METABOLISM OF PHENANTHRENE BY THE RAT AND THE GUINEA PIG

A Dissertation

Presented to The Faculty of

The Department of Biochemical and Biophysical Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

Khingkan Lertratanangkoon

May, 1984

DEDICATION

To: my academic parents, Drs. Marjorie G. and Evan C. Horning

my academic advisor, Dr. Brian S. Middleditch,

whose support, encouragement and understanding are bound here as firmly as the very pages.

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To these, and to the many others, I am very grateful.

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ABSTRACT

The metabolism of phenanthrene has been studied in the rat and the guinea pig, with the aim of seeking evidence in whole animals of the formation of dihydrodiol epoxides and/or diepoxides as metabolic intermediates and of comparing the bivalent sulfur metabolites excreted by the two species.

After the administration of phenanthrene (50 mg/kg, ip) to young adult male rats and guinea pigs, a series of oxygenated- and bivalent sulfur-containing metabolites were isolated and identified by GC and GC/MS. Thirty-four oxygenated metabolites were isolated after enzymatic hydrolysis of the urine samples from the rat, whereas only twenty-one were detected in guinea pig urine. The major metabolites in both species were identified as 9,10- and 1,2-dihydrodiols, indicating that both K-region and non-K-region pathways are highly operative *in vivo*. Several triols and tetrols were also isolated as urinary metabolites, suggesting that dihydrodiol epoxides and/or diepoxides are involved in the metabolism of phenanthrene by the rat and the guinea pig.

Besides the oxygenated metabolites, seven methylthio metabolites were isolated from the neutral fraction of hydrolyzed rat urine, whereas only two were detected in guinea pig urine. The major methylthio metabolite excreted by each species was 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene. This was observed as a second-day metabolite in the rat, and its appearance was accompanied by 9-hydroxyphenanthrene.

Acidic urinary metabolites derived from glutathione conjugates are species-dependent. The major metabolite in hydrolyzed rat urine was N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine. In the guinea pig, the major bivalent sulfur acid was S-(9-hydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid, but N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine was also present. The excretion of S-(9-hydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid by the guinea pig may be due to incomplete acetylation or to a combination of acetylation and deacetylation of cysteine or cysteinylglycine adducts.

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amu	atomic mass unit
BSA	N,O-bis(trimethylsilyl)acetamide
^{14}C	carbon-14
$\underline{d}_{18}$-BSA	bis(trimethyl-$\underline{d}_9$-silyl)acetamide
DNA	deoxyribonucleic acid
eV	electron volt
FAB/MS	fast atom bombardment/mass spectrometry
g	gram
G	glycerol
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
GSH	glutathione, reduced form
^{3}H	tritium
HPLC	high performance liquid chromatography
hr	hour
Hyd	hydrolyze
ip	intraperitoneal
kV	kilovolt
m	meter
$M^{+\cdot}$	molecular ion
mA	milliampere
mCi	millicurie

ME-TMS	methyl ester-trimethylsilyl
mg	milligram
MH^+	protonated molecular ion
ml	milliliter
mm	millimeter
mmole	millimole
MU	methylene unit
µA	microampere
µCi	microcurie
µl	microliter
µM	micromole
m/z	mass-to-charge ratio
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
RNA	ribonucleic acid
TLC	thin layer chromatography
TMS	trimethylsilyl
UDP	uridine diphosphate

CHAPTER I

INTRODUCTION

INTRODUCTION

I. ARENE OXIDES: METABOLITES OF POLYCYCLIC AROMATIC HYDROCARBONS

Interest in the metabolic fate of polycyclic aromatic hydrocarbons was stimulated by the discovery in the 1930's of the carcinogenic activity of certain members of this class. A number of polycyclic aromatic hydrocarbons are formed during the incomplete combustion of organic materials and thus are widespread atmospheric pollutants. Dibenzo[a,h]-anthracene was identified as the first chemical of this kind to have carcinogenic activity (Kennaway and Hieger, 1930), and benzo[a]pyrene was found to be the principal carcinogen in coal tar (Cook et al., 1933). The subsequent identification of a number of polycyclic aromatic hydrocarbons with carcinogenic activity resulted in numerous attempts to draw structure-activity relationships and to define mechanisms of action. Fieser (1938) suggested that hydrocarbon metabolites could effect a chemical lesion by binding to cellular constituents, and several studies established that hydrocarbon-protein covalent binding did occur (Doniach et al., 1943; Miller, 1951; Heidelberger and Weiss, 1951).

Inasmuch as the hydrocarbons themselves do not possess reactive functional groups, it was evident that metabolic activation was necessary before they could react with cellular macromolecules. The enzymes thought most likely to be involved in this metabolic activation were the mono-oxygenases which are present in the endoplasmic reticulum of most cells, and this hypothesis was supported by the results of experiments in

which [^{3}H]-labeled hydrocarbons were incubated with rat liver microsomal systems (which contain the mono-oxygenase) in the presence of DNA (Grover and Sims, 1968; Gelboin, 1969). Earlier, based on an extensive series of metabolism studies, Boyland (1950) postulated that arene oxides of polycyclic aromatic hydrocarbons were involved in the binding of hydrocarbons with cellular macromolecules, and Jerina et al. (1968) provided the first direct demonstration of arene oxide formation.

The concept of metabolite-binding as a necessary, but not sufficient, criterion for the induction of cancer (Miller, 1970; Miller and Miller, 1974) has become a fundamental tenet of cancer research, and DNA seems to be the critical target (Brookes, 1966), although RNA and protein have not been excluded. Numerous studies established that polycyclic hydrocarbon metabolites will bind covalently to DNA and RNA (for example, Brookes and Lawley, 1964; Grover and Sims, 1968; Gelboin, 1969) both in vivo and in vitro and, although arene oxides were attractive as reactive metabolites that could explain this binding, the structures of the bound metabolites remained unknown at that time.

II. REACTIONS OF K-REGION EPOXIDES WITH NUCLEIC ACIDS

Some 20 years after the view that epoxides are primary metabolites of polycyclic hydrocarbons was first proposed (Boyland, 1950), the formation of epoxides of phenanthrene, benz[a]anthracene, 7-methylbenz[a]-anthracene, 7,12-dimethylbenz[a]anthracene, pyrene, benzo[a]pyrene, and dibenz[a,h]anthracene was directly demonstrated in rat and hamster liver

microsomal systems (Grover et al., 1971a, 1972; Keysell et al., 1973; Wang et al., 1972; Selkirk et al., 1971, 1975). As these first-discovered epoxides were all K-region epoxides, attention was naturally focused on the properties of epoxides formed at this reactive region. The K-region epoxides of these hydrocarbons were relatively easy to synthesize, and could be readily obtained labeled with tritium. Grover and Sims (1970) showed that the K-region epoxides of phenanthrene and dibenz[a,h]anthracene reacted with DNA, RNA and histone in the absence of any metabolizing systems (conditions under which the parent hydrocarbons and their respective K-region dihydrodiols did not react). They further showed that these epoxides, and also the K-region epoxides of benz[a]-anthracene and 7-methylbenz[a]anthracene, reacted mainly with the purine moieties of nucleic acids: they reacted to a greater extent with DNA and RNA, in aqueous ethanol solution, than with a purinic acid and, in similar experiments with polyribonucleotides, they were reactive towards poly(G), less reactive towards poly(A), poly(X) and poly(I), and did not react appreciably with poly(U) or poly(C) (Grover and Sims, 1973).

K-Region epoxides were found to cause the malignant transformation of rodent cells in culture (Grover et al., 1971b), to be mutagenic in a variety of systems including mammalian cells (Huberman et al., 1971), bacteria (Ames et al., 1972) and bacteriophage T_2h^+ (Cookson et al., 1971) and they appeared to have the requisite chemical reactivity to qualify them for the role of ultimate carcinogenic forms of polycyclic aromatic hydrocarbons. They are generally weaker carcinogens than their

parent hydrocarbons (Miller and Miller, 1967; Boyland and Sims, 1967; Sims, 1967; Van Duuren et al., 1967; Flesher et al., 1976), however, and it was eventually shown that they were not the major metabolites involved in the binding of aromatic hydrocarbons to nucleic acids in vivo (Baird et al., 1973, 1976).

Baird et al. (1975) found that DNA-bound products of benzo[a]pyrene metabolism did not arise via the K-region epoxide, either in mouse embryo cell cultures that had been treated with [^{3}H]-labeled benzo[a]-pyrene or when the hydrocarbon was metabolized by rat liver microsomal fractions in the presence of DNA (King et al., 1975). Blackburn et al. (1974) arrived at the same conclusion from studies of the extent of loss of tritium from labeled benzo[a]pyrene that became bound to the DNA of mouse kidney cells.

Thus, despite the ready formation of the K-region epoxide from benzo[a]pyrene in liver-metabolizing systems (Keysell et al., 1973), and the covalent reactions that occur between this epoxide and nucleic acids in neutral solution (Baird et al., 1975), no hydrocarbon-nucleoside products derived from the K-region epoxide have been found when benzo[a]-pyrene is metabolized in the presence of DNA.

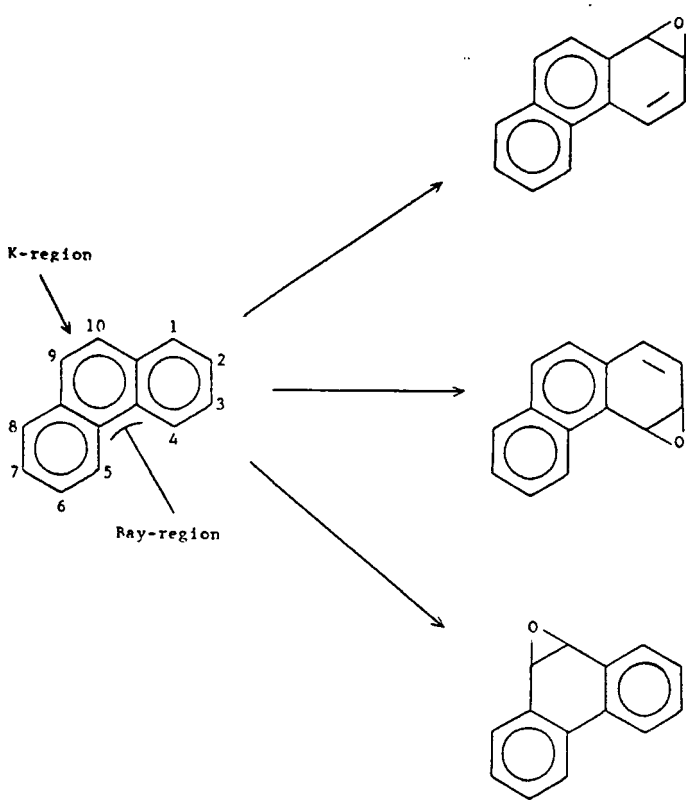
III. NON-K-REGION EPOXIDES AND DIOL-EPOXIDES AS REACTIVE METABOLITES OF POLYCYCLIC AROMATIC HYDROCARBONS

Although K-region epoxides do not appear to be involved in the carcinogenic action of polycyclic aromatic hydrocarbons, the possibility

of the involvement of epoxides of some other sort was not ruled out by the experimental results. Simple non-K-region epoxides did not seem likely candidates as ultimate carcinogens, however, because the hydrocarbon-nucleoside products formed in cells that had been treated with the parent hydrocarbon were more polar than those that were derived from the reaction of a simple epoxide with DNA (Baird et al., 1975, 1973). It was known that non-K-region dihydrodiols, which are indeed more polar than simple epoxides, were formed by rat liver preparations, and that these compounds underwent further metabolic conversion to even more polar products (Booth et al., 1973), possibly via epoxide intermediates. Borgen et al. (1973) found that benzo[a]pyrene-7,8-dihydrodiol was the substrate that was metabolized most extensively in liver microsomes preparations to form DNA-bound products. Sims et al. (1974) provided evidence that benzo[a]pyrene-7,8-diol-9,10-epoxide was responsible for the binding to DNA by showing that an epoxide of benzo[a]pyrene-7,8-dihydrodiol, on reaction with DNA, led to the same profile of nucleoside adducts as was produced when cells in culture were exposed to benzo[a]-pyrene. Both diastereoisomeric diol epoxides are highly mutagenic toward bacterial and mammalian cells (Conney et al., 1977; Wislocki et al., 1976a; Huberman et al., 1976; Newbold and Brookes, 1976; Wood et al., 1976a); they are much more active than the mutagenic K-region arene oxides.

IV. PHENANTHRENE AS SIMPLEST POLYCYCLIC AROMATIC HYDROCARBON WITH DIFFERENT TYPES OF EPOXIDE METABOLITES

The metabolism of phenanthrene in mammalian and bacterial systems has been the subject of several previous reports (Boyland and Wolf, 1950; Boyland and Sims, 1962; Jerina et al., 1976a; Koreeda et al., 1978; Nordqvist et al., 1981). Phenanthrene is of particular interest because of its widespread distribution in the environment and also because it is the simplest member of the polycyclic aromatic hydrocarbon series to have both a K-region and a bay-region (scheme 1). The latter is an essential structural feature in the "Bay-Region Theory" (Conney et al., 1978) which



Scheme 1. Epoxidation of phenanthrene.

has been used to link mutagenic and carcinogenic activity with the structure of polycyclic aromatic hydrocarbon metabolites. Phenanthrene is considered to have no carcinogenic activity (Dipple, 1976), although the diastereoisomeric bay-region diol-epoxides exhibited dose-dependent mutagenic activity in strains TA-98 and TA-100 of Salmonella typhimurium. (±)-1β,2α-Dihydroxy-3α,4α-epoxy-1,2,3,4-tetrahydrophenanthrene was more mutagenic in strain TA-100 and in Chinese hamster V79 cells than was (±)-1β,2α-dihydroxy-3β,4β-epoxy-1,2,3,4-tetrahydrophenanthrene. Both diol-epoxides had low but essentially equivalent activity in strain TA-98. The bay-region 3,4-epoxy-1,2,3,4-tetrahydrophenanthrene was from seven to sixty times more mutagenic than was (±)-1β,2α-dihydroxy-3α,4α-epoxy-1,2,3,4-tetrahydrophenanthrene in bacterial and mammalian cells and was from eight to seventeen times more mutagenic than was 1,2-epoxy-1,2,3,4-tetrahydrophenanthrene, the non-bay-region tetrahydroepoxide of phenanthrene (Wood et al., 1979). These mutagenic dihydrodiol epoxides have not been found as urinary metabolites.

As a result of an early investigation into the metabolism of phenanthrene by the rat and the rabbit (Boyland and Sims, 1965), it was suggested that epoxidation was the initial step in the metabolism of phenanthrene (scheme 1). They demonstrated that 9,10-epoxy-9,10-dihydrophenanthrene was converted by the rat and the rabbit into trans-9,10-dihydroxy-9,10-dihydrophenanthrene and its glucuronide and/or sulfate conjugates, and into N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine. Subsequent studies have shown that epoxide hydrolase

(EC 3.3.2.3) converts phenanthrene 9,10-oxide to 9,10-dihydroxy-9,10-dihydrophenanthrene, and that glutathione transferase (EC 2.5.1.18) converts the epoxide to S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-glutathione (Boyland and Sims, 1962).

Epoxidation at the 1,2- and 3,4-positions of phenanthrene is also known to occur. Metabolism *via* the hydrolase converts the 1,2- and 3,4-epoxides to *trans*-1,2-dihydroxy-1,2-dihydro- and *trans*-3,4-dihydroxy-3,4-dihydrophenanthrene, respectively (Boyland and Sim, 1962). Inasmuch as the 1,2-dihydrodiol has a bay-region double bond, it may be metabolized further to a dihydrodiol epoxide. In this dissertation study, several triols and tetrols were identified as rat and guinea pig urinary metabolites of phenanthrene suggesting that mutagenic dihydrodiol epoxides might have been formed *in vivo*. The related 9-, 1-, 2-, 3- and 4-hydroxyphenanthrenes and their sulfate conjugates have been found as urinary metabolites of the rat and the rabbit after dosage with phenanthrene (Boyland and Sims, 1962). 1,2- and 9,10-Dihydroxyphenanthrenes were also identified as urinary metabolites.

V. REMOVAL OF EPOXIDES FROM BIOLOGICAL SYSTEMS

(A). By Epoxide Hydrolase

The formation of arene oxides of many drugs, carcinogens and other environmental chemicals are made possible by a remarkable microsomal electron transport system whose terminal oxidase is cytochrome P_{450}. The interaction of molecular oxygen and NADPH-supplied electrons with the

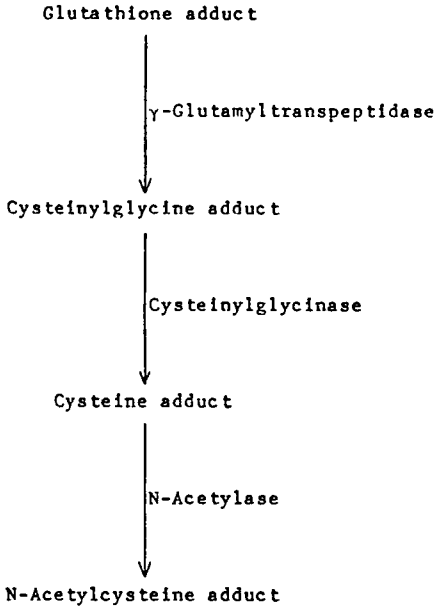
iron of this cytochrome results in the formation of a highly active oxygen species which can oxidize exogenous substrates at otherwise poorly reactive sites. In the case of benzo[a]pyrene, the reactive electrophiles thus formed can undergo a number of further metabolic transformations, some of which serve to deactivate and eliminate the active intermediate, others of which may potentiate its toxicity. Arene oxides can react with water to form a dihydrodiol. This hydration is catalyzed by epoxide hydrolase (Oesch, 1979), and is normally considered a step of detoxification. In addition to its role in the metabolism and excretion of nonpolar, xenobiotic substrates (Daly et al., 1972), epoxide hydrolase may also protect against the adverse effects of certain drug metabolites. Arene oxides are, for example, potent mutagens toward bacterial and mammalian cells (Ames et al., 1972; Huberman et al., 1971; Wood et al., 1975), have been implicated as hepatotoxic metabolites in vivo (Brodie et al., 1971), transform cells in culture (Grover et al., 1971b) and are carcinogens in vivo (Jerina and Daly, 1974; Sims and Grover, 1974; Levin et al., 1976). In the absence of further metabolic activation, the dihydrodiols produced from these arene oxides by epoxide hydrolase are either weak or inactive as mutagens (Jerina and Daly, 1974; Sims and Grover, 1974; Wood et al., 1976b; Wislocki et al., 1976b; Conney et al., 1977; Jerina et al., 1976b) and cell-transforming agents (Grover et al., 1971b). In the specific case, however, when the dihydrodiol thus formed is on an angular benzene ring, as is the case for benzo[a]pyrene 7,8-diol, a further activation can take place. A second oxygenation by

microsomal monooxygenases produces a so-called "vicinal diol-epoxide". These diol epoxides were first recognized in the mid-1970s to be the single most potent carcinogenic metabolites of benzo[a]pyrene. These diol epoxides are apparently poor substrates for epoxide hydrolase, and their deactivation *via* hydrolysis to tetrols is primarily non-enzymatic. The formation of inactive glutathione conjugates of these diol epoxides has been proposed, but not directly demonstrated.

Arene oxides can undergo spontaneous isomerization to form phenols. These phenols are substrates for UDP-glucuronyl- and sulfo-transferases and the highly polar glucuronides and sulfates formed are inactive and readily excreted.

(B). By Glutathione S-Transferase

Glutathione (GSH) conjugation is an important route of metabolism for a variety of xenobiotics (Chasseaud, 1976 and references cited therein), including many drugs, insecticides and herbicides; these conjugates are metabolized to the corresponding mercapturic acids, which are conjugates of N-acetylcysteine. The initial step in mercapturic acid biosynthesis involves conjugation of electrophilic metabolites with the endogenous nucleophile GSH (Bray et al., 1959a; Barnes et al., 1959; Boyland and Chasseaud, 1969a; Hutson, 1970; Wood, 1970). This reaction is catalyzed by glutathione S-transferases (Chasseaud, 1973, 1974), which appear to function as cellular protection enzymes (Chasseaud, 1973,1974; Boyland and Chasseaud, 1968, 1969b). The GSH conjugates are further metabolized in steps (scheme 2) to N-acetylcysteine conjugates by



Scheme 2. Mercapturic acid biosynthesis.

(a) removal of the γ-glutamyl moiety by γ-glutamyl transpeptidase,
(b) removal of the glycine moiety by cysteinylglycinase, and
(c) N-acetylation of the cysteine conjugate by N-acetylase. In contrast to other major conjugation processes, such as sulfation or glucuronidation, GSH conjugation does not require the initial formation of high energy intermediates involving ATP, although the synthesis of GSH from its component amino acids (Jocelyn, 1972; Binkley et al., 1959) and N-acetylation of the cysteine conjugate do utilize ATP.

The first reports of the urinary excretion of mercapturic acids were published over a century ago (Baumann and Preusse, 1879; Jaffe,

1879), but the mechanism for their formation was not conclusively established until much later when it was shown that GSH was the major, and possibly the only, source of the cysteine moeity present in mercapturic acids. The supporting evidence for this concept has been thoroughly reviewed (Barnes et al., 1959; Boyland and Chasseaud, 1969a; Wood, 1970) and more recent work continues to confirm the biosynthetic route proposed by Bray et al. (1959a) and Barnes et al. (1959).

Recent studies (Mio, 1979; Tateishi et al., 1978; Stillwell et al., 1980) have shown that GSH adducts may be metabolized to a series of sulfur-containing acids, and to mercaptoethylamine derivatives, in addition to mercapturic acids. Mio has proposed that mercaptoethylamine and mercaptoacetic acid adducts are formed from GSH adducts via cysteine adducts.

In earlier studies of sulfur-containing metabolites of phenanthrene, Boyland and Sims (1962, 1965) found that rats and rabbits metabolized phenanthrene to a number of hydroxydihydromercapturic acids. A metabolite which appeared to be a glycine conjugate was also detected in urine but was not fully identified. Species differences in the metabolism of phenanthrene were observed in 1959-60 (Bray et al., 1959b; Bray and James, 1960), and it was concluded that the guinea pig did not excrete mercapturic acids. In recent reports, small amounts of N-acetylcysteine adducts (mercapturic acids) were found as urinary metabolites in the guinea pig following the administration of naphthalene (Stillwell et al., 1982), phenanthrene (Lertratanangkoon et al., 1982a) or styrene

oxide (Nakatsu et al., 1983). These effects are believed to be due solely to species differences in acetylation and/or deacetylation of cysteine adducts. If GSH transferase reactions occur in the guinea pig in the same fashion as in other animals, L-cysteine adducts of arene oxides should be excreted directly or should be converted into metabolites of the cysteine adducts of, for example, mercaptopyruvic, mercaptolactic and mercaptoacetic acids.

In addition to sulfur-containing acidic metabolites, methylthio metabolites have been reported. This class of metabolites has been found predominantly in cases where glutathione conjugation is important in the metabolism of foreign compounds. The introduction of a methylthio group into an aromatic compound was first described by the Millers and their associates (Scribner et al., 1965; Miller, 1970). More recently, methylthio metabolites have been reported for several other foreign compounds including a series of methylthio metabolites of styrene oxide (Nakatsu et al., 1983), phenanthrene (Lertratanangkoon et al., 1982a), naphthalene (Stillwell et al., 1978a,b), tetrachlorobiphenyl (Mio et al., 1976), halobenzenes (Kitamura et al., 1977, 1978; Mizutani et al., 1978; Lertratanangkoon et al., 1982b) and biphenyl (Halpaap et al., 1978) and also for a number of drugs including phenacetin (Focella et al., 1972), 2-acetamido-4-chloromethylthiazole (Chatfield and Hunter, 1973), caffeine (Kamei et al., 1975), propanolol (Walle, 1977), bromazepam (Tateishi and Shimizu, 1976), 1-allyl-3,5-diethyl-6-chlorouracil (Kaul et al., 1976), 3-[5-nitro-2-furyl-2-(2-furyl)-acrylamide] (Ou et al., 1977), clozapine

(Stock and Spiteller, 1977), mecloqualone (Stillwell, 1977), acetaminophen (Klutch et al., 1978) and carbamazepine (Lertratanangkoon and Horning, 1982).

Studies of thioether metabolites of aromatic compounds are complicated by the fact that metabolites of the dihydrohydroxy series can often be readily converted to aromatic compounds by dehydration. It was first recognized by Boyland and Sims in 1958 that the aromatic N-acetylcysteine derivative of naphthalene, isolated in earlier work, was formed as a product of the dehydration of a hydroxydihydro N-acetylcysteine metabolite. In a later study, Jeffrey and Jerina (1975) showed that rearrangement of the sulfur group can accompany the dehydration of 1-hydroxy-2-S-glutathionyl-1,2-dihydronaphthalene and related compounds. In our laboratory we have found that very careful isolation, derivatization and chromatographic procedures must be used in order to detect non-aromatic metabolites and to prevent their dehydration to aromatic analogs. Under these conditions, the identification of methylthio metabolites is often a valuable tool in assessing the structure of reactive intermediates in metabolic pathways.

The origin of the methylthio group is uncertain, but several pathways have been proposed for the formation of these compounds. The attachment of a methylthio group, derived from methionine or N-acetylmethionine, was proposed by the Millers and their associates. In their studies, methylthio metabolites were isolated as alkaline degradation products of liver proteins from rats administered 2-acetylaminofluorene

and structurally related compounds. Administration of radiolabeled methionine (^{3}H or ^{35}S) confirmed that the methylthio group was derived from methionine.

A second mechanism has been proposed in which the precursors of the methylthio-containing metabolites are the corresponding cysteine or N-acetylcysteine conjugates (Chatfield and Hunter, 1973; Bakke et al., 1976; Tateishi et al., 1978). It was shown in animal studies that administration of the cysteine or N-acetylcysteine conjugates of selected compounds leads to the excretion of methylthio-containing metabolites in urine (Bakke et al., 1976; Chatfield and Hunter, 1973). Isolation and characterization of a cleavage enzyme designated "cysteine conjugate β-lyase" from rat liver has recently been accomplished by Tateishi and co-workers (Tateishi et al., 1978). This enzyme cleaved cysteine conjugates of the aromatic compounds 2,4-dinitrobenzene and bromobenzene, but S-alkyl derivatives of cysteine did not serve as substrates, nor did the GSH conjugate of 2,4-dinitrobenzene. These authors proposed a pathway which involves cleavage of the thioether bond in cysteine conjugates of aromatic compounds to yield the thiols, which are methylated by S-adenosylmethionine to form methylthio ethers.

A similar pathway has been proposed by Mio and co-workers in which the methylthio metabolites found as urinary products of halogenated aromatic compounds are also thought to be derived from the corresponding GSH conjugates. The GSH moiety is proposed to be successively converted in the liver and kidney to a series of intermediates (cysteinylglycine,

cysteine, mercaptopyruvate, mercaptolactate and mercaptoacetate conjugates) and also to the thiol metabolites which are transmethylated by S-adenosylmethionine (Kitamura et al., 1978). Mio and co-workers have isolated various metabolites, including methylthio derivatives, from the urine of animals treated with 1,3,5-trichlorobenzene, 1,3-dichlorobenzene and bromobenzene (Kitamura et al., 1978).

Another mechanism has been proposed in which the GSH conjugation products excreted in the bile of experimental animals are cleaved in the gastrointestinal tract. This cleavage process is thought to be catalyzed by a C-S lyase enzyme of the intestinal microorganisms or intestinal tissues (Bakke et al., 1980).

In a recent report of bivalent sulfur metabolites of phenanthrene (Lertratanangkoon et al., 1982a), a sulfonium ion pathway was proposed for the formation of 9-hydroxy-10-methylthio-9,10-dihydro- and 9-hydroxyphenanthrene by the rat and the guinea pig.

VI. OBJECTIVE OF DISSERTATION

This research was carried out in order to identify metabolites of phenanthrene in the uninduced rat and guinea pig, and to study pathways both for the formation of epoxide metabolites of phenanthrene and for their removal from the animal. Earlier whole animal studies were carried out without the aid of modern analytical techniques, and were largely concerned with products derived only from phenanthrene 9,10-oxide. Recent studies of phenanthrene metabolism have involved the microsomal

oxidation of intermediates, but have not included whole animal work. There are several specific problems relating to arene oxide formation, and to arene oxide metabolism, that are not yet fully resolved, and that are best studied through whole animal experimental work. The specific objectives were as follows.

(1). To determine if all metabolites of phenanthrene can be accounted for in terms of epoxidation at one of three positions (1,2-, 3,4- or 9,10- in scheme 1), followed by additional metabolic transformations, or if one or more metabolites are apparently formed through "direct hydroxylation" step.

