CONTRASTING PATHWAYS FOR ACTIVATION OF TWO CYCLOPENTAFUSED PAH IN THE AMES ASSAY: IDENTIFICATION OF METABOLITES OF BENZ[j]ACEANTHRYLENE AND CYCLOPENTAFUSED BENZO[e]PYRENE

by

Karyn O. Newcomb
(Under the direction of Dr. L. M. Ball)

ABSTRACT

To determine the contribution of the diol-epoxide to the genotoxicity of benz[j]aceanthrylene (BjAA), the mutagenic activity of BjAA and its bay-region metabolites were evaluated in the Ames assay. BjAA, the 9,10-oxide, and the 9,10-dihydrodiol are indirect acting mutagens with specific activities of 12.9 rev/nmole, 5.6 rev/nmol, and 9.4 rev/nmole, respectively. The 9,10-dihydrodiol-7,8-oxide was direct acting having the same activity as the parent compound, BjAA.

Metabolism studies using Aroclor 1254 induced rat liver S9 were conducted on the BjAA-9,10-dihydrodiol metabolite. Four metabolites were isolated. Three metabolites were identified as 1,2,9,10-tetrahydro-1,2,9,10-tetrahydroxy-BjAA, 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxy-BjAA and the 9,10-dihydroxy-BjAA. The fourth metabolite was tentatively identified as BjAA-9,10-dihydrodiol-2-one.

Similarly, the metabolism of cyclopentafused benzo[e]pyrene (cpBeP) was also investigated to determine whether the previously observed mutagenic activity of this compound was due to metabolism at the bay-region or the etheno bridge. Only one major metabolite was observed, the

3,4-dihydrodiol cpBeP. Additional minor metabolites were isolated, but not enough material was available to identify them.

Oxidation occurs at the cyclopentafused ring for both BjAA and cpBeP when incubated with rat liver S9. This structure is of importance when considering mutagenic activation of a compound, and is an exception to the bay-region theory proposed by Jerina and colleagues.

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I. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants generated in combustion processes (Kamens, et al., 1987; Lo, et al., 1978). Several examples are shown in figure 1. Whether these compounds are potential public health hazards, causing cancer, depends on the chemical dose received, and its distribution within the body (Boulos, et al., 1986; Jeffrey, 1985). The distribution is influenced by the compound's metabolism, and the stability and reactivity of the metabolites produced.

Cytochrome P-450, an enzyme predominantly found in the liver, is known to be involved in xenobiotic metabolism. Studies have indicated that this enzyme oxidizes PAHs forming electrophilic species. These metabolites may then interact with cellular macromolecules, such as protein or DNA. If the damage is not repaired these interactions can ultimately result in tumor formation.

The prediction of biologically active metabolites is directed towards the structural features of PAHs. The relationship between metabolism at the K- and bay-regions, shown in figure 1, and biological activity is known for several compounds, including benz[a]anthracene (BaA) and benzo[e]pyrene (BeP). The mutagenic activity, determined by the Ames assay, indicates that the bay-region metabolites are highly reactive, and lead to the ultimate

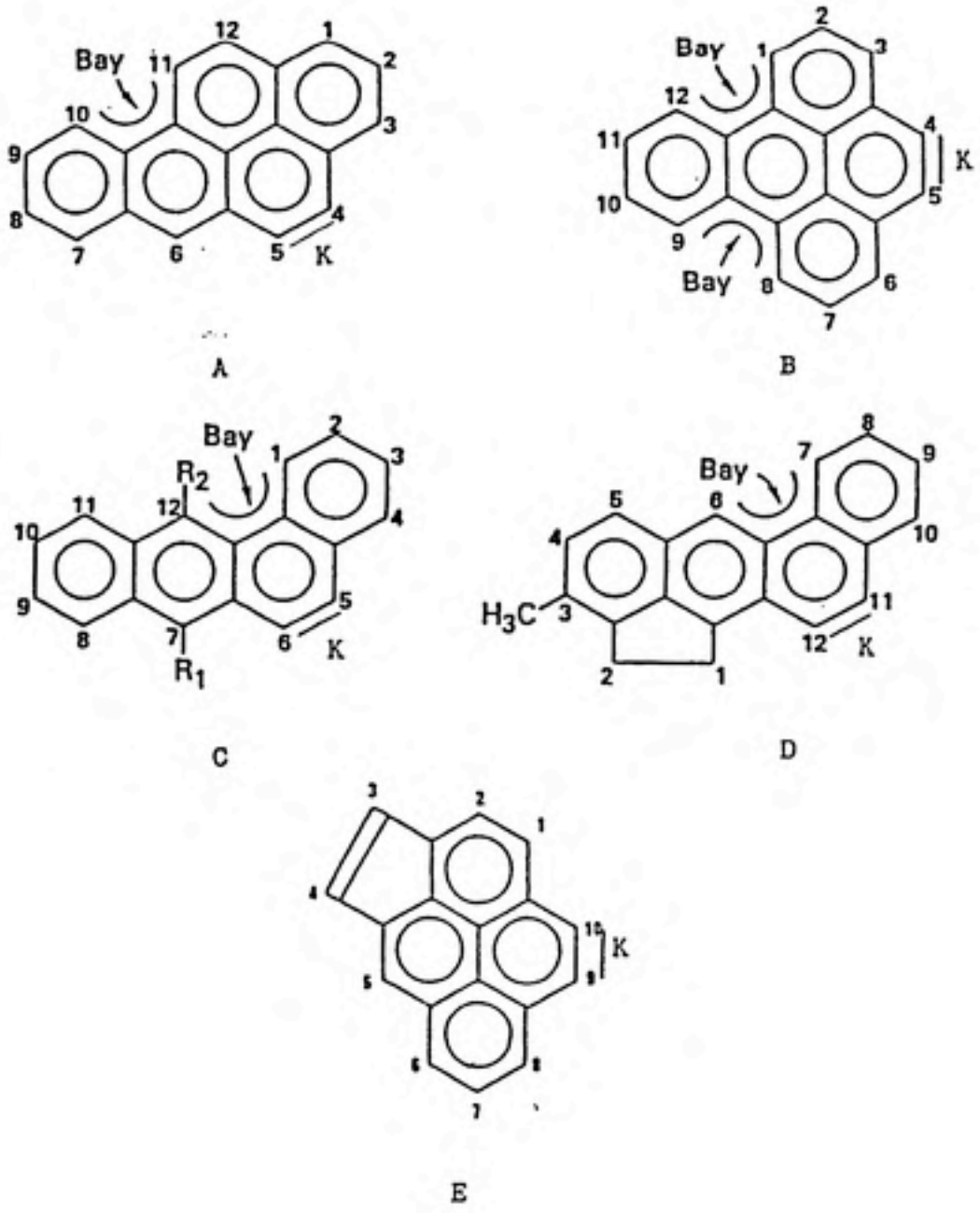
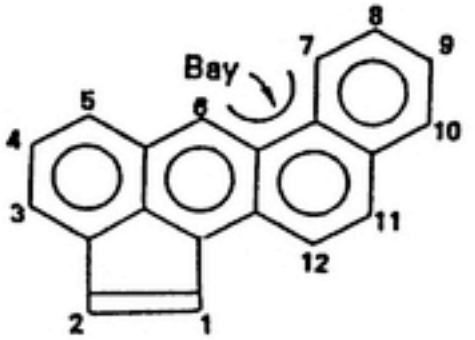


FIGURE 1: The polycyclic aromatic hydrocarbons shown, A) benzo[a]pyrene, B) benzo[e]pyrene, C) benz[a]anthracene, D) 3-methylcholanthrene, and E) cyclopenta[c,d]pyrene, are environmental contaminants found in diesel exhaust and wood smoke (Gold, et al., 1980; Thakker, et al. 1981)

carcinogenic metabolites (Jerina, et al., 1978).

Derivatives of BaA and BeP, benz[j]aceanthrylene (BjAA) and cyclopentafused benzo[e]pyrene (cpBeP), respectively, are shown in figure 2. BjAA and cpBeP are the focus of this study. BjAA is unique in that it has three structural features, the K-region, bay-region and a peripheral etheno bridge. CpBeP has two regions of potential activity, the bay-region and a peripheral etheno bridge. The peripheral etheno bridge or cyclopentafused ring may be subject to electrophilic attack and undergo oxidation, ultimately forming a mutagenic metabolite. The resulting active electrophile could then interact with DNA (Harvey, et al., 1988a). Gold and colleagues (1988) postulated that a reactive intermediate of this ring most likely forms the major metabolic pathway of cyclopentafused compounds.

Studies performed by Nesnow and colleagues (1984) showed that BjAA incubated with Aroclor 1254 induced S9 rat liver microsomes generated several metabolites. The trans-1,2-dihydrodiol was the major metabolite comprising 60% of the total metabolites formed (Nesnow, et al., 1988). The trans-9,10- and 11,12-dihydrodiol were formed contributing to approximately 6% and 3% of the total, respectively. The 10-hydroxy-BjAA was also observed (Nesnow, et al., 1984).



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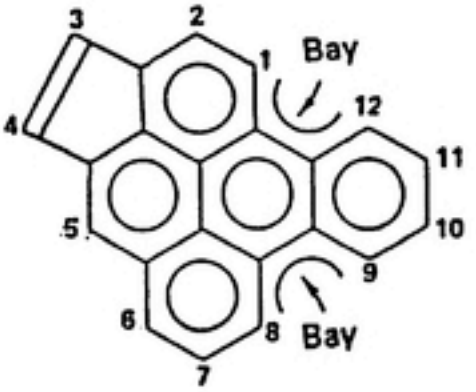

B

FIGURE 2: Benz[j]aceanthrylene, (A), and cyclopentafused benzo[e]pyrene (B).

BjAA metabolism has also been studied in mouse embryo fibroblasts, C3H10T½CL8 cells where this compound had been found to initiate morphological transformation. The major metabolite formed, the BjAA-9,10-dihydrodiol, comprises 55% of the total. The BjAA-1,2-dihydrodiol was only a minor metabolite consisting of 14% of the total metabolites (Mohapatra, et al., 1987). Since the BjAA-9,10-dihydrodiol was so prominant and could be a precursor to the bay-region diol-epoxide, we initiated mutagenicity studies on the bay-region metabolites to determine if this was a possible route of metabolic activation for BjAA.

This study involves mutagenicity testing of BjAA and its bay-region metabolites by the Ames assay to determine if the morphological transformation observed in the C3H10T½CL8 cells could be explained by oxidation of the BjAA bay-region. Metabolism studies with rat liver S9 will be conducted on the BjAA-9,10-dihydrodiol to determine what metabolites may be formed by Aroclor induced rat liver isozymes.

Previous studies by Ball and colleagues (1991) have indicated that cpBeP shows significantly greater mutagenic activity in the Ames assay than BeP. Metabolites of cpBeP generated by rat liver S9 will be identified in this study to determine how the addition of the cyclopentafused ring affects the metabolite profile, which may explain the increase in mutagenic activity observed.

II. BACKGROUND

II.A. ENVIRONMENTAL SOURCES

PAHs have been found in the environment due to natural occurrences such as forest fires. As a result of these fires, PAHs are deposited on the soil, and may contaminate nearby river and marine sediments from water runoff (Lo, et al., 1978). However the major source of PAH contamination occurs through activities of man. Fossil fuels are used for 90% of the nation's energy. Combustion is usually the last process in converting fossil fuels to energy. An inefficient combustion process is a major source of PAH.

Benzo[a]pyrene (BaP), a well studied PAH, is often used as a measure of PAH contamination. Over 1300 tons of this compound were released into the environment per annum in the United States (Levin et al., 1978). The processes that release this particular PAH, in addition to other PAHs are refuse burning, 600 tons BaP, heat and power generation, 500 tons BaP, coke production, 200 tons BaP, and gasoline-powered motor vehicles 22 tons BaP (Boulos, et al., 1986; Lo, et al., 1978).

PAHs are also released in wood smoke and cigarette smoke (Lo, et al., 1978). These compounds are sorbed onto particulate matter present in the air, where they may be

inhaled by humans. This is one route of human exposure.

Another route of human exposure is ingestion. Potable water supplies have been contaminated with PAH present in tar used to coat water pipes. Also, PAHs present in air may deposit onto food stuff, and into soil where plant roots may absorb these compounds. Many foods such as fresh vegetables (2.8-24.5 ppb BaP), coffee (3.9 ppb BaP), and even vegetable oils (0.4-1.3 ppb BaP), contain low concentrations of PAH. PAHs are also generated in cooking, especially in flame and charcoal broiled meats, trace-50.4 ppb BaP (Boulos, et al., 1986; Lo, et al., 1978). Once PAHs are absorbed into the body, they may interact with cellular components after metabolic transformation and are thus said to be biological agents.

An association between cancer and PAHs generated from fossil fuel sources is well documented in epidemiological studies involving coke oven workers, gas works operators and coal liquefaction workers. An increase in cancer incidence has also been documented in urban and industrialized areas with higher energy emissions compared to rural areas (Guerin, et al., 1978).

II.B. CARCINOGENICITY

Chemical carcinogenicity is a multistep process involving three stages: initiation, promotion and progression (Levi, 1987; Weinstein, 1988). The initiating

chemical is an electrophile damaging DNA by causing base substitution, deletions and chromosome translocations, amplifications and transpositions. Although mutations occur at high frequency, they are usually repaired. Unrepaired or misrepaired mutations will result in irreversible damage. Protooncogenes, tumor suppressor genes and transcriptional regulatory genes are involved in controlling normal cell growth and differentiation. These genes are often the genetic targets of the initiation phase (Weinstein, 1988).

Promoters are compounds that act at stages subsequent to initiation. These compounds are generally not electrophilic, and do not interact with DNA. However post-initiation treatment with low concentrations of promoter can induce carcinogenesis at much lower doses of initiator than in the absence of promoter (Levi, 1987). Studies with phorbol esters, a class of compounds that are highly effective as promoters, show that these molecules do not bind to DNA, but bind to membrane-associated receptors producing effects at the epigenetic level (Weinstein, 1988). The effects of promoter binding is reversible at an early stage. Continuous low level exposure is needed, suggesting that there is a threshold level that must be reached in order for the promoter to have an effect.

The effectiveness of a chemical in causing carcinogenesis depends on several factors: the rate of interaction between the chemical and DNA, as well as the

rate of interaction between the chemical and competing cellular nucleophiles, such as RNA or proteins; whether detoxification of the chemical occurs, and to what extent; and the stability of the active intermediate during transport across the membrane and the probability of it reaching a genetic target (Weisburger, 1980).

Brookes and Lawley (Conney, 1982; DePierre, et al., 1978) using several PAHs, including BaP, observed a positive correlation between carcinogenic activity of the compound and the degree to which it bound to DNA. This observation was also confirmed by Buty and colleagues (1976). However no correlation was observed between carcinogenicity and the chemical binding to protein. It is thought that arene oxides of PAHs bind to DNA and transform normal cells into cancer cells (DePierre, et al., 1978).

II.C. STRUCTURE-ACTIVITY RELATIONSHIPS

Understanding the relationship between molecular structure and biological activity would be very helpful in predicting the mutagenic/carcinogenic activity of new compounds. Several theories, the most important being the bay-region theory, were developed to attain this goal.

II.C.1 K-Region Theory

Pullman and Pullman suggested that the reactivity of the molecule was the key in relating chemical structure and carcinogenic activity. Earlier studies by Boyland and

colleagues (Pullman, et al., 1955) demonstrated that metabolic transformation took place at carbons adjacent to the most reactive regions of the molecule. The K-region reacting with a cellular macromolecule caused perhydroxylation of the M-region, figure 3, resulting in dihydrodiols as intermediates in metabolism.

Pullman and Pullman (1955) compared theoretical calculations of bond energies with biological activity of several compounds. For a compound to have carcinogenic activity it needed an active K-region, however this was not sufficient. If the compound also contained a L-region, this region would need to be less reactive or inactive to increase the carcinogenic activity of the compound.

Attempts to show if the K-region arene oxides were proximate carcinogens or ultimate carcinogens, were unsuccessful in the early 1970's (Bresnick, 1976). However, Jerina and colleagues (1978) observed that molecules containing bay-regions usually had a K-region as well, which might explain the correlation between the K-region and biological activity seen by the Pullmans.

II.C.2 Bay-Region Theory

Previous studies have shown that arene oxides, dihydrodiol precursors, were the primary oxidative metabolites of PAHs (Lehr, 1982). Metabolism studies indicated that the BaP-7,8-dihydrodiol is further metabolized to the BaP-7,8-dihydrodiol-9,10-oxide, a highly

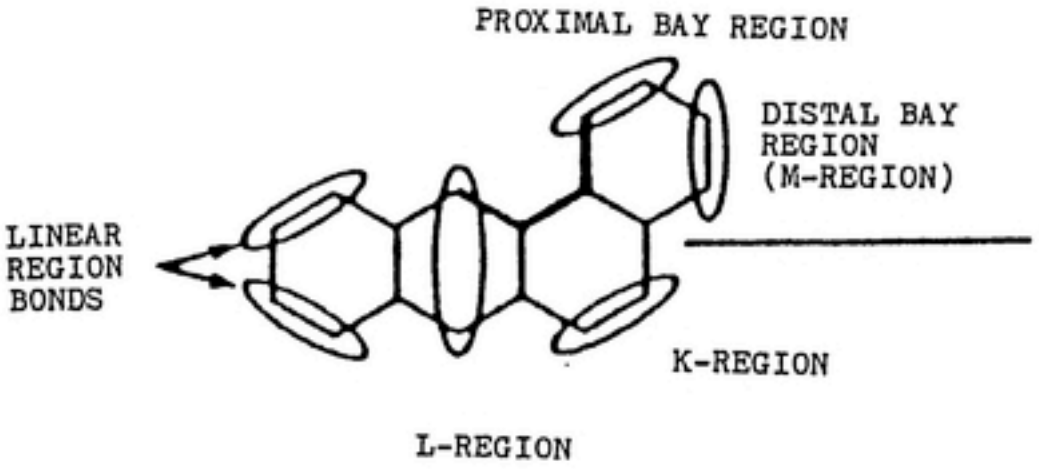

FIGURE 3: The K-, L-, and bay-regions are important in determining structure activity relationships of PAHs (Chu, 1980).

reactive metabolite that binds to DNA (Lehr, 1982; Jerina, et al., 1978; Wood, et al., 1976). The diol-epoxides were highly reactive, and compounds forming this structure display substantial biological activity. Also a unique structural feature was observed for the BaP-7,8-dihydrodiol-9,10-oxide. The epoxide was located on a saturated, angular benzo-ring which formed part of the bay-region of the molecule (Jerina, et al., 1978).

The bay-region became the critical structural feature accounting for the mutagenic/carcinogenic activity of some PAHs (Jerina, et al., 1978; Tsang and Griffin, 1979). Jerina and colleagues (Conney, 1982) who developed the theory were able to predict the proximate and ultimate carcinogenic metabolites of PAH. Examples conforming to this theory are BaA, 3-methylcholanthrene (3-MC), chrysene and dibenzo[a,i]pyrene. However as Tsang and Griffin (1979) observed, the presence of a bay-region oxide may be necessary, but this structural feature is not sufficient for carcinogenic activity as seen from the example, phenanthrene, which contains a bay-region, but is not carcinogenic. Tsang and Griffin (1979) also indicated that cyclopenta[c,d]pyrene (CPP) was shown to be highly mutagenic, but did not contain a bay region.

In the bay-region theory, the resonance stabilization energy for the carbonium ion formed on oxide ring opening, $\overset{+}{\Delta}E_{deloc}/\beta$, is calculated. The most favorable carbonium ion is

usually the benzylic atom on the saturated, terminal ring forming part of the bay region. The larger the value of $\Delta E_{deloc}/\beta$, the more readily the epoxide precursor forms the carbonium ion (Jerina, et al., 1978). The ion appears to be more readily formed for bay-region diol-epoxides, than for non-bay-region epoxides and diol-epoxides. The stability of the carbonium ion formed from the epoxide in the bay-region vs. the non-bay-region may in part account for the biological and chemical activity seen with compounds containing bay-regions. Compounds containing substitutuents and methylene bridges adjacent to the bay-region may increase the stability of the carbonium ion, thereby increasing the activity of the compound (Rice, et al., 1988). The ease of carbonium ion formation as reflected in $\Delta E_{deloc}/\beta$ values may be used in a qualitative ranking of PAHs for carcinogenicity (Jerina, et al., 1978).

Many compounds appear to follow the bay-region theory. However, Jerina and colleagues (1978) suggest caution in applying this theory, since several elements were not considered in the quantum mechanical calculations. Factors not included were the influence of the detoxification of a compound by enzyme systems, and the delivery to and reaction with the critical target or receptor (Jerina, et al., 1978). Tsang and Griffin (1979) also mention that the calculations do not take into account substituents on the saturated or aromatic angular benzo-rings. Experiments have shown that

substituents on the angular benzo-ring tend to decrease the activity of the molecule as a result of inhibiting epoxide formation. Methyl groups in the bay-region on the same angular ring will lower tumorigenic activity (Rice, et al., 1988). However, substituents on other carbons on the molecule may block these sites from metabolism, thereby increasing metabolism at the benzo-ring of the compound (Tsang & Griffin, 1979; Jerina, et al., 1978). Methyl groups at sites other than the angular ring protruding into the bay-region may promote association of the metabolite with DNA or effect the metabolism of the PAH (Harvey, et al., 1988a; Hoffman, et al., 1981; Smithgall, et al., 1988).

II.C.3 Di-Region Theory

Discrepancies in the bay-region theory have been seen concerning the correlation between biological activity and carbonium ion delocalization energies (Lehr, 1982). Benzo[a]naphthacene and dibenzo[a,j]naphthacene are two compounds that are not biologically active, but the calculated delocalization energies are greater than the delocalization energies calculated for BaP. Other exceptions are benzo[c]chrysene and dibenz[a,j]anthracene which exhibit biological activity, but have smaller delocalization energies than compounds that are marginally active or nonactive (Qianhuan, 1985).

In lieu of these short comings of the bay-region theory, Qianhuan (1985) has suggested a theory which takes several regions of the molecule (including the bay- and K-regions) into consideration. The di-region theory suggests that two active centers need to be present in the PAH molecule in order to have complete carcinogenic activity. Figure 4 shows the regions of the molecule considered in this theory. Experimental evidence indicates that the sites of lowest to highest delocalization energies are L, K, E' followed by E.

In addition, Qianhuan (1985) suggested that the optimum distance between the active centers in the molecule should be 2.80-3.00 Å, the distance between two negative centers in the DNA double helix. Experimental evidence has shown that carcinogenic molecules interact with DNA via electrophilic intermediates that have resulted from metabolism of the parent compound. Qianhuan concluded that mutagens such as DMBA (dimethylbenzanthracene) induced cross linking of complementary bases of different DNA strands.

II.D. CYTOCHROME P-450

Cytochrome P-450 is involved in the metabolism of drugs, steroids, pesticides and PAHs (Estabrook, et al., 1978). This enzyme converts hydrophobic compounds to hydrophilic compounds through a variety of reactions such as hydroxylation of the aromatic rings, nitrogen and sulfur

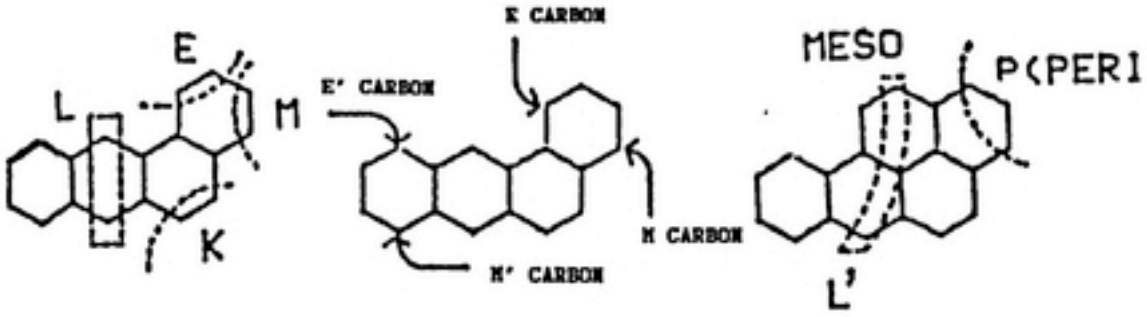

FIGURE 4: The regions shown above are important in the di-region theory. This theory suggests that two active centers need to be present in the molecule in order to have complete carcinogenic activity (Qianhuan, 1985).

oxidation, nitrogen, sulfur and oxygen dealkylations, oxidative deamination and nitrogen reduction to name a few (Yang, et al., 1987; Hodgson, 1987).

Cytochrome P-450 monooxygenase system (MO), or mixed function oxidases (MFOs), consists of two enzymes, NADPH cytochrome P-450 reductase and cytochrome P-450, the latter enzyme contains a heme group (DePierre, et al., 1978). The MFOs are found predominantly in the endoplasmic reticulum of the liver, but are also present in lower concentrations in the kidney, brain, and cells near portals of entry in the lung, intestine and skin (Hodgson, 1987). The name P-450 was derived from the reduced form of the carbon monoxide derivative which has an absorption maximum at 450 nm. Separation and purification of many isozymes of cytochrome P-450 has been accomplished. The absorbance maximas of the CO bound reduced form have ranged from 447-452 nm. These isozymes differ in structure and show independent and overlapping specificities for various substrates (DePierre, et al., 1978).

