CHARACTERIZATION OF CYCLOPENTA[*cd*]PYRENE-DNA ADDUCTS *IN VITRO* AND IN MICE *IN VIVO*

by

CHING-HUNG HSU

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Signature of Author _____

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Certified by ____
          /
                                        Steven R. Tannenbaum
                                        Thesis Supervisor

Accepted by
                                                      Peter C. Dedon
                               Chair, Department Graduate Committee

This doctoral thesis has been examined by a committee of the Division of Toxicology as follows:

Professor Gerald N. Wogan _____ Chairman

Professor Steven R. Tannenbaum _____ Thesis Supervisor

Professor Peter C. Dedon _____

Professor John E. Essigmann _____

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Abstract

Exposure to chemical carcinogens is believed to be an important etiological factor in human cancer. Carcinogen-DNA adducts are considered to represent the initiating events leading to cancer. This dissertation was undertaken to determine the DNA adduct formation by cyclopenta[*cd*]pyrene (CPP), a ubiquitous environmental carcinogen, in vitro and in mice.

Calf thymus DNA was reacted in vitro with CPP 3,4-epoxide (CPPE) or with its metabolites, 3,4-dihydroCPP-3,4-diol (CPP-3,4-diol) and 4-hydroxy-3,4-dihydroCPP (4-OH-DCPP), activated with sulfotransferase. The results demonstrated that the two major CPPE-derived adducts were formed by guanine, while minor adducts were formed by adenine and cytosine. Specifically, the major dG adduct formed in DNA was identified as *cis*-3-(deoxyguanosin-N2-yl)-4-hydroxy-3,4-dihydroCPP (3-dG-4-OH-DCPP) and the minor dG adduct seems likely to be its *trans* isomer. Adenosine adducts were presumably formed by aralkylation of the N6 position since they co-chromatographed with regio-specifically synthesized standards. Sulfotransferase activation of *trans*-CPP-3,4-diol yielded two adducts that were identical to the products resulting from the reaction of CPPE with DNA, while *cis*-CPP-3,4-diol gave very low covalent binding. Adducts formed by sulfotransferase activation of 4-OH-DCPP were identical to the diastereomeric products generated from the reaction of synthetic 4-NaO$_3$S-O-DCPP with dG. These results indicate that guanine is predominantly the site of CPP adduct formation in DNA, regardless of the identity of the reactive metabolite, and that the 4-hydroxy-3-dG adducts can arise by reaction of DNA with either CPPE or sulfotransferase-activated *trans*-CPP-3,4-diol.

In CPP-treated CD-1 mice, four and six peaks were detected in the DNA digests of liver and lung samples, respectively, by HPLC. Cryogenic fluorescence spectroscopic analysis indicated that all peaks were derived from CPPE. These peaks were also identified by their chromatographic similarity to synthetic CPP metabolites and adduct standards formed in vitro. The results suggest that adducts

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detected in liver DNA digests are *trans*-3-dG-4-OH-DCPP, two diastereomers of *cis*-3-dG-4-OH-DCPP, and *trans*-3-deoxyadenosinyl-4-hydroxy-3,4-dihydroCPP (3-dA-4-OH-DCPP). Adducts detected in lung samples are probably *trans*-3-dG-4-OH-DCPP, two diastereomers of *cis*-3-dG-4-OH-DCPP, *cis*-CPP-3,4-diol, *cis*-3-dA-4-OH-DCPP, and 4-OH-DCPP. The detection of CPP-3,4-diol and 4-OH-DCPP suggests that the formation of unstable adducts by CPP. Furthermore, both CPPE and sulfotransferase-activated *trans*-CPP-3,4-diol could be the major ultimate carcinogenic metabolites of CPP, while sulfotransferase-activated 4-OH-DCPP could be a minor one.

Thesis Supervisor: Dr. Steven R. Tannenbaum
Professor of Chemistry and Toxicology

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List of Abbreviation

3MCA	3-methylcholanthrene
4-OH-DCPP	4-Hydroxy-3,4-dihydroCPP
6TG	6-thioguanine
^{35}S-TBM-NHS	t-butoxycarbonyl-L-[^{35}S]methionine,N-hydroxysuccinimidyl ester
AFB$_1$	aflatoxin B$_1$
BaP	benzo[a]pyrene
BF	benzo[ghi]fluoranthene
B[j]A	benzo[j]aceanthyrlene
BPDE	BaP-7,8-dihydrodiol-9,10-epoxide
BSTFA	N,O-bis-(trimethylsilyl)trifluoroacetamide
CHO	Chinese hamster ovary
cisplatin	cis-diamminedichloroplatinum(II)
CPAA	cyclopent[hi]aceanthrylene
CPAP	cylcopent[hi]acephenanthrylene
CPP	cyclopenta[cd]pyrene
CPP-3,4-diol	3,4-Dihydroxy-3,4-dihydroCPP
CP-PAHs	cyclopenta-fused PAHs
CPPE	CPP 3,4-epoxide
DCNP	2,6-dichloro-4-nitrophenol
DGGE	denaturing gradient gel electrophoresis
DHCPP	3,4-Dihydrocyclopenta[cd]pyrene
DHEA	dehydroepiandrosteone
DMBA	7,12-Dimethylbenz[a]anthracene
DMF	dimethylformamide
ECD or EC	electrochemical conductance detection
ELISA	enzyme-linked immunosorbent assay
FA	fluoranthene
FLN	fluorescence line-narrowing
GC/FID	GC/flame ionization detection
GC/NPD	GC-nitrogen phosphorus detection
hifi-PCR	high-fidelity polymerase chain reaction
HPRT	hypoxanthine phosphoribosyltransferase
ISB	immuno-slot blot technique
LIF	Laser Induced Fluorescence
MS/MS	tandem mass spectrometry
PAH	Polycyclic aromatic hydrocarbon
PAPS	3'-Phosphoadenosine-5'-phosphosulfate
PCP	pentachlorophenol
PMO	perturbation molecular orbital
PMT	photomultiplier tubes
RIA	radioimmunoassay
SFS	synchronous fluorescence spectrometry
USERIA	ultrasensitive enzymatic radioassay

1.   Introduction

Exposure to chemical carcinogens is believed to be an important etiological factor in human cancer. Carcinogen-DNA adducts are considered to represent the initiating events leading to mutations and malignant transformation, which ultimately lead to cancer. The determination of DNA adduct formation and levels in vivo, therefore, can reveal both the DNA damage and exposure to the carcinogens. In addition, addressing the DNA adduct formation of the major metabolic products of a carcinogen in vitro can provide information about the roles that these metabolites may play in the formation of DNA adducts in vivo.

Cyclopenta[cd]pyrene (CPP, Figure 2.1) is a widespread environmental pollutant produced by incomplete combustion. It is found in, for example, carbon black (Wallcave et al., 1975), cigarette smoke (Snook et al., 1977), automobile exhaust (Stenberg et al., 1983), and rural and urban air particulate (Grimmer et al., 1980; Nielsen, 1983) and generally co-occurs with benzo[a]pyrene (BaP). CPP may be as much as 10- to 20 fold more abundant than BaP (Grimmer et al., 1977; Grimmer, 1979; Skopek et al., 1979). It shows high mutagenicity upon bioactivation in both bacterial and mammalian cells (Wood et al., 1980; Raveh et al., 1982; Eisenstadt and Gold, 1978; Cavalieri et al., 1981b). In addition, CPP is a strong inducer of adenocarcinoma in newborn mice (Busby et al., 1988), adenomas in weanling A/J mice (Nesnow et al., 1994), and is carcinogenic in mouse skin bioassays (Wood et al., 1980; Raveh et al., 1982). CPP was more potent than BaP in at least three studies including its mutagenicity in bacteria, induction of malignant tumors, and tumorigenicity of A/J mouse lung (Eisenstadt and Gold, 1978; Busby et al., 1988; Nesnow et al., 1994).

CPP is one of a series of cyclopenta-fused polycyclic aromatic hydrocarbon (PAH), which, if lacking a bay region, may be metabolically activated by epoxidation of the fixed double bond in the 5-membered ring. The resulting 3,4-

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epoxide of CPP is often considered to be the ultimate carcinogen, though evidence for diol epoxides as reactive electrophiles has also been presented (Sahali et al., 1992; Kwon et al., 1992). The principal adducts formed by CPP 3,4-epoxide (CPPE) with DNA, both in vivo and in vitro, have been characterized as products of reaction with guanine (Nesnow et al., 1994; Beach et al., 1993; Beach and Gupta, 1994). While the structures of the guanine adducts have not yet been elucidated, they are commonly thought to be the 4-hydroxy-3-(N2-guanyl)dihydroCPP. The first part of this dissertation describes detailed information regarding the regio- and stereo-chemistry of guanine adduct formation by CPPE.

Two important metabolites of CPPE, 3,4-dihydroCPP-3,4-diol (CPP-3,4-diol) and 4-hydroxy-3,4-dihydroCPP (4-OH-DCPP), have been shown to react with DNA and to be mutagenic when activated by sulfotransferase. The first of these would be expected to produce the same adduct as CPPE. 4-OH-DCPP would yield a unique adduct but there is no certainty that it would be separated from dG-CPP under the chromatographic conditions used to detect in vivo adducts (Nesnow et al., 1994; Beach and Gupta, 1994). The second part of this dissertation characterizes the binding of these two secondary metabolites with DNA and determines if bases other than guanine are alkylated by CPPE.

In the third and final section, the identification of adducts formed in a mouse model is reported along with the inference of adduct-forming metabolites of CPP. To our knowledge this is the first report to investigate the formation of PAH-DNA adducts in vivo by laser-induced fluorescence spectroscopy without adduct hydrolysis.

2.   Literature Survey

2.1   Polycyclic aromatic hydrocarbons

2.1.1. Source and composition

Polycyclic aromatic hydrocarbons (PAHs) contain two or more fused benzene rings in angular, linear or cluster arrangements.   They can be produced by incomplete combustion or high temperature pyrolytic processes involving fossil fuels (Badger, 1962), and by definition PAHs consist of only C and H. PAHs were one of the first atmospheric contaminants to be identified as being carcinogenic. These compounds are ubiquitous in the urban atmosphere. Therefore, they have undergone considerable examination. Recently, attention has focused on the possible adverse effects on human health of these chemical carcinogens in the atmospheric environment (Baek et al., 1991).

Several environmental pathways including diet can contribute to human exposure to PAHs (Basu and Saxena, 1978; Lijinsky and Ross, 1967; Baek et al., 1991; Higginson and Muir, 1976). However, the occurrence of PAHs in ambient air has caused the most concern due to the size of risk population and the continuous exposure, especially in urban, suburban and industrial areas. The concentration of PAHs in ambient air, PAH distribution between the gaseous and particulate phases, and the size of the PAH particles are some factors that are associated with the extent of human exposure to PAHs. In addition, the evaluation of the physical and chemical characteristics of airborne PAHs as well as the identification of the major emission sources of PAHs are two major parameters for assessing the significance of PAHs in ambient air.

PAH emissions from human activities are the predominant source even though some PAHs can arise from the natural combustion such as volcanic eruptions

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and forest fires (Nikolaou et al., 1984). The sources of PAH can generally be divided into mobile and stationary categories. Vehicular petrol and diesel engines are the major contributors in the mobile category. Within the stationary category, a wide variety of combustion processes, including residential heating, industrial activities (e.g., aluminum production and coke manufacture), incineration and power generation (Hangerbrauck et al., 1967; United States National Academy of Sciences, 1972), may all produce high atmospheric PAH concentrations in the vicinity of the major emitters (Baek et al., 1991; United States National Academy of Sciences, 1983).

The composition of PAHs produced from different pyrolytic processes varies. It depends upon the combustion conditions and the fuel type (Blumer, 1976; Westerholm et al., 1988). For instance, high BaP concentrations seem to arise from low temperature combustion (Freeman and Cattell, 1990). Other factors such as the emission rate of the PAH source and the local climatic conditions can also make certain contribution to the atmospheric PAHs (Harkov and Greenberg, 1985). Nevertheless, it has been estimated that the stationary sources account for approximately 90% of the total PAH emissions with the remainder produced by mobile source. But in urban or suburban areas, where major stationary sources are not present, mobile sources contribute the majority of PAHs (Baek et al., 1991; United States National Academy of Sciences, 1983).

BaP was the first identified carcinogenic PAH found in coal tar (Cook et al., 1933). Because of its early identification, BaP has been investigated extensively and used as a model compound for PAHs. However, it is only one of many carcinogenic PAHs including benzo[a]anthracene, benzo[b]fluoranthene, indeno[cd]pyrene, 7,12-dimethylbenzo[a]anthracene (DMBA), and dibenzo[ae]pyrene. Therefore, using BaP as the sole or even a main indicator to assess the impact of human exposure to PAH mixtures on health obviously raises question. In soot extract, for example, CPP (Figure 2.1), fluoranthene (FA) and

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perylene occur in greater amounts than BaP and account for the major mutagenicity of the soot extract using a bacterial forward mutation assay (Kaden et al., 1979). CPP and FA also exhibit potent biological effects and have been recognized as potential health hazards, such as in human cell forward mutation assays (Skopek et al., 1979). Identification of all the components of PAH mixtures is time-consuming. Thus, it seems practical to select known index substances for the health effects posed by PAHs. This approach should be valid if it is based on the amount/concentration and genotoxicity of each PAH compound from different sources.

CPP belongs to a unique class of cyclopenta-fused PAHs (CP-PAHs). It has been identified, for instance, in coal-tar pitch (Wallcave, 1969), carbon black (Wallcave et al., 1975), and cigarette smoke (Snook et al., 1977). The level of CPP in gasoline auto exhaust condensates is 8 to 25 times higher than that of BaP (Tong and Karasek, 1984; Grimmer et al., 1977; Grimmer, 1979). In a diesel engine exhaust analysis, the amount of CPP has been shown to be equal to the sum of BaP, benzo[e]pyrene and perylene (Schuetzle et al., 1981). In the mixture of kerosene soot extract, CPP is also the major contributor of mutagenicity (Skopek et al., 1979). Being more mutagenic and structurally intriguing, CPP, at concentrations equal to or higher than that of BaP , may be a good indicator of health risk associated with exposure to PAH mixtures.

2.1.2 Metabolism of cyclopenta[cd]pyrene

The ultimate carcinogenic form of CPP is not known since no DNA adduct structures have yet been determined. Epoxidation of the 3,4 double bond which forms the cyclopenta ring appears to be the major microsomal metabolic pathway (Eisenstadt et al., 1981; Gold and Eisenstadt, 1980; Sahali et al., 1992; Kwon et al., 1992). Therefore, it has been suggested that the 3,4-epoxide may be the ultimate carcinogen derived from CPP. This hypothesis was also supported by the

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preponderance of 3,4-dihydrodiol among the products of microsomal metabolism (Eisenstadt et al., 1981; Gold and Eisenstadt, 1980; Sahali et al., 1992; Kwon et al., 1992) and by perturbation molecular orbital (PMO) calculations (Fu et al., 1980).

The PMO approach to CPP has shown that the 3,4 double bond of the penta-fused ring of CPP possesses the highest bond order and is thus the most olefinic double bond. The olefinic characteristic of the highly localized double bond renders the five-member ring of CPP very suspectable to epoxidation (Fu et al., 1980). Also, the carbonium ion resulting from the ring opening of the penta ring epoxide can be stabilized by the pyrene of CPP. Similarly, the carbonium ion derived from CPP 3,4-epoxide (CPPE) also appears in the bay region of tetrahydrobenzo-ring epoxies of PAHs (Figure 2.2) (Eisenstadt and Gold, 1978). The mutagenicity of several oxygenated derivatives of CPP, however, casts some uncertainty on the role of CPPE as a DNA-binding metabolite. For instance, *cis*-3,4-dihydroxy-3,4-dihydroCPP (CPP-3,4-diol) is more mutagenic than 3-hydroxy-3,4-dihydroCPP. The isomeric *trans*-3,4-diol and the related 4-hydroxy and 3 or 4-keto derivatives are also potent mutagens using *Salmonella typhimurium* strain TM677 forward mutation assay (Santella et al., 1985).

Incubation of CPP with rat liver microsomal system has been reported to yield two major metabolites, the *trans*-3,4-dihydrodiol and the *trans*-9,10-dihydrodiol (Gold and Eisenstadt, 1980). The formation of 3,4-dihydrodiol suggested that 3,4-epoxide is transiently formed, and the formation of a 9,10-dihydrodiol indicated that oxidation is not limited to the 3,4 double bond in CPP.

Since CPP is a mouse carcinogen, the metabolism of CPP by both mouse and human live microsomes was determined (Sahali et al., 1992). This study identified additional CPP metabolites from which the identities of genotoxic alkylating species might be deduced. The major metabolite was still *trans*-3,4-diol as previously found

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in rat liver microsomal incubation. In addition, cis-3,4-diol, 4-hydroxy-3,4-dihydroCPP (4-OH-DCPP), and 4-oxo-3,4-dihydroCPP were observed. Two extra pairs of metabolites were characterized as isomeric tetrahydrotetrols and dihydrotriols. The trans-3,4-dihydrodiol functionality was found in the dihydrotriols. Further investigation also showed that either 3,4- or 9,10-trans-dihydrodiol could serve as the precursor of the tetrahydrotetrols. The updated metabolic pathway of CPP, summarized from this study, is shown in Figure 2.3.

Oxidation of CPP by mouse and human microsomes and selected cytochrome P450 enzymes has been investigated recently and a basis for interspecies comparison was established (Kwon et al., 1992). All the CPP metabolites identified from the mouse liver microsomal incubations were also observed in human samples. However, three human microsomal samples produced different amounts of these metabolites. The microsomes derived from genetically engineered cells containing specific cytochrome P450 isozyme cDNAs were employed to examine the variation among human samples. These studies suggested the possibility of the 3,4-dihydrodiol 9,10-epoxide and the 9,10-dihydrodiol 3,4-epoxide of CPP.

2.1.3   Biological effects of CPP

The biological effects of CPP have not to date been studied extensively especially when compared to BaP. Studies show that biological activity of a number of PAHs results from metabolism to bay region diol-epoxides, which are capable of forming covalent adducts at nucleophilic sites within DNA. CPP has evoked considerable interest because it has a wide environmental distribution and it lacks a bay region.

Cyclopenta-fused PAH (CP-PAH) is a unique class of PAHs that is abundant in the effluent from a number of different combustion systems and is also present

in airborne particulate matter (Gold, 1975; Yergey et al., 1982; Prado et al., 1981; Nesnow et al., 1981; Cavalieri et al., 1981b; Nesnow et al., 1984). Many CP-PAHs have been revealed to be mutagenic and animal carcinogenic and thus potentially pose a significant health threat to humans (Gold, 1975; Nesnow et al., 1981; Nesnow et al., 1984). CPP, one of several $C_{18}H_{10}$ CP-PAH products from different combustors and pyrolyzers operated over a wide range of conditions, was found to be the most important bacterial mutagen emitted from a jet-stirred/plug-flow reactor (Prado et al., 1981). Recently, three of the other $C_{18}H_{10}$ CP-PAHs including cylcopent[*hi*]acephenanthrylene (CPAP), cyclopent[*hi*]aceanthrylene (CPAA) and benzo[*ghi*]fluoranthene (BF) were also identified. These four $C_{18}H_{10}$ isomers, together with BaP, were tested in bacterial and human cell mutagenicity assays. In a forward mutation assay using *Salmonella typhimurium*, CPP, BF and CPAA were roughly twice as mutagenic as BaP, whereas CPAP was only slightly active. In a human cell mutagenicity assay using MCL-3 cells, a derivative of AHH-1 TK$^{+/-}$ cells containing high P450 1A1 activity, CPP and CPAA were strongly mutagenic but less active than BaP, while CPAP and BF were inactive at the dose levels tested (Lafleur et al., 1993).

The 3,4-epoxide of CPP has been synthesized and shown to be a powerful direct-acting mutagen to *Salmonella typhimurium* (Cavalieri et al., 1981b; Gold et al., 1979). It was also suspected as the ultimate mutagenic metabolite of the parent molecule as mentioned before. Again, the confirmation of the 3,4-epoxide as a primary metabolite and ultimate mutagen was mainly based on the identification of *trans*-3,4-diol as a major metabolic product from various enzymatic incubations (Eisenstadt et al., 1981; Gold and Eisenstadt, 1980; Sahali et al., 1992; Kwon et al., 1992).

3,4-Dihydrocyclopenta[*cd*]pyrene   (DHCPP),   the   3,4-dehydrogenated derivative of CPP, is a constituent of coal tar pitch and also widely distributed in

the environment (Wallcave et al., 1975; Wallcave, 1969). In an earlier study, it was examined for its carcinogenicity, mutagenicity and DNA binding compared with CPP and its derivatives (Cavalieri et al., 1981b). In a bacterial mutation assay, DHCPP was several times less mutagenic than CPP. Nonetheless, DHCPP exhibited greater mutagenic activity than pyrene, indicating that the five-member ring contributes to the activity of DHCPP. The oxygenated derivatives of DHCPP at the 3 and 4 positions, including 3- and 4- ketone and hydroxy CPP, showed the same relatively low mutagenicity as DHCPP. The cis-3,4-diol was approximately as mutagenic as CPP and BaP. In contrast, trans-3,4,-diol was the least mutagenic compound in these CPP and its derivatives. Since its level of tumor-initiating activity is similar to that of benzo[a]anthracene, CPP was considered to be a weak tumor initiator in mouse skin (Wood et al., 1980). DHCPP was inactive in both initiation-promotion and repeated application tumorigenicity experiments on mouse skin. Although less active than BaP, CPP was moderately potent as a complete carcinogen in mouse skin (Cavalieri et al., 1981; Cavalieri et al., 1983). Interestingly, most of the CPP-induced neoplasms were malignant (Busby et al., 1988) and some metastasized to lung and lymph nodes (Cavalieri et al., 1983). The tumorigenic inactivity of DHCPP suggests that the carcinogenic effect of CPP may be due to formation of the predicted ultimate derivative CPPE. The involvement of epoxidation of 3,4 double bond on CPP is also strengthened by the high binding of CPP to mouse skin nucleic acids in vivo, whereas binding of DHCPP was insignificant (Cavalieri et al., 1981b).

Other biological effects of CPP include its mutagenicity to V79 cells (Raveh et al., 1982), L5178Y mouse lymphoma cells (Gold et al., 1980) and Chinese hamster ovary (CHO) cells (Krolewski et al., 1986). CPP was also shown to be a strong mutagen in human diploid lymphoblast (Skopek et al., 1979; Crespi and Thilly, 1984) and it transformed mammalian cells and caused sister chromatid exchanges (Eisenstadt and Gold, 1978). CPP was also reported to induce fibrosarcoma in CFW mice (Neal and Trieff, 1972).

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In conclusion, CPP was highly mutagenic, when compared to BaP (Wood et al., 1980), and was carcinogenic in mouse skin tumorigenicity assays with (Wood et al., 1980; Cavalieri et al., 1981) and without (Raveh et al., 1982; Cavalieri et al., 1981) subsequent promotion. In newborn mouse lung adenoma bioassay, CPP was a strong inducer of malignant lung tumors and resulted in synergistic malignant tumor induction with BaP (Busby et al., 1988). Although CPP was thought to be less carcinogenic than BaP, based on mouse skin studies, the observed skin cytotoxicity and inverse dose-tumorigenicity relationships at the doses of CPP commonly used clouded the data (Cavalieri et al., 1981; Cavalieri et al., 1983). In contrast, CPP exhibited five times more tumorigenic activity than BaP in the A/J mouse lung adenoma system. Additionally, the high malignancy index of CPP in the newborn mouse assay (Busby et al., 1988) and its potent effect in mixtures on tumor multiplicity and metastases (Cavalieri et al., 1983) warrant its consideration as a potent carcinogen.