(2). To determine if metabolism by way of K-region (9,10-) epoxidation of phenanthrene occurs to a much greater extent than non-K-region (1,2- or 3,4-) epoxidation, or if both routes are followed to approximately the same extent.

(3). To look for evidence, in whole animal studies with uninduced animals, of the formation of dihydrodiol epoxides and/or diepoxides as metabolic intermediates.

(4). To compare urinary bivalent sulfur acids, derived from phenanthrene and excreted by the rat and guinea pig, in relation to the report that guinea pigs do not excrete mercapturic acids.

(5). To compare methylthio metabolites excreted by the rat and guinea pig, and to examine possible pathways of formation of these sulfur-containing metabolites of arene oxides.

CHAPTER II

MATERIALS

MATERIALS

I. CHEMICALS

Chemicals and reference compounds were obtained from the following sources: phenanthrene, 9-phenanthrol, 9-chlorophenanthrene, 9,10-phenanthrenequinone, 2- and 3-acetylphenanthrenes, m-chloroperoxybenzoic acid, diethylaniline and Diazald (Aldrich Chemical Co., Milwaukee, Wisconsin); pyridine and ammonium carbonate (Mallinckrodt, Inc., St. Louis, Missouri); glass-distilled methanol, ethyl acetate and tetrahydrofuran (Burdick & Jackson Labs., Muskegon, Michigan); sodium acetate and HPLC-grade acetic acid (J. T. Baker Chemical Co., Philipsburg, New Jersey); concentrated sulfuric acid and magnesium sulfate (Fisher Scientific Co., Fair Lawn, New Jersey); bis(trimethylsilyl)acetamide (BSA) (Pierce Chemical Co., Rockford, Illinois); bis(trimethyl-d_9-silyl)acetamide (MSD Isotopes, Rahway, New Jersey); Glusulase (Endo Labs., Garden City, New York); Aquasol (New England Nuclear Corp., Boston, Massachusetts); thioglycolic acid (mercaptoacetic acid; 70% aqueous solution), reduced glutathione, L-cysteine hydrochloride and N-acetyl-L-cysteine (Sigma Chemical Co., St. Louis, Missouri); methanethiol (Eastman Organic Chemicals, Rochester, New York); methoxylamine hydrochloride (Supelco, Inc., Bellefonte, Pennsylvania); trideuteromethoxylamine hydrochloride (Regis Chemical Co., Morton Grove, Illinois); Gas Chrom Q, 3% OV-17 (80-100 mesh) and 1% SE-30 (100-120 mesh) column packings (Applied Science Div., State College, Pennsylvania); SE-30 liquid phase (General

Electric, Waterford, New York). A sample of trans-1,2-dihydroxy-1,2-dihydrophenanthrene was generously provided by Dr. Franz Oesch. The 1- and 4-keto-1,2,3,4-tetrahydrophenanthrenes were gifts from Dr. G. W. Griffin.

Unless stated otherwise, all reagents and materials were purchased from suppliers offering the highest commercially available grades.

II. RADIOLABELED COMPOUND

[9-^{14}C]Phenanthrene, specific activity 19.3 mCi/mmole, was purchased from the Radiochemical Centre, Amersham, United Kingdom.

III. ANIMALS

A. Rats

Adult male Sprague Dawley Rats were obtained from Timco Breeding Laboratories, Houston, Texas.

B. Guinea pigs

Young adult male Hartley guinea pigs were obtained from Camm Research Institute, Wayne, New Jersey.

CHAPTER III

METHODS

METHODS

I. SYNTHETIC METHODS

A. Oxygen Series

1. (±)-Phenanthrene 9,10-oxide

This epoxide was synthesized from phenanthrene and m-chloroperoxybenzoic acid by the procedure of Ishikawa et al. (1977).

2. cis- and trans-9,10-Dihydroxy-9,10-dihydrophenanthrene

Ten milligrams of phenanthrene 9,10-oxide were dissolved in 2 ml of tetrahydrofuran and 2 ml of distilled water. Twenty microliters of glacial acetic acid were added and the solution was allowed to stand at room temperature for 3 hr. The products were extracted twice with 5-ml portions of ethyl acetate. The combined extracts were washed with 0.5 ml of sodium bicarbonate solution (8%) and then with 0.5 ml of distilled water. The solvent was evaporated under a nitrogen stream and the residue was dissolved in 200 µl of methanol for purification by HPLC. The HPLC analyses were carried out by 10-min linear gradient elution on a semipreparative C_{18}-µBondapak column (Waters Assoc., Milford, Massachusetts). The solvent system consisted of solvent A: methanol/water/acetic acid (20:80:0.1, v/v) and solvent B: methanol/water/acetic acid (80:20:0.1, v/v). The percentage of B varied from 10 to 90 with a flow rate of 1.2 ml/min. Two major products which were not completely separated were eluted from the HPLC column at retention times of 22 and 23 minutes. The products were pooled and the solvent was removed (Rotovap).

The residue was dissolved in methanol and an aliquot (1/10) was analyzed by GC and GC/MS. The GC and GC/MS properties (TMS derivative) of the synthetic products and the metabolites isolated from rat and guinea pig urine were identical. The major product (MU=20.23) was the trans(±)-9,10-dihydroxy-9,10-dihydrophenanthrene and the minor product (MU=20.17) was the cis(±)-9,10-dihydroxy-9,10-dihydrophenanthrene (Lertratanangkoon et al., 1982a).

3. 9,10-Dihydroxyphenanthrene

9,10-Dihydroxyphenanthrene (TMS derivative) was synthesized by silylation of the corresponding 9,10-phenanthrenequinone. One milligram of 9,10-phenanthrenequinone was dissolved in 50 µl of pyridine and silylated with 50 µl BSA. After heating at 60°C for 1 hr, an aliquot was analyzed by GC and GC/MS. Two components were observed on a capillary SE-30 column. The GC and GC/MS properties of the minor component (MU=22.09) and a metabolite isolated from rat and guinea pig urine were identical. The metabolite was identified as 9,10-dihydroxyphenanthrene. The major component was identified as unchanged 9,10-phenanthrenequinone.

4. Dimethoxime Derivative of 9,10-Phenanthrenequinone

One milligram of 9,10-phenanthrenequinone was dissolved in 100 µl of a pyridine solution of methoxylamine hydrochloride (50 mg/ml). After heating at 60°C for 1.5 hr, the pyridine was evaporated to dryness under a nitrogen stream. The residue was treated with 0.5 ml of isooctane, and the isooctane solution which contained the products was

evaporated to dryness (nitrogen stream) and the residue was dissolved in 30 µl of pyridine, and 50 µl of BSA was added. The reaction mixture was heated at 60°C for 1 hr and an aliquot was analyzed by GC and GC/MS. Two major products were characterized as dimethoxime derivatives by GC and GC/MS. The mass spectra (MO derivatives) of both products were identical. The GC and GC/MS of one of the products (MU=22.10) and the metabolite isolated from rat and guinea pig urine were identical. The synthetic products were the dimethoxime derivatives of 9,10-phenanthrene-quinone.

5. 1- and 4-Hydroxyphenanthrenes

The 1- and 4-phenanthrols were synthesized from the corresponding 1- and 4-keto-1,2,3,4-tetrahydrophenanthrenes by bromination with bromine in ether, and followed by treatment with diethylaniline according to the procedure described by Mosettig and Burger (1935).

6. 2-Hydroxyphenanthrene

2-Phenanthrol was synthesized by Baeyer-Villiger oxidation of the readily accessible 2-acetylphenanthrene. A two-fold excess of m-chloroperoxybenzoic acid (3.8 g) was added to a solution of 1.6 g of 2-acetylphenanthrene in 20 ml of chloroform. The reaction mixture was stirred at room temperature in the dark for 24 hr and the precipitate formed in the reaction was separated. The chloroform solution was washed with 8% aqueous sodium carbonate solution and water, and finally dried over anhydrous sodium sulfate. After filtration, the solvent was removed under reduced pressure and the residue of crude acetate was dissolved in

10 ml of methanol and hydrolyzed with 2 ml of 6N sodium hydroxide. The reaction mixture was diluted with an equal volume of water and acidified to give the phenolic product which was subsequently recrystallized from ethanol (0.9 g; yield 50%). The GC and GC/MS properties of the TMS derivatives of the synthetic 2-phenanthrol and the metabolite (MU=21.23) isolated from rat and guinea pig urine were identical.

7. 3-Hydroxyphenanthrene

This phenanthrol was synthesized from 3-acetylphenanthrene by the procedure used to synthesized 2-phenanthrol. The GC and GC/MS properties of the TMS derivatives of 3-phenanthrol and a metabolite (MU=20.88) isolated from rat and guinea pig urine were identical.

B. Sulfur Series

1. Sulfonium Adducts from Phenanthrene 9,10-oxide and N-Acetyl-L-methionine

Ten milligrams (51.5 μM) of phenanthrene 9,10-oxide was added to a solution of N-acetyl-L-methionine (49 mg; 258 μM) in 1 ml of acetone. After heating at 37°C for 2 hr, the solvent was evaporated to dryness under a nitrogen stream. The residue was dissolved in 0.2 ml of methanol for purification by HPLC. The HPLC analyses were carried out by a 40 min gradient elution on an analytical C_{18}-μBondpak column. The same solvent system as those described in part A-2 of the synthetic methods (p. 23) was used. Four major products were observed on an HPLC separation (Fig. 1). Individual fractions were collected and the solutions were lyophilized. Each residue was dissolved in 0.1 ml of methanol for

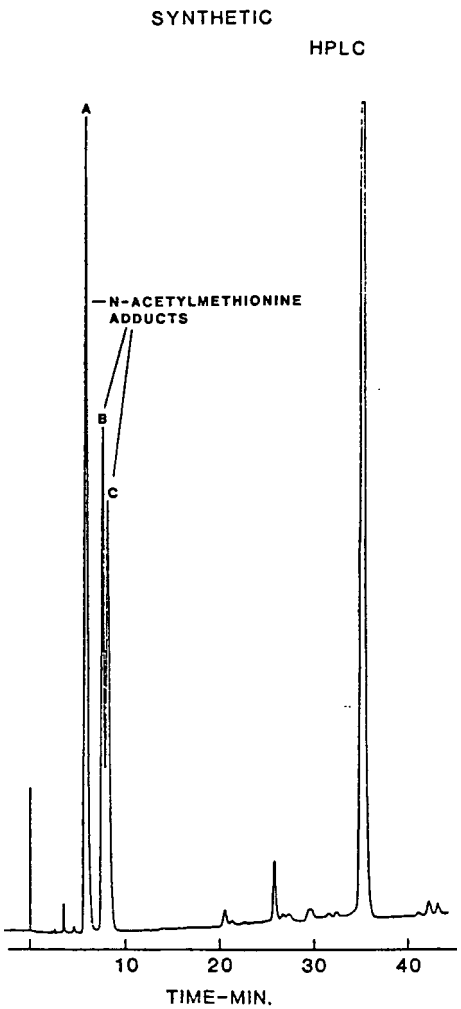


Figure 1. HPLC separation of synthetic N-acetylmethionine adducts from phenanthrene 9,10-oxide and N-acetylmethionine.

analyses by FAB/MS or GC/MS. The FAB mass spectra of the first three fractions (A-C, Fig. 1) with the retention times of 5.5, 7 and 7.5 minutes were almost identical (Fig. 2). Molecular ions were observed at m/z 386 which corresponded to the addition of a proton to the zwitterionic structure of the sulfonium compounds resulting from the reaction of phenanthrene 9,10-oxide and N-acetyl-L-methionine. A FAB mass spectrum

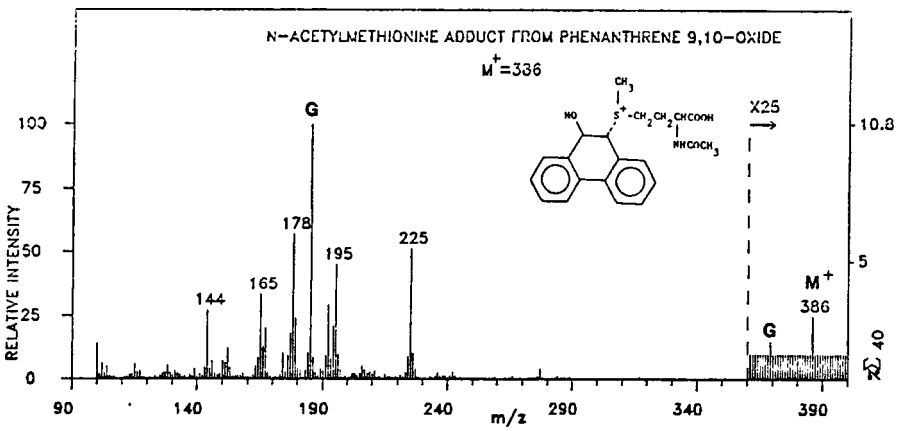


Figure 2. FAB mass spectrum of synthetic N-acetylmethionine adduct from phenanthrene 9,10-oxide and N-acetylmethionine.

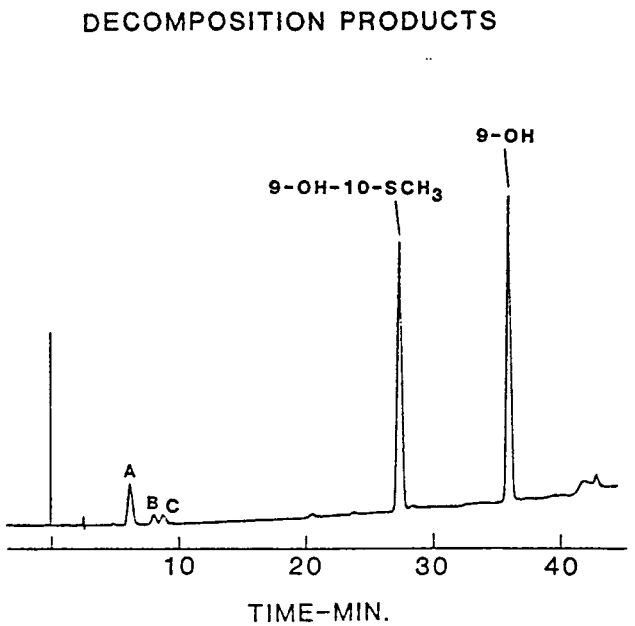


Figure 2A. HPLC separation of the decomposition products of N-acetylmethionine adducts from phenanthrene 9,10-oxide and N-acetylmethionine.

of N-acetyl-L-methionine showed an MH^+ ion at m/z 192 and fragment ion at

m/z 144 (MH^+). These ions were present in all FAB mass spectra of the sulfonium compounds (Sheng et al., 1984).

The fourth major peak (Fig. 1) was identified as a mixture of unchanged phenanthrene 9,10-oxide and 9-hydroxyphenanthrene.

Studies of the decomposition of sulfonium compounds from phenanthrene 9,10-oxide and N-acetylmethionine showed that a major route of spontaneous decomposition in methanol or acetone solution is to the 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene and 9-hydroxyphenanthrene (Fig. 2A).

2. trans-S-(9-Hydroxy-9,10-dihydro-10-phenanthryl)glutathiones

Phenanthrene 9,10-oxide (9.7 mg; 0.05 μM) was dissolved in 0.6 ml of ethanol/tetrahydrofuran (2/1) solution and one equivalent of reduced glutathione (15.5 mg; 0.05 μM) in 0.2 ml of 0.75N sodium hydroxide was added. The reaction mixture was heated at 60°C for 3 hr and the volume was then reduced to approximately 0.2 ml under reduced pressure. The final volume was diluted to 1 ml with sodium bicarbonate solution (8%) and extracted twice with 7-ml portions of ethyl acetate. After separation of the organic phase, the remaining aqueous layer was acidified with 12N sulfuric acid to pH 3.5. The acidified aqueous phase was then transferred to an Amberlite XAD-2 column (1.5 cm x 20 cm). The column was washed with 60 ml of glass-distilled water and the products were eluted with 60 ml of methanol. The methanol was concentrated under reduced pressure (Rotovap) to approximate 1 ml for further purification by HPLC. The HPLC analyses were carried out by a 40-min linear gradient

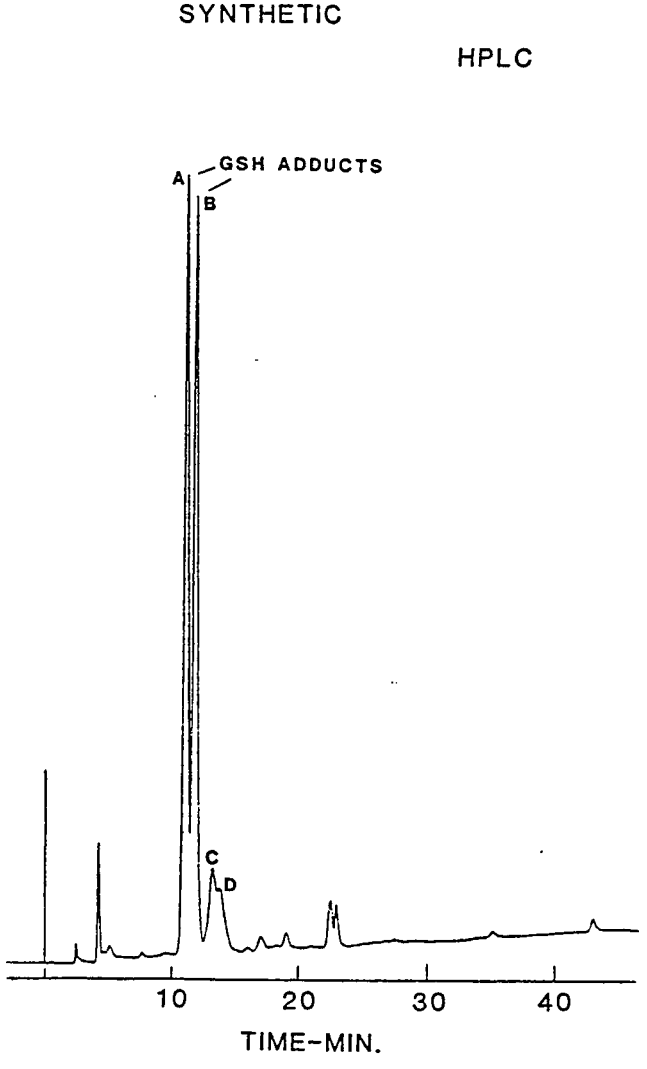


Figure 3. HPLC separation of synthetic trans-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)glutathiones.

elution on an analytical C_{18}-μBondapak column (Waters Assoc., Milford, Massachusetts). The same solvent system as those described in part A-2 of the synthetic methods (p. 23) was used. Two major products (A and B, Fig. 3) which were not completely separated were eluted from the HPLC column at retention times of 12 and 13 minutes. Individual products were

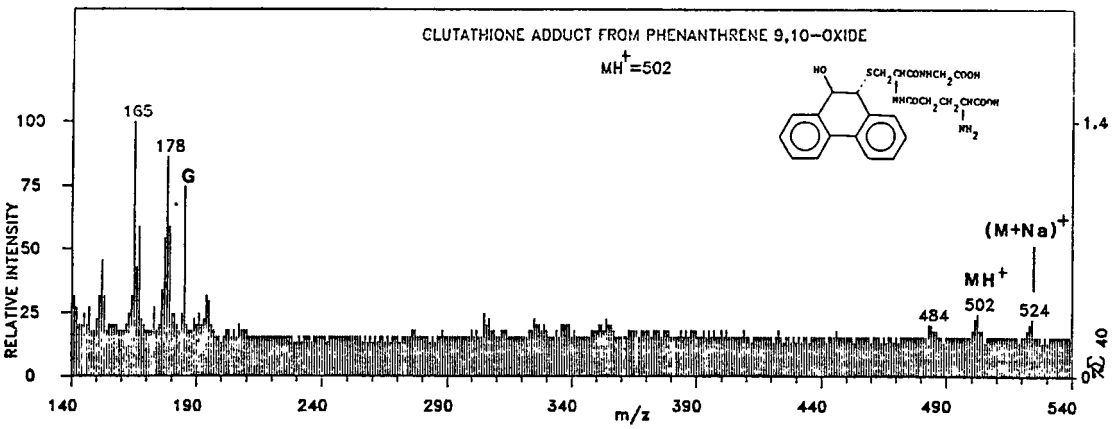


Figure 3A. FAB mass spectrum of trans-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)glutathione.

collected and pooled, and the solvent was taken to dryness under reduced pressure (Rotovap) followed by lyophilization. Each product was dissolved in 0.4 ml of methanol and an aliquot was analyzed by FAB/MS. In the mass spectra from both products (Fig. 3A), molecular ions were observed at m/z 502 (MH^+). This ion corresponded to the addition of the glutathione moiety to the hydroxydihydrophenanthrene structure. An $[M+Na]^+$ ion was also observed at m/z 524. A radical ion corresponding to phenanthrene was present at m/z 178 ($M^{+\cdot}$) and an MH^+ ion for glutathione was at m/z 308. The two major products are the expected diastereoisomers formed by trans ring opening. The HPLC and FAB/MS properties of the synthetic glutathione adducts formed from phenanthrene 9,10-oxide were identical with those of two metabolites isolated from the bile of rats treated with phenanthrene. The biliary metabolites have been assigned the trans configuration. Therefore the synthetic products are the diastereoisomeric trans-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)gluta-

thiones. Two additional pairs of products present in the synthetic sample were not identified. It is possible that peaks C and D (Fig. 3) are diastereoisomers formed by a cis ring opening.

3. trans-S-(9-Hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteines

These compounds were synthesized from phenanthrene 9,10-oxide and L-cysteine hydrochloride by the procedure of Boyland and Sims (1965). L-Cysteine hydrochloride (8.2 mg; 52 μM) and sodium bicarbonate

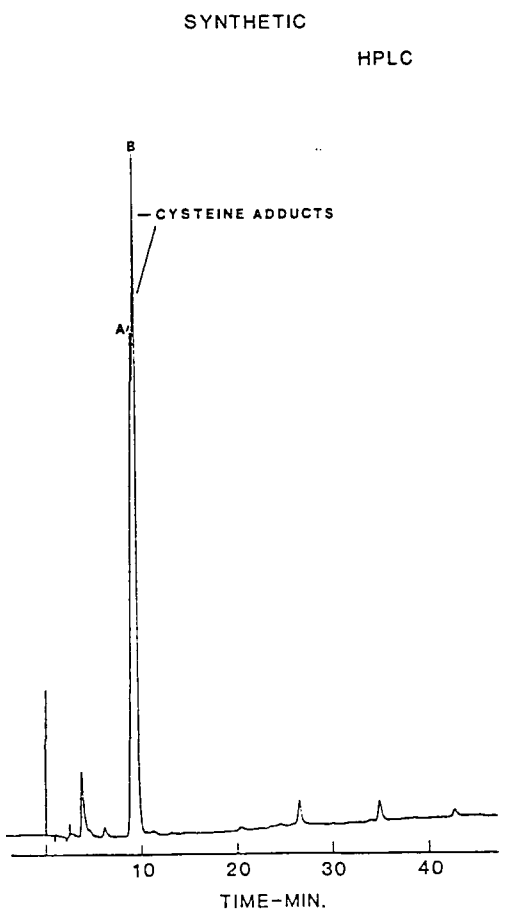


Figure 4. HPLC separation of synthetic trans-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteines.

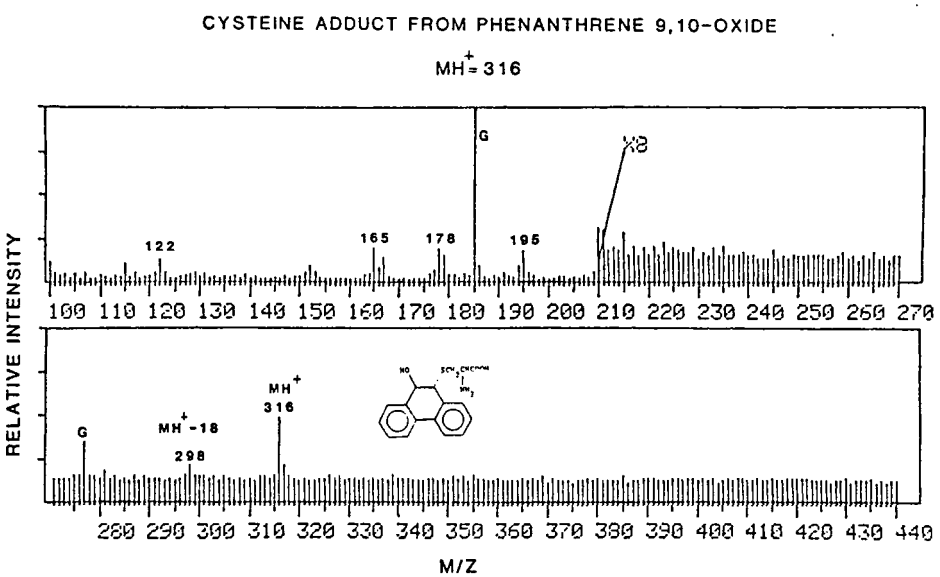


Figure 4A. FAB mass spectrum of trans-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine.

(8.8 mg; 105 μM) were dissolved in 1 ml of glass-distilled water and added to a solution of phenanthrene 9,10-oxide (10 mg; 52 μM) in 1 ml of acetone. The mixture was heated under reflux for 3 hr. Upon termination of the reaction, the acetone was evaporated under a nitrogen stream and the remaining aqueous solution was extracted twice with 5-ml portions of ethyl acetate. The ethyl acetate extracts were separated and the aqueous phase was acidified with 12N sulfuric acid to pH 2. The acidified aqueous phase was transferred to an Amberlite XAD-2 column and the products were isolated as described in part A-2 of the synthetic methods (p. 23). Two major products were eluted from an HPLC (analytical C_{18}-μBondapak) column at 9.5 and 10 minutes (Fig. 4). Each product was collected and pooled and analyzed by FAB/MS. Both mass spectra were

identical (Fig. 4A) with a protonated molecular ion present at m/z 315 (MH^+). An ion corresponding to the cysteine moiety was also present at m/z 122 (MH^+). These synthetic products were the diastereoisomers formed by trans ring opening and have been assigned the structure trans-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine. These products have the same HPLC properties as two metabolites isolated from the bile of rats treated with phenanthrene.

4. N-Acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine

An equimolar mixture (0.15 μM) of N-acetyl-L-cysteine (24 mg) and sodium hydroxide (9 mg) was dissolved in 0.5 ml of glass-distilled water. The mixture was added to a solution of phenanthrene 9,10-oxide (19.4 mg; 0.1 μM) in 0.4 ml of ethanol/tetrahydrofuran (1:1) solution. The reaction mixture was heated at 60°C for 3 hr and the solvent was evaporated under reduced pressure (Rotovap). The residue was dissolved in 1 ml of glass-distilled water and extracted by the ammonium carbonate-ethyl acetate procedure (Horning et al., 1974) to removed unchanged phenanthrene 9,10-oxide and other neutral by-products formed during the reaction. After separation of the organic phase, the aqueous phase was acidified with 12N sulfuric acid to pH 3 and extracted three times with 5-ml portions of ethyl acetate. The ethyl acetate was evaporated under a nitrogen stream and the residue was dissolved in 0.5 ml of methanol. A small aliquot was analyzed by HPLC as described earlier. A single peak was observed with a retention time of 16 min on

an analytical C_{18}-μBondapak column. Inasmuch as the product was quite pure, further purification by HPLC was not necessary, but another aliquot of the methanolic solution was used directly for derivatization. Following 5-min methylation with diazomethane (prepared from Diazald), the solvent was evaporated under a nitrogen stream and the residue was dissolved in 10 μl of pyridine and silylated with 10 μl of BSA. The solution was heated at 60°C for 1 hr, and an aliquot was analyzed by GC and GC/MS. The HPLC, GC and GC/MS properties of the synthetic product and a metabolite isolated from rat and guinea pig urine were identical. The metabolite was assigned the structure trans-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine.

5. trans-S-(9-Hydroxy-9,10-dihydro-10-phenanthryl)mercapto-acetic acid

Twenty-five microliters of mercaptoacetic acid (70%) solution were added to 1 ml of tetrahydrofuran. The solution was dried over 25 mg of magnesium sulfate for 5 min. Eight milligrams of phenanthrene 9,10-oxide was added and the mixture was allowed to stand for 20 hr at room temperature. After centrifugation, an aliquot of the mixture was removed and evaporated (nitrogen stream). The residue was dissolved in 0.5 ml of methanol and treated with diazomethane for 15 min at room temperature. The solution was evaporated under a nitrogen stream and the residue was dissolved in 15 μl of pyridine and silylated with 15 μl of BSA. After heating the solution for 1 hr at 60°C, an aliquot was analyzed by GC and GC/MS. The mass spectra of the major product (MU=23.80) and

the ME-TMS derivative of a metabolite isolated from rat and guinea pig urine were identical. The metabolite was assigned the structure trans-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid.