A well known scheme of cytochrome P-450 catalyzed reactions is shown in figure 5, although the intermediates in some of the steps have not been completely elucidated (Estabrook, et al., 1978). The oxidized form of cytochrome P-450 (FeIII) interacts with the substrate to form an enzyme-substrate complex. The substrate is thought to bind

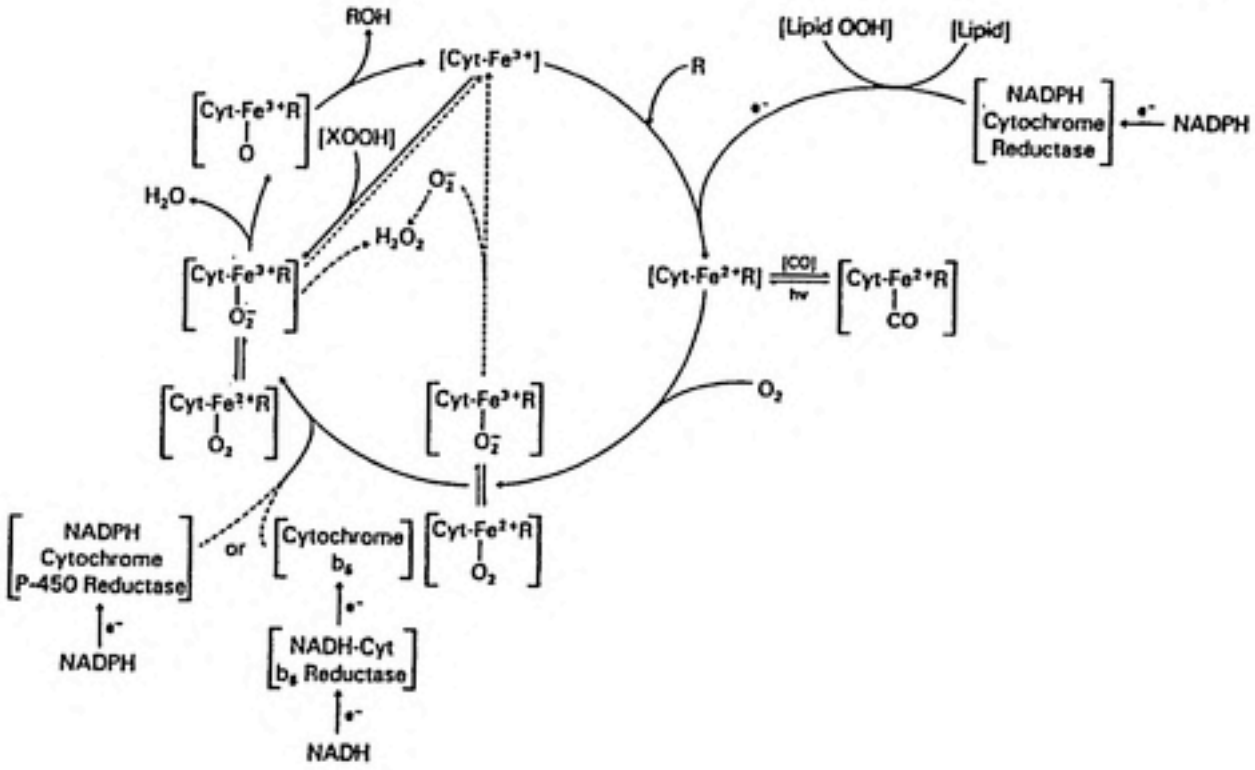

FIGURE 5: The schematic indicates the events that occur for cytochrome P-450 monooxygenations. This enzyme is involved in the oxidation of PAHs, and the formation of the ultimate carcinogenic metabolite (Hodgson & Levi, 1987; p. 55).

loosely at the heme moiety. A series of oxidation-reduction reactions then occur. First, one electron is transferred from NADPH cytochrome P-450 reductase to the enzyme-substrate complex. Molecular oxygen combines with the reduced enzyme-substrate complex. Transfer of a second electron from NADPH or NADH cytochrome b_5 reductase results in reductive cleavage of the oxygen-oxygen bond, yielding H_2O and an oxygen atom which is transferred from the iron center to the substrate. The enzyme and oxidized substrate then dissociate, restoring the enzyme to the ferric form (Hodgson, 1987).

Experiments performed by Conney and colleagues (1982) involved the treatment of rats with benzopyrene prior to exposure to several other organic compounds. They showed that the liver microsomal enzymes responsible for hydroxylation of benzopyrene, hydroxylated some of the organic compounds tested, but not all of them. Additional studies (Conney, 1982) showed that purified hepatic cytochrome P-450 of control rats and rats treated with methylcholanthrene and phenobarbitol, in the presence of excess NADPH cytochrome P-450 reductase exhibit different substrate specificities. These results suggest that isozymic monooxygenases are present in the liver.

In fact, we now know that many isozymes of cytochrome P-450 exist, and they have overlapping substrate specificities. These cytochromes, as suggested in Conney's

studies above, are inducible. Whitlock and colleagues (1973) have shown that PAHs stimulate synthesis of the enzyme at the level of transcription and that protein synthesis is also controlled by a regulatory protein. Buty and colleagues (1976) demonstrated with several PAHs, including BaA, BaP and 3-MC, that induction of the MFO system, and tumor initiating capability of a compound were unrelated. As a result, the generation of PAH induced cytochrome P-450 has become a very useful tool in the study of carcinogenesis.

II.E. PAH METABOLISM

Epoxide formation occurs by the addition of an oxygen atom catalyzed by cytochrome P-450, to an arene bond. In 1950 Boyland and colleagues (Grover, 1986) proposed that dihydrodiols, phenols and mercapturic acids were formed from epoxide precursors. Jerina and colleagues showed that napthalene oxide was the necessary intermediate in the metabolism of napthalene to napthol, diol and the glutathione conjugate (DePierre, et al., 1978). Other studies with phenanthrene, BaA and BaP also support these results (Grover, 1986).

Arene oxides may occur in the bay-region. Results from molecular orbital studies indicate that the distal region carbon-carbon bond has greater electron density than the proximal region bond (figure 3), and therefore favors arene

oxide formation. Depending on the stability and reactivity of the metabolite and its affinity to the enzyme, the substrate may undergo a variety of reactions as shown in figure 6. The epoxide may undergo hydrolysis via epoxide hydrolase to form a trans-dihydrodiol (Path A); conjugation with glutathione (Path B) and be detoxified; or rearrangement to a phenol (Path C) and be detoxified by subsequent conjugation with glutathione (Chu, 1980; Kadlubar and Hammons, 1987). It has also been demonstrated that arene oxides of BaP, BaA and 3-MC may be reduced back to the parent hydrocarbon when incubated with rat liver microsomes. This process is NADPH dependent and inhibited by oxygen (DePierre, et al., 1978).

The trans-dihydrodiol is the key product in the metabolic pathway. This molecule may be detoxified by conjugation with glucuronic acid (Path A2), or be reduced to a catechol and subsequently oxidized to a quinone (Path A3). The trans-dihydrodiol may also form the ultimate carcinogen, the diol-epoxide (Path A1), as a result of further oxidation by cytochrome P-450. This metabolite may still be detoxified via glutathione conjugation. However diol-epoxides are of concern, since an increase in stability of the carbocation enhances the carcinogenicity of the molecule, and its liklihood of binding to DNA (Chu, 1980; Kadlubar and Hammons, 1987) and microsomal and nuclear protein (Bresnick, 1976).

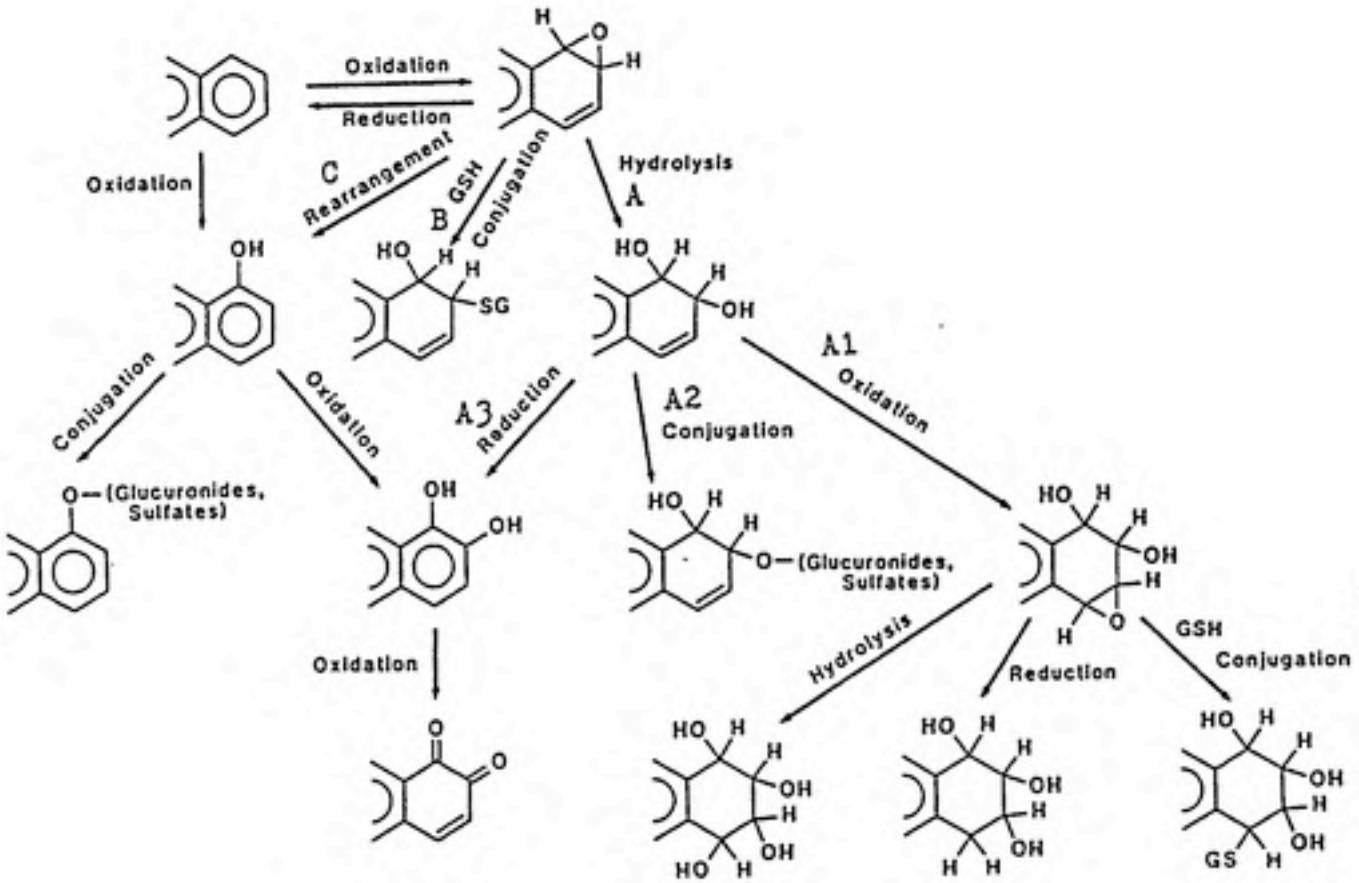

FIGURE 6: The diol-epoxide is a very reactive intermediate in PAH metabolism. This diagram demonstrates several pathways leading to the detoxification and production of the ultimate carcinogen upon formation of the diol-epoxide (Kadlubar, et al., 1987; p.92).

Additional studies by Grover and Sims, and independently by Heidelberger (1975), demonstrated that epoxides bind more readily to cellular macromolecules than the corresponding parent hydrocarbon or dihydrodiol metabolite. Grover and Sims (Heidelberger, 1975) from earlier studies showed that K-region epoxides of DMBA and phenanthrene reacted covalently with DNA and histones. Grover and colleagues (DePierre, et al., 1978) also showed that K-region epoxides were formed after incubation with lung microsomes from 3-MC treated animals. These epoxides reacted with glutathione (Grover, 1986). Dihydrodiol and phenol metabolites have also been observed (DePierre, et al., 1978). Other studies have shown epoxides to be electrophilic, cytotoxic, mutagenic and capable of inducing cell transformation. However the potency of these epoxides is still less than their parent compounds (Kadlubar and Hammons, 1987).

Mechanisms proposed for phenol formation are isomerization of epoxides, dehydration of dihydrodiols, breakdown products of dihydrodiol conjugates, and reactions between water and radical cations. Although few examples were cited for these proposals, the most popular mechanism was the spontaneous isomerization of epoxides to phenols (Grover, 1986). The high electron density of the L-region, and the ease of formation of phenols, increases the

probability of quinones occurring in this region, and thereby detoxifying the compound (Miller, et al., 1974).

II.E.1. Benzo[a]pyrene

Benzo[a]pyrene was extensively studied to determine its metabolites, and their mutagenicity and carcinogenicity, figure 7 (Conney, 1982). In the presence of purified hepatic cytochrome P-450, BaP metabolites that were generated consisted of phenols and quinones. Upon the addition of epoxide hydrolase, three trans-dihydrodiols, 4,5-, 7,8-, and 9,10- appeared, decreasing the concentration of phenolic metabolites. The BaP-4,5-oxide metabolite was present in the initial incubation, appearing on the chromatogram. After epoxide hydrolase was added, BaP-4,5-oxide was no longer observed. Conney and colleagues (1982) reported that the metabolite was a potent mutagen, producing a low incidence of tumors when applied to mouse skin and did not exhibit a dose-response relationship (Wood, et al., 1976). This metabolite was demonstrated to be a weak tumor initiator as well. Although BaP-4,5-oxide is a potent mutagen, in the presence of epoxide hydrolase and glutathione-s-transferases, deactivation of the metabolite probably occurs at a rate which limits the interaction between the compound and the genetic material of the skin (Wood, et al., 1976).

Further work varying the ratio of cytochrome P-450 and epoxide hydrolase resulted in changes to the profile of

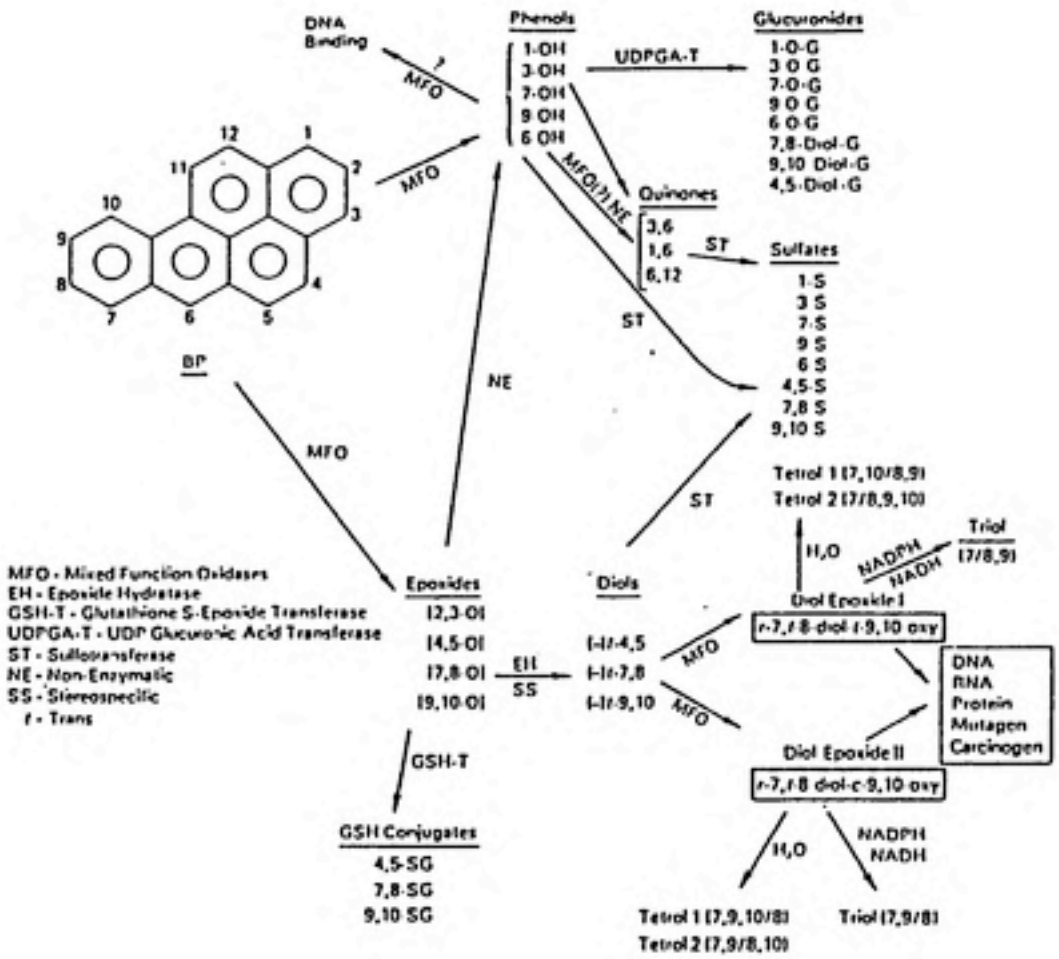

FIGURE 7: The metabolism of benzo[a]pyrene by cytochrome P-450 and other enzymes located in the liver (Yang, et al. 1978; p.210).

oxides and dihydrodiols (Conney, 1982). In the presence of epoxide hydrolase, the epoxide gave rise to dihydrodiols, while phenols were formed in the absence of epoxide hydrolase. In addition, this evidence indicates that epoxide hydrolase is involved in the detoxification pathway as well as in the formation of ultimate carcinogens.

Weak mutagenic activity was seen for the 7,8- and 9,10-oxides. However the 7,8-dihydrodiol-9,10-epoxide exhibited the highest mutagenic activity of all the metabolites of BaP in S. typhimurium TA98 and TA100 and mammalian cell assays. In the bacterial strains, the 7,8-dihydrodiol-9,10-epoxide-BaP 2 (syn isomer) was a more potent mutagen than the 7,8-dihydrodiol-9,10-epoxide-BaP 1 (anti isomer). The opposite result was observed in mammalian cell assays (Conney, 1982; Wood, et al., 1976). Unlike the 4,5-dihydrodiol, the 7,8-dihydrodiol was shown to be a potent tumor initiator in mouse skin. The strong carcinogenic activity of the 7,8-oxide and 7,8-dihydrodiol is a result of the conversion to the highly reactive 7,8-dihydrodiol-9,10-oxide (Conney, 1982).

Epoxides of BaP are the key metabolites involved in DNA binding (Singer, et al., 1983). The first evidence of metabolic activation of PAH was reported by E. C. Miller in the early 1950's (Conney, 1982). Application of BaP to mouse skin resulted in covalent binding of BaP metabolites to protein. Guengerich and colleagues (1989) observed that

human liver microsomes can oxidize the 7,8-dihydrodiol to metabolites that bind to DNA. Weinstein's group (Conney, 1982) demonstrated that the BaP-7,8-dihydrodiol-9,10-oxide isomer 2 was shown to bind to DNA in cultured cells. This result was also demonstrated by Sims and colleagues (Conney, 1982). In addition Weinstein's group reported that the BaP-7,8-dihydrodiol-9,10-oxide isomer 1 bound to a greater extent than isomer 2; and that the major adduct formed was a covalent bond between isomer 1 and the N2 position of guanine (Conney, 1982; Singer, et al., 1983). Minor DNA adduct products have been detected as well. BaP metabolites were observed to bind at the N7-guanine, O6-guanine, N6-adenine and the N4-cytosine positions within the DNA (Osborne, 1985).

II.E.2. Benzo[e]pyrene

Benzo[e]pyrene is a symmetrical hydrocarbon with a K-region and two identical bay-regions. Little to no tumorigenic activity was seen in mice or rats for this compound (Conney, 1982; Jacob, et al., 1985). The main metabolite formed is the trans-4,5-dihydrodiol in rat liver homogenates, figure 8, and embryonic cells of mice and hamsters. This compound is also the predominant metabolite formed in untreated rat liver microsomes. The 3-hydroxy- and 4-hydroxy-BeP have been identified as well. The putative proximate carcinogen, the trans-9,10-dihydrodiol, was shown to be a minor metabolite in hamster embryo cells

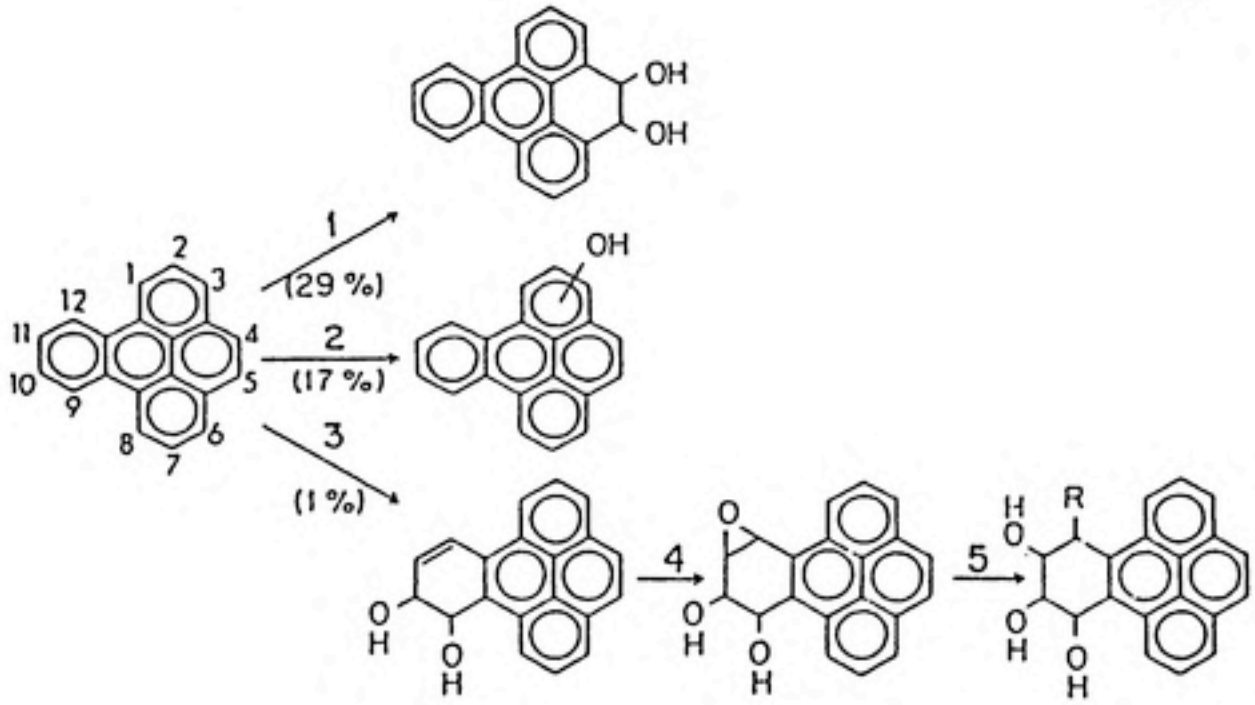

FIGURE 8: Benzo[e]pyrene predominantly forms the K-region dihydrodiol when incubated with methylcholanthrene induced hepatic microsomes; only a small amount, 1%, of the proximate carcinogen, the 9,10-trans-dihydrodiol, is formed (MacLeod, et al., 1980).

and rat liver microsomes (Jacob, et al., 1985). This metabolite was usually further metabolized to the 4,5,9,10-tetrahydro-4,5,9,10-tetrahydroxy-BeP, and not the ultimate carcinogen, the 9,10-dihydrodiol-11,12-oxide. Incubation of BeP with uninduced rat and human liver microsomes, resulted in 1% and 12% yields of the 9,10-dihydrodiol, respectively (Jacob, et al., 1985).

The 9,10-dihydrodiol-11,12-oxide was made synthetically, and its mutagenic activity was tested. The actual mutagenic activity of this compound was lower than expected from the quantum mechanical calculations (MacLeod, et al., 1980). This weakly active ultimate carcinogen can increase the number of pulmonary tumors in mice and the number of hepatic tumors occurring in male mice (Conney, 1982).

Many investigators have attempted to determine why BeP has low mutagenic activity compared to BaP. MacLeod and colleagues (1980) answered this question by comparing the metabolism rate of the two compounds. When both compounds were incubated with purified or reconstituted mixed function oxidase systems, they had comparable metabolism rates. These results indicated that BeP could be used by the mixed function oxidase system as a substrate. MacLeod and colleagues concluded that the initial site of oxidation was different. BaP oxidation resulted in formation of dihydrodiols in the bay-region (7,8 or 9,10 positions),

whereas BeP oxidation resulted in formation of dihydrodiols in the K-region (4,5 position). PAH induced cytochrome P-450 incubated with BeP resulted in a slightly greater yield of the BeP-9,10-dihydrodiol. The low yield of this bay-region metabolite precursor may explain the low mutagenic and carcinogenic activity of this compound (Jacob, et al., 1985).