2.1.4 Mutational spectra of CPP

CPP induces mutations in various systems. The spectrum of basepair substitution mutations induced in *lacI* gene of a *uvrB⁻* strain of *Escherichia coli* by CPPE as well as by baP-7,8-dihydrodiol-9,10-epoxide (BPDE) was determined. Either BPDE or CPPE induced about 10% of nonsense mutations in all *lacI* gene mutations, suggesting that basepair substitutions are a large fraction of the mutational events caused by these species in this system. Both carcinogens produced the major G:C→T:A and the minor A:T→T:A transversions. One potential mechanism for these induced transversion induction at G:C sites might involve carcinogen binding to the exocyclic amino group of guanine in the template strand followed by a rotation of the modified base around its glycosydic bond from the *anti* to the *syn* conformation. Then, an imine tautomer of adenine could pair with these modified bases (Eisenstadt et al., 1982).

In the most recent CPP-induced mutation report, four independent cultures of a human cell line expressing cytochrome P450 1A1 (cell line MCL-15) were treated with CPP, and mutants at the hypoxanthine phosphoribosyltransferase (HPRT) locus were selected against 6-thioguanine (6TG). The mutations were examined by the combination of high-fidelity polymerase chain reaction (hifi-PCR) and denaturing gradient gel electrophoresis (DGGE). In detail, the third exon of the HPRT gene was first amplified from 6TG-resistant cells using the hifi-PCR. Then, the amplified fragment was analyzed by DGGE to separate mutant sequences from the wild-type sequence. Finally, mutant bands were excised from the gel, amplified using PCR and sequenced. Sixteen different mutations were identified and consisted mostly of the G to C transversion and a single G deletion. Six mutations occurred within a run of six guanines. The majority of transversions induced by CPP involved a guanine or an adenine, which is similar to the data previously reported for the racemic mixture of BaP in human TK6 cell line. This observation also suggested that the mechanisms of mutation induced by CPP may be similar to those induced by BaP (Keohavong et al., 1995), as it observed in the *lacI* gene of *Escherichia coli* treated with CPPE or BPDE mentioned above (Eisenstadt et al., 1982).

The mutational spectrum in Ki-*ras* oncogene from CPP-induced tumor in strain A/J mice lung was also explored. Ki-*ras* codon 12 mutation analyses of the DNA from tumors taken from CPP dose groups displayed the following mutations: GGT→CGT (50%); GGT→GTT (15%); GGT→TGT (25%); GGT→GAT (10%). Using this A/J mouse lung adenoma model, CPP is five times more tumorigenic than BaP. This is different from the result that CPP was a considerably less potent mouse skin tumorigen or tumor initiator when compared to that of BaP. Therefore, it is suggested that the increased activity of CPP may be due to the unique induction of the GGT→CGT on Ki-*ras* codon 12 mutation (Nesnow et al., 1994). The related results observed with another CP-PAH, benzo[*j*]aceanthyrlene (b[j]A), also sustained

this hypothesis (see below).

B[j]A is a demethylated analogue of 3-methylcholanthrene (3-MCA) with an unsaturated cyclopenta ring. It produced 16 to 60 fold more A/J mouse lung tumors at equivalent doses than did BaP, and it induced a similar mutational spectrum as CPP did in A/J mouse lung tumors (Mass et al., 1993). The mutations induced by B[j]A were GGT→CGT (65%), GGT→GTT (30%), and GGT→TGT (4%). In contrast, BaP induced GGT→TGT (56%), GGT→GTT (25%), and GGT→GAT (19%) mutations. 3-MCA was shown to be activated primarily via bay-region diol epoxide since its cyclopenta ring is saturated and not easily epoxidated (Yang et al., 1990). In fact, 3-MCA induced similar mutational patterns in Ki-*ras* codon 12 of mouse cells as BaP did (Chen and Herschman, 1989). Previous studies have also been suggested that the cyclopenta ring of B[j]A could be activated (Nesnow et al., 1988) and was the major site of DNA-adduct formation (Nesnow et al., 1991), even thought B[j]A contains a bay-region. Therefore, the GGT→CGT mutations produced by B[j]A here were proposed to be the results of cyclopenta-ring derived adducts (Mass et al., 1993). This conclusion is also consistent with the results observed with CPP mentioned above (Nesnow et al., 1994).

2.2   Covalent adducts as dosimeters

People are exposed to chemicals from environmental, occupational and life-style sources. It has been estimated that the majority of human cancers are attributable to environmental factors such as contaminants in air and food, and personal habits such as smoking and dietary patterns (Perera and Weinstein, 1982; Basu and Saxena, 1978; Lijinsky and Ross, 1967; Baek et al., 1991; Higginson and Muir, 1976). Therefore, one approach to understanding cancer from these sources is to identify the carcinogens to which we are exposed and to categorize them in terms of their relative carcinogenic risk. Clearly, determining if a compound is a

human carcinogen, studying it epidemiologically, and preventing exposure to it can minimize cancer. In fact, more than 50 substances or mixtures have been identified based on this system (Tomatis et al., 1989). Epidemiological studies have been successful in cases where specific exposures to high concentrations of compounds have been involved (e.g., workplace exposures). However, traditional epidemiological techniques have certain limitations, such as their difficulty investigating the individual contributions of single chemicals in highly complex exposure situations (e.g., urban pollution). Therefore, this limitation has been one of the stimuli for the recent development of "biomonitoring" or "molecular dosimetry" techniques (Farmer, 1994).

Molecular dosimetry refers to the assessment of the internal dose of a toxic compound by measurement of the extent of chemical interaction of the compound with biological macromolecules, including proteins and nucleic acids. Such evaluation takes into account individual differences in absorption, metabolism, distribution, and excretion. Therefore, determination of the dose of active compound that reaches the target site of action within the body permits one to estimate the risk associated with the exposure.

The molecular dosimetry approach has been most extensively developed for exposure to genotoxic carcinogens. These carcinogens have the common characteristic of being able to react with DNA to form covalently bound products, or adducts. In some cases, these compounds may be acting directly on DNA to yield an adduct. In many other cases, a metabolic mechanism is required to convert the chemical to a reactive species that can then interact with DNA. Adduct formation is considerably site-selective within the DNA chain even for simple alkylating agents such as methylating agents (Dolan et al., 1988). In addition, adduct formation at specific sites in DNA is shown to cause the activation of oncogenes (Zarbl et al., 1985) and may also result in suppression of tumor-suppressor genes (Hsu et al., 1991; Bressac et al., 1991).

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Adduct formation with carcinogens also occurs with proteins and it is not considered to be as relevant to the carcinogenic processes as the formation of DNA adducts. However, substantial emphasis has been placed on protein adducts, mainly because of their availability and long lifetime as with hemoglobin.

Two kinds of mechanisms have been proposed to explain how macromolecular interactions lead to neoplastic changes (Foulds, 1969) and may still be valid today. Genetic mechanisms assume that neoplasia is due to the alteration in the cell genome. Epigenetic mechanisms assume that neoplastic changes are caused by a change in expression of the genetic compliment of a cell without altering its genotype.

2.2.1   DNA adducts vs. protein adducts

The degree of binding of a carcinogen or its metabolite(s) to a given cellular protein or DNA varies from one carcinogen to another. This could depend upon the stability of the carcinogens or their metabolites and their chemical and physical reactivity with these cellular macromolecules (Montesano, 1990).

Determination of the suitable target molecule for carcinogen exposure will be based on the experimental investigation. The degree of binding of the adduct is the primary consideration. The percentages of an oral dose of 15 chemical carcinogens binding to rat hemoglobin vary more than 300-fold (Pereira and Chang, 1981). For instance, 4-aminobiphenyl bound to hemoglobin is a valuable marker of exposure to this carcinogen from cigarette smoke (Bryant et al., 1987; Bryant et al., 1988), but the binding of aflatoxin B_1 (AFB_1) to hemoglobin is some 50-fold less efficient than to albumin after a single exposure to rats (Wild et al., 1988). Instead, AFB_1-albumin adduct determination has offered important findings on human exposure to this carcinogen and on the relationship between adduct levels

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and intake of food contaminated with AFB_1 (Gan et al., 1988; Montesano, 1990; Wild and Montesano, 1989).

The other parameter to consider is the stability of the adduct. For protein adducts, this is determined primarily by the half-life of the protein. Hemoglobin has a lifetime of approximately 125 days. Adduct measurements, therefore, should provide information on cumulative past exposure. In contrast, albumin can provide exposure information relevant to the past 2-3 months since it has a half-life of about 25 days. This assumes that the modified protein has the same half-life as the parent molecule. In fact, it appears that hemoglobin has a longer half-life than 4-aminobiphenyl-substituted hemoglobin (Green et al., 1984). No indication of enzymatic repair is involved in protein adducts (cf. DNA adducts).

Lately, other non-blood proteins such as histones and collagen are being used to investigate tissue-specific carcinogen-protein adduct formation (Groopman et al., 1980; MacLeod et al., 1980; Skipper et al., 1994; Peng, 1993). Histones are small positively-charged proteins that bind DNA. It has been estimated that the half lives of mouse liver and brain histones are as much as 20% of the lifespan of a mouse. Thus, investigating histones from a chemical target tissue offers a more realistic assessment of exposure. Recent data has also suggested the role of histones in gene regulation (Grunstein, 1992; Wolffe, 1992). Collagen is a structural protein of extracellular matrix that consists of one or more domains having the conformation of a collagen triplex helix. It comprises about one third of the total mass of protein in vertebrates. Like histone, collagen also possesses a longer half-life in comparison to albumin and hemoglobin and it can be tissue-specific (Skipper et al., 1994).

It is worth noting that the situation of patients undergoing chemotherapy may provide an unique opportunity to evaluate some of these questions discussed above if biological samples can be obtained without increasing the discomfort of the patient. Generally, the patient is exposed to high and defined doses of drugs which

could allow dosimetry data to be established (Montesano, 1990).

The modification of DNA is generally recognized as an important step in the carcinogenic process; therefore information on DNA adducts as biomarkers is considered to have better predictive value than protein adducts. However, the detection of the low levels of adduct in human cells (generally less than one modification in 10 million normal deoxynucleosides) is technically demanding (Montesano, 1990). Measurements can be made of DNA adducts in cells of accessible tissues (e.g, white blood cells, biopsy, or autopsy samples). In addition, another approach to monitoring DNA adducts takes advantage of the fact that adducts are removed from cellular DNA and are excreted in urine (Shuker and Farmer, 1992).

It is generally believed that DNA adducts result in mutations, which ultimately lead to cancer. Several mechanisms have been proposed to rationalized the mutations based on the results obtained from bulky mutagens/carcinogens. These mechanisms can be classified into three categories: (1) a miscoding mechanism in which the adduct is misinformational, (2) a noncoding mechanism in which the adduct is noninformational, and (3) indirect mechanisms in which the adducts may an intermediate in the formation of another lesion that induces mutations such as an apurinic site (Loechler, 1989; Loechler et al., 1995).

2.2.2   Hemoglobin adducts of CPP

Since mouse and human microsomes exhibited similar CPP metabolic profiles, the hemoglobin adducts induced by CPP in mice in vivo has been investigated (Kwon, 1992). CPPE has been shown to be the derivative that binds to mouse hemoglobin using fluorescence-line narrowing spectroscopy. The major hemoglobin adduct formed by the single dose of CPP was identified to be histidine

substituted. In experiments with multiple doses of CPP (every three days for 14 days), this adduct was not detected. Instead, a labile ester CPPE-derived adduct was obtained by the presence of dihydrodiol.

2.2.3 Methods for measuring carcinogen-DNA adducts

The analytical techniques used in human biomonitoring through DNA adducts include physicochemical methods, immunochemical methods, ^{32}P-postlabelling method, and ^{35}S-postlabelling method.

2.2.3.1 Physicochemical Techniques

DNA adducts can be analyzed by GC and HPLC in conjunction with different detection techniques. For example, GC with mass spectrometry (GC/MS) may be the most commonly used approach to detect and identify carcinogen-DNA adducts and has a resolving power of 1 adduct per 10^7 nucleotides. The application of GC/MS to human biomonitoring, however, has been limited mostly to the analysis of DNA repair products in urine. HPLC with electrochemical conductance detection (HPLC/ECD or HPLC/EC) has been used to detect 8-hydroxy-2'-deoxyguanosine in urine. This analysis is also adopted generally to monitor oxidative damage of DNA caused by endogenous oxidants (Van Welie et al., 1992). In addition, HPLC with fluorescence detection was employed to measure the low levels of BaP-DNA adducts (Farmer, 1994).

Other techniques used for urinalysis include HPLC-fluorescence, tandem mass spectrometry (MS/MS), GC/MS, and immunochemical procedures, in addition to HPLC/EC. GC/MS and enzyme-linked immunosorbent assay (ELISA) followed by immunoaffinity purification could also quantify adducts effectively in urinalysis

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(Prevost et al., 1990; Friesen et al., 1991; Farmer, 1994; Prevost et al., 1993).

N-3-alkyladenines and N-7-alkylguanines are two unstable DNA adducts in nucleic acid and undergo spontaneous depurination. Investigation of these products in urine, thus, provide a noninvasive and convenient way to monitor recent DNA damage caused by alkylating carcinogens (Marnett and Burcham, 1993). For example, N-7-methylguanine has been detected in urine by GC-MS, GC-flame ionization detection (GC/FID) and GC-nitrogen phosphorus detection (GC/NPD). In rats, the levels of urinary N-7-methylguanine was used to monitor exposure to certain N-nitroso compounds, nitrosatable drugs such as aminopyrine and cimetidine, and alkylating agents such as methyl methanesulfonate (Shuker et al., 1984; Farmer et al., 1986; Bailey et al., 1987; Farmer, 1994; Van Welie et al., 1992). In humans, the quantitation of alkylating products derived from methylating agents, ethylating agents, and AFB_1 has also been subjected to urinalysis. Most notably, the concentration of urinary aflatoxin B1-N7-guanine adducts were recently demonstrated to be associated with the risk of developing hepatocellular carcinoma in a case-control study in China (Ross et al., 1992).

The use of fluorescence assays for the detection of DNA adducts is particularly applicable to exposure to aflatoxin and PAHs (Farmer, 1994). These means as well as mass spectrometry generally require hydrolysis of the DNA followed by chromatographic or immunoaffinity purification of the adduct or one of its hydrolysis products. For instance, synchronous fluorescence spectrometry (SFS) has been used to detect BaP tetrol derived from acid hydrolysis of BaP-DNA adducts, and the sensitivity of this technique is about 1 adduct per 10^7 nucleotides that is similar to GC/MS measurements (Weston et al., 1989; Vahakangas et al., 1985). One disadvantage of this technique is that the resolving power of SFS is often not sufficient to determine single compounds of interest in complex mixtures. However, specificity may be enhanced by organic extraction and HPLC before quantitation (HPLC/SFS) (Weston et al., 1989; Farmer, 1994; dell'Omo and

Lauwerys, 1993).

The newly developed fluorescence line-narrowing (FLN) techniques are also being used for PAH-DNA adduct analysis (Jeffrey, 1991; Jankowiak et al., 1988). This laser-induced cryogenic (77 or 4.2 ^{0}K) FLN spectroscopy together with fluorescence quenching is a highly specific approach for the study of carcinogen-DNA adducts and has been suggested as a tool for human biomonitoring (Jankowiak and Small, 1991). The principles of FLN for analysis of DNA- and globin-PAH adducts have been reviewed (Personov, 1983; Jankowiak and Small, 1989). Recent applications of FLN to the analysis of macromolecular and nucleoside adducts include the investigation of stereoisomeric DNA adducts derived from BPDE (Lu et al., 1991; Jankowiak et al., 1990b; Jankowiak and Small, 1989), elucidation of different DNA binding configurations for stereoisomeric BPDE adducts with the aid of fluorescence quenching (Lu et al., 1991; Jankowiak et al., 1990b), conformational studies of in vivo BaP radical cation pathway (Zamzow et al., 1989; Rogan et al., 1990), human hemoglobin adduct formation by BaP (Jankowiak et al., 1990a), and human lung DNA and histone adducts from BaP (Jankowiak and Small, 1991; Ozbal et al., 1995). The disadvantages of FLN as well as other fluorescence assays are that the number of adducts that can be analyzed is limited and that cross-reactivity with other fluorophores, leading to difficulties in quantitation, is possible (Farmer, 1994). In one recent study, FLN was able to detect 5 BaP adducts per 10^8 bases using 1 ug of human lung DNA (Ozbal et al., 1995).

2.2.3.2 Immunochemical Techniques

Immunoassays use mono- and poly- clonal antibodies to detect DNA adducts. The basic principle of an immunoassay is the coupling of the antigen or antibody to a fluorescent dye or an enzyme. Then, quantification of substrate conversion by the amount of bound fluorescence or the enzyme is a measure of the amount of

antigen present. At least 20 specific polyclonal and monoclonal antibodies against carcinogen-DNA adducts are available (Santella, 1988; Anon, 1989). Antibodies are employed in a variety of competitive and noncompetitive, single- or multistep assays, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), ultrasensitive enzymatic radioassay (USERIA), and the immuno-slot blot technique (ISB). The sensitivities of these techniques may reach 1 adduct per 10^8 normal nucleotides, but the quantitative demands for sample size may be relatively large, for example, up to 500ug DNA. Nevertheless, an advantage of utilizing immunoassays to analyze DNA adducts is that they are more applicable for routine and relatively low-cost screening of large human populations (Farmer, 1994).

The best known problem associated with the operation of immunoassays is cross-reactivity of the antibody with adducts of chemically-related composition. This property, on the other hand, serves another role as the basis for immunoaffinity chromatography because of the broad-spectrum of antibodies. This affinity chromatography is commonly adopted to be the preliminary purification step for adducts prior to their examination by physicochemical or postlabelling techniques (Farmer, 1994).

BaP-DNA adducts have been detected using ELISA and USERIA (Perera et al., 1982; Shamsuddin et al., 1985; Van Welie et al., 1992; Perera et al., 1986). Other carcinogens where immunological detection of their DNA adducts have been used include aflatoxin B_1, 4-aminobiphenyl, 2-actetylaminofluorene, cis-diamminedichloroplatinum (II) (cisplatin), and certain alkylating agents (Farmer, 1994).

2.2.3.3 ^{32}P-Postlabelling Technique

Currently, the ^{32}P-postlabelling system is the most sensitive technique for

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determining DNA adducts (Gupta, 1985; Gupta et al., 1982). Although it is not adduct-specific, an extreme sensitivity is achieved. At best, it can reach the detection of 1 adduct per 10^{10}-10^{11} unmodified nucleotides, or ~1 adduct per cell. It is particularly useful for the measurement of adducts derived from exposure to complex mixtures of unknown carcinogens. Generally, purified DNA (1 to 10ug) is subjected to enzymatic digestion and forms deoxyribonucleotide-3'-monophosphates followed by isolating. A subsequent ^{32}P transfer from adenosine [r-^{32}P]triphosphate to the 5' position is catalyzed by T4 polynucleotide kinase to result in deoxyribonucleotide-3',5'-[5'-^{32}P]biphosphates. Then, radiolabelled adducts are isolated by multi-directional TLC to produce a fingerprint of the modified nucleotides. Finally, adducts are detected and quantified using autoradiography. Sensitivity may be improved by extracting the digested DNA adducts with 1-butanol before labelling and by removing the hydrophobic adducts. In some cases, HPLC was adopted to substitute for TLC (Beach and Gupta, 1992; Farmer, 1994; Van Welie et al., 1992).

The ^{32}P-postlabelling assay has been used to detect DNA adducts in several human tissue and blood samples in terms of quantifying total adduct levels. It has been applied to mixed exposure situations such as smoking (Randerath et al., 1986; Everson et al., 1986; Dunn and Stich, 1986), foundry work (Phillips et al., 1988; Hemminki et al., 1988), or coke plant operations (Hemminki et al., 1990). Many classes of chemical carcinogen have also been monitored by the ^{32}P-postlabelling method, and they include PAHs, nitroPAHs, aromatic amines, alkylating agents, mycotoxins, chemotherapeutic agents, and active oxygen radical generators (Randerath et al., 1985).

2.2.3.4 ^{35}S-Postlabelling Technique

Although ^{32}P-postlabelling is a sensitive technique for DNA adduct detect

ion in tissue DNA samples and widely applicable to compounds of unknown structure (Reddy et al., 1984; Randerath et al., 1989), accurate quantitation of adducts levels is complex because the recovery of adducted nucleotide 3'-monophosphates and their kinase substrate activities vary substantially. Therefore, its application to specific exposure cased has been restrict.

A new postlabelling technique, ^{35}S-postlabelling, was developed for carcinogen-DNA adducts and attempted to address some problems that inherit from aforementioned techniques. Briefly, DNA is digested enzymatically and adducted nucleosides are separated from unmodified nucleosides by either HPLC or immunoaffinity purification. Subsequently, adducted nucleosides are subjected to acylation with t-butoxycarbonyl-L-[^{35}S]methionine, N-hydroxysuccinimidyl ester (^{35}S-TBM-NHS) for identification and quantification. Alternatively, adducted nucleosides can be acylated as a mixture, after that total adduct levels can also be determined by HPLC separation, and in parallel, specific carcinogen adducts can be determined by immunoaffinity purification.

^{35}S-postlabelling was designed to be sensitive, specific, quantitative, and applicable to carcinogens representing diverse chemical classes. In fact, studies have shown that this technique has the detection limit on the order of tenths of attomoles (Sheabar et al., 1994b). In addition, a linear correlation was obtained between the amounts of N-(2'-deoxyguanosin-8-yl)-4-aminobiphenyl introduced to the reaction and the total amount of TBM-acylated products formed (Sheabar et al., 1994a). Taken together, ^{35}S-postlabelling has a potential as an analytical method for the detection and quantification of DNA adducts in human target tissues or accessible surrogate cells.

2.2.4   Covalent modification of DNA

The chemistry of carcinogen-DNA interaction is not fully understood, even though a few aspects of reactivity towards different nucleophiles have been summarized (Swain and Scott, 1953; Ross, 1962; Lawley, 1976; Ehrenberg and Osterman-Golkar, 1980). Most of these reviews regarding these chemical interactions seem to apply well to simple alkylating agents.

Lawley and Brookes used methylating agents to model the more complex reactions of antitumor alkylating agents, and defined much of the basic chemistry of nucleic acid alkylation (Lawley and Brookes, 1963). This is relatively complex because the sites of reaction on some nucleic acid components vary depending on whether the target molecule is a base, a nucleoside, a component of RNA or a component of DNA. For instance, the 3-position of adenine is the primary site for alkylation in double stranded DNA. For adenosine or adenine residues in RNA or single stranded DNA, however, the principal site is the 1-position. The 7-position of guanine is the major site for alkylation in guanine nucleosides, RNA, and DNA. By the mid-1960's, the most susceptible site for alkylation in DNA was showed to be N-7 of guanine residues in the major groove of the helix followed in decreasing order by N-3 of adenine, N-1 of adenine, N-3 of cytosine and N-7 of adenine residues.

Another complexity of alkylation chemistry is that ring nitrogen alkylated products are relatively unstable. For example, at neutral and acid pH, the glycosidic bond in 7-alkylguanine nucleotides and the 3- and 7-alkyladenine nucleotides in DNA is easily hydrolyzed. At alkaline pH, however, 7-alkyldeoxyguanosines are opened in the imidazole ring to give triaminopyrimidine derivatives and 1-alkyldeoxyadenosines undergo a rearrangement to 6-alkylaminopurine deoxyribonucleosides (Moschel et al., 1979; Qian and Dipple, 1995; Macon and Wolfenden, 1968). Thus, biological effects of these ring nitrogen-substituted products can be influenced by chemical transformations secondary to the initial DNA alkylation. Notably, the exocylcic amino groups are not effectively targete

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d by the alkylating agents (Hemminki, 1983; Dipple, 1995).