6. trans-9-Hydroxy-10-methylthio-9,10-dihydrophenanthrene

a. By Methanethiol Procedure

Thirty milliliters of a 2% solution of sodium hydroxide was added to a mixture of 300 mg of phenanthrene 9,10-oxide and 1 g of methanethiol. After stirring the solution for 1 hr under nitrogen. the products were extracted with ether. The extract was dried over anhydrous potassium carbonate and concentrated under reduced pressure. The products were separated by TLC (diethyl ether), and the major band was eluted to yield a colorless viscous oil. After derivatization with BSA, an aliquot was analyzed by GC and GC/MS. The GC/MS analysis indicated that two products with the same MU value (20.64) on an SE-30 capillary column were present. A complete separation of the two products, however, was obtained on a 3% OV-17 column. The major product (approximately 90% by GC analysis) was assigned the structure 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene. NMR data indicated a trans configuration. The GC and GC/MS properties (TMS derivatives) of the synthetic methylthio compound and a metabolite (MU=20.64) isolated from rat and guinea pig urine were identical. The minor product was identified as 9-phenanthrol by comparison with a reference sample.

b. By Sulfonium Adduct Procedure

9-Hydroxy-10-methylthio-9,10-dihydrophenanthrene and

9-hydroxyphenanthrene were found as spontaneous decomposition products of the corresponding synthetic sulfonium compounds which were prepared by the reaction of N-acetyl-L-methionine with phenanthrene 9,10-oxide. These decomposition products were easily separated from the mixture either by extraction from an alkaline solution or by HPLC. The GC and GC/MS properties (TMS derivatives) of the methylthio compound formed by the sulfonium pathway were identical to those of a metabolite (MU=20.64) isolated from rat and guinea pig urine.

II. ADMINISTRATION OF PHENANTHRENE

A. Urinary Studies

1. Rats

Male Sprague-Dawley rats (190 to 220 g) were maintained on a 12-hr light-dark cycle and had free access to food and water. Each rat received a single dose of phenanthrene (50 mg/kg) labeled with [9-^{14}C]-phenanthrene (5 μCi). The compounds were dissolved in 0.5 ml of ethanol/corn oil (1/9) solution and injected ip. The animals were placed in individual metabolism cages with water but no food during the 0-24 hr urine collection; however, rat chow was fed during the 24-48 and 48-72 hr collections. Each urine was diluted with a measured amount of glass-distilled water and stored at -20°C until analyzed.

2. Guinea Pigs

A similar study was carried out with male Hartley guinea pigs (450-700 g) with the same dosage (50 mg/kg) of phenanthrene and

[9-^{14}C]phenanthrene. Because guinea pig urine is alkaline, the urine was acidified with glacial acetic acid to pH 4.5-5.0 before storing at -20°C.

B. Biliary Studies

Male Sprague-Dawley rats (270-300 g) were anesthetized with ether and their common bile ducts were cannulated with PE-10 tubing. The cannula was secured and brought subcutaneously through an opening under the abdomen. The opening was sutured and, before placing the rats in the restraining cages, an ip injection with phenanthrene (50 mg/kg) labeled with 5 μCi of [9-^{14}C]phenanthrene dissolved in 0.5 ml of corn oil/ethanol (1/9) solution was made. Bile samples were collected up to 24 hr and the total volumes were recorded. The samples were stored at -20°C until analyzed.

III. ADMINISTRATION OF METABOLITES OF PHENANTHRENE

A. Oxygenated Metabolites

Phenanthrene 9,10-oxide (60 mg/kg), 1,2-dihydroxy-1,2-dihydrophenanthrene (8 mg/kg) and 9,10-dihydroxy-9,10-dihydrophenanthrene (18 mg/kg) dissolved in 0.5 ml of corn oil were administered individually by ip injection to male Sprague-Dawley rats (220-310 g). 2-, 3- and 9-Hydroxyphenanthrenes (50 mg/kg) dissolved in 0.2 ml of dimethylsulfoxide, were injected individually into rats. Because of the limited quantity of the synthetic 1-hydroxyphenanthrene, only 1.6 mg was administered ip to a male Sprague-Dawley rat (200 g) in 0.2 ml of dimethylsulfoxide. The animals were placed in individual metabolism

cages with water but no food, and 0-24 hr urine samples were collected. The urine samples were stored at -20°C until analyzed.

B. Bivalent Sulfur Metabolites

1. Rat

Synthetic N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (1-2 mg) labeled with [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine was administered to male Sprague-Dawley rats (250 g). Rats were placed in individual metabolism cages with water but no food, and 0-24 and 24-48 hr urine samples were collected. The urine samples were stored at -20°C until analyzed.

2. Guinea Pig

A similar study was carried out with a male Hartley guinea pig (530 g) with the same dosage (1-2 mg) of N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine labeled with [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine.

IV. RECOVERY STUDIES

The extent of the recovery of phenanthrene metabolites from rat and guinea pig urine and bile samples was determined by administration of phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 μCi). Urine samples were collected for 3 days, whereas only 24-hr bile samples were collected from the rat. ^{14}C-Radioassays were carried out with a Tracor Analytic model 6892 liquid scintillation counter. Appropriate aliquots of the whole urine or bile samples and urine or bile extracts were

counted in Aquasol scintillation fluid.

V. ISOLATION OF METABOLITES FROM URINE

A. Unconjugated Neutral Metabolites

The unconjugated neutral metabolites were extracted from urine (usually 1/5 of the 0-24, 24-48 and 48-72 hr urine samples) by the ammonium carbonate-ethyl acetate procedure (Horning et al., 1974). The ethyl acetate solution was evaporated to dryness (nitrogen stream) and the residue was dissolved in 1 ml of methanol and stored at -20°C until analyzed by GC and GC/MS.

B. Total Urinary Neutral Metabolites

Neutral metabolites present in the unconjugated form and as glucuronide and/or sulfate conjugates were isolated from urine after enzymatic hydrolysis. Following the addition of 0.2 g of sodium acetate to an aliquot of urine (usually 1/5 of the 0-24, 24-48 and 48-72 hr urine samples), the pH was adjusted to 4.7 with glacial acetic acid and 0.2 ml of Glusulase was added. After incubation for 17 hr at 37°C, phenanthrene and its neutral metabolites (including the aglycones liberated by the enzymatic hydrolysis) were extracted by the ammonium carbonate-ethyl acetate procedure. The ethyl acetate extracts were evaporated to dryness (nitrogen stream) and the residue was dissolved in 1 ml of methanol. An aliquot (usually 1/2) was derivatized for analysis by GC and GC/MS.

C. Acidic Metabolites (Ethyl Acetate-Soluble)

After extraction of the urine sample by the ammonium carbonate-

ethyl acetate procedure, the aqueous layer was cooled in an ice bath and carefully acidified to pH 2-3 with cold 12N sulfuric acid. The urine sample was saturated with sodium chloride and extracted with ethyl acetate. The extract was washed with 0.5 ml of glass-distilled water and the ethyl acetate was evaporated under a nitrogen stream. The residue was dissolved in 1 ml of methanol and a small aliquot was examined for radioactivity; the remainder was stored at -20°C for further analysis by HPLC and GC/MS.

D. Water-Soluble Metabolites

Approximately 20% of the radioactivity excreted in rat urine remained in the acidified aqueous phase after extraction by ethyl acetate. The aqueous phase which contained the ethyl acetate-insoluble metabolites was lyophilized to dryness. The residue was suspended in methanol (3 x 5 ml) and centrifuged to removed the insoluble materials. The methanolic solutions were combined and concentrated to a small volume under a N_2 stream, and were subsequently used for HPLC analysis. The identification of the metabolites was based on radioactivity measurement and the fractions which contained radioactivity were collected from a semi-preparative HPLC column. Fractions were pooled and the solutions were taken to dryness under reduced pressure. Each residue was dissolved in methanol and an aliquot was analyzed by FAB/MS.

VI. ISOLATION OF METABOLITES FROM BILE

The same methods which were used for the isolation of metabolites

from rat and guinea pig urines were used to isolate neutral, acidic (ethyl acetate soluble) and water-soluble metabolites from rat bile.

VII. QUANTITATION

A. Neutral Metabolites

For quantification of neutral metabolites, a known amount of 9-chlorophenanthrene, the internal standard, was added to the urine sample or to the hydrolysate before extraction. An external standard, eicosane, was added to the extract before derivatization. Because most of the metabolites to be quantified were not available as pure compounds for the determination of response factors, a response factor of 1 with respect to the internal standard was assumed.

B. Acidic Metabolites

Inasmuch as the bivalent sulfur acids can undergo thermal degradation, quantification of the acidic metabolites was based on radioactivity measurement of the fractions collected from an HPLC analytical C_{18}-μBondapak column.

VIII. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

μBondapak-C_{18} reversed phase analytical columns (30 x 0.39 cm) and semipreparative columns (30 x 0.78 cm) from Waters Assoc. (Milford, Massachusetts.) were used. HPLC analyses were carried out by linear gradient elution with a dual solvent-delivery system (Waters model 6000A) and a solvent programmer (Waters model 440) set at 254 nm.

OmniScribe recorders (Houston Instruments, Austin, Texas) were employed. An automatic fraction collector (LKB model 7000) equipped with an electronic control board (Nakatsu, 1981) was used for collecting fractions eluted from the HPLC column.

The acidic metabolites were quantified by HPLC on an analytical C_{18}-μBondapak column. A 40-min linear gradient was used with a column pressure of 1100-1200 psi and a flow rate of 1.2 ml/min. The solvent system consisted of a mixture of solvent A: methanol/water/acetic acid (20:80:0.1, v/v) and solvent B: methanol/water/acetic acid (80:20:0.1, v/v), in which the proportion of B varied from 10-90%. The individual metabolites were collected after an injection of an appropriate volume of the methanolic extract of acidic metabolites, and the amount of each metabolite was determined by radioactivity measurements.

A semipreparative C_{18}-μBondapak column was used when larger quantities of metabolites were isolated for GC/MS analysis. The acidic metabolites extracted from a 24-hr urine sample were dissolved in 100-150 μl of methanol. After centrifugation of the sample, several injections of 30-40 μl each were made onto the HPLC column. The same solvent system was used for the separations; however, the gradient time was reduced to 35 min. The flow rate was 1.2 ml/min with a column pressure of 600-700 psi. The fractions collected from a semipreparative column were examined for radioactivity and only the individual fractions with radioactivity were pooled. The solvent was removed from each pooled sample and, after lyophilization of the aqueous remainder, the residue of each fraction was

transferred with methanol to a calibrated screw-capped centrifuge tube. An aliquot was derivatized for analysis by GC and GC/MS.

IX. PREPARATION OF DERIVATIVES

A. Neutral Metabolites

The neutral metabolites were converted to TMS derivatives. An aliquot (usually half of the final methanolic solution) was evaporated to dryness in a 1-ml Reacti-vial by a nitrogen stream. The dry residue was dissolved in 25 µl of pyridine and silylated with 25 µl of BSA or $\underline{d}_{18}$-BSA for 1 hr at 60°C. An aliquot (1-2 µl) was analyzed by GC and GC/MS.

B. Acidic Metabolites

The acidic metabolites were converted to ME-TMS derivatives by treating an aliquot of the final methanolic solution with diazomethane (prepared from Diazald) for 15 min at room temperature. After removal of the methanol and excess reagents with a nitrogen stream, the dry residue was dissolved in 25 µl of pyridine and silylated with 25 µl of BSA for 1 hr at 60°C. An aliquot was analyzed by GC and GC/MS.

X. GAS CHROMATOGRAPHIC ANALYSES

Gas chromatographic analyses were carried out with a 60-m SE-30 glass open tubular capillary column (German et al., 1973) or with a glass column (3.7 m x 2 mm) packed with 3% OV-17 on 80-100 mesh Gas-Chrom Q. A flame ionization detector was used. The GC separations were temperature programmed and MU values were determined with n-alkanes as reference

compounds.

XI. GAS CHROMATOGRAPHY/MASS SPECTROMETRY

Identifications of neutral and acidic (ethyl acetate-soluble) metabolites were based upon GC/MS analyses with an LKB 9000/PDP-12 system, operated in the electron-impact ionization mode. Glass columns, 3 m x 2 mm, containing 1% SE-30 on 80-100 mesh Gas-Chrom Q, and 1.85 m x 2 mm, containing 3% OV-17 on 80-100 mesh Gas-Chrom Q, were used in the temperature programmed analyses. The ion source temperature was 250°C; the ionizing current was 60 μA; the electron energy was 70eV.

XII. FAST ATOM BOMBARDMENT/MASS SPECTROMETRY

A modified Finnigan 1015 mass spectrometer was employed. A saddle field FAB-11F-GG ion gun (Ion Tech Ltd., Teddington, England) was attached to the housing at the gas chromatographic inlet site, and an open source was constructed. Argon was used as the neutral gas. The probe was stainless steel; the angle of incidence of the argon beam with the probe tip was 60°. The ion gun voltage was 4 kV; the limiting current was 2 mA and the equivalent ion current of the neutral beam was 20 μA. All samples were dissolved in methanol and added to a glycerol matrix on the probe tip. An RDS-Nermag unit (Nermag Co., Reuil-Malmaison, France) was used as a data system.

CHAPTER IV

RESULTS

RESULTS

RAT

I. URINARY METABOLITES OF PHENANTHRENE IN THE RAT

A. Recovery of Radioactive Metabolites from Urine

The extent of recovery of phenanthrene and its metabolites from rat urine was determined by administration of phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 μCi). Of the administered dose, 35 ± 10% was excreted during 0-24 hr; 19 ± 4% in the 24-48 hr period and 8 ± 2% in the 48-72 hr urine sample. The average excretion observed for seven rats in 72 hr was 62 ± 12% of the administered dose. Before enzymatic hydrolysis of the 0-24 hr urine sample; 8 ± 1% of the radioactivity excreted in urine was extractable as neutral metabolites. This accounted

Table 1

Percentage of recovery of radioactive metabolites of phenanthrene from rat urine

	0-24 hr	24-48 hr	48-72 hr	Total
Total Urine % of administered dose	34.6 ± 10.5	19.1 ± 4.1	7.9 ± 2.0	61.6 ± 11.9
Non-Hydrolyzed Urine neutral metabolites*	8.1 ± 1.0	11.3 ± 2.8	11.2 ± 3.0	
Hydrolyzed Urine neutral metabolites*	46.5 ± 5.0	51.2 ± 6.0	49.8 ± 10.5	
acidic metabolites*	32.8 ± 2.5	23.8 ± 2.9	25.4 ± 6.4	

* % of total radioactivity excreted in urine.
Each rat was given unlabeled phenanthrene (50 mg/kg) and [9-^{14}C]phenanthrene (5 μCi; specific activity 19.3 mCi/mmole). Results are expressed as mean values ± standard deviations from 7 rats.

for 2.8% of the administered dose. After enzymatic hydrolysis with Glusulase, 46 ± 5% of the radioactivity excreted in urine was extracted as neutral metabolites by the ammonium carbonate-ethyl acetate procedure. Following acidification of the enzymatic hydrolysate, 33 ± 3% of the radioactivity excreted in urine was recovered as ethyl acetate-soluble acids. Approximately 20% of the radioactivity excreted in urine remained in the acidified aqueous phase after hydrolysis. These data are summarized in table 1.

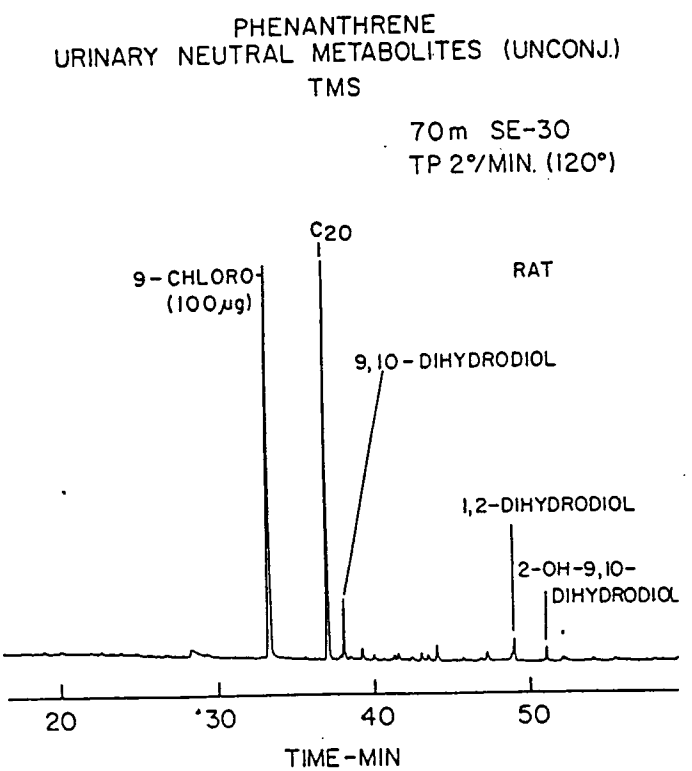


Figure 5. Gas chromatographic separation of the TMS derivatives of unconjugated phenanthrene metabolites isolated from 0-24 hr urine collected from a rat administered phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 uCi).

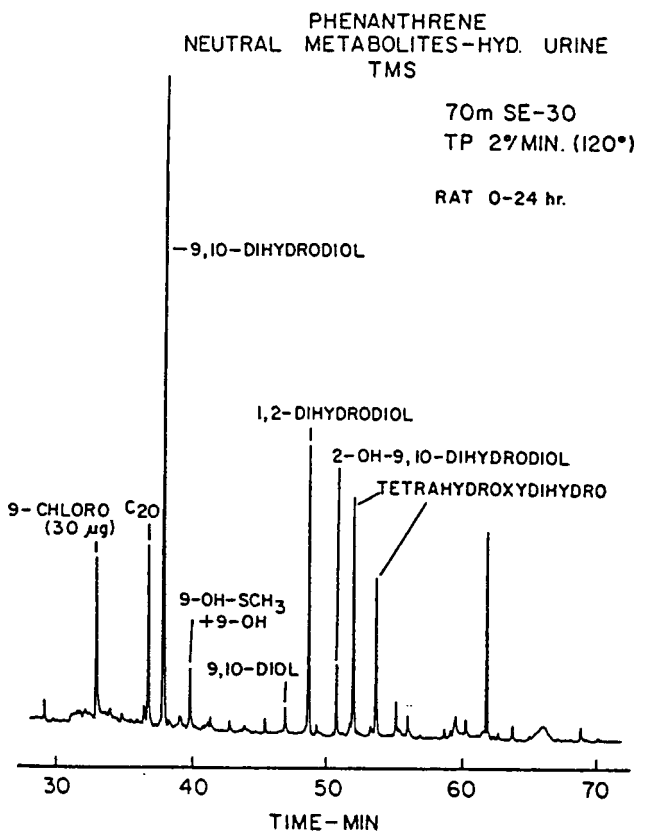


Figure 6. Gas chromatographic separation of the TMS derivatives of phenanthrene metabolites isolated from a hydrolyzed urine collected from a rat administered phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 µCi).

B. Urinary Neutral Metabolites

Phenanthrene metabolites are excreted in rat urine primarily as glucuronide and/or sulfate conjugates. Figures 5 and 6, respectively, show gas chromatographic separations of the TMS derivatives of the urinary metabolites before and after enzymatic hydrolysis. A series of oxygenated- and sulfur containing metabolites was isolated and identified by GC and GC/MS. Due to the very large number of metabolites present,

Table 2

Urinary oxygenated metabolites of phenanthrene in the rat

Compounds*	MU	M^+	$(M-15)^+$	Major ions		
Phenanthrene I	17.33	178(100)	--	176(17)	89(15)	76(14)
4-Phenanthrol II	20.14	266(100)	251(69)	235(54)	117(19)	117.5(17)
9-Phenanthrol III	20.63	266(100)	251(59)	235(20)	117.5(13)	117(11)
3-Phenanthrol IV	20.88	266(100)	251(66)	125.5(22)	165(13)	176(13)
1-Phenanthrol V	20.93	266(100)	251(74)	235(31)	117.5(13)	165(11)
2-Phenanthrol VI	21.23	266(100)	251(85)	125.5(22)	176(15)	165(12)
9,10-Phenanthrene-quinone VII	22.10	266(100)	251(1)	206(37)	235(35)	190(18)
9,10-Dihydroxy-phenanthrene VIII	22.09	354(100)	339(4)	266(30)	236(8)	147(8)
Dihydroxy-phenanthrene IX**	23.58	354(100)	339(6)	266(27)	236(22)	147(6)
3,9-Dihydroxy-phenanthrene X**	23.90	354(100)	339(16)	191(12)	251(6)	147(6)
2,9-Dihydroxy-phenanthrene XI**	24.09	354(100)	339(22)	147(14)	253(6)	266(6)
cis-9,10-Dihydroxy-9,10-dihydrophenanthrene XII	20.17	356(35)	341(27)	147(100)	266(23)	178(13)
trans-9,10-Dihydroxy-9,10-dihydrophenanthrene XIII	20.23	356(29)	341(25)	147(100)	266(24)	178(17)
trans-3,4-Dihydroxy-3,4-dihydrophenanthrene XIV	20.53	356(21)	341(<1)	191(100)	147(29)	253(29)
cis-1,2-Dihydroxy-1,2-dihydrophenanthrene XV	21.87	356(6)	341(<1)	191(100)	147(27)	253(26)
trans-1,2-Dihydroxy-1,2-dihydrophenanthrene XVI	22.49	356(11)	341(2)	191(100)	147(18)	253(15)
Trihydroxy-phenanthrene XVII	24.74	442(100)	427(7)	354(76)	339(25)	147(17)
Trihydroxy-phenanthrene XVIII	24.95	442(100)	427(4)	354(25)	282(22)	147(16)
4,9,10-Trihydroxy-9,10-dihydrophenanthrene XIX	21.77	444(78)	429(37)	147(100)	354(35)	341(33)
x,3,4-Trihydroxy-3,4-dihydrophenanthrene XX	22.34	444(59)	429(4)	191(100)	147(40)	354(23)
3,9,10-Trihydroxy-9,10-dihydrophenanthrene XXI	22.47	444(89)	429(39)	147(100)	354(89)	341(18)
2,9-10-Trihydroxy-9,10-dihydrophenanthrene XXII	22.95	444(100)	429(18)	147(80)	354(41)	253(12)

(Continued on next page)

Table 2 (continued)

Compounds*	MU	M^+	$(M-15)^+$	Major ions		
x,y,9,10-Tetrahydroxy-9,10-dihydrophenanthrene XXIII	23.20	532(100)	517(10)	147(50)	442(31)	443(25)
Tetrahydroxydihydrophenanthrene XXIV**	23.58	532(1)	517(<1)	354(100)	266(27)	236(22)
Tetrahydroxydihydrophenanthrene XXV**	23.90	532(3)	517(1)	354(100)	147(22)	191(21)
Tetrahydroxydihydrophenanthrene XXVI**	24.00	532(3)	517(<1)	354(100)	191(37)	339(22)
Tetrahydroxydihydrophenanthrene XXVII**	24.09	532(61)	517(11)	354(100)	443(89)	147(85)
Tetrahydroxydihydrophenanthrene XXVIII**	24.74	532(8)	517(<1)	442(100)	354(76)	339(25)
Tetrahydroxydihydrophenanthrene XXIX**	24.95	532(20)	517(<1)	442(100)	354(25)	281(22)
Tetrahydroxydihydrophenanthrene XXX**	25.51	532	517			
Tetrahydroxytetrahydrophenanthrene XXXI**	23.90	534	519			
Tetrahydroxytetrahydrophenanthrene XXXII	25.59	534(7)	519(4)	191(100)	147(17)	444(11)
Tetrahydroxytetrahydrophenanthrene XXXIII	25.68	534(5)	519(2)	191(100)	147(19)	267(6)
Pentahydroxydihydrophenanthrene XXXIV**	25.66	620	605			

Relative intensities of the ions are listed in parentheses.

*Trimethylsilyl ether derivatives.

**Mixed spectra.

the oxygenated and sulfur-containing metabolites will be described separately.

1. Oxygenated Metabolites of Phenanthrene in the Rat

Twenty-four oxygenated metabolites not previously reported were identified. The structures of several of the metabolites were confirmed by comparison with synthetic compounds and also by comparison with

metabolites isolated from rat urines after administration of 9,10-epoxy- and 1-, 2-, 3- and 9-hydroxyphenanthrene and 9,10-dihydroxy-9,10-dihydro- and 1,2-dihydroxy-1,2-dihydrophenanthrene. Gas chromatographic and mass spectrometric properties of the urinary oxygenated metabolites are summarized in table 2.

Dihydrodiol Metabolites

The major neutral metabolite in rat urine (Figs. 5 and 6) is the previously reported trans-9,10-dihydroxy-9,10-dihydrophenanthrene (XIII) (Lertratanangkoon et al., 1982a; Boyland and Wolf, 1950; Young, 1947). This metabolite, which was excreted primarily as its glucuronide and/or sulfate conjugate accounted for 5.0 ± 1.6% of the dose administered to the rat. A cis-9,10-dihydroxy-9,10-dihydrophenanthrene (XII) was also present but in trace amounts in the hydrolyzed urine. The mass spectra of the cis- and trans-9,10-dihydrodiols were identical. A molecular ion present at m/z 356 (TMS derivatives) shifted 18 amu to m/z 374 in the mass spectra of the deuterated analog, indicating the presence of two trimethylsilyloxy groups on a dihydrophenanthrene structure. The base peak of these K-region dihydrodiols at m/z 147 (Fig. 7) was assigned structure a

$$(CH_3)_3Si\text{-}O^{+}=Si(CH_3)_2$$

a: m/z 147

This ion shifted to m/z 162 in the deuterated analogs. An intense ion at

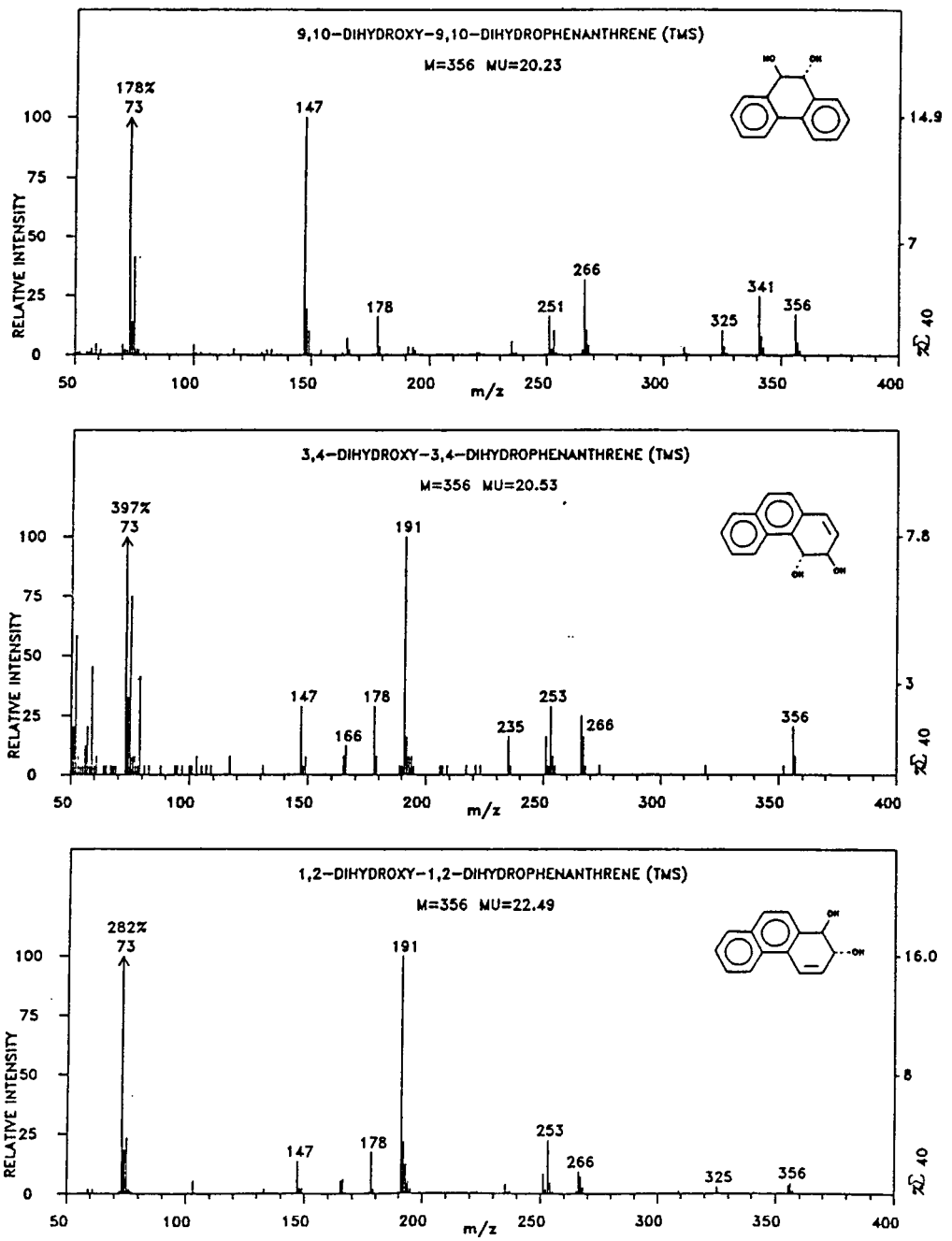


Figure 7. Comparison of the mass spectra of the TMS derivatives of 9,10-, 3,4- and 1,2-dihydrodiols (metabolites XIII, XIV and XVI).

m/z 266 (m/z 275 in the mass spectrum of the deuterated analog) was due to loss of trimethylsilanol (90 amu) from the molecular ion. A radical ion corresponding to phenanthrene was present at m/z 178. The structures of metabolites XII and XIII were confirmed by comparison of the GC and GC/MS properties of the TMS derivatives with the authentic samples obtained from hydrolysis of phenanthrene 9,10-oxide. When phenanthrene 9,10-oxide was administered to rats, the major urinary metabolite was trans-9,10-dihydroxy-9,10-dihydrophenanthrene (XIII).