II.E.3 Cyclopenta[c,d]pyrene

The bay-region theory does not account for the biological activity of this compound, since it does not possess a bay-region. The five-membered ring is highly susceptible to epoxidation due to the olefinic nature of the double bond, and $\Delta E_{deloc}/\beta$ of the C3 carbonium ion resulting from epoxide ring opening is identical to that from BaP diol-epoxide (Eisenstadt, et al., 1978).

CPP was tested over the standard spectrum of S. typhimurium strains used in the Ames assay. The specific activity was 174 rev/nmole in TA1537, and the amount of S9 needed to produce an effect in this assay was approximately one-tenth of the amount needed for BaP (Eisenstadt, et al., 1978). However, unlike the ultimate carcinogen of BaP, a dramatic decrease was observed in the mutagenicity of CPP upon addition of epoxide hydrolase (Wood, et al., 1980).

The predicted ultimate carcinogen of CPP is the 3,4-oxide. This metabolite is direct acting and highly reactive in the Ames assay (Wood, et al., 1980). In strain

TA100, the specific activity of this metabolite was calculated to be 1440 rev/nmole (Gold, et al., 1980a).

The CPP-3,4-oxide transforms C3H10T½CL8 cells, but with less activity than BaP. This assay is a morphological transformation assay which has been documented to respond to chemical carcinogens, including PAHs (Gold, et al., 1980b). The 3,4-oxide is a weak tumor initiator, similar in activity to BaA (Wood, et al., 1980), but only one-twentieth as active as BaP (Raveh, et al., 1982).

Since the activity of CPP cannot be explained by the formation of a bay-region diol epoxide, Eisenstadt and Gold (1978) suggested the activity may be governed by the formation of a stable benzylic carbonium ion. The resulting electrophile would interact with cellular macromolecules yielding biological activity (Eisenstadt, et al., 1978; Gold, et al., 1980a).

II.E.4. Benz[a]anthracene

Based on the bay-region theory, Jerina and colleagues predicted the proximate and ultimate carcinogenic metabolites of BaA to be the 3,4-dihydrodiol and the 3,4-dihydrodiol-1,2-oxides 1 and 2, respectively (Conney, 1982; Wood, et al., 1976). Incubation of BaA with purified hepatic cytochrome P-450 and epoxide hydrolase resulted in the trans-5,6- and 8,9-dihydrodiols as the major metabolites, and the trans-3,4-dihydrodiol as a minor metabolite (Conney, 1982). Also, small amounts of the 1,2-

and 11,12-dihydrodiols have been observed (Singer, et al., 1983). The 3,4-dihydrodiol was at least 10 times more mutagenic than BaA and the other trans-dihydrodiol metabolites in S. typhimurium strain TA100 with S9 activation (Conney, 1982).

BaA is a weak skin tumor initiator, and noncarcinogenic in many in vitro cell transformation systems and in vivo animal tumorigenesis assays (Slaga, et al., 1978). When tested as a tumor initiator in mouse skin, the 3,4-dihydrodiol was shown to be 5-10 times more active than BaA and the trans-1,2-, 5,6-, 8,9- and 10,11-dihydrodiols of BaA (Conney, 1982; Slaga, et al., 1978). Since the 3,4-dihydrodiol is distal to the bay-region the high level of activity of this metabolite can be explained by secondary metabolism to the bay-region diol-epoxide (Wood, et al., 1976).

The 8,9-dihydrodiol-10,11-oxide 2 (anti isomer) and the 3,4-dihydrodiol-1,2-oxide 2 (anti isomer) were shown to bind covalently to DNA when BaA was applied to mouse skin. Both dihydrodiols react with the 2-amino group of guanine (Singer, et al., 1983). The 8,9-dihydrodiol-10,11-oxide also reacted with deoxyadenosine (Hemminiki, 1980). The 3,4-dihydrodiol-1,2-oxide isomer 2 has greater mutagenic and carcinogenic activity of the two metabolites, and appears to be a tumor initiator, more tumorigenic than BaA, initiating lung and skin tumors in mice (Conney, 1982, Slaga, 1978).

However both metabolites are probably responsible for the biological activity of BaA (Hemminiki, 1980). Singer and Grunberger (1983) state the significance of the mutagenic activity of the 8,9-dihydrodiol-10,11-oxide is not clear; and that its activity is a failure of the bay-region theory to predict all the metabolites that may bind to DNA.

II.E.5. 3-Methlycholanthrene and 3-Methylcholanthrylene

3-Methylcholanthrene is similar to BaA with the exceptions of a methyl group at the 3 position, and a saturated 5-membered ring fused between positions 7 and 8 of BaA. When this compound was incubated with cytochrome P-450 and epoxide hydrolase, the two major metabolites formed were the 1-hydroxy- and the 2-hydroxy-3-MC. Only a small amount of the proximate carcinogen, the 9,10-dihydrodiol, was formed (Conney, 1982; Singer, et al., 1983; Jacobs, et al., 1983). In contrast, Li and colleagues (1983) have claimed that the 9,10-dihydrodiol is one of the major metabolites. When the 1-hydroxy-, 2-hydroxy- (Conney, 1982) and the 2-oxo-3-MC (Shou, et al., 1990) were incubated with cytochrome P-450 and epoxide hydrolase, a larger amount of the corresponding 9,10-dihydrodiol was formed. Additional metabolites formed included the 4,5-, 7,8-, and the 11,12-trans-dihydrodiols and 1,2-diol (Singer, et al., 1983).

In S. typhimurium strain TA98 with S9, the 1-hydroxy- was 10 times more mutagenic than 3-MC, the 3-MC-2-one showed greater activity than 3-MC, and the 2-hydroxy- had similar

activity as 3-MC. The 11,12-dihydrodiol was inactive (Wood, et al., 1978). The 9,10-dihydrodiol was the most mutagenic of the 3-MC metabolites in both bacterial and mammalian systems. This metabolite was a more active tumor initiator than the 4,5-, 7,8-, and 11,12-dihydrodiols (Conney, 1982), and had similar activity to 3-MC (Shou, et al., 1990).

The 9,10-dihydrodiol-7,8-oxide was the ultimate carcinogen formed from 3-MC (Conney, 1982; Jacobs, 1983; Li, et al., 1983). Eight major DNA adducts and one minor adduct were isolated by HPLC from mouse skin incubated with ^{3}H-3-methylcholanthrene. The large number of adducts are most likely due to the 9,10-dihydrodiol-7,8-oxide and the hydroxylated derivatives that were isolated in the metabolism studies (Singer, et al., 1983). DNA adducts covalently bound at either the 7, 8, 9 or 10 positions of the hydrocarbon, similar to BaP, were observed in cultured mouse embryo cells and mouse skin (Osborne, 1985).

Osborne (1986) identified two of the DNA adducts from the incubation of hamster DNA with the 9,10-dihydrodiol-7,8-oxide. The metabolite binding at the N2 position of guanine was the major adduct. The second adduct, a minor product, resulted from the diol-epoxide binding at the 7 position of guanine. Incubation of the ultimate carcinogen with RNA isolated from E. coli resulted in both guanosine and adenosine adducts (Osborne, 1985).

3-Methylcholanthrylene contains a double bond at the 1 and 2 positions. This compound is a minor metabolite of 3-MC, and a potent mutagen in the Ames assay in both S. typhimurium TA98 and TA100. 3-Methylcholanthrylene may be further metabolized into 3-methylcholanthrene-2-one, a known carcinogen, figure 9 (Yang, et al., 1990). Yang and colleagues (1990) also predict that the potential ultimate carcinogenic metabolite of 3-MC and 3-methylcholanthrylene is the 3-methylcholanthrene-1,2-oxide, having greater carcinogenic activity than BaP or BaA.

II.F. ANALYTICAL

II.F.1. Ames Mutagenicity Assay

In an attempt to correlate mutagenesis with carcinogenesis, specifically cancer as a result of somatic mutations, Dr. Bruce Ames developed an assay with a genetically altered bacterial strain of S. typhimurium (Ames, et al., 1975). Originally three strains of S. typhimurium TA1535, TA1537 and TA1538, all histidine auxotrophs, were employed in standard screening assays to detect base pair substitution and frameshift mutations. TA1535, predecessor to TA100, contains a mutation in the hisG gene for the first enzyme of histidine biosynthesis. DNA sequence analysis performed by Barnes and colleagues (Maron, et al., 1983) indicated that the amino acid proline was substituted for leucine in the "wild type" gene. These

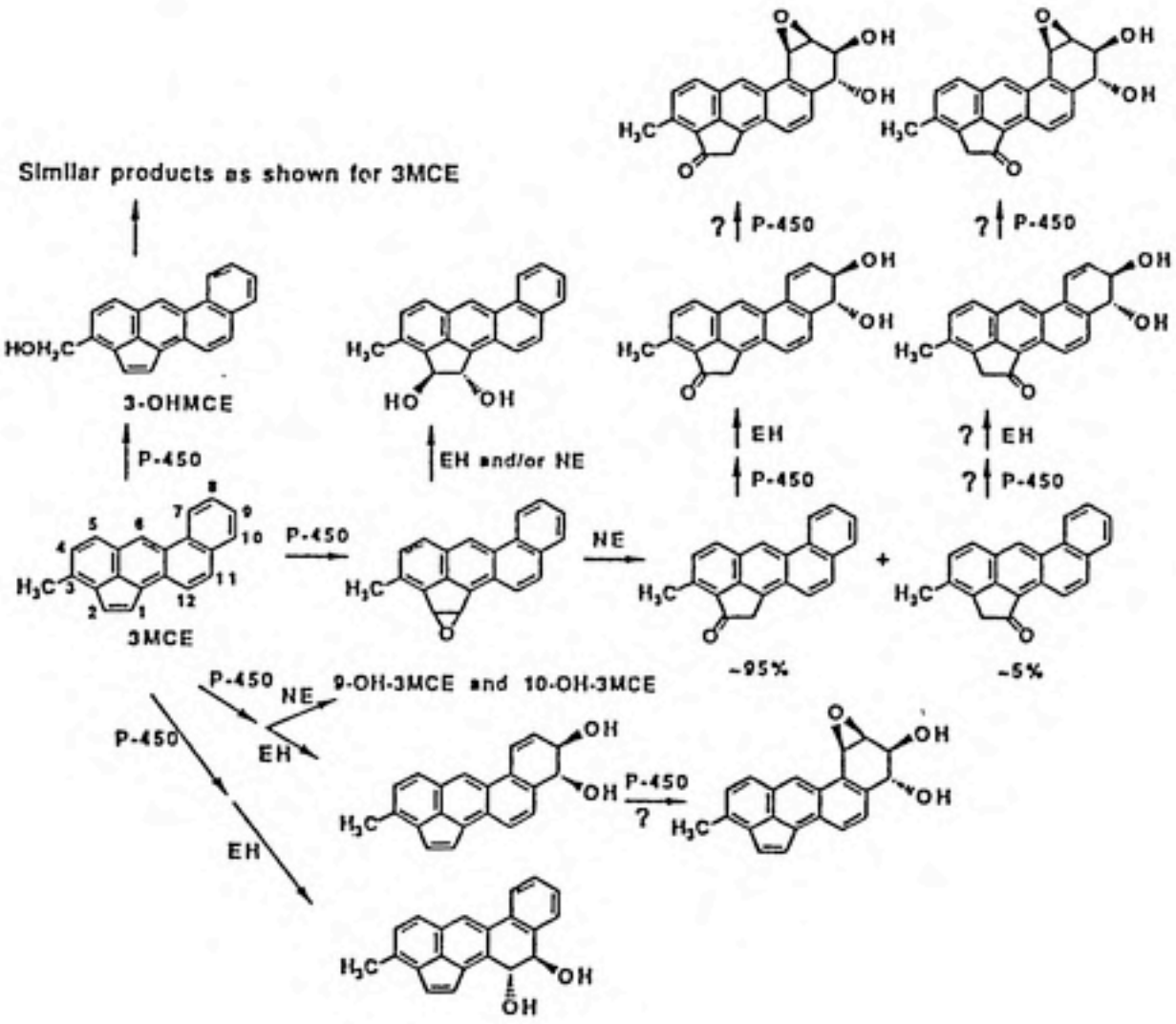

FIGURE 9: A proposed pathway by Yang and colleagues (1990) for the metabolism of 3-methylcholanthrene by rat liver microsomes.

strains detect compounds causing base pair mutations mostly at G-C pairs. In strain TA1538, predecessor to TA98, the hisD3052 mutation is a -1 deletion, near a sequence of eight repetitive GC residues. The correction in the misreading of the nucleotide sequence is termed frameshift and results in histidine synthesis. Strain TA1537 also detects frameshift mutations.

Strains TA100 and TA98 differ from their respective parent strains by the addition of an R factor plasmid, pkm101. The plasmid increases the sensitivity of the bacteria to mutagens by enhancing an error prone DNA repair system. A gene coding for ampicillin resistance is also present on the plasmid, and is used in determining the presence of the plasmid (Claxton, et al, 1982).

The culture grown for the assay is made from a single colony picked from a master plate made from frozen stock cultures. The S. typhimurium contain two additional mutations, uvrB and rfa, whose presence is also verified before use. An increase in sensitivity of the bacterial strain results from a deletion in the gene coding for DNA excision repair, uvrB, which prevents mutations from being repaired. The deletion extends through the biotin gene, therefore biotin is also needed for growth. The absence of growth after exposure to ultraviolet light (UV) is indicative of the presence of the gene (Claxton, et al., 1982; Maron, et al., 1983).

The permeability of the cell membrane to larger molecules results from the rfa mutation, a partial loss in the lipopolysaccaride layer in the cell membrane. The presence of a zone of inhibition around a crystal violet soaked filter paper added to freshly plated cells, suggests the crystal violet is entering the cells and killing them; thereby indicating the presence of the rfa mutation (Claxton, et al., 1982; Maron, et al., 1983).

Whether the test compound is a direct acting mutagen, interacting with cellular components, or an indirect acting mutagen, requiring metabolic activation prior to interacting with cellular components, can be determined in the Ames assay. The S9 (9000g supernatent) liver homogenate, containing cytochrome P-450 mixed function oxidases and cytosolic enzymes may also be added to the top agar with the test compound. The liver is induced with a compound similar to those tested to increase the specific cytochrome P-450 isozymes that are responsible for the compounds metabolism. Aroclor 1254, a polychlorinated biphenyl mixture, is quite commonly used in the induction of cytochrome P-450 mixed function oxidases, since it induces a broad-range of P-450 isozymes. This PAH has been shown to increase the hepatic cytochrome P-450 concentration 45-60 fold (Conney, 1982). Hamster hepatic S9 preparations appear to be the most efficient systems for activation of aromatic amines, nitrosamines and many PAH (Phillipson, et al., 1989).

The procedure for the Ames assay is very straightforward. The test compound, culture, S9 and other agents to be tested are added to melted top agar pre-cooled at 45° C. The mixture is quickly poured into a petri dish containing solidified minimal agar. The test compound is added to the top agar, since it will not readily diffuse through solid agar. The agar contains only a trace amount of histidine to initiate cell growth. A faint background lawn appears on the plate, and observation of this lawn is one method of monitoring bacterial integrity in the assay. A mutation needs to occur in the histidine gene, resulting in histidine prototrophy, in order for colonies to form on the plate. Plates in the assay are done in duplicate and incubated at 37° C for 48 hours. Standards used in the assay with TA98 are 2-anthramine, as a positive control with S9 for indirect activity, and 2-nitrofluorene as the positive control for direct acting activity (Claxton, et al., 1982).

Correlation of mutagenicity with carcinogenicity has often been attempted on the basis of the Ames assay. Positive mutagenic results imply that the test compounds are possible animal or human mutagens/carcinogens. When Ames first developed the test in the 1970's, 85-90% of the carcinogenic compounds tested were mutagenic (Ames, et al., 1975). In 1983 further testing by the Ames group indicated that 83% of the carcinogenic compounds tested were mutagenic. Tennant and colleagues (Tennant, et al., 1987)

attempted to determine which short term tests singly or in combination would yield results most similar to large scale animal assays. None of the four short term tests: Ames assay, sister chromatid exchanges, mouse lymphoma cell mutagenesis or chromosome aberration proved to be superior. The Ames assay is often chosen not only because it is inexpensive, widely available or technically easy, but also for the sizable literature available. The Ames assay results indicated that 62% of the known carcinogenic compounds tested (by rodent assays) were mutagenic. Unfortunately, of the other three tests, none was found to be complementary to the Ames assay. The Ames assay was the least sensitive of the four tests. The inclusion of all four assays in a battery of tests would only increase the correlation of mutagenicity to carcinogenicity to 67%. Although a short term assay with the above advantages would be helpful in replacing the high cost, labor intensive animal studies, a single short term assay cannot adequately test the many mechanisms involved in carcinogenesis. A battery of short term assays may be inadequate in testing these mechanisms as well (Tennant, et al., 1987).

II.F.2. High Pressure Liquid Chromatography

High pressure liquid chromatography (HPLC) is used to separate mixtures of less volatile compounds between two phases, the stationary phase and the mobile phase. The sample interacts with the stationary phase, the column, and

is retained until a suitable mobile phase or solvent is run through the column. Several types of chromatography exist for HPLC, reverse or normal phase are the most common. Reverse phase consists of a nonpolar material for the stationary phase and a polar mobile phase, normal phase chromatography is the opposite of reverse phase. In separating PAH mixtures reverse phase chromatography is used and the more polar PAHs are eluted first from the column.

Column packing material for reverse phase columns often consist of octadecylsilane, also known as ODS or C18, an alkane with 18 carbons. However columns containing eight carbons or shorter alkyl chains may also be used. The stationary phase is chemically bonded to silica as a support.

HPLC can give both qualitative and quantitative information. The retention time comparison between a standard and unknown, or co-elution of a standard with an unknown may be helpful in determining structures of unknown compounds. Calibration curves of detector response made with standards of known concentrations may also be used to determine the concentration of the unknown, since peak area is proportional to the amount of sample injected (Meyer, et al., 1988).

II.F.3. Mass Spectrometry

Mass spectrometry (MS) is used to determine the molecular weight of a compound, and may also provide useful

mass spectrometer. Nonvolatile molecules are often retained in the mist. The mist droplets need to have an initial charge which effects the ionization of the compound during rapid vaporization. With thermospray injection, ionization occurs during the volatilization of the solvent and the sample.

When the vaporized sample enters the mass spec, it is bombarded by an electron beam. Energy from the electrons is transferred to the sample. Generally, the sample has enough energy to eject an electron, resulting in a residual positive charge. This species is termed the molecular ion and denoted as M^+ or $M^\cdot$ (Watson, 1985).

II.F.4. Nuclear Magnetic Resonance Spectrometry

Nuclear magnetic resonance is useful in determining the structure of an unknown compound, for instance in metabolism studies, where oxidation may have occurred on the molecule. An ideal sample should contain at least a 3 mM concentration (Bovey, et al., 1988). However for samples that are of very low concentrations, multiple scans are accumulated, stored in the computer and compiled to obtain the final spectrum (Harris, 1986).

The sample is dissolved in a deuterated solvent such as acetone, methanol or chloroform and then placed in the homogeneous part of the magnetic field, and the molecules undergo Larmor precession (Bovey, et al., 1988). The resonance of the protons are independent of the solvent the

structural information from the fragmentation pattern. This technique requires only nanomoles of sample. Ionization, 70 eV, by electron impact is one of the important techniques applied in MS analysis (Watson, 1985).

Sample injection into the mass spectrometer may be achieved by a variety of methods. A direct probe, where the sample is placed in a glass ampule mounted on the end of a probe is one method. The probe is then placed against the ionization chamber wall, when heated, sample vapors travel a short distance to the ionization chamber. The short distance between the probe and ionization chamber is the primary advantage of the direct probe sample inject. However it is important that the sample be relatively free of contaminants to obtain a clean spectrum (Watson, 1985).

Instruments designed to clean up samples have been interfaced with mass spectrometers, the most common technique is gas chromatography, GC/MS. Recently liquid chromatography (LC) has been used for obtaining mass spectra of more polar compounds not amenable to separation by gas chromatography. A thermospray technique of sample injection has solved two major problems of LC/MS, the reduced pressure in the mass spec due to the vaporized LC solvent; and the generation of nonvolatile compound ions (Watson, 1985). After leaving the column, the solvent and sample are partially or completely vaporized upon heating. The result is a combination of heated vapor and mist which enters the

sample is dissolved in, the sample concentration and temperature (Rahman, 1986). A sample often has nuclei in several different chemical environments. Different chemical environments result in different frequencies of absorption of isotopes in solution. These differences in resonances are referred to as chemical shifts. Chemical shifts are expressed in ppm or delta with reference to a standard. Nuclei may be shielded from the external magnetic field due to nearby electrons (Harris, 1986). For example, a sample containing hydroxy groups will shield a nearby proton from the magnetic field resulting in the shifting of the signal upfield to lower ppm values (Jackman, et al., 1969).

The positions of the signals may be dependent on the spectrometer operating frequencies and therefore should not be used when comparing spectra. Also these positions vary with the solvent used. However, coupling constants, denoted as J and measured in Herz, reflect the splitting between adjacent protons into doublet and triplet signals, etc. They depend on the chemical environment the nucleus is in, and are not affected by the variation in spectrometer operating frequency (Harris, 1986). As a result coupling constants are characteristic of structural features and useful in the structure determination of the sample; they may be used when comparing spectra.

The NMR is calibrated with a standard trimethylsilane, $Si(CH_3)_4$ or TMS. The chemical shift is usually reported as

the difference of absorption between the peak of interest and the TMS standard (Bovey, et al., 1988). Also the chemical shifts of a variety of solvents used in the NMR are known, and may also be used as an internal reference for the sample.

III. EXPERIMENTAL

III.A. MATERIALS

The test compounds, benz[j]aceanthrylene (BjAA), BjAA-9,10-oxide, BjAA-7,8-oxide-9,10-dihydrodiol and BjAA-9,10-dihydrodiol (BjAA-9,10-dhd) used in the Ames mutagenicity assay, and the BjAA-9,10-dihydrodiol and cyclopentafused benzo[e]pyrene (cpBeP), used in the metabolite generation procedure were synthesized by Dr. Ramiah Sangaiah at the University of North Carolina.

The S. typhimurium strain TA98 used in the Ames mutagenicity assay was acquired from Dr. Bruce Ames, University of California at Berkeley. The reagents needed in preparing the minimal and top agar were agar-agar, gum from U.S Biochemical Corp. (Cleveland, OH), dextrose from E.M. Science (Cherry Hill, NJ) and histidine and biotin from Fisher Scientific (Fair Lawn, NJ). The Vogel-Bonner medium E (50X) included in the agar preparation consisted of magnesium sulfate, citric acid, potassium phosphate, monobasic, and ammonium phosphate all purchased from Fisher Scientific. The overnight cultures were grown in Oxoid Nutrient Broth #2 from Oxoid Ltd. (Basingstoke, Hants, England). The S9 mix consisted of Aroclor 1254 induced rat liver from Mol Tox (College Park, MD), lot #258, protein

concentration 38.7 mg/ml, and $NADP^+$ and ß-D-glucose-6-phosphate from Boehringer-Mannheim (West Germany), magnesium chloride from J.T. Baker Chemical (Phillipsburg, NJ), potassium chloride from Fisher Scientific, and 1X phosphate buffered saline, pH 7.5 from the tissue culture facility at the Lineberger Cancer Research Center at the University of North Carolina.

Controls used in the Ames assay were 2-anthramine from Sigma Chemical Co. (St. Louis, MO) and 2-nitrofluorene from Aldrich Chemical Co. (Milwaukee, WI). The controls and test compounds were dissolved in dimethyl sulfoxide (DMSO) purchased from Fisher Scientific.

The Aroclor induced rat liver S9 used to generate metabolites from the BjAA-9,10-dihydrodiol and cpBeP was obtained from Mol Tox, lot# 258, 264 and 312 with the following protein concentrations 38.7 mg/ml, 39.8 mg/ml and 43.7 mg/ml, respectively. The potassium phosphate buffer, pH 7.5 was made from potassium phosphate, dibasic, from Mallinckrodt (Paris, KY) and potassium phosphate, monobasic, from Fisher Scientific. The pH of the buffer was measured with an American pH I meter from American Scientific Products (McGaw Park, IL). After the incubation, samples were extracted with HPLC grade ethyl acetate purchased from Baxter Health Care Corp. (Muskegon, MI) and HPLC grade acetone from Fisher Scientific.

Solvents used in high pressure liquid chromatography (HPLC) were HPLC grade methanol purchased from Baxter Healthcare Corporation, and distilled/deionized water courtesy of Dr. Mark Sobsey at the University of North Carolina. The distilled/deionized water was generated from a Dacor water system (Durham, NC) consisting of 1 µm millipore prefilter, a carbon resin, two deionizing resins, a macro reticular column to remove residual Cl^- and particulates and a 2 µm millipore postfilter.

The naphthalene-1,2-diol and the 1,2-naphthoquinone used for NMR analysis were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI).