The history of chemical carcinogenesis is closely related to aromatic amin es, a major class of arylaminating agents, which were recognized as causes of bladder cancer in dye workers. For most of aromatic amines, N-hydroxylation a nd esterification have been considered necessary for their carcinogenicity. Other carcinogenic arylaminating agents include aminoazo dyes, the nitroaromatics, and the heterocyclic aromatic amines found in trace amounts in cooked meats and fis h (Hemminki, 1983). Although the site of substitution on nucleosides by the arylaminating agents in the major product varies, it seems initially that the substituted sites on deoxyribonucleosides form a pattern that is very different fro m that displayed by the alkylating agents. The C-8 atom and the exocylcic amin o groups of the purine nucleosides, especially deoxyguanosine, are the major targ ets for the arylaminating agents. In contrast, these sites are unaffected by alkylat ing agents (Dipple, 1995).

A recent study has suggested that the aromatic amine adducts modified at C-8 of deoxyguanosine arise from a 7-substituted deoxyguanosine precursor (Humphreys et al., 1992). This evidence implies that the 7-position of deoxyguanosine residues may be a common reactive center for both the alkylatin g and arylaminating agents.

Carcinogens that transfer an aralkyl group to DNA include the large grou p of PAHs and those nitroaromatics that are activated through the diol epoxide mechanism. The reactions of the hydrocarbon diol epoxides with DNA result primarily in modification of the exocyclic amino group of deoxyguanosine and deoxyadenosine residues (Hemminki, 1983; Dipple, 1995).

The sites of reaction of each of the three categories of agent discussed ab ove, including alkylating, arylaminating, and aralkylating agents, are summarized

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in Figure 2.4.

2.2.4.1 Nucleic acid adducts of PAHs

The studies concerning nucleic acid adducts are few as compared to carcinogenicity bioassays and metabolic studies with PAHs. Two diol epoxides of benz[a]anthracene, 3,4-diol-1,2-epoxide, which possesses the epoxide group adjacent to the bay-region and 8,9-diol-10,11-epoxide, which is a non-bay region diol epoxide, were reacted with DNA in aqueous solution in vitro. Then, DNA was digested enzymatically and the adducts were analyzed by HPLC. The major adducts, formed by both diol epoxides, were modified at N2 position of deoxyguanosine (Hemminki et al., 1980). This type of adduct has also been observed in vivo after dosing benzo[a]anthracene (Hemminki, 1983).

Nucleic acid adducts formed in vitro by BaP-7,8-diol-9,10-epoxide (BPDE), a bay-region diol epoxide, were characterized the following substitution sites: 90% of N2-G, 5% of N6-A, and 1% at possible N4-C (Straub et al., 1977; Meehan et al., 1977). Using the (-) isomer of anti-BaP-diolepoxide, the N7-G and O6-G derivatives were also detected and suggested, respectively (Osborne et al., 1981). In contrast, no N7 or O6-G adduct was detected in DNA from BaP-treated mammalian cells. This might be due to the fact that (+) anti-BaP-diolepoxide was the major metabolite in cells. Few authors have found that BaP-diolepoxide may also react with DNA and form phosphotriesters (Koreeda et al., 1976; Gamper et al., 1977). N2-G adducts were also identified in several in vivo samples, such as cultured cells (Feldman et al., 1978), tissue cultures (Atrup et al., 1978; Harris et al., 1979), and mouse skin (Koreeda et al., 1978), after administration of BaP (Hemminki, 1983).

One-electron oxidation with formation of radical cations is another major

mechanism of PAH activation leading to carcinogenesis (Cavalieri and Rogan, 1985; Cavalieri and Rogan, 1992). Cavalieri and Rogan have suggested that PAHs are predominantly activated by either the diolepoxide pathway or the one-electron oxidation pathway or by both (Cavalieri and Rogan, 1992). Structure determination of biologically-formed PAH adducts provides evidence about the mechanism of activation. Identification and quantitation of the DNA adducts formed by 3-MCA-induced rat liver microsomes demonstrated that BaP (Moncada et al., 1991) and DMBA (RamaKrishna et al., 1992a) are activated primarily by one-electron oxidation to form adducts that are lost by depurination. In the case of BaP, the depurination adducts formed by one-electron oxidation, 8-(benzo[a]pyren-6-yl)guanine (BaP-6-C8G), BaP-6-N7G, and BaP-6-N7A, constitute 80% of all the adducts, whereas the depurination adduct formed by BPDE, BPDE-10-N7A, accounts for only 0.5%. The major stable DNA adducts, or BPDE-10-N2G, accounts for only 15% of the adducts. Recent results demonstrate that in mouse skin, BaP-DNA adducts (Rogan et al., 1993) and DMBA-DNA adducts (Devanesan et al., 1993) are also predominantly formed via one-electron oxidation.

The structural and functional aspects of DNA modification by BaP at N2 of G has been investigated and the data demonstrated that BaP is located in the narrow groove of DNA (Grunberger and Weinstein, 1979). In contrast to N2-G adducts, BaP-N6-A adducts appear to localize to the major groove of DNA (Jeffrey et al., 1979).

Some halogenated derivatives of PAHs have been used as model compounds to study nucleic acid binding of PAHs. The correlations between DNA binding and carcinogenesis of 7-bromomethylbenz[a]anthracene and 7-bromomethyl-12-methylbenz[a]anthracene, which form their reactive carbonium ions spontaneously, have been investigated. These compounds produced similar major products, N2-G and N6-A adducts, in DNA in vitro (Rayman and Dipple, 1973). However, the level of binding is lower with 7-bromomethyl-12-methylbenz[a]anthracene, which

is more carcinogenic than 7-bromomethylbenz[a]anthracene. This contradiction between carcinogenic potency and the level of DNA binding is not due to the differential biological stabilities of the adducts (Dipple and Schultz, 1979). The in vitro modification positions by 7-bromomethylbenz[a]anthracene depend on the solvent used. In aprotic solvents a higher preference towards ring nitrogens is noted as compared to exocyclic nitrogens in aqueous solvents (Dipple et al., 1971; Shapiro and Shiuey, 1976). In 50% dioxane, 6-chloromethylbenzo[a]pyrene reacted with guanosine and formed N7- and N2- aralkyl derivatives as major and minor products, respectively. In similar conditions, the products with adenosine were N-1 (major) and N6 (minor) derivatives, and with cytidine were N3 (major) and N4 (minor) derivatives (Hemminki, 1983; Royer et al., 1979).

In summary, the reactions of the PAH diol epoxides with DNA result predominately in modification of the exocyclic amino groups of dG and dA [minor products at N7 of dG (Cheh A.M. et al., 1993) and amino group of dC (Chadha et al., 1989) have been characterized in isolated cases]. The distribution of PAHs over the target sites varies considerably with the structure of the PAHs. Diol epoxides derived from planar PAHs react primarily at the amino group of dG in DNA. However, diol epoxides derived from PAHs that are substantially distorted from planarity, by a substituent methyl group in the bay region or by the presence of a fjord region, react mainly at the amino groups of both dG and dA (Dipple, 1994). As mentioned above, some PAHs also react with DNA extensively through one-electron oxidation process (Rogan et al., 1993; Dipple, 1995).

2.3   Intercalation vs covalent binding to DNA

In addition to covalent binding of electrophilic metabolites of PAHs, the formation of non-covalent interaction complexes of PAHs and DNA was also studied (Geacintov et al., 1984). Generally, when the reaction rates of PAH diol

epoxides with DNA dominate over those in the external aqueous solution, the level of covalent binding is independent of the intercalative binding, which precedes the covalent binding. Whereas, if DNA does not enhance or catalyze the rates of reaction of diol epoxides, such as in high salt buffers, the level of covalent binding to DNA can be predicted to be directly proportional to the intercalative binding. This relationship also applies to the condition when there are efficient competitive reaction pathways in the solution external to DNA. In either of these two cases, the ability of a diol epoxide to intercalate DNA prior to covalent binding can be a critical factor of estimating the biological activity of its parent molecule (Geacintov, 1986).

The salt concentration in a cell is approximately 150 mM. This should be sufficient for a given diol epoxide to form an intercalative complex with DNA prior to covalent binding (Geacintov et al., 1984). Additionally, enzymes, lipoproteins, and cellular nucleophiles may also compete with DNA to react with PAH diol epoxides. One study has shown that the mutagenicity and tumorigenicity of 1-alkyl derivatives of BaP depend strongly on their ability of intercalating with DNA (Harvey et al., 1985). As the bulkiness of the substituent increases (methyl, ethyl, isopropyl, and tert-isobutyl) a significant drop in the biological activities were observed. This drop was not attributable to differences in metabolic activation. In vitro physical binding experiments were consistent with the notion that bulky inert side groups inhibit intercalation of PAHs to DNA (Geacintov, 1986; Michaud et al., 1983).

2.4   Sulfonation in chemical carcinogenesis

Sulfonation is used here to replace the commonly used but less precise term sulfation for the sulfotransferase-catalyzed transfer of the sulfo group ($-SO_3H$, $-SO_3^-$) (Benkovic and Hevey, 1970) from the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) (Robbins and Lipmann, 1957) to the oxygen and nitrogen

atoms of -C-OH, -N-OH, and -NH groups in foreign and physiological substrates (Mulder and Jakoby, 1990). The products of these transfers are sulfuric acid esters (also called sulfates, or sulfooxy- or sulfonyloxy- derivatives) or sulfamides (sulfamates). Sulfonation has been used to describe the conjugation of substrates by sulfotransferase and PAPS (Miller and Surh, 1995).

O- and *N*-sulfonation produce many water-stable sulfuric acid esters and amides, such as heparin and estrogen sulfates, endogenously (Mulder and Jakoby, 1990). Many foreign compounds have also shown to be detoxified in mammals by metabolism to sulfates. These sulfates are believed to pass across membranes easily and then are excreted in urine (Williams, 1959). Recently, chemical carcinogens have also been investigated for sulfonation. For several cases these agents are activated to form sulfuric acid esters or covalently react with cellular macromolecules to from adducts. Detoxification of chemical carcinogens by the sulfonation pathway was also observed (Miller and Surh, 1995).

Evidence for formation of sulfuric acid esters is mainly obtained from the isolation and characterization of cellular molecule adducts in reactions that were dependent on PAPS and showed inhibition of sulfotransferase activities by pentachlorophenol (PCP), 2,6-dichloro-4-nitrophenol (DCNP), or dehydroepiandrosteone (DHEA). One direct evidence was obtained by isolating the unstable sulfuric acid esters of 7-hydroxymethylbenz[*a*]anthracene from medium of treated cells (Watabe et al., 1986). PCP and DCNP are inhibitors of the phenol sulfotransferases, and DHEA is a competitive substrate of the hydroxy-steroid sulfotransferases.

2.4.1   Metabolic activation of PAHs by sulfonation

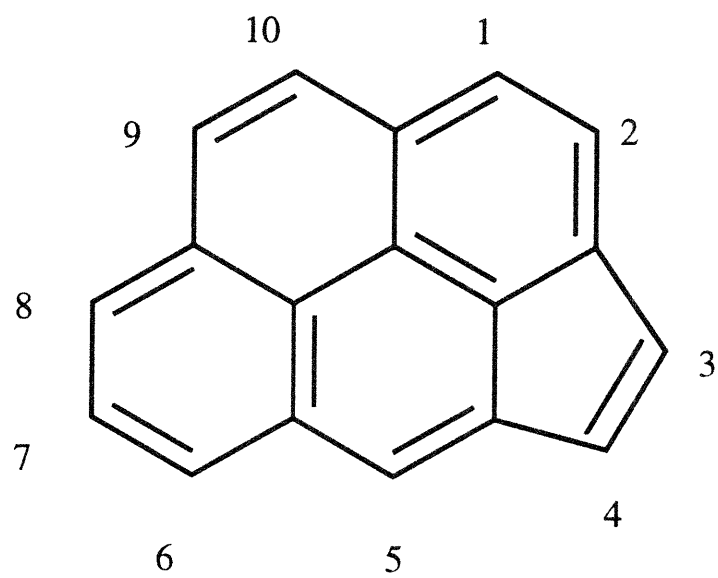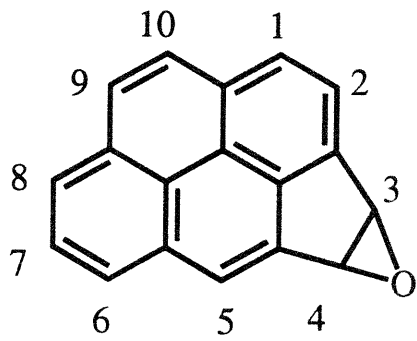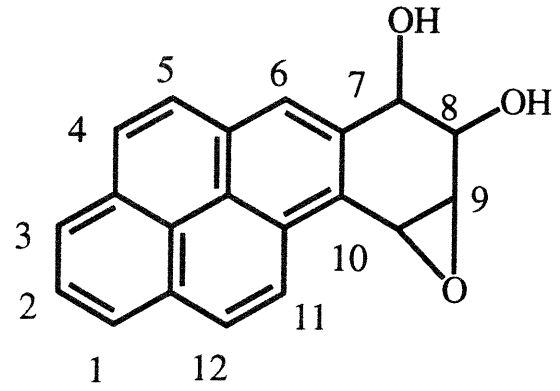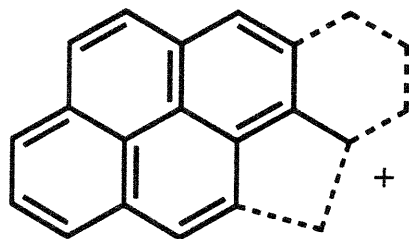2.4.1.1 Methyl-substituted aromatic hydrocarbons

Figure 2.1 CPP

CPPE

BPDE

Carbonium ion

Figure 2.2 Benzylic carbonium ion resulting
from the opening of CPPE or BPDE

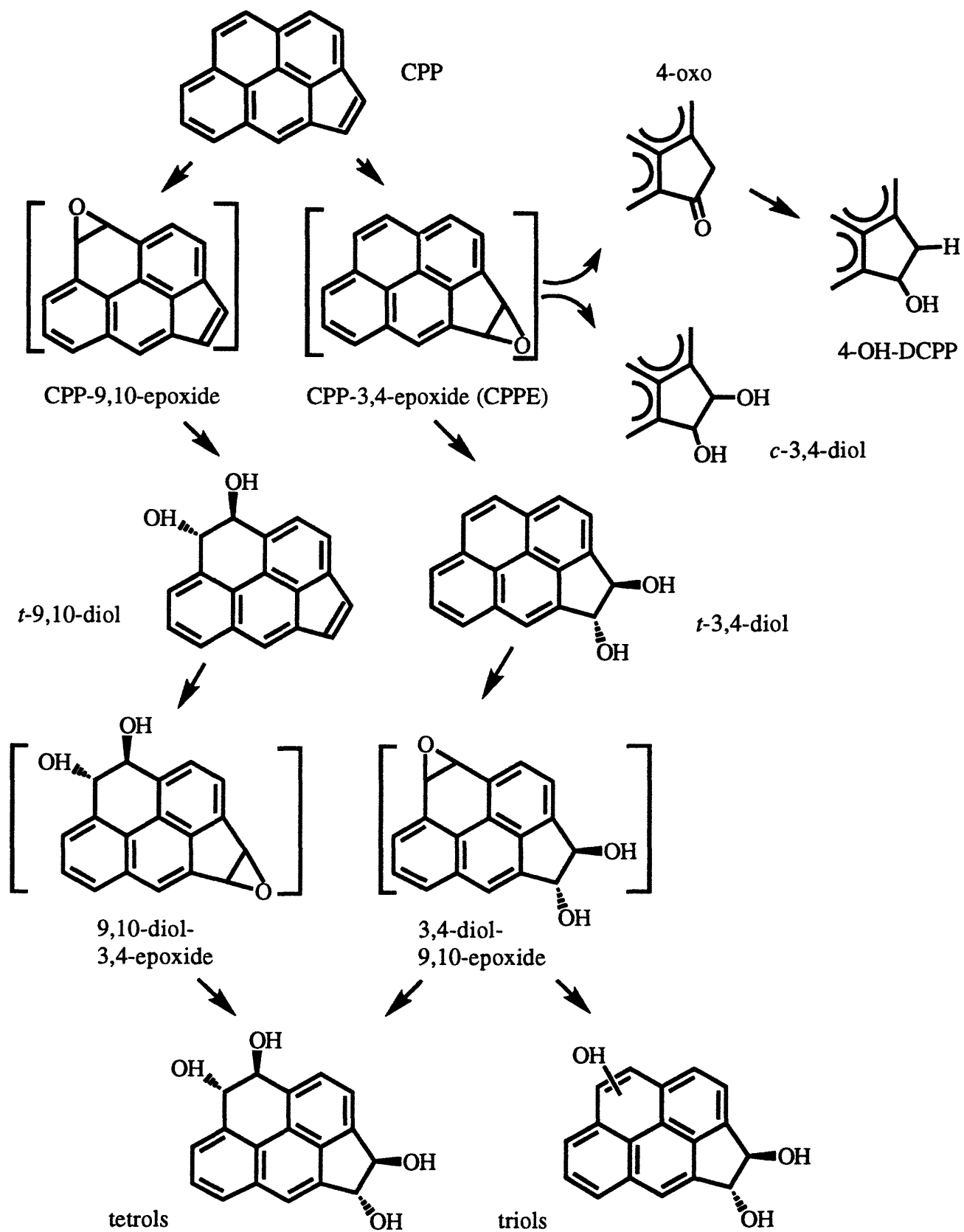Figure 2.3 Metabolic pathway of cyclopenta[*cd*]pyrene

I,II
O
I, (II)
HN 1 6 5 7
2 4 8 II
H₂N 3 N 9 N
II,III sugar

II,III
NH₂
I, (II)
I N 1 6 5 7
2 4 8 II
3 9 N
N I sugar

III
NH₂
N 3 4 5
2 6
O 1 N
I sugar

I
O
CH₃
HN 3 4 5
2 6
O 1 N
I sugar

Figure 2.4 Sites of substitution of DNA bases by genotoxic carcinogens. Sites modified by alkylating agents are marked by the numerbal I, those modified by arylaminating agents by a II and those modified by aralkylating agents by a III. Since it has been suggested that C-8 substituted arylamino adducts may have arisen from N-7 substituted precursors (Humphreys et al., 1992), the arylaminating agents are listed parenthetically at the 7-position of the purines.

3.   Structure of the most abundant DNA adduct formed by
cyclopenta[cd]pyrene epoxide

3.1   Introduction

The resulting 3,4-epoxide of CPP (Figure 3.1) is often considered to be the
ultimate carcinogen, though evidence for diol epoxides as reactive electrophiles has
also been presented (Sahali et al., 1992; Kwon et al., 1992). The principal adducts
formed by CPPE with DNA, both in vivo and in vitro, have been characterized as
products of reaction with guanine (Nesnow et al., 1994; Beach et al., 1993; Beach
and Gupta, 1994). Structures of the guanine adducts are commonly thought to be
the 4-hydroxy-3-(N2-guanyl)dihydroCPP structures shown in Figure 3.1. The
present study was undertaken to provide detailed information regarding the regio-
and stereo-chemistry of guanine adduct formation by CPPE. This information is
expected to be helpful in understanding the mechanisms of CPP-induced
genotoxicity.

3.2   Materials and Methods

Chemicals

CPP and [G-^{3}H]CPP were prepared by Midwest Research Institute (Kansas City, MO) and Chemsyn Science Laboratory (Lenexa, KS), respectively, and obtained from the National Cancer Institute Chemical Carcinogen Repository. CPPE was prepared from CPP by oxidation with dimethyldioxirane in methylene chloride as described previously (Kwon et al., 1992). CPP-3,4-diols were prepared by acid hydrolysis of CPPE followed by HPLC to isolate the *trans-* and *cis-* isomers (Sahali et al., 1992).   Calf thymus DNA containing [8-^{3}H]guanine or [1',2'-^{3}H]deoxyguanosine was prepared with the Nick Translation System of Promega (Madison, WI), using the supplier's protocol.   [^{3}H]dGTPs used for the Nick Translation were the products of Amersham Life Science (Arlington Heights, IL). DNAse I (from bovine pancreas) was purchased from Sigma Chemical Co. (St. Louis, MO) and spleen exonuclease (calf), snake venom exonuclease (*Crotalus durissus*), and alkaline phosphatase (calf intestine) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).   Partisil 40 ODS-3 bulk chromatography medium was obtained from Whatman Labsales (Hillsboro, OR). All other chemicals and solvents used were reagent grade.

Instrumentation

Fast atom bombardment mass spectra were obtained by the spectroscopy facility of the Chemistry Department at M.I.T. using a Finnigan MAT system 8200 with a Xe$^+$ gun operated at 8 kV. Samples were prepared in a matrix of 3-nitrobenzyl alcohol.   Electrospray ionization mass spectra were acquired with a Finnigan TSQ 7000. Nitrogen was used as sheath (80 psi) and auxillary (20 psi) gas. A potential of 4.5 kV was applied to the ESI needle.   The metal capillary was maintained at 220 °C. Mass spectrometer parameters were optimized to obtain

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maximum sensitivity without sacrificing unit resolution. Tube lens and capillary voltages were voltages were optimized for maximum response at m/z 510. ^{1}H-NMR spectra were recorded using a Bruker AM-400 spectrometer. A Hewlett-Packard HP1090 chromatograph equipped with diode-array detector was used for analytical and preparative HPLC. Columns were Nucleosil C18 from Alltech Associates, Inc. (Deerfield, IL) and had the dimensions 4.6 x 250 mm for analytical separations and 7.8 x 250 mm for preparative work; flow rates were 1 and 3 mL/min, respectively. The solvent program was 50% CH_3OH in H_2O for 5 min, followed by a linear gradient to 80% CH_3OH at 55 min and to 100% CH_3OH at 60 min.

Preparation of Adducts

Deoxyguanosine (500 mg) was dissolved in Tris-HCl buffer (0.05 M, pH 7.4, 250 mL) at 37 °C. To this was added, with vigorous stirring, a solution of CPPE in DMSO (5 mL). The CPPE was prepared by epoxidation of 50 mg of CPP and used immediately thereafter. Reaction was allowed to proceed for 2 hrs before isolation of the adducts. The entire reaction mixture was applied to a column of Partisil 40 ODS-3 (25 g) packed in methanol and equilibrated with H_2O prior to use. Elution with 10% CH_3OH in H_2O removed unreacted deoxyguanosine. Two fractions with characteristic pyrene absorption spectrum were obtained by elution with 60% and 70% CH_3OH in H_2O, respectively. These two fractions were combined and evaporated to dryness. The residue was partitioned between H_2O and benzene and the benzene fraction was discarded. The aqueous phase was then extracted with ethyl acetate. Adducts were isolated from the ethyl acetate phase by HPLC using conditions described above. An additional, smaller amount of adducts was also isolated from the aqueous phase by HPLC.

DNA adducts were obtained by treating calf thymus DNA (1 mg/mL) in Tris-HCl buffer (0.05 M, pH 7.4) with CPPE (20 nmol/mg DNA, based on amount of CPP used) at 37 °C. After 2 hr the reaction was terminated by extraction with

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ethyl acetate and the DNA was precipitated with ice-cold 95% ethanol. Precipitated DNA was rinsed successively with 75% ethanol, 100% ethanol, acetone and diethyl ether. DNA was redissolved in Tris-HCl buffer (0.04 M, pH 8.5) containing 10 mM $MgCl_2$ at 0.5-1 mg/mL and digested with DNAse I (200 U/mg DNA), spleen exonuclease (0.01 U/mg), snake venom exonuclease (0.5 U/mg), and alkaline phosphatase (10 U/mg) at 37 °C for 3 hr. Adducts were extracted from the aqueous phase with $CHCl_3$/*n*-propanol (7:3 v/v).