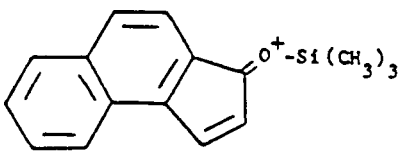
Large amounts of a third dihydrodiol (XVI), trans-1,2-dihydroxy-1,2-dihydrophenanthrene, were present in hydrolyzed rat urine (Fig. 6). A small amount was detected unconjugated in the 0-24 hr urine samples (Fig. 5). The mass spectrum of metabolite XVI was quite different from the spectra of the cis- and trans-9,10-dihydrodiols (Fig. 7). Although a molecular ion was also present at m/z 356 (TMS derivative), the base peak in the mass spectrum of metabolite XVI was observed at m/z 191 (structure b).

$$(CH_3)_3Si\text{-}O^{+}{=}CH\text{-}O\text{-}Si(CH_3)_3$$

b: m/z 191

This ion, which is characteristic of vicinal dihydrodiols, has been observed many times in the mass spectra of cyclohexadienediol metabolites of aromatic compounds (Horning et al., 1976a,b). The 1,2-dihydrodiol (XVI), a non K-region metabolite, exhibits a greater tendency to undergo

a ring-contraction fragmentation (Wong et al., 1979) than the 9,10-dihydrodiol (K-region). The resulting ion (intensity = 15%) observed at m/z 253 (M-103) was assigned structure c.



c: m/z 253

This ion shifted 9 amu to m/z 262 in the mass spectrum of the deuterated TMS derivative, indicating the presence of one hydroxyl group in the rearranged structure. An analogous ion was also observed in the mass spectra of the cis- and trans-9,10-dihydrodiols (XII and XIII), but at lower intensities (7%). Metabolite XVI was assigned the structure trans-1,2-dihydroxy-1,2-dihydrophenanthrene (Oesch, 1973) by comparison with the GC and GC/MS properties of the synthetic compound. Metabolite XVI accounted for 2.5 ± 0.8% of the dose administered to the rat.

Trace amounts of a fourth dihydrodiol (XV) were detected in hydrolyzed rat urine. A molecular ion was present at m/z 356 in the mass spectrum of the TMS derivative and the base peak was observed at m/z 191. The mass spectrum of metabolite XV was identical to that of trans-1,2-dihydroxy-1,2-dihydrophenanthrene (XVI) with a methylene unit value (MU=21.87) slightly lower than that of metabolite XVI (MU=22.49). Metab-

olite XV was tentatively assigned the structure cis-1,2-dihydroxy-1,2-dihydrophenanthrene.

The mass spectrum of a fifth dihydrodiol (XIV) was almost identical to that of the cis- and trans-1,2-dihydroxy-1,2-dihydrophenanthrenes (XV and XVI). This metabolite was tentatively assigned the structure trans-3,4-dihydroxy-3,4-dihydrophenanthrene, a previous reported metabolite of phenanthrene (Boyland and Sims, 1962). A molecular ion was present at m/z 356 (TMS derivative) and the base peak was observed at m/z 191 (Fig. 7). Metabolite XIV was detected in trace quantities in hydrolyzed rat urine.

Monohydroxy Metabolites

All five possible monohydroxy metabolites (1-, 2-, 3-, 4- and 9-hydroxyphenanthrene: II-VI) were isolated from rat urine. The mass spectra of the five phenolic metabolites (TMS derivatives) were quite similar (Fig. 8). The molecular ions at m/z 266 were the base peaks. This ion shifted 9 amu to m/z 275 in the deuterated analogs. Characteristic ions were observed at m/z 251 ($M-CH_3$) and m/z 235 ($M-CH_3-CH_4$) in all five mass spectra. The intensities of the ions at m/z 235 varied according to the position of the hydroxyl group on the phenanthrene ring. In the mass spectra of 1-, 4- and 9-hydroxyphenanthrene, these ions (structures d), which shifted 3 amu in the deuterated analog, were accompanied by doubly-charged ions of high intensities (13-17%) at m/z 117.5. In contrast, doubly-charged ions of high intensities (22%) at m/z 125.5 as well as metastable ions at m/z 236.8 ($251^2/266$) were observed in

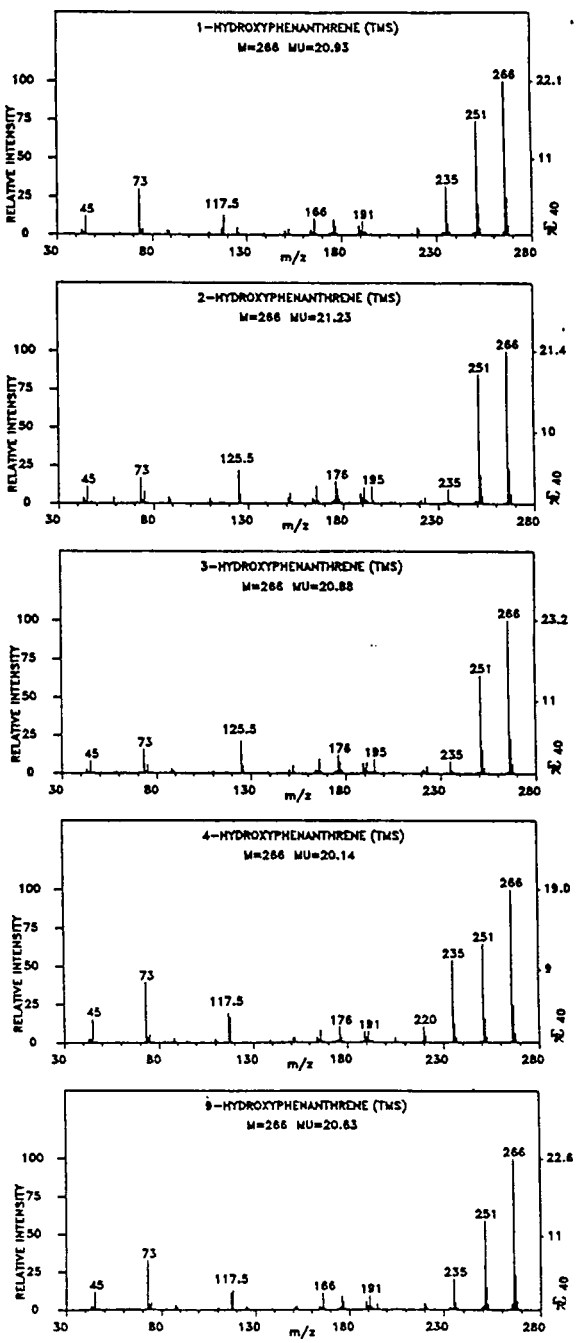
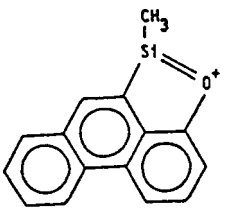
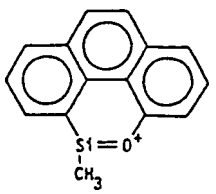


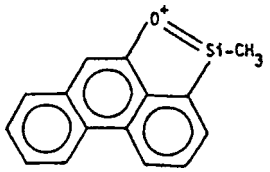
Figure 8. Comparison of the mass spectra of the TMS derivatives of 1-, 2-, 3-, 4- and 9-hydroxyphenanthrenes (metabolites II-VI).



(From 1-OH)



(From 4-OH)



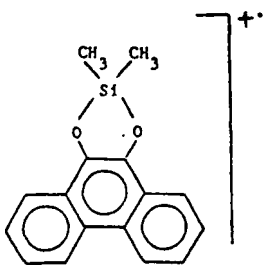
(From 9-OH)

d: m/z 235

the spectra of 2- and 3-hydroxyphenanthrenes. Thus, 1- and 3-hydroxyphenanthrenes could be identified readily on the basis of their mass spectra although they barely separated on a 70 m SE-30 capillary column.

Dihydroxy Metabolites

Four diols (VIII-XI, Table 2) were isolated from hydrolyzed rat urine. The molecular ions at m/z 354 were the base peaks in all four mass spectra (TMS derivatives). These ions shifted 18 amu to m/z 372 in the mass spectra of the deuterated TMS analogs, indicating the presence of two hydroxyl groups on the phenanthrene ring. Diol VIII was identified as 9,10-dihydroxyphenanthrene by comparison of the GC and GC/MS properties of the TMS derivative of VIII with those of the synthetic derivative prepared from 9,10-phenanthrenequinone. An intense ion present at m/z 266 (M-88, structure e) was due to loss of tetramethylsilane from the molecular ion. This characteristic ion shifted 6 amu to m/z 272 in the d_9-TMS analog, indicating that vicinal hydroxyl groups are



e: m/z 266

present (Halpaap et al. 1978).

Trace quantities of three additional diols (IX-XI, Table 2) were isolated from rat urine. Metabolites X and XI were found as urinary products of 9-hydroxyphenanthrene and, therefore, one of the hydroxyl groups must be at position 9 on the phenanthrene ring. Following ip administration of 3-hydroxyphenanthrene to rat, metabolite X was isolated from urine. Inasmuch as metabolite X was the only product from both 3- and 9-hydroxyphenanthrene, it was assigned the structure of 3,9-dihydroxyphenanthrene. Metabolite XI was also identified as a urinary metabolite of 2-hydroxyphenanthrene, and therefore metabolite XI was tentatively assigned the structure of 2,9-dihydroxyphenanthrene. The positions of the hydroxyl groups for metabolite IX could not be determined.

Quinone Metabolite

9,10-Phenanthrenequinone (VII) was also present in hydrolyzed urine samples. The quinone was identified as the dimethoxime derivative (Fig. 9). The molecular ion was observed at m/z 266. This ion shifted 6

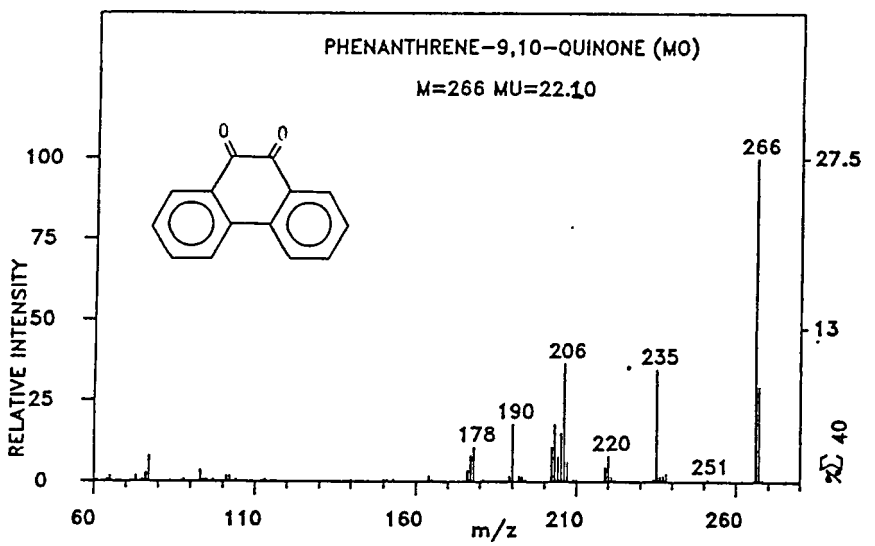


Figure 9. Mass spectrum of 9,10-phenanthrenequinone as dimethoxime derivative.

amu to m/z 272 when the derivative was prepared with deuterated methoxylamine hydrochloride, indicating the presence of two methoxime groups on a phenanthrene ring. A characteristic ion was present at m/z 235 (M-31). An intense ion at m/z 206 was due to loss of CH_2NH from the m/z 235 ion. The GC and GC/MS properties of metabolite VII and the dimethoxime derivative of authentic 9,10-phenanthrenequinone were identical. This quinone may have been formed non-enzymatically by oxidation of 9,10-dihydroxyphenanthrene (VIII).

Trihydroxy Metabolites

Two aromatic trihydroxyphenanthrenes (XVII and XVIII, Table 2) were isolated after enzymatic hydrolysis of rat urine. Molecular ions at m/z 442 were the base peaks in the mass spectra of both triols, which

shifted 27 amu to m/z 469 in the mass spectra of the deuterated samples, indicating the presence of three trimethylsilyloxy groups in the molecules. Characteristic ions were also present at m/z 427 ($M-CH_3$) and m/z 354 (M-88). The positions of the hydroxyl groups could not be determined but ions of high intensity were present at m/z 354 (M-88), suggesting that at least two of the hydroxyl groups were vicinal.

Trihydroxydihydro Metabolites

Following the enzymatic hydrolysis of rat urine, four hydroxydihydrodiol metabolites (XIX-XXII, Table 2) were isolated. Molecular ions were observed at m/z 444 (TMS derivatives) in all four mass spectra. When derivatives were prepared with deuterated BSA, the molecular ion shifted by 27 amu to m/z 471, indicating the presence of three trimethylsilyloxy groups on a dihydrophenanthrene nucleus. Inasmuch as ions of high abundance were not present at m/z 191 in the mass spectra of metabolites XIX, XXI and XXII, these metabolites are probably derivatives of 9,10-dihydroxy-9,10-dihydrophenanthrene. Metabolites XIX, XXI and XXII were also isolated from hydrolyzed rat urine following the administration of either phenanthrene 9,10-oxide or 9,10-dihydroxy-9,10-dihydrophenanthrene, confirming that they are derivatives of 9,10-dihydroxy-9,10-dihydrophenanthrene. Metabolites XXI and XXII were also isolated as urinary products after ip administration of 3- and 2-hydroxyphenanthrene, respectively. Therefore, metabolite XXI was assigned the structure 3,9,10-trihydroxy-9,10-dihydrophenanthrene whereas metabolite XXII was assigned the structure 2,9,10-trihydroxy-9,10-dihydrophenanthrene.

Metabolite XXII was excreted free and as a conjugate, with the total excretion accounting for 0.5 ± 0.2% of the dose administered to the rat. Metabolite XIX, which is also a derivative of 9,10-dihydroxy-9,10-dihydrophenanthrene, is believed to be either 1,9,10- or 4,9,10-trihydroxy-9,10-dihydrophenanthrene. Inasmuch as all three hydroxydihydrodiol metabolites (XIX, XXI and XXII) were derivatives of 9,10-dihydroxy-9,10-dihydrophenanthrene; their methylene unit values should resemble or have the same order as that of the hydroxyphenanthrenes (Table 2). If this assumption is correct, metabolite XIX should have the structure 4,9,10- rather than 1,9,10-trihydroxy-9,10-dihydrophenanthrene.

The mass spectrum of metabolite XX, which differed from other hydroxydihydrodiols described thus far, had a base peak at m/z 191 (TMS derivative), indicating that the metabolite must have either a 1,2- or 3,4-dihydrodiol structure. Inasmuch as the methylene unit value of metabolite XX (MU=22.34) was lower than that of the 1,2-dihydrodiol (MU=22.49), it was likely that metabolite XX had the structure x,3,4-trihydroxy-3,4-dihydro- rather than x,1,2-trihydroxy-1,2-dihydrophenanthrene.

Tetrahydroxydihydro Metabolites

Eight tetrahydroxydihydrophenanthrene metabolites (XXIII-XXX, table 2) were isolated from rat urine. Significant quantities of metabolite XXIII (1.38 ± 0.6% of the administered dose) were detected after enzymatic hydrolysis of urine samples (Fig. 6). A molecular ion was observed as base peak at m/z 532. This metabolite is not a derivative of

either a 1,2- or 3,4-dihydrodiol because a peak of high abundance was not observed at m/z 191. Metabolite XXIII was tentatively assigned the structure x,y,9,10-tetrahydroxy-9,10-dihydrophenanthrene.

Metabolites XXV and XXX were present in rat urine after administration of 9,10-phenanthrene oxide and XXX was also a metabolite of 9,10-dihydroxy-9,10-dihydrophenanthrene. Therefore, both XXV and XXX must be derivatives of 9,10-dihydroxy-9,10-dihydrophenanthrene.

Metabolites XXV-XXX were present in trace quantities (less than 0.1% of the administered dose and less than 2% of the total neutral metabolites isolated from hydrolyzed urine).

Tetrahydroxytetrahydro Metabolites

Three tetrahydroxytetrahydrophenanthrenes (XXXI-XXXIII, table 2) were isolated after enzymatic hydrolysis of rat urine. The mass spectra of the TMS derivatives were almost identical, with molecular ions present at m/z 534 (Fig. 10). The base peaks were observed at m/z 191, indicating that the dihydrodiols were derivatives of either a 1,2- or

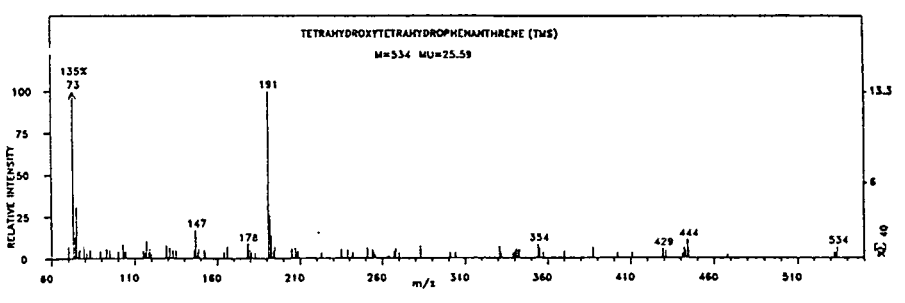
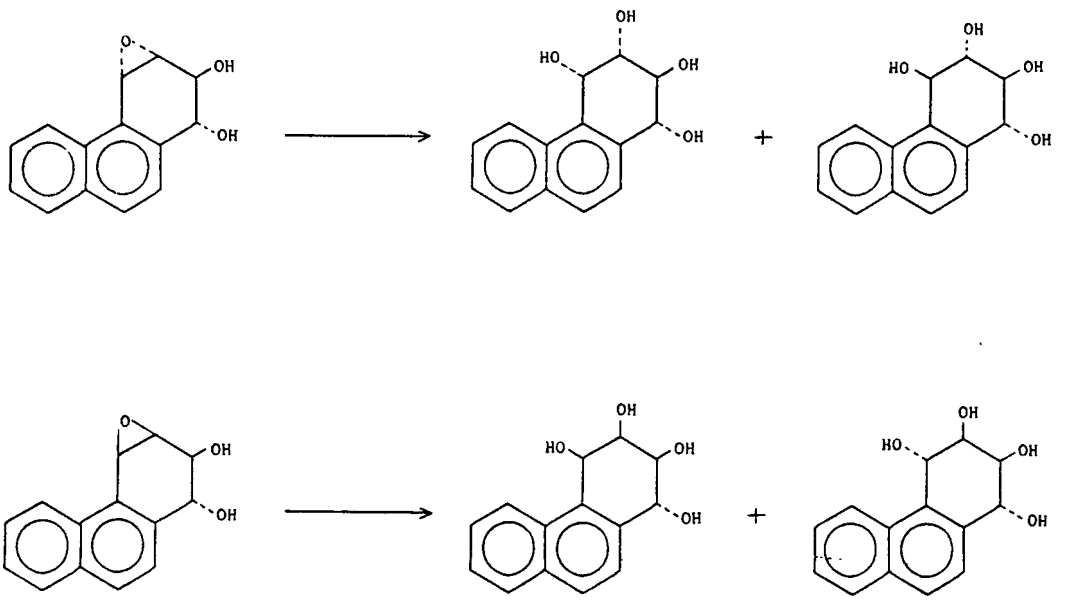


Figure 10. Mass spectrum of the TMS derivative of metabolite XXXII.



Scheme 3. Bay-region diol epoxides of phenanthrene and the tetrols which result by cis and trans attack of water at the benzylic epoxide carbon atom.

3,4-dihydrodiol. Inasmuch as four different isomeric tetrahydrotetrols (Scheme 3) were found as microsomal (from 3-methylcholanthrene-treated rat) metabolites of 1,2-dihydrodiol (Nordqvist et al., 1981), metabolites XXXI-XXXIII are presumably three of these four isomeric compounds. Definitive structures, however, could not be assigned.

Pentahydroxydihydro Metabolite

A pentahydroxydihydrophenanthrene (XXXIV) was isolated in trace amount after enzymatic hydrolysis of rat urine. A molecular ion was observed at m/z 620 in the mass spectrum of the TMS derivative. This ion shifted 45 amu to m/z 665 in the mass spectrum of the deuterated analog, indicating that five hydroxyl groups were present on a dihydrophenan-

threne structure. The definitive positions of the hydroxyl groups, however, could not be determined.

2. Bivalent Sulfur Metabolites

The profile (Fig. 11) of neutral metabolites excreted on Day 2 was quite different from the profile of neutral metabolites excreted on Day 1. the major neutral metabolites in the 0-24 hr hydrolyzed urine were 9,10-dihydroxy-9,10-dihydro- and 1,2-dihydroxy-1,2-dihydrophenanthrene (metabolites XIII and XVI, table 2). The major neutral metabolites in the 24-48 hr hydrolyzed urine were, in addition to decreased amounts of the 9,10- and the 1,2-dihydrodiols, 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene (XXXV) and 9-phenanthrol. The last two metabolites were excreted in very small amounts during the first 24 hr. The 9-hydroxy-10-methylthio-9,10-dihydro- and 9-hydroxyphenanthrene were not separated on an SE-30 capillary column, but were completly resolved on a 3% OV-17 column (Fig. 11A) for structure studies by mass spectrometry.

In addition to metabolite XXXV, six methylthio metabolites (XXXVI-XXXXI) were excreted in trace amounts (<0.1% of the administered dose). The gas chromatographic and mass spectrometric properties of these neutral bivalent sulfur metabolites (as TMS derivatives) are listed in table 3.

The mass spectrum of the TMS derivative of 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene (XXXV), a new metabolite of phenanthrene, is shown in Fig. 12. The base peak was at m/z 267 (M-47), corresponding to loss of the methylthio (CH_3S) group from the molecular ion. The

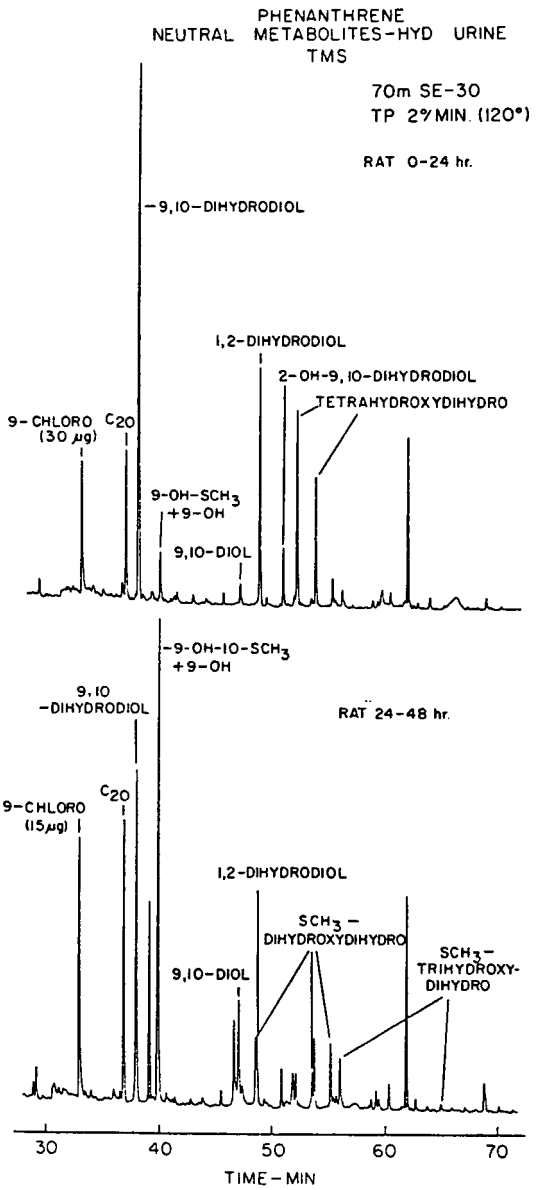


Figure 11. GC analyses of the TMS derivatives of phenanthrene metabolites isolated from hydrolyzed 0-24 and 24-48 hr rat urine samples. The GC analyses were carried out on an SE-30 capillary column.

molecular ion shifted by 9 amu in the d_9-TMS analog, indicating the presence of one trimethylsilyloxy group in the molecule. An intense ion

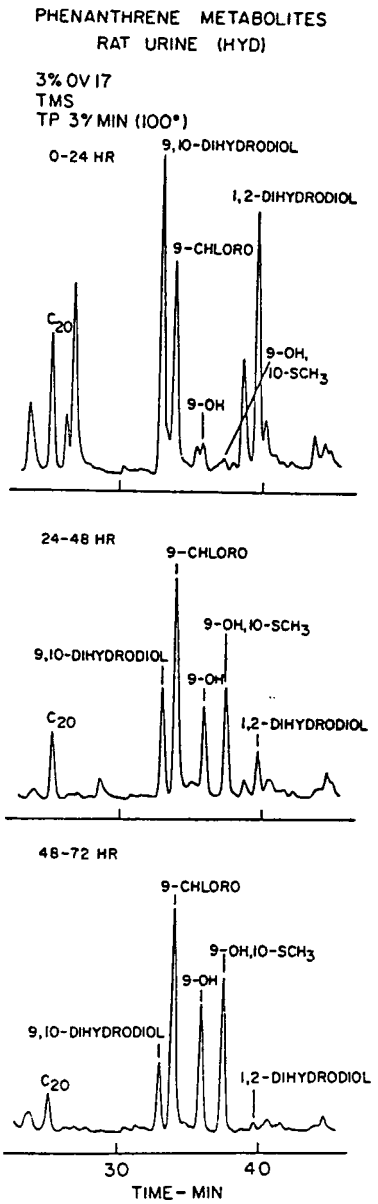


Figure 11A. GC analyses of the TMS derivatives of phenanthrene metabolites isolated from hydrolyzed 0-24, 24-48 and 48-72 hr rat urine samples. The GC analyses were carried out on a 3% OV-17 column.

at m/z 266 (M-48) was due to loss of CH_3SH, and an ion at m/z 251 was due to loss of both CH_3SH and a methyl radical originating from the tri-

Table 3

Urinary neutral bivalent sulfur metabolites of phenanthrene in the rat

Compounds*	MU	M^+	$(M-15)^+$	Major ions		
9-Hydroxy-10-methylthio-9,10-dihydrophenanthrene XXXV	20.63	314(22)	299(2)	267(100)	178(59)	266(28)
Methylthiodihydroxy-dihydrophenanthrene XXXVI	22.23	402(21)	387(3)	355(100)	235(30)	251(21)
Methylthiodihydroxy-dihydrophenanthrene XXXVII	23.59	402(23)	387(3)	355(100)	354(78)	267(36)
Methylthiodihydroxy-dihydrophenanthrene XXXVIII**	23.87	402	387			
Methylthiotrihydroxy-dihydrophenanthrene XXXIX	24.25	490(13)	475(6)	443(100)	354(48)	147(26)
Methylthiotrihydroxy-dihydrophenanthrene XXXX	26.33	490(63)	475(8)	443(100)	147(63)	442(63)
Bis(methylthio)trihydroxy-dihydrophenanthrene XXXXI**	25.76	536	521			

Relative intensities of the ions are listed in parentheses.

*Trimethylsilyl ether derivatives.

