary metabolite of MBOCA during arylsulfatase hydrolysis in 0.2 M Tris buffer, pH 7.1, using 2 μg of metabolite. ● 0.05, ▲ 0.5 units of enzyme, ◆ inactive enzyme, ■ no enzyme. Percent reaction mixture refers to the 2 μg of metabolite used. N = 3-5. Standard error bars are shown or are within the symbol.

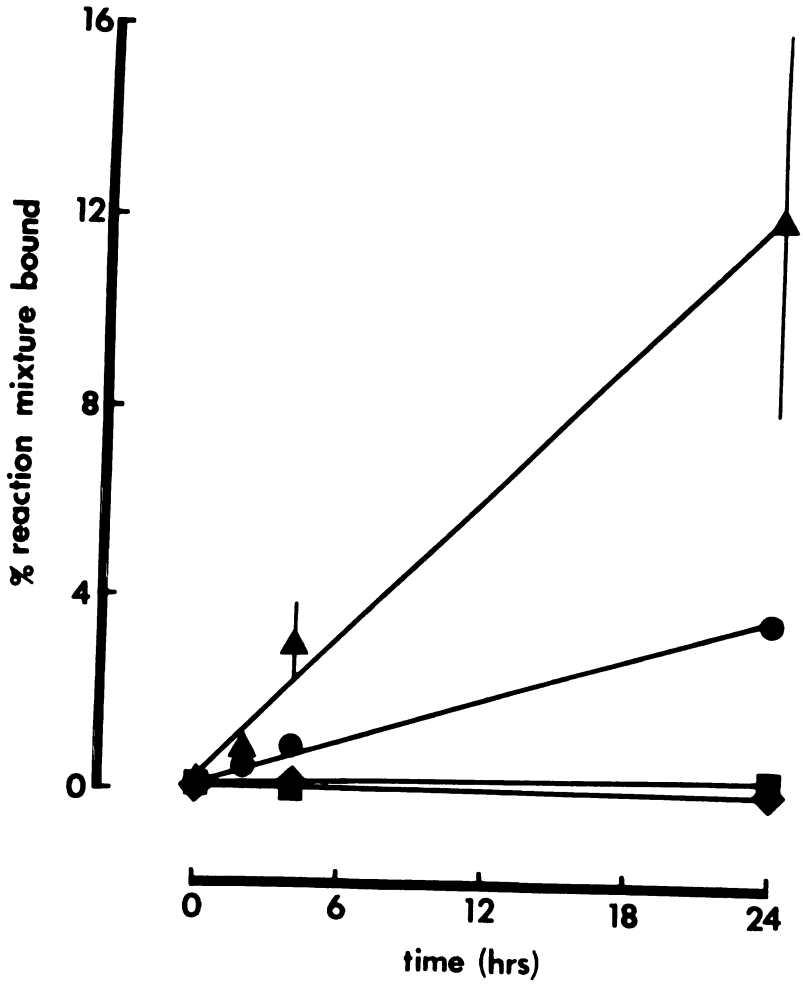

Figure 5

Figure 7. DNA binding of the major urinary metabolite of MBOCA during arylsulfatase hydrolysis in 0.2 M Tris buffer with 2 μg metabolite and 1 mg/ml DNA. ● 0.05, ▲ 0.5 units of enzyme, ◆ inactive enzyme, ■ no enzyme. N=3. Standard error bars are shown or are within the symbol.

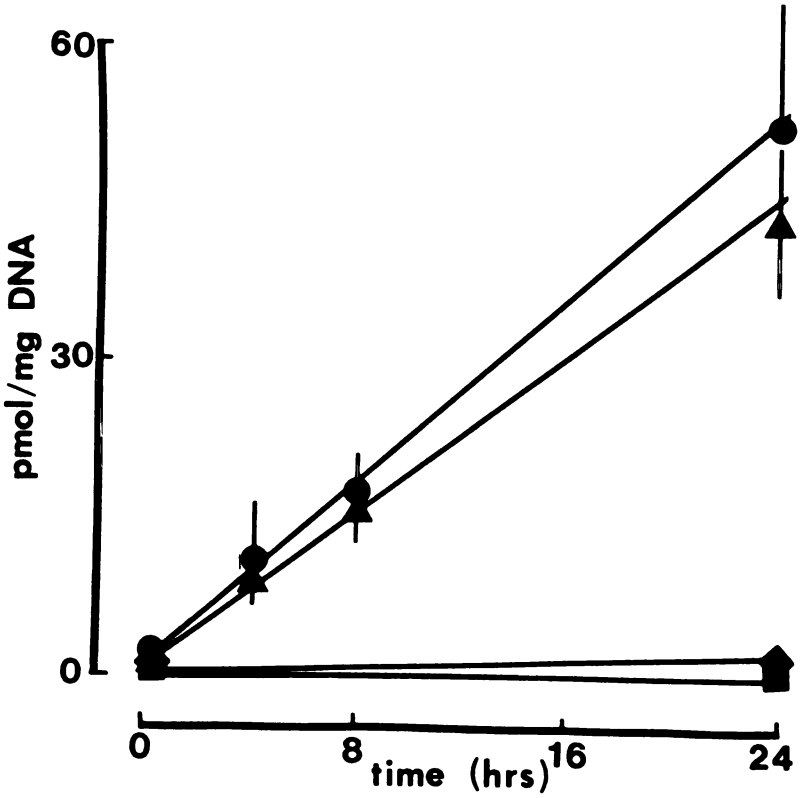

Figure 7

TABLE 2

Mutagenicity[a] of the Major Urinary Metabolite of MBOCA

	Concentration (μg/plate)						
	0.1	1.0	10.0	20.0	50.0	NE[b]	E[c]
No Preincubation							
no./plate[d]	6	10	10	9	n.d.[e]	11	9
no./10^8 colonies	11	17	21	21	n.d.	19	16
viability[f]	1.04	1.21	0.90	0.84	1.22	1.17	1.10
With Preincubation							
no./plate	8	6	7	7	n.d.	8	7
no./10^8colonies	22	18	20	16	n.d.	17	14
viability	0.73	0.68	0.74	0.91	0	0.93	0.99

[a] *Salmonella typhimurium* TA1538 was used.

[b] NE is without arylsulfatase and without substrate.

[c] E is with arylsulfatase and without substrate.

[d] Number of mutant colonies.

[e] Not determined.

[f] (colonies x 10^8)/ml.

the 1 hr incubation was cytotoxic as demonstrated by the absence of colonies on nutrient agar plates. This cytotoxicity of arylamines at high concentrations has been observed previously (Ann Aust, personal communication).

B. In Vitro Incubations

1. Preliminary investigation of metabolism

An HPLC system was developed to separate the MBOCA metabolites produced *in vitro* in tissue slices. Retention time provided an initial characterization and a handle for discussion of those metabolites with unknown structure. The slice incubations provided a spectrum of metabolites. Liver incubations produced 7 metabolites (Figure 8) with retention times of 9.5, 10.1, 11.5, 12.2, 13.5, 23.5, and 25.5 minutes. Renal slice incubations produced 6 metabolites (Figure 9) with retention times of 9.5, 12.2, 15.5, 22 (or 21.5), 23.5, and 25.5 min. Metabolites from liver and kidney were consistent from one animal to the next, except for the peak at 13.5 in liver, which was sometimes missing. There were metabolites with identical retention times in both organs (9.5, 12.2, 23.5, and 25.5 min) and metabolites unique to each organ. The peak at 25.5 min corresponded to the previously identified major canine urinary metabolite, the ortho-hydroxy sulphate of MBOCA. The HPLC separation method allowed further preliminary investigation of metabolism and later study of the structure of these metabolites and their role in macromolecular binding.

In a preliminary experiment, the production of metabolites and macromolecular binding were investigated over a range of tissue weights

Figure 8. HPLC chromatogram of metabolites from a hepatic tissue slice incubation. Metabolite peaks are identified by retention time. A 35 min linear gradient from 23-42.5% acetonitrile, 0.0025 M PIC A at 3 ml/min was used on a 5μ Radial compression Nova-pak column (Waters Associates).

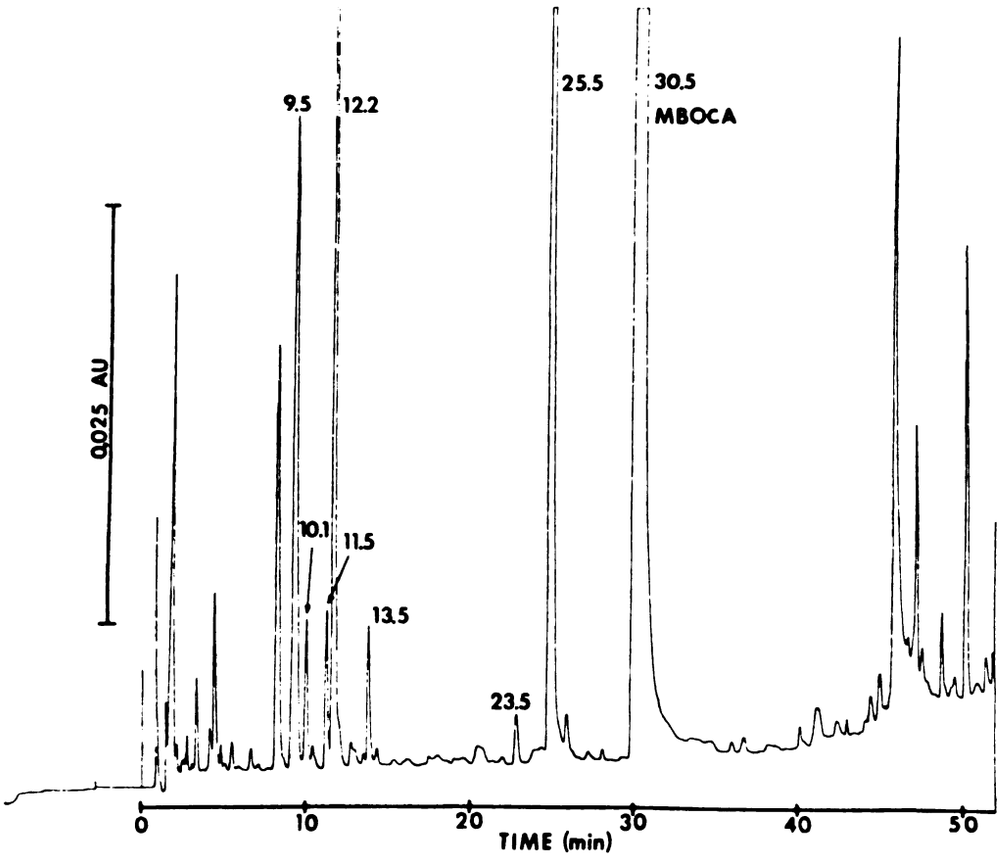
9.5
12.2
25.5
30.5
MBOCA
.025 AU
10.1
11.5
13.5
23.5
0
10
20
30
40
50
TIME (min)

Figure 8

Figure 9. HPLC chromatogram of metabolites from a renal cortical tissue slice incubation. Metabolite peaks are identified by retention time. A 35 min linear gradient from 23-42.5% acetonitrile, 0.0025 M PIC A at 3 ml/min was used on a 5μ Radial compression Nova-pak column (Waters Associates).

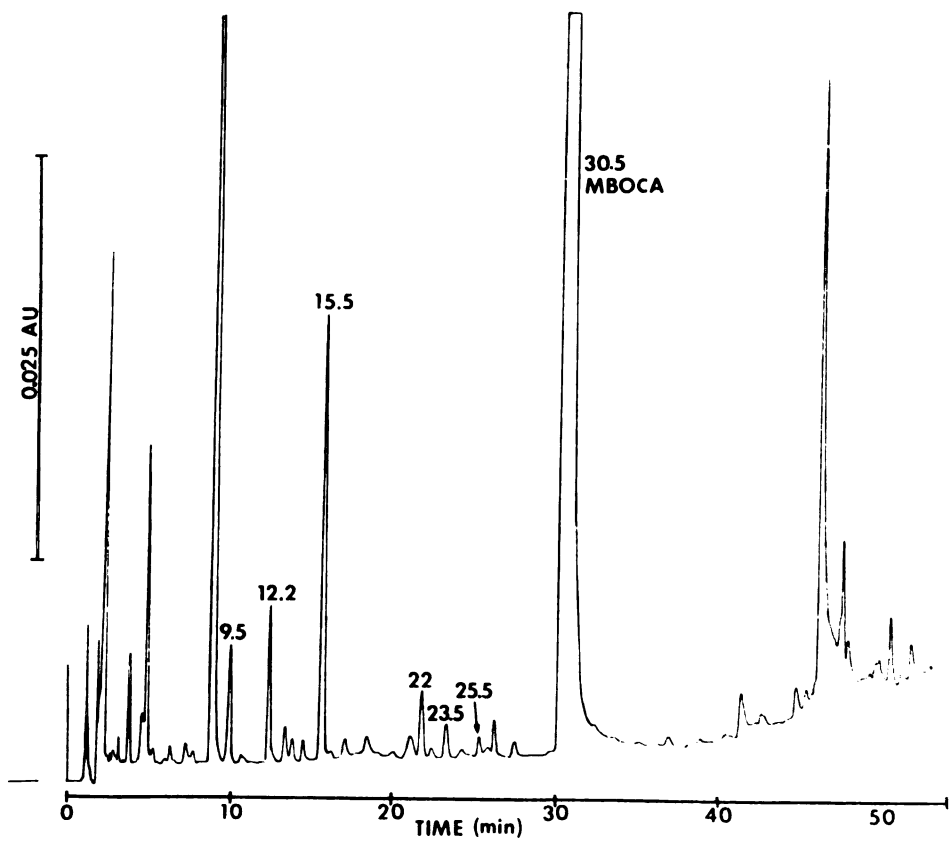

Figure 9

and at two substrate concentrations (data not shown). Metabolites from 50, 100, and 200 mg liver increased at both 0.05 and 0.10 μmol/ml ^{14}C-MBOCA over the 90 min incubation. Metabolites from 100, 200, and 400 mg renal cortex also increased at both concentrations of substrate over a 120 min incubation period. The amount of metabolite at any timepoint was greatest using 200 mg liver or 400 mg kidney and 0.1 μmol/ml ^{14}C-MBOCA. These conditions were used in later studies for elucidation of metabolites. In this preliminary experiment, macromolecular binding in renal and hepatic slices increased linearly over the time periods studied. Binding based on pmol/mg did not differ within a tissue, regardless of the substrate or the enzyme concentration.