3.3   Results

Reaction of [^{3}H]-CPPE with DNA under the conditions used in this study resulted in a level of DNA base modification of one adduct per 15,000 nucleotides. This level of binding is about six-fold lower than that reported by others (Beach et al., 1993), but the ratio of CPPE:DNA was at least 20-fold smaller. Our results are thus comparable and also suggest that adduct formation is more efficient at lower CPPE:DNA ratios. Dependency of adduct yield in this manner has been observed and discussed previously in the reactions of PAH metabolites (Geacintov, 1986) as well as aflatoxin B_1 epoxide (Raney et al., 1990) with DNA, and has been ascribed to a mechanism in which intercalation of the carcinogen precedes covalent bond formation.

HPLC analysis of the enzymatic digest of [^{3}H]-CPPE-adducted DNA is illustrated in Figure 3.2. Four peaks of radioactivity were observed, not including the early peak at 4-6 min. This more polar material may include products such as phosphate adducts; in any case, it was not investigated further. Two minor peaks, with retention times corresponding to *trans-* and *cis*-CPP 3,4-diol, were present in the chromatogram. The presence of diols in the digest is likely the result of incomplete extraction of the reaction mixture. The remaining two peaks (F1 and F2) were considered likely to be adducts. To test this assumption, [^{3}H]-guanine was incorporated into DNA and the DNA was reacted with nonradioactive CPPE. Only the two peaks of radioactivity corresponding to F1 and F2 were observed in the HPLC analysis of the enzyme digest, as shown in Figure 3.3. This result is consistent with the two major peaks representing adducts of guanine, as expected, and the two minor peaks being diols.

Greater quantities of the putative adducts were required for spectrochemical characterization than could be obtained through the reaction of CPPE with DNA. We thus attempted to obtain the same adducts by reaction of CPPE with

deoxyguanosine. This reaction yielded a complex mixture of products as detected by absorbance at 275 and 342 nm, including two very closely eluting peaks with essentially the same retention time as that of F2. These two peaks separated cleanly from the large excess of *cis*-diol that eluted just after them.

Several procedures were attempted in an effort to purify micromole amounts of the two UV peaks corresponding to F2. Ultimately, a combination of low resolution ODS column chromatography to remove deoxyguanosine, benzene extraction to remove the least polar impurities including unreacted CPP and the 4-ketone, extraction of the adducts and diols as well as some other impurities into ethyl acetate, and repeated semi-preparative HPLC proved satisfactory and each compound was obtained essentially free of the other. These are now designated F2a and F2b.

FAB-MS analysis of both F2a and F2b gave the expected molecular weight for a product formed by alkylation of dG by CPPE ($MH^+ = 510$, $MNa^+ = 532$) as well as a fragment ion at m/z 394. ESI-MS similarly yielded molecular and sodium adduct ions at m/z 510 and 532, respectively. Fragmentation by cleavage of the N9-C1' bond with retention of charge by the guanine (m/z 394) was also observed in ESI-MS, and yielded the base peak in this case. This distinctive fragmentation pathway has been observed previously in the FAB (Annan et al., 1990) as well as electrospray (Chaudhary et al., 1995) mass spectra of 2'-deoxyribonucleosides.

We were unable to obtain adequate separation of diol from other material in the F1– *trans*-diol region of the chromatogram of CPPE-deoxyguanosine adducts. UV spectral analysis indicated that this region may have contained at least one adduct since spectra with a characteristic increase in absorbance at 250-270 nm were observed on the leading edge of the *trans*-diol peak. Material eluting in this region of the chromatogram was collected for ESI-MS analysis on the premise that if an

adduct molecular ion could be detected in the mixture, then a mass spectrum could be obtained by tandem MS. Good spectra were in fact recorded without resorting to tandem MS, since the contaminating dihyrodiol did not undergo significant ionization under ESI conditions. Ions at m/z 510 (MH^+), 532 (MNa^+), and 394 were the most abundant ions in the spectra, which resembled the spectra of the *cis* adducts very closely.

The two adducts, F2a and F2b gave ^1H spectra which were essentially identical indicating that the two compounds were diastereomers arising from the two enantiomers of CPPE reacting in the same manner with deoxyguanosine. Chemical shifts and coupling constants are listed in Table 3.1. The three structural features of the adducts that needed to be defined were (1) the stereochemistry of opening of the epoxide ring of the CPPE, (2) the linkage site on deoxyguanosine, and (3) the linkage site on the CPP moiety. No attempt was made to assign the absolute configurations of the CPP moiety in the diastereomers. NMR studies on one diastereomer were carried out in a DMSO-d_6 solution containing a small amount of H_2O which lead to rapid exchange of OH protons with the HDO signal. The other diastereomer was exhaustively dried prior to dissolution in high purity DMSO-d_6 leading to the OH signals being in slow exchange so that coupling to vicinal carbon-bound protons could be observed.

Signals for the protons on the CPP nucleus could be assigned on the basis of observable couplings. Protons H6, H7, and H8 represent an AMX system in which H7 is a doublet of doublets due to coupling to vicinal protons H6 and H8; the coupling constants to the two protons differ only slightly such that H7 appeared as a triplet. H6 and H8 show long range coupling to each other. The signal for H5 is a singlet. H9 and H10 are isochronous due to the high degree of symmetry in the K region of the molecule. On the other hand, the signals for H1 and H2 are significantly separated from one another and appear as doublets (J = 7.7 Hz). H3 and H4 have similar but non-identical chemical shifts. One of them is a doublet of

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doublets due to coupling to the other one and also to the amino proton. Both coupling constants are ~7 Hz causing the signal to appear as a triplet. The other CH appears as a doublet (J = 7.7 Hz) under conditions of rapid proton exchange of the hydroxyl group but under more anhydrous conditions becomes an approximate triplet (J_{CH-OH} = 6.3 Hz).

The stereochemistry of ring opening was assigned as *cis* on the basis of the 7.7 Hz vicinal coupling constant. In five-membered rings with a high degree of planarity, *cis* protons have a low torsional angle and show substantial coupling whereas *trans* protons have a torsional angle near 90° and have minimal coupling. The *cis* and *trans* 3-methoxy-4-hydroxy derivatives of CPP arising form methanolysis of CPPE show vicinal coupling constants of 6.0 and 1.7 Hz, respectively.

Assignments of the CH-N and CH-O as H3 and H4, respectively, were made on the basis of long-range couplings to H2 and H5. Resolution enhancement of the doublet assigned to H2 by Gaussian multiplication revealed additional long-range coupling (1.0 Hz) which collapsed on irradiation of the CH-N signal, establishing that the CH-N was H3. Similar resolution enhancement of the signal assigned to H5 showed it to be a 1.1 Hz doublet; irradiation of the CH-O signal collapsed this doublet establishing that the CH-O was H4. Consequently, it can be concluded that attack by deoxyguanosine had occurred at C3 of CPPE.

The site of attachment to deoxyguanosine could be assigned as N2 on the basis of vicinal coupling of H3 to an amino proton; the amino proton appeared at δ7.11 which is consistent with NH signals of other PAH derivatives of deoxyguanosine linked to N2. Reaction at N1, N7, O6 of guanine or the hydroxyl groups of deoxyribose would have led to isomeric structures which lacked the coupled NH signal. Those structures would have retained the 2-NH_2 group; however, no signal was detected which could be ascribed an NH_2 group. Additional

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evidence supporting the N2 assignment included the fact that the protons on N1 and O3' and O5' were still present; the guanine 1-NH signal was observed at $\delta10.88$ and the 3' and 5' hydroxy groups of deoxyribose could be observed under conditions of slow exchange as a doublet ($\delta5.2$) and triplet ($\delta5.14$), respectively.

3.4   Discussion

Our results provide definitive evidence that the major adducts formed by reaction of CPPE with deoxyguanosine *in vitro* are the two diastereoisomers of *cis*-3-(deoxyguanosin-2-yl)-4-hydroxy-3,4-dihydroCPP, and further indicate that they are also the major products formed when CPPE reacts with DNA. In a preliminary publication the adducts were tentatively assigned as FAPy derivatives arising from hydrolysis of N7 adducts (Hsu et al., 1995). The structure was based primarily on ^{1}H NMR data that turned out to be compromised by the presence of an impurity in the sample that made it appear that the NH_2 group on C2 of the purine was still intact. Mass spectral data were inconsistent with this assignment. In addition, the ^{1}H NMR spectra of purified material strongly support the deoxyguanosine nucleus still being intact. FAPy structures exist as mixtures of rotomers around in the nitrogen-formamide and nitrogen-pyrimidine bonds leading to ^{1}H spectra which would be composites of the individual spectra of four rotomers for each CPP diastereomer. The two diastereomers were well-defined single species which showed no tendency to equilibrate to additional forms on standing.

Preferential reaction at N2 of deoxyguanosine is consistent with the reaction preference of bay region PAH diol epoxides. The site of reaction of CPPE with nucleophiles has generally been presumed to be C3 based upon calculations of the relative stabilities of C3 and C4 carbocations of CPP. In addition, Gold and coworkers have reported that nucleophilic attack by ammonia occurs exclusively at C3 of CPPE (Sangaiah et al., 1992). However, to our knowledge no detailed NMR or other structural studies of CPPE adducts have previously been made which prove that C3 is in fact the preferred site of reaction.

A second radioactive fraction F1 was also observed in the enzymatic digest of DNA that had reacted with CPPE. This fraction appeared to contain one or more deoxyguanosine adducts, since it was observed when [^{3}H]-deoxyguanosine was

introduced into the DNA. It seems likely that one or both of the diastereoisomers of *trans*-3-(deoxyguanosin-N2-yl)-4-hydroxy-3,4-dihydroxyCPP are present in F1. F1 has the same chromatographic relationship to the *trans*-dihydrodiol as the *cis* adduct has to the *cis*-dihydrodiol. The ESI mass spectra of the adduct(s) obtained from the dGuo reaction, which elute(s) in the same region as F1, exhibit the same molecular and fragment ion as the do the spectra of the *cis* adducts. Although these ions are unlikely to be unique to the N2 adduct structure, the MS data are at least consistent with it, and support the idea that reaction of CPPE with DNA forms one or both *trans* adduct isomers as well as the *cis* isomers.

The mechanism for preferential formation of a *cis* adduct deserves further attention. It is possible that the *cis* adduct is arising from attack of chloride ion or other nucleophiles on CPPE to form a *trans* product which then reacts with the nucleosides to give the *cis* product. Chloride ion was 0.05 M in the reaction mixtures of CPPE with both the nucleoside and calf thymus DNA. Meehan has found evidence for a transient chlorohydrin in the formation of *cis* deoxyguanosine adducts of the diol epoxide of BaP (Wolfe et al., 1994).

This study was designed to identify the major adducts that form in the reaction of CPPE with DNA. Whether the same adducts predominate in vivo when living organisms are exposed to CPP depends on whether CPPE is indeed the major metabolite formed that reacts with DNA. Recent evidence indicates that it is (Beach et al., 1993; Beach and Gupta, 1994), so the adducts formed in vivo should be those described in this study.

3.5   Summary
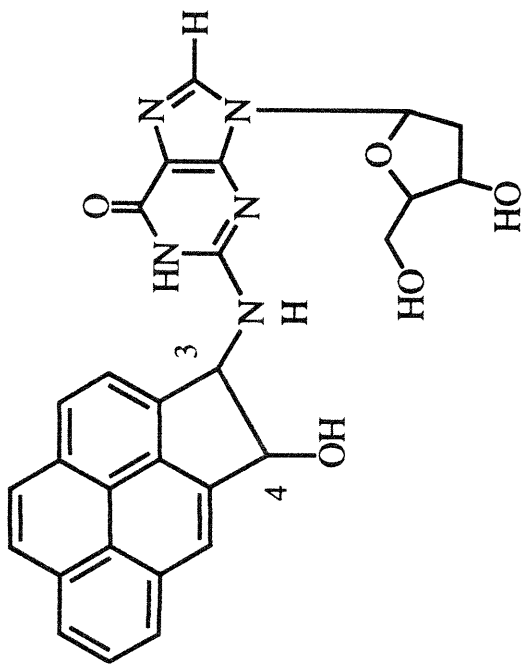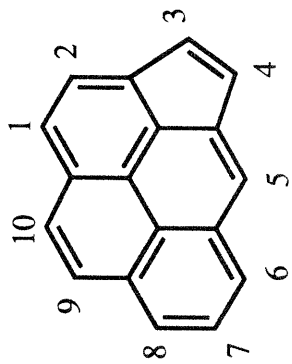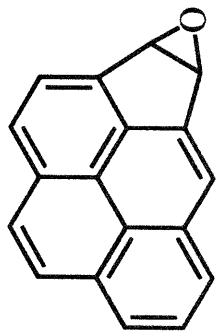
Cyclopenta[cd]pyrene (CPP) is a ubiquitous environmental pollutant whose 3,4-epoxide (CPPE) is generally regarded as its ultimate carcinogenic metabolite. The present study was undertaken to determine the structures of major DNA adducts formed by CPPE in vitro. Comparison of these adducts with adducts formed in vivo may be useful for elucidating the actual mechanisms of DNA damage by CPP in living organisms.   Calf thymus DNA was reacted with [^{3}H]-CPPE and deoxynucleoside adducts were obtained by enzymatic digestion of the adducted DNA.   These adducts were compared chromatographically with the products obtained by reaction of CPPE with 2'-deoxyguanosine (dG), which yielded two dG adducts that were identified by ^{1}H-NMR and FAB-MS as diastereoisomers of cis-3-(deoxyguanosin-N2-yl)-4-hydroxy-3,4-dihydroCPP.   Other products that may have included the isomeric trans-N2-dG adduct were formed in the reaction, but attempts to purify them were hindered by the high proportion of trans-3,4-diol present.   The major adduct fraction in the DNA digest, accounting for over 70% of the total, was chromatographically indistinguishable from the two cis dG-N2 adducts.   A second DNA adduct fraction was observed, which appeared also to be formed by reaction with guanine as indicated by experiments in which DNA containing [^{3}H]-guanine was reacted with unlabelled CPPE.   The results confirm that guanine is the major target in DNA for reaction with CPPE and are the first spectrochemical evidence to show that dG-N2 is the nucleophilic partner in the reaction.
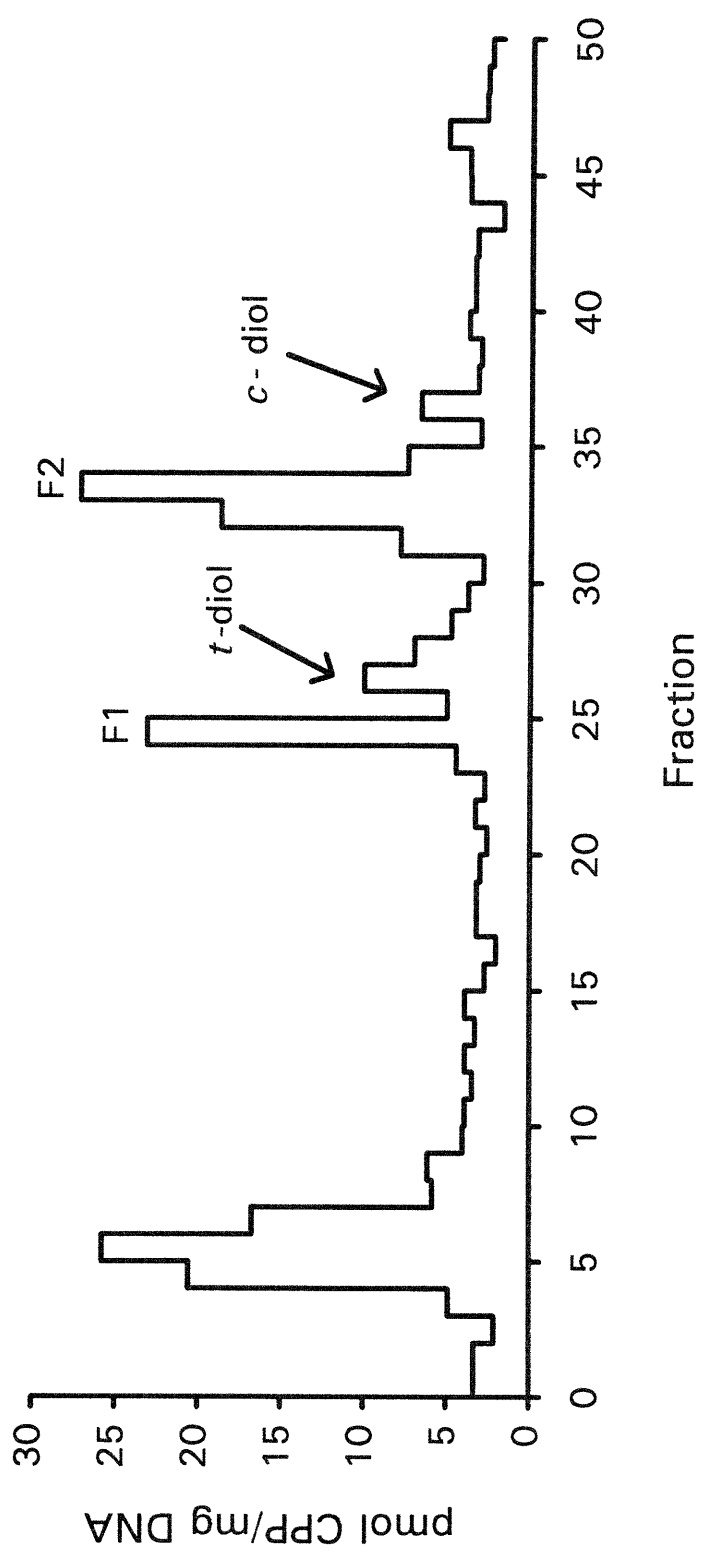
Figure Legends

Figure 3.1   Structures of CPP, its 3,4-epoxide, and adducts formed by reaction of the epoxide with the N2 of guanine.

Figure 3.2   Radiochromatogram obtained from the reversed-phase HPLC analysis of the enzymatic digest of calf thymus DNA after reaction with [^{3}H]-CPPE. Indicated retention times of the *cis*- and *trans*-3,4-diols that are CPPE hydrolysis products were determined by analysis of standards.

Figure 3.3   Radiochromatogram obtained from the reversed-phase HPLC analysis of the enzymatic digest of [8-^{3}H]-guanyl calf thymus DNA after reaction with CPPE. Indicated retention times of the *cis*- and *trans*-3,4-diols were determined by UV detection of residual product.
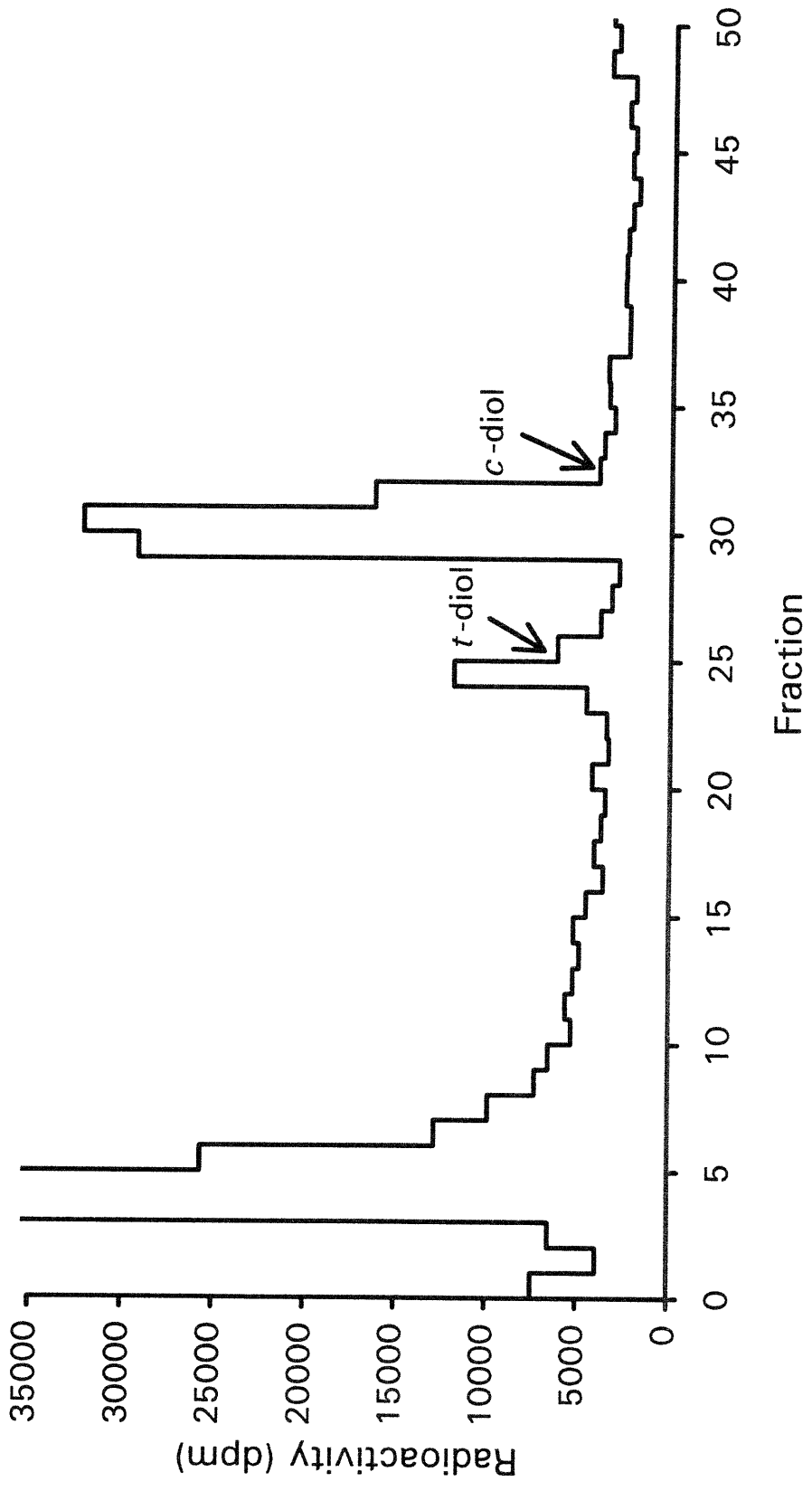
Table 1. ^{1}H NMR Spectrum of CPPE-Deoxyguanosine Adduct[a]

	Chemical Shift (δ)	Multiplicity
Gua 1-NH	10.88	s
CPP H8[b]	8.36	dd
CPP H1	8.28	d
CPP H6[b]	8.27	dd
CPP H9	8.19	s
CPP H10	8.19	s
CPP H5	8.15	d
CPP H2	8.12	dd
CPP H7	8.08	dd
Gua H8	8.04	s
Gua 2-NH	7.11	d
CPP 4-OH[c]	6.32	d
Deoxyribose H1'	6.28	dd
CPP H3 (CH-N)	6.14	dd
CPP H4 (CH-O)[d]	5.84	dd
Deoxyribose 3'-OH[c]	5.20	d
Deoxyribose 5'-OH[c]	5.14	t
Deoxyribose H3'[e]	4.36	dddd
Deoxyribose H4'	3.79	ddd
Deoxyribose H5'[f]	3.55	ddd
Deoxyribose H5''[f]	3.49	ddd
Deoxyribose H2'	2.70	ddd
Deoxyribose H2''	2.29	ddd

$J_{1,2}$=7.8 Hz, $J_{2,3}$=1.0, $J_{3,4}$=6.6, $J_{3,NH}$=7.8, $J_{4,OH}$=6.3, $J_{4,5}$=1.1

$J_{6,7}$=7.7^C, $J_{6,8}$=0.8, $J_{7,8}$=7.3^C, $J_{9,10}$=n.d., $J_{1',2'}$=7.7, $J_{1',2''}$=6.3

$J_{2',2''}$=13.2, $J_{2',3'}$=6.0, $J_{2'',3'}$=3.1, $J_{3',4'}$=3.0, $J_{3',OH}$=5.2, $J_{4',5'}$=5.0

$J_{4',5''}$=6.4, $J_{5',5''}$=11.6, $J_{5',OH}$=~5.1, $J_{5'',OH}$=~5.1

[a] Spectra were recorded of solutions in DMSO-d$_6$ on a Bruker AM 400 spectrometer at 27°C.
The values shown are for the adduct that eluted last from the reverse phase HPLC column.
In no case did chemical shifts of the two diastereomers differ by more than 0.05 ppm.
[b] Assignments may be reversed.
[c] Not observed under conditions of fast proton exchange.
[d] Collapsed to a doublet under conditions of fast proton exchange.
[e] Collapsed to a doublet of doublets of doublets under conditions of fast proton exchange.
[f] Collapsed to a doublet of doublets under conditions of fast proton exchange.