III.B. METHODS

III.B.1. Ames Mutagenicity Assay Procedure

BjAA and several other metabolites thought to be responsible for its mutagenicity were tested in the Ames assay. The assay procedure is a modification of the EPA HERL Report (Claxton, et al., 1982) and is described briefly. The master plates were prepared from frozen stock cultures that were stored at -80°C. The S. typhimurium strain TA98 stock was removed from the freezer, quickly thawed, and used to inoculate nutrient broth and incubated for approximately 16 hours at 37°C. Master plates were prepared from this culture and incubated for 48 hours at 37°C. Integrity of the bacterial strain was tested for

ampicillin resistance (presence of the R factor plasmid), uvrB and rfa mutations, and histidine and biotin auxotrophy according to the EPA HERL Report (Claxton, et al., 1982). Acceptable master plates were securely wrapped in foil and in plastic, and stored at 4°C. New master plates were prepared every 4-6 weeks.

S9 concentrations of 774 µg/ml (50%), 1548 µg/ml (100%), 2322 µg/ml (150%) and 3096 µg/ml (200%) were used in the experiments based on preliminary experiments performed by Ravinder Singh in Dr. Ball's lab. The S9 was prepared with 92.4 mg of $NADP^+$ and 42.4 mg of glucose-6-phosphate dissolved in 10 ml of sterile 1X phosphate buffered saline (PBS). This solution was sterilized by passage through a 0.22 µ filter into a sterile flask on ice. To the sterile solution, 600 µl of salt solution, 1.65M KCl+0.4M $MgCl_2$, was added followed by the appropriate S9 concentration [600 µl (50%), 1.2 ml (100%), 1.8 ml (150%) and 2.4 ml (200%)]. Sterile 1X PBS was added to yield a final volume of 30 ml.

Five concentrations of PAH, 0.5 µg, 1.0 µg, 5.0 µg, 10.0 µg and 20.0 µg, dissolved in sterile DMSO were tested. The nutrient broth was inoculated with strain TA98 S. typhimurium from a master plate, and allowed to grow 14-16 hours at 37°C. The culture was then placed on ice prior to and during the assay. Melted top agar containing 100 µl of culture, 100 µl of test compound and 0.5 ml of S9, when appropriate, was mixed and added to the minimal agar

plate. After the top agar had solidified, plates were inverted and incubated at 37° C. After 48 hours the plates were counted or refrigerated and counted at a later time. Assays for each compound were done in duplicate or triplicate on at least two separate days.

Control plates for spontaneous background counts contained 100 µl DMSO in lieu of the test compound in the presence of 50% and 100% S9, and in the absence of S9. Plate counts ranged from 20-50 colonies/plate. 2-Anthramine in sterile DMSO, 0.5 µg/plate with 0.5 ml 100% S9, and 2-nitrofluorene in sterile DMSO, 3 µg/plate, without S9 served as the positive controls.

The number of colonies were averaged and standard deviations were calculated for each dose. Dose-response curves were generated for each compound. From the slope of the linear portion of the dose-response curve, the specific activity of the compound was calculated and recorded in revertants/nmole.

III.B.2. Metabolite Generation Procedure

To determine the oxidative metabolites formed in BjAA-9,10-dihydrodiol and cpBeP by cytochrome P-450 a modified procedure of Nesnow and colleagues (Nesnow, et al., 1984) was utilized. The incubation mixture contained 5 µmole $NADP^+$, 22.5 µmole B-D-glucose-6-phosphate, 15 µmole $MgCl_2$, 250 µM potassium phosphate buffer, pH 7.5 and approximately 300 nmole polycyclic aromatic hydrocarbon

(PAH) dissolved in 100-200 µl of acetone in a 5.0 ml volume. Aroclor 1254 induced rat liver S9 was added in a concentration of 1.0 mg/ml (approximately 26 µl) for BjAA-9,10-dihydrodiol and 4.0 mg/ml (approximately 104 µl) for cpBep. Five milliliters of incubation mixture was aliquoted into a 25 ml Erlenmeyer flask and incubated in a Techne SB16 Shaking water bath at 37°C for 15 minutes. For the BjAA-9,10-dihydrodiol large scale incubations, 3.0-3.5 mg/incubation of compound was used. The small incubations consisted of 0.5-1.0 mg/incubation. Two incubations of approximately 3.6 mg/incubation were done for cpBeP.

The incubation mixtures in the 25 ml Erlenmeyer flasks were pooled for each compound and extracted 1:1 with ethyl acetate:acetone (2:1), three separate times. The organic fraction was concentrated by rotary evaporation (Buchi Rotavapor, Flawil, Switzerland) to a 1-5 ml volume. The sample was then transferred to a scintillation vial. The round bottom flask was rinsed with either methanol, acetone or ethyl acetate and the rinse was added to the scintillation vial. The sample was evaporated to dryness under nitrogen.

Control incubations were done on a smaller scale, less than 250 µg of compound, following the above procedure without the addition of the rat liver S9. Samples were then analyzed by HPLC.

The above incubation procedure was modified for optimal dihydrodiol dehydrogenase activity to obtain a high yield of the BjAA-9,10-dihydrodiol peak 7 for identification purposes (Vogel, et al., 1980). This second incubation mixture consisted of 5 µmole $NADP^+$, Aroclor 1254 induced rat liver S9, 5.0 mg/ml (approximately 130 µl), 100 mM glycine buffer, pH 9.0, and 300 nmoles of PAH (dissolved in 50 µl acetone) in a 1.0 ml volume. The tubes were placed in a shaking water bath for 15 minutes at 37°C. The pH was checked before and after shaking, and ranged from 8.4-8.6. The amount of compound incubated at one time ranged from 0.5-1.5 mg. The control incubation consisted of one tube of the same components with the exception of the rat liver S9.

The tubes were pooled and extracted 1:1 with ethyl acetate:acetone (2:1), three separate times. The organic fraction was concentrated under nitrogen, and separated by HPLC.

III.B.3. High Pressure Liquid Chromatography

The HPLC system consisted of an Isco Model 2300 HPLC pump with a Model 2360 gradient programmer (Lincoln, NE) and a six valve Rheodyne injector (Cotati, CA) with 20 µl, 100 µl and 500 µl injection loops. HPLC profiles were recorded on a Spectra Physics SP4270 integrator (San Jose, CA), 0-1 V full scale, with an attenuation x64, and chart speed of 0.5 cm/min.

A reverse phase Zorbex C8 column, 9.4 mm x 250 mm, from DuPont Instruments (Wilmington, DE) and a pellicular ODS precolumn, 4.6 mm x 50 mm were used in separating and purifying the BjAA-9,10-dihydrodiol and cpBeP metabolites. The operating pressure ranged from 1500-3000 psi and the flow rate was 2.0 ml/min.

A Perkin-Elmer spectrophotometric detector LC85B with LC autocontrol (Norvalk, CT) monitored column eluate at 254 nm. Stop flow capability allowed peaks of interest to be scanned from 230-430 nm in an absorbance range of 0.8-5.16 cm. This information was recorded with a Perkin-Elmer 561 recorder, chart speed 30 mm/min.

The HPLC gradient systems used in metabolite separation were:

BjAA-9,10-dhd	50% methanol:50% water	10'
	gradient to 100% methanol	20'
	100% methanol	10'
cpBeP	60% methanol:40% water	15'
	gradient to 80% methanol:20% water	2'
	80% methanol:20% water	10'
	gradient to 100% methanol	2'
	100% methanol	10'

All samples were collected manually with the aid of an Isco Retriever III fraction collector, concentrated under nitrogen and stored at -80° C.

III.B.4. Mass Spectrometry

Mass spectrometry was used to determine the structure and molecular weight of the BjAA-9,10-dihydrodiol and cpBeP metabolites. With the assistance of Guy Lambert of

Environmental Health Research and Technology (EHRT) at Research Triangle Park, NC, mass spectra for metabolite peaks 3, 4 and 5 from the BjAA-9,10-dihydrodiol, and peaks A and B from the cpBeP incubations were obtained using a HPLC system coupled with a mass spectrometer via a thermospray interface. A mass spectrum of Peak 7 from the BjAA-9,10-dihydrodiol was obtained by direct probe with the assistance of Dean Marbury at the UNC Mass Spec Lab in the School of Public Health.

The pump and gradient programmer of the HPLC system were Series 8000 DuPont Instruments (Wilmington, DE) with a Rheodyne injector 7125 (Cotati, CA) and 50 µl sample loop. The columns used were Beckman (San Ramon, CA) and Phenomonex (Rancho Palos Verdes, CA) C18 columns, 2.0mm x 250mm, with a flow rate of 0.5 ml/min.

The mass spectrometer was an Extrel ELQ 400, with an electron impact source and ionizing voltage of 70 eV, located at EHRT in Research Triangle Park, NC. The source temperature was 225°C and the nebulizer temperature ranged from 120°-125 °C. The Extrel ELQ was calibrated with perchlorotributylamine.

The mass spectrometer in the UNC Mass Spec Lab in the School of Public Health was a VG-70-250-SEQ used with an electron impact source and an ionizing voltage of 70 eV. The source temperature was 275°C and the probe temperature was increased from 30°-300 °C at approximately 50°C per

minute. The VG-70-250-SEQ was calibrated with perfluorokerosene.

III.B.5. Nuclear Magnetic Resonance Spectrometry

The samples proton number and their positions were determined by nuclear magnetic resonance. NMR spectral analysis of the isolated metabolite fractions were performed by Dr. Dave Harris at the Chemistry Department, University of North Carolina. Metabolite peaks 3, 4, 5 and 7 from the BjAA-9,10-dihydrodiol and peak A from the cpBeP incubations were dissolved in deuterated acetone, 99.5 atom %D (Aldrich Chemical Co., Milwaukee, WI and Sigma Chemical Co., St. Louis, MO). Spectra were recorded on a Varian XL 400 at 400 MHz operated under the following conditions: spectral width 5299 Hz, ACQ Time 2.832 sec, pulse width 29-30^0, spin rate 20 Hz. The number of transients varied between 18000-20000.

IV. RESULTS

IV.A. Mutagenicity of Benz[j]aceanthrylene Metabolites

In the Ames mutagenicity assay, the spontaneous background counts for strain TA98 in this study averaged 33 rev/plate in the absence of S9 and 43 rev/plate in the presence of 100% S9. The compound 2-nitrofluorene at 3 µg/plate was used as a positive control in the absence of S9, with an average of 299 rev/plate. 2-Anthramine at 0.5 µg/plate served as the positive control with S9 (0.5 ml of 100% S9) and averaged 592 rev/plate.

The BjAA-9,10-oxide (a 1:1 mixture of 9,10-oxide and 10-phenol) and its metabolites, the 9,10-dihydrodiol and the 9,10-dihydrodiol-7,8-oxide were tested along with the parent compound, BjAA at concentrations of 0.5, 1.0, 5.0, 10.0 and 20.0 µg/plate with S9 concentrations of 0, 774, 1548, 2322, and 3096 µg/ml at each dose. Dose-response curves are shown in figure 10 and 11 (100% S9 shown).

The parent compound, the 9,10-oxide and the 9,10-dihydrodiol are indirect mutagens with specific activities of 0.1 rev/nmole, 0.2 rev/nmole and 0.7 rev/nmole, respectively. The 9,10-dihydrodiol-7,8-oxide was a direct acting mutagen with a specific activity of 10 rev/nmole. The 9,10-dihydrodiol-7,8-oxide exhibited

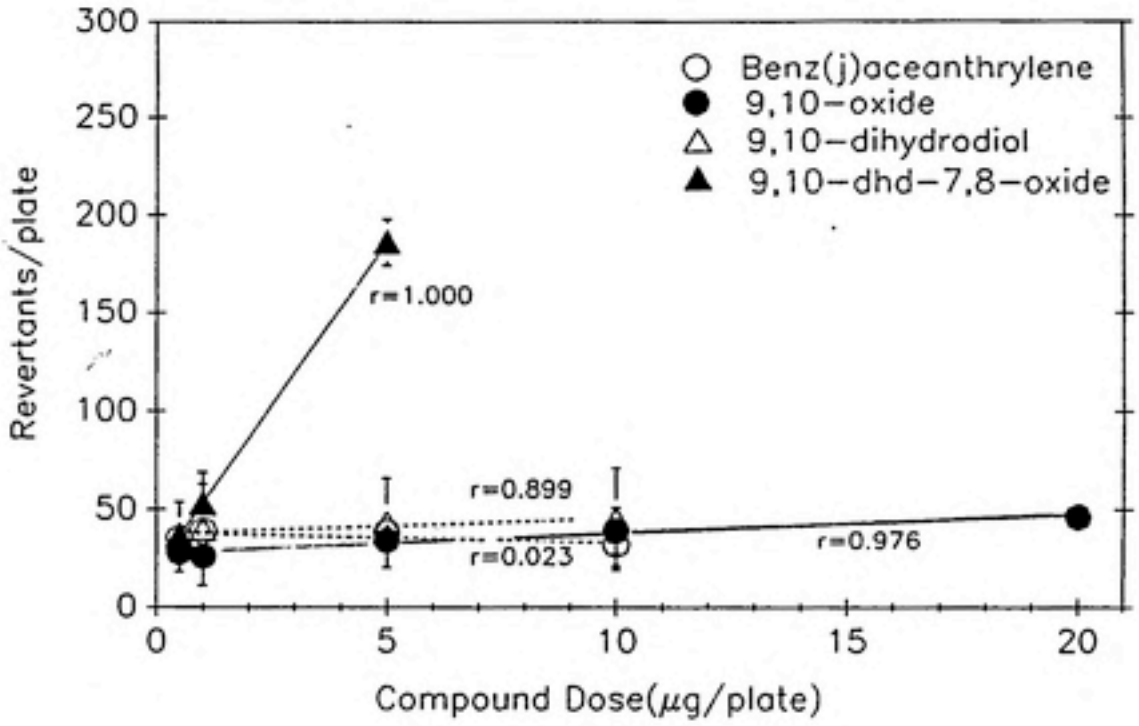

FIGURE 10: Mutagenicity of benz[j]aceanthrylene and several metabolites in S. typhimurium strain TA98 in the absence of S9. Revertants/nmole were calculated from the linear portion of the dose-response curve.

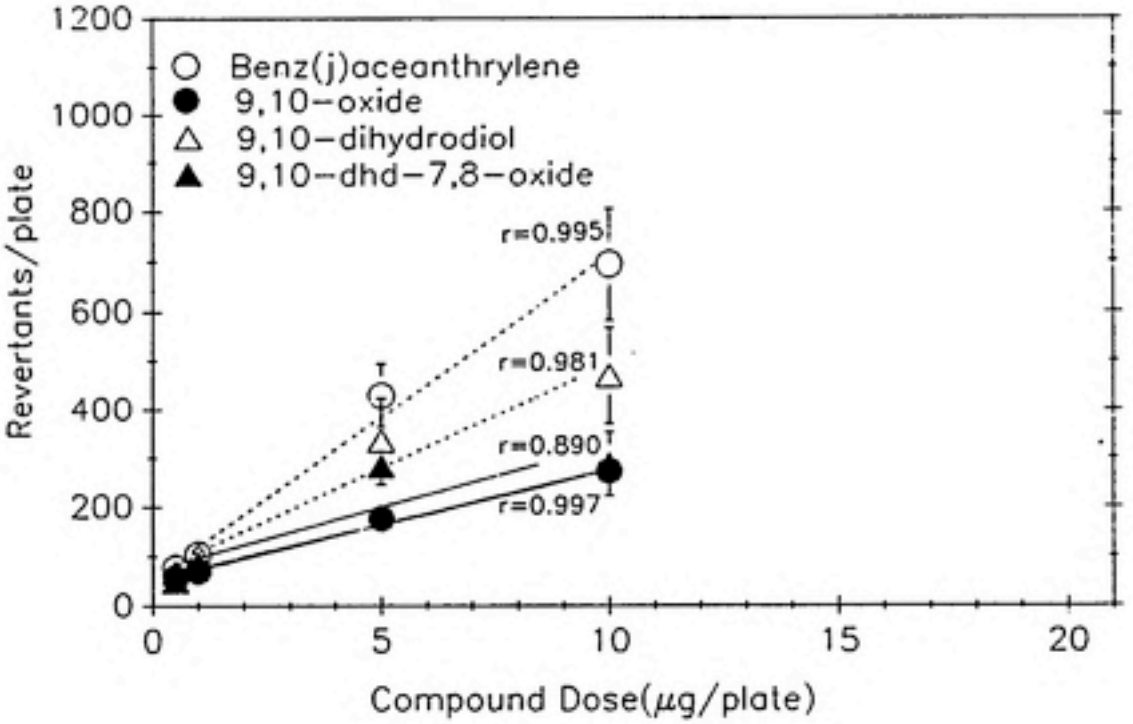

FIGURE 11: Mutagenicity of benz[j]aceanthrylene and several metabolites in S. typhimurium strain TA98 in the presence of S9, 0.8 mg/plate. Revertants/nmole were calculated from the linear portion of the dose-response curve.

toxicity to the cells at concentrations above 5 µg/plate, table 1.

Upon addition of S9, all the compounds exhibited mutagenicity. The 9,10-oxide showed moderate activity at 5.6 rev/nmole, the 9,10-dihydrodiol was active at 9.4 rev/nmole and the parent compound and the 9,10-dihydrodiol-7,8-oxide had similar activity at 12.9 rev/nmole. The 9,10-dihydrodiol-7,8-oxide, again, exhibited toxicity to the cells at concentrations above 5 µg/plate.

The specific activity of the compounds were also calculated at the other S9 concentrations, 0.7, 2.3 and 3.1 mg/ml, table 2. The compound was fifty-percent as active at 0.7 mg/ml as compared with 1.6 mg/ml S9 concentration. Comparison of the mean revertants/nmole at the 1.6, 2.3 and 3.1 mg/ml S9 concentrations showed no significant difference for $\alpha=0.01$. Indicating that the specific activity of the compounds did not increase with the addition of S9 above the 1.6 mg/ml concentration.

IV.B. Metabolism of the Benz[j]aceanthrylene-9,10-dihydrodiol

The BjAA-9,10-dihydrodiol was incubated with Aroclor 1254 induced rat liver S9 following a modified procedure of Nesnow and colleagues (1984). The organic fractions were separated by HPLC using the BjAA-9,10-dhd gradient program as described in the methods section. For comparison, a

DOSE ug/plate	BENZ(j)ACEANTHRYLENE -S9	BENZ(j)ACEANTHRYLENE +S9	9,10-OXIDE -S9	9,10-OXIDE +S9	9,10-DIHYDRODIOL -S9	9,10-DIHYDRODIOL +S9	9,10-DHD-7,8-OXIDE -S9	9,10-DHD-7,8-OXIDE +S9
	35(8)	76(16)	28(5)	58(15)	36(18)	67(33)	37(7)	51(13)
	39(8)	103(28)	26(3)	71(4)	40(29)	100(35)	53(10)	86(4)
	38(6)	429(65)	34(3)	176(14)	43(23)	335(89)	186(12)	283(16)
	32(11)	694(111)	39(12)	272(22)	45(26)	469(98)	172(20)	287(65)
	38(11)	915(148)	46(9)	350(47)	75(45)	543(111)	93(6)	148(49)
REV/NMOL	0.1 +0.1	12.9(2)	0.2(0.02)	5.6(1.5)	0.7(0.4)	9.4(3)	10.0(1.1)	12.9(5.9)
r	0.023	0.995	0.976	0.997	0.899	0.981	1.000	0.890

TABLE 1: Mutagenicity of benz[j]aceanthrylene metabolites in S. typhimurium strain TA98 in the presence of S9, 0.8 mg/plate.

DOSE	BENZ(j)ACEANTHRYLENE			9,10-OXIDE			9,10-DIHYDRODIOL			9,10-DIHD-7,8-OXIDE		
(µg/plate)	50X	150X	200X	50X	150X	200X	50X	150X	200X	50X	150X	200X
0.5	58(8)	64(6)	63(5)	76(29)	64(8)	56(5)	64(22)	68(27)	67(23)	41(10)	43(11)	38(8)
1.0	102(17)	71(*)	74(1)	98(46)	68(11)	63(5)	79(30)	109(42)	103(30)	60(14)	71(10)	56(8)
5.0	509(23)	337(20)	333(8)	172(76)	183(14)	184(6)	199(72)	402(74)	397(61)	221(11)	264(44)	268(45)
10.0	538(105)	679(69)	615(46)	212(76)	267(24)	304(14)	252(50)	590(130)	606(101)	239(28)	314(30)	335(42)
20.0	598(14)	1214(24)	1144(42)	240(45)	427(17)	522(35)	258(28)	603(112)	569(133)	124(36)	158(66)	190(46)
REV/NMOL	6.9[1]	14.8[1]	14.0[1]	2.8(0.4)	4.9(0.3)	6.8(0.02)	4.3(2)	7.9(1)	7.7(1)	5.9(0.4)	8.6(1)	9.8(1)
r	0.91	0.99	0.99	0.97	0.99	0.99	0.97	0.99	0.99	0.92	0.95	0.96

(*) Only 1 point at this dose.
[1] Results of 1 Ames assay.

TABLE 2: Mutagenicity of benz[j]aceanthrylene metabolites in S. typhimurium strain TA98 in the presence of S9 at the following concentrations, 0.4 mg/plate (50X), 1.15 mg/plate (150X), and 1.5 mg/plate (200X).

control incubation consisting of the same components except S9 is shown in figure 12. As expected, the major peak in this profile is the unmetabolized 9,10-dihydrodiol.

In the presence of S9, seven peaks were observed in the HPLC profile, figure 13. Peaks 1 and 2, retention times 6.9' and 7.4', respectively, elute shortly after the solvent peak. Based upon the UV spectra and mass spectra (data not shown) it did not appear that these peaks were associated with the 9,10-dihydrodiol; and were probably other components extracted from the incubation.

UV spectra, figure 14, were monitored between 230 and 430 nm, and are shown for peaks 3, 4, 5, 6 and 7. Peak 6 is the unmetabolized 9,10-dihydrodiol and elutes at 31.7'. When the sample is concentrated and dissolved in methanol it appears dark orange in color. This peak was identified from the UV spectrum and LC/MS, figure 15. The MS gives a strong molecular ion at m/z 286 with losses of 18 and 16, indicative of water and oxygen (McLafferty, 1980), which appears to be characteristic of dihydrodiols in this system.

The NMR data, of an authentic sample of the 9,10-dihydrodiol (Sangaiah, et al., 1984), along with data obtained for the 9,10-dihydrodiol metabolites in this report are shown in table 3. Signals for the protons in the 9 and 10 positions were located upfield, not in the aromatic region of the spectrum (>6ppm), those corresponding to the

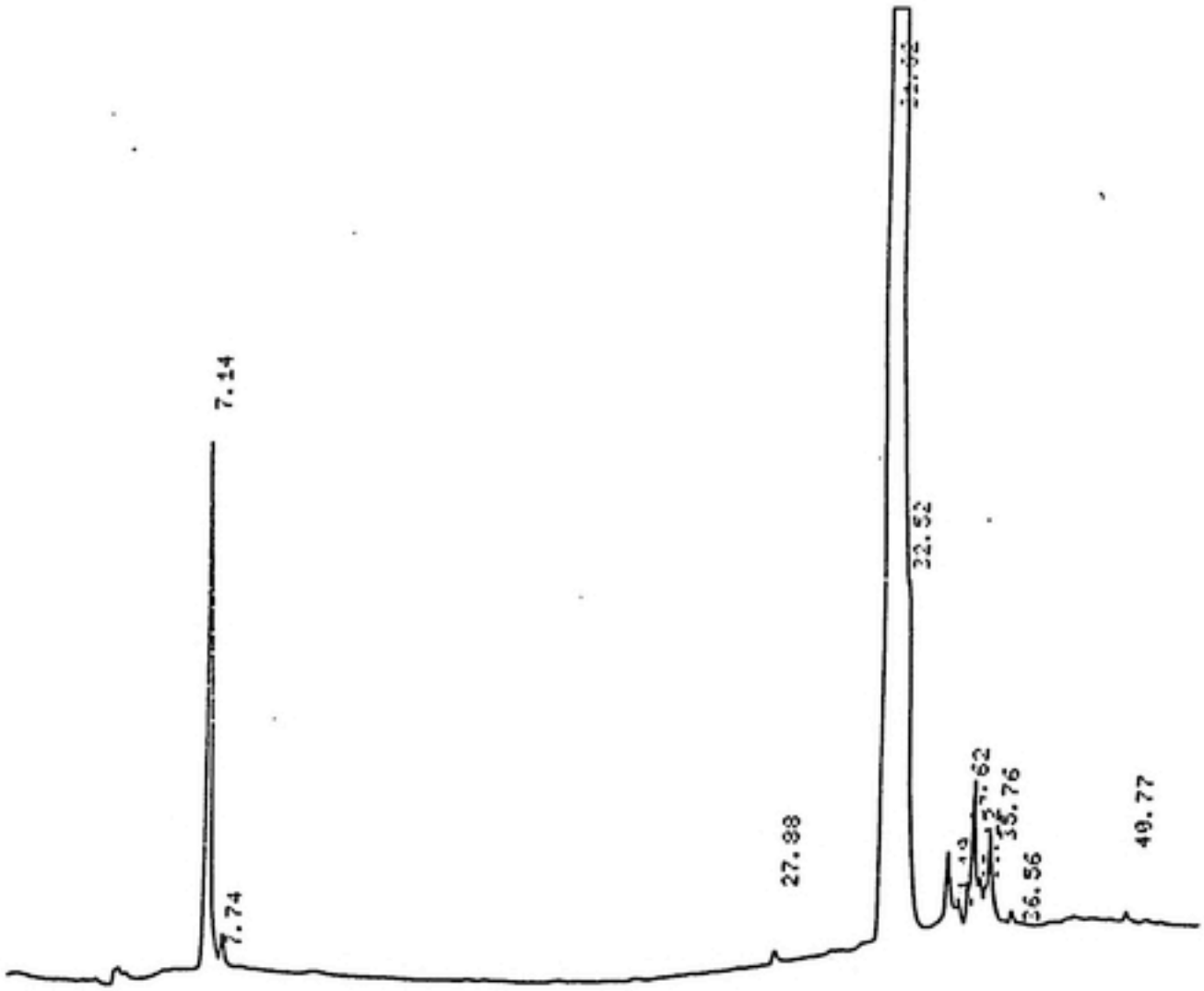

FIGURE 12: HPLC profile of the benz[j]aceanthrylene-9,10-dihydrodiol control incubation. The control incubation consisted of the compound and cofactors without S9.