**Mixed spectra.

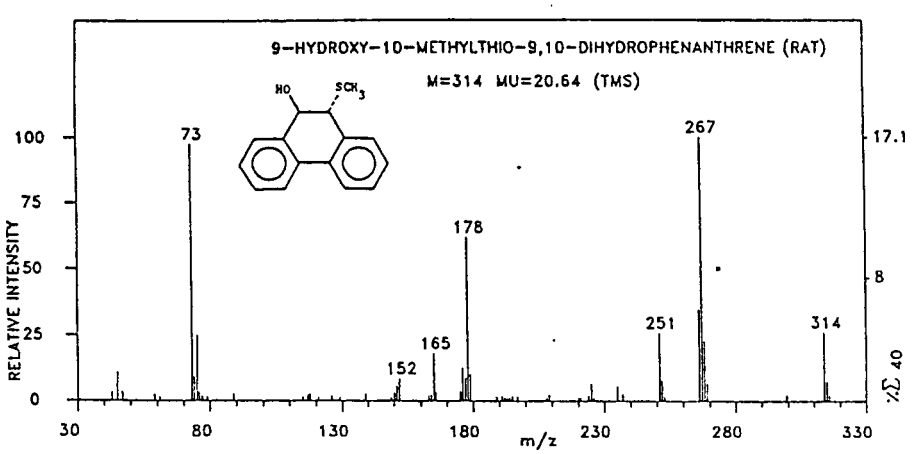


Figure 12. Mass spectrum of the TMS derivative of 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene (metabolite XXXV).

methylsilyloxy group. An ion of high abundance was present at m/z 178, corresponding to the phenanthrene radical ion. The structure of this metabolite was confirmed by synthesis. Metabolite XXXV was excreted as the unconjugated neutral metabolite and also as a glucosiduronic acid derivative (to be described later in the acidic metabolite section) that was slow to hydrolyze.

The structures of metabolites XXXVI, XXXVII and XXXVIII were not fully determined, but these compounds are methylthiodihydroxydihydrophenanthrenes. In the mass spectrum of each metabolite, a molecular ion was present at m/z 402, and the base peak at m/z 355 (M-47) corresponds to loss of CH_3S from the molecular ion. An ion of high intensity was also present at M-48 ($M-CH_3SH$). The losses of 47 and 48 amu from the molecular ion indicate that the methylthio group is on a nonaromatic ring; when methylthio groups are present on an aromatic ring, a characteristic ion is present at M-46 ($M-CH_2S$) (Lertratanangkoon and Horning, 1982; Halpaap et al., 1978). An ion of high abundance was not found at m/z 191. The 191 amu ions are the base peaks in the mass spectra of the TMS derivatives of the 1,2- and 3,4-dihydrodiols. Metabolites XXXVI, XXXVII and XXXVIII are evidently not derivatives of 1,2- or 3,4-dihydrodiols of phenanthrene, but have a 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene structure. The position of the second hydroxyl group in the molecule could not be determined from the mass spectra of the TMS derivatives. Metabolites XXXVI, XXXVII and XXXVIII are three of the four possible (1-, 2-, 3- and 4-hydroxy) derivatives of trans-9-hydroxy-10-

methylthio-9,10-dihydrophenanthrenes with an additional phenolic hydroxyl group.

Two methylthiotrihydroxydihydrophenanthrenes (XXXIX and XXXX) were isolated from rat urine. A molecular ion was present at m/z 490 in the mass spectra of both TMS derivatives, and a base peak at m/z 443 (M-47) corresponded to the loss of CH_3S from the molecular ion. If these metabolites are similar in general structure and origin to methylthiotrihydroxydihydro metaboltes from naphthalene, they are two of the isomers of a tetrahydrophenanthrene with a methylthio, a hydroxyl and a carbonyl group in the same ring, and are derived from either the 1,2:3,4-dioxide or a related dihydrodiol epoxide. They may also be catechols with a 9-hydroxy-10-methylthio-9,10-dihydro structure.

Metabolite XXXXI was identified as a di(methylthio)trihydroxydihydrophenanthrene. The molecular ion of the TMS derivatives was at m/z 536. Loss of 46 amu from the molecular ion indicated that one of the methylthio groups was present on an aromatic ring. Ions present at M-93 were due to loss of $S{=}CH_2$ and CH_3S from the molecular ion, and ions at M-94 were due to the loss of $S{=}CH_2$ and CH_3SH from the molecular ion. A definitive mass spectrum was not obtained for this metabolite.

C. Urinary Acidic (Ethyl Acetate-Soluble) Metabolites

Six acidic bivalent sulfur metabolites were isolated from hydrolyzed rat urine. These acids accounted for 33 ± 2% of the radioactivity in the 0-24 hr urine samples; this was equivalent to 11% of the administered dose.

Table 4

Urinary acidic bivalent sulfur metabolites of phenanthrene in the rat

Compounds*	MP	M^+	$(M-15)^+$	Major ions		
S-(9-Hydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid XXXXII	23.80	372(0.4)	357(<0.2)	267(100)	266(81)	178(55)
2-Hydroxy-S-(9'-hydroxy-9',10'-dihydro-10'-phenanthryl)-3-mercaptopropanoic acid XXXXIII	25.85	474(0.5)	459(0.8)	267(100)	261(45)	178(42)
S-(x,9-Dihydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid XXXXIV	26.42	460(14)	445(4)	355(100)	267(56)	354(48)
N-Acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine XXXXV	28.41	443(0.6)	428(2.6)	267(100)	178(62)	266(47)
N-Acetyl-S-(x,9-dihydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine XXXXVI	29.47	531(6)	516(5)	355(100)	354(66)	266(41)
(9-Glucuronyl-10-methylthio-9,10-dihydro)phenanthrene XXXXVII	31.72	648(0.3)	633(0.9)	178(100)	225(87)	224(33)

Relative intensities of the ions are listed in parentheses.

*Methyl ester-trimethylsilyl ether derivatives.

The gas chromatographic and mass spectrometric properties of these bivalent sulfur metabolites isolated from rat urine are summarized in table 4. The mass spectra of ME-TMS derivatives of the acidic metabolites possess characteristic ions which permit the identification of the metabolites.

The major acidic metabolite excreted by the rat (Fig. 13) was N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (XXXXV). The mass spectrum of the ME-TMS derivative is shown in Fig. 14. A molecular ion of low intensity (0.6%) was observed at m/z 443. The base peak at m/z 267 (M-176) is due to loss of N-acetylcysteine. The ion at m/z 178 is the radical ion of phenanthrene. An intense ion at m/z 353 (M-90) corresponds to loss of trimethylsilanol, and an ion at m/z 294 (M-90-59) corresponds to loss of trimethylsilanol and either H_2NCOCH_3 or $COOCH_3$ from the molecular ion. The ion at m/z 144 (relative intensity 16%) is a characteristic fragment ion which is present in the spectra of

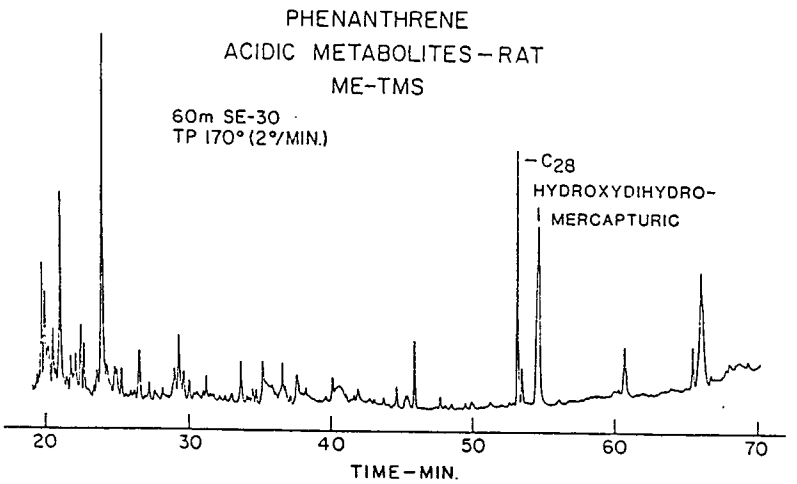
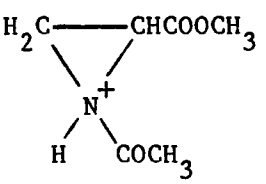


Figure 13. GC analysis of the ethyl acetate-soluble urinary acids excreted by the rat during 0-24 hr after ip injection of phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 μCi).

mercapturic and hydroxydihydromercapturic acids (ME derivatives) and probably has structure f.



f: m/z 144

The GC and GC/MS properties of the hydroxydihydromercapturic acid derivatives prepared by synthesis and the derivative of metabolite XXXXV were identical.

Metabolites XXXXII-XXXXIV and XXXXVI were present in trace quantities in hydrolyzed rat urine. The mass spectrum of the ME-TMS derivative of S-(9-hydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid (XXXXII) exhibited a molecular ion of low intensity (0.4%) at

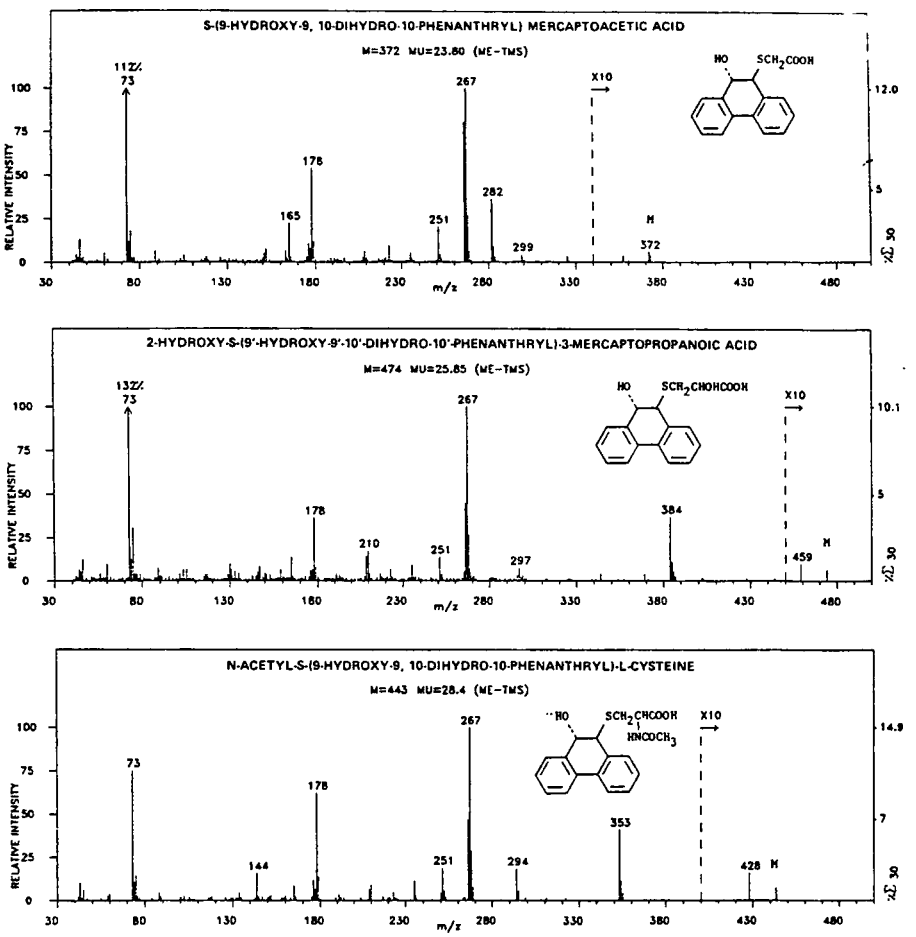


Figure 14. Comparison of the mass spectra of metabolites XXXXII, XXXXIII and XXXXV.

m/z 372 (Fig. 14). The base peak at m/z 267 (M-105) corresponded to loss of SCH_2COOCH_3, and an intense ion at m/z 266 (M-106) was due to loss of the methyl ester of mercaptoacetic acid ($HSCH_2COOCH_3$) from the molecular ion. Losses of 105 and 106 amu from a molecular ion are characteristic of ME-TMS derivatives of mercaptoacetic acids of this kind. Fragment ions at m/z 282 (M-90) and 299 (M-73) are due to loss of $(CH_3)_3SiOH$ and CH_2COOCH_3, respectively, from the molecular ion (Fig. 14). Mass spectra

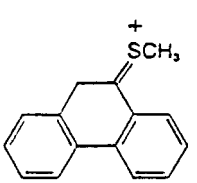
and chromatographic properties of the derivative of an authentic sample prepared by synthesis and of metabolite XXXXII isolated from urine were identical.

Metabolite XXXXIII was identified by GC/MS as 2-hydroxy-S-(9'-hydroxy-9',10'-dihydro-10'-phenanthryl)-3-mercaptopropanoic acid. The mass spectrum (Fig. 14) contained a molecular ion of low abundance at m/z 474. The base peak observed at m/z 267 (M-207) corresponded to loss of the $SCH_2CH(OTMS)COOCH_3$ (mercaptolactic) moiety from the molecular ion. The loss of 207 amu from the molecular ion is characteristic of ME-TMS derivatives of mercaptolactic acids of this kind. A radical ion corresponding to phenanthrene was present at m/z 178, and the ion at m/z 384 (M-90) was due to loss of trimethylsilanol from the molecular ion.

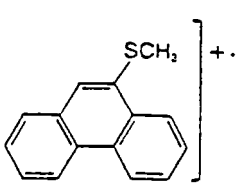
Metabolite XXXXIV was present in only trace amounts in rat urine. The molecular ion at m/z 460 is 88 amu higher than the molecular ion of metabolite XXXXII, indicating the presence of an additional trimethylsilyloxy group. The mass spectrum of metabolite XXXXIV was similar to that of XXXXII. The base peak in the spectrum was observed at m/z 355 (M-105) and an ion of high abundance was present at m/z 354 (M-106). This metabolite was, therefore, characterized as S-(x,9-dihydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid. The position of the additional hydroxyl group cannot be assigned on the basis of the mass spectral analysis. Inasmuch as the 1,2-epoxide forms more readily than the 3,4-epoxide, the hydroxyl group is most likely on C-1 or C-2 of the phenanthrene ring.

Metabolite XXXXVI was also detected in rat urine. The molecular ion at m/z 531 in the mass spectrum of the ME-TMS is 88 amu higher than the molecular ion of the hydroxydihydromercapturic acid (XXXXV), indicating the presence of an additional trimethylsilyloxy group. An ion of low intensity present at m/z 355 (M-176) is due to loss of N-acetylcysteine, and this indicates the presence of two trimethylsilyloxy groups. Compound XXXXVI was therefore assigned the structure N-acetyl-S-(x,9-dihydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine. The additional hydroxyl group may be on C-1 or C-2 of the phenanthrene ring.

Metabolite XXXXVII was isolated after enzymatic hydrolysis of rat urine. This metabolite accounted for 10% of the radioactivity in the acidic fraction (ethyl acetate-soluble acids) of rat urine samples. The mass spectrum of the ME-TMS derivative of metabolite XXXXVII showed a very weak molecular ion at m/z 648; the base peak was observed at m/z 178, corresponding to the phenanthrene radical ion. Characteristic ions at m/z 423 as well as ions at m/z 217, 317 and 333 indicated that the metabolite was a glucuronide. An ion of high abundance at m/z 225 corresponded to the ion g and the ion at m/z 224 probably has the radical ion structure h.



g: m/z 225



h: m/z 224

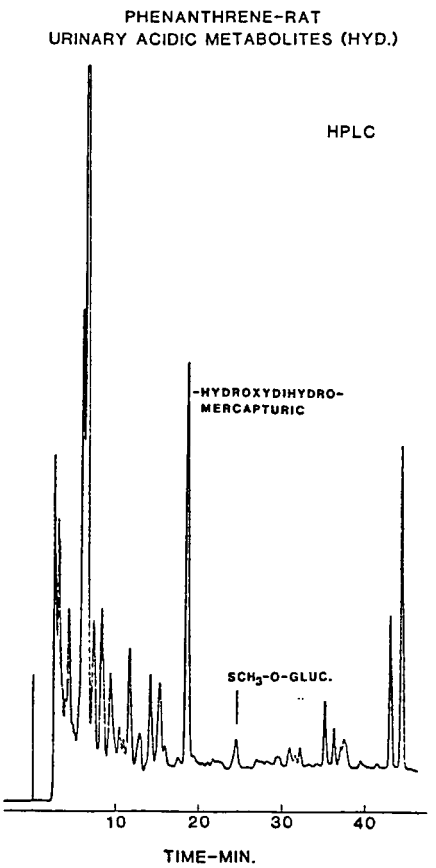


Figure 15. HPLC separation of acidic metabolites (ethyl acetate-soluble) isolated from a hydrolyzed urine collected from a rat administered phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 μCi).

The ions at m/z 225 and 423 represent the two halves of the molecule; fragmentation occurred with retention of charge primarily on the methylthiophenanthryl moiety. This metabolite was the glucuronide of 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene.

The methylthio-O-glucuronide (XXXXVII) was not readily hydrolyzed by Glusulase (pH 4.7) when present in a urinary matrix, although glucuronides without a methylthio group are cleaved. When metabolite XXXXVII was isolated from hydrolyzed urine by semipreparative

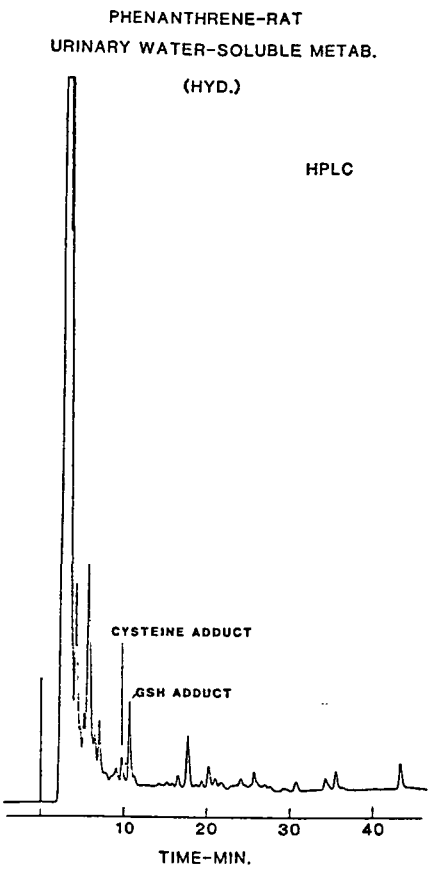


Figure 16. HPLC separation of water-soluble metabolites isolated from a hydrolyzed urine collected from a rat administered phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 μCi).

HPLC, and subsequently treated with Glusulase (pH 4.7 for 17 hr), approximately 90% of the purified methylthio-O-glucuronide was hydrolyzed to 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene and only 10% was recovered as the intact methylthio-O-glucuronide.

Inasmuch as the bivalent sulfur acids can undergo thermal degradation during GC separation, quantification of the acidic metabolites was based on radioactivity measurements of the fractions collected from a HPLC analytical C_{18}-μBondapak column. Figure 15 shows a typical HPLC

separation of the acidic metabolites isolated from hydrolyzed rat urine.

D. Urinary Water-Soluble Metabolites

The HPLC separation of the water-soluble metabolites isolated from hydrolyzed rat urine is shown in Fig. 16. The metabolites were identified as S-(9-hydroxy-9,10-dihydro-10-phenanthryl)glutathione and S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine by comparison of their FAB mass spectra with those of the synthetic compounds. Some other components were also isolated but they could not be identified.

II. URINARY METABOLITES OF PHENANTHRENE 9,10-OXIDE IN THE RAT

A. Neutral Metabolites

When phenanthrene 9,10-oxide was administered ip to rats, the major urinary metabolite isolated after enzymatic hydrolysis was the trans-9,10-dihydroxy-9,10-dihydrophenanthrene (Fig. 17). This metabolite had the same GC and GC/MS properties as those of the synthetic compound and a metabolite (XIII) isolated from rat urine after ip administration of phenanthrene. A significant quantity of 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene (XXXXV) was also detected along with the only possible monohydroxy metabolite of phenanthrene 9,10-oxide, 9-hydroxyphenanthrene (III).

Two aromatic diols were identified. One of these diols was identified as 9,10-dihydroxyphenanthrene, which had the same GC and GC/MS properties as those of metabolite VIII isolated from hydrolyzed rat urine after ip administration of phenanthrene. The structure of the second

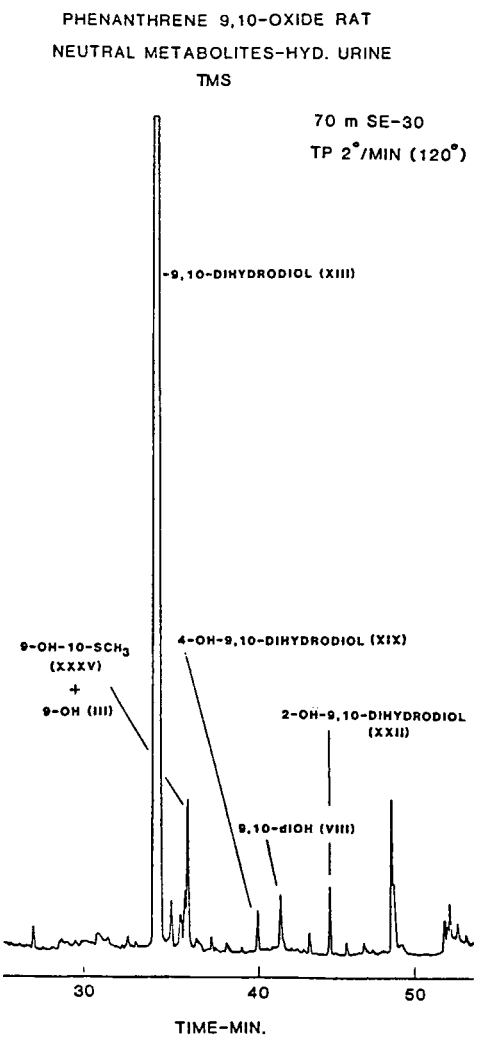


Figure 17. Gas chromatographic separation of the TMS derivatives of neutral metabolites isolated from a hydrolyzed urine collected from a rat administered phenanthrene 9,10-oxide (60 mg/kg, ip).

diol could not be determined directly from the mass spectrum; however, this metabolite was also identified as a urinary product after ip administration of 3- and 9-hydroxyphenanthrene to the rat. Inasmuch as this diol was a product from both 9- and 3-hydroxyphenanthrene and phenanthrene 9,10-oxide, it was assigned the structure of 3,9-dihydr-

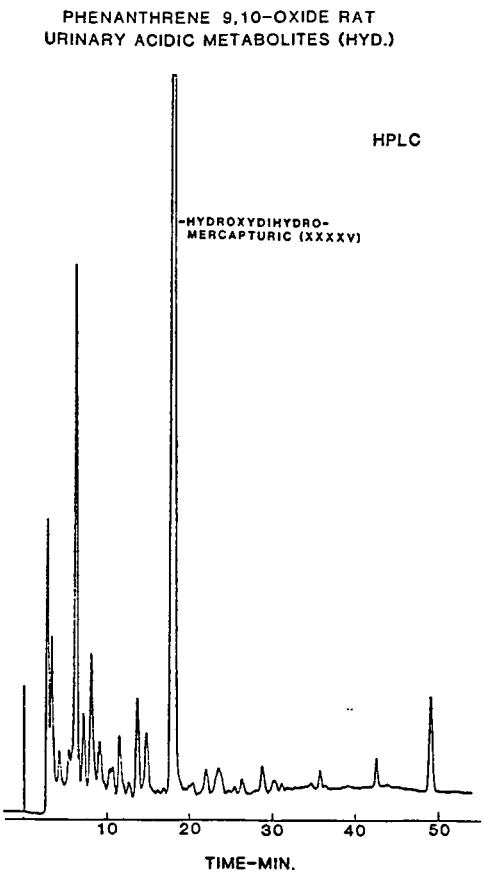


Figure 18. HPLC separation of acidic metabolites (ethyl acetate-soluble) isolated from a hydrolyzed urine collected from a rat administered phenanthrene 9,10-oxide (60 mg/kg, ip).

oxyphenanthrene. A trace quantity of this diol was also isolated as a urinary metabolite (X) of phenanthrene.

Three different hydroxydihydrodiol metabolites of phenanthrene (XIX, XXI and XXII) were detected as urinary metabolites of phenanthrene 9,10-oxide by the rat. These metabolites were identified as 4,9,10-, 3,9,10- and 2,9,10-trihydroxy-9,10-dihydrophenanthrene, respectively.

B. Acidic (Ethyl Acetate-Soluble) Metabolites

The profile of acidic metabolites isolated from rat urine after

ip administration of phenanthrene (Fig. 15) and phenanthrene 9,10-oxide (Fig. 18) were almost identical. In both profiles, the major acidic bivalent sulfur metabolite was identified as N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (XXXXV). Trace quantities of the hydroxydihydromercaptoacetic acid (XXXXII) and hydroxydihydromercaptolactic acid (XXXXIII) were also present in the profile in Fig. 18.

III. URINARY METABOLITES OF 9,10-DIHYDROXY-9,10-DIHYDROPHENANTHRENE IN THE RAT

The profile of the urinary neutral metabolites after ip administration of 9,10-dihydroxy-9,10-dihydrophenanthrene (Fig. 19) was quite similar to that of the phenanthrene 9,10-oxide (Fig. 17). A large amount of the 9,10-dihydroxy-9,10-dihydrophenanthrene was excreted unchanged. Trace quantities of three different trihydroxydihydro metabolites (4,9,10-, 3,9,10- and 2,9,10-trihydroxy-9,10-dihydrophenanthrene) and an aromatic diol (9,10-dihydroxyphenanthrene) were isolated; however, 9-phenanthrol and 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene were not detected.

IV. URINARY METABOLITES OF 9-HYDROXYPHENANTHRENE IN THE RAT

A significant quantity of the administered 9-hydroxyphenanthrene was excreted in urine as glucuronide and/or sulfate conjugates. Five different hydroxylated metabolites of 9-hydroxyphenanthrene were detected as urinary products after enzymatic hydrolysis (Fig. 20). The major

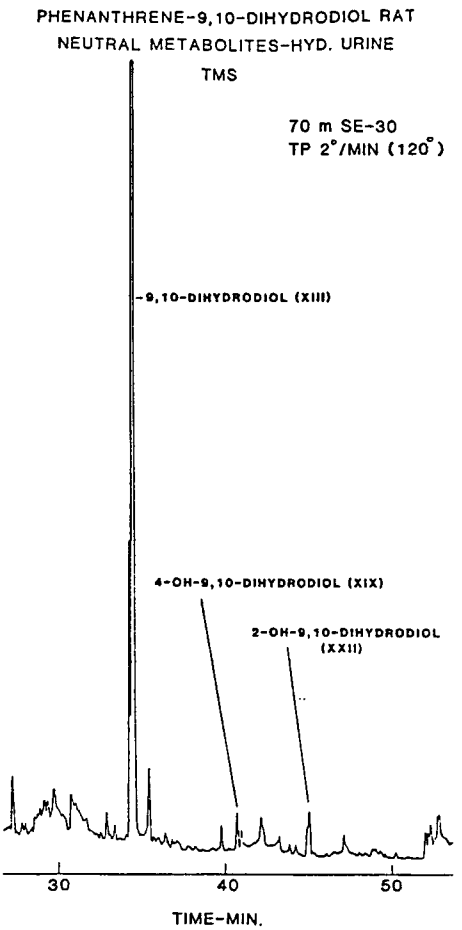


Figure 19. Gas chromatographic separation of the neutral metabolites (TMS derivatives) isolated from hydrolyzed rat urine after administration of 9,10-dihydroxy-9,10-dihydrophenanthrene (18 mg/kg, ip).

metabolite was identified as 9,10-dihydroxyphenanthrene by comparison of the GC and GC/MS properties with the reference sample. The other dihydroxy compounds (peaks A-D, Fig. 20) were excreted in trace quantities. Metabolites B and D in Fig. 20 were also isolated as urinary products of 3- and 2-hydroxyphenanthrene, respectively. Therefore, metabolite B was assigned the structure 3,9-dihydroxyphenanthrene whereas metabolite D was assigned the structure 2,9-dihydroxyphenanthrene. Both B and D were also

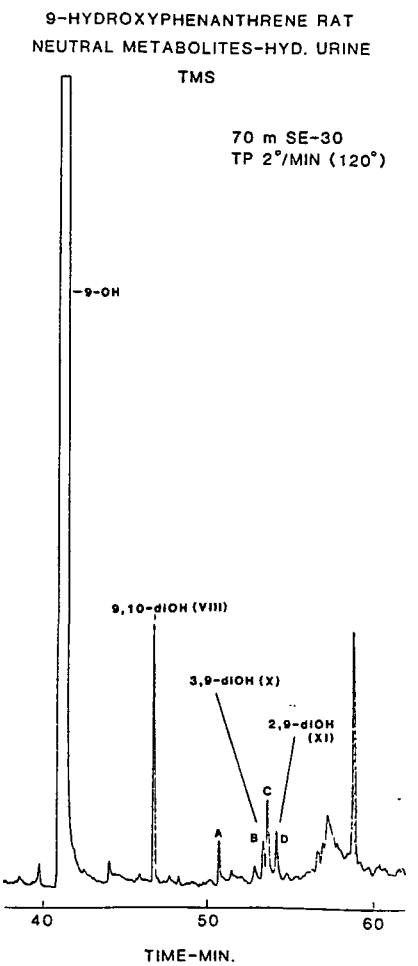


Figure 20. Gas chromatographic separation of the TMS derivatives of neutral metabolites isolated from a hydrolyzed urine collected from a rat administered 9-hydroxyphenanthrene (50 mg/kg, ip).

isolated as urinary metabolites after ip administration of phenanthrene to the rat (metabolite X and XI in Table 2, respectively).