4.   DNA adduct formation by metabolites of cyclopenta[cd]pyrene in vitro

4.1   Introduction

CPPE has been proposed as the ultimate carcinogenic form of CPP and it has been shown that the major DNA adducts formed in vivo co-chromatograph on TLC with dG adducts formed by the reactions of CPPE in vitro. These studies have not provided definitive evidence that dG-CPP arises solely through reaction with CPPE in vivo, nor have they precluded the possibility that other bases are alkylated by CPPE or other reactive intermediates produced from CPP. Two important metabolites of CPPE, CPP-3,4-diol and 4-OH-DCPP, have been shown to react with DNA and to be mutagenic when activated by sulfotransferase. The first of these would be expected to produce the same adduct as CPPE. 4-OH-DCPP would yield a unique adduct but there is no certainty that it would be separated from dG-CPP under the chromatographic conditions used to detect in vivo adducts. The present study was thus undertaken to characterize the binding of these two secondary metabolites with DNA and to determine if bases other than guanine are alkylated by CPPE.

4.2 Materials and methods

Chemicals and Enzymes

CPP and [G-^{3}H]CPP were products of the Midwest Research Institute (Kansas City, MO) and the Chemsyn Science Laboratory (Lenexa, KS), respectively, and were obtained from the National Cancer Institute Chemical Carcinogen Repositories. CPPE and [^{3}H]CPPE were prepared from CPP with dimethyldioxirane in dichloromethane (Kwon et al., 1992). CPP-3,4-diol and [^{3}H]CPP-3,4-diol were made from CPPE by acid hydrolysis and purified to separate the *trans*- and *cis*-isomers. [^{3}H]4-OH-DCPP was obtained by [^{3}H]NaBH$_4$ reduction (New England Nuclear Products, Boston, MA) of 4-oxo-DCPP, which was prepared by rearrangement of CPPE in benzene. The sulfuric acid ester of 4-OH-DCPP was prepared as the sodium salt by *N,N'*-dicyclohexylcarbodiimide-mediated coupling of sulfuric acid to 4-OH-DCPP in dimethylformamide (DMF) (Surh et al., 1993). Radiolabeled calf thymus DNA containing [8-^{3}H]guanine, [1',2',2,8-^{3}H]adenine, or [1',2',5-^{3}H]cytosine, was prepared using the Nick Translation System of Promega (Madison, WI) to incorporate selected [^{3}H]dNTPs, which were obtained as the ammonium salts from Amersham Life Science (Arlington Heights, IL). Calf thymus DNA was sonicated and extracted with phenol prior to use. The amount of labeled dNTPs incorporated into the DNA was determined by the DE81 filter-binding assay (Sambrook et al., 1989). The typical percentage of incorporation ranged from 25% to 45%. The enzymes for digestion of the modified calf thymus DNA by CPP metabolites were DNase I (from bovine pancreas), phosphodiesterase II (from calf spleen), snake venom phosphodiesterase (from *Crotalus durissus*), and alkaline phosphatase (from calf intestine). All these enzymes were products of Boehringer Mannheim Biochemicals (Indianapolis, IN), except DNase I, which was purchased from Sigma Chemical Co. (St. Louis, MO). The digestion of modified DNA has been described in previous section. Rat liver cytosol was prepared from postmitochondrial supernant fractions supplied by Molecular Toxicology, Inc.

(Annapolis, MD). All other chemicals and solvents used were reagent grade.

Preparation and Isolation of Modified DNA

Calf thymus DNA was treated with CPPE (20 nmol/mg DNA) in a 50 mM Tris-HCl buffer (pH 7.4) at 37°C for 2 hr. CPP-3,4-diol and 4-OH-DCPP were incubated with calf thymus DNA (20 nmol/mg DNA) in the presence of rat liver cytosol and 3'-phosphoadenosine-5'-phophosulfate (PAPS) at 37°C for 2 hr. Each reaction mixture was extracted twice with a half volume of water-saturated ethyl acetate to remove unbound CPP derivatives, and DNA was isolated by ethanol precipitation. Phenol/chloroform extraction was performed to remove cytosolic proteins in the sulfotransferase-mediated activation reactions (Surh et al., 1993). To every 50 ug of nick translated DNA, 13.3 nmol of various CPP metabolites were added.

Synthesis of trans-3-(deoxyadenosin-N6-yl)-4-hydroxy-3,4-dihydroCPP

CPPE was allowed to react with liquid NH_3 and one equivalent of H_2O as described (Sangaiah et al., 1992). This procedure has been reported to yield trans-4-amino-3-hydroxy-3,4-dihydroCPP, but when the product was analyzed by GC-MS it was found to be a mixture of two isomers in nearly equal amounts. The isomers were inseparable by HPLC. GC-MS was conducted with a Hewlett Packard 5971A equipped with a standard EI source and 15 mm x 0.2 mm HP1 fused silica capillary column. bis-Trimethylsilyl derivatives were prepared by treating with a mixture of N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), pyridine, and acetonitrile (5:2:2, v:v) for 1.5 hr at 60°. Spectra obtained from each isomer were nearly identical: m/z 403 (M^+), 388 (M^+-CH$_3$), 330 (M^+-TMS), 313 (M^+-TMSOH), 240 (M^+-TMS-TMSOH). Pentafluoropropionamide derivatives were prepared by treatment with pentafluoropropionic anhydride, saturated trimethylamine in hexane, and

dichloromethane (1:10:50, v:v) for 1.5 hr at room temperature. This derivative of each isomer also gave virtually identical spectra: m/z 387 (M^+-H_2O), 240 (M^+-H_2O-COC_2F_5).

The reaction of CPPE with NH_3 can yield a maximum of 4 isomers, depending on the position of nucleophilic attack as well as the stereochemistry of ring opening. Under the reaction conditions used there is little possibility of *cis* attack on the epoxide. Thus the isomers must be positional, one being *trans*-3-amino-4-hydroxy-3,4-dihydroCPP and the other *trans*-4-amino-3-hydroxy-3,4-dihydroCPP. Since we were unable to separate the two by HPLC, the mixture was used for the remainder of the synthesis.

trans-3-(Deoxyadenosin-N6-yl)-4-hydroxy-3,4-dihydroCPP and *trans*-4-(deoxyadenosin-N6-yl)-3-hydroxy-3,4-dihydroCPP were obtained by the reaction of *trans*-CPPAA with 6-chloropurine 2'-deoxyriboside in refluxing ethanol for 48 hr as described for the syntheses of acenaphthene and aceanthrene oxide adducts (Bartczak et al., 1989). HPLC analysis of the products revealed only one with the expected pyrene-like UV spectrum. This product gave the correct mass by ionspray-MS: m/z 494 (MH^+) and 516 (MNa^+). Spectra were acquired with a PE SCIEX Ionspray-API1 LC/MS system (Toronto, Canada). The product is presumed to be an unresolvable mixture of positional isomers since the amino alcohol was a mixture.

Chromatography

The DNA digests were analyzed by reverse phase HPLC (Hewlett Packard HP 1090, equipped with a diode array detector, Palo Alto, CA) with a fluorescence detector (Spectroflow 980, ABI analytical Inc., CA). Excitation was at 341nm and a 370nm cut off filter was used on the emission. Columns (4.6 mm x 250 mm or

66

7.8 mm x 250 mm) were Nucleosil C18 from Alltech Assoc. Inc. (Deerfield, IL) and the following gradient for separating CPP metabolites and DNA adducts at the flow rate of 1 ml/min or 3 ml/min, depending on column size, was used: an isocratic phase of 50% methanol in water for 5 min, linear gradient to 80% methanol at 55 min, and to 100% at 60 min. To isolate CPPAA from the reaction mixture, an acetonitrile/water gradient containing 0.05% trifluoroacetic acid (TFA) was applied. The solvent program was a 3-min isocratic phase of water followed by a linear gradient to 50% acetonitrile in water at 25 min, and to 100% at 40 min. For radioactive samples, the eluting fractions were collected every minute, and the radioactivity of each fraction was measured upon addition of liquiscint (National Diagnostics, Atlanta, GA) in a Beckman LS6000 counter (Fullerton, CA).

4.3   Results and Discussion

Characterization of DNA adducts formed by serveral different CPP metabolites was undertaken in this study. In agreement with an earlier report (Beach and Gupta, 1994), our study shows that CPPE preferentially reacts with guanine than with other bases. We also demonstrate here that two other major CPP metabolites, 4-OH-DCPP and *trans*-CPP-3,4-diol, predominantly yield deoxyguanosinyl adducts.

The covalent binding of [^{3}H]CPPE to DNA in vitro resulted in a level of DNA base modification equal to one hydrocarbon adduct per 15,000 nucleotides. The HPLC radiochromatogram shown in Figure 4.1 reveals that two major products are formed of the enzyme digest of this adducted DNA. The components eluting before 10 min on the profile were not investigated further in this study since they were too polar to be deoxynucleoside adducts. Trace amounts of *trans*- and *cis*-CPP-3,4-diol were observed.

In order to establish the identity of the base(s) that react with CPPE, specific radiolabelled deoxyribonucleotides were introduced into the DNA by nick translation prior to its reaction with nonradioactive CPPE. Previous studies show that thymine is a minor site, at best, of modification of DNA by PAHs, so it was not investigated (Hemminki, 1983; Dipple, 1995). The nick translated [^{3}H]DNA was treated with CPPE and digested for HPLC analysis. The results from CPPE-treated [^{3}H-dG]DNA (Figure 4.2) indicated that the two major fractions produced by [^{3}H]CPPE-treated DNA (F1 and F2 in Figure 4.1) are CPP-dG adducts. Further incubations of CPPE with the nick-translated [^{3}H-dA]DNA (Figure 4.3) and [^{3}H-dC]DNA (Figure 4.4) showed that CPPE also bound covalently to A and C in DNA, but on the order of 10 and 100 times lower, respectively, than it bound to G. This comparative binding ratio of individual bases in DNA was determined by dividing the total radioactivity of the adducted peaks resolved in HPLC by the total

radioactivity injected. Results from experiments with [^{3}H-dA]DNA and [^{3}H-dC]DNA provide additional evidence that the two major CPPE-DNA adducts are deoxyguanosine-specific since the products formed migrated differently from F1 and F2 on HPLC.

The later eluting adduct fraction (F2 in Figure 4.1) of the reaction of [^{3}H]CPPE and DNA co-migrated with authentic diastereomeric *cis*-3-(deoxyguanosin-N2-yl)-4-hydroxy-3,4-dihydroCPP, obtained from the reaction of CPPE and dG. The earlier eluting adduct fraction (F1 in Figure 4.1) was presumed to be the corresponding *trans-* isomer for the reasons discussed (also see below), but this assumption remains unproven.

The two fractions obtained in the reaction of [^{3}H-dA]DNA with CPPE (Figure 4.3) can be identified, by a similar line of reasoning, as *cis-* and *trans-* isomers of 3-(deoxyadenosin-N6-yl)-4-hydroxy-3,4-dihydroCPP. A1, the earlier eluting peak, co-migrated with the product obtained by reacting *trans*-CPPAA with 6-chloropurine-2'-deoxyriboside. This reaction product was almost certainly composed of a mixture of 3,4- and 4,3- isomers since the amino alcohol from which it was made was an inseparable mixture, but the *trans* geometry and N6 position of substitution in the purine ring, which are structural elements that would be expected to have a pronounced influence on HPLC retention time, are unambiguous in this synthetic strategy. Thus, the identification of A1 is reasonably certain. A2 is presumed to be the *cis* isomer of the same adduct, since it elutes later (see below) and since exclusive formation of the *trans* isomer is unlikely in view of the predominance of the *cis*-dG adduct.

Only one fraction (C1 in Figure 4.4) was obtained from the reaction of CPPE and [^{3}H-dC]DNA. No evidence was obtained for its structure, but it may be speculated that the stereochemistry of CPPE ring opening in this adduct is *cis*

because *cis* adducts were the predominant adducts observed with guanine and adenine in DNA. In addition, the retention time of this adducted fraction is consistent with *cis* stereochemistry (see below).

Adduct identification in this study was based on reactivity considerations and on chromatographic comparisons. Highly reactive PAH epoxides and diol epoxides react predominantly by the S_N1 mechanism at neutral pH, typically leading to a mixture of *cis* and *trans* products. For example, F2 was shown to contain *cis* adducts by co-chromatography with authentic material. F1 was, therefore, considered to be the corresponding *trans* isomer since it is reasonable to assume that both *cis* and *trans* opening of the epoxide ring are likely occur. Also, in several other cases it has been established that *trans* disubstitued dihydroPAHs elute earlier than their corresponding *cis* forms on reverse phase HPLC. Examples here include CPP-3,4-diols (Sahali et al., 1992), 3-methoxy-4-hydroxy-3,4-dihydroCPP derivatives (Hsu et al., 1996c), and others (Delclos et al., 1988; Bigger et al., 1978; Roy et al., 1991). Accordingly, the earlier retention value of F1 also supported its *trans* assignment. Based on these considerations, A2 was then presumed to be the *cis* form since A1 was shown to be the *trans* deoxyadenosinyl adduct. The findings reported here are consistent with the idea that adducted dG or G always elutes earlier than adducted dA or A on reverse phase HPLC when DNA is modified by PAHs (Canella et al., 1992; Babson et al., 1986; Cheh A.M. et al., 1993; Li et al., 1993; Misra et al., 1992; Jennette et al., 1977; Straub et al., 1977; Chadha et al., 1989; Melikian et al., 1988; Lurie et al., 1988; Phillips et al., 1981; Mlcoch et al., 1993; Alexandrov et al., 1988; Rojas and Alexandrov, 1986; Cheng et al., 1989; Nair et al., 1989; Marletta and Szuba, 1988; Carmella and Hecht, 1987; Surh et al., 1989; Surh et al., 1991a; Surh et al., 1990). To our knowledge, this generalization has never been stated previously, but it appears true for a number of cases. C1 was assigned *cis-* geometry for the following two reasons. Substituted dC or C should elute only slightly earlier than substituted dG or G on reverse phase HPLC (Surh

et al., 1989; Straub et al., 1977; Jennette et al., 1977) and other CPPE-derived DNA adducts in this study were found to be predominately *cis* adducts.

Sulfotransferase activation was used to generate DNA adducts from CPP-3,4-diols and 4-OH-DCPP. Liver cytosol from male rats was used in this study, since it has the highest sulfotransferase activity for 4-OH-DCPP (the only CPP derivative tested) when compared to that of male mice, female mice and rats (Surh et al., 1993). Not surprisingly, no adduct was detected by HPLC-fluorescence or by radiochromatography in the sulfotransferase-mediated reaction of *cis*-CPP-3,4-diol modified DNA: this isomer has previously been shown to give only very low levels of sulfotransferase-dependent DNA binding (Surh et al., 1993). In contrast, sulfotransferase-dependent binding of [^{3}H]*trans*-CPP-3,4-diol gave one hydrocarbon adduct per 50,000 nucleotides. In this experiment, two radioactive fractions with the retention times of F1 and F2 were observed (DIOL1 and DIOL2 in Figure 4.5), suggesting that *trans*- and *cis*-3-(deoxyguanosin-N2-yl)-4-hydroxy-3,4-dihydroCPP were the products formed. As a partial test of this inference, *trans*-3,4-diol was reacted with nick-translated [^{3}H-dG]DNA. The same two radioactive fractions were obtained (DIOL1 and DIOL2 in Figure 4.6), confirming that reaction takes place with guanine. Although the adducts obtained in the sulfotransferase-mediated binding of CPP-3,4-diol co-chromatograph with known adducts, their stereochemical characterization remains incomplete because it cannot be determined whether the 3- or 4-hydroxyl group of CPP-3,4-diol is sulfated by the enzyme prior to attack DNA. Thus, the minor dG adduct (DIOL1) might be one or both of *trans*-3-(deoxyguanosin-N2-yl)-4-hydroxy-3,4-dihydroCPP and*trans*-4-(deoxyguanosin-N2-yl)-3-hydroxy-3,4-dihydroCPP, and the major dG adduct might be a mixture of the corresponding *cis* isomers.

Sulfotransferase-activated *trans* CPP-3,4-diol and CPPE apparently produced similar ratios of stereoisomers in their reaction with guanine, suggesting that the

same reactant is involved. The reactant could be the fully formed C3-carbocation produced by epoxide ring-opening or by heterolysis of the C3-sulfate bond. If the nucleophilic partner in these reactions were involved in the transition state, different stereochemical outcomes would be expected because the bond being broken in CPPE is *cis* to the final C4-hydroxyl group, while the bond being broken in the sulfate ester is *trans*. This inference that the fully formed C3-carbocation is the ultimate reactant in both cases is not unreasonable, but we note that another interpretation is possible: the common reactant could be CPPE itself, formed by the action of sulfotransferase on the *trans* diol. CPPE has been synthesized by the action of the tosyl chloride and base on the *trans* diol (Sahali et al., 1990). Depending on the conditions within the active site of the sulfotransferase, it is conceivable that the same intramolecular displacement (of a sulfate rather than a sulfonate group) could take place. This mechanism would explain the lack of reactivity of *cis*-CPP-3,4-diol. Of course, it may be that the *cis* diol is not a good substrate for the enzyme. The common stereochemical course of the reactions preludes determining which reactant predominates in vivo without additional information.

The covalent binding of 4-OH-DCPP to DNA in the presence of rat cytosol and PAPS resulted in a level of DNA modification equal to one hydrocarbon adduct per 2,500 nucleotides. Figure 4.7 shows the HPLC-radioactivity profile of the products obtained from the reaction of $[^3H]$4-OH-DCPP and DNA in the sulfotransferase-mediated incubation. With fluorescence detection, two closely eluting peaks were apparent in the region of OH2. These correspond to the products obtained from reaction of synthetic 4-NaO$_3$S-O-DCPP or sulfotransferase-activated 4-OH-DCPP with deoxyguanosine. The two adducts in fraction OH2 are thus presumed to be a diastereomeric pair of adducts formed by nucleophilic substitution of the sulfate group by guanine. The minor component (OH1 in Figure 4.7), not always observed in the radiochromatogram, eluted from the column at

approximately 32 min. This more polar fraction was formed in the absence of DNA or dG and so is assumed not to be an adduct. Two products were obtained from attempted reaction of 4-NaO$_3$S-O-DCPP with deoxyadenosine and they eluted around 45 min on our HPLC system.

The present study is the first to report DNA adduct formation by secondary metabolites of CPP. It also concludes that the same major DNA adducts are formed by CPPE and sulfotransferase-activated *trans* CPP-3,4-diol. These findings suggest that in addition to CPPE, CPP-3,4-diol may also be involved in CPP-DNA adduct formation in vivo (Nesnow et al., 1994; Beach and Gupta, 1994). Adducts derived from 4-OH-DCPP were obtained and distinguished on our HPLC system from CPPE-derived adducts. Guanine residues were found to be the primary targets of DNA modification by the various CPP metabolites that we tested. The availability of the adducts resulting from this work and use of our chromatographic conditions should aid the identification of electrophilic species of CPP in vivo.

bases in DNA directly to produce the benzylic DNA adducts efficiently (Okuda et al., 1989; Surh et al., 1987; Surh et al., 1991a; Surh et al., 1989; Surh et al., 1990; Watabe et al., 1985; Watabe et al., 1987). For example, some sulfooxymethyl PAHs yielded higher amounts of DNA adducts than did the corresponding parent hydroxymethyl PAHs when administrated intraperitoneally to infant rats (Surh et al., 1987; Surh et al., 1991a; Surh et al., 1989; Surh et al., 1990). The carcinogenicity of 6-sulfooxymethylBaP was also found to be higher than those of 6-hydroxymethylBaP and BaP (Surh et al., 1991b). As tumor initiators, 9-sulfooxymethyl-10-methylanthracene and 1-sulfooxymethylpyrene were more active than the parent agents (Surh et al., 1990).

2.4.1.2 Cyclopenta-fused aromatic hydrocarbons

4-OH-DCPP and CPP-3,4-diols contain secondary benzylic hydroxyl groups in the cyclopenta ring. Significant covalent DNA binding of these CPP metabolites were observed in the presence of rodent cytosol and PAPS, and the binding could be inhibited by both DCNP and DHEA (Surh et al., 1993). These results indicated that either the phenol sulfotransferase or hydroxy-steroid sulfotransferase was capable to activate these compounds.

The bay-region diol epoxides are generally recognized as the ultimate carcinogenic metabolites of a large group of PAHs. However, adducts formed from enzymatic one-electron oxidations of BaP and DMBA have also been characterized and their carcinogenesis is of great interest (RamaKrishna et al., 1992b; Devanesan et al., 1992; RamaKrishna et al., 1992a). For methyl-substituted PAHs, hydroxylation of *meso*-methyl groups with subsequent formation of reactive benzylic esters bearing a good leaving group such as acetate, phosphate, or sulfate has been proposed as a possible or an alternative mechanism of activation, carcinogenesis, and DNA binding of these hydrocarbons (Flesher and Sydnor, 1971; Flesher and Sydnor, 1973).

Methyl-substituted PAHs that have been studied using sulfotransferase activation include 7-hydroxy-12-methylbenz[*a*]anthracene (Watabe et al., 1982), a major metabolite of 7,12-dimethylbenz[*a*]anthracene, 7,12-dihydromethylbenz[*a*]anthracene (Watabe et al., 1987), 7-hydroxymethylbenz[*a*]anthracene (Watabe et al., 1986), 5-hydroxymethylchrysene (Okuda et al., 1989), 6-hydroxymethylBaP (Surh et al., 1989), 9-hydroxymethyl-10-methylanthracene (Surh et al., 1990), and 1-hydroxymethylpyrene (Surh et al., 1991a; Surh et al., 1990). All of these PAHs exhibited mutagenicity using Ames test and formed benzylic adducts with dG and dA in calf thymus DNA in the presence of rodent liver cytosol and PAPS. DHEA substantially inhibited their mutagenicity and DNA binding but PCP and DCNP did not. This information together with the result that purified hydroxysteroid sulfotransferase showed high catalytic activity for hydroxymethyl PAHs (Ogura et al., 1990) suggested hydroxy-steroid sulfotransferase may be responsible for the sulfotransferase-mediated activation of these compounds.