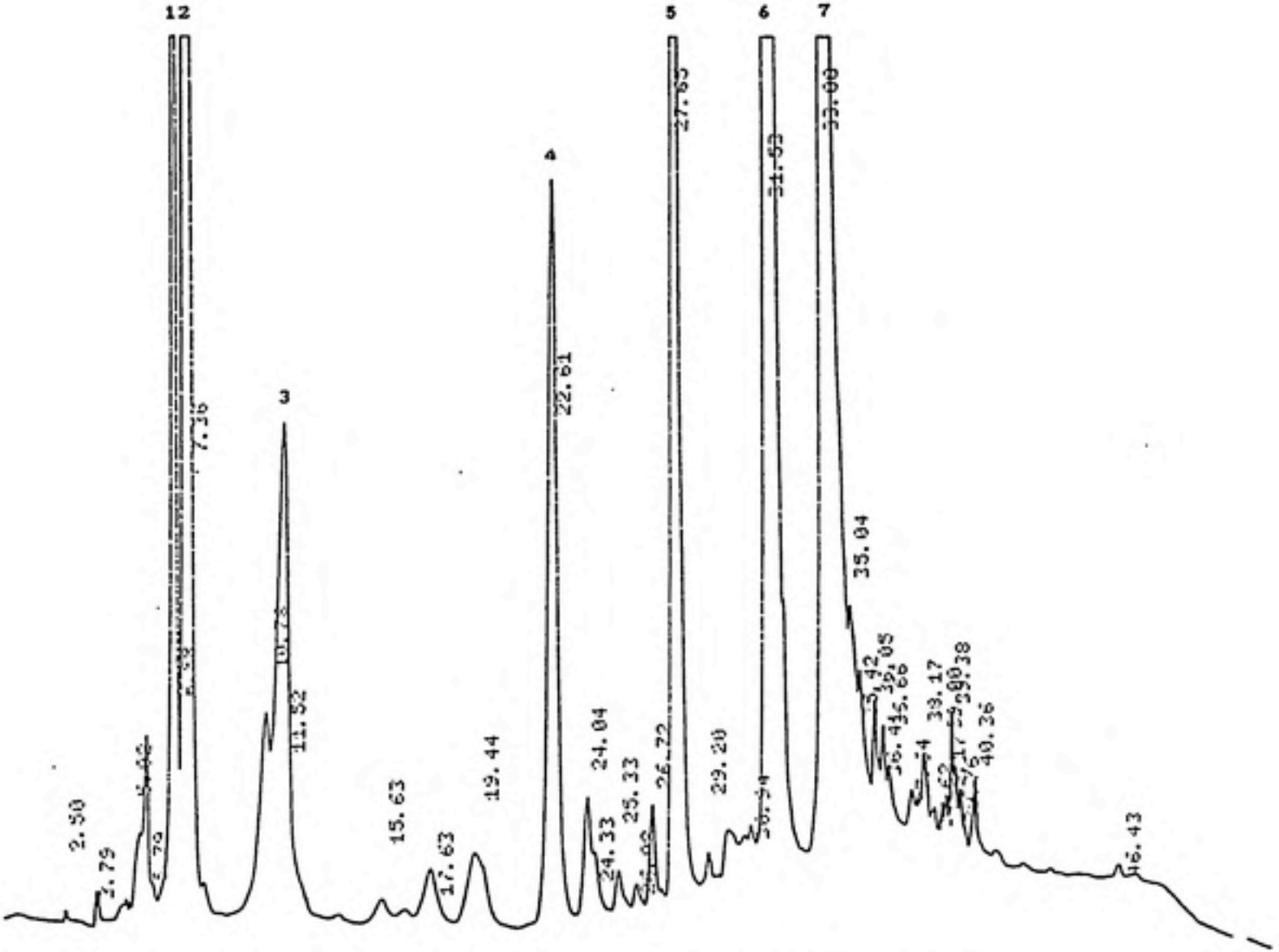

FIGURE 13: HPLC profile of the benz[j]aceanthrylene-9,10-dihydrodiol incubated with rat liver S9. Peaks 3, 4, 5 and 7 were identified as metabolites of the 9,10-dihydrodiol (Peak 6).

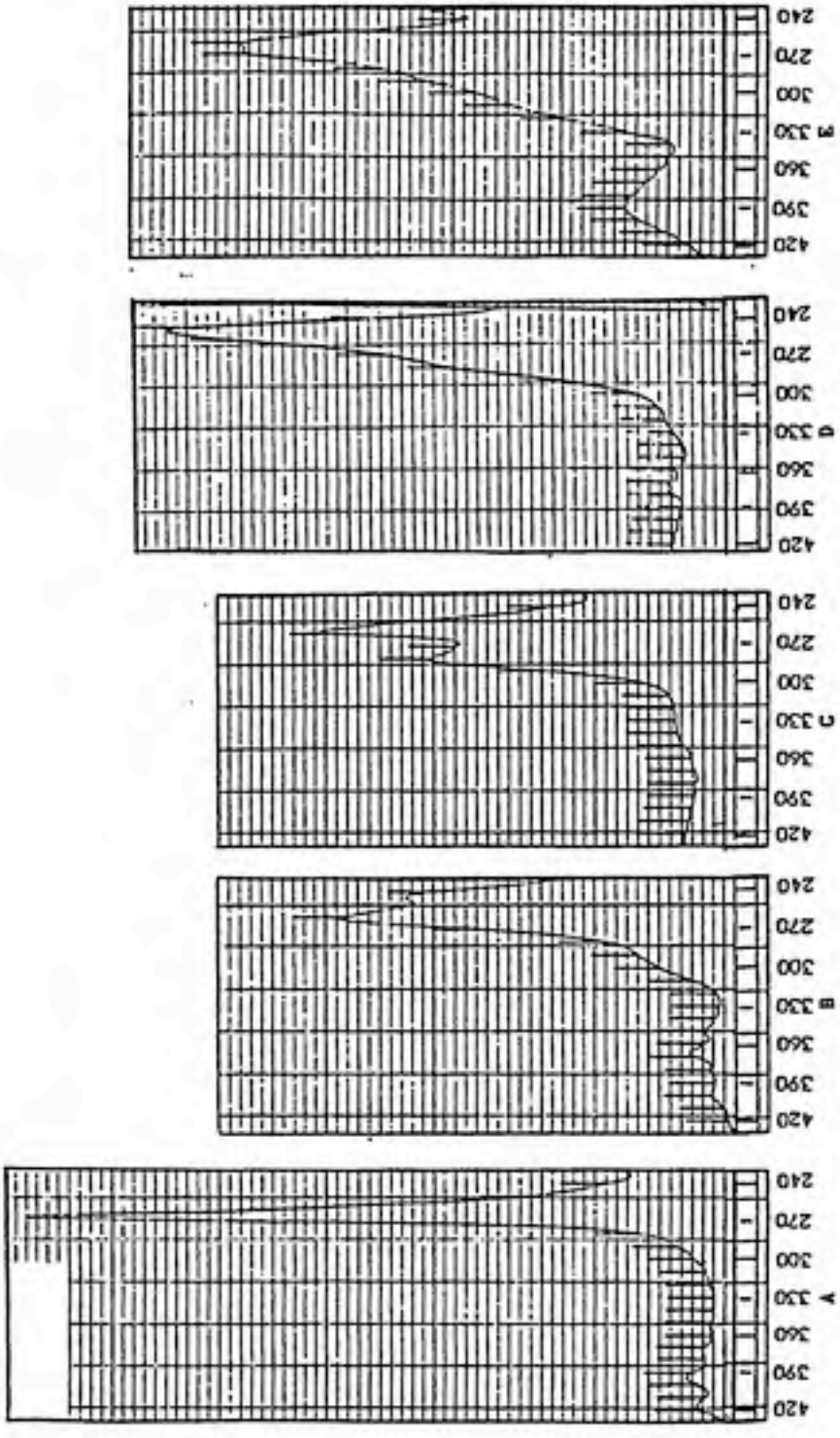

FIGURE 14: UV spectra, 230-430 nm, of the benz[j]aceanthrylene-9,10-dihydrodiol and its metabolites, (A) peak 3b, (B) peak 4a, (C) peak 5, (D) peak 6, (E) peak 7.

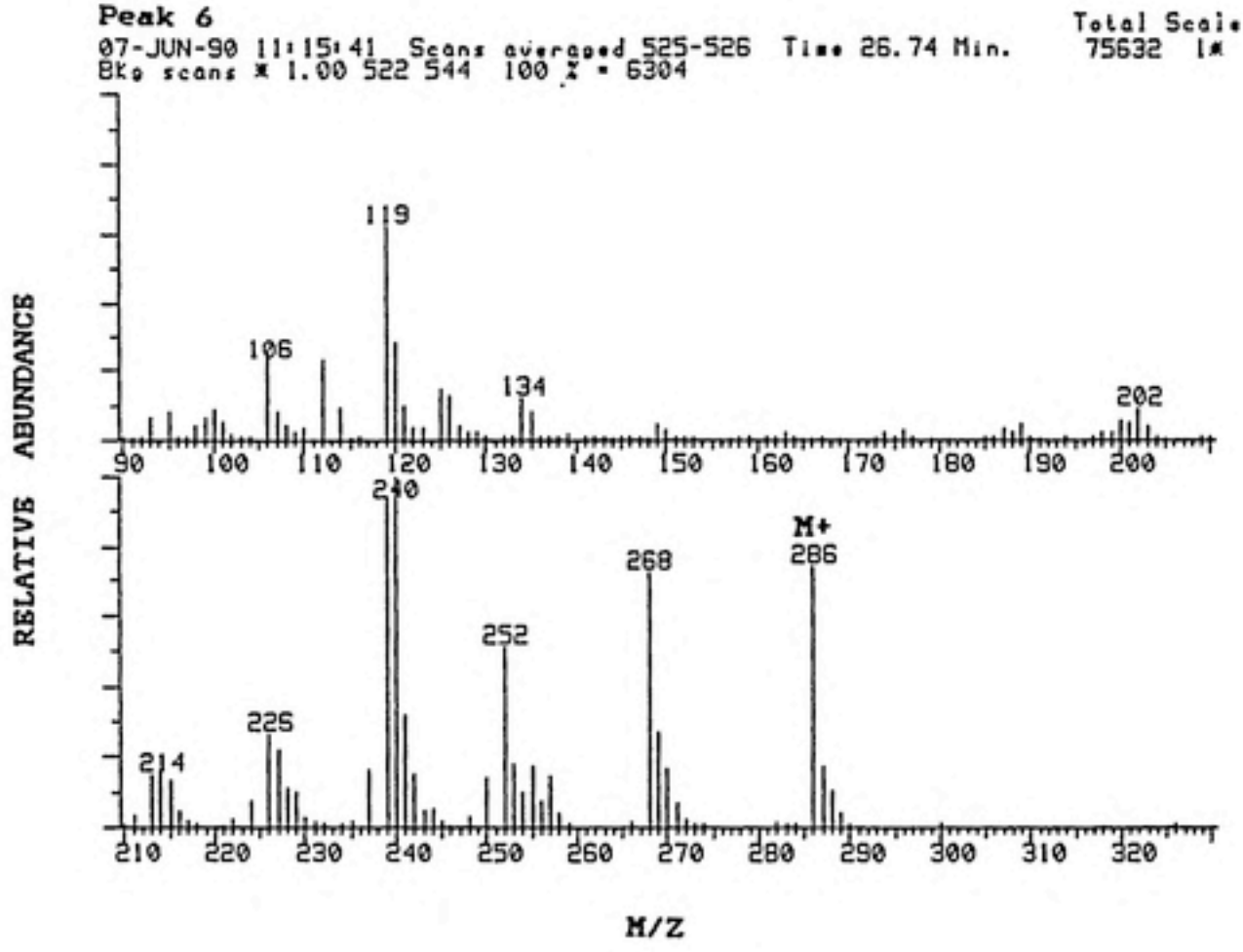

FIGURE 15: Mass Spectrum of the benz[j]aceanthrylene-9,10-dihydrodiol, mw. 286.

	BjAA-9,10-Dihydrodiol		BjAA-1,2,9,10-Tetrol(Peak 3B)			BjAA-7,8,9,10-Tetrol(Peak 4A)			BjAA-9,10-Dihydrodiol-2-One(Peak 5)			BjAA-9,10-Diol(peak 7)		
	Signal	PPM	Signal	PPM	J	Signal	PPM	J	Signal	PPM	J	Signal	PPM	J
H1	d	7.75	-	-	-	d	7.72	5.25	-	-	-	d	7.82	5.39
H2	d	7.2	-	-	-	d	7.15	5.00	-	-	-	d	7.28	5.18
H3	d	7.9	d	7.4	6.76	d	7.89	6.47	d	7.9	6.79	d	8.0	-
H4	t	7.7	t	7.5	8.30	t	7.65	-	t	7.8	8.32	t	7.65	5.50
H5	d	8.4	d	7.92	8.45	d	8.35	8.08	d	8.4	8.23	d	8.2	8.41
H6	s	9.0	s	8.75	-	s	9.0	-	s	8.95	-	s	9.6	-
H7	dd	7.5	dd	7.45	10.16	-	-	-	dd	7.5	10.06	m	8.0	-
H8	dd	6.35	dd	6.25	9.90	-	-	-	dd	6.3	10.08	-	-	-
H9	d	4.55	-	-	-	-	-	-	d	4.55	11.07	-	-	-
H10	d	4.95	-	-	-	-	-	-	d	4.95	11.65	-	-	-
H11/H12	d,d	8.15/8.05	d,d	8.45/7.9	9.06	d,d	8.1/7.91	8.72/8.41	d,d	8.05/8.0	9.06/8.99	d,m	8.65/8.0	8.85/-

TABLE 3: NMR data for benz[j]aceanthrylene-9,10-dihydrodiol and several metabolites.

etheno bridge protons H1 and H2 are prominent at 7.75 and 7.2 ppm, and a vinylic resonance at 6.35 ppm corresponding to H8. This information will be helpful in identifying the metabolites present in this section.

Peak 3 was resolved into two peaks, A and B with retention times 10.8' and 11.5', respectively. Both samples when concentrated were colorless. Not enough of peak 3A could be isolated for MS and NMR analysis. However peak 3B was collected and analyzed by MS and NMR. A molecular ion of 320, figure 16, and major fragments with m/z ratios of 302, 284, 268 and 252, corresponding to two losses of 18 and 16, suggest that the molecule has two dihydrodiols functionalities.

The NMR spectrum of peak 3B, figure 17 was compared with peak 6, the unmetabolized 9,10-dihydrodiol. The number of protons in the spectrum was based on the integration of H6, in the peak 3B spectrum, as one proton. An impurity in the sample may be responsible for the apparent singlet, x, seen at 8.5 ppm. An impurity in the solvent, probably $CHCl_3$, is seen at 7.35 ppm in spectrum peak 3B and all the NMR spectra presented in this report. Protons in the 9 and 10 positions did not appear in the aromatic region of the spectrum as expected. Signals corresponding to protons at the 1 and 2 positions were also not discernable. From the given data it was concluded that peak 3B was the 1,2,9,10-tetrahydro-1,2,9,10-tetrahydroxy-BjAA.

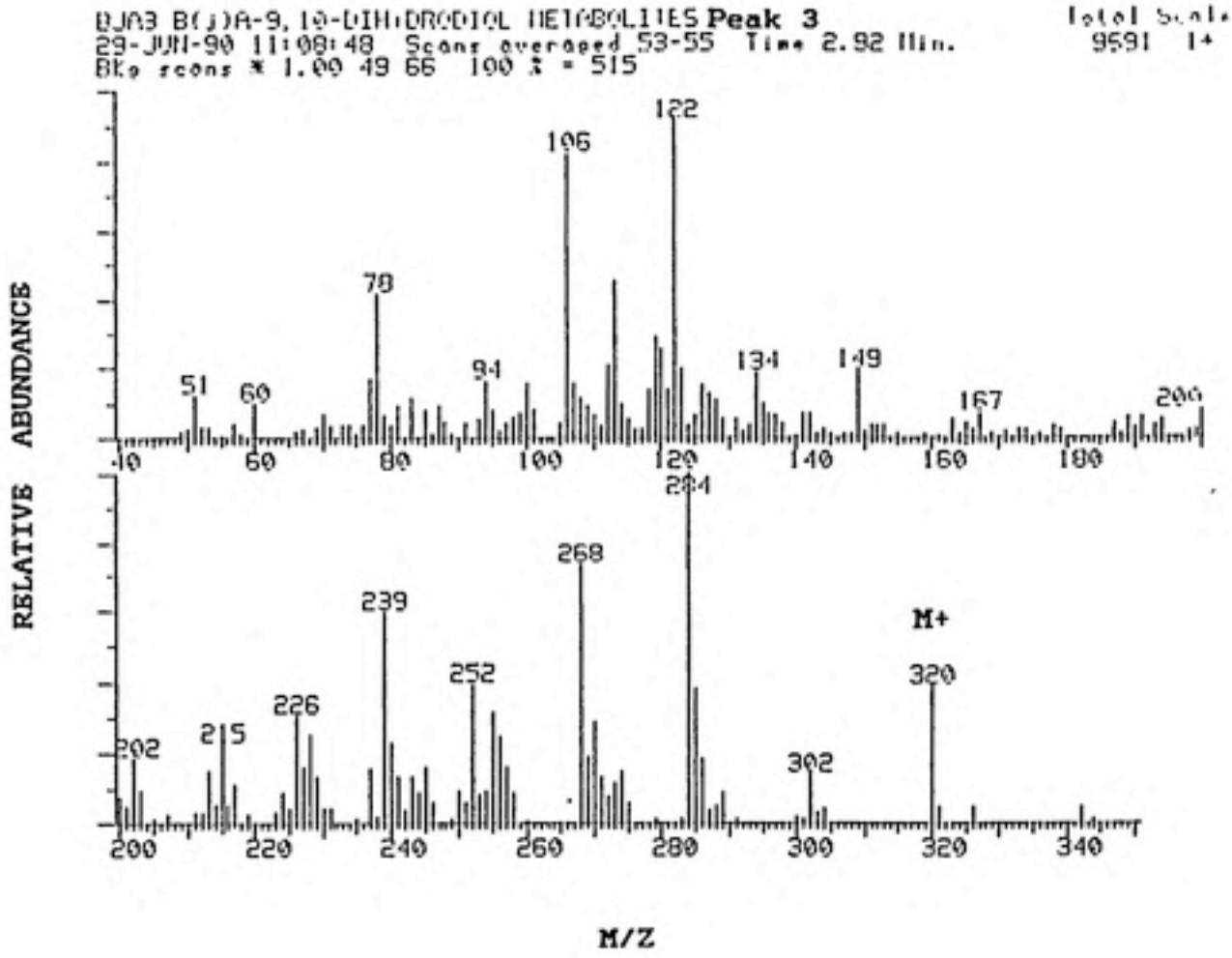

FIGURE 16: Mass spectrum of peak 3b, the 1,2,9,10-tetrahydro-1,2,9,10-tetrahydroxy-benz[j]aceanthrylene.

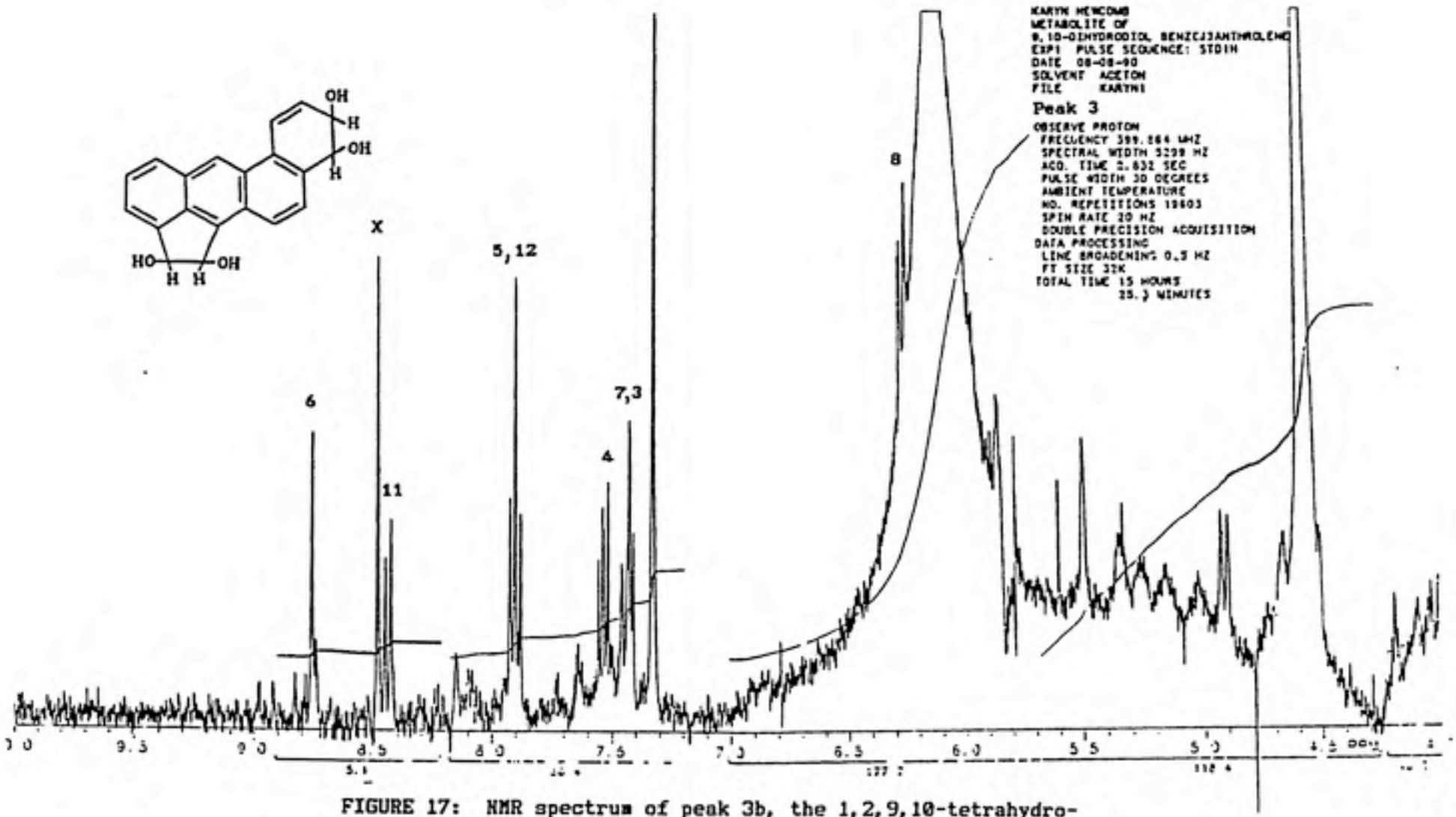

FIGURE 17: NMR spectrum of peak 3b, the 1,2,9,10-tetrahydro-1,2,9,10-tetrahydroxy-benz[j]aceanthrylene.

Peak 4 elutes at 22.6' and is colorless when dissolved in methanol. Upon subsequent cleanup, this peak separated into two peaks. Only peak 4A could be isolated in a high enough concentration to obtain LC/MS, figure 18, and NMR, figure 19, information. Like peak 3B, the molecular ion for peak 4A was also 320. Losses of 16 and 18 resulted in m/z ratios of 304, 286, 268 and 252 suggest that this molecule is also a tetrol. Comparison of the NMR profile, of peak 4A with the 9,10-dihydrodiol indicated that protons 7, 8, 9 and 10 were not present in the aromatic region. From both MS and NMR results it was concluded that this sample was the 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxy-BjAA.