The substrate concentration dependence of macromolecular binding was investigated using 200 mg hepatic slices and 400 mg renal cortical or medullary slices. In liver, binding increased linearly to 0.1 μmol/ml MBOCA (Figure 10). Both renal cortex and medulla also demonstrated increased binding to 0.1 μmol/ml MBOCA (Figure 11). Macromolecular binding in the liver was much higher than the kidney, considering the differences in time (60 min vs. 90 min) and mg tissue used. Binding in the renal medulla was about 20% of the renal cortex.

Macromolecular binding was further investigated in a time dependent manner using 200 mg liver or renal cortex. Figure 12 demonstrates that binding was linear over the time periods studied and that renal cortical binding was about 30% that in the liver. Thus, under the conditions of the experiment, liver slices metabolized ^{14}C-MBOCA to a reactive intermediate 3-4 times faster than the renal cortex.

Figure 10. MOBCA concentration-dependent macromolecular binding in hepatic tissue slice incubations. Incubations were 60 min and contained 200 mg liver. ▲ Sample, ● inactivated control. Samples N=3. Standard error bars are within the symbols. Controls N=2.

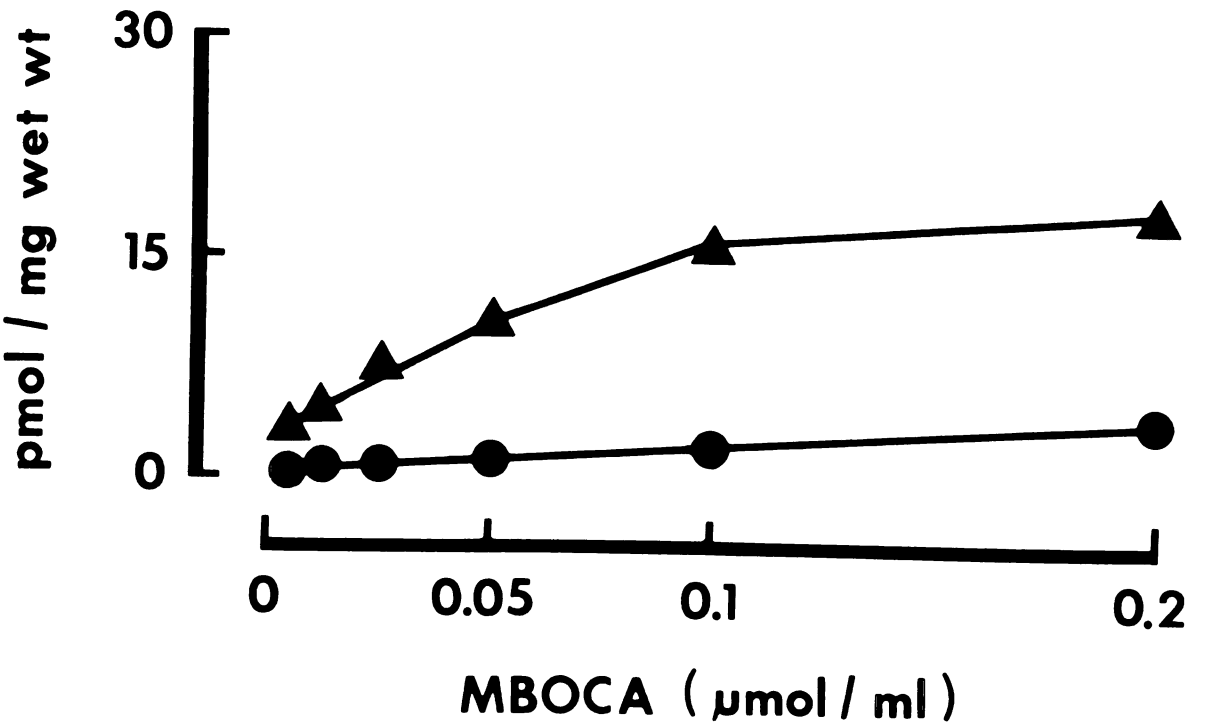

Figure 10

Figure 11. MBOCA concentration-dependent macromolecular binding in renal cortical and medullary tissue slice incubations. Incubations were 90 min and contained 400 mg tissue. ◆ Renal cortex. ■ Renal medulla. ● Inactivated control. Samples N=3. Standard error bars are shown or are within the symbol. Controls N=2 except kidney medulla 0.025 μmol/ml where n=1 and 0.1 μmol/ml where n=3.

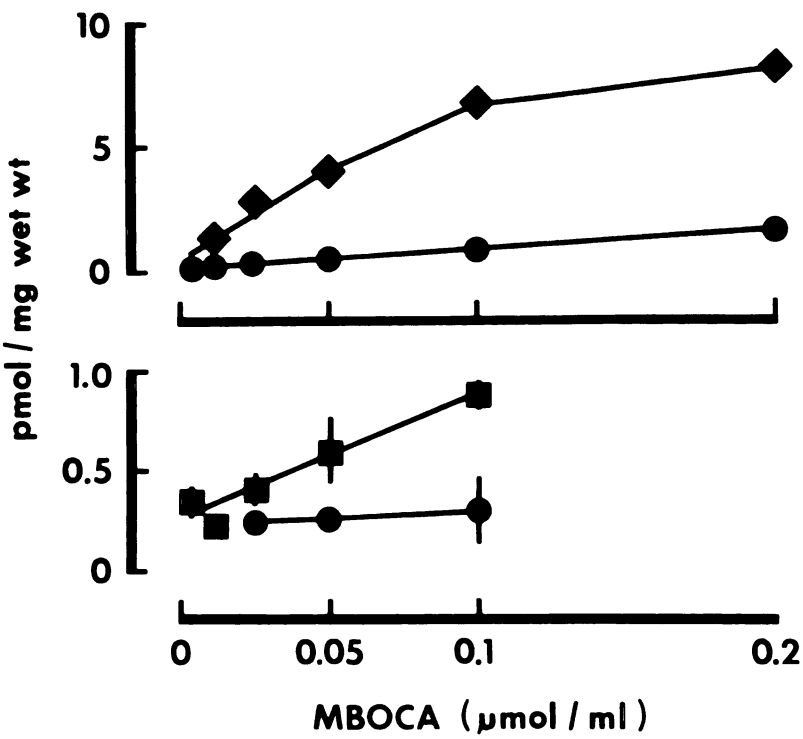

Figure 11

Figure 12. Time-dependent macromolecular binding of MBOCA in tissue slices. Incubations used 200 mg tissue and 0.1 μmol/ml ^{14}C-MBOCA. ▲Liver. ◆ Renal cortex. N=3 except renal cortex at 120 min where N=2. Standard error bars are shown or are within the symbol.

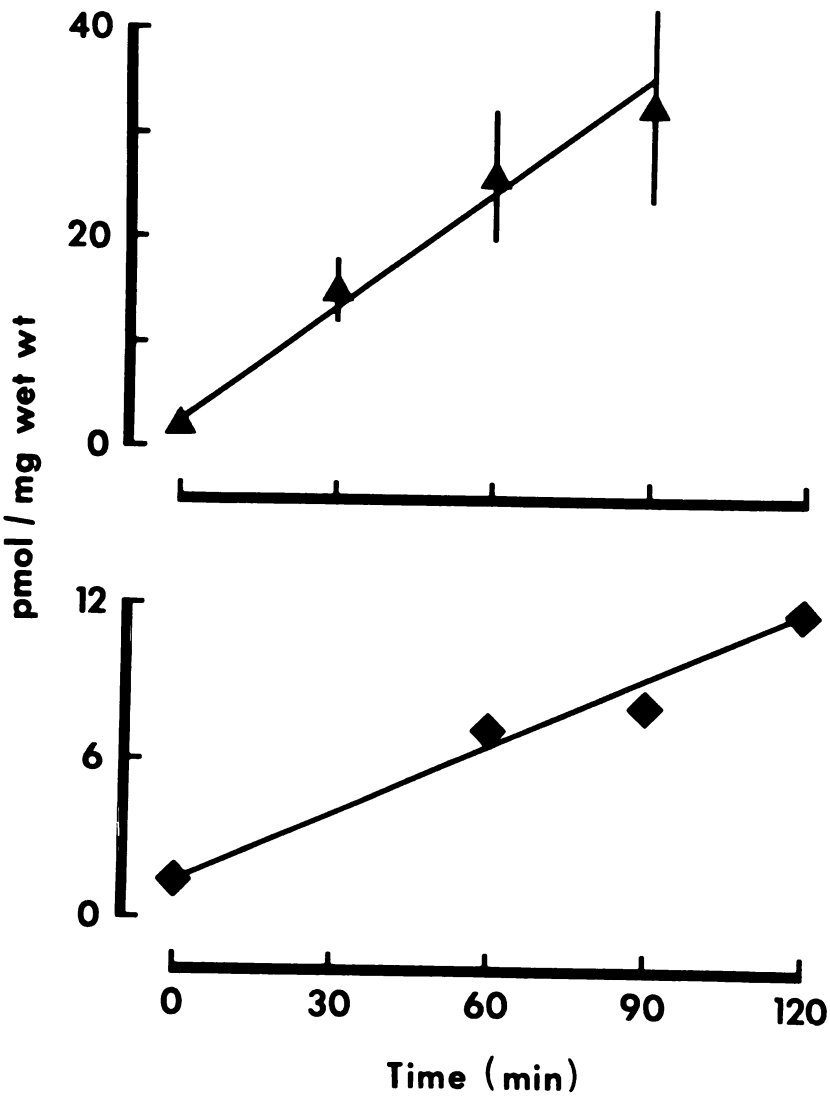

Figure 12

The time-dependent appearance of selected metabolites was also investigated in these initial experiments (Figures 13 and 14). These metabolites, quantifiable at all timepoints studied, increased in liver and renal cortex during the 90 or 120 min period. Thus, the peaks appeared to be the product of time-dependent metabolism.

These initial experiments demonstrated that MBOCA was metabolized by canine liver and kidney. Metabolites stable enough for isolation were characterized by their HPLC retention time and reactive metabolites were measured by macromolecular binding. These and later studies indicated that by 60 min metabolism of 0.1 μmol/ml ^{14}C-MBOCA in 200 mg liver slices reached 5-10%, the HPLC peak at 25.5 min being 80% of that. In 90 min incubations, 400 mg renal cortical slices metabolized ^{14}CMBOCA 3-5%, the peak at 15.5 min being 25-40% of that. These concentrations and timepoints produced enough of each major metabolite to quantify and were therefore used in further studies.

The renal medulla did not consistently produce metabolites identifiable by either UV absorbance or DPM during preliminary investigations or in later studies with altered incubation conditions. Therefore, metabolism in the renal medulla was detected only by macromolecular binding. Elucidation of metabolites and post-incubation analyses were done on hepatic and renal cortical incubations.

2. Elucidation of metabolites - sulfation

Arylamines form two types of conjugates containing a sulphur atom, sulphates and sulphamates. Formation of these conjugates was investigated *in vitro* by addition of $Na_2{}^{35}SO_4$ and cold MBOCA. Two MBOCA metabolites in liver, at 23.5 and 25.5 min (Figure 15) and three MBOCA metabolites in kidney, at 22, 23.5, and 25.5 min (Figure 16) were

Figure 13. Appearance of metabolites in hepatic tissue slice incubations. Incubations contained 100 mg tissue and 0.05 μmol/ml ^{14}C-MBOCA. The ordinate refers to nmol metabolite produced. ▲ peak at 9.5 min, ●peak at 25.5 min. N=3. Standard error bars are shown or are within the symbol.

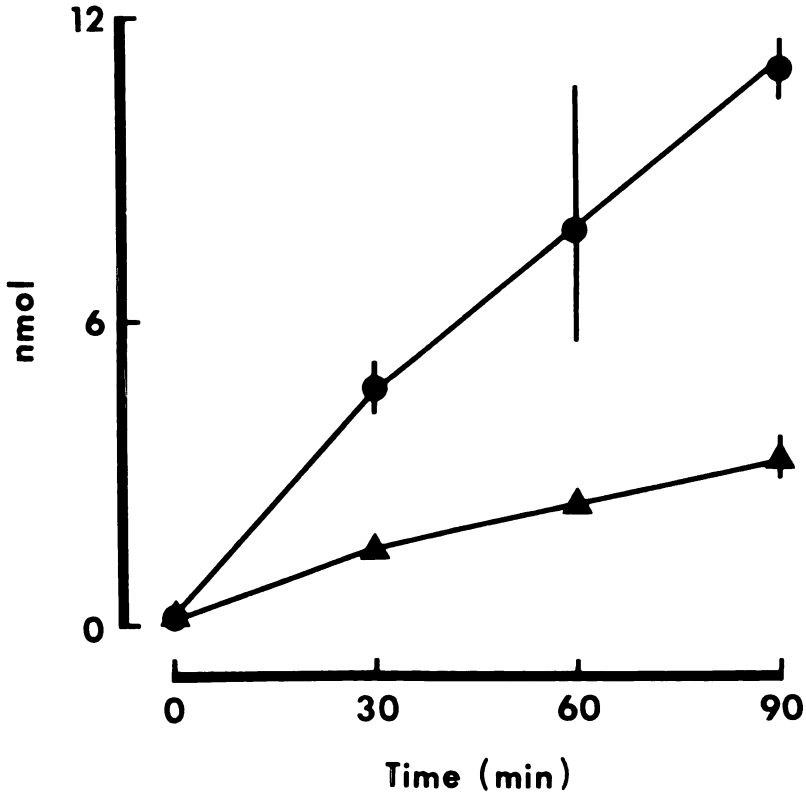

Figure 13

Figure 14. Appearance of metabolites in renal cortical tissue slice incubations. Incubations contained 400 mg tissue and 0.1 μmol/ml ^{14}C-MBOCA. The ordinate refers to nmol metabolite produced. ▲ peak at 9.5 min. ● peak at 15.5 min. N=2.