The chemically synthesized sulfuric acid esters of these hydroxymethyl PAHs are extremely potent mutagens and can react with guanine, adenine, and cytosine

4.4   Summary

Cyclopenta[*cd*]pyrene (CPP) is a common carcinogenic environmental contaminant. Although the 3,4-epoxide (CPPE) is recognized as a potent mutagen, other metabolites arising from CPPE also exhibit the ability to react with and cause mutations in DNA. In this study, we compared these other metabolites to CPPE with respect to the adducts formed in their reaction with DNA. Calf thymus DNA was reacted in vitro with CPPE or with its metabolites, 3,4-dihydroCPP-3,4-diol (CPP-3,4-diol) and 4-hydroxy-3,4-dihydroCPP (4-OH-DCPP), activated with sulfotransferase. The adducts formed were analyzed by HPLC with fluorescence detection following enzymatic digestion of DNA to deoxynucleosides. Radiolabeling of specific DNA bases prior to reaction demonstrated that the two major CPPE-derived adducts were formed by guanine, while minor adducts were formed by adenine and cytosine. The major dG adduct formed in DNA was identified as *cis*-3-(deoxyguanosin-N2-yl)-4-hydroxy-3,4-dihydroCPP and the minor dG adduct seems likely to be its *trans* isomer. Adenosine adducts were presumably formed by aralkylation of the N6 position since they co-chromatographed with the regio-specifically synthesized standard, which was prepared by reaction of *trans*-3,4-dihydroCPP amino alcohol with 6-chloropurine 2'-deoxyriboside. Sulfotransferase activation of *trans*-CPP-3,4-diol yielded two adducts that were identical to the products resulting from the reaction of CPPE with DNA, while *cis*-CPP-3,4-diol gave very low covalent binding. Two adducts formed by sulfotransferase activation of 4-OH-DCPP were identical to the diastereomeric products generated from the reaction of synthetic 4-NaO$_3$S-O-DCPP with dG. These results indicate that guanine is predominantly the site of CPP adduct formation in DNA, regardless of the identity of the reactive metabolite, and that the 4-hydroxy-3-dG adducts can arise by reaction of DNA with either CPPE or sulfotransferase-activated *trans*-CPP-3,4-diol.

Figure Legends

Figure 4.1   Radiochromatogram obtained from the reversed-phase HPLC analysis of the enzymatic digest of calf thymus DNA after reaction with [³H]CPPE. Indicated retention times of the *cis*- and *trans*-3,4-diols that are CPPE hydrolysis products were determined by analysis of standards. The fraction eluting before 10 min on the profile was not investigated further since they were too polar to be deoxynuceloside adducts. F1 was considered to be *trans*-3-dG-4-OH-DCPP and F2 was shown to contain *cis*-3-dG-4-OH-DCPP.

Figure 4.2   Radiochromatogram obtained from the reversed-phase HPLC analysis of the enzymatic digest of [8-³H]guanyl calf thymus DNA after reaction with CPPE. Indicated retention times of *cis*- and *trans*-3,4-diols were determined by UV detection of residual product. G1 was considered to be *trans*-3-dG-4-OH-DCPP and G2 was shown to contain *cis*-3-dG-4-OH-DCPP.

Figure 4.3   Radiochromatogram obtained from the reversed-phase HPLC analysis of the enzymatic digest of [1',2',2,8-³H]adenyl calf thymus DNA after reaction with CPPE. Indicated retention times of *cis*- and *trans*-3,4-diols were determined by UV detection of residual product. A1 was shown to contain *trans*-3-dA-4-OH-DCPP and A2 was considered to be *cis*-3-dA-4-OH-DCPP.
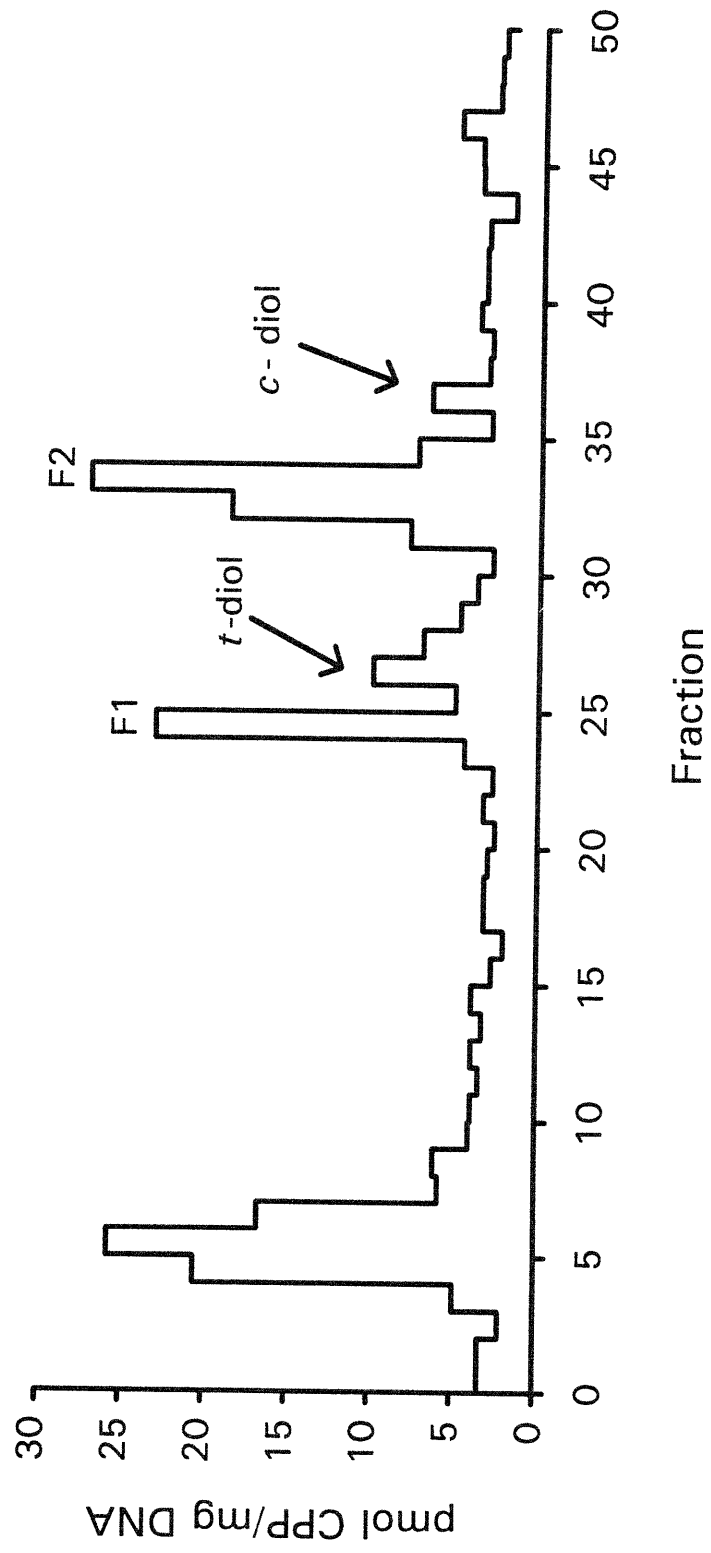
Figure 4.4   Radiochromatogram obtained from the reversed-phase HPLC analysis of the enzymatic digest of [1',2',5-³H]cytosinyl calf thymus DNA after reaction with CPPE. Indicated retention times of *cis*- and *trans*-3,4-diols were determined by UV detection of residual product. C1 was thought to be *cis*-3-dC-4-OH-DCPP.
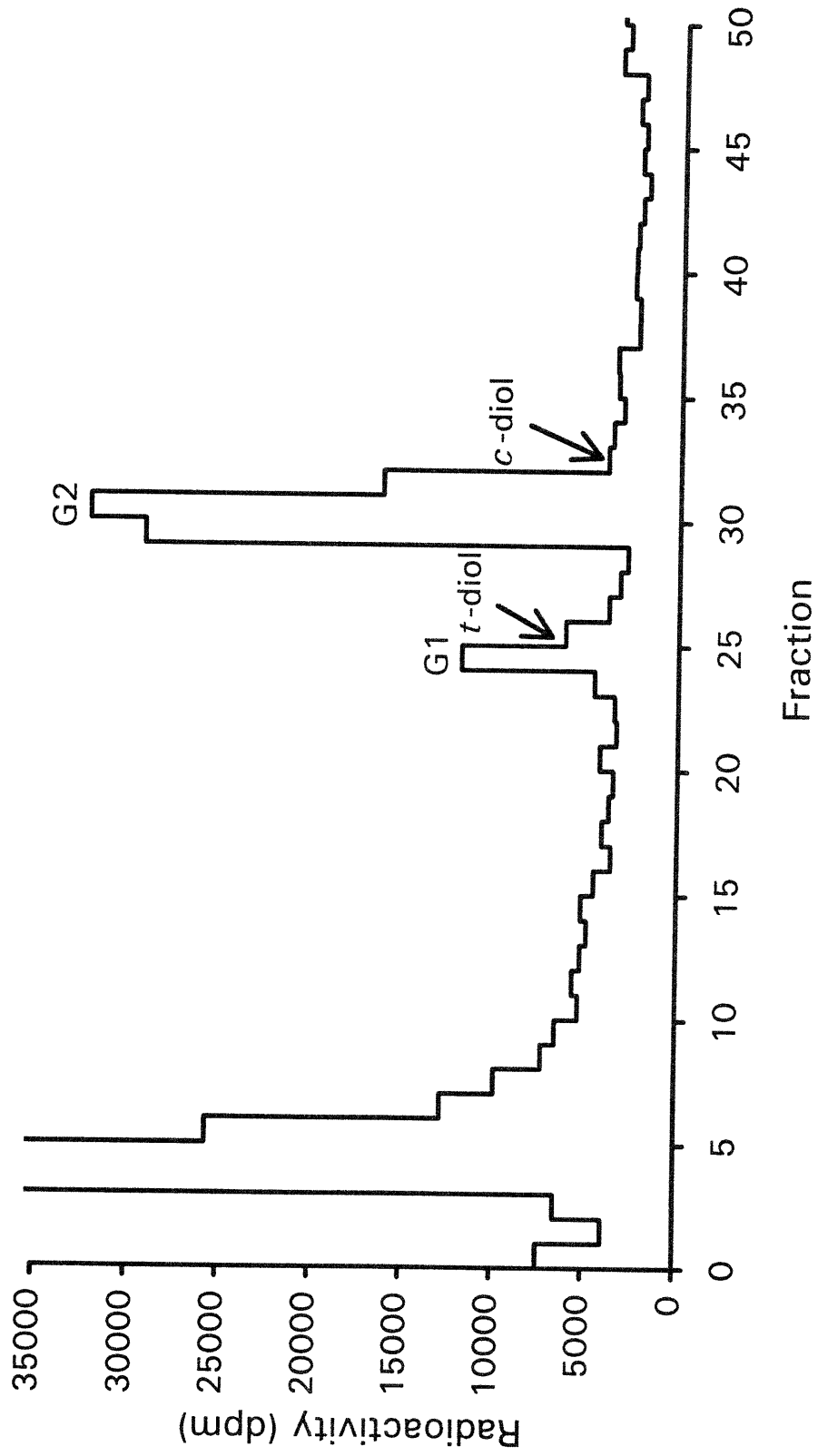
Figure 4.5   Radiochromatogram obtained from the reversed-phase HPLC analysis of the enzymatic digest of calf thymus DNA after reaction with sulfotransferase-activated [³H]*trans*-CPP-3,4-diol. Indicated retention times of the *cis*- and *trans*-3,4-diols were determined by analysis of standards. The fraction eluting before 10 min on the profile was not investigated further since they were too polar to be deoxynuceloside adducts. DIOL1 was considered to be the mixture of *trans*-3-dG-4-OH-DCPP and trans-3-OH-4-dG-DCPP and DIOL2 was assigned to be the mixture of *cis*-3-dG-4-OH-DCPP and *cis*-3-OH-4-dG-DCPP by comparison to the *cis*-3-dG-4-OH-DCPP standards.
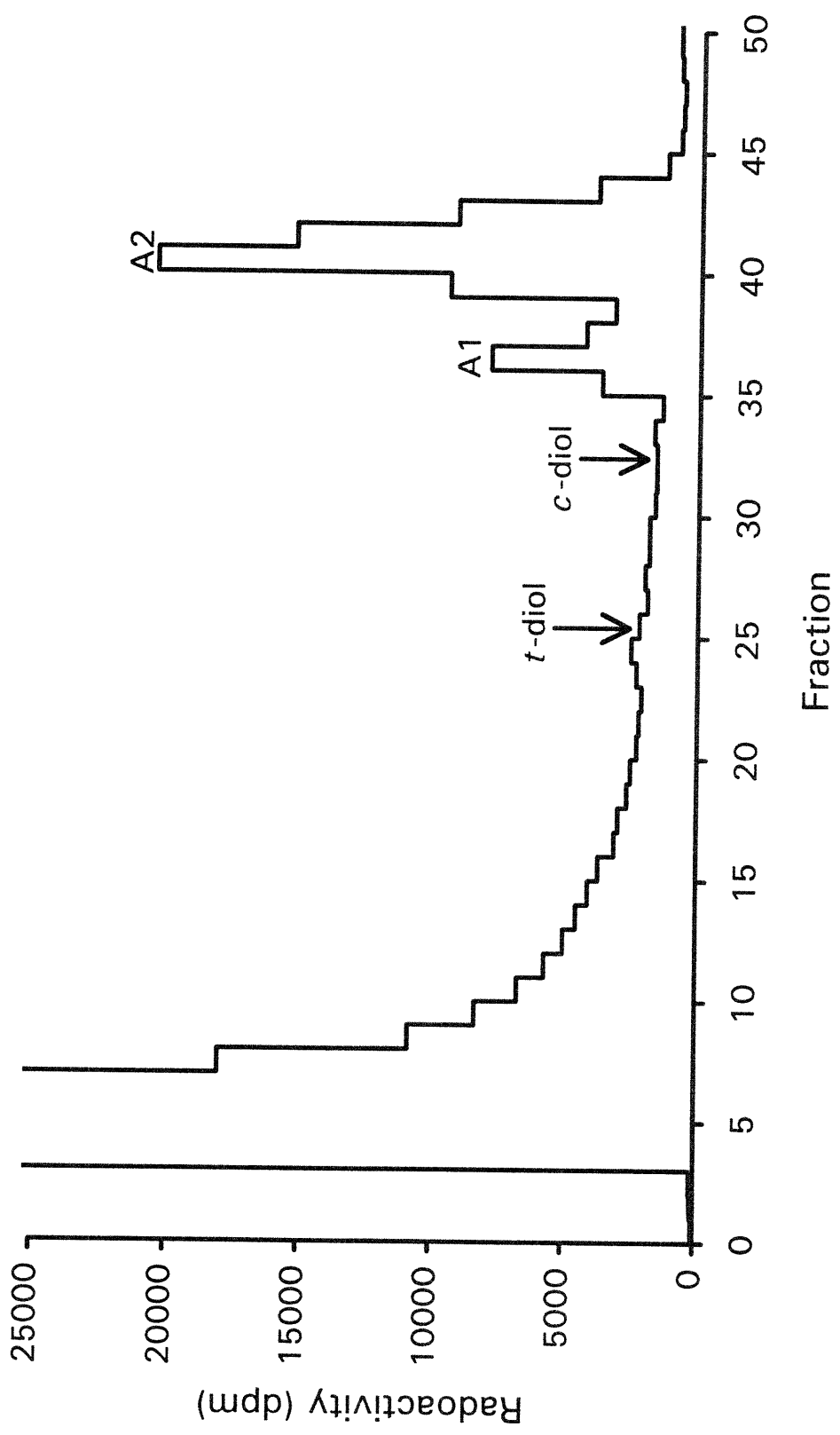
Figure 4.6   Radiochromatogram obtained from the reversed-phase HPLC analysis of the enzymatic digest of [8-³H]guanyl calf thymus DNA after reaction with sulfotransferase-activated *trans*-CPP-3,4-diol. Indicated retention times of *cis*- and *trans*-3,4-diols were determined by UV detection of residual product. DIOL1 was considered to be the mixture of *trans*-3-dG-4-OH-DCPP and trans-3-OH-4-dG-DCPP and DIOL2 was assigned to be the mixture of *cis*-3-dG-4-OH-DCPP and *cis*-
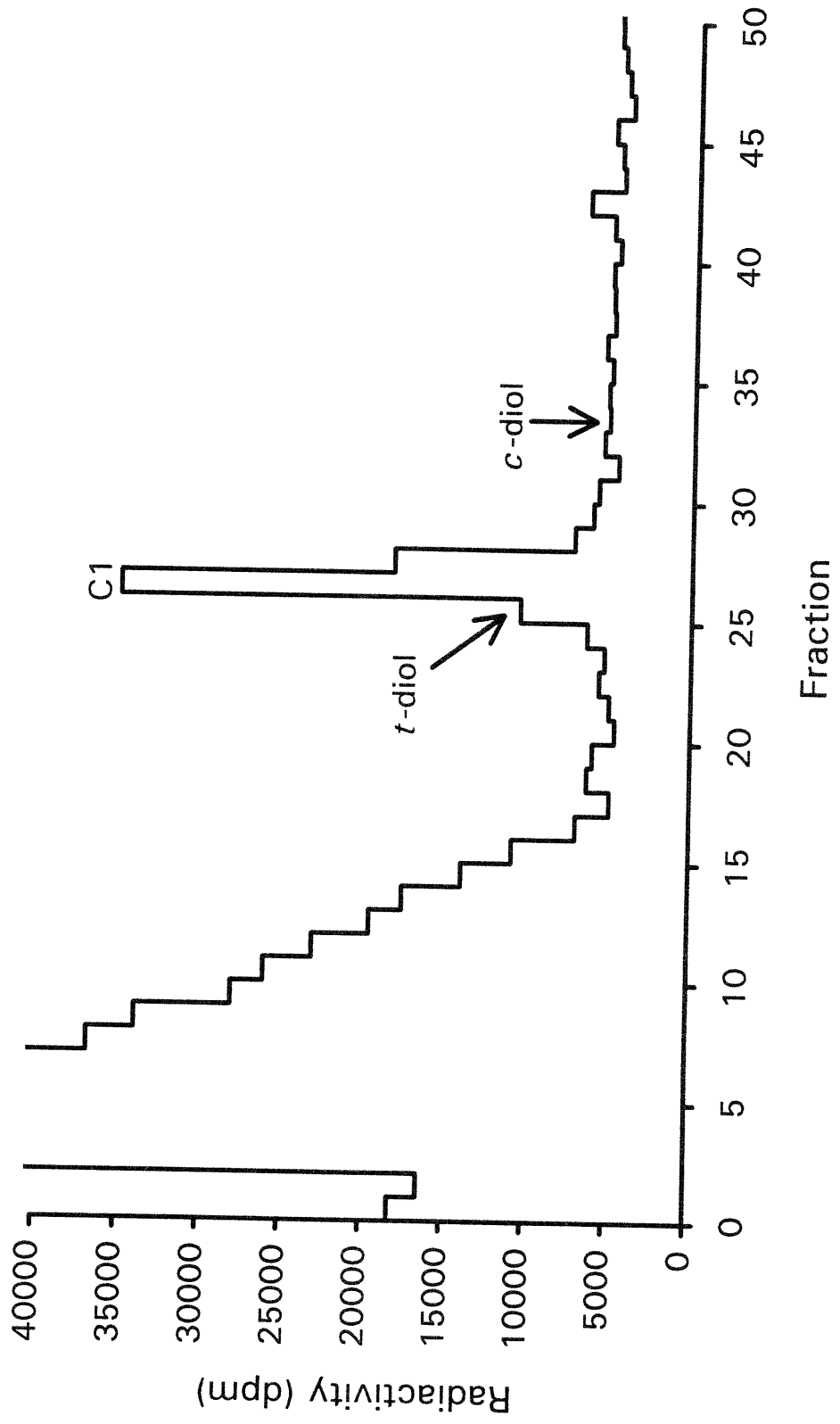
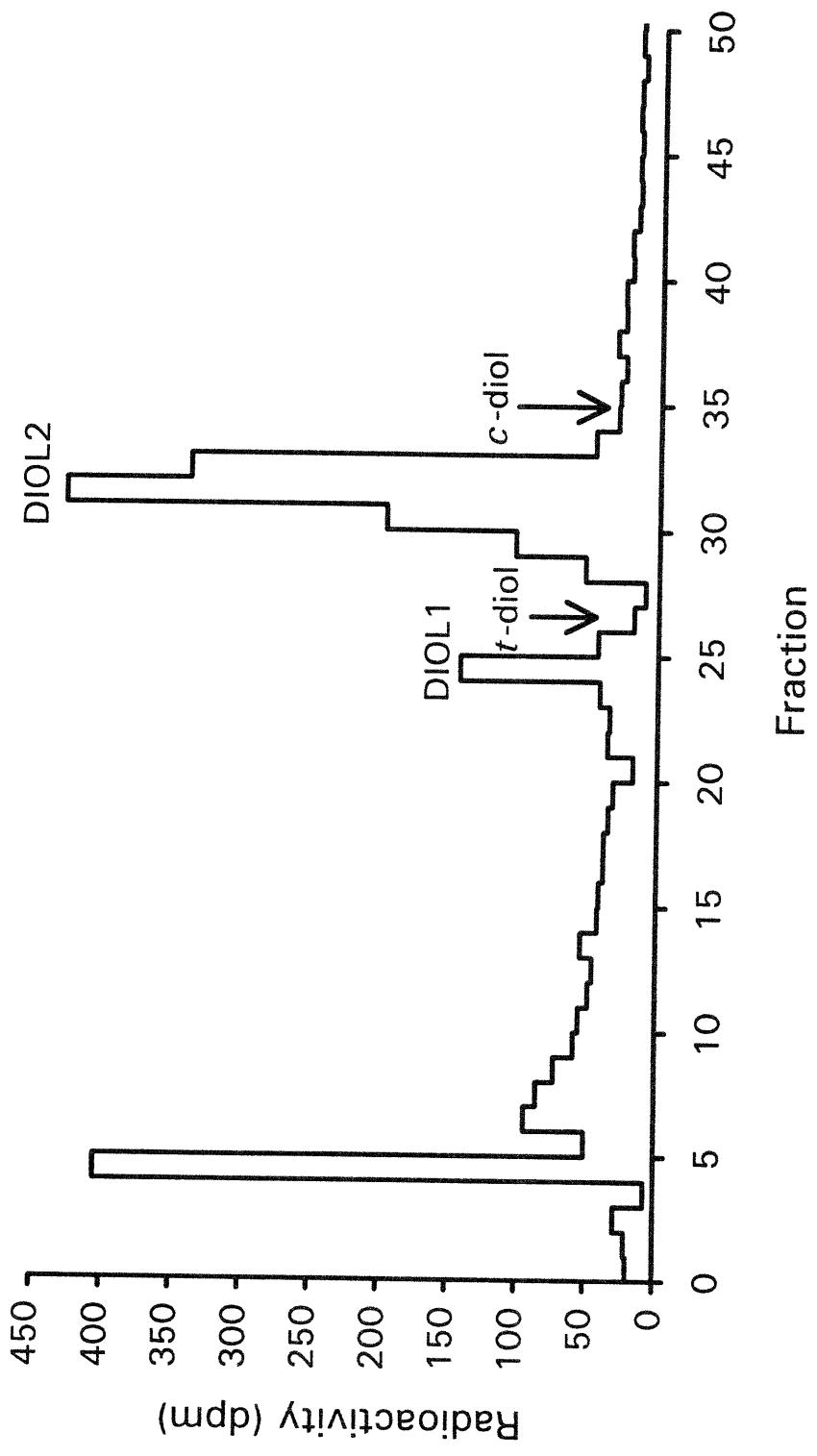3-OH-4-dG-DCPP by comparison to the *cis*-3-dG-4-OH-DCPP standards.

Figure 4.7   Radiochromatogram obtained from the reversed-phase HPLC analysis of the enzymatic digest of calf thymus DNA after reaction with sulfotransferase-activated [^{3}H]4-OH-DCPP. Indicated retention time of the 4-OH-DCPP were determined by analysis of standard. OH1 was not formed in the absence of DNA or dG and so is assumed not to be an adduct. OH2 was proved to be 4-dG-DCPP by comparing it to the reaction products of 4-NaOSO$_3$-DCPP and individual nucleosides (dA and dG).