Peak 5 eluted at 27.6'; and when concentrated and dissolved in methanol was a lemon yellow color. From the LC/MS analysis, figure 20, the molecular ion was 302 with m/z ratios of 284, 268, 256 and 239 corresponding to losses of 18 (H_2O), 34 (H_2O_2), 46 (H_2CO_2) and 63 (CH_3O_3). The NMR spectra for peak 5, figure 21, indicated that the signals for the etheno protons, 1 and 2, were not present in the aromatic region, however a singlet integrating to two protons was apparent upfield at 4.2 ppm. This suggests that the cyclopentafused ring is saturated. Also in this spectrum well resolved resonances corresponding to the 9 and 10 protons, and their respective hydroxyl protons, can be discerned clearly between 4.5 and 5.0 ppm. From the MS data, three oxygens are present in the molecule. Therefore

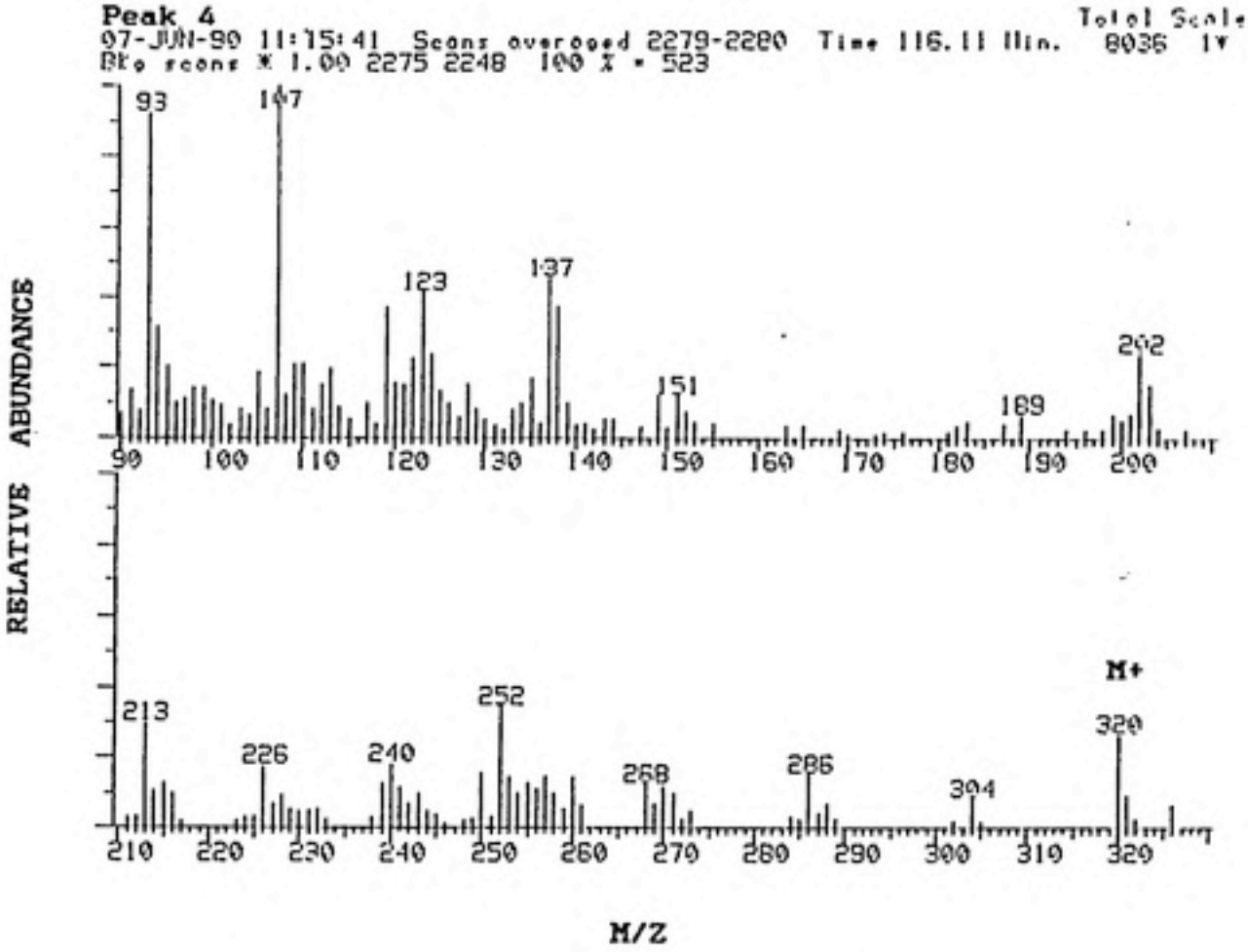

FIGURE 18: Mass spectrum of peak 4a, the 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxy-benz[j]aceanthrylene.

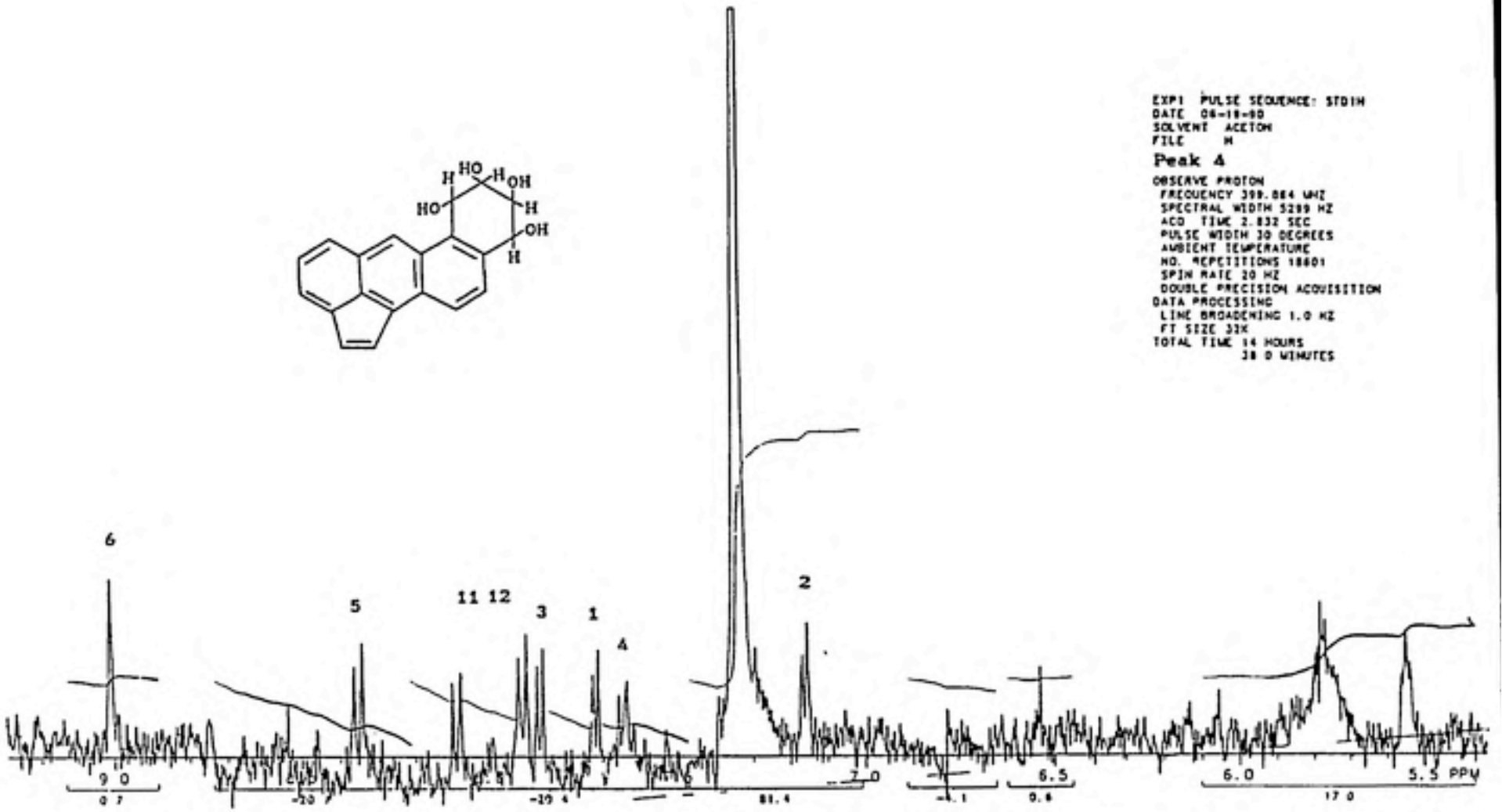

FIGURE 19: NMR spectrum of peak 4a, the 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxy-benz[j]aceanthrylene.

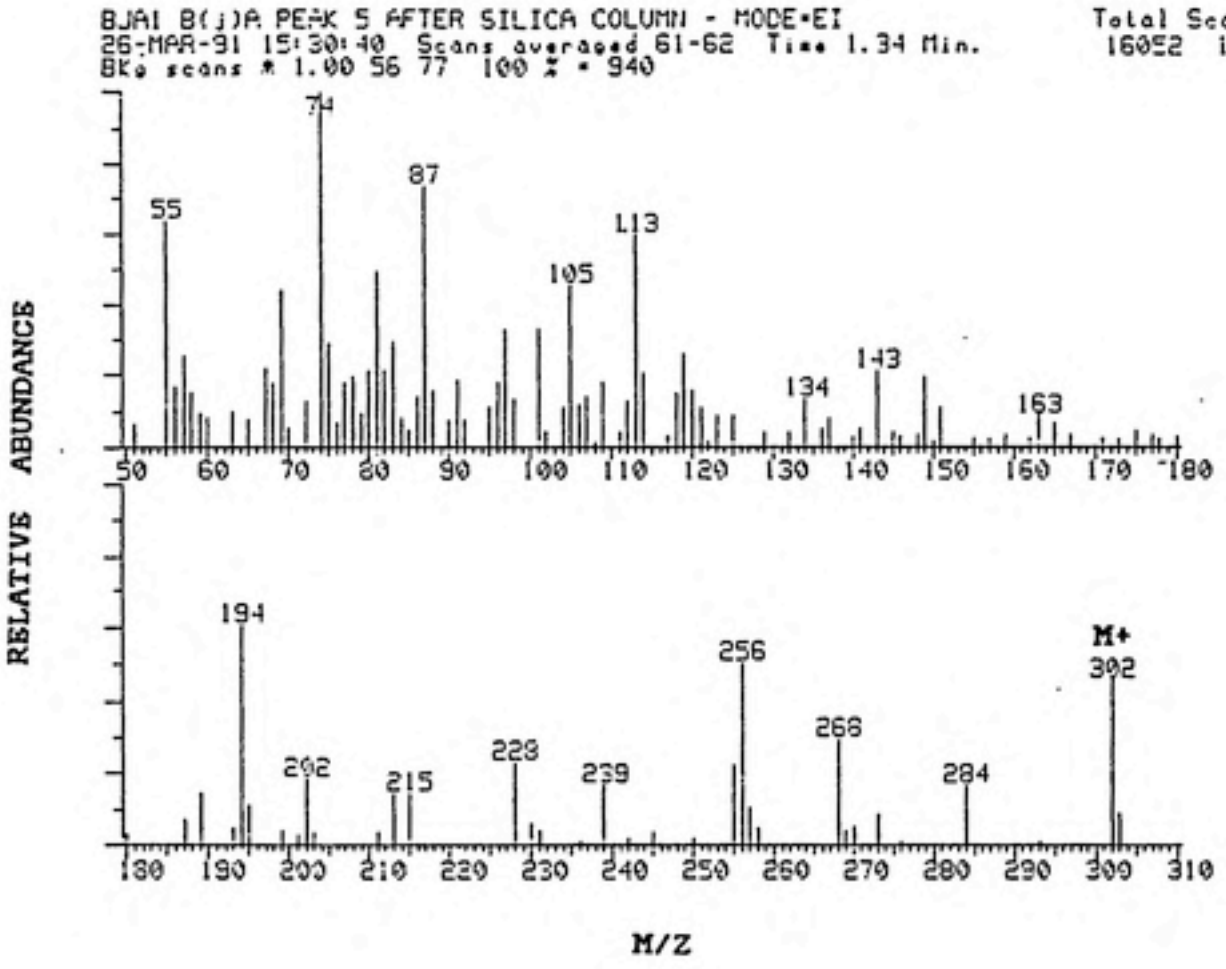

FIGURE 20: Mass spectrum of peak 5, the benz[j]aceanthrylene-9,10-dihydrodiol-2-one.

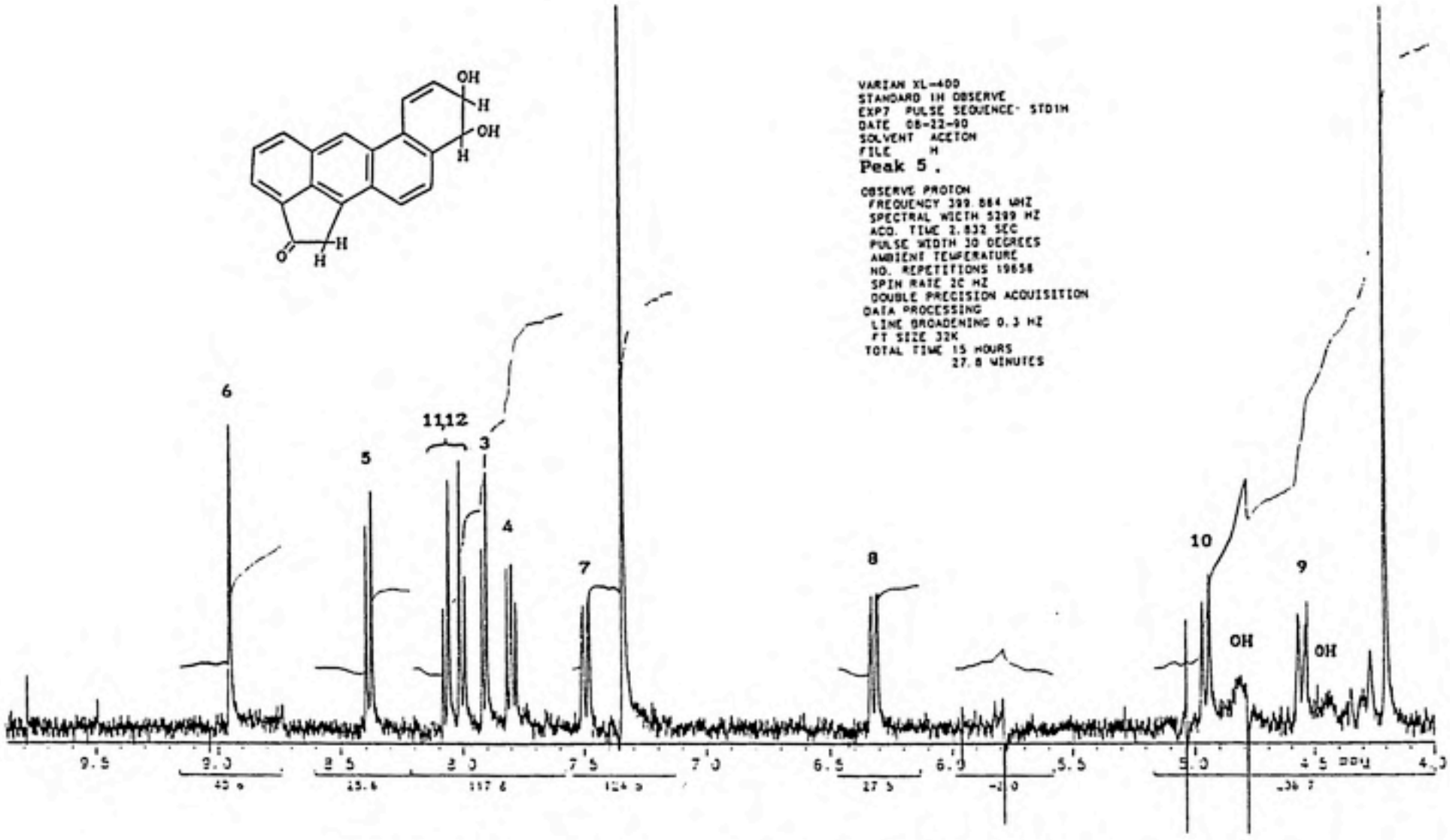

FIGURE 21: NMR spectrum of peak 5, the benz[j]aceanthrylene-9,10-dihydrodiol-2-one.

one oxygen is present at the 1 or 2 position. M/Z ratios of 268 and 256 indicate a loss of an H_2O_2- and CH_3O_2-, respectively. This fragmentation pattern suggests that a ketone exists at either the 1 or 2 position. The presence of a ketone at the 1 or 2 positions should effect the location of signals of protons adjacent (positions 12 or 3) and opposite (positions 6 or 5) the substituent. Yang and colleagues (1990) have reported that the 3-MC-9,10-dihydrodiol-2-one conformation has been observed in higher concentrations than the 3-MC-9,10-dihydrodiol-1-one in rat liver microsomal metabolism. Pending additional NMR analysis and comparison with suitable model components, we have tentatively identified this peak as BjAA-9,10-dihydrodiol-2-one by analogy with the metabolism of 3-MC.

Peak 7 eluted after the 9,10-dihydrodiol, at 34'. When concentrated this sample was a yellow-brown color. The UV spectrum is shown in figure 14. Several attempts were made to obtain MS data for this peak using both LC/MS and direct probe. Contamination from the 9,10-dihydrodiol and phthalates made it difficult to obtain a clear spectrum. However, a m/z of 284 is present in both MS methods. Results of the direct probe method are shown in figure 22. Peaks at m/z ratios 266 and 255 were observed suggesting losses of 18, H_2O, and 29, CHO. Due to the difficulty of separating peak 7 from the parent compound, the molecular ion, 286, of the parent compound is also observed in this

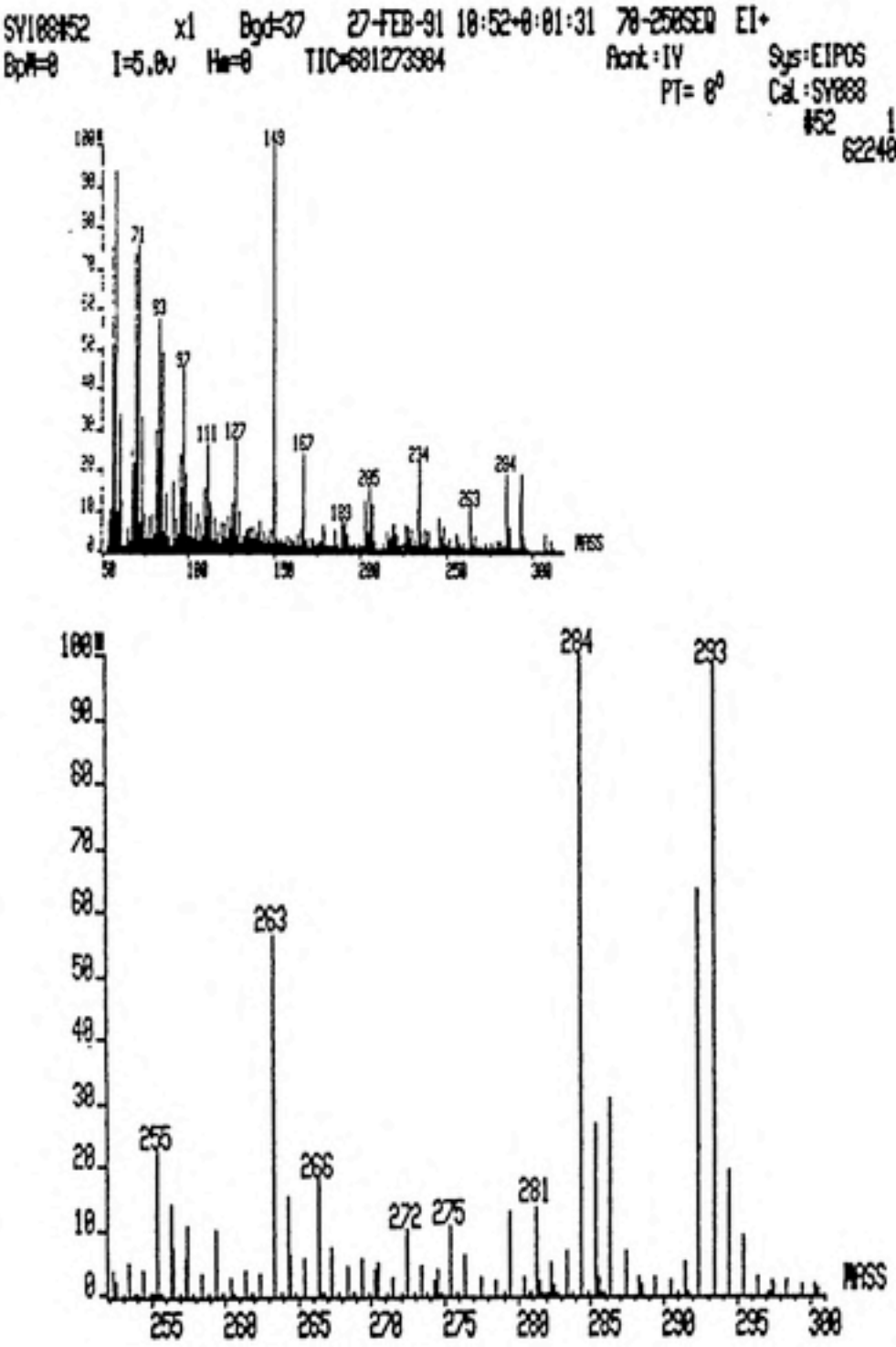

FIGURE 22: Mass spectrum of peak 7, 9,10-dihydroxy-benz[j]aceanthrylene.

spectrum. Analysis of single ion chromatograms from other MS samples indicate that m/z ratios of 293 and 275 appear to be a result of phthalate contamination. It is thought that m/z ratio at 263 is also due to this contaminant as well.

The NMR spectrum for peak 7 is shown in figure 23. As expected, signals corresponding to H9 and H10 are not obvious. In addition, the vinylic resonance at 6.35 ppm characteristic of H8 is not apparent.

The HPLC profile indicates that this sample is less polar than the parent compound, therefore peak 7 would contain only 1 or 2 oxygens. The possibility of a phenol in the 9 or 10 position was ruled out by comparison to UV spectra and NMR data of authentic 9-hydroxy-BjAA and 10-hydroxy-BjAA. Therefore the possibility of reduction of the dihydodiol functionality to a diol or an ortho-quinone was investigated.

An NMR spectrum of the BjAA-9,10-quinone from the synthesis of the 9,10-dihydrodiol was available from Dr. Sangaiah. The proton resonances are given in table 4, and do not match those of peak 7. Notably, the vinylic resonance corresponding to H8 is apparent at 6.65 ppm.

To obtain additional compound for comparison and further analysis, another incubation procedure was initiated which optimized conditions for the enzyme dihydrodiol dehydrogenase. Several reports on compounds incubated with

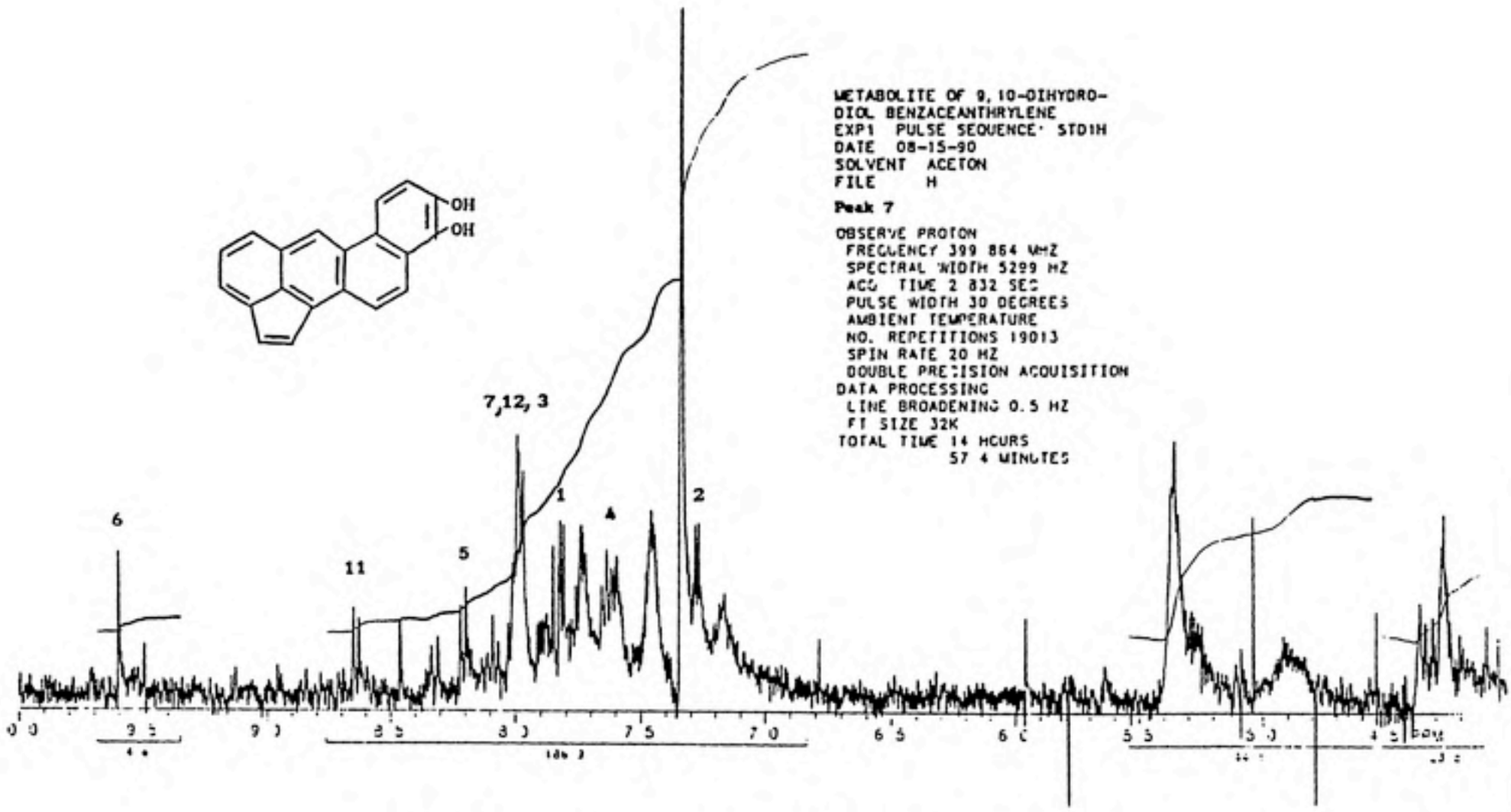

FIGURE 23: NMR spectrum of peak 7. The data suggests this metabolite may be 9,10-dihydroxy-benz[j]aceanthrylene.