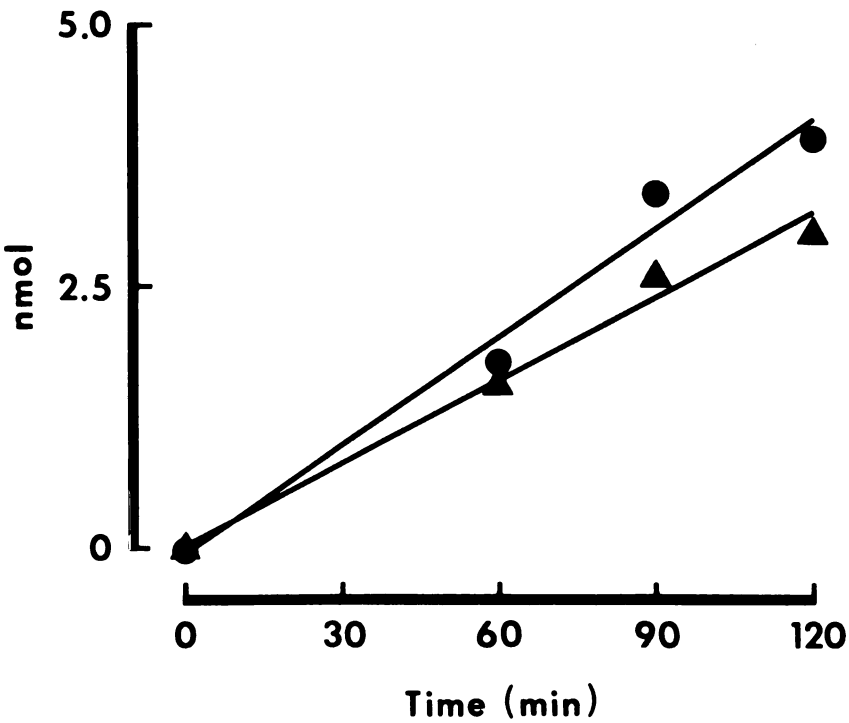

Figure 14

Figure 15. Labeling of hepatic metabolites with $Na_2{}^{35}SO_4$. Plot of the LSS analysis of one minute or peak fraction collections during a 35 min linear HPLC gradient from 23-42.5% acetonitrile, 0.0025 M PIC A at 3 ml/min. A 5μ Radial compression Nova-pak column was used.

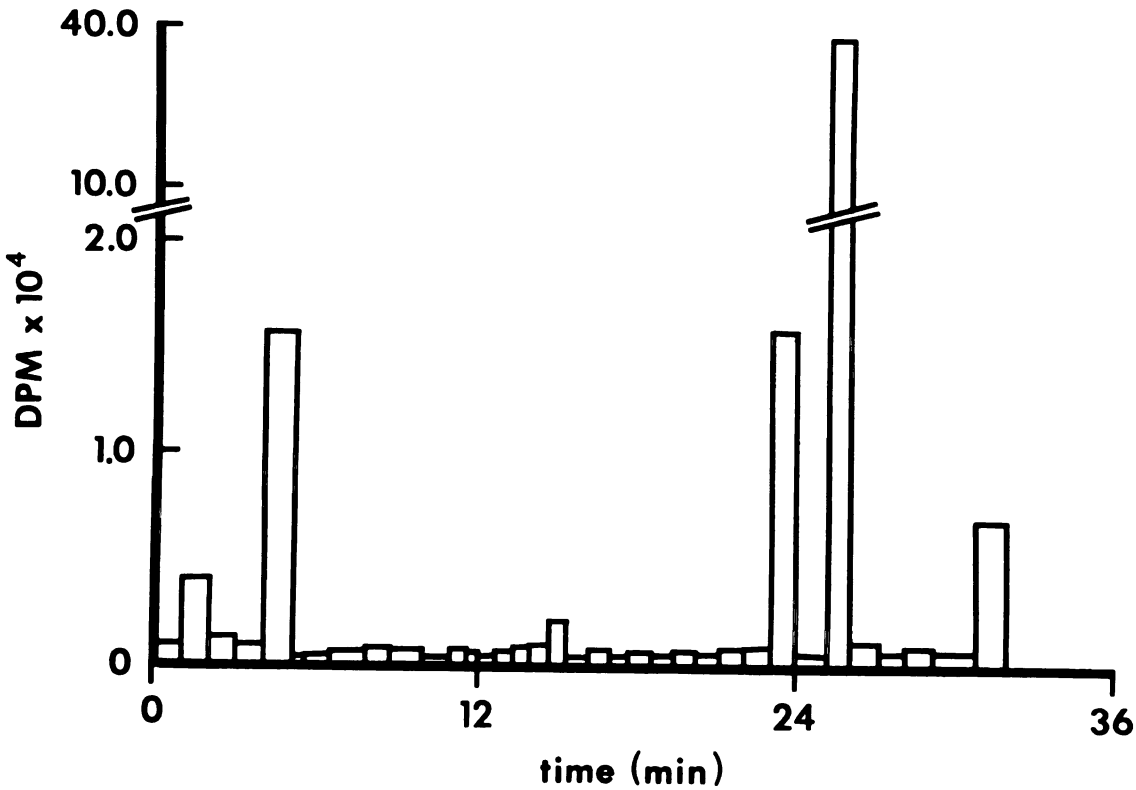

Figure 15

Figure 16. Labeling of renal cortical metabolites with $Na_2{}^{35}SO_4$. Plot of the LSS analysis of one minute or peak fraction collections during a 35 min linear HPLC gradient from 23-42.5% acetonitrile, 0.0025 M PIC A at 3 ml/min. A 5μ Radial compression Nova-pak column was used.

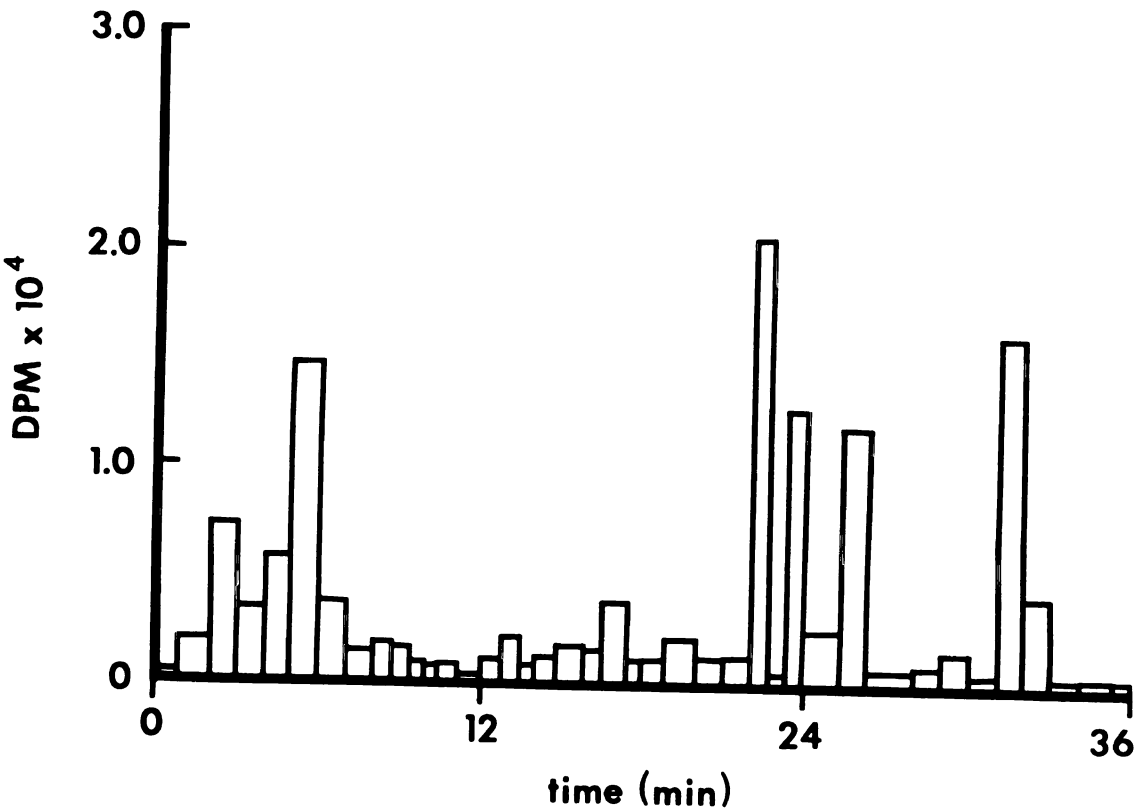

Figure 16

labeled. The peak at 25.5 min had been structurally identified as the ortho-hydroxy sulphate (see A). The DPM at 5 min did not correspond to an MBOCA metabolite produced in reactions with ^{14}C-MBOCA. The DPM associated with the parent compound at 30.5 min were due to carryover from a previous injection. In the duplicate experiment these MBOCA DPM represented 0.2-0.3% of the DPM added to a ^{14}C-MBOCA incubation. This study indicated that the three peaks eluting 22-25.5 min were sulphate or sulphamate conjugates of MBOCA.

The contribution of sulphation to metabolism and binding was investigated using p-nitrophenyl sulphate (PNPS) to stimulate sulfation. Concentrations of 0.05-4 mM PNPS have been reported to stimulate sulphation of 1-naphthol up to 275% in isolated rat liver cells (Schwarz, 1980). In a preliminary experiment with canine tissue slices, 4 mM PNPS inhibited metabolism in general and therefore 0-2 mM concentrations were used. PNPS at 0.1 or 0.5 mM significantly inhibited formation of metabolites at 9.5, 10.1 and 12.2 min in liver (Figure 17) but metabolites at 23.5 and 25.5 were unchanged. Because of its strong U.V. absorption, elution of PNPS interfered with detection and collection of MBOCA metabolite at 11.5 min. In experiments at 2 mM PNPS it interfered with collection of peaks at 10.1 and 12.2 min as well. In the kidney, PNPS stimulated the peak at 9.5 min, inhibited formation of the peak at 15.5 min and did not alter other metabolites significantly (Figure 18). Macromolecular binding was unchanged in liver and kidney (Figure 19). This study demonstrated that although PNPS could alter production of some metabolites in tissue slices, it did not alter binding, nor did it increase production of the conjugates at 22-25 min, one previously

Figure 17. Production of metabolites by hepatic tissue slices in the presence of p-nitrophenyl sulphate. ☐ 0.1 mM, ▤ 0.5 mM, ▨ 2.0 mM. N=3 except (a) where N=2. Standard error bars are shown. * Significantly different from control, $p<0.05$. Control values were 2.45±0.16 (9.5 min), 0.96±0.12 (10.1 min), 1.08±0.33 (11.5 min), 2.62±0.54 (11.5 min), 0.43±0.07 (23.5 min) and 13.0±2.02 (25.5 min) nmol/200 mg tissue.

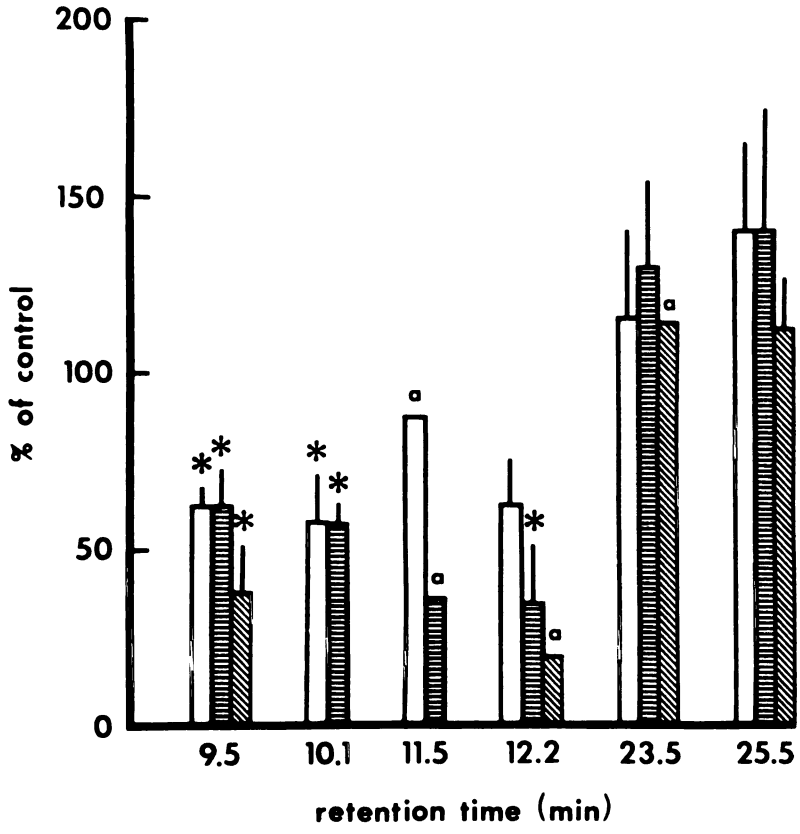
200
150
100
50
0
% of control
9.5
10.1
11.5
12.2
23.5
25.5
retention time (min)

Figure 17

Figure 18. Production of metabolites by renal cortical tissue slices in the presence of p-nitrophenyl sulphate. □ 0.1 mM, ▤ 0.5 mM, ▨ 2.0 mM. N=3 except (a) where N=2. Standard error bars are shown. * Significantly different from control, $p \leq 0.05$. Control values were 0.87 ± 0.07 (9.5 min), 1.72 ± 0.74 (12.2 min), 3.26 ± 0.86 (15.5 min), 1.16 ± 0.24 (21.5 min), 1.16 ± 0.53 (23.5 min), 0.98 ± 0.40 (25.5 min) nmol/400 mg tissue.