OH2

OH1

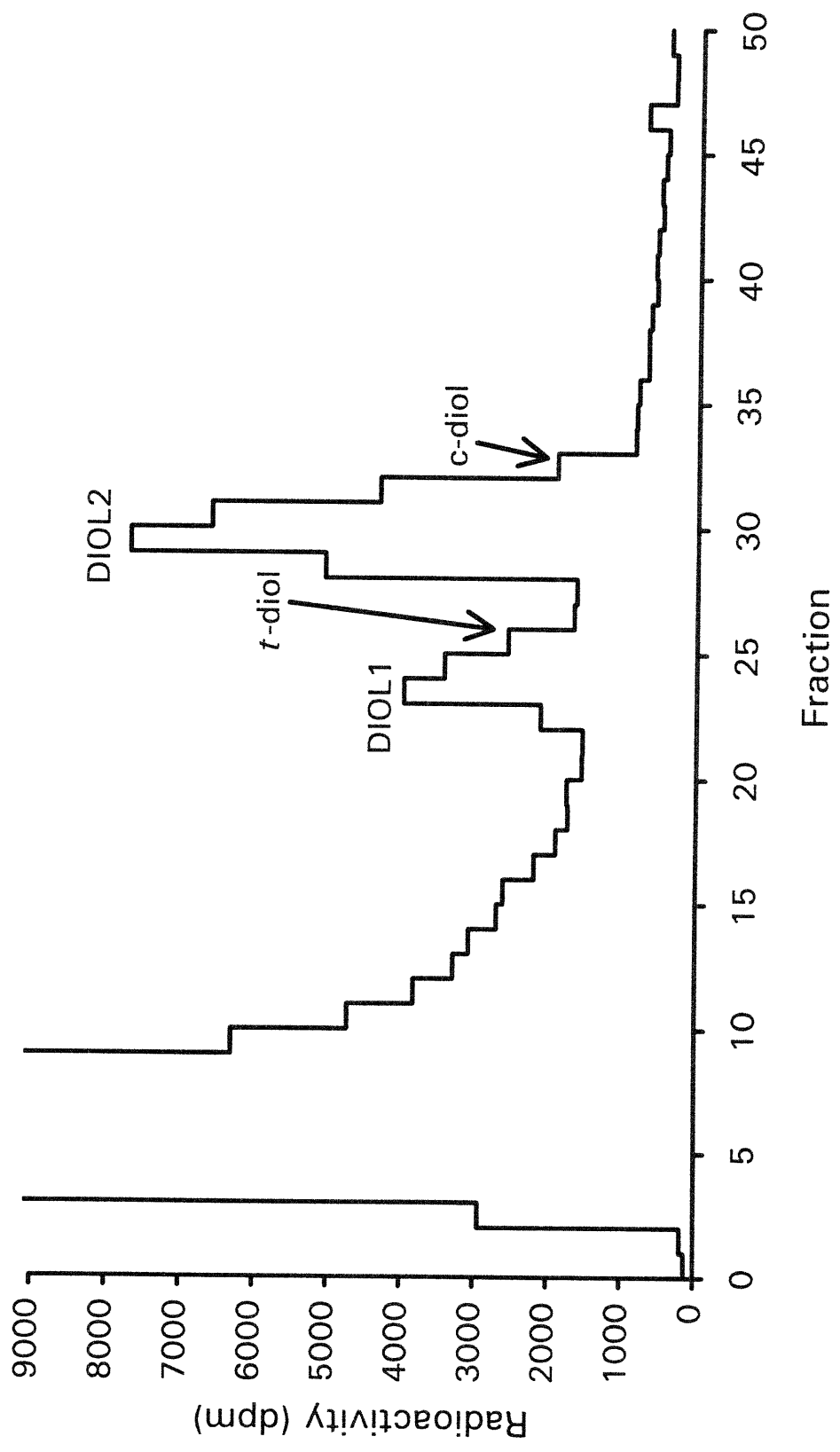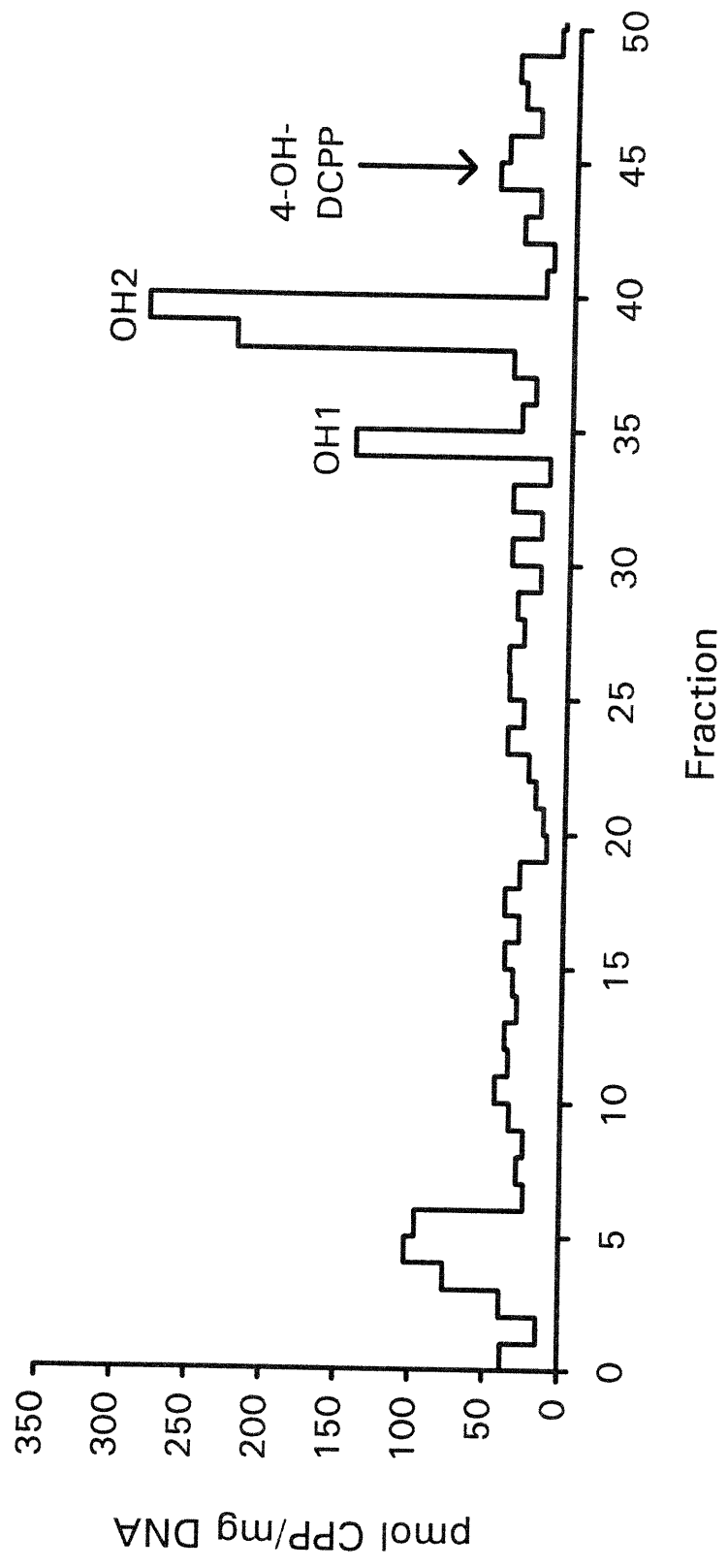4-OH-
DCPP

Fraction

pmol CPP/mg DNA

5. Characterization of cyclopenta[*cd*]pyrene-DNA adducts formed in vivo

5.1 Introduction

Using ^{32}P-postlabelling analysis, Nesnow *et al.* found that DNA adducts from CPP-treated A/J mouse lung tumor were CPPE-derived guanines (Nesnow et al., 1994). Beach and Gupta reported that in CPP-treated Sprague-Dawley rat lung, B6C3F1 mouse lung, and NIH Swiss mouse skin DNA, adducts were also formed by CPPE (Beach and Gupta, 1994). In previous section, *trans*-CPP-3,4-diol and 4-OH-DCPP were shown to reacted with DNA when activated by sulfotransferase and the same major CPP-dG adducts were yielded by either CPPE or *trans*-CPP-3,4-diol. Although adducts derived from 4-OH-DCPP were separated from CPPE-adducted dG adducts under the chromatographic conditions described here, there is no certainty that they would be distinguished in the in vivo studies (Nesnow et al., 1994; Beach and Gupta, 1994). In addition, the possibility of forming adducts by the diolepoxides of CPP deserves further investigation.

This present study was undertaken to identify DNA adducts formed in a mouse model. The identification of these adducts was conducted by comparing them to CPP metabolites and adduct standards using an HPLC-LIF system combined with a cryogenic fluorescence spectroscopy. Accordingly, adduct-forming metabolites of CPP were deduced. To our knowledge this is the first report to investigate the formation of PAH-DNA adducts in vivo by this kind of system without adduct hydrolysis.

5.2   Materials and Methods

Materials

Male CD-1 mice were obtained from Charles River Associates (Wilmington, MA) a week before treatment. CPP and [G-^{3}H]CPP were products of the Midwest Research Institute (Kansas City, MO) and the Chemsyn Science Laboratory (Lenexa, KS), respectively, and were obtained from the National Cancer Institute Chemical Repository.   CPPE   was   prepared   from   CPP   with   dimethyldioxirane   in dichloromethane (Kwon et al., 1992). CPP-3,4-diols were made from CPPE by acid hydrolysis and purified to separate the *trans* and *cis* isomers. 4-OH-DCPP was obtained by NaBH$_4$ reduction of 4-oxo-DCPP, which was prepared by rearrangement of CPPE in benzene. The enzymes for digestion of DNA were DNase I (from Bovine pancreas), snake venom phosphodiesterase (from *Crotalus durissus*), and acid phosphatase (from Potato). All these enzymes were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN), except DNase I which was purchased from Sigma Chemical Co. (St. Louis, MO). Rat liver cytosol was prepared from postmitochondrial supernatant fractions supplied from Molecular Toxicology, Inc. (Annapolis, MD). QIAGEN Genomic-tip 500/G were from QIAGEN Inc. (Chatsworth, CA). All other chemicals and solvents used were of reagent grade.

Adduct Preparation

In vivo adducts.   Male CD-1 mice weighing approximately 25-30 g were housed in suspended polycarbonate cages containing corncob bedding prior to CPP treatment. Animals were given one dose of [^{3}H]CPP (7 mg/kg body weight, specific activity: 588 dpm/pmol) in 100 ul corn oil intraperitoneally. Three days after the administration, animals were sacrificed and livers and lungs were removed. The tissues were stored at -70 °C for subsequent DNA isolation. Isolation of DNA

from the livers and lungs of CPP-treated mice that has been described elsewhere (Gorelick et al., 1989). Briefly, the tissues were thoroughly homogenized and membranes disrupted by the addition of detergent. After extensive shearing, the samples were digested with proteinase K and RNase A followed by organic extraction. Finally, DNA was precipitated using ethanol and then subjected to enzymatic digestion to deoxynucleoside adducts (Surh et al., 1989) prior to HPLC analysis.

In vitro adducts. Calf thymus DNA was treated with CPPE (20 nmol/mg DNA) in a 50 mM Tris-HCl buffer (pH 7.4) at 37 °C for 2 hr. CPP-3,4-diol and 4-OH-DCPP were incubated with calf thymus DNA (20 nmol/mg DNA) in the presence of rat liver cytosol and 3'-phosphoadenosine-5'-phophsulfate (PAPS) at 37 °C for 2 hr (Surh et al., 1993). Each reaction mixture was extracted twice with a half volume of water-saturated ethyl acetate to remove unbound CPP derivatives. Then, DNA was isolated by ethanol precipitation followed by enzymatic digestion to dexoynucleoside adducts (Surh et al., 1989) prior to HPLC analysis. Phenol/chloroform extraction was used to remove cytosolic proteins in the sulfotransferase-mediated activation reactions (Surh et al., 1993).

Chromatography and Detection System

Separations were carried out by HPLC (Hewlett Packard HP 1090, Palo Alto, CA) with a Nucleosil C18 column (4.6 mm x 250 mm, Alltech Assoc. Inc., Deerfield, IL) at a flow rate of 1 ml/min. The elution program used was: an isocratic phase of 50% methanol in water for 5 min, followed by a linear gradient to 80% methanol at 55 min, and then to 100% at 60 min. To resolve in vivo DNA digests, HPLC combined with a Laser Induced Fluorescence (LIF) detector was utilized. This involved irradiating the HPLC eluent in a fused silica capillary (100 um diameter) with a 30 mW CW Helium/Cadmium laser at 325 nm. The fluorescence emission was then focused with a 40X microscope objective on to the

entrance slit of a 0.22 m Spex Monochrometer. Two photomultiplier tubes (PMT) set up in a coincidence circuit were used to measure the fluorescence intensity. The pyrene fluorescence intensity was determined as the intensity at 400 nm (signal) minus the intensity at 450 nm (reference). This was then amplified using a Lock-in amplifier (SciTec Instruments, UK) and was monitored on an IBM compatible PC with a data acquisition board and appropriate software.

Cryogenic LIF spectroscopy

Cryogenic LIF and fluorescence line-narrowed emission spectra were recorded with a system that has been described elsewhere with minor modifications (Day et al., 1992). The main modification to the system was the replacement of the diode-array detector with a cooled intensified CCD-array detector (Princeton Instruments, Trenton NJ). Briefly, laser light was generated with a Nd-YAG laser whose second harmonic at 532 nm pumped a dye laser. The output of the dye laser was double by using a KDP crystal to generate laser wavelengths in the range of 300-400 nm. Typical pulse energies were in the range of 100 uJ to 1 mJ/pulse at 10 Hz and the pulse width was 5 ns. Fluorescence from the sample was collected by a 5 cm focal length lens and focused in a 0.32 m monochrometer using a 15 cm focal length lens. The fluorescence was detected with an intensified CCD-array detector on an IBM compatible PC. Excitation into the origin of the first excited state, S_1, results a line-narrowed spectrum and is known as fluorescence line narrowing (FLN). As one goes higher in the S_1 state or into the S_2 state, the line narrowing effects are reduced and spectra become broad due to the increased vibrational level density. In our experiments, excitation of pyrene-based compounds at 346 nm (S_2 excitation) resulted in an intense fluorescence peak centered at 378 nm. S_1 excitation was achieved by tuning the laser to 355 nm and resulted in a sharp double peak around 376 nm. To avoid amorphous crystal formation at cryogenic temperatures the DNA digests were dissolved in a glass matrix of 5 parts glycerol, 4 parts sample dissolved in water and 1 part ethanol. The sample (max.

30 ul) was then placed in a quartz capillary and sonicated for 15 minutes to remove dissolved gasses. The samples were then rapidly cooled in the cryostat to $4^{\circ}K$ with liquid helium. Collection of the fluorescence spectra was gated by 90 ns after the laser pulse to reduce laser scattering. The final fluorescence spectra were obtained by the cumulative addition of spectra over 20 minutes.

5.3   Results

CD-1 mice were used in this study because their susceptibility to CPP carcinogenesis (Wood et al., 1980; Cavalieri et al., 1981a). They were dosed with 7 mg CPP/kg in our experiments. This dose is slightly lower than those used in previous in vivo CPP-DNA adduct studies (10-50 mg CPP/kg) (Beach et al., 1991; Beach and Gupta, 1994; Nesnow et al., 1994). Our results indicated that CPP adduct levels in lung DNA were 1.5 times higher than in liver DNA (4.6 pmol/mg lung DNA vs. 3.0 pmol/mg liver DNA). Similar tissue preference was also observed in Sprague-Dawley rats 5 days after treatment with a single dose of 10 mg CPP/kg (2.6 pmol/mg lung DNA vs. 1.1 pmol/mg liver DNA) (Beach et al., 1991). In contrast, the level of DNA binding in liver tissue was at least 10 times greater than in lung tissues of CPP-treated Balb/c mice over a 15-day period (3 CPP mg/kg, max. 0.5 pmol/mg lung DNA vs. max. 8.3 pmol/mg liver DNA) (Kwon, 1992).

The HPLC radiochromatogram of digested tissue DNA has an elevated background in the region in which our synthesized in vitro CPPE-DNA adducts elute (not shown here). However no distinct fraction could be obtained from the radiochromatogram due to the low in vivo CPP binding levels. This indicated that no CPP diolepoxide-derived DNA adducts were formed in vivo. If DNA adducts were formed by CPP diolepoxides, it would be expected that these adducts would elute earlier in our reversed-phase HPLC system due to their greater hydrophilicity. However, even the injection of as much as 1 mg of digested liver DNA failed to show any radioactivity in the regions where more polar CPP diolepoxide-adducted nucleosides would be expected to elute. These results are consistent with in vivo CPP-DNA adduct studies carried out by ^{32}P-postlabelling assays (Beach and Gupta, 1994; Nesnow et al., 1994).

CPP-DNA adducts formed in CD-1 mice were examined using HPLC with LIF detection. In liver and lung DNA digests, four peaks (P1-P4 in Figure 5.1) and

six peaks (P1-P6 in Figure 5.2) were detected respectively (in contrast, no peaks were detected in animals dosed with corn oil). Fractions obtained from the DNA digests were collected and analyzed by cryogenic LIF Spectroscopy. cis-3-dG-4-OH-DCPP was used as a standard for the fluorescence spectroscopic analysis. The results demonstrated that all peaks were from pyrene-based compounds since they all exhibited pyrene-specific spectra. This is consistent with the conclusion that adducts were not formed by diolepoxides of CPP (Beach and Gupta, 1994; Nesnow et al., 1994). Figures 5.3 and 5.4 show S_2 and S_1 spectra of an adducted peak. Finally, all peaks were further characterized by their chromatographic similarity to synthetic CPP metabolites and adduct standards formed in vitro by reacting calf thymus with CPPE or with trans-CPP-3,4-diol and 4-OH-DCPP, upon the activation of sulfotransferase.

The sensitivity of this HPLC-LIF system was determined to be on the order of tenths of femtomoles by injecting different amounts of CPP-3,4-diols (Figure 5.5) and synthesized CPP-DNA adducts. This sensitivity could be further enhanced with appropriate optimization. In our experiments less than 30 ug of digested DNA proved to be sufficient for the detection of the major in vivo CPP-DNA adducts.

Because there are many factors which affect the retention properties of a compound, such as the room temperature, CPP-3,4-diols were used as internal standards and reference peaks when necessary. P5 and P6 of Figure 5.2 and P4 of Figure 5.1 were identified by chromatographic similarities to 4-OH-DCPP, 4-dG-DCPP, and CPP-dA adducts. CPP-3,4-diols were not used as internal standards in assisting the identification of these peaks since the fractions have very different chromatographic properties. CPP-dA adduct standards were obtained by reacting CPPE with [^{3}H]adenyl calf thymus DNA (Hsu et al., 1996b).

Our results suggest that P1 in liver DNA digests (Figure 5.1) is trans-3-dG-4-OH-DCPP since it has similar chromatographic characteristics to as the minor dG

adduct obtained from the reaction of CPPE with [^{3}H]guanyl calf thymus DNA (Hsu et al., 1996b); P2 and P3 are a *cis* diastereomeric pair of 3-dG-4-OH-DCPP since they co-migrate with the two major products prepared from CPPE reacting with dG (Hsu et al., 1996a); and P4 is *trans*-3-dA-4-OH-DCPP. Similarly, peaks found in lung samples (Figure 5.2) are *trans* 3-dG-4-OH-DCPP (P1), two diastereomers of *cis*-CPP-3-dG-4-OH-DCPP (P2 and P3), *cis*-CPP-3,4-diol (P4), *cis*-3-dA-4-OH-DCPP (P5), and 4-OH-DCPP (P6).

The relative amounts of material in each peak was estimated by integrating the peak areas. A standard curve was constructed using injections of standards with known concentrations. The concentration of adduct in each peak was then estimated using this standard curve. The fluorescence intensity of adducts was normalized to that of *trans*-CPP-3,4-diol since the fluorescence characteristics of different compounds is known to vary greatly. This data is presented in Table 5.1 which shows the relative amount of each adduct as a percentage of the total adducts for a given sample.

5.4   Discussion

This present investigation was undertaken to identify the CPP-DNA adducts formed using a CPP-carcinogenesis susceptible mouse model. Based on these results, major electrophilic metabolites of CPP were deduced. Previous studies using ^{32}P-postlabelling techniques suggest that the CPP-DNA adducts formed in vivo were derived from CPPE and they appeared to be dG-substituted (except in one study a minor adduct was found to be dA-substituted) (Beach and Gupta, 1994; Nesnow et al., 1994). Our results lead to the conclusion that all detected in vivo adducts are CPPE derivatives, however, trans-CPP-3,4-diol and 4-OH-DCPP, upon activation of sulfotransferase, may also be responsible for DNA-binding. Additionally, the detection of CPP metabolites suggests the formation of unstable adducts.

The possibility of formation of the 3,4-dihydrodiol 9,10-epoxide and the 9,10-dihydrodiol 3,4-epoxide of CPP was proposed by the study of oxidation of CPP using mouse and human microsomes and selected cytochrome P450 enzymes (Sahali et al., 1992; Kwon et al., 1992). They are, however, not the adduct-forming species in this study. In fact, the major hemoglobin adduct formed by a single dose of CPP in CD-1 mice was shown to be CPPE-substituted histidine. Also, a labile ester adduct of CPPE appeared when mice were dosed with CPP every three days for 40 days by the presence of CPP-3,4-diols (Kwon, 1992).

trans-CPP-3,4-diol, the principal metabolite of CPP (Gold and Eisenstadt, 1980; Eisenstadt et al., 1981; Sahali et al., 1992), may play a role in the DNA modification of CPP. DNA adducts found in CPP-treated Sprague-Dawley rat liver were concluded to be adducted by CPPE (Beach and Gupta, 1994). Our findings suggest that these adducts may also be formed by sulfotransferase-activated trans-CPP-3,4-diol, since same major DNA adducts were formed by either CPPE or trans-CPP-3,4-diol in the presence of Sprague-Dawley rat liver cytosol and PAPS (Hsu

et al., 1996b). Similarly, some adducts, such as P1, P2, and P3 in Figure 5.1, could be formed by either CPPE or sulfotransferase-activated *trans* CPP-3,4-diol or by both, since high sulfotransferase activity was found in hepatic cytosol of CD-1 mice for another benzylic hydroxyl metabolite of 3,4-dihydroCPP, 4-OH-DCPP (Surh et al., 1993).

P6 of Figure 5.2 exhibits similar retention property as 4-OH-DCPP. This result suggests that sulfotransferase-activated 4-OH-DCPP in CD-1 mice may also serve as an adduct-forming species of CPP. 4-OH-DCPP is a better substrate for sulfotransferases when compared to CPP-3,4-diols (Surh et al., 1993). In addition, a higher level of DNA binding was observed when 4-OH-DCPP was incubated with human liver post-mitochondrial supernatant in the presence than in the absence of PAPS (Surh and Tannenbaum, 1996). Thus, it would be worthwhile to further investigate the role of 4-OH-DCPP in CPP carcinogenesis.