BjAA-9,10-Quinone

	Signal	PPM
H1	d	7.80
H2	d	7.25
H3	d	8.0
H4	t	7.75
H5	d	8.60
H6	s	9.40
H7	m	8.20
H8	d	6.65
H9	-	-
H10	-	-
H11	d	8.85
H12	m	8.20

TABLE 4: NMR data for BjAA-9,10-quinone in CD_3COCD_3.

dihydrodiol dehydrogenase have indicated that both diols (Vogel, et al., 1980) and quinones (Penning, et al., 1990) may be generated from this procedure. The HPLC profile and UV spectra are shown in figure 24 and 25, respectively. The UV spectrum and the column retention time of the new peak, matched that of peak 7 from the original metabolite generation procedure. Attempts to obtain MS and NMR data were foiled due to the rapid degradation (hours) of this sample.

Since the second incubation procedure was unsuccessful due to the instability of the compound, it was decided to look at NMR spectra of quinones and diols of other PAH. By comparing NMR spectra of diols and quinones, variation in signal position of adjacent protons to the diol and quinone could be observed. The naphthalene-1,2-diol and 1,2-naphthoquinone were both commercially available. Upon obtaining these compounds, NMR analysis was performed. Results are shown in table 5. The position of H3 adjacent to the diol and quinone did vary between the spectra. The position of H3 in the diol spectrum was 7.25 ppm, whereas the position in the quinone spectrum was 6.40 ppm. In the presence of the diol, H3 shifted approximately 0.9 ppm downfield.

Comparing the BjAA-9,10-quinone NMR spectrum to the peak 7 spectrum, a signal for H8 is not observed for the latter. However two undefined peaks are present in the

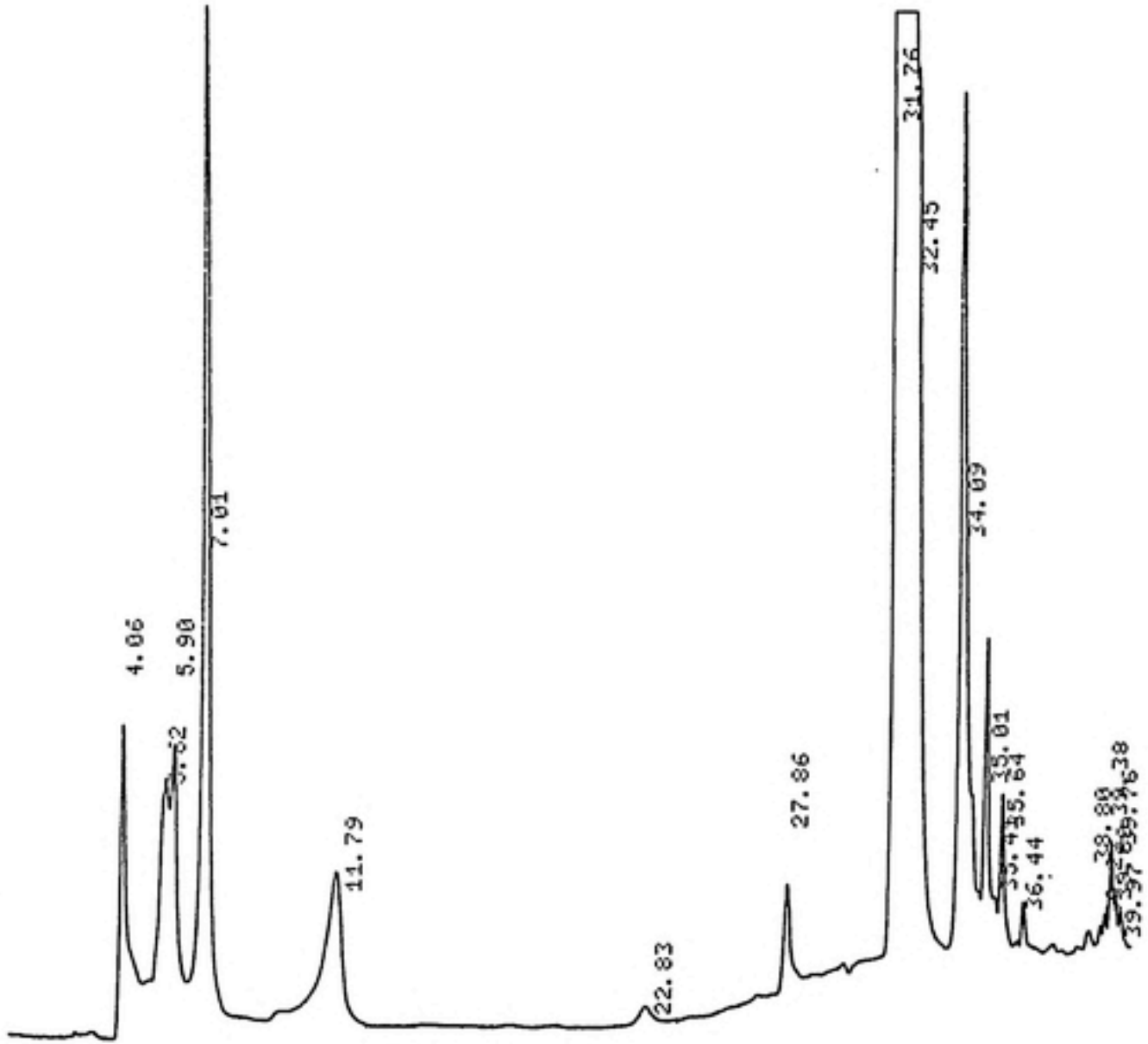

FIGURE 24: HPLC profile of the 9,10-dihydrodiol incubated with rat liver S9, under conditions which favored increased dihydrodiol dehydrogenase activity.

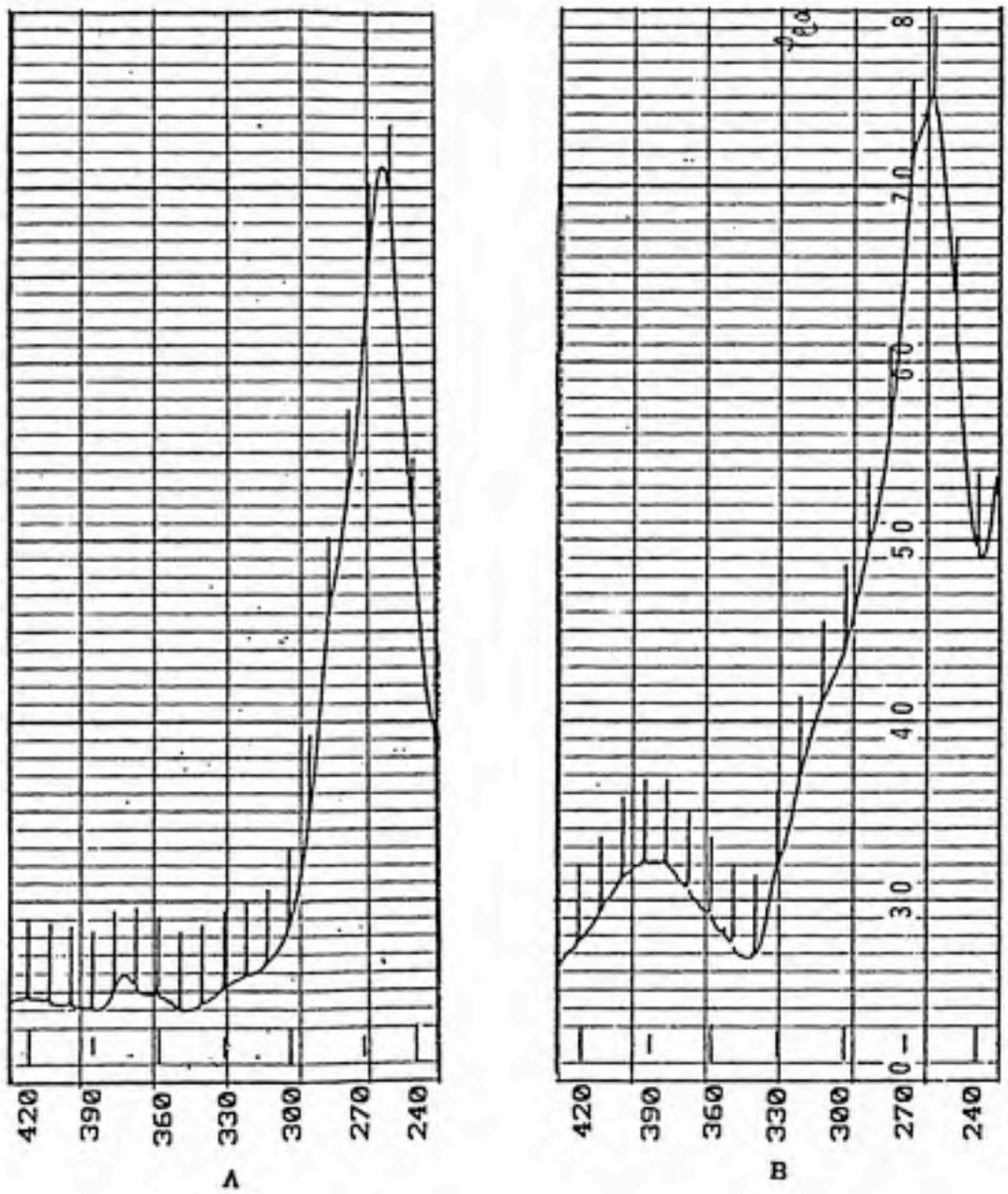

FIGURE 25: UV spectra, 230-430 nm, of the (A) benz[j]aceanthrylene-9,10-dihydrodiol and (B) unknown (peak 7) from the incubation that favored dihydrodiol dehydrogenase activity.

	Naphthalene-1,2-Diol		1,2-Naphthoquinone	
	PPM	J	PPM	J
OH1	7.93	-	-	-
OH2	7.77	-	-	-
H3	7.25	-	6.40	10.1
H4	7.72	8.16	7.61	10.1
H5	8.10	8.48	7.74	7.44
H6	7.30	-	7.57	-
H7	7.38	-	7.57	-
H8	8.22	8.34	8.01	7.12

TABLE 5: Comparison of NMR spectra of naphthalene-1,2-dione and 1,2-naphthoquinone.

peak 7 spectrum at 7.45 and 7.75 ppm. From the 1,2-naphthalene-diol and 1,2-naphthoquinone spectra, a diol formed at the 9 and 10 position of BjAA could shift H8 downfield about 0.9 ppm into proximity of the two undefined signals. From the MS and NMR data comparison, it was proposed that peak 7 is the 9,10-dihydroxy-BjAA.

IV.C. Metabolism of cyclopentafused benzo[e]pyrene

CpBeP was incubated according to the same procedure as used for the BjAA-9,10-dihydrodiol. The control sample contained all components, except S9. The HPLC profile is shown in figure 26. As expected only one major peak was seen, the unmetabolized cpBeP.

The HPLC profile of cpBeP in the presence of S9, figure 27, contained several peaks, with only one major peak, A, in addition to the parent compound, B. Peaks at 11.9', 12.4', 16', 18.2' and 22' were collected, however not enough sample was available for MS and NMR analysis. UV spectra of the minor metabolites (data not shown) suggested that oxidation had occurred at positions other than the cyclopentafused bond.

Peak B, the unmetabolized parent compound, cpBeP, eluted at 39.6' in the cpBep HPLC gradient program. When concentrated this compound is red-orange in color. This peak was identified by its UV spectra, figure 28, and its LC/MS, figure 29. The MS indicated a strong molecular ion

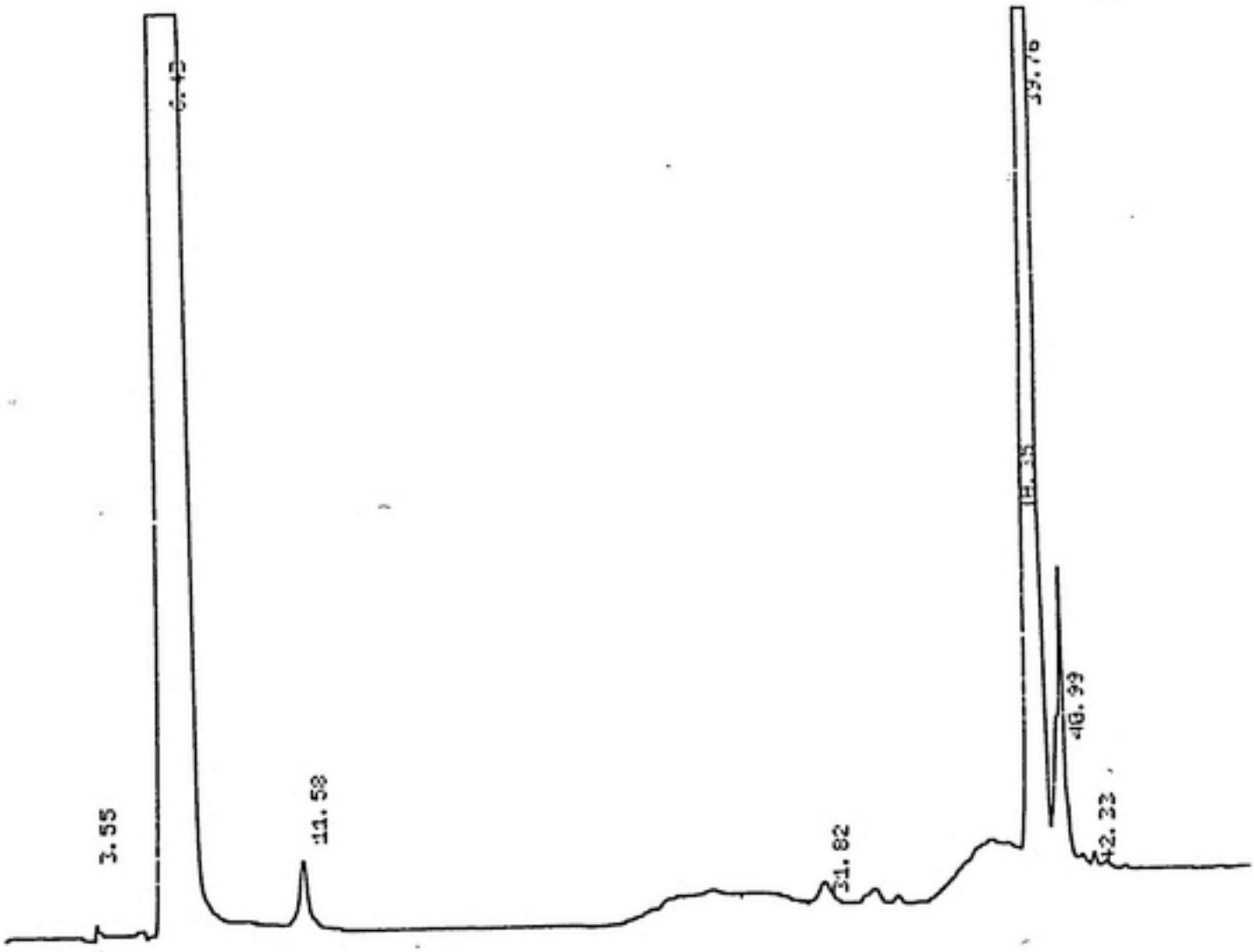

FIGURE 26: The HPLC profile of the cyclopentafused benzo[e]pyrene control incubation. The control incubation consisted of the compound and cofactors without S9.

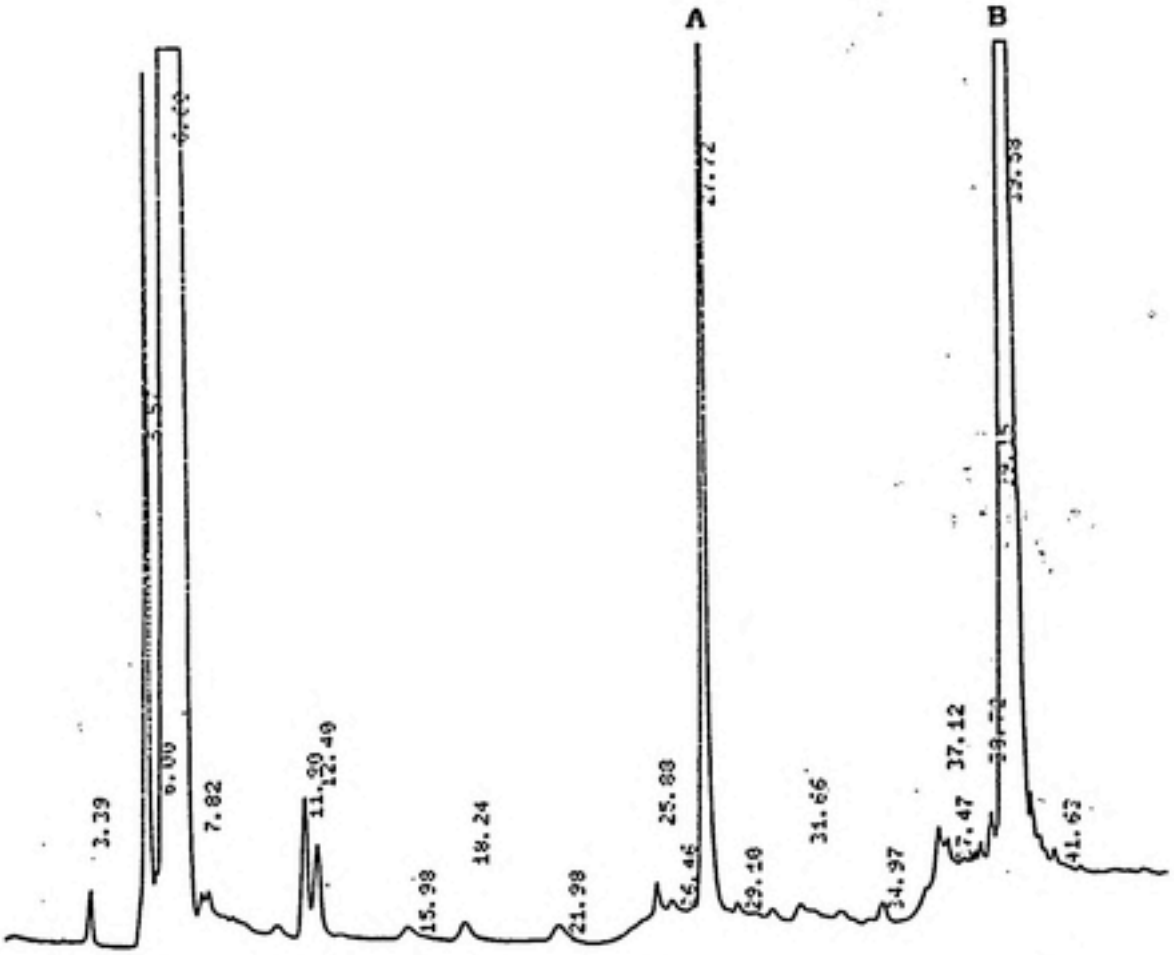

FIGURE 27: The HPLC profile of cyclopentafused benzo[e]pyrene incubated with rat liver S9.

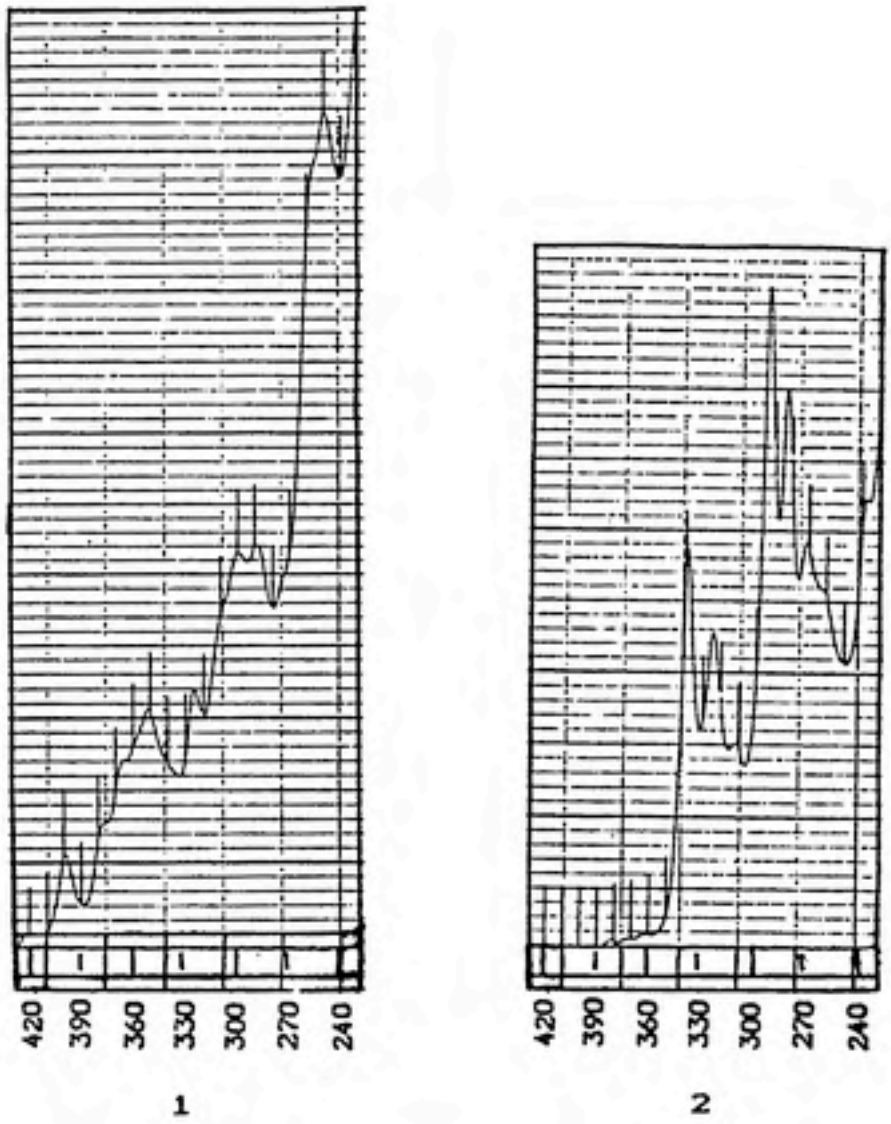

FIGURE 28: UV spectra, 230-430 nm, of the (1) cyclopentafused benzo[e]pyrene and the major metabolite, the (2) cyclopentafused benzo[e]pyrene-3,4-dihydrodiol.