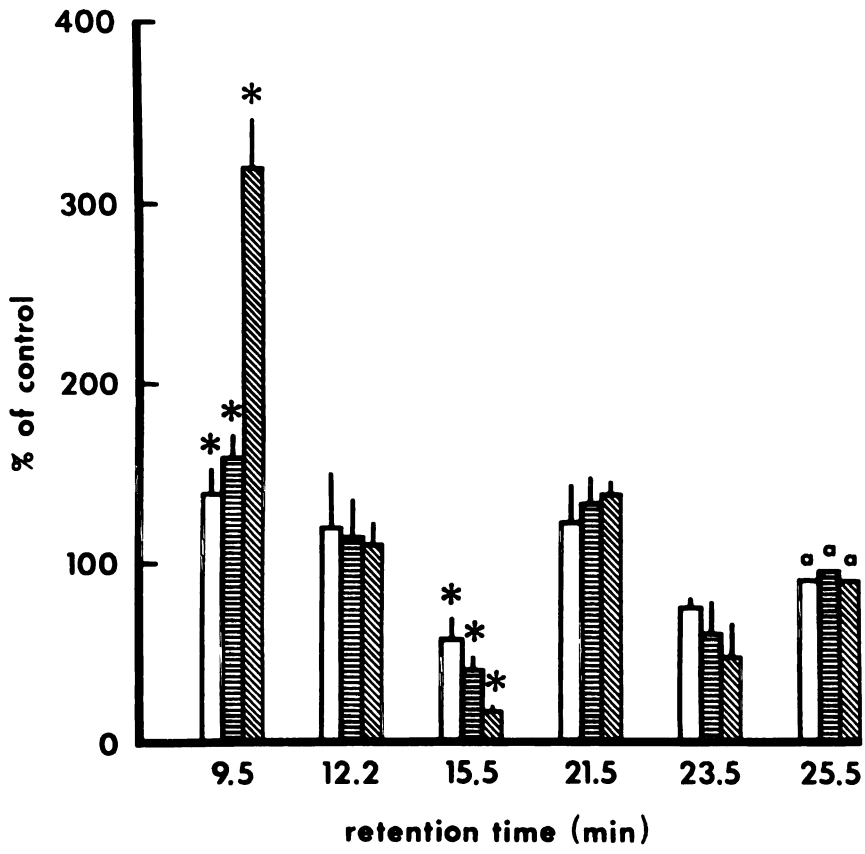
400
300
200
100
0
% of control
9.5
12.2
15.5
21.5
23.5
25.5
retention time (min)

Figure 18

Figure 19. Macromolecular binding in tissue slices in the presence of p-nitrophenyl sulphate. ▲ Liver. ◆ Kidney. N=3. Standard error bars are shown.

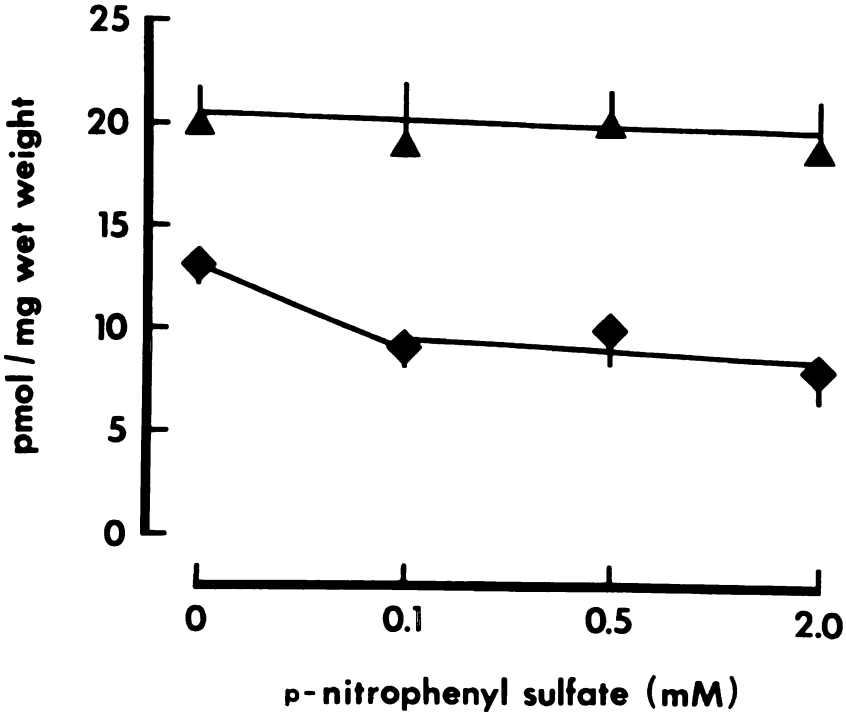
25
20
15
10
5
0
pmol / mg wet weight
0
0.1
0.5
2.0
p-nitrophenyl sulfate (mM)

Figure 19

identified as a sulphate and all previously labeled with ^{35}S. Thus, it did not seem to enhance sulphation. This study indicated that peaks at 10.1, 11.5, 12.2 and 15.5 min in liver or kidney were not sulphate conjugates, they were decreased by the treatment. The peak at 9.5 min was an enigma, increasing in the kidney and decreasing in the liver.

The contribution of sulphation to metabolism and binding was further investigated using 2,6-dichloro-4-nitrophenol (DCNP). DCNP is less toxic than pentachlorophenol and is a selective inhibitor of sulphation (Mulder and Scholtens, 1977). DCNP did not affect production of any metabolites in liver or kidney slices nor did it effect binding at the two lowest concentrations used, 0.1 and 1.0 μM (Figures 20-22). At 10 and 100 μM DCNP, metabolism was inhibited nonselectively in both organs and binding was decreased. The exception was the peak at 11.5 min in liver which was increased at all concentrations of DCNP. Thus, DCNP did not selectively inhibit isolatable sulphates or binding but did enhance a metabolite of unknown structure at 11.5 min.

3. Elucidation of metabolites - glucuronidation and glucosidation

The role of glucuronidation and glucosidation in MBOCA metabolism was investigated by two separate means, first by the use of ^{14}C-glucose to label conjugates and second, by use of D(+)galactosamine to inhibit their formation. Addition of ^{14}C-glucose was unsuccessful at selectively labeling conjugates containing a glycosyl or glucuronosyl moiety. At the lower concentration (3.7×10^7 DPM, 1.29 μmol) no metabolites in liver or kidney were labeled. At a five times higher concentration, used in hepatic slices, metabolite peaks at 9.5, 12.2, and 25.5 were labeled. There were DPM at 5 min and at 30.5 min (MBOCA) as well (data not shown). DPM at 30.5 min were carryover from a previous

Figure 20. Production of metabolites by hepatic tissue slices in the presence of 2,6-dichloro-4-nitrophenol. ▭ 0.1 μM, ▤ 1.0 μM, ▨ 10.0 μM, ■ 100 μM. N=2. Control values were 3.80 (9.5 min), 1.85 (10.1 min), 1.84 (11.5 min), 5.98 (12.2 min), 4.10 (23.5 min), and 20.15 (25.5 min) nmol/200 mg tissue.

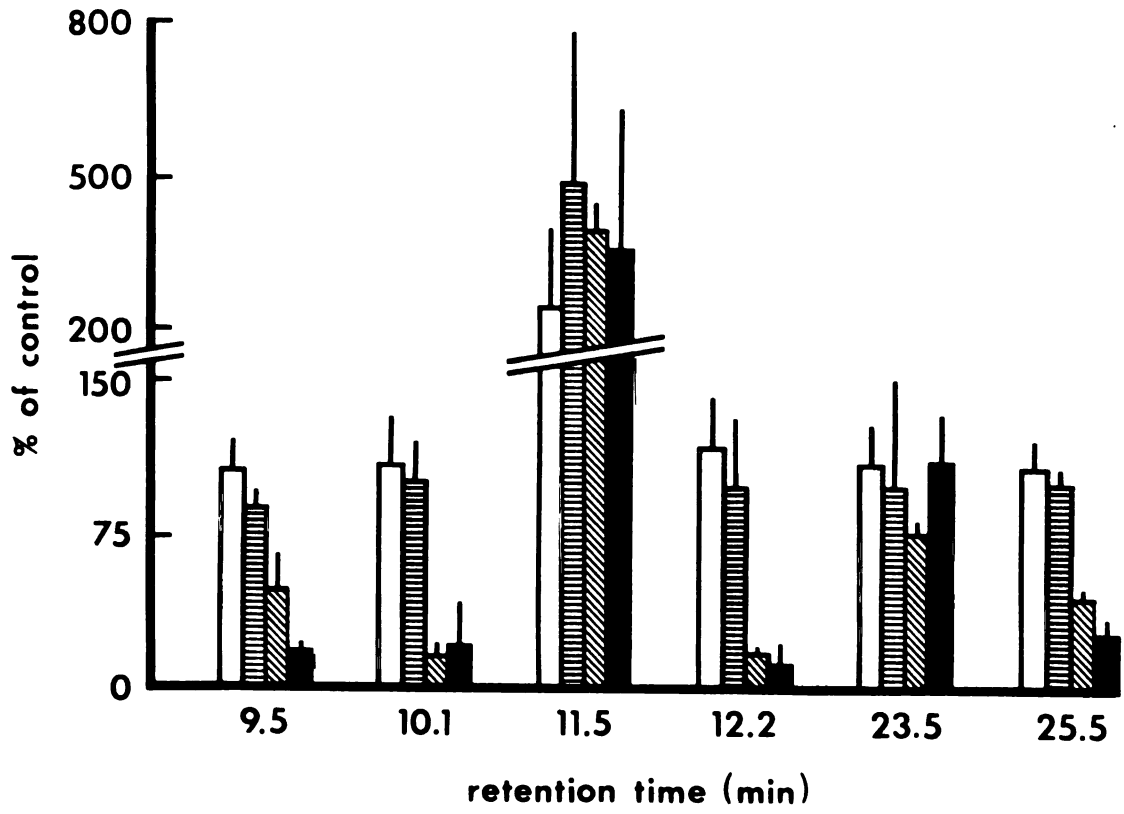
800
500
200
150
75
0
% of control
9.5
10.1
11.5
12.2
23.5
25.5
retention time (min)

Figure 20

Figure 21. Production of metabolites by renal cortical tissue slices in the presence of 2,6-dichloro-4-nitrophenol. ▭ 0.1 μM, ▤ 1.0 μM, ▨ 10 μM, ■ 100 μM. N=3. Standard error bars are shown. * Significantly different from control. $p<0.05$. Control values were 1.29±0.29 (9.5 min), 3.46±0.48 (12.2 min), 4.38±0.85 (15.5 min), 1.36±0.20 (21.5 min), 1.02±0.36 (23.5 min), 1.12±0.19 (25.5 min) nmol/400 mg tissue.

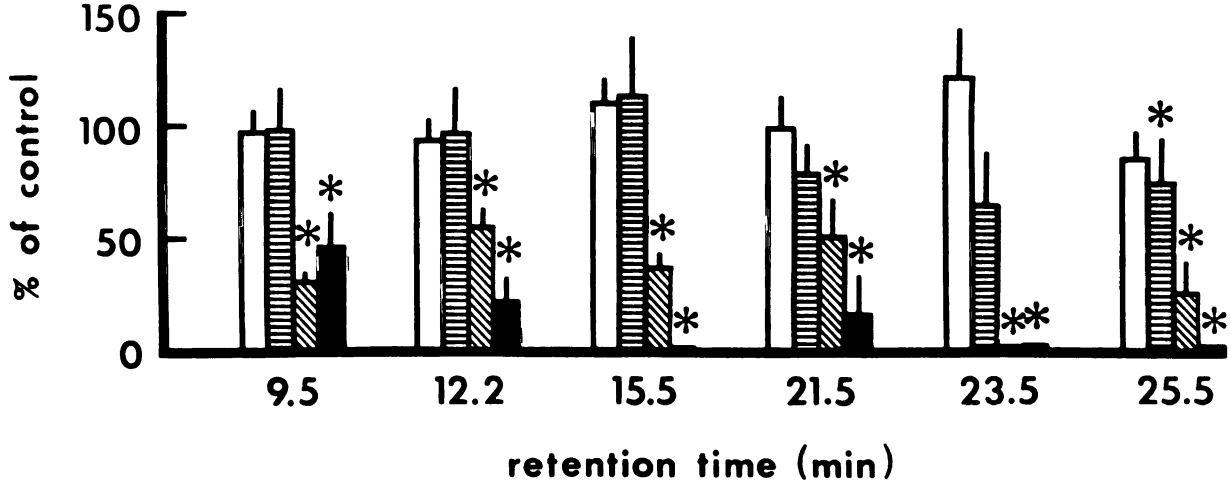
150
100
50
0
% of control
9.5
12.2
15.5
21.5
23.5
25.5
retention time (min)

Figure 21

Figure 22. Macromolecular binding in tissue slices in the presence of 2,6-dichloro-4-nitrophenol. (μM) ▲ Liver. ◆ Kidney cortex. N=3. Standard error bars shown or are within the symbol. ✱Significantly different from control, $p \leq 0.05$.

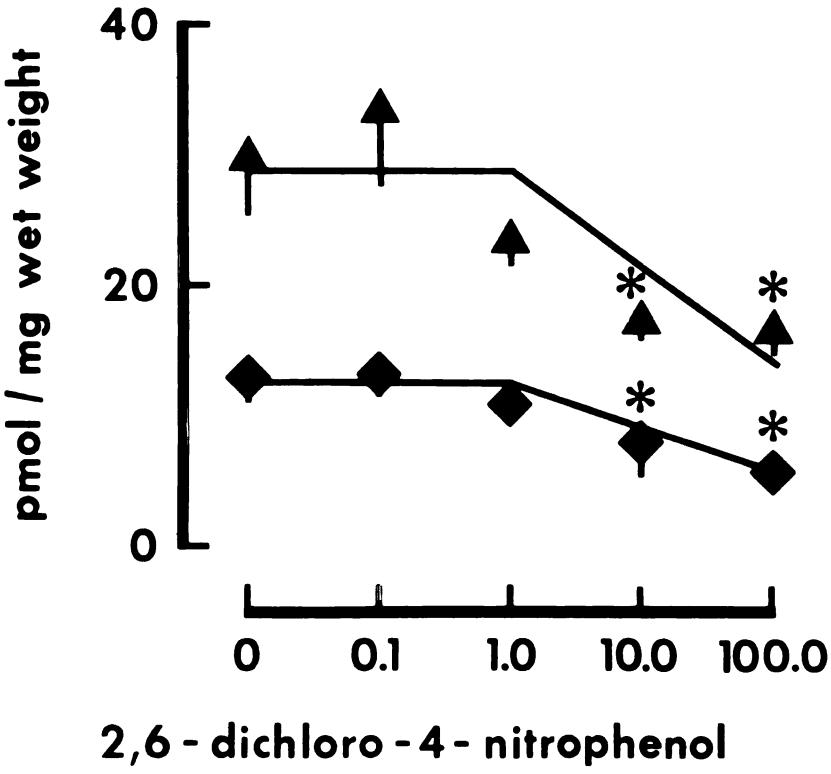
40
20
0
pmol / mg wet weight
0
0.1
1.0
10.0
100.0
2,6 - dichloro - 4 - nitrophenol

Figure 22

injection. The peak at 25.5 min, structurally identified as a sulphate (see A) had the highest amount of DPM (20 times other metabolites). This apparent non-specific association of ^{14}C-glucose radioactivity with a known sulfate metabolite precluded further interpretation of the data on unknown peaks. D(+)galactosamine inhibited formation of the metabolite peaks at 9.5, 10.1, and 12.2 min in liver (Figure 23) and 9.5 and 12.2 min in kidney (Figure 24) but did not effect macromolecular binding (Figure 25). This indicated that the peaks at 9.5, 10.1 and 12.2 contained glucuronosyl or glycosyl moieties and were not involved in the majority of macromolecular binding.