LIF Spectroscopy provides the sensitivity and selectivity required by this study. The sensitivity of the HPLC-LIF system was determined to be on the order of tenths of femtomoles. This detection limit is comparable to that of radioactive postlabelling techniques and can be further improved with proper optimization. A linear correlation existed between the amount of *trans*-CPP-3,4-diol injected on the column and the integrated area of the HPLC-LIF peak over a range of 5 fmol to 1 pmol (Figure 5.5). This provides the means to estimate concentrations of adducts and metabolites. Notably, this system offers the capability of detecting CPP metabolites which was not possible in previous studies employing postlabelling techniques (Beach and Gupta, 1994; Nesnow et al., 1994). The detection of CPP-3,4-diol and 4-OH-DCPP suggest the formation of unstable CPP-DNA adducts, such as N7-dG or phosphate adducts. As a test of this inference, DNA isolated from the reaction of CPPE-treated [^{3}H]guanyl calf thymus DNA was heated and resulted in two modified fractions were detected on our HPLC-radiochromatogram. Further investigation of these fractions showed that they were chromatographically distinct

from the *trans-* and *cis-* dG adduct standards that were obtained from the enzymatic digestion of this reaction mixture (Hsu et al., 1996c). These findings might be due to the depurination of N7-dG adducts upon heating or acid-catalysis (Humphreys and Gurngerich, 1991). QAIGEN Genomic-tip was employed to ensure that CPP metabolites were not caused by cleavage of stable CPP-DNA adducts during the DNA isolation.

Cryogenic fluorescence spectroscopy (4° K) eliminates the problems of spectral broadening and quenching seen in room temperature fluorescence. Additionally, fluorescence line narrowing (FLN) spectroscopy can provide detailed structural information. Line-narrowed spectra are obtained by exciting a compound into the origin of the first excited state,S_1. However, in FLN spectroscopy only a small population of molecules are actually excited making this a rather insensitive spectroscopic method. S_2 excitation, on the other hand, provides less structural information but is extremely sensitive. The laser was tuned to 355 nm and 346 nm for S_1 and S_2 excitation of pyrene-based compounds. Excitation of pyrene-based compounds at 346 nm (S_2 excitation) results in an intense fluorescence emission peak at 378 nm, whereas excitation at 355 nm (S_1 excitation) results in two sharp peaks around 376 nm. A survey of similar non-pyrene containing PAHs such as benzo[*a*]anthracene, chrysene, and phenanthrene showed these fluorescence characteristics to be unique to pyrene containing compounds. An intense fluorescence peak at 378 nm was observed in all fractions assayed upon S_2 excitation. Subsequent analysis of selected fractions by FLN spectroscopy further confirmed the presence of a pyrene moiety.

Our results here illustrate the utility of the combination HPLC-LIF and cryogenic fluorescence spectroscopy in characterizing carcinogen-DNA adducts. This combination analysis also allows sensitive and specific investigation of metabolites and protein adducts derived from fluorescent xenobiotics. However, a quantitative measurement may rely on the availability of various of standards due

to the differences in fluorescence characteristics.

In conclusion, our findings confirm that CPPE is the major DNA adduct-forming metabolite of CPP. Additionally, we have (1) completed the structural assignments of in vivo DNA adducts with the use of adduct standards and sensitive detection system, (2) suggested the formation of unstable adducts due to the detection of CPP metabolites, and (3) implicated sulfotransferase-activated 4-OH-DCPP and trans-CPP-3,4-diol as additional adduct-forming species of CPP. The last conclusion may also apply to previous studies (Beach and Gupta, 1994; Nesnow et al., 1994). Moreover, this study represents the first adduct investigation of in vivo PAH adduct formation with using LIF analysis without adduct hydrolysis. It also promises the possibility of using the utility of the system to monitor carcinogen exposure in humans.

5.5   Summary

Cyclopenta[*cd*]pyrene (CPP) is a ubiquitous environmental pollutant. This present study was undertaken to investigate the formation of the CPP-DNA adducts using a mouse model and to determine the ultimate carcinogenic metabolite(s) of CPP. Male CD-1 mice were sacrificed 3 days postdosing i.p. with 7 mg/kg CPP, and DNA adduct levels in lung and liver tissues were measured. Adduct levels were found to be approximately 1.5 times higher in liver samples compared to lung tissue (4.6 pmol/mg lung DNA vs. 3.0 pmol/mg liver DNA). Deoxynucleoside adducts obtained by enzymatic digestion of DNA were analyzed by HPLC coupled with Laser Induced Fluorescence (LIF) detection. HPLC separation of the DNA digests resulted in four and six peaks of liver and lung samples, respectively. Further analysis indicated that all peaks were derived from CPP 3,4-epoxide (CPPE) since they all exhibited pyrene-specific spectra when analyzed by a cryogenic LIF Spectroscopy. Identification of these peaks was also carried out by comparing their chromatographic properties to synthetic CPP metabolites and adduct standards formed in vitro. These results suggest that adducts detected in liver DNA digests are *trans*-3-deoxyguanosinyl-4-hydroxy-3,4-dihydroCPP (3-dG-4-OH-DCPP), two diastereomers of *cis*-3-dG-4-OH-DCPP, and *trans*-3-deoxyadenosinyl-4-hydroxy-3,4-dihydroCPP (3-dA-4-OH-DCPP). Similarly, results suggest that adducts detected in lung samples are *trans*-3-dG-4-OH-DCPP, two diastereomers of *cis*-3-dG-4-OH-DCPP, *cis*-CPP-3,4-diol, *cis*-3-dA-4-OH-DCPP, and 4-OH-DCPP. The detection of CPP-3,4-diol and 4-OH-DCPP suggests the formation of unstable DNA adducts of CPP. Furthermore, both CPPE and sulfotransferase-activated *trans*-CPP-3,4-diol could be the major reactive ultimate electrophilic and carcinogenic metabolites of CPP, while sulfotransferase-activated 4-OH-DCPP could be a minor one.
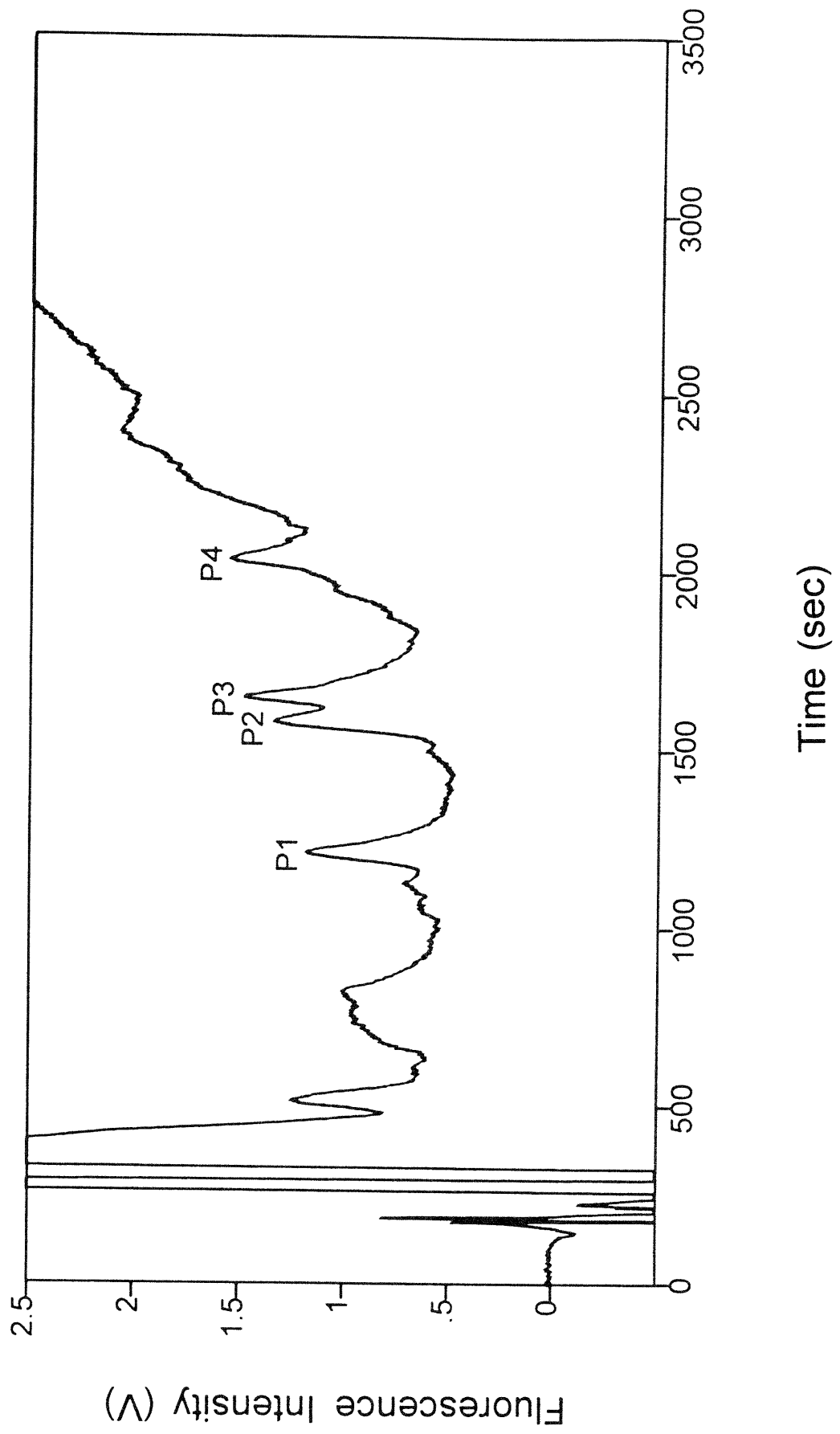
Figure Legends

Figure 5.1   Chromatographic profile of liver DNA from CPP-treated mice three days after one dose. One hundred ug liver DNA from a CPP-treated mouse was enzymatically digested and was analyzed by HPLC-LIF system as described in Materials and Methods.   P1 is *trans*-3-dG-4-OH-DCPP, P2 and P3 are a *cis* diastereomeric pair of 3-dG-4-OH-DCPP, and P4 is *trans*-3-dA-4-OH-DCPP, tentatively.
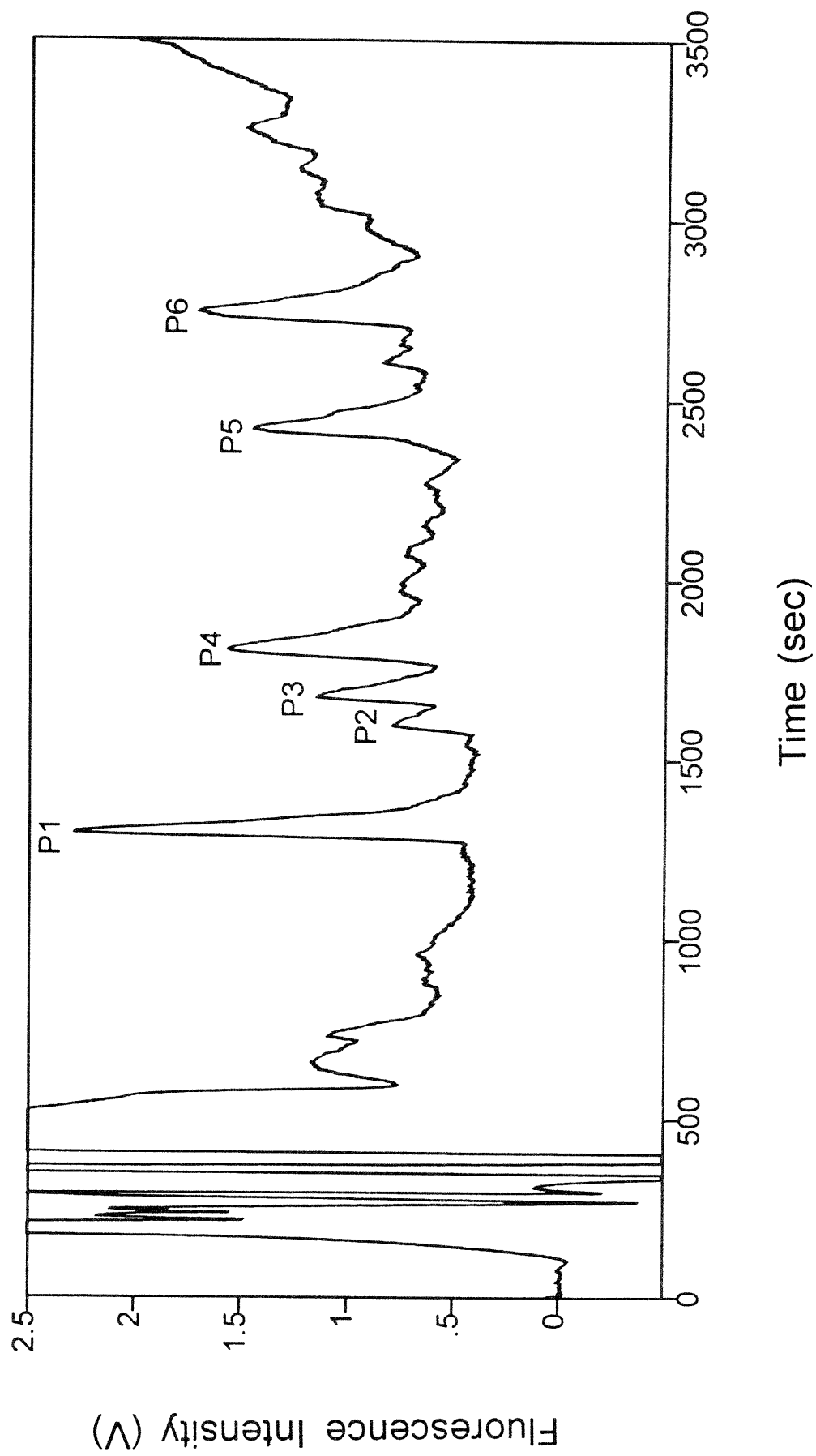
Figure 5.2   Chromatographic profile of lung DNA from CPP-treated mice three days after one dose. Fifty ug lung DNA from a CPP-treated mouse was enzymatically digested and was analyzed by HPLC-LIF system as described in Materials and Methods.   P1 is *trans*-3-dG-4-OH-DCPP, P2 and P3 two diastereomers of *cis*-CPP-3-dG-4-OH-DCPP, P4 is *cis*-CPP-3,4-diol, P5 is *cis*-3-dA-4-OH-DCPP, P6 is 4-OH-DCPP, tentatively.

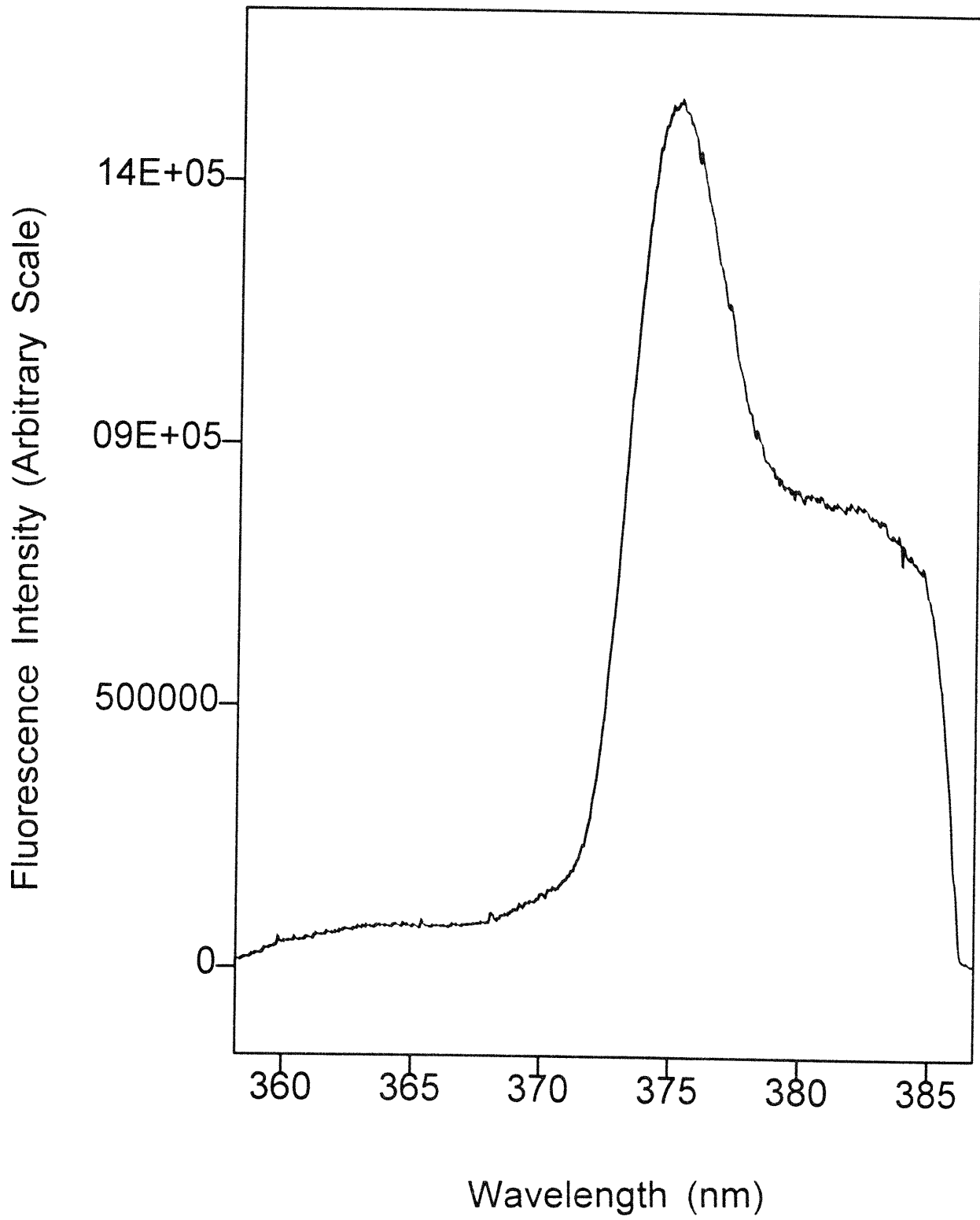Figure 5.3 S_2 spectra (excitation at 346 nm, T=4 K) of P1 of liver DNA.

Figure 5.4 S_1 spectra (excitation at 355 nm, T=4 K) of P1 of liver DNA.

Figure 5.5   Correlation between the amount of *trans*-CPP-3,4-diol injected on the column and the integrated area of the HPLC-LIF peak over a range of 5 fmol and 1 pmol.

$y = 4.92 + 0.44x$ $R^2 = 0.97$

Peak Area (Arbitrary Scale)

t-CPP-3,4-diol conc. (femtomoles)

Table 5.1 Realtive Percentages of Products
in Liver and Lung Smaples

Peaks	Liver percentage of total	Peaks	Lung Percentage of total
P1	32	P1	51
P2	21	P2	4
P3	32	P3	14
P4	15	P4	10
		P5	13
		P6	8

6.   Proposals for future research

It is recommended that the following research be undertaken to further develop and extend the research and results presented above:
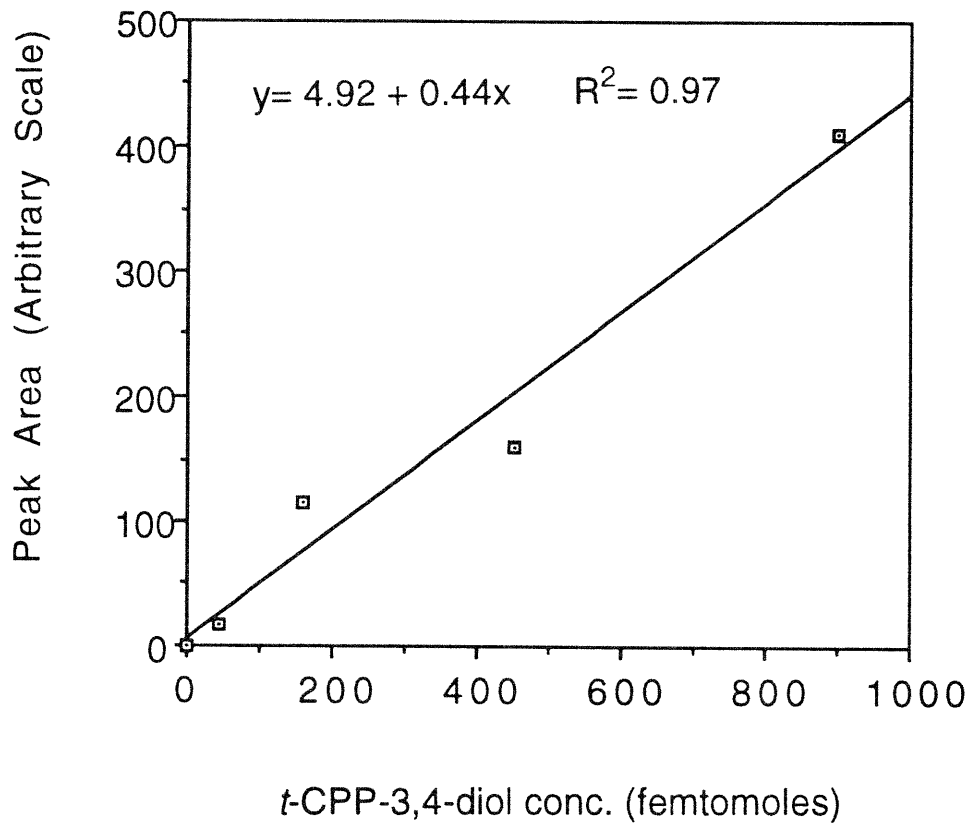
1) Structural elucidation of the unstable CPP-DNA products

Characterization of the primary in vitro adduct, CPP-N2-dG, enables the development of appropriate analytical methods for quantifying in vivo CPP-DNA adducts. There are some products derived from CPP-dosed tissue DNA that seem likely to be dihydrodiols. Therefore, structural elucidation of unstable derivatives will provide further insights into the processes which activate and detoxify CPP and those that repair the damage caused by in vivo covalent DNA lesions. Additionally, estimate of these adduct levels may aid the evaluation of CPP genotoxicity.

2) Comparative studies on rates of formation and removal of CPP lesions in DNA of target and non-target tissues

The organ specificity of CPP carcinogenesis in the mice may be exploited to gain insight as to which, if any, DNA lesion caused by CPP is a causative factor in carcinogenesis. The time-dependence of adduct formation and removal is important to study in order to characterize the possible use of CPP-DNA adducts as monitors of PAH exposure. To approximate chronic human exposure, a study of the formation and persistence of CPP-DNA adducts as a function of the dosing regimen is also necessary.

3) In vitro physical binding experiments

The possible role of intercalative binding and adduct formation in mutagenesis and carcinogenesis initiated by PAH diol epoxides has been studied and discussed (Geacintov, 1986; Harvey et al., 1985). In a cellular environment, the ability of a given PAH diol epoxide derivative to form intercalative non-covalent complexes with DNA prior to covalent binding is considered to be an important factor. Therefore, intercalative binding is an important factor in determining the

biological activities of PAHs.

4) <u>Laser spectroscopic studies of CPP-DNA structure types</u>

Considerable progress on the elucidation of structurally different types of adducts has been made using spectroscopic techniques including fluorescence quenching. The BPDE-DNA adducts have been classified as type I and II. Type I refers sites in which the aromatic plane of pyrene appears to be nearly parallel (with 25-30^0) to the planes of the unmodified DNA bases. Such adducts may be viewed as interior (i.e., quasi-intercalated). Type II sites are characterized by a lower degree of intercalation between the pyrenyl moiety and the bases and, therefore, may be viewed as exterior. The result obtained here may have the potential for distinguishing between CPP adduct formation in DNA and for monitoring the fates of CPP adducts as a function of time in repair-competent cells. It has been speculated that type I adducts are more efficiently repaired.

5) <u>Attempt the application of the methodology described in this report to samples obtained from humans</u>

The profile of CPP-DNA adduct formed in vitro as well as in mice and the availability of a sensitive and reproducible method for adduct detection and quantification should permit analysis DNA from exposed humans.

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