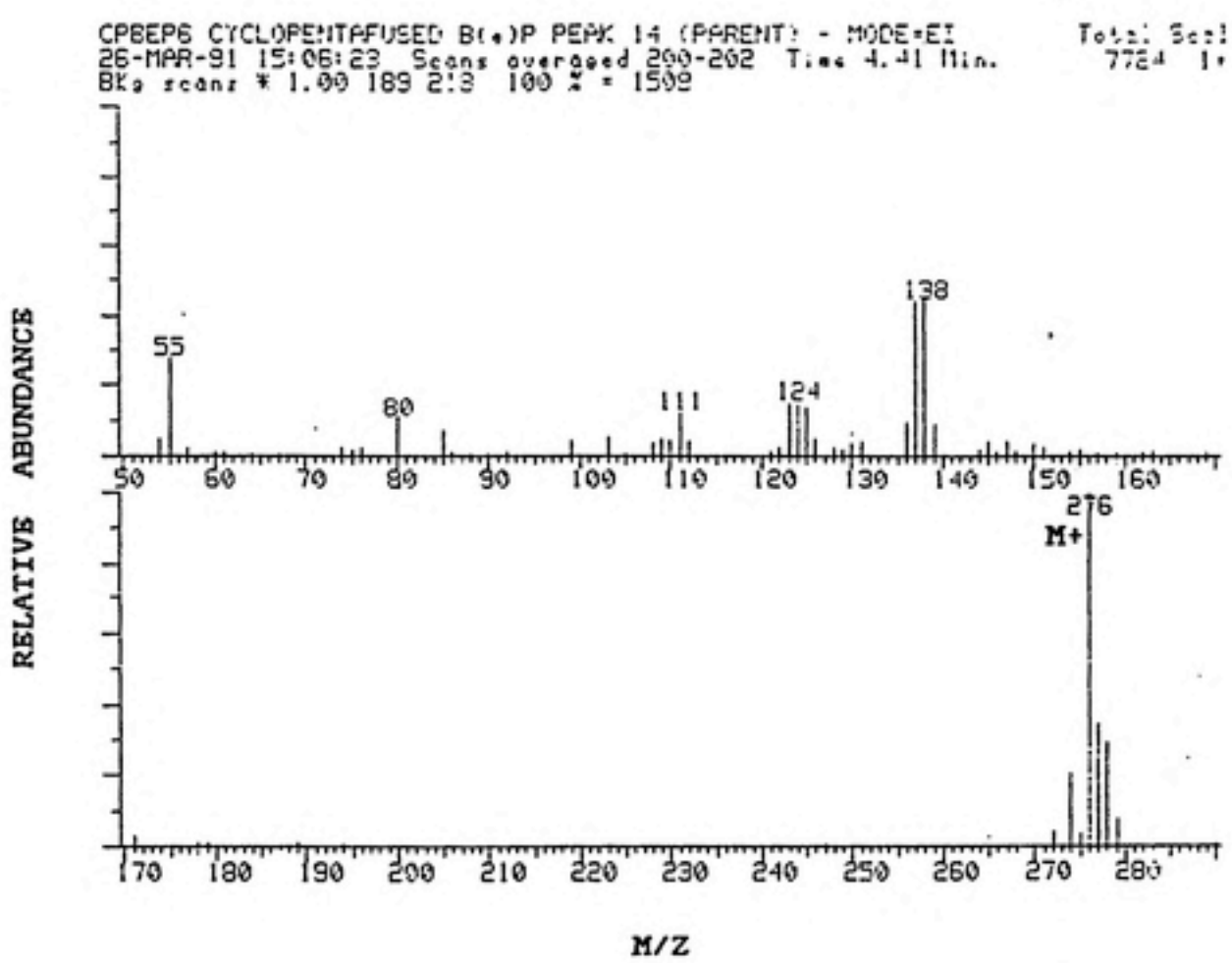

FIGURE 29: Mass spectrum of peak B, the cyclopentafused benzo[e]pyrene, mw. 276.

at m/z ratio 276. Little fragmentation is seen in this spectrum, characteristic of unsubstituted PAH. NMR analysis was performed using a 500 MHz instrument. The sample was dissolved in CD_2Cl_2 and the results are shown in table 6. This information will be useful in determining proton positions of other cpBeP metabolites.

Peak A eluted at 27.7'. When concentrated this sample was a lemon yellow color. UV spectra were recorded from 230 to 430 nm, figure 28. The LC/MS, figure 30, indicated a molecular weight at 310 with the following losses 18 (H_2O), 34 (H_2O_2), 47 (CH_3O_2) and 58 ($C_2H_2O_2$) yielding m/z ratios of 292, 276, 263 and 252, respectively. This data indicates the compound contains two oxygens. The losses of 18 and 16 indicate that the molecule may be a dihydrodiol. The additional loss of carbon suggests dihydrodiol is on the cyclopentafused ring. The NMR spectrum for peak A, figure 31, was compared with the parent compound. The proton assignments are shown in table 6. Signals corresponding to the etheno bridge protons 3 and 4 were not present in the aromatic region of the NMR spectrum. This evidence supports the speculation made from the MS data. It was concluded that this compound was the cpBeP-3,4-dihydrodiol.

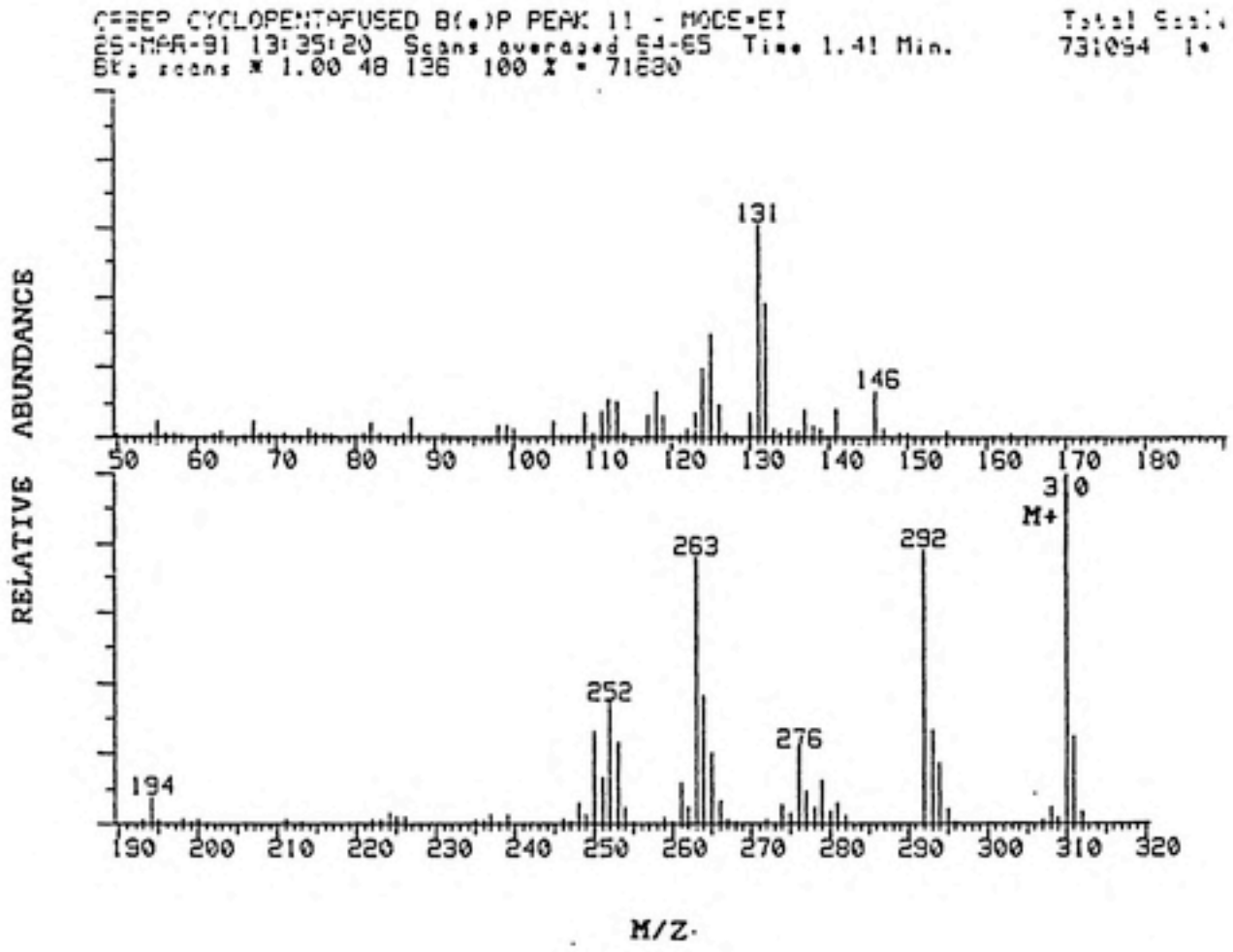

FIGURE 30: Mass spectrum of peak A, the cyclopentafused benzo[e]pyrene-3,4-dihydrodiol.

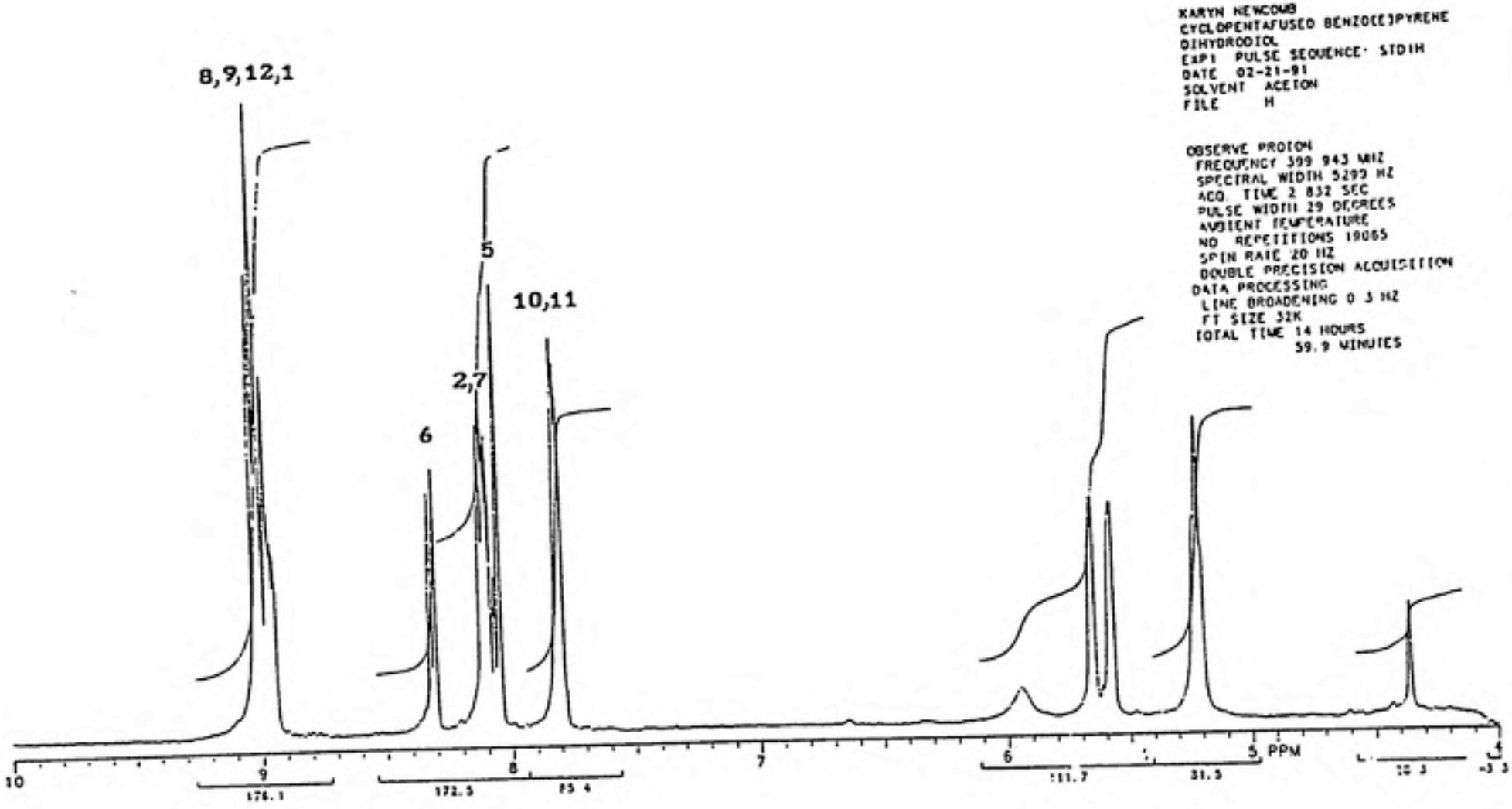

FIGURE 31: NMR spectrum of peak A, the cyclopentafused benzo[e]pyrene-3,4-dihydrodiol.

	Cyclopentafused Benzo[e]pyrene		cpBeP-3,4-Dihydrodiol		
	Signal	PPM	Signal	PPM	J
H1	d	8.75	m	9.0	-
H2	d	8.15	m	8.1	-
H3	d	7.43	-	-	-
H4	d	7.26	-	-	-
H5	s	8.35	s	8.05	-
H6	d	8.40	d	8.31	7.69
H7	t	8.05	m	8.1	-
H8	d	8.95	m	9.0	-
H9	t	8.89	m	9.0	-
H10/H11	m	7.75	m	7.8	-
H12	t	8.80	m	9.0	-

TABLE 6: NMR data of cyclopentafused benzo[e]pyrene in CD_2Cl_2 at 500 MHz, and the cyclopentafused benzo[e]pyrene-3,4-dihydrodiol in CD_3COCD_3 at 400 MHz.

V. DISCUSSION

PAHs are activated through monooxygenation by cytochrome P-450 and may undergo further reactions with cellular macromolecules such as DNA, RNA and proteins (Gold, et al., 1988). Structure-activity relationships are useful in determining whether mutagenic/carcinogenic effects may result from the metabolism of a compound.

The bay-region theory predicts chemical reactivity as well as biological activity of compounds containing bay-regions, based on carbonium ion formation (Jerina, 1977). As previously discussed these ions are readily formed on the saturated benzo-rings in the bay-region (Jerina, 1977). However the addition of other structural features on the molecule, such as the cyclopentafused ring, may effect the stability, hence reactivity of the carbonium ion formed in the bay-region. Our study demonstrates this point. The cyclopentafused ring of both BjAA and the cpBeP is oxidized by Aroclor treated rat liver S9 preferentially to other sites. Therefore activation via this metabolic pathway will predominate in any assay system containing this exogenous metabolic activtion system.

Research has shown that the ease of carbonium ion formation is related to the carcinogenic activity of the compound, however this relationship is not linear (Fu, et

al., 1980). A threshold for carcinogenic activity has been suggested by Gold and colleagues (1988) as a result of comparing delocalization energies and biological activity. This threshold was indicated to be $\Delta E_{deloc}/\beta > 0.7$.

V.A. Mutagenicity of Benz[j]aceanthrylene Metabolites

BjAA is unique in that it contains a bay-region, K-region and cyclopentafused ring, offering several sites of attack for cytochrome P-450. In our study, Ames assay results with S. typhimurium strain TA98 indicate the specific activity of BjAA to be 12.9 rev/nmol at 0.75 mg/plate of S9. Mutagenic activity of BaA in TA100 induced with Aroclor 1254 S9 is 11 rev/nmole (McCann, et al., 1975). This compound was also shown to be mutagenic in TA98 as well, although the specific activity was not given. The addition of the cyclopentafused ring in a non-bay-region of the compound may result in a slight increase in biological activity.

The ease of carbonium ion formation has been predicted for potential metabolites of BaA and BjAA, and biological activities have been measured and shown in table 7. The BaA-3,4-dihydrodiol-1,2-oxide appears to be the metabolite responsible for the mutagenic activity of the parent compound as discussed previously. The $\Delta E_{deloc}/\beta$ calculated, correctly predicted this result. Oxides formed on the cyclopentafused ring of BjAA were predicted to be more

COMPARISON OF MUTAGENIC ACTIVITY AND DELOCALIZATION ENERGIES OF BaA AND BjAA

TABLE 7

BaA METABOLITES[1]	ΔE_{deloc} /β	MUTAGENIC ACTIVITY	REFERENCES
3,4-dhd-1,2-oxide	0.766	1650 rev/nmol	Lehr, 1978
1,2-dhd-3,4-oxide	0.628	NA[2]	Lehr, 1978
10,11-dhd-8,9-oxide	0.572	55 rev/nmol	Jerina, 1978
8,9-dhd-10,11-oxide	0.526	50 rev/nmol	Conney, 1982

BjAA METABOLITES[3]	ΔE_{deloc} /β	MUTAGENIC ACTIVITY	REFERENCES
1,2-oxide	0.879	245 rev/nmol	Sangaiah, 1985; Bartczak, 1987
9,10-dhd-7,8-oxide	0.734	10 rev/nmol	Gold, 1988; this study

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[1] results from S. typhimurium strain TA100
[2] not available
[3] results from S. typhimurium strain TA98

mutagenic then the bay-region oxide. Ames assay results show that the 1,2-oxide is more than 40 times as mutagenic as the 9,10-oxide. These results suggest that the BjAA-1,2-dihydrodiol would be the metabolite responsible for the mutagenic activity of BjAA. Nesnow and colleagues (in press) have confirmed that both BjAA-1,2-oxide and the diol-epoxide interact with DNA forming adducts with 2'-deoxyguanosine and 2'-deoxyadenosine.

V.B. Metabolism of 9,10-dihydrodiol Benz[j]aceanthrylene

Although this metabolite has been shown to be a minor route of metabolic activation with rat liver S9 metabolism studies and S. typhimurium mutagenicity assays, it has been shown to be the major route of metabolic activation for C3H10T%CL8 cells. Therefore it was of interest to look at the metabolic pathway of this compound.

Four metabolites were identified from the BjAA-9,10-dihydrodiol incubation with rat liver S9 as described in the methods section. NMR and MS analysis confirmed that three of the metabolites, the 1,2,9,10-tetrahydro-1,2,9,10-tetrahydroxy-BjAA, the 7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxy-BjAA and the 9,10-dihydrodiol-BjAA-2-one, were the result of oxidation by cytochrome P-450 of the BjAA-9,10-dihydrodiol. As suggested by the specific activity of the 9,10-dihydrodiol-7,8-oxide incubated with S9 further oxidation at the 1,2 position of the molecule would result

in greater activity. The formation of the tetrol epoxides, the BjAA-1,2,9,10-tetrol-7,8-oxide and the BjAA-7,8,9,10-tetrol-1,2-oxide, as the ultimate carcinogenic metabolites was suggested by Nesnow and colleagues (in press). The 9,10-dihydrodiol-2-one forming a 7,8-oxide could also be an ultimate carcinogenic metabolite as suggested from the metabolism studies by Shou and colleagues (1990) with 3-MC-2-one.

Peak 7 appeared to be the 9,10-dihydroxy-BjAA from NMR comparison. Dihydrodiol dehydrogenase present in mouse, guinea pig, rat and human liver is another enzyme involved in the metabolism of xenobiotics (Penning, et al., 1990). Monooxygenation of PAHs by cytochrome P-450 results in epoxides that are normally detoxified by epoxide hydrolase. However in situations where epoxide hydrolase may not inactivate the epoxide, such as epoxides formed in the bay-region opposite a dihydrodiol or when the dihydrodiol is present in large concentrations, dihydrodiol dehydrogenase may be involved in detoxification. Experiments performed by Glatt and colleagues (1979) indicate a decrease in mutagenic activity of BaP when pure dihydrodiol dehydrogenase was added to 3-MC induced rat liver microsomes in the Ames assay. In strain TA98, the mutagenic activity of BaP decreased 30-60% confirming that dihydrodiol dehydrogenase was involved in detoxification of BaP.

Previous studies have demonstrated that benzenedihydrodiol incubated with pure dihydrodiol dehydrogenase resulted in the benzenediol (Glatt, et al., 1979). Other studies by Penning and colleagues (1990) suggest that pure dihydrodiol dehydrogenase incubated with several trans-dihydrodiols such as chrysene-1,2-dihydrodiol, BaP-7,8-dihydrodiol and BaA-3,4-dihydrodiol result in the formation of ortho-quinones. The series of reactions postulated for this oxidation is shown in figure 32. The quinones that are formed may bind with glutathione, thereby resulting in detoxification of the PAH.

Therefore peak 7 from the BjAA incubation is not a cytochrome P-450 metabolite, but results from oxidation by another enzyme, such as dihydrodiol dehydrogenase present in the rat liver S9 fraction. The 9,10-dihydroxy-BjAA intermediate may be nonenzymatically converted to the quinone or other detoxification products in the isolation and purification process, explaining the difficulty experienced in identifying this compound.

V.C. Mutagenicity of Cyclopentafused Benzo[e]pyrene

The mutagenicity of cpBeP was determined by Ball and colleagues (1991) and compared with BeP in S. typhimurium strain TA98 and TA100. The delocalization energies and mutagenicity results for cpBeP and BeP are shown in table 8. As mentioned earlier, BeP does not follow the bay-region

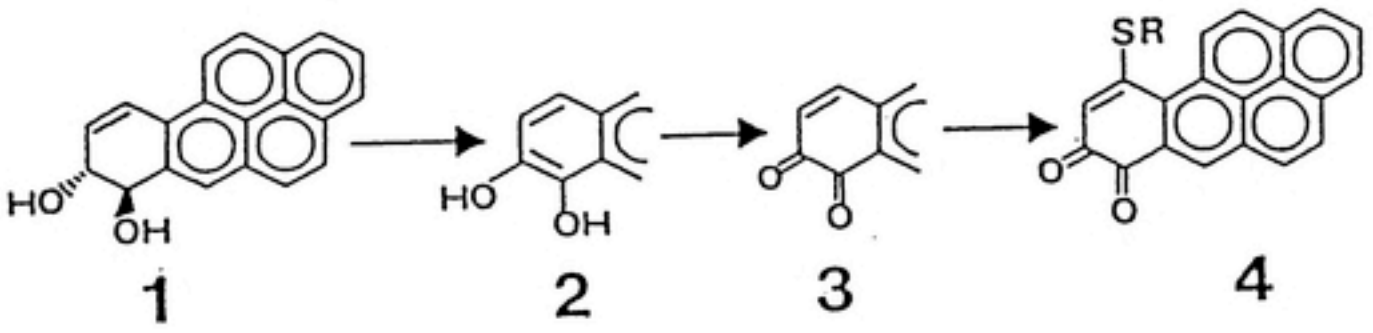

FIGURE 32: Pathway of detoxification of the <u>trans</u>-dihydrodiol by dihydrodiol dehydrogenase resulting in a diol intermediate (Penning, et al., 1990).

COMPARISON OF MUTAGENIC ACTIVITY AND DELOCALIZATION ENERGIES OF BeP, CPP AND cpBeP
TABLE 8

COMPOUND	$\Delta E_{deloc}/\beta$	MUTAGENIC ACTIVITY	REFERENCES
Benzo[e]pyrene(C12)	0.714	0.6 rev/nmol[1] 4.4 rev/nmol[2]	Jerina, 1977; Ball, 1991
Cyclopenta[c,d]pyrene (C1)	0.794	174 rev/nmol[3]	Sangaiah, 1988
Cyclopentafused Benzo[e]pyrene(C1)	0.736	22.8 rev/nmol[1]	Fu, 1980 Ball, 1991

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[1] reported for S. typhimurium strain TA98
[2] reported for S. typhimurium strain TA100
[3] reported for S. typhimurium strain TA1537

theory. Even though the molecule contains two bay-regions, and the delocalization energy of carbon 12 is above the "carcinogenic threshold level", the predominant metabolite occurs in the K-region. Only small amounts of the ultimate carcinogen is formed, the BeP-9,10-dihydrodiol-7,8-oxide, and this compound exhibits weak mutagenic activity in strain TA98 and moderate activity in TA100.

The effects of a cyclopentafused ring on a compound not containing a bay-region, with a similar structure as BeP and cpBeP, may be observed for cyclopenta[c,d]pyrene. The delocalization energy of carbon 1 on this molecule is 0.794, well above the threshold for genotoxic activity. The ultimate carcinogenic metabolite, 3,4-dihydrodiol-1,2-epoxide is readily formed, and is shown to be highly mutagenic with a specific activity of 1440 rev/nmole in S. typhimurium strain TA100.

These results suggest addition of a cyclopentafused ring on BeP would increase the mutagenic activity of the compound. CpBeP is mutagenic in TA98, table 8, however it is ten fold less active than CPP. The difference in mutagenic activity may be explained in the metabolite profile of cpBeP incubated with rat liver S9.

V.D. Metabolism of Cyclopentafused Benzo[e]pyrene

CpBep was incubated with Aroclor 1254 induced rat liver S9 as described in the methods section. Since the K-region

of the molecule is blocked due to the addition of the cyclopentafused ring, metabolism would be directed to either the cyclopentafused ring or the bay-regions. The HPLC profile indicated one major metabolite, identified as the cpBeP-3,4-dihydrodiol by MS and NMR analysis. Several additional minor metabolites were isolated, however, not enough sample was available for MS and NMR analysis.

The localization of electron density at the carbon-carbon double bond of the cyclopentafused ring directs metabolism, providing an alternative site for metabolic activation for cpBeP. The use of quantum mechanical calculations to correctly identify the chemically reactive and biologically active portion of the molecule has been successful. Therefore cpBeP is an exception to the bay-region theory.

Determining the ultimate carcinogenic metabolites of PAHs is unlikely to be accomplished by a single theory. The use of delocalization energies in the bay-region theory has been successful in predicting mutagenic metabolites, although exceptions do exist. Continuing research with compounds containing several structural regions, as studied here, with the assistance of quantum mechanical calculations may help in understanding the routes of metabolic activation of PAHs.

V.E. Recommendations

V.E.1 Benz[j]aceanthrylene-9,10-dihydrodiol

Additional work on the BjAA-9,10-dihydrodiol peak 5 needs to be done to determine if the ketone is located on the 1 or 2 position of the molecule. The location of the signal in the NMR will vary for adjacent and opposite protons to the oxo- substituent. Comparing NMR spectra of similar compounds such as BjAA-1-one and BjAA-2-one, or aceanthrylene-1-one and aceanthrylene-2-one, may be helpful in determining the identity of this metabolite.

The highly mutagenic metabolite BjAA-1,2-oxide generated by rat liver S9, warrants further study. The specific activity of this compound is over ten fold greater than the parent compound. It is likely that tetrol-epoxides are formed in this pathway as suggested for the bay-region diol-epoxide by Nesnow and colleagues (in press). A metabolite profile of BjAA-1,2-oxide incubated with rat liver S9 could confirm the formation of tetrol-epoxides.

V.E.2 Cyclopentafused Benzo[e]pyrene

Several minor metabolites were isolated from the incubation of cpBeP with rat liver S9. Determining the identity of these compounds, whether they resulted from oxidations in just the bay-region, or both the bay-region and on the cyclopentafused ring would be of interest. Possible mutagenicity and metabolism studies with

synthesized cpBeP-3,4-dihydrodiol may be more helpful in the latter case, since metabolite yields may be increased.

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