4. Elucidation of metabolites - glutathione conjugation (N=1, data not shown)

A preliminary investigation of the role of glutathione in MBOCA metabolism was made. Diethyl maleate, which reacts directly with glutathione and methionine sulfoximine, which inhibits γ-glutamylcysteine synthetase (Meister, 1983) were used. Preincubation with diethyl maleate reduced nonprotein sulfhydryls in liver 10% at 1 mM and 30% at 5 mM and in kidney 25% at 1 mM and 55% at 5 mM. Methionine sulfoximine did not decrease nonprotein sulfhydryls in either tissue during the 10 min preincubation.

In this preliminary experiment diethyl maleate inhibited production of metabolites in hepatic slices by 50-60% at 0.2 and 1 mM and by 100% at 5 mM. In renal cortical slices metabolite production was inhibited 40-60% for peaks at 9.5, 23.5 and 25.5 min at all concentrations. Methionine sulfoximine had no effect on metabolite production in hepatic slices but depressed peaks 9.5 and 23.5 min in renal slices. Neither inhibitor altered binding in hepatic or renal slices.

Figure 23. Production of metabolites by hepatic tissue slices in the presence of D(+)galactosamine. ☐ 0.6 mM, ▤ 3.0 mM, ▧ 15 mM. N=3 except (a) where N=1 and (b) where N=2. Standard error bars are shown. *Significantly different from control, $p \leq 0.05$. Control values were 3.07±0.98 (9.5 min), 1.17±0.18 (10.1 min), 0.95±0.38 (11.5 min), 2.96±0.22 (12.2 min), 0.42±0.04 (13.5 min), 0.58±0.18 (23.5 min) and 14.83±1.30 (25.5 min) nmol/200 mg tissue.

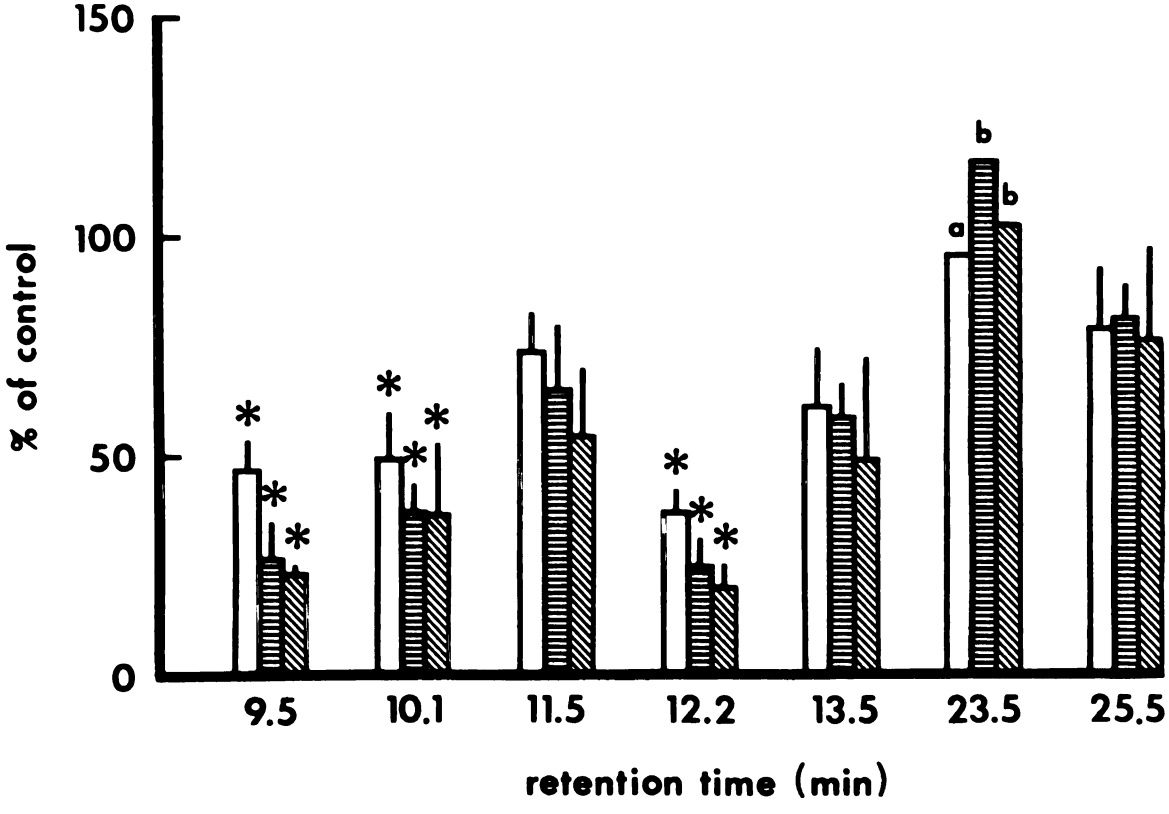
150
100
50
0
% of control
9.5
10.1
11.5
12.2
13.5
23.5
25.5
retention time (min)
a
b
b

Figure 23

Figure 24. Production of metabolites by renal cortical tissue slices in the presence of D(+)galactosamine. ☐ 0.6 mM, ▤ 3.0 mM, ▧ 15 mM. N=3 except (a) where N=2. Standard error bars are shown. * Significantly different from controls, $p \leq 0.05$. Control values were 0.744±0.13 (9.5 min), 1.62±0.39 (12.2 min), 3.27±0.93 (15.5 min), 1.08±0.23 (21.5 min), 0.55±0.03 (23.5 min), and 1.05±0.56 (25.5 min) nmol/400 mg tissue.

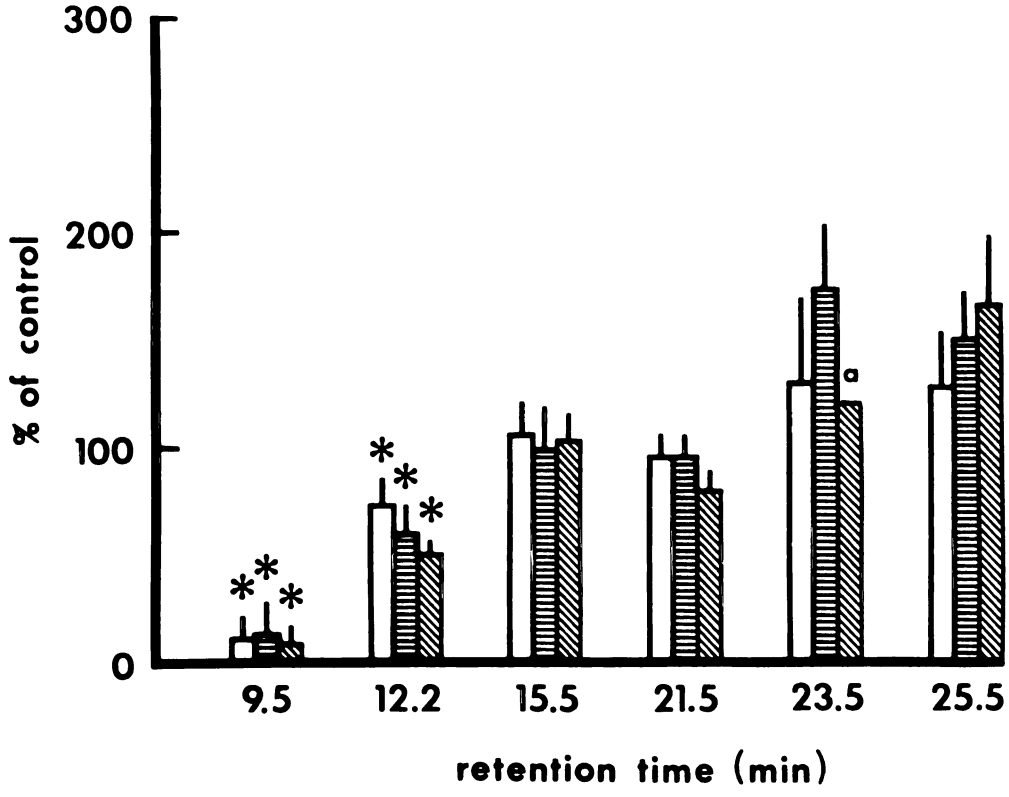
300
200
100
0
% of control
9.5
12.2
15.5
21.5
23.5
25.5
retention time (min)
a

Figure 24

Figure 25. Macromolecular binding in tissue slices in the presence of D(+)galactosamine. ▲Liver. ◆ Kidney cortex. N=3. Standard error bars are shown or are within the symbol.

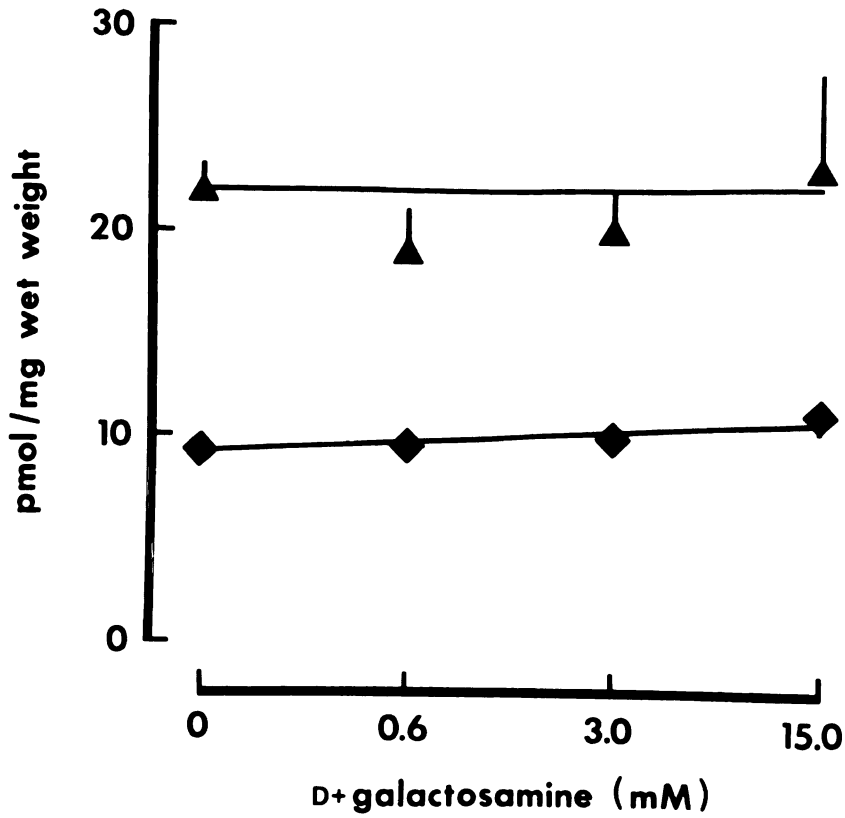

Figure 25

These preliminary data indicated that neither inhibitor would be effective in hepatic slices *in vitro* and neither would help elucidate the mechanism of production of reactive metabolites binding to macromolecules. Although either might be useful for some further study of metabolism in renal cortical slices, neither inhibited the peak at 15.5 min, the major renal cortical slice metabolite and one that proved refractory to post-incubation analyses. For these reasons, this avenue of investigation was not pursued.

5. Post-incubation analyses

Four types of hydrolysis tests were done on post-incubation extracts to further characterize the metabolites in terms of the type of conjugate. N-Glucuronides, N-hydroxy-N-glucuronides and sulphamates are sensitive to citric acid hydrolysis at pH 3-4 (Kadlubar *et al.*, 1978; Radomski *et al.*, 1977). These hydrolysis conditions decreased the peaks at 9.5, 10.1, and 12.2 in liver incubation extracts and 9.5, 12.2, and 23.5 in kidney incubation extracts (Figures 26 and 27).

O-Glucuronides and N-hydroxy-N-glucuronides are sensitive to β-glucuronidase hydrolysis (Kadlubar *et al.*, 1977, 1978; Radomski *et al.*, 1973, 1977). β-Glucuronidase decreased the peaks at 10.1, 11.5, and 12.2 min in liver and at 12.2 min in kidney incubation extracts (Figures 20 and 21).

None of the metabolites was sensitive to hydrolysis at 37°C for 24 hours, a method used to detect N-glucuronides and N-hydroxy-N-glucuronides (Radomski *et al.*, 1977; Lynn *et al.*, 1983). The peak at 9.5 min in liver and kidney incubation extracts was later tested at room temperature in buffer at pH 6.8 and decomposed to MBOCA over a 48 hour period.

Figure 26. Post-incubation hydrolysis of metabolites produced by hepatic tissue slices. Arylsulfatase 4-8 units, β-glucuronidase 500-1000 units, heat 37°C, 24 hr, citric acid pH 3.5-4.0. N=3 except for heat where N=2. Standard error bars shown.