The definitive position of the second hydroxyl group on the phenanthrene ring of metabolites A and C in Fig. 20 could not be assigned.

V. URINARY METABOLITES OF 1-HYDROXYPHENANTHRENE IN THE RAT

When 1-hydroxyphenanthrene was administered ip to rats, several oxygenated metabolites were detected after enzymatic hydrolysis of the urine samples (Fig. 21). These metabolites included two different tri-

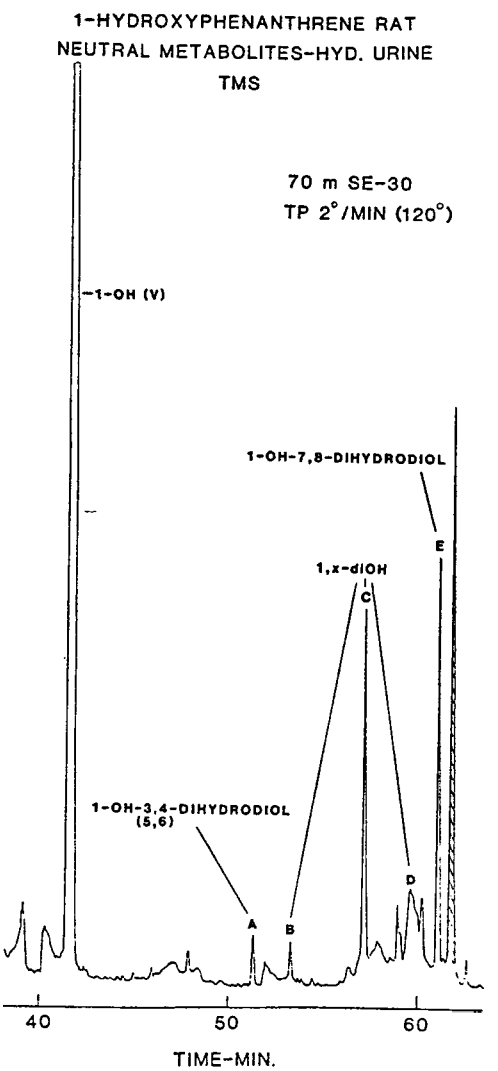


Figure 21. Gas chromatographic separation of the TMS derivatives of neutral metabolites isolated from a hydrolyzed urine collected from a rat administered 1-hydroxyphenanthrene (1.6 mg/rat, ip).

hydroxydihydro- and three different dihydroxyphenanthrenes; however, none of these metabolites was detected as a urinary metabolite after ip administration of phenanthrene to the rat.

The major metabolite (peak E) in Fig. 21 was tentatively assigned the structure of 1,7,8-trihydroxy-7,8-dihydrophenanthrene. The base peak in the mass spectrum of the TMS derivative was observed at m/z 191, which suggested that the dihydrodiol must be at the 3,4- or 5,6- or 7,8-position. Inasmuch as the retention time of this metabolite was quite long (MU=25.91) on an SE-30 column, suggesting that metabolite E could not have either a 3,4- or 5,6-diol moiety, it must have the structure 1,7,8-trihydroxy-7,8-dihydrophenanthrene.

The mass spectrum of the second trihydroxydihydrophenanthrene (peak A) was almost identical to that of peak E; however, the retention time (MU=23.09) was approximately 2.5 methylene units earlier than that of metabolite E. The position of the dihydrodiol of this metabolite could not be determined but it must have the structure of either 1,3,4-trihydroxy-3,4-dihydro- or 1,5,6-trihydroxy-5,6-dihydrophenanthrene.

Three different dihydroxy metabolites (peaks B-D) were isolated but could not identified. Inasmuch as they were the metabolites of 1-hydroxyphenanthrene; they must have the structure of 1,x-dihydroxyphenanthrenes.

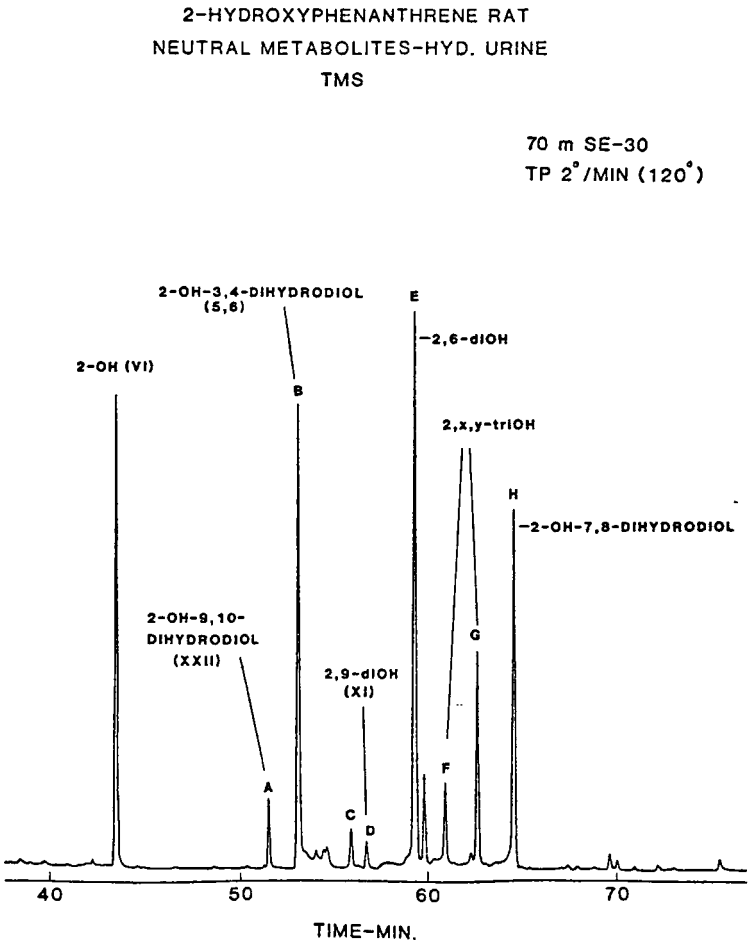


Figure 22. Gas chromatographic separation of the TMS derivatives of neutral metabolites isolated from a hydrolyzed urine collected from a rat administered 2-hydroxyphenanthrene (50 mg/kg, ip).

VI. URINARY METABOLITES OF 2-HYDROXYPHENANTHRENE IN THE RAT

When 2-hydroxyphenanthrene was administered ip to the rat it was metabolized more extensively than the 1- and 9-hydroxyphenanthrenes described thus far. Figure 22 shows the urinary neutral metabolites of 2-hydroxyphenanthrene after enzymatic hydrolysis. The major urinary metabolite was identified as a dihydroxyphenanthrene (peak E, Fig. 22). This metabolite was also found as a urinary product after ip administra-

tion of 3-hydroxyphenanthrene to the rat (to be described in a later section). Inasmuch as metabolite E (Fig. 22) was a diol formed from both 2- and 3-hydroxyphenanthrene, it could have a structure either of 2,3- or 2,6- (equivalent with 3,7-) dihydroxyphenanthrene. The mass spectrum of the TMS derivative of metabolite E, however, did not have a high abundance ion at M-88 which is a characteristic of adjacent hydroxyl groups. Therefore, metabolite E was not likely to have the structure 2,3- but was 2,6-dihydroxyphenanthrene. This metabolite was not a urinary product when phenanthrene was administered to rats.

Small quantities of metabolites C and D (Fig. 22) were also identified as dihydroxyphenanthrenes. Metabolite D was also identified as a urinary product when 9-hydroxyphenanthrene was administered ip to the rat. Inasmuch as metabolite D was a urinary metabolite of 2- and 9-hydroxyphenanthrene, metabolite D was assigned the structure of 2,9-dihydroxyphenanthrene. A trace quantity of this metabolite was also isolated as a urinary product when phenanthrene was administered ip to the rat. The structure of the other dihydroxy metabolite (C, Fig. 22) could not be determined.

Three different hydroxydihydrodiol metabolites (A, B and H, Fig. 22) were isolated after enzymatic hydrolysis of the urine samples. Inasmuch as an ion of high abundance was not present at m/z 191, metabolite A must be a derivative of the 9,10-dihydrodiol, and must have the structure of 2,9,10-trihydroxy-9,10-dihydrophenanthrene. Metabolite A was also isolated from hydrolyzed rat urine following the administration

of either the 9,10-phenanthrene oxide or 9,10-dihydroxy-9,10-dihydrophenanthrene, confirming that they are derivatives of 9,10-dihydroxy-9,10-dihydrophenanthrene. A significant amount of metabolite A was also isolated as a urinary product after ip administration of phenanthrene.

The mass spectra of metabolites B and H in Fig. 22 were quite different from the mass spectrum of metabolite A. In the mass spectra of metabolites B and H, the base peaks were observed at m/z 191 indicating that the dihydrodiol must be at the 3,4-, 5,6- or 7,8-position; however, since the retention time of metabolite B was quite low on an SE-30 column, metabolite B was tentatively assigned the structure of 2,3,4-trihydroxy-3,4-dihydro- or 2,5,6-trihydroxy-5,6-dihydrophenanthrene. Metabolite H was presumed to have the structure of 2,7,8-trihydroxy-7,8-dihydrophenanthrene.

Two different trihydroxy metabolites (peaks F and G, Fig. 22) were isolated from rat urine after ip administration of 2-hydroxyphenanthrene. Definitive structures could not be determined.

VII. URINARY METABOLITES OF 3-HYDROXYPHENANTHRENE IN THE RAT

Following enzymatic hydrolysis, the major urinary neutral metabolite of 3-hydroxyphenanthrene was identified as 3,9,10-trihydroxy-9,10-dihydrophenanthrene (peak A, Fig. 23), by comparison of the GC and GC/MS properties with a metabolite isolated from rat urine after ip administration of phenanthrene 9,10-oxide and 9,10-dihydroxy-9,10-dihydrophenanthrene. A significant quantity of this metabolite was also identified as

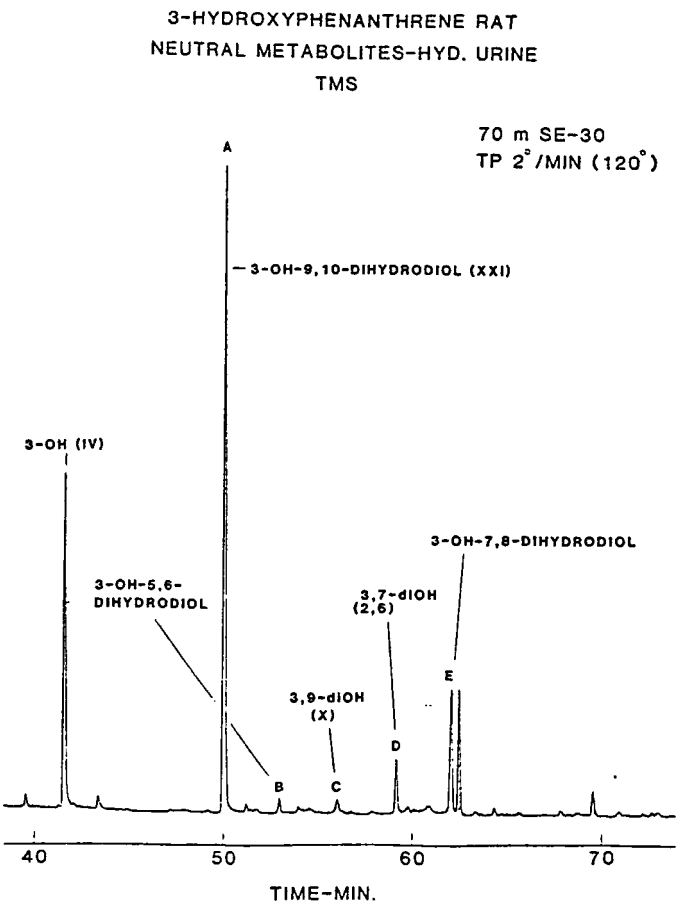


Figure 23. Gas chromatographic separation of the TMS derivatives of neutral metabolites isolated from a hydrolyzed urine collected from a rat administered 3-hydroxyphenanthrene (50 mg/kg, ip).

a urinary product when phenanthrene was administered ip to the rat (metabolite XXI, table 2).

Peaks B and E in Fig. 23 were also identified as dihydrodiol metabolites of 3-hydroxyphenanthrene; however, their mass spectra were quite different from the mass spectrum of 3,9,10-trihydroxy-9,10-dihydrophenanthrene (peak A, Fig. 23). The base peaks in the mass spectra of peaks B and E were observed at m/z 191 which suggested that the dihydrodiol must be at the non K-region (1,2- or 5,6- or 7,8-position). By

studying the retention times of peaks B and E on an SE-30 column, peak E was tentatively assigned the structure of 3,7,8-trihydroxy-7,8-dihydrophenanthrene whereas peak B was assigned the structure of 3,5,6-trihydroxy-5,6-dihydrophenanthrene.

Two different hydroxylated metabolites of 3-hydroxyphenanthrene were isolated (peaks C and D, Fig. 23). Peak D, which was also a metabolite of 2-hydroxyphenanthrene, was tentatively assigned the structure 2,6-dihydroxyphenanthrene (3,7- is equivalent to 2,6-positions on the phenanthrene ring). Following ip administration of 9-hydroxyphenanthrene to the rat, a dihydroxy metabolite which had the same GC and GC/MS properties as metabolite C (Fig. 23) was isolated. Inasmuch as peak C (Fig. 23) was a metabolite of both 3- and 9-hydroxyphenanthrene, it was assigned the structure 3,9-dihydroxyphenanthrene. A trace amount of this metabolite was also present when phenanthrene was administered ip to the rat (metabolite X, table 2).

VIII. URINARY METABOLITES OF 1,2-DIHYDROXY-1,2-DIHYDROPHENANTHRENE IN THE RAT

Few or no metabolites were formed when 1,2-dihydroxy-1,2-dihydrophenanthrene was administered ip to the rat. Only trace quantities of 1- and 2-hydroxyphenanthrene could be detected. These metabolites could be formed non-enzymatically by dehydration of the administered 1,2-dihydroxy-1,2-dihydrophenanthrene.

IX. URINARY METABOLITES OF N-ACETYL-S-(9-HYDROXY-9,10-DIHYDRO-10-PHENANTHRYL)-L-CYSTEINE IN THE RAT

The fate of N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine in the rat was followed by the administration of synthetic N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (1-2 mg) labeled with [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (0.01 μCi). Approximately 55% of the administered dose was excreted in urine during the 0-24 hr and 11% in the 24-48 hr sample. After enzymatic hydrolysis of the urine samples with Glusulase, 9% of the radioactivity excreted during 0-24 hr was extracted as neutral metabolites by the ammonium carbonate-ethyl acetate procedure. Following acidification of the enzymatic hydrolysate, 68% of the radioactivity excreted in urine (0-24 hr) was recovered as ethyl acetate-soluble acids. These data are summarized in table 5.

Table 5

Percentage of recovery of radioactive metabolites of N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine from rat urine

	0-24 hr	24-48 hr
Total Urine		
% of administered dose	55	11
Hydrolyzed Urine		
neutral metabolites	9*(5)	
acidic (ethyl acetate-soluble)	68*(37)	

* % of total radioactivity excreted in urine.
Numbers in parentheses represented % of administered dose. Rat was given N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (1-2 mg, ip) and [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (0.01 μCi).

A. Neutral Metabolites

The profile of the neutral metabolites excreted on Day 1 is shown in Fig. 24. Only two neutral metabolites were detected and they were identified as 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene and 9-hydroxyphenanthrene by comparison of their GC and GC/MS properties with reference samples.

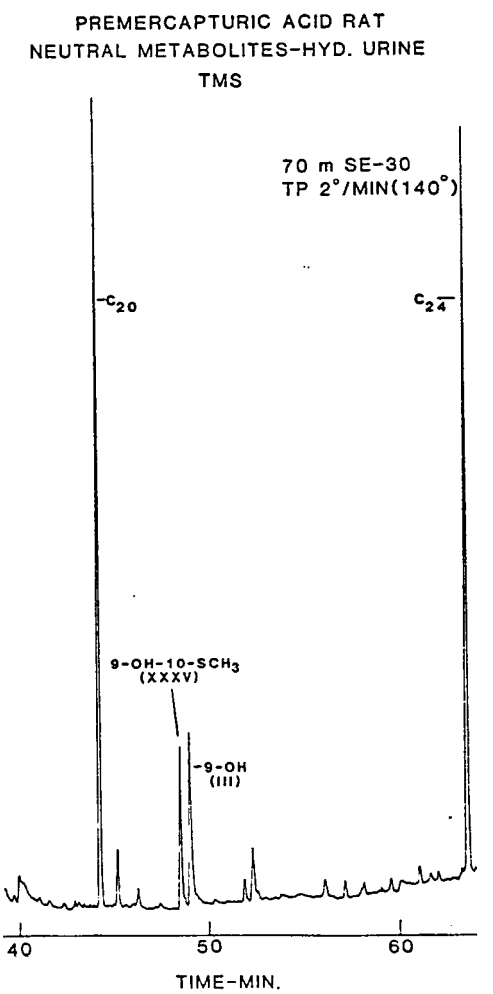


Figure 24. GC separation of the TMS derivatives of neutral metabolites isolated from a hydrolyzed urine collected from a rat administered N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (1-2 mg) labeled with [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (0.01 μCi).

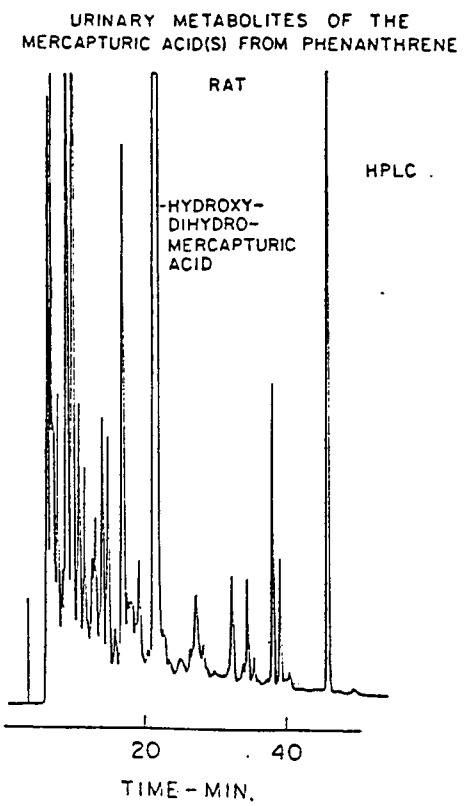


Figure 25. HPLC separation of acidic metabolites (ethyl acetate-soluble) isolated from a hydrolyzed urine collected from a rat administered N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (1-2 mg) labeled with [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (0.01 μCi).

B. Acidic (Ethyl Acetate-Soluble) Metabolites

The major acidic metabolite isolated after enzymatic hydrolysis was identified as the unchanged N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (Fig. 25). This bivalent sulfur acid accounted for 37% of the administered dose.

Table 6

Percentage of recovery of radioactive metabolites of phenanthrene from rat bile

	0-24 hr
Total Bile	
% of administered dose	68.9 ± 5.9
Hydrolyzed Bile	
neutral metabolites*	24.7 ± 1.3
acidic (ethyl acetate-soluble) metabolites*	16.0 ± 3.9
water-soluble metabolites*	57.5 ± 7.1

* % of total radioactivity excreted in urine
Each rat was given phenanthrene (50 mg/kg) and [9-^{14}C]phenanthrene (5 μCi; specific activity 19.3 mCi/m mole). Results are expressed as mean values ± standard deviations from 4 rats.

X. BILIARY METABOLITES OF PHENANTHRENE IN THE RAT

A. Recovery of Radioactive Metabolites from Rat Bile

The degree of recovery of phenanthrene and its metabolites from rat bile following cannulation was determined by administering phenanthrene (50 mg/kg) labeled with 5 μCi of [9-^{14}C]phenanthrene. Of the administered dose, 69 ± 6% was excreted in the 0-24 hr bile sample. After enzymatic hydrolysis with Glusulase, 25 ± 1% of the radioactivity excreted in the bile was extracted as neutral metabolites by the ammonium carbonate-ethyl acetate procedure. Following acidification of the enzymatic hydrolysate, 16 ± 4% of the radioactivity excreted in the bile was recovered as ethyl acetate-soluble acids. Approximately 58 ± 7% of the radioactivity excreted in bile remained in the acidified aqueous phase after hydrolysis. These data are summarized in Table 6.

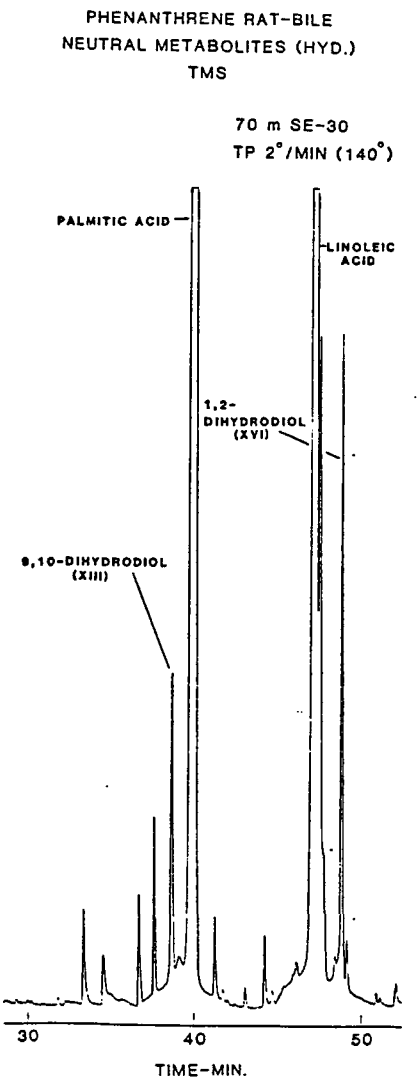


Figure 26. GC separation of neutral metabolites (TMS derivatives) isolated from a bile sample collected from a rat administered phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 μCi).

B. Biliary Neutral Metabolites

A typical gas chromatographic analysis of the neutral metabolites (TMS derivatives) isolated after enzymatic hydrolysis of the bile sample is shown in Fig. 26. The major metabolites were identified as

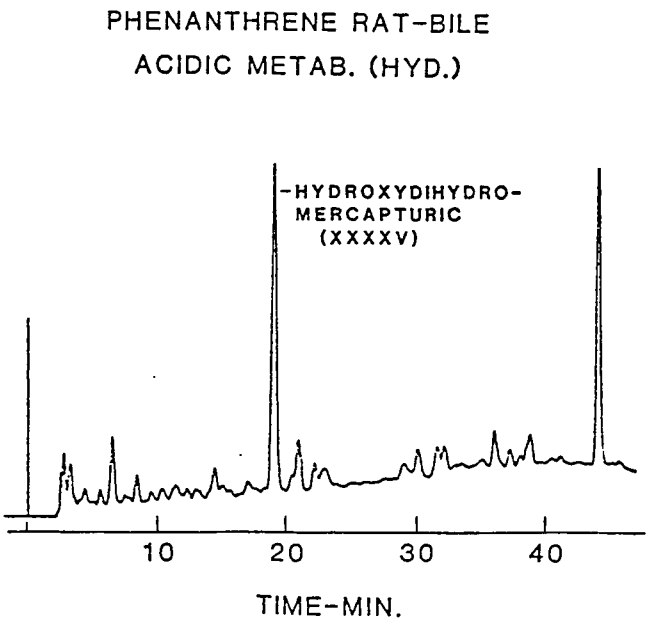


Figure 27. HPLC separation of acidic (ethyl acetate-soluble) metabolites isolated from a hydrolyzed bile sample obtained from a rat administered phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 μCi).

1,2-dihydroxy-1,2-dihydro- and 9,10-dihydroxy-9,10-dihydrophenanthrene by comparison of their GC and GC/MS properties with the reference samples.

C. Biliary Acidic (Ethyl Acetate-Soluble) Metabolites

The only acidic metabolite isolated from hydrolyzed rat bile (0-24 hr collection) was identified as N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (Fig. 27). This metabolite accounted for 16% of the total radioactivity excreted in the bile, which was equivalent to 11% of the administered dose. No other bivalent sulfur acids or the (9-glucuronyl-10-methylthio-9,10-dihydro)phenanthrene were present.

D. Biliary Water-Soluble Metabolites

When the metabolites of phenanthrene in the acidified aqueous phase were examined, several sulfur-containing metabolites were isolated

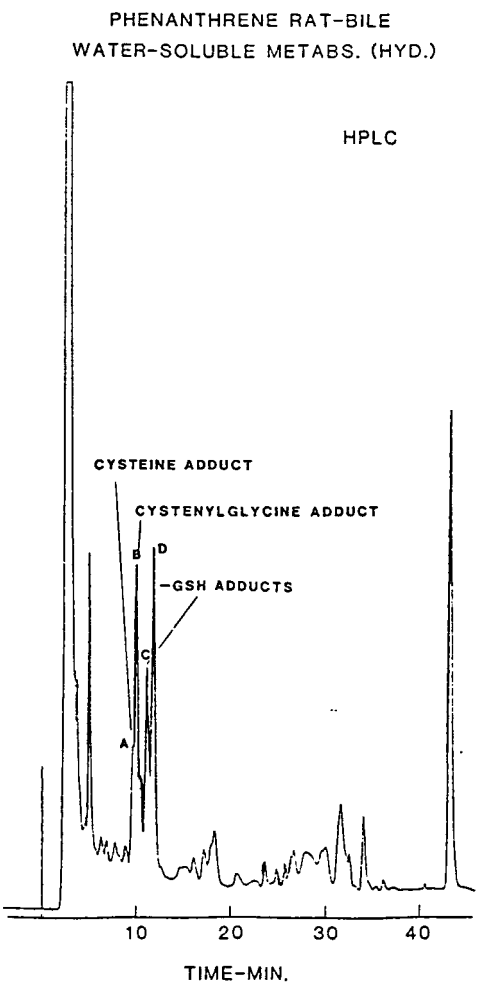


Figure 28. HPLC separation of water-soluble metabolites isolated from a bile sample obtained from a rat administered phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5μCi).

and a few were identified. The isolation was based on the separation of individual fractions by a semi-preparative HPLC column and the identification was based on FAB/MS. The major metabolites (peaks C and D, Fig. 28) were identified as diastereoisomeric trans-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)glutathione by comparison of the HPLC and FAB/MS properties with the synthetic samples. Although the FAB/MS data of

fractions A and B (Fig. 28) showed that they were mixed spectra of several components, ions which corresponded to cysteine and cysteinylglycine adducts of phenanthrene 9,10-oxide were present. Definitive mass spectra could not be obtained.

GUINEA PIG

I. URINARY METABOLITES OF PHENANTHRENE IN THE GUINEA PIG

A. Recovery of Radioactive Metabolites from Urine

When phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 μCi) was administered ip to four guinea pigs, 67 ± 12% of the administered dose was recovered in the 0-24 hr, 13 ± 1% in the 24-48 hr and 5 ± 1% in the 48-72 hr urine samples. Neutral (unconjugated) metabolites accounted for 13 ± 0.1% of the metabolites excreted in urine in 0-24 hr. After enzymatic hydrolysis of 0-24 hr urine samples with Glusulase, 81 ± 2% of the radioactivity excreted in urine was recovered as neutral metabolites and 14 ± 2% was recovered as acidic metabolites. Less than 5% of the radioactivity excreted in urine remained in the aqueous phase. These data are summarized in table 7.

B. Urinary Neutral Metabolites

The studies with [9-^{14}C]phenanthrene (4 guinea pigs) showed very little difference between guinea pigs in terms of the profiles of urinary metabolites. Typical gas chromatographic analyses of the neutral metabolites (TMS derivatives) recovered from the unhydrolyzed and hydrolyzed guinea pig urines are showed in Figs. 29 and 30, respectively. A

Table 7

Percentage of recovery of radioactive metabolites of phenanthrene from guinea pig urine

	0-24 hr	24-48 hr	48-72 hr	Total
Total Urine				
% of administered dose	67.3 ± 11.6	13.3 ± 1.0	4.7 ± 0.6	85.3 ± 12.3
Non-Hydrolyzed Urine				
neutral metabolites*	12.8 ± 0.1	15.7 ± 1.5	13.6 ± 2.1	
Hydrolyzed Urine				
neutral metabolites*	81.3 ± 1.9	72.0 ± 2.3	68.0 ± 2.0	
acidic metabolites*	14.3 ± 1.9	18.5 ± 1.5	25.0 ± 1.0	

* % of total radioactivity excreted in urine.
Each guinea pig was given unlabeled phenanthrene (50 mg/kg) and [9-^{14}C]phenanthrene (5 μCi; specific activity 19.3 mCi/m mole). Results are expressed as mean values ± standard deviations from 4 guinea pigs.

series of oxygenated and two sulfur-containing metabolites were isolated and identified by GC and GC/MS.