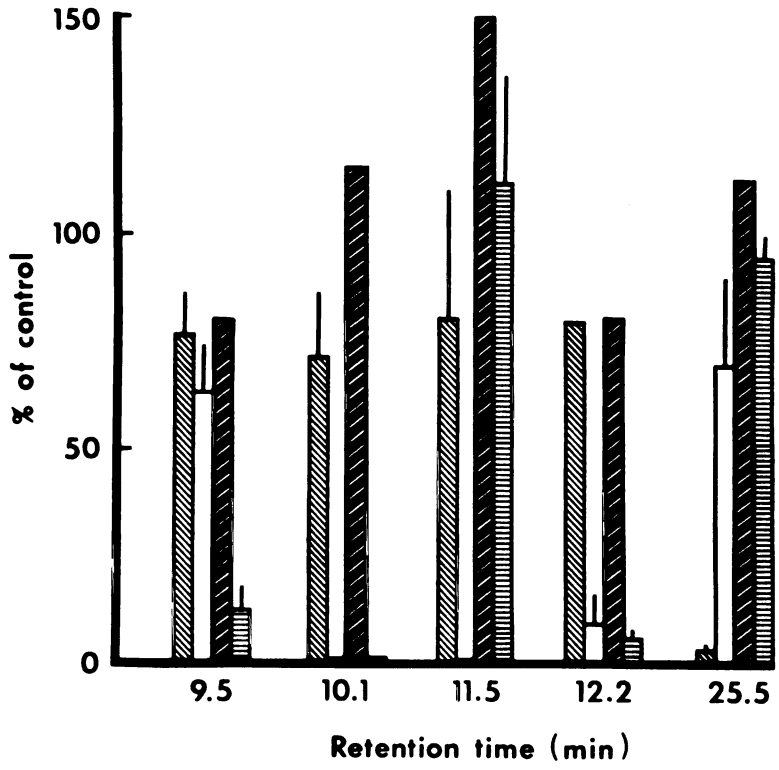

Figure 26

Figure 27. Post-incubation hydrolysis of metabolites produced by renal cortical tissue slices. ▨ Arylsulfatase 4-8 units, ▭ β-glucuronidase 500-1000 units, ■ heat, 37°C, 24 hr, ▤ citric acid pH 3.5-4.6. N=3 except heat where N=2. Standard error bars shown.

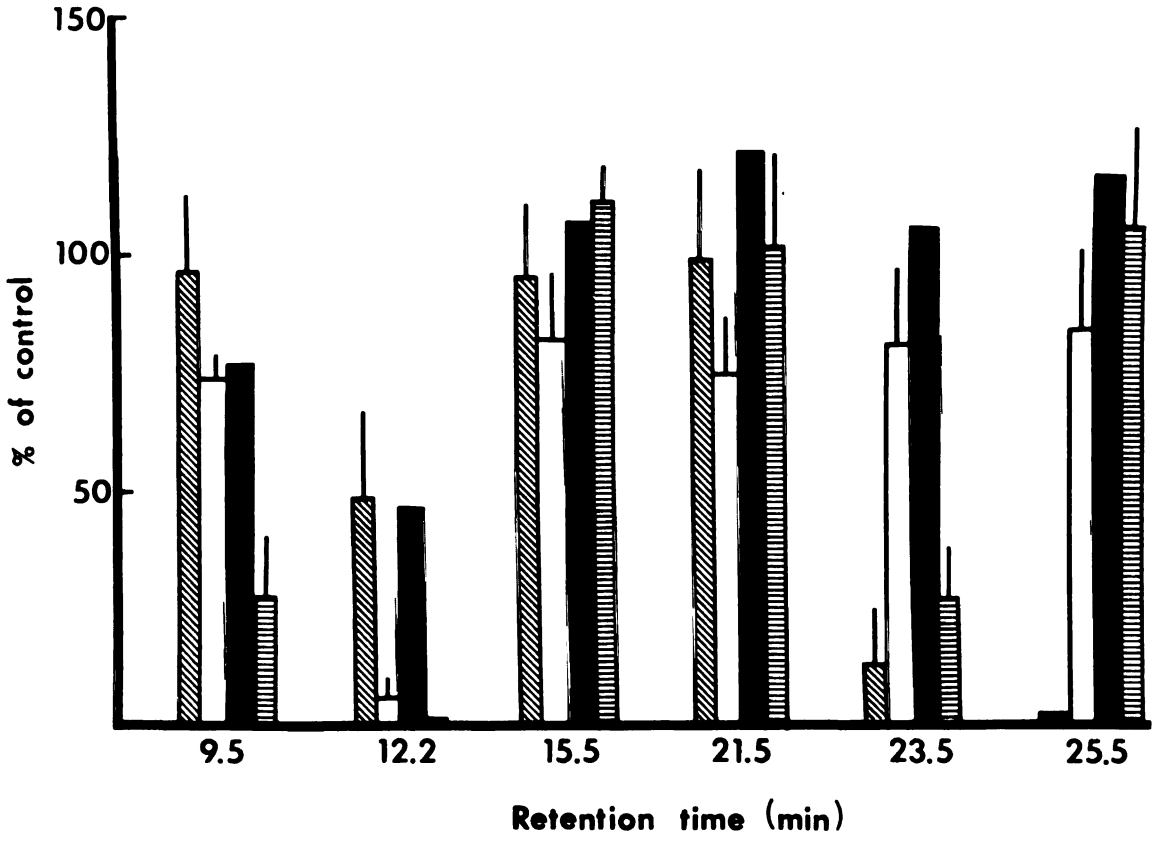
150
100
50
% of control
9.5
12.2
15.5
21.5
23.5
25.5
Retention time (min)

Figure 27

Arylsulphatase, which hydrolyzes the sulphate esters of aromatic phenols, decreased the peak at 25.5 from liver as expected and the peaks at 23.5 and 25.5 from kidney incubation extracts.

6. Mass spectrometry of metabolites from in vitro incubations

FAB-MS of the peaks at 9.5 min (liver) and 15.5 min (kidney) was unsuccessful. There was residual PIC A reagent which interfered with detection of metabolites. In addition, there may not have been enough metabolite to produce a spectrum. Previous FAB-MS of the major canine urinary metabolite required 10-20 μg, or about 4 times the amount used here.

EI-MS of the carbohydrate moiety of the hydrolyzed peak at 9.5 min demonstrated that peak 9.5 min was a glucose conjugate. The mass spectrum (Figure 28) of the TMS derivative was that of the penta-TMS of glucose and did not correspond to the same derivative of glucuronic acid, galactose or mannose. In addition, the gas chromatographic retention times of the α and β anomers were identical to those of glucose, but not mannose or galactose on two separate GLC liquid phases (OV17 in Figure 29).

7. Renal medullary metabolism

Renal medullary metabolism was detected by macromolecular binding. Although HPLC and LSS detection of metabolites was attempted there were no peaks detected by UV absorbance or LSS. Arachadonic acid, substrate for prostaglandin synthase, significantly enhanced binding at 1 mM but inhibitors indomethacin and aspirin had no effect (Figure 30).

Figure 28. EI-MS of the penta-TMS derivatized carbohydrate moiety from the 9.5 min HPLC peak and a glucose standard. The derivative was analyzed by gas chromatography-Mass spectrometry on a Finnigan 3200 at 70 eV. Chromatography was carried out on 1% OV17 using 30 ml/min helium and a temperature program of 140-260°C at 12°C/min beginning 0.5 min after injection.

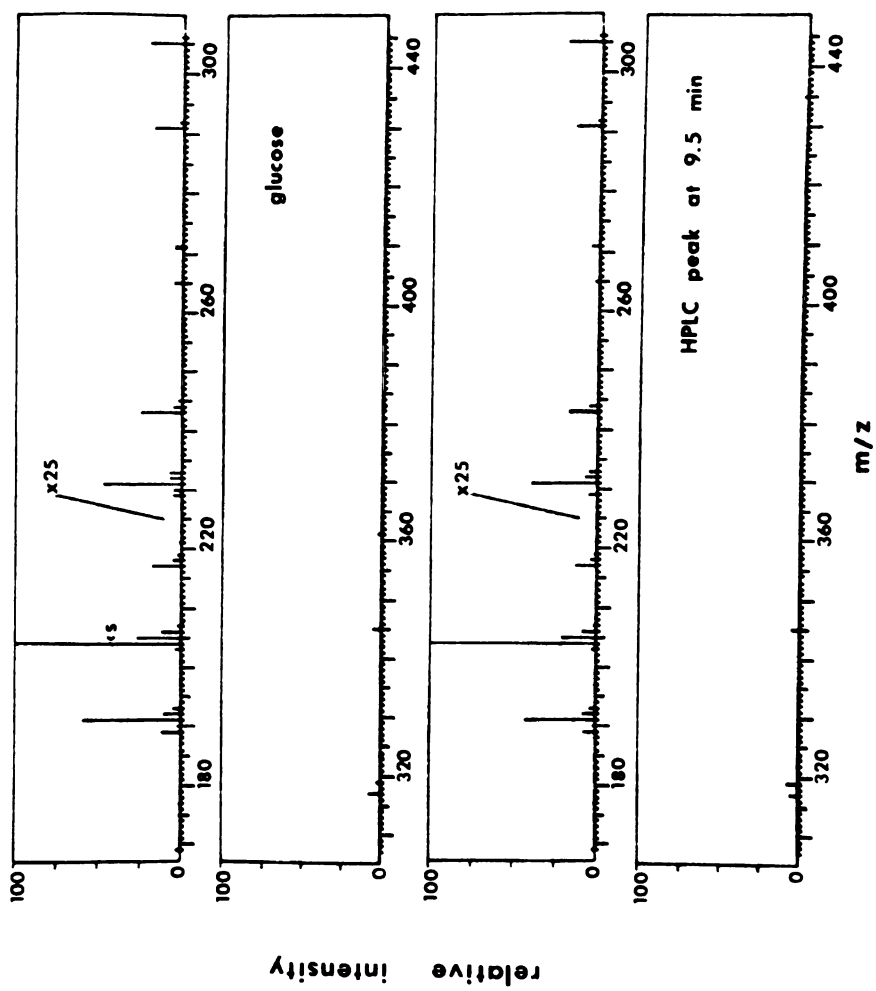
relative intensity
m/z
glucose
HPLC peak at 9.5 min
x25
x25
100
0
180
220
260
300
320
360
400
440

Figure 28

Figure 29. Selected ion chromatogram of the penta-TMS derivative carbohydrate moiety from the 9.5 min HPLC peak and mannose, galactose and glucose standards. Chromatography was carried out on 1% OV17 using 30 ml/min helium and a temperature program of 140-260°C at 12°C/min beginning 0.5 min after injection.

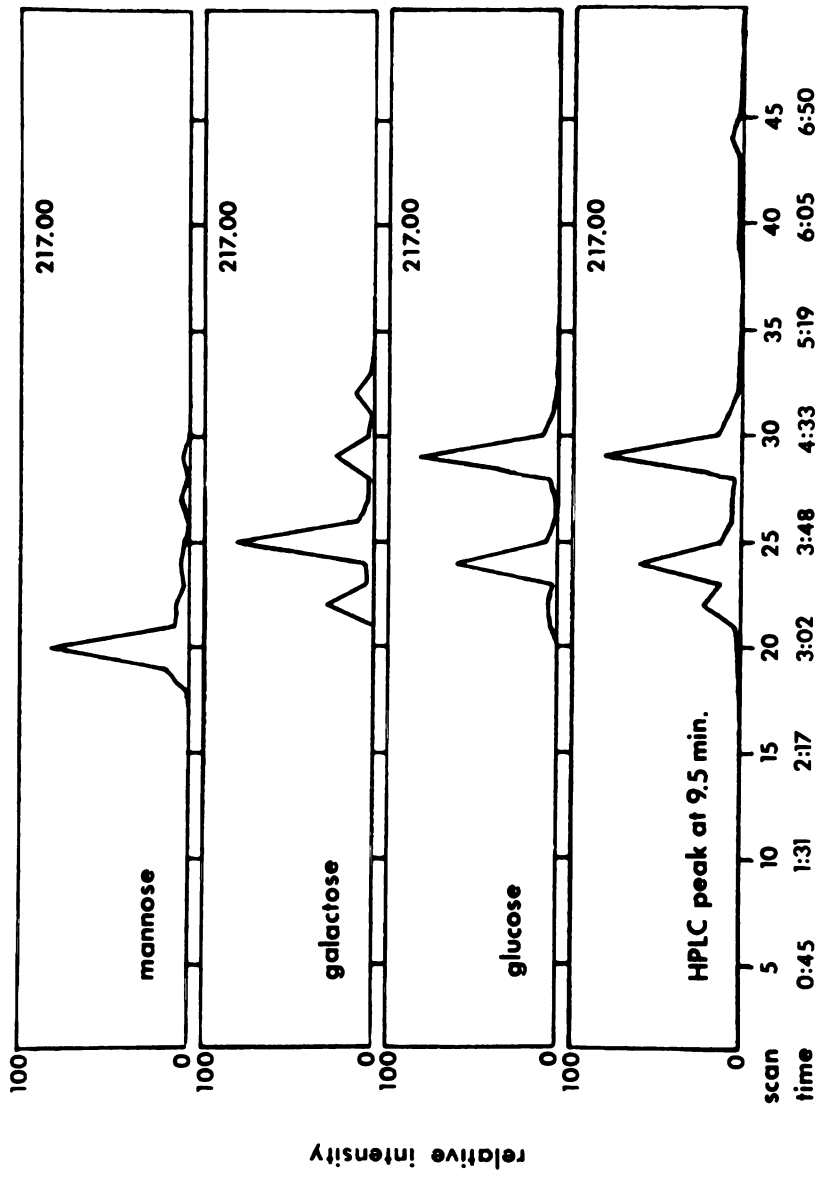
relative intensity
100
0
mannose
217.00
galactose
glucose
HPLC peak at 9.5 min.
scan
time
0:45
1:31
2:17
3:02
3:48
4:33
5:19
6:05
6:50
5
10
15
20
25
30
35
40
45

Figure 29

Figure 30. Macromolecular binding in renal medullary slices. N=4 except (a) where N=5 and (b) where N=3. Standard error bars are shown. *Significantly different from control, $p \leq 0.05$.

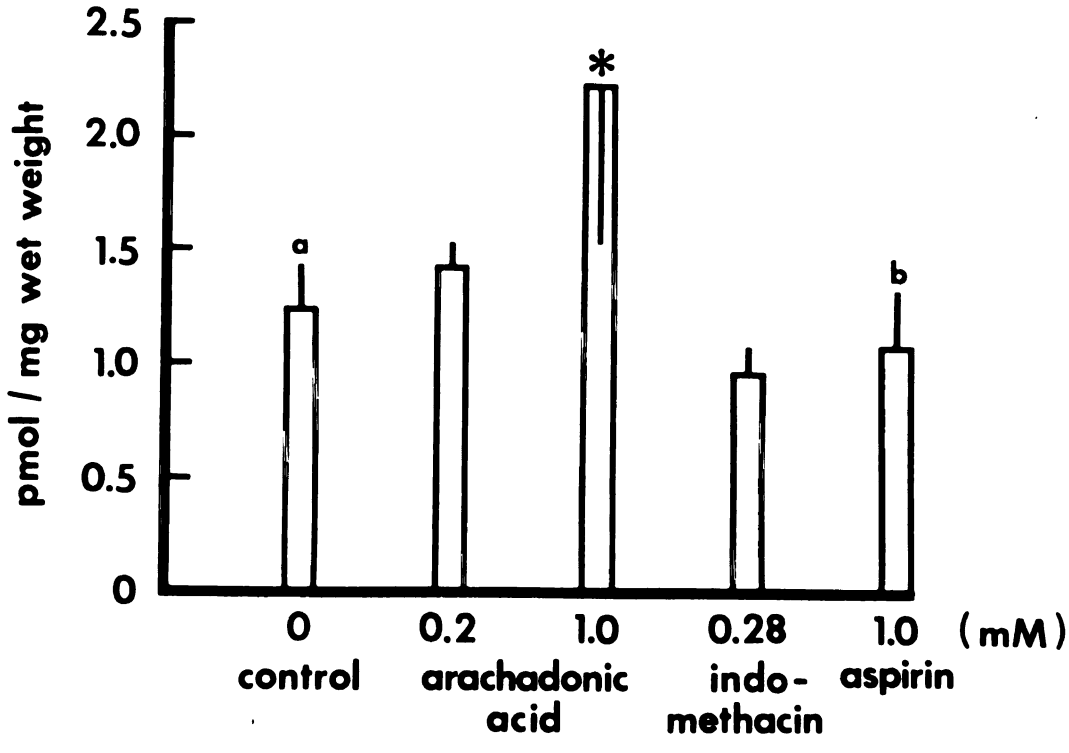
2.5
2.0
1.5
1.0
0.5
0
pmol / mg wet weight
a
*
b
0
control
0.2
arachadonic acid
1.0
0.28
indo-methacin
1.0
aspirin
(mM)

Figure 30

DISCUSSION

A. Characterization of the Major Metabolite in Canine Urine

The major metabolite of MBOCA in canine urine was an ortho-hydroxy sulphate, the same type of major metabolite excreted by dogs after administration of other arylamine bladder carcinogens. The metabolite represented about 75% of the excreted radioactivity (Figure 2) and was hydrolyzed by arylsulfatase in a time and enzyme concentration-dependent manner (Figure 3) but not by β-glucuronidase or citric acid.

The EI spectrum indicated that the metabolite was ring hydroxylated. It provided evidence of the addition of 5 substituents during derivatization and transesterification, possible only if one substituent was added to a ring position. The molecular ion data obtained from FAB agreed with the molecular weight of a hydroxylated MBOCA sulfate with K^+ counter ion and K^+ adduct (Figure 4). Proton NMR data (Figure 5 and Table 1) also supported the proposed structure by providing evidence that the ring substitution was ortho to an amine. These data demonstrated that the major metabolite of MBOCA in dog urine was 5-hydroxy-3,3'-dichloro-4,4'-diamino-diphenylmethane-5-sulfate.

Several other aromatic amine carcinogens are excreted in urine primarily as the ortho-sulfate conjugate. Wiley (1938) found that dogs excreted 2-naphthylamine as the sulfuric acid ester of 2-amino-1-naphthol. The major canine urinary metabolite of 4-aminobiphenyl was

identified as 4-amino-3-diphenylyl sulfate (Bradshaw and Clayson, 1955) and was hydrolyzed to 3-hydroxy-4-aminobiphenyl. Benzidine was excreted from dogs primarily as 3-hydroxybenzidine sulfate (Sciarini and Meigs, 1958; Troll and Nelson, 1958). That MBOCA had a similar major urinary metabolite indicated that it was metabolized in a similar manner.

Metabolism of arylamines can result in mutagenic activation. However, in the present study the major metabolite of MBOCA was not mutagenic in Salmonella typhimurium TA1538 under conditions of aryl-sulphatase hydrolysis (Table 2). The assay conditions were harsh, as demonstrated by the loss of viability in the Tris buffer and may have determined the outcome of the test. TA1538, a frameshift mutant has been used successfully to determine the mutagenicity of 2AF, 4AB, BZ, 2AAF (Ames et al., 1973a; Fouarge et al., 1982), N-hydroxy-2AF (Ames et al., 1973b), and N-hydroxy-2-AAF (White et al., 1983) but was not as sensitive as the base pair mutants TA100 and TA1535 to 2NA and MBOCA (Ames et al., 1973a; McCann et al., 1975). TA1535 was selected for this study due to its sensitivity to arylamine mutagens, its low background reversion rate and its superior survival under the incubation conditions. The limited quantity of substrate precluded further evaluation of the mutagenicity of the major metabolite in other tester strains.

Bacterial mutagenicity may be a specialized measure of reactivity. Reversion frequency depends on the tester strain and the activation system. Moreover, quantitative determination of carcinogenesis cannot be made from the test results (International Commission, 1982). The DNA adduct responsible for mutagenicity may be different from the adduct involved in carcinogenicity. The only DNA lesion identified in TA1538

treated with 2AAF was a 2AF adduct (Beland et al., 1982). This was the same as the major adduct in rat tissues and the only one identified in female rats and in tissues not sensitive to 2AAF carcinogenicity (Beland et al., 1982). In addition, the reversion rate for 4AB, 2AF and 2NA (2AF>2NA>4AB) did not correlate with carcinogenicity in the urinary bladder (4AB>2AF>2NA) but rather with the amount of mutagen bound per 10^6 nucleotides (Kadlubar et al., 1982). Although bacterial mutagenicity may be used as a screening method for carcinogens, it may be a measure of DNA binding under physiological conditions.

The ortho-hydroxy sulphate of MBOCA bound to both DNA and protein in vitro upon hydrolysis with arylsulfatase (Figures 6 and 7). This indicated that the ortho-hydroxy MBOCA metabolite was a reactive compound, detoxified by conjugation with the sulphate. Its role in bladder carcinogenesis may be limited by urinary hydrolysis and Boyland and Williams (1960) demonstrated that human urinary arylsulfatase activity did not hydrolyze 2-amino-1-naphthol sulphate. Arylsulphatase A and B are excreted in the urine (Dodgson and Spencer, 1957) and increased arylsulfatase A activity has been observed in patients with urologic cancer (Kosugi et al., 1983). This has been further investigated because aromatic sulphates are hydrolyzed by the A form (Kosugi et al., 1983). The increased activity was due to an arylsulfatase A isozyme not detected in normal urine (Kosugi et al., 1983) and may reflect an increase in arylsulfatase activity due to the malignancy (Maru et al., 1980). Thus, arylamine sulphates may not be hydrolyzed by the urinary enzyme and increased urinary arylsulfatase activity may be a marker for the urologic carcinoma and not a participant.

The major MBOCA metabolite in canine urine, the ortho-hydroxy sulphate, was a detoxication product with a structure comparable to the major canine urinary metabolite of other arylamine bladder carcinogens. This demonstrated that MBOCA could be metabolized in a manner similar to those arylamines. It was of interest to determine the roles that liver and kidney played in the production of this and other conjugates and the roles they played in production of reactive metabolites.

B. In Vitro Metabolism of MBOCA by Liver and Kidney Slices

In order to study the potential roles of individual organs in MBOCA metabolism, they must be isolated from total body metabolic capacity. This can be done by using purified enzymes, isolated liver cell or renal tubule preparations, tissue slices, or organ perfusion (Jones et al., 1980). Broken cell, microsomal and purified enzyme preparations produce Phase I metabolites unless cosubstrates for Phase II metabolism are added. This does not take advantage of cellular compartmentalization and limits the variety of conjugated metabolites produced at one time. Whole cell and organ preparations offer the advantage of obtaining a complement of Phase II metabolites. Tissue slices were used in this investigation because they provided a rapid, convenient method of obtaining a whole cell preparation capable of producing a spectrum of conjugated metabolites. Unlike organ perfusion, tissue slices may be used to study several sets of experimental conditions simultaneously and without possible carryover in metabolism or binding. Tissue slices have limitations, however. Slice thickness and media hinder oxygen diffusion and can alter metabolism (Cohen and Ramm, 1976). In addition, xenobiotic delivery is not the same as in vivo. In spite of these

limitations tissue slices represented a convenient method for investigating MBOCA metabolism.

An HPLC system was developed to separate metabolites in order to investigate conjugate structure and the effects of various compounds on their production. Most arylamine conjugates are glucuronides or sulphates and are ionized in aqueous media. An ion-pair reagent such as tetrabutylammonium phosphate (PIC A) combines with the ion, forming a non-ionic pair which is retained on a reversed-phase column. Reversed phase chromatography was used because the more polar metabolites eluted prior to the parent compound, MBOCA (Figures 8 and 9). This method separated three MBOCA conjugates sensitive to β-glucuronidase (Figure 26) demonstrating the power of the technique and allowing investigation of these metabolites individually. These three metabolites (10.1, 11.5, 12.2 min) did not change in parallel during experiments to study the production of reactive metabolites and their separation was essential to their structural elucidation.

MBOCA metabolism to reactive products was determined in terms of macromolecular or nonextractable binding. Metabolism can produce reactive products that are extremely unstable and bind to the organelle in which they are produced. They may also migrate within the cell and react with cell membranes, proteins, RNA and DNA (Gillette and Pohl, 1977). A correlation between covalent binding and carcinogenesis has been demonstrated for related alkylating agents, azo dyes, and polycyclic hydrocarbons (Brookes, 1977) and covalent binding may play a role in mutagenesis and cell necrosis as well (Cohen, 1983). Nonextractable binding has been used to evaluate formation of these reactive species. It includes covalent binding and high affinity noncovalent binding but

omits lipid bound metabolites that are extracted with organic solvents (Gillette and Pohl, 1977). Although it may therefore underestimate total covalent binding, it is a simple, rapid, and widely used method. Nonextractable binding is used here to determine covalent binding.

1. Macromolecular binding in hepatic and renal tissue slices

Macromolecular binding of ^{14}C-MBOCA occurred in both liver and kidney, demonstrating that the renal cortex and medulla metabolized MBOCA to reactive products. ^{14}C-MBOCA binding increased with time (Figure 12) and enzyme concentration to 0.1 μmol/ml (Figures 10 and 11). It was not an artifact of incubation with tissue slices as demonstrated by the low binding in acetonitrile-poisoned controls (Figures 10 and 11). Increasing the substrate concentration to 0.2 μmol/ml produced no further increase in binding and the 0.1 μmol/ml conditions (200 mg liver, 60 min; 400 mg kidney, 90 min) were used for further study.

Renal medullary metabolism was low. Rapp et al. (1980) demonstrated a linear increase of BZ binding to rabbit medullary slices up to 6 pmol/mg at 0.2 mM arachadonic acid. In the present study MBOCA binding to canine medullary slices was not increased at 0.2 mM and reached just 2.2 pmol/mg at 1.0 mM arachadonic acid (Figure 30). These differences may be due to differences in the tissues from rabbit and dog or the difference in incubation conditions. However, MBOCA was not metabolized to tRNA binding species by prostaglandin synthase in vitro under conditions where BZ and 2AF were highly bound (Morton et al., 1983). Thus, MBOCA may not be as readily cooxidized as other aryl-amines.

Macromolecular binding was investigated further in the kidney cortex and liver. Alteration of incubation conditions by addition of D(+)galactosamine or PNPS had little effect on binding but did alter metabolite production. D(+)galactosamine inhibits glucuronidation two ways. It traps uridine nucleotides as UDP-galactosamine and that compound inhibits UDP-glucose dehydrogenase (Schwarz, 1980). Trapping uridine nucleotides could decrease glucosidation as well. D(+)galactosamine inhibited production of metabolites in liver (Figure 23 - 9.5, 10.1 and 12.2 min) and kidney (Figure 24 - 9.5 and 12.2 min) without altering macromolecular binding (Figure 25). This indicated that these metabolites were not involved in macromolecular binding and were detoxication products.

PNPS also decreased metabolites in liver (Figure 17 - 9.5, 10.1, 12.2) and kidney (Figure 18 - 15.5 min) without altering binding (Figure 19). PNPS contains sulphate group potential comparable to that of the metabolic sulphate donor, 3'-phosphoadenosine-5'-phosphosulphate (PAPS) and can enhance the endogenous pool of PAPS by phenolsulphotransferase mediated transulfation of 3'-phosphoadenosine-5'-phosphate (Schwartz, 1980). Concentrations of 0.05-4 mM PNPS have been reported to stimulate sulphation of 1-naphthol up to 275% in isolated rat liver cells (Schwartz, 1980). In the present experiment PNPS did not enhance production of any metabolite previously labelled with ^{35}S (Figures 15 and 16, vs. 17 and 18). Phenolsulphotransferase is a high affinity, low capacity enzyme (Koster et al., 1982b) and may have been saturated under the incubation conditions. The peak at 9.5 min increased over 300% in the kidney and decreased significantly in liver without increasing or

decreasing binding. Thus, it was not a reactive metabolite. These experiments indicated that peaks at 10.1, 12.2, and 15.5 min were also unreactive.