1. Oxygenated Neutral Metabolites

Twenty-one oxygenated metabolites of phenanthrene which were isolated from rat urine and described in the previous section (I-XVI, XX-XXII, XXX and XXXI, table 2) were also found as urinary metabolites from the guinea pig. The gas chromatographic and mass spectrometric properties of these oxygenated metabolites are summarized in Table 8. These metabolites included five different phenanthrols (II-VI, Table 8), four dihydroxyphenanthrenes (VIII-XI), one phenanthrenequinone (VII), five dihydrodiols (XII-XVI), three hydroxydihydrodiols (XX-XXII), one tetrahydroxydihydro- (XXX) and one tetrahydroxytetrahydrophenanthrene (XXXI).

The profile of the unconjugated neutral metabolites from

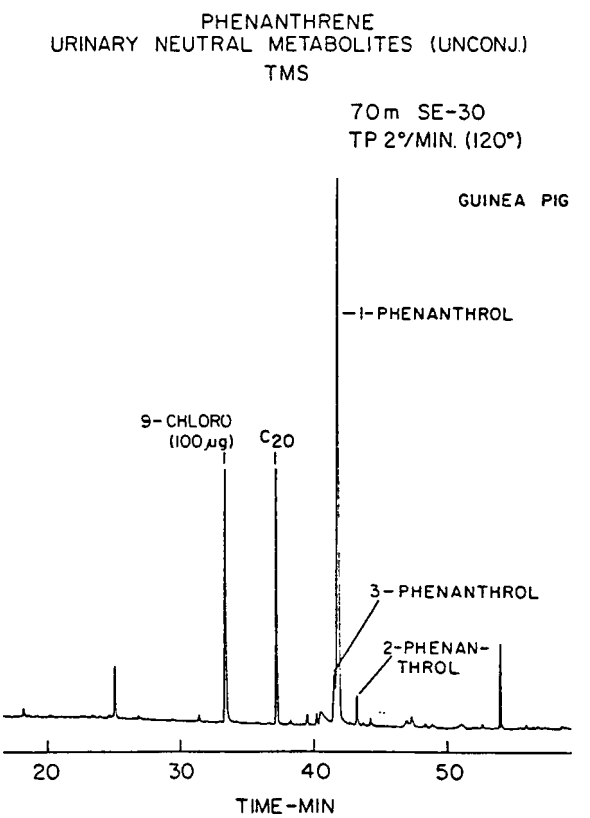


Figure 29. GC separation of the TMS derivatives of unconjugated phenanthrene metabolites isolated from 0-24 hr urine collected from a guinea pig administered phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenenthrene (5 μCi).

guinea pig urine (Fig. 29) was quite different from that of the rat (Fig. 5). The major metabolite in guinea pig urine was identified as 1-hydroxyphenanthrene (V, Table 8), whereas none could be detected in the unconjugated fraction from rat urine. This unconjugated metabolite accounted for approximately 11% of the dose administered to the guinea pig. A significant quantity of metabolite V was also excreted as glucuronide and/or sulfate conjugates. The total excretion of 1-hydroxyphenanthrene accounted for 16 ± 0.6% of the dose administered to the

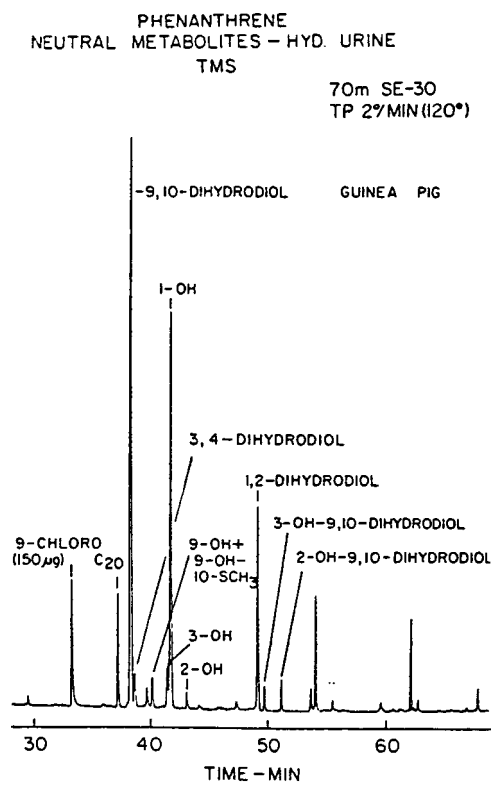


Figure 30. GC separation of the TMS derivatives of phenanthrene metabolites isolated from a hydrolyzed urine collected from a guinea pig administered phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 μCi).

guinea pig.

Small quantities of 2- and 3-hydroxyphenanthrenes (VI and IV, respectively) were also detected in the unconjugated and conjugated fractions. Following enzymatic hydrolysis of the urine sample, trace amounts of 4- and 9-hydroxyphenanthrenes (II and III, respectively) were detected.

The major urinary metabolite after enzymatic hydrolysis was identified as trans-9,10-dihydroxy-9,10-dihydrophenanthrene (XIII). This

Table 8

Urinary oxygenated metabolites of phenanthrene in the guinea pig

Compounds*	MU	M^+	$(M-15)^+$	Major ions		
Phenanthrene I	17.33	178(100)	--	176(17)	89(15)	76(14)
4-Phenanthrol II	20.14	266(100)	251(69)	235(54)	117(19)	117.5(17)
9-Phenanthrol III	20.63	266(100)	251(59)	235(20)	117.5(13)	117(11)
3-Phenanthrol IV	20.88	266(100)	251(66)	125.5(22)	165(13)	176(13)
1-Phenanthrol V	20.93	266(100)	251(74)	235(31)	117.5(13)	165(11)
2-Phenanthrol VI	21.23	266(100)	251(85)	125.5(22)	176(15)	165(12)
9,10-Phenanthrene-quinone VII	22.10	266(100)	251(1)	206(37)	235(35)	190(18)
9,10-Dihydroxy-phenanthrene VIII	22.09	354(100)	339(4)	266(30)	236(8)	147(8)
Dihydroxy-phenanthrene IX**	23.58	354(100)	339(6)	266(27)	236(22)	147(6)
3,9-Dihydroxy-phenanthrene X**	23.90	354(100)	339(16)	191(12)	251(6)	147(6)
2,9-Dihydroxy-phenanthrene XI**	24.09	354(100)	339(22)	147(14)	253(6)	266(6)
cis-9,10-Dihydroxy-9,10-dihydrophenanthrene XII	20.17	356(35)	341(27)	147(100)	266(23)	178(13)
trans-9,10-Dihydroxy-9,10-dihydrophenanthrene XIII	20.23	356(29)	341(25)	147(100)	266(24)	178(17)
trans-3,4-Dihydroxy-3,4-dihydrophenanthrene XIV	20.53	356(21)	341(<1)	191(100)	147(29)	253(29)
cis-1,2-Dihydroxy-1,2-dihydrophenanthrene XV	21.87	356(6)	341(<1)	191(100)	147(27)	253(26)
trans-1,2-Dihydroxy-1,2-dihydrophenanthrene XVI	22.49	356(11)	341(2)	191(100)	147(18)	253(15)
x,3,4-Trihydroxy-3,4-dihydrophenanthrene XX	22.34	444(59)	429(4)	191(100)	147(40)	354(23)
3,9,10-Trihydroxy-9,10-dihydrophenanthrene XXI	22.47	444(89)	429(39)	147(100)	354(89)	341(18)
2,9-10-Trihydroxy-9,10-dihydrophenanthrene XXII	22.95	444(100)	429(18)	147(80)	354(41)	253(12)
Tetrahydroxydihydro-phenanthrene XXX**	25.51	532	517			
Tetrahydroxytetrahydro-phenanthrene XXXI**	23.90	534	519			

Relative intensities of the ions are listed in parentheses.

*Trimethylsilyl ether derivatives.

**Mixed spectra.

metabolite accounted for 28 ± 2% of the dose administered to the guinea pig. A trace amount of the cis-isomer (XII) was also detected.

A significant quantity of trans-1,2-dihydroxy-1,2-dihydrophenanthrene (XVI) was isolated after enzymatic hydrolysis of the urine samples with Glusulase. This metabolite accounted for 9 ± 2% of the dose administered to the guinea pig. A trace quantity of cis-1,2-dihydroxy-1,2-dihydrophenanthrene (XV) was also present.

The fifth dihydrodiol was identified as trans-3,4-dihydroxy-3,4-dihydrophenanthrene (XIV). Approximately 1.5% of the administered dose was accounted for by this metabolite in guinea pig urine whereas only a trace quantity could be detected from the rat (described previously).

Small quantities of four different dihydroxyphenanthrenes (VIII-XI) were isolated from guinea pig urine after enzymatic hydrolysis of the urine samples. These metabolites were also detected in rat urine.

9,10-Phenanthrenequinone (VII) was also detected along with the 9,10-dihydroxyphenanthrene from guinea pig urine. This quinone was detected as its dimethoxime derivative, which had the same GC and GC/MS properties as the reference sample and a metabolite from rat urine.

Three trihydroxydihydrophenanthrenes (XX-XXII) were isolated after enzymatic hydrolysis of the urine sample with Glusulase. These metabolites were identified as x,3,4-trihydroxy-3,4-dihydro-, 2,9,10-trihydroxy-9,10-dihydro- and 3,9,10-trihydroxy-9,10-dihydrophenanthrene, respectively, by comparison of their GC and GC/MS properties with those

of metabolites isolated from rat urine (metabolites XX-XXII, Table 2).

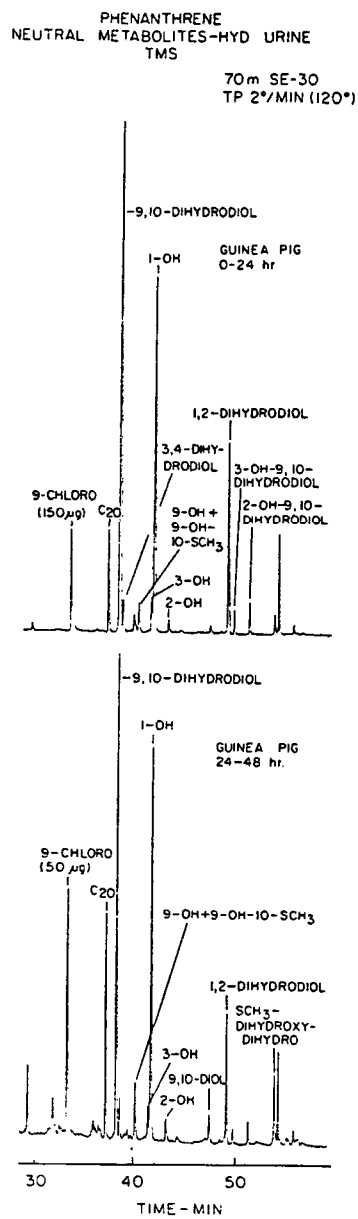


Figure 30A. GC analyses of the TMS derivatives of phenanthrene metabolites isolated from hydrolyzed 0-24 and 24-48 hr guinea pig urine samples.

Table 9

Urinary neutral bivalent sulfur metabolites of phenanthrene in the guinea pig

Compounds*	MU	M^+	$(M-15)^+$	Major ions		
9-Hydroxy-10-methylthio-9,10-dihydrophenanthrene XXXV	20.63	314(22)	299(2)	267(100)	178(59)	266(28)
Methylthiodihydroxy-dihydrophenanthrene XXXVII	23.59	402(23)	387(3)	355(100)	354(78)	267(36)

Relative intensities of the ions are listed in parentheses.

*Trimethylsilyl ether derivatives.

One tetrahydroxydihydro- (XXX) and one tetrahydroxytetrahydrophenanthrene (XXXI) were isolated but could not be identified. Both of these metabolites were detected in trace quantities in both rat and guinea pig urine.

Although the total quantity of urinary neutral metabolites (Table 7) excreted on Day 2 (16% of administered dose) was less than that obtained on Day 1 (54% of administered dose), profiles of the neutral metabolites were quite similar (fig. 30A).

2. Bivalent Sulfur Metabolites

In the previous section, a series of neutral bivalent sulfur metabolites was isolated and identified from rat urine (XXXV-XXXXI, Table 3). Only two of those neutral bivalent sulfur metabolites (XXXV and XXXVII, Table 9) were detected in extracts of hydrolyzed guinea pig urine. Both XXXV and XXXVII were minor metabolites in the 0-24 and 24-48 hr urine samples, and were not present in the 48-72 hr samples.

C. Urinary Acidic (Ethyl Acetate-Soluble) Metabolites

Six acidic bivalent sulfur metabolites (ethyl acetate-soluble acids) were isolated from guinea pig urine. These bivalent sulfur acids accounted for 14 ± 2% of the radioactivity in the administered dose.

The profiles of acidic metabolites of the rat (Fig. 13) and the guinea pig (Fig. 31) were quite different. The major acid in the 0-24 hr rat urine was the N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (XXXXV). None of the other bivalent sulfur acids accounted for more than 1-4% of the radioactivity in that fraction. In contrast, the major bivalent sulfur acid excreted in guinea pig urine was S-(9-hydr-

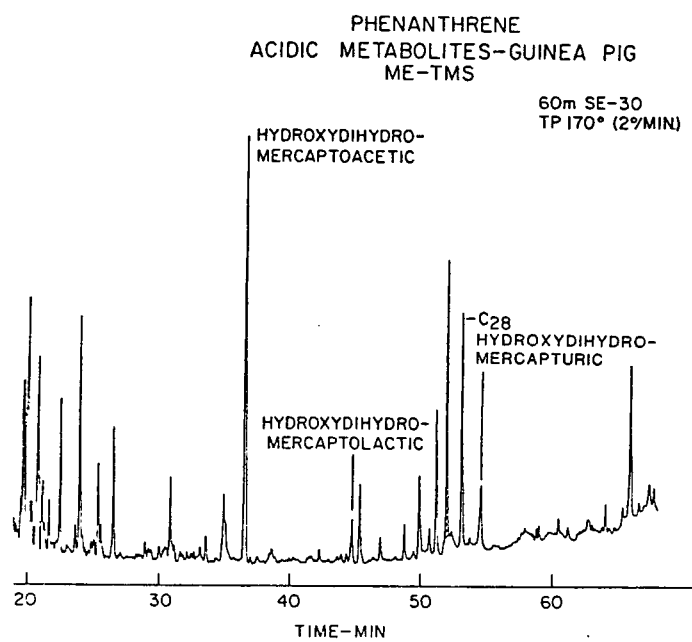


Figure 31. GC analysis of the ethyl acetate-soluble urinary acids excreted by the guinea pig during 0-24 hr after ip administration of phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 μCi).

oxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid (XXXXII). 2-Hydroxy-S-(9'-hydroxy-9',10'-dihydro-10'-phenanthryl)-3-mercaptopropanoic acid (XXXXIII) and N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (XXXXV), however, were present in significant quaintities [19% and 14%, respectively, of the S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-mercaptoacetic acid].

Inasmuch as 95-98% of the radioactivity present in hydrolyzed guinea pig urine was recovered by extraction (Table 7), the recovery of hydroxydihydromercaptoacetic, hydroxydihydromercaptolactic and hydroxy-dihydromercapturic acids, the methylthio-O-glucuronide and the neutral metabolites, was essentially quantitative as far as isolation from urine is concerned. The more polar acidic metabolites excreted in rat urine (5-20% of the urinary radioactivity) and which are not extracted by ethyl acetate were either not present in guinea pig urine or were excreted in only very small amounts (2% or less of administered dose).

The gas chromatographic and mass spectrometric properties of acidic bivalent sulfur metabolites isolated from guinea pig urine are summarized in Table 10.

The major acidic metabolite excreted by the guinea pig was S-(9-hydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid (XXXXII, Table 10). The GC and GC/MS properties of the ME-TMS derivative of metabolite XXXXII and a derivative of an authentic sample prepared by synthesis were identical. The mass spectrum of this metabolite is shown in Fig. 14.

Table 10

Urinary acidic bivalent sulfur metabolites of phenanthrene in the guinea pig

Compounds*	MU	M^+	$(M-15)^+$	Major ions		
S-(9-Hydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid XXXXII	23.80	372(0.4)	357(<0.2)	267(100)	266(81)	178(55)
2-Hydroxy-S-(9'-hydroxy-9',10'-dihydro-10'-phenanthryl)-3-mercaptopropanoic acid XXXXIII	25.85	474(0.5)	459(0.8)	267(100)	261(45)	178(42)
S-(x,9-Dihydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid XXXXIV	26.42	460(14)	445(4)	355(100)	267(56)	354(48)
N-Acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine XXXXV	28.41	443(0.6)	428(2.6)	267(100)	178(62)	266(47)
(9-Glucuronyl-10-methylthio-9,10-dihydro)phenanthrene XXXXVII	31.72	648(0.3)	633(0.9)	178(100)	225(87)	224(33)

Relative intensities of the ions are listed in parentheses.

*Methyl ester-trimethylsilyl ether derivatives.

Metabolite XXXXIII (Table 10) was present in trace quantity in the hydrolyzed guinea pig urine. The GC and GC/MS properties of this metabolite were identical to those of 2-hydroxy-S-(9'-hydroxy-9',10'-dihydro-10'-phenanthryl)-3-propanoic acid, a metabolite isolated from hydrolyzed rat urine. The mass spectrum of the ME-TMS derivative is shown in Fig. 14.

Metabolite XXXXIV was present in only trace amounts in guinea pig urine. The molecular ion at m/z 460 is 88 amu higher than the molecular ion of metabolite XXXXII (Table 10), indicating the presence of an additional trimethylsilyloxy group. The same metabolite was also isolated from hydrolyzed rat urine. This metabolite was assigned the partially defined structure S-(x,9-dihydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid. The position of the additional hydroxyl group cannot be assigned on the basis of the mass spectral analysis. Inasmuch as the 1,2-epoxide forms more readily than the 3,4-epoxide and 1-hydroxyphenanthrene was the major urinary neutral metabolite from guinea pig

urine, the hydroxyl group is likely to be on C-1 or C-2 of the phenanthrene ring.

A significant quantity of N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (XXXXV) was detected in hydrolyzed guinea pig urine (Fig. 31). This metabolite was the major bivalent sulfur acid in rat urine (described in previous section). The GC and GC/MS properties of the TMS derivatives of metabolite XXXXV and those of the authentic sample prepared by synthesis were identical.

The GC and GC/MS properties (ME-TMS derivative) of metabolite XXXXVII (Table 10) were identical to those of a metabolite isolated from rat urine, the methylthio-O-glucuronide. Significant amounts of this

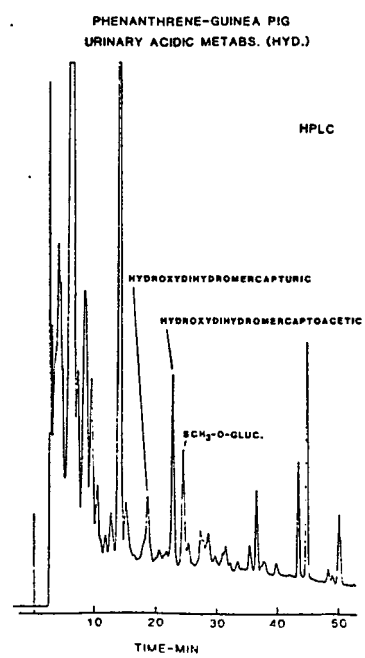


Figure 32. HPLC separation of the acidic metabolites (ethyl acetate-soluble) isolated from hydrolyzed urine collected from a guinea pig administered phenanthrene (50 mg/kg) labeled with [9-^{14}C]phenanthrene (5 μCi).

metabolite were detected in guinea pig urine after enzymatic hydrolysis with Glusulase.

The bivalent sulfur acids described thus far can undergo thermal degradation during GC separaion. Quantification of these acidic metabolites was therefore based on radioactivity measurement of the fractions collected from a HPLC analytical C_{18}-μBondapak column. Figure 32 shows a typical HPLC separation of the acidic metabolites isolated from hydrolyzed guinea pig urine.

II. URINARY METABOLITES OF N-ACETYL-S-(9-HYDROXY-9,10-DIHYDRO-10-PHENANTHRYL)-L-CYSTEINE IN THE GUINEA PIG

The recovery of N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine from guinea pig urine was followed by administering (ip) N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (1-2 mg) labeled with [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (0.01 μCi). Urine samples were collected for 2 days. Approximately 87% of the administered dose was excreted in urine during 0-24 hr and 11% during 24-48 hr samples. After enzymatic hydrolysis of the urine sample with Glusulase, 26% of the radioactivity excreted in the 0-24 hr sample was extracted as neutral metabolites by the ammonium carbonate-ethyl acetate procedure. These neutral metabolites accounted for 23% of the administered dose. Following acidification of the enzymatic hydrolysate, 30% of the radioactivity in urine was recovered as ethyl acetate-soluble acids, equivalent to 26% of the administered dose.

Table 11

Percentage of recovery of radioactive metabolites of N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine from guinea pig urine

	0-24 hr	24-48 hr
Total Urine		
% of administered dose	87	11
Hydrolyzed Urine		
neutral metabolites	26*(23)	
acidic (ethyl acetate-soluble)	30*(26)	

* % of total radioactivity excreted in urine.
Numbers in parentheses represented % of administered dose. Guinea pig was given N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (1-2 mg, ip) and [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (0.01 μCi).

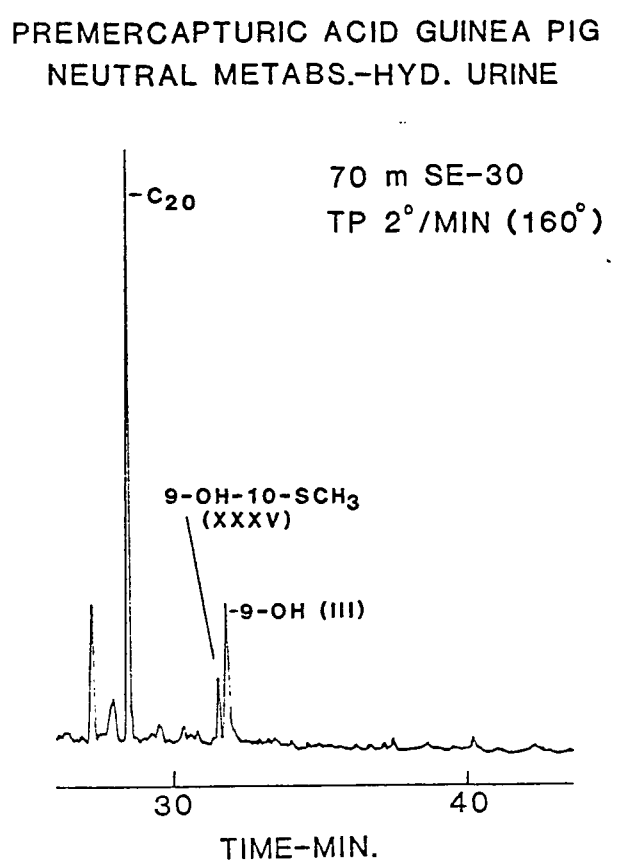


Figure 33. GC separation of the TMS derivatives of neutral metabolites isolated from a hydrolyzed urine collected from a guinea pig administered N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (1-2 mg) labeled with [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (0.01 μCi).

These data are summarized in Table 11.

A. Neutral Metabolites

Significant amounts of 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene (XXXV) and 9-hydroxyphenanthrene (III) were detected after enzymatic hydrolysis of the 0-24 hr sample (Fig. 33).

B. Acidic (Ethyl Acetate-Soluble) Metabolites

Following ip administration of synthetic N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine to the guinea pig, several urinary acidic bivalent sulfur metabolites were detected after enzymatic

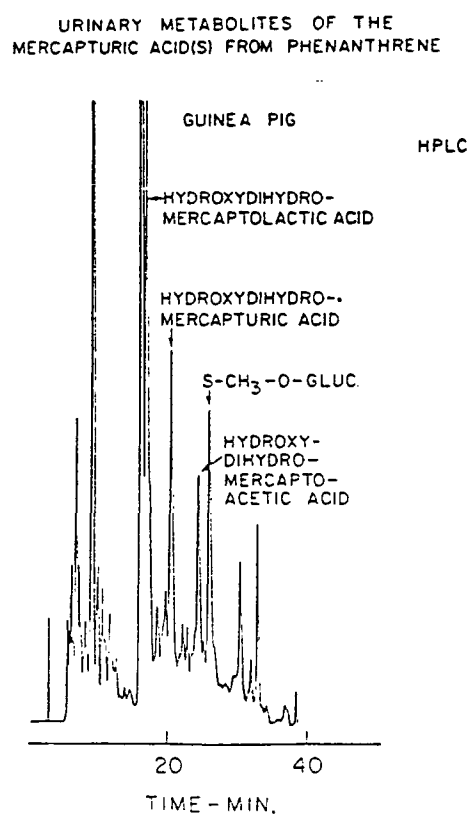


Figure 34. HPLC separation of acidic metabolites (ethyl acetate-soluble) isolated from a hydrolyzed urine collected from a guinea pig administered N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (1-2 mg) labeled with [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (0.01 μCi).

Table 12

Percentage of recovery of radioactive acidic bivalent sulfur metabolites of N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine from guinea pig urine

	0-24 hr
N-Acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (XXXXV)	37.7*(9.8)
(9-Glucuronide-10-methylthio-9,10-dihydro)phenanthrene (XXXXVII)	30.0*(7.8)
S-(9-Hydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid (XXXXII)	23.8*(6.2)
2-Hydroxy-S-(9'-hydroxy-9',10'-dihydro-10'-phenanthryl)-3-mercaptopropanoic acid (XXXXIII)	12.4*(3.2)

*% of radioactivity in the acidic extract (ethyl acetate-soluble).
Numbers in parentheses represented % of administered dose. Guinea pig was given N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (1-2 mg, ip) and [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (0.01 μCi).

hydrolysis of the urine sample with Glusulase (Fig. 34). Approximately 10% of the administered dose of N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine was excreted unchanged in urine. The acidic bivalent sulfur metabolites were identified as 9-glucuronyl-10-methylthio-9,10-dihydrophenanthrene (XXXXVII), S-(9-hydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid (XXXXII) and 2-hydroxy-S-(9'-hydroxy-9',10'-dihydro-10'-phenanthryl)-3-mercaptopropanoic acid (XXXXIII). These metabolites accounted for 8, 6 and 3%, respectively, of the administered dose. These data are summarized in Table 12.

C. Water-Soluble Metabolites

Inasmuch as 44% of the radioactivity present in urine could not be extracted by ethyl acetate, attempts were made to identify the urinary water-soluble metabolites of N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phen-

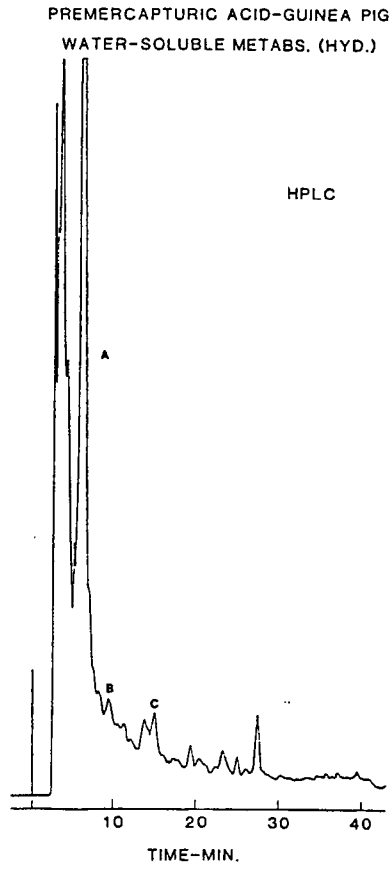


Figure 35. HPLC separation of water-soluble metabolites isolated from a hydrolyzed urine collected from a guinea pig administered N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (1-2 mg) labeled with [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (0.01 μCi).

anthryl)-L-cysteine by the guinea pig. Three different fractions (Fig. 35) which contained radioactivity were collected from an HPLC C_{18}-μBonda-pak column and each was pooled. Each fraction was taken to dryness under reduced pressure. Fraction B which had the same HPLC retention time as the synthetic S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine was analyzed by FAB/MS. This fraction presumably contained the deacylated product of N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine;

however, a definitive mass spectrum could not obtained. The failure to obtained a definitive FAB/MS was due to low concentration of administered dose [1-2 mg of N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine].