Inhibition of sulphation was also used to investigate MBOCA metabolite reactivity and structure. DCNP is a selective inhibitor (Koster et al., 1982a) active towards phenol, harmol, or N-hydroxyphenacetin sulphation (Mulder, 1981). Glucuronidation is not inhibited, however. Thus, inhibition of phenolsulphotransferase activity towards harmol in the rat is reflected in an increased production of harmol glucuronide (Koster et al., 1982b). In the present investigation, DCNP had no effect on binding in either liver or kidney at low concentrations but inhibited binding at 10 and 100 μM (Figure 22) where production of all but one metabolite fell (Figures 20 and 21). The metabolite at 11.5 min increased up to 500% without altering binding, demonstrating that it was unreactive. The depression of metabolite production and concomitant decrease in binding indicated that metabolism was necessary for binding. It is possible that the depression of metabolism was a result of inhibition of oxidative phosphorylation and commitant depletion of cellular ATP. DCNP inhibited rat mitochondrial respiration at 2 μM in our laboratory (Karl Ebner, personal communication).

The experiments on alteration of incubation conditions demonstrated that metabolism was necessary for binding and indicated that hepatic and renal metabolites eluting at 9.5, 10.1, 11.5, 12.2 and 15.5 min were detoxication products. These metabolites were more sensitive to altered incubation conditions and were produced in greater quantity than those eluting 22-25.5 min except for the MBOCA orthohydroxy sulphate, peak 25.5 min, which was the major metabolite in liver tissue

slices. The structures of other metabolites characterized by HPLC were further elucidated using post-incubation hydrolysis.

2. Elucidation of metabolites in renal and hepatic tissue slices

The post-incubation experiments, in concert with previous experiments, aided in elucidation of the structure of MBOCA metabolites from liver and kidney. Post-incubation hydrolyses indicated that peaks from liver and kidney with the same HPLC retention time were identical. This was also true for experiments on alteration of incubation conditions, except for the peak at 9.5 min in the presence of PNPS. Metabolites from both organs are therefore considered together, in their elution sequence.

The peak at 9.5 min was identified as an N-glucoside. The peak was sensitive to citric acid hydrolysis at pH 3.5-4.2 (Figures 26 and 27), a characteristic of N-conjugates. It decomposed to the parent compound on standing at room temperature, indicating that MBOCA was not oxidized prior to conjugation. It was not labeled by ^{35}S (Figures 15 and 16) and was decreased by D(+)galactosamine (Figures 23 and 24) an inhibitor of glucuronidation and glucosidation. Further, the peak was not sensitive to β-glucuronidase or arylsulfatase which hydrolyze O-conjugates (Figures 26 and 27). These data indicated that the compound was a glucose or glucuronic acid MBOCA N-conjugate. The EI-MS of the liberated sugar following hydrolysis at room temperature (Figure 28) demonstrated a glucose moiety and identified the structure as an N-glucoside.

The peak at 10.1 min was sensitive to both citric acid hydrolysis and β-glucuronidase (Figure 26), a property of N-hydroxy-N-glucuronides (Kadlubar et al., 1977, 1978). It was inhibited by PNPS (Figure

17), insensitive to arylsulfatase (Figure 26) and not labeled by ^{35}S (Figure 15) indicating that it was not a sulphate. In addition, it was inhibited by D(+)galactosamine (Figure 23). This evidence supports an N-hydroxy-N-glucuronide structure. The 10.1 min MBOCA metabolite may also contain more than one conjugate. Hydrolysis of one moiety would result in disappearance of the peak.

The peak at 11.5 min appeared to be an O-glucuronide because it was insensitive to citric acid hydrolysis but sensitive to β-glucuronidase (Figure 26). In addition, it was enhanced up to 500% by DCNP (Figure 20), a compound known to enhance the glucuronidation of phenols while inhibiting sulphation (Koster et al., 1982b). However, there was no apparent decrease in the sulphate conjugates eluting 22-25 min in the presence of DCNP. This discrepancy may be due to the relative proportions of metabolites. The ortho-hydroxy sulphate represented the major metabolite in liver slice metabolism and a small decrease in sulfation would not be easily discerned. Since the peak at 11.5 was small, the increase was readily observed.

Like the peak at 10.1 min, the peak at 12.2 min was decreased by PNPS in the liver (Figure 17), was insensitive to arylsulfatase (Figures 26 and 27) and was not labeled by ^{35}S (Figures 15 and 16) indicating that it was not a sulphate and did not contain a sulfur atom. It was sensitive to both citric acid and β-glucuronidase (Figures 26 and 27), evidence in support of an N-hydroxy-N-glucuronide structure and was decreased in both liver and kidney by D(+)galactosamine (Figures 23 and 24). Unlike the peak at 10.1 min, the peak at 12.2 min was produced in both liver and kidney. It was produced in quantities great enough to be isolated and further elucidation of this structure is warranted.

The experiments were not able to define the structure of the peaks at 15.5 and 22 min. Both were produced in renal slice incubations only. The peak at 15.5 min was not labeled by ^{35}S (Figure 16) was decreased by PNPS (Figure 18), and was insensitive to arylsulfatase (Figure 27). It was not affected by β-glucuronidase, heat or citric acid (Figure 27). For these reasons, it is probably not a glucuronide, glucoside, sulphate or sulphamate. It is produced in large enough quantities to make further structural elucidation possible. The peak at 22 min was labeled by ^{35}S (Figure 16) but was unaffected by other incubation or post-incubation treatments. This metabolite was present in very small quantities, making further characterization difficult.

The peak at 23.5 may have been a sulphate-sulphamate double conjugate or a combination of an N-glucuronide and sulphate. It was sensitive to citric acid, a characteristic of N-conjugates, and to arylsulfatase, characteristic of o-sulphates (Figures 27). In addition, it was labeled with ^{35}S (Figures 15 and 16). It was produced in quantities too small to evaluate further.

The peak at 25.5 min was the ortho-hydroxy sulphate characterized earlier after extraction from canine urine. It was labeled by ^{35}S (Figures 15 and 16) and, not surprisingly, was sensitive to arylsulfatase in post-incubation hydrolysis (Figures 26 and 27). In the slice incubation studies it represented the major metabolite in liver but was produced in only small quantities in the kidney.

Elucidation of MBOCA metabolite structures demonstrated that both liver and kidney produced glucuronide and sulphate conjugates in vitro. As outlined in the Introduction, ortho-hydroxy glucuronides and

sulphates have been identified for BZ, 1NA, 2NA, and 4AB (Wiley, 1983; Clayson and Ashton, 1956; Sciarni and Meigs, 1958; Clayson et al., 1959; Booth et al., 1955; Sciarini and Meigs, 1961), sulphamates for BZ, 1NA, and 2NA (Boyland et al., 1957; Clayson et al., 1959) and N-hydroxy-N-glucuronides for 2NA and 4AB (Kadlubar et al., 1981; Radomski et al., 1977). Characterization of MBOCA metabolites from dog liver and kidney indicated that an o-glucuronide and an o-sulphate were formed. A sulphamate was not identified although the peak at 23.5 min may contain this moiety. One of the two peaks with identical responses to the incubation and post-incubation studies (10.1 and 12.2 min) may have represented an N-hydroxy-N-glucuronide but only one of them was formed in the liver. Further elucidation of their structures may provide insight into hepatic and renal formation of the conjugate of a proximate bladder carcinogen. The studies indicated that MBOCA metabolism was similar to that of other arylamines.

One MBOCA metabolite, identified as an N-glucoside, appeared to be unique in the spectrum of arylamine metabolites. The N-glucoside has not been reported as a metabolite for other arylamines. The formation of 2NA-N-glucoside was observed in rat tissue slice incubations containing 0.2% glucose and in a 0.2% glucose solution without slices but not in slice incubations without added glucose (Booth et al., 1955). The media used in MBOCA metabolism did not contain glucose. In addition, in the presence of PNPS, production of this peak varied in opposite directions in liver and kidney, making nonmetabolic production unlikely. Glucosidation can supplement glucuronidation in mammalian systems when the aglycon load is high (Dutton, 1980) as the UDP-glucosyltransferases are normal cellular constituents in liver and kidney,

with steroids and bilirubin as the natural substrates (Dutton, 1980). It is conceivable that the UDP-glucose dehydrogenase, necessary for conversion of UDP-glucose to UDP-glucuronic acid, may have been inhibited under the incubation conditions. Further investigation is warranted to determine whether the N-glucoside is a product of slice incubations only.

MBOCA metabolites formed in the tissue slices included several detoxication products which could be hydrolyzed in the bladder. The N-glucoside (at 9.5 min) was sensitive to mild acid hydrolysis and decomposed to MBOCA. It may also decompose in the urine, releasing MBOCA which could then be metabolized by the urothelium. Thus, both liver and kidney contained a route of metabolism for delivery of the lipophilic parent compound to the bladder. The minor, ^{35}S-labeled metabolite at 23.5 min was also labile in acidic media but the product of that hydrolysis is unknown. o-Glucuronides may be hydrolyzed by urinary β-glucuronidase. The o-glucuronides of 2-acetamino-6-naphthol, 2-acetamido-1-naphthol, 1-naphthol, and 2-amino-1-naphthol were hydrolyzed by human urinary β-glucuronidase (Boyland and Williams, 1960). Whether the arylamine aglycons are reactive or undergo further metabolism is not known.

SUMMARY AND CONCLUSIONS

The purposes of this investigation were 2-fold: (1) to determine the structure and reactivity of the major urinary metabolite of MOBCA in the dog and (2) to test the hypothesis that the liver and kidney play a role in arylamine metabolism, using MBOCA as a model. The dog was used as a model species because it is sensitive to arylamine-induced bladder cancer.

The similarity of MBOCA metabolism to known bladder carcinogens was assessed initially by determining the structure and binding characteristics of the major urinary metabolite in dogs. Detailed enzymatic, chemical, and instrumental analyses including arylsulfatase, β-glucuronidase, and citric acid hydrolyses, EI-MS, FAB-MS, and NMR determined that the metabolite was an ortho-hydroxy sulphate. It was therefore similar to the major urinary metabolites in dogs of 4AB, BZ, and 2NA (Wiley, 1938; Bradshaw and Clayson, 1955; Sciarini and Meigs, 1958; Troll and Nelson, 1958). Binding studies indicated that the ortho-hydroxy sulphate of MBOCA was inert but upon hydrolysis with arylsulfatase it bound to both DNA and protein, in vitro. Thus, the oxidized metabolite was reactive and sulphate conjugation was a detoxification mechanism.

The major urinary metabolite was also the major metabolite produced by liver slices but was a minor metabolite in slices from the kidney

cortex. Both organs produced a variety of metabolites *in vitro* including an N-glucuside, metabolites sensitive to β-glucuronidase, the ortho-hydroxy sulphate, and another metabolite sensitive to arylsulfatase. Arylamine metabolism usually results in production of an N-glucuronide, not an N-glycoside. This product has not been reported as a metabolic product of other carcinogenic arylamines *in vivo* or *in vitro* but has been reported as a metabolite *in vivo* of the non-carcinogenic arylamine 1NA (Clayson and Ashton, 1956). Whether or not this difference in metabolism is physiologically important can be debated. Both metabolites are sensitive to mild acid hydrolysis which would release the parent compound in the bladder where it could then be metabolically activated by the urothelium. However, if differences in metabolism are reflected in differences in carcinogenicity, then the production of the MBOCA N-glycoside may be a reflection of its low carcinogenicity. It is therefore important to determine whether or not the MBOCA-N-glycoside is produced by dogs *in vivo* and whether production of an arylamine N-glycoside can be used as an index of carcinogenicity.

A metabolite from liver and kidney slice incubations, identified as peak 12.2 min by its HPLC retention time, had properties of an N-hydroxy-N-glucuronide. Importantly, it was sensitive to citric acid hydrolysis, a property of Nconjugates, and was sensitive to β-glucuronidase, a property of o-glucuronides and N-hydroxy-N-glucuronides (Kadlubar *et al.*, 1977, 1978). The N-hydroxy-N-glucuronide has been proposed as the hepatic metabolite which, when excreted in the urine, decomposes to the hydroxylamine, a proximate carcinogen. Production of the N-hydroxy-N-glucuronide by the kidney could make it an important site of metabolism of arylamine bladder carcinogens.

Liver and kidney tissue slices produced metabolites unique to each organ. The liver produced a metabolite (at 11.5 min) sensitive to β-glucuronidase and whose production was enhanced in the presence of DCNP, an inhibitor of phenol sulphation known to enhance phenol glucuronidation (Koster et al., 1982a). Thus, it appeared to be an o-glucuronide. Liver slices produced a second, uncharacterized metabolite at 13.5 min which was not observed in all experiments. The kidney also produced two unique metabolites neither of which was identified. One at 15.5 min, the major metabolite in kidney slice incubations, was insensitive to all hydrolysis methods used. It may be an unusual metabolite and warrants further investigation. The other, at 22 min, was produced in such small quantities that characterization was difficult.

The kidney medulla did not produce HPLC identifiable metabolites on incubation with MBOCA and macromolecular binding was used to determine metabolism to reactive products. Prostaglandin synthase mediated arylamine cooxidation has been proposed as a route of metabolism in the kidney medulla. Binding was low, even in the presence of the prostaglandin synthase substrate arachadonic acid, and inhibitors failed to decrease binding. Morton et al. (1983) reported low tRNA binding by MBOCA using microsomal prostaglandin synthase to cooxygenate, thus MBOCA may not be a good substrate.

Macromolecular binding was also used to determine the production of reactive metabolites in liver and kidney cortex slices. Binding did not change under altered incubation conditions including inhibition of glucuronidation and glucosidation with D(+)galactosamine and addition of PNPS to stimulate sulfation. Binding decreased in concert with the decrease in metabolism in general on incubation with DCNP. Incubation

with D(+)galactosamine, PNPS and DCNP did significantly alter 5 MBOCA metabolites from kidney or liver and demonstrated that they were detoxication products.

These studies were therefore successful in demonstrating that both liver and kidney can play a role in metabolism of arylamine bladder carcinogens to conjugated and reactive products. Because of its high concentration of oxidizing and conjugating enzymes, the liver has been considered the primary source of arylamine metabolites and the source of the purported proximate carcinogen, the hydroxylamine. The kidney cortex receives a high blood flow and contains the enzymes required for oxidation and conjugation of arylamines, albeit at a lower concentration than the liver. The present investigation demonstrated for the first time that the kidney cortex has the potential to produce reactive and conjugated metabolites. Thus, the kidney cortex must be added to the potential sites of metabolism for arylamine bladder carcinogens.

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