CHAPTER V

DISCUSSION

DISCUSSION

There are many similarities in the metabolism of all polycyclic aromatic hydrocarbons that have been studied. The initial step is the formation of one or more arene oxides; variations in immediate products, and in secondary metabolites, are dependent upon the structure of the hydrocarbon and upon differences in species, strain, sex and degree and type of enzyme induction in different animals. Phenanthrene is a useful compound for studying these processes, inasmuch as the 9,10-oxide is converted into 9,10-dihydrodiol metabolites that are relatively stable with respect to proton-catalyzed dehydration reactions. Metabolites from the 1,2-oxide, in contrast, have properties that resemble those of compounds derived from non-K-region epoxides of polycyclic aromatic hydrocarbons, and structures of this kind are of interest because they include some moderately strong carcinogens (for example, the 7,8-dihydrodiol-9,10-oxides of benzo[a]pyrene). Phenanthrene is the simplest polycyclic aromatic hydrocarbon that provides metabolites derived from both types of epoxide.

Epoxides are removed from biological systems through the action of epoxide hydrolase(s) (EC 3.3.2.3) and S-glutathione transferase(s) (EC 2.5.1.18). Hydrolytic ring opening leads to oxygen series metabolites; the initial products from arene oxides are dihydrodiols. These compounds may be excreted directly or after conjugation, and they may also be metabolized further to dihydrodiol epoxides. Phenols are believed to be

formed by spontaneous isomerization of epoxides, or by "direct hydroxylation". Sulfur series metabolites include acids derived from glutathione conjugates (mercapturic acids are the best known compounds of this kind) and also methylthio analogs of dihydrodiols and phenols. There are differences of opinion about the origin of methylthio metabolites. The view has been expressed that these compounds are derived entirely from glutathione conjugates, but we believe that there is a second pathway which involves sulfonium compounds as intermediates. This is discussed later.

Preliminary Studies. A preliminary study was carried out to establish that phenanthrene was metabolized without difficulty by the guinea pig as well as by the rat. At the same dose (50 mg/kg), the rat excreted 35 ± 10% of the administered dose in the first 24 hr; the guinea pig excreted 67 ± 12% of the dose during the same time. After hydrolysis of 0-24 hr urine samples, 16% of the administered dose was recovered as neutral metabolites from rat urine, and 54% was recovered as neutral metabolites from guinea pig urine. These data indicate that the first-day metabolism of phenanthrene in the guinea pig, as indicated by urinary excretion of metabolites, is faster than in the rat, and shows emphasis upon pathways leading to neutral products (primary the 9,10-dihydrodiol and the 1,2-dihydrodiol) which are excreted free and as glucuronides and/or sulfate conjugates. The proportion of dose excreted as ethyl acetate-soluble acids was about the same in the two species.

Figures 36 and 37, respectively, show the comparisons of the gas chromatographic separations of urinary neutral metabolites of the rat and guinea pig before and after enzymatic hydrolysis.

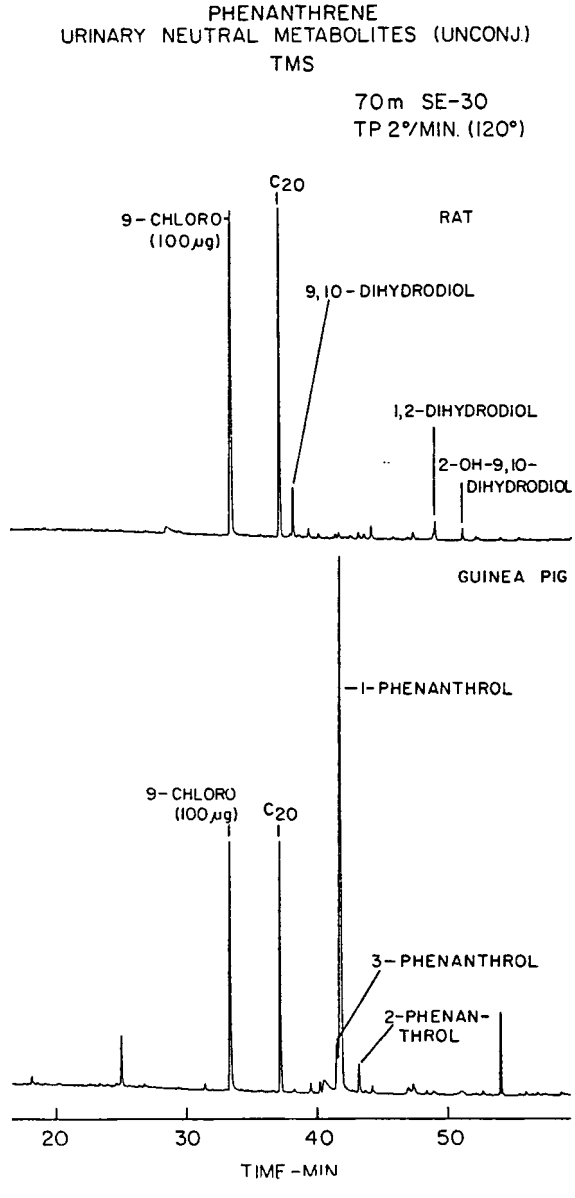


Figure 36. Comparison of the GC analyses of the TMS derivatives of unconjugated neutral metabolites isolated from 0-24 hr urine samples collected from a rat and a guinea pig after ip administration of phenanthrene (50 mg/kg).

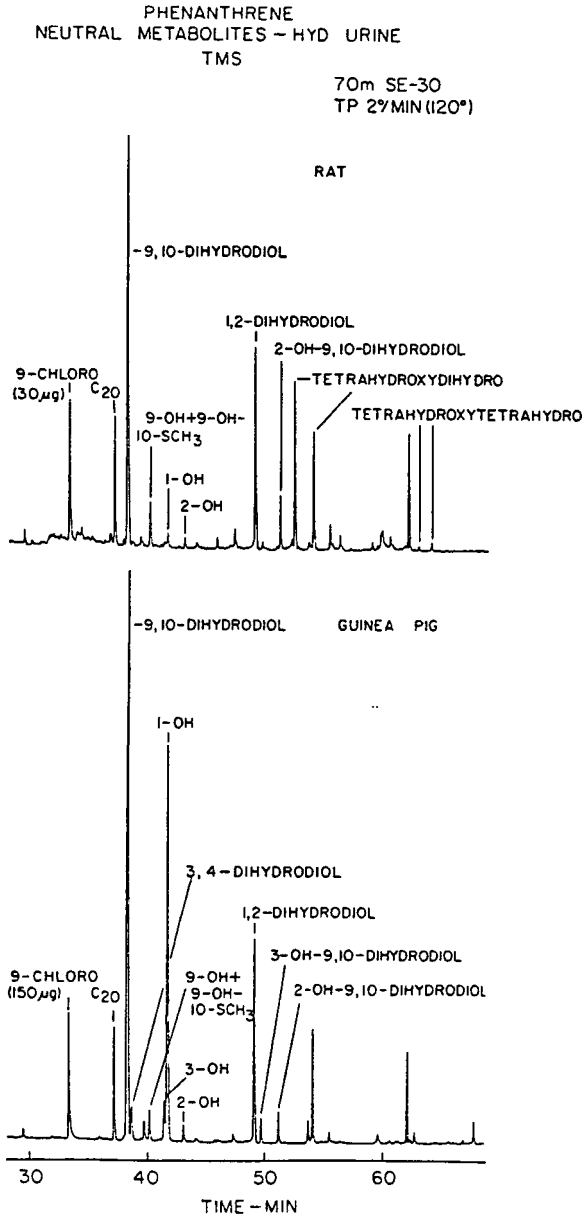


Figure 37. Comparison of the GC analyses of the TMS derivatives of neutral metabolites isolated from hydrolyzed urine samples collected from a rat and a guinea pig after ip administration of phenanthrene (50 mg/kg).

Oxygen Series Metabolites

Rat and guinea pig metabolized phenanthrene predominantly by

epoxidation at the 9,10- (K-region) and 1,2- (non-K-region) positions. The dihydrodiols to be expected from these oxides are the corresponding trans-9,10-dihydroxy-9,10-dihydro- and trans-1,2-dihydroxy-1,2-dihydrophenanthrene. Although these dihydrodiols are the major products in the urine from both rat and guinea pig treated with phenanthrene, the percentage of excretion in terms of administered dose was quite different in both species. Approximately 28% of the administered dose was accounted for by 9,10-dihydroxy-9,10-dihydrophenanthrene in the guinea pig whereas only 5% was excreted in this form by the rat. Similar results were observed for the 1,2-dihydroxy-1,2-dihydrophenanthrene. These results indicated that the microsomal enzyme, epoxide hydrase, is much more active in the guinea pig than in the rat.

Phenanthrene 9,10-oxide is a good substrate for epoxide hydrase and glutathione S-transferase. When synthetic phenanthrene 9,10-oxide was administered ip to rat, the major urinary neutral metabolite was 9,10-dihydroxy-9,10-dihydrophenanthrene and the major acidic metabolite was N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine.

In addition to the trans-9,10- and trans-1,2-dihydrodiols, very small amounts of the cis-9,10- and cis-1,2-dihydrodiol were also present. The mechanism leading to their formation was unclear. They may be formed by the same mechanism as the cis-1,2-dihydrodiol of naphthalene (Horning et al., 1980). Jerina and co-workers (Jerina et al., 1971; Jeffrey et al., 1975) proposed that 1,2-peroxides and the corresponding cis-dihydrodiols are products of bacterial metabolism of aromatic compounds, and

this is supported by ^{18}O experiments with benzene (Gibson et al., 1970).

Epoxidation at the 3,4-position (non-K-region) of the phenanthrene nucleus is also known to occur. In this dissertation, significant amounts of the trans-3,4-dihydroxy-3,4-dihyrophenanthrene was detected in hydrolyzed guinea pig urine whereas only trace amounts were detected in the rat. The epoxidation at this non-K-region was shown to be a minor pathway (in comparison with the K-region, 9,10- and non-K-region, 1,2-oxide) in both rat and guinea pig.

Monohydric phenols. Qualitative and quantitative differences were observed in the profiles in Figs. 36 and 37 in terms of the urinary excretion of 1-hydroxyphenanthrene by the rat and guinea pig. 1-Hydroxyphenanthrene, a major metabolite in guinea pig urine, is excreted free (Fig. 36) and as glucuronide and/or sulfate conjugates (Fig. 37). The total excretion of 1-hydroxyphenanthrene by the guinea pig accounted for 16% of the administered dose. This phenanthrol was present as a minor metabolite in rat urine.

The mechanism of the formation of 1-hydroxyphenanthrene by the rat and the guinea pig is unclear. It may be due to two entirely different mechanisms by the two species. When 1,2-dihydrodiol was administered ip to the rat, and urinary metabolites of this compound were examined, the major metabolite was the unchanged 1,2-dihydodiol and only trace amounts of 1- and 2-hydroxyphenanthrene were detected. This obsevation may prove to be the mechanism of formation of the 1- and 2-hydroxyphenanthrene by the rat after ip administration of phenanthrene; however, spontaneous

isomerization could have happened.

Our experience with dihydrodiols suggested that there was no possibility that 1-phenanthrol (guinea pig) was an artifact of the isolation process. Inasmuch as 16% of the administered dose was accounted for by this phenanthrol in guinea pig urine, this metabolite may be an example of a "direct" enzymatic hydroxylation process.

The 3- and 4-hydroxyphenanthrenes are probably formed by dehydration of the 3,4-dihydrodiol.

The formation of 9-hydroxyphenanthrene is quite different from the other phenanthrols. The 9-phenanthrol is a typical second- and third-day metabolite in the rat and the time course of appearence of 9-phenanthrol does parallel the formation of 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene. This suggests that some common intermediates are involved. Our present hypothesis is that glutathione and/or mercapturic acid and methionine adducts are intermediates in the formation of 9-phenanthrol and 9-hydroxy-10-methylthio-9-10-dihydrophenanthrene.

Tetrahydrotetrols. Three tetrahydroxytetrahydrophenanthrenes were isolated from rat urine. The GC/MS data indicated that the positions of the hydroxyl groups must be at the non-K-region (1,2- or 3,4-). Nordqvist et al. (1981) reported the formation of diol epoxides as liver microsomal metabolites from 3-methylcholanthrene treated rats, and four different isomeric tetrols were identified. In our experiment, three tetrahydrotetrols were formed as urinary metabolites in the rat after ip administration of phenanthrene. These tetrols are presumably formed in

vivo by the second step oxidation of the 1,2-dihydrodiol to form diol epoxide; however, the diepoxide pathway was not excluded.

Sulfur Series Metabolites

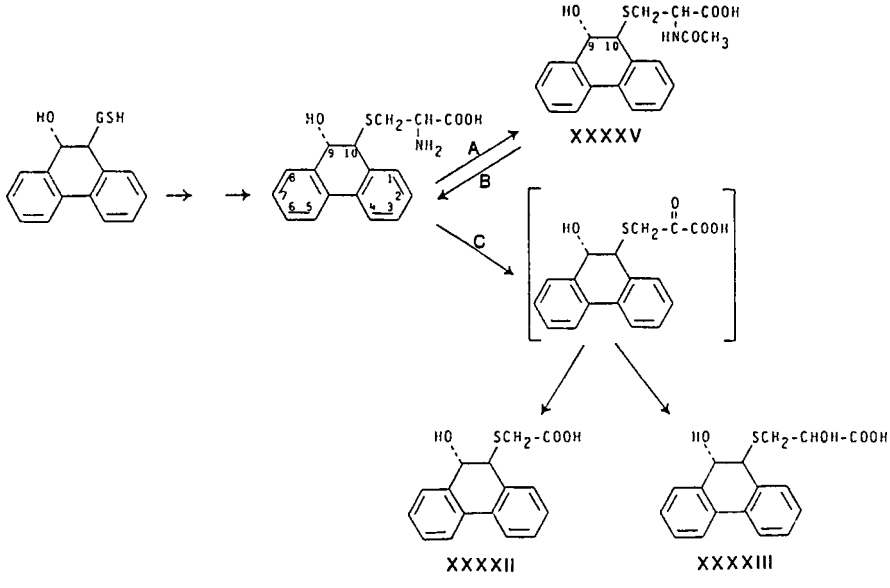
A. Acidic Bivalent Sulfur Metabolites

Both qualitative and quantitative differences were observed in the profiles of acidic bivalent sulfur metabolites obtained from rat (Fig. 13) and guinea pig (Fig. 31) urine.

Six acidic bivalent sulfur metabolites were isolated from rat urine (table 4). The major metabolite was the hydroxydihydromercapturic acid, N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (XXXXV). Only trace quantities of hydroxydihydromercaptoacetic (XXXXII) and hydroxydihydromercaptolactic (XXXXIII) acids (1-4% of the radioactivity in the acid fraction) were detected, and none of the other acids, excluding the glucuronide (XXXXVII), accounted for more than 1-4% of the radioactivity in this fraction. In contrast, the major acidic bivalent sulfur metabolite in guinea pig urine was the hydroxydihydromercaptoacetic acid, S-(9-hydroxy-9,10-dihydro-10-phenanthryl)mercaptoacetic acid (XXXXII). Significant amounts of the hydroxydihydromercapturic (XXXXV) and the hydroxydihydromercaptolactic acid (XXXXIII) were also excreted. These results indicated that the earlier reports (Bray and James, 1960; Bray et al., 1959b) that the guinea pig is unable to synthesize mercapturic acids are not strictly correct. It is not possible, in experiments of the type described here, to determine if the relatively low urinary

excretion of the hydroxydihydromercapturic acid by the guinea pig is due to incomplete acetylation at a single site, or to a combination of acetylation and deacetylation reactions at different sites. The isolation and identification of the hydroxydihydromercaptoacetic and hydroxydihydromercaptolactic acids suggests that the expected L-cysteine adduct was converted in the guinea pig by oxidative deamination to the corresponding hydroxydihydromercaptoacetic acid or reduced to a hydroxydihydromercaptolactic acid (scheme 4).

If acetylation does not occur, metabolism follows pathway C; however, if the excretion of hydroxydihydromercaptoacetic and hydroxydihydromercaptolactic acids is the result of deacetylation, their metabo-



Scheme 4. Proposed pathways for the formation of the bivalent sulfur acids.

lism follows pathway A ⟶ B ⟶ C.

When synthetic N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine was administered ip to the guinea pig, extensive deacetylation of hydroxydihydromercapturic acid occurred [S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine was formed] and hydroxydihydromercaptoacetic (6% of the administered dose) and hydroxydihydromercaptolactic acids (3% of administered dose) as well as hydroxydihydromercapturic acid (10% of administered dose) were excreted in urine (Fig. 34). When the same hydroxydihydromercapturic acid was administered ip to the rat, the only bivalent sulfur acid recovered from the urine was the unchanged hydroxydihydromercapturic acid (Fig. 25). These experiments indicate that deacetylation enzyme(s) are more active in the guinea pig than in the rat, and that there are species-specific effects that determine the nature of the urinary acidic bivalent sulfur metabolites from phenanthrene. Other studies (Nakatsu et al., 1983; Stillwell et al., 1980, 1982) have confirmed these effects for epoxide metabolism in general. There is no indication in this or other studies, however, that this circumstance reflects species-specific effects in glutathione adduct formation. On the contrary, these effects are believed to be due solely to species differences in acetylation and deacetylation processes for cysteine adducts.

A similar pathway was proposed by Mio (1979) for the metabolism of glutathione adducts formed from trichlorobenzene and related polychloroaromatic compounds. In the studies of Mio, however, the L-cysteine

moiety was attached to an aromatic ring, whereas in our study the L-cysteine moiety is attached to a nonaromatic ring. Mercaptopyruvic, mercaptoacetic and mercaptolactic acid adducts were identified by Mio (1979).

Microsomal acetyltransferases that N-acetylate S-substituted L-cysteine are usually present in both liver and kidney. The excretion of an S-substituted hydroxydihydromercaptoacetic acid by the guinea pig, as the major acidic bivalent sulfur urinary metabolite of phenanthrene, suggests that this enzyme system is absent or relatively inactive in the guinea pig kidney. The fact that some hydroxydihydromercapturic acid is excreted, however, indicates that partial acetylation occurs in the liver, kidney, or other tissues. The formation of glutathione adducts apparently proceeds as usual (Jerina and Bend, 1977). Although it is difficult to provide proof in all instances we believe, along with Boyland and Sims (1958, 1962, 1965), that biological enzyme-mediated pathways from arene oxides to sulfur-series acids end with structures of the dihydro type, rather than with aromatized compounds, and that the isolation of aromatic mercapturic, mercaptoacetic and mercaptolactic acids is due to nonenzymatic proton-catalyzed dehydration reactions that occur during isolation processes when solutions are acidified (and particularly after acidification and heating, as was the practice in early metabolic studies).

B. Methylthio Metabolites

When the metabolism of phenanthrene was reinvestigated in detail in our laboratory using GC and GC/MS procedures, seven methylthio metabolites were isolated and identified in rat urine (table 3) and two of these metabolites were also isolated in guinea pig urine (table 9). These methylthio metabolites are analogs of the dihydrodiols which are derived *via* epoxide intermediates. This was clearly established in the studies of sulfur-series and oxygen-series metabolites of naphthalene in the rat (Stillwell et al., 1978a). Methylthio metabolites have not, however, to our knowledge been described in any of the many studies of carcinogenic polycyclic aromatic hydrocarbon metabolism that have been carried out in the past decade. It would be surprising if the metabolic pathway from arene oxide to methylthio compounds was specific for naphthalene, and in this investigation several methylthio metabolites of phenanthrene were found. The major compound was 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene (XXXV), an analog of 9,10-dihydroxy-9,10-dihydrophenanthrene. We believe that this pathway of metabolism is general for arene oxides, but that in whole animal experiments the rate of formation of methylthio compounds is slower than most rates of formation of dihydrodiols and glutathione adducts. If this view is correct, methylthio metabolites will be found as urinary excretion products in more than trace amounts only when the rates of formation of dihydrodiols and glutathione adducts are depressed.

The obvious structural relationships between mercapturic, mercap-

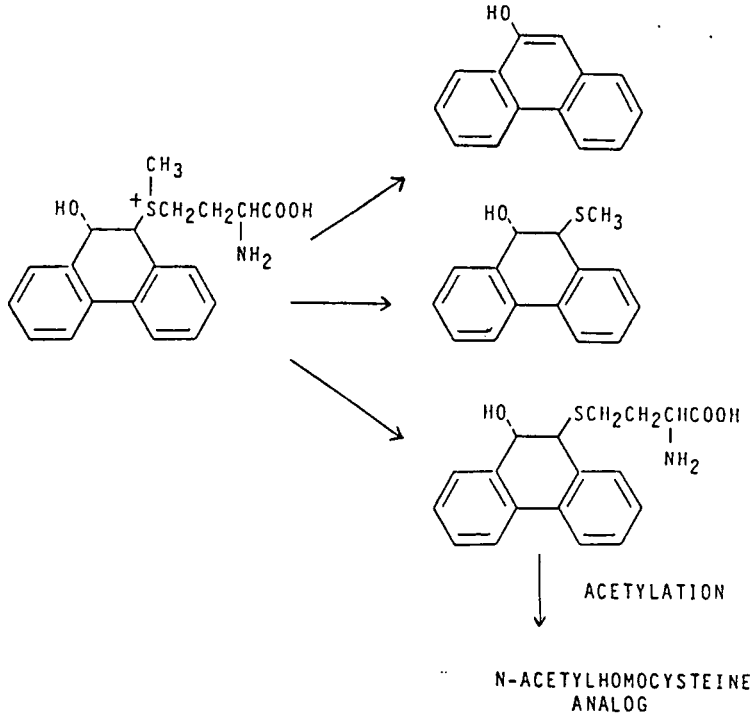
topyruvic, mercaptoacetic and mercaptolactic acids, and methylthio metabolites, suggest that all of these compounds might well be derived from glutathione adducts, with cysteine adducts as the key intermediates. Both Mio (1979) and Tateishi and co-workers (Tateishi et al., 1978; Tateishi and Shimizu, 1980) proposed that cysteine adducts are cleaved in the liver to the corresponding thiols, and that the thiols are methylated to form methylthio metabolites. The compounds under study were aromatic halogenated compounds; the thiols were thiophenols, and the corresponding sulfoxides and sulfones were also present as urinary metabolites. The possible significance of the enterohepatic circulation of metabolites, with the participation of gut microorganisms, was emphasized by Bakke et al. (1981a,b), and in recent study by Bakke et al. (1982), an analogous pathway was defined, with the lyase reaction occurring for mercapturic acids as a gut microflora reaction. A lyase reaction was found by Suzuki et al. (1982) for the cysteine conjugate of bromobenzene. These routes may be considered as well established, but they may or may not be the major route(s) involved in the formation of methylthio analogs of dihydrodiols.

In order to estimate the contribution of the N-acetylcysteine adduct of phenanthrene to the formation of methylthio metabolites, synthetic N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine (XXXXV) labeled with [9-^{14}C]-N-acetyl-S-(9-hydroxy-9,10-dihydro-10-phenanthryl)-L-cysteine was administered ip to the rat. Data for the recovery of radioactive metabolites from urine are summarized in table 5.

Almost equal amounts of 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene and 9-hydroxyphenanthrene were excreted as neutral metabolites (Fig. 24). Each of these metabolites accounted for 9% of the total radioactivity present in urine. Much of the unchanged XXXXV was recovered in the acidic extract (68% of the total radioactivity in urine); the hydroxydihydromercaptoacetic (XXXXII) and hydroxydihydromercaptolactic acid (XXXXIII) were not detected (Fig. 25). The ratio of 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene to hydroxydihydromercapturic acid excreted in urine in this experiment (0.04) is less than the ratio obtained in the experiment when phenanthrene was administered to the rat (0.24).

Similar results were also observed when the synthetic N-acetylcysteine adduct of phenanthrene was administered ip to the guinea pig (tables 11 and 12). These results suggest that formation of methylthio metabolites from mercapturic acid or cysteine conjugates is not a major pathway.

In our experiments, methylthio metabolites from phenanthrene are typical second- and third-day metabolites in the rat; dihydrodiols and products derived from glutathione adducts are early metabolites that are excreted in diminished amounts during the second and third 24-hr periods after a single dose. This effect is shown in Fig. 11. The time course of appearance of 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene does not parallel the formation of products from glutathione addition to the epoxide (suggesting that the sulfur atom is not from glutathione), and 9-phenanthrol follows the same time course of appearance as the methyl-



Scheme 5. Proposed pathways via methionine adduct.

thio metabolite. This suggests that a common intermediate is involved. Our present hypothesis is that methylthio compounds are formed from arene oxides by methionine addition (scheme 5). The sulfonium ion derived from phenanthrene 9,10-oxide, shown in scheme 5, would be expected to dissociate to form three products: 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene, 9-phenanthrol and the L-homocysteine analog of the 9-hydroxy-9,10-dihydromercapturic acid (XXXXV). These three products will not necessarily be formed in equal amounts. For phenanthrene, approximately the same amounts of 9-phenanthrol and 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene are excreted at the same time by the rat, and in

increasing amounts during the second day, while the excretion of the 9,10-dihydrodiol and products of glutathione addition are decreasing in quantity.

Studies of the cleavage of synthetic sulfonium compounds prepared from phenanthrene 9,10-oxide and N-acetylmethionine showed that the major route of decomposition is to 9-hydroxy-10-methylthio-9,10-dihydrophenanthrene and 9-hydroxyphenanthrene.

This hypothesis is of interest from both mechanistic and practical viewpoints. It should not be inferred that all arene oxide $\longrightarrow$ phenol conversions are accompanied by the formation of methylthio metabolites, but this mechanism may explain the origin of m-bromophenol as a major metabolite of bromobenzene, along with o- and p-isomers. A reexamination of bromobenzene metabolism, essentially as carried out by Lau et al. (1980) but directed to sulfur-series metabolites which are analogs of dihydrodiols, should show methylthiohydroxydihydro metabolites, if proton-catalyzed elimination reactions can be minimized during the isolation process.

Several mechanisms have been proposed for the formation of methylthio metabolites. A 3-thiomethyl metabolite of 2-acetamidofluorene was isolated by the Millers and their associates (DeBaun et al., 1970; Miller et al., 1968; Lotlikan et al., 1966), and was considered to arise from sulfonium ion formation with methionine (possibly in a polypeptide structure). Mizutani et al. (1978) suggested that o-, m- and p-methylthiobromobenzenes (isolated as aromatized metabolites of bromobenzene)

were formed through intermediate sulfonium ions of undefined but not macromolecular structure. The pathways proposed by Mio (1979) and by Tateishi and co-workers (Tateishi et al., 1978; Tateishi and Shimizu, 1980) involved the methylation of thiophenols, and may not apply to arene oxide reactions leading to dihydroaromatic products. Calder et al. (1974) viewed methionine as the source of the methylthio group in the 3-methylthio metabolite of acetaminophen (Klutch et al., 1978; Focella et al., 1972). The methyl group is derived from methionine for metabolites of oxides of aromatic hydocarbons (Stillwell et al., 1978b).

Metabolic switching, in instances where multiple alternative pathways of metabolism are present, is a well established phenomenon (Horning et al., 1976c, 1978, 1979). A decrease in rate in a major pathway may result in a normally minor product becoming a significant metabolite. In the case of arene oxide reactions, a decrease in the rate of glutathione adduct formation, or dihydrodiol formation, might well result in the appearance of products arising from reaction with methionine.

If the appearance of methylthio metablites in significant amounts, along with specific phenols formed via sulfonium ion intermediates, signalizes glutathione depletion, diminished glutathione S-transferase activity, or diminished epoxide hydrolase activity, with the consequent onset of reactions leading to cell damage, it may become possible to monitor the effects of some cytotoxic substances by analyses of blood or urine for methylthio or related metabolites. An absence of these

metabolites would indicate a state of protection against cell damage because of rapid arene oxide metabolism, while the appearance of methylthio or related compounds would indicate a lowered rate of adduct or dihydrodiol formation and inadequate protection. It is not known if the administration of methionine would have a protective effect in these instances through the increased formation of methylthio metabolites.

The results of these experiments demonstrate differences in metabolic response by the young adult male Sprague-Dawley rat and the young adult male Hartley guinea pig when phenanthrene is administered. The two major differences in response in sulfur series metabolites may, however, be due to two entirely different causes. The difference in sulfur-series urinary acids is presumably due largely or entirely to reduced acetyltransferase activity for S-substituted L-cysteine adducts in the guinea pig kidney. The difference in methylthio adduct formation, and 9-phenanthrol formation, may be due to a greater degree of circulating 9,10-oxide in the rat, with respect to the guinea pig, under the condition of these experiments.

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