To the Graduate Council:

I am submitting herewith a thesis written by Gregory N. Tjossem entitled "The In Vivo Interaction of Activated Benzo(a)pyrene with DNA Phosphates." I have examined the final copy of this thesis for form and content and recomend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

Lee R. Shugart

Lee R. Shugart, Major Professor

We have read this thesis
and recommend its acceptance:

Robert B. Cumming

Accepted for the Council:

The Graduate School

THE IN VIVO INTERACTION OF ACTIVATED BENZO(a)PYRENE WITH DNA PHOSPHATES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Gregory N. Tjossem

December 1983

ACKNOWLEDGMENTS

I am extremely grateful for the guidance and never ending help of my thesis advisor, Dr. Lee Shugart. I also would like to give a very special thanks to Jerry Hall, Barbara Chastain, and John Kao. Their presence in the lab made my experience there a most rewarding and enjoyable one. I am indebted to Bob Cumming for his willingness to serve on my committee and to the Biology Division of the Oak Ridge National Laboratory for allowing me to conduct my studies there. A final thanks to Helen, whose moral support and friendship was always there.

ABSTRACT

The ultimate carcinogenic metabolite of benzo(a)-pyrene (BP) is believed to be the anti isomer of the 7,8-diol-9,10-epoxide (BPDE I). BPDE I binds primarily to the guanine base in DNA, although several other adducts have also been reported. The binding of the diol epoxide to the phosphodiester linkage in DNA is believed to lead to DNA strand scission. In vitro studies, however, show little or no binding at this site.

In the present study the in vivo binding of the diol epoxides of BP to the phosphates in DNA were measured using the high sensitivity of fluorescence. BPDE-DNA was isolated from the dorsal skins of mice each exposed to a single treatment of BP. The BPDE-phosphate adducts were then chemically or enzymatically hydrolyzed to selectively release the diol epoxide from the phosphate in the from of highly fluorescent tetrols. The tetrols were separated by HPLC and quantitated by fluorescence.

The chemical and enzymatic methods employed were unable to yield any detectable amounts of tetrol release from the BPDE-phosphate adducts. The proportion of BPDE modifications at the phosphodiester site are, therefore, below the lower limits of detection (2 %). The possibility of other BP metabolites binding to the phosphate backbone have not been ruled out.

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LIST OF ABBREVIATIONS

BP	benzo(a)pyrene
BPDE	anti and syn isomers of benzo(a)pyrene 7,8-diol-9,10-epoxide
BPDE I	anti isomer of benzo(a)pyrene 7,8-diol-9,10-epoxide
BPDE II	syn isomer of benzo(a)pyrene 7,8-diol-9,10-epoxide
CIP	chloroform/isoamyl alcohol/phenol
HPLC	high pressure liquid chromatography

I. INTRODUCTION

Polycyclic aromatic hydrocarbons, such as benzo(a)-pyrene (BP), are widespread contaminants of the air, water, and soil. They are formed by the free-radical conjugation of alkenes during the incomplete combustion of fossil fuels (Selkirk, 1980). BP, therefore, is a major exhaust component of transportation, industrial energy sources, and refuse burning. It is becoming widely accepted that the covalent binding of carcinogens to cellular macromolecules, particularly nucleic acids, is the initial critical in the process of carcinogenesis (Miller and Miller, 1974; Weinstein et al., 1978). Since BP does not possess a reactive group, it must be metabolically activated before it can react with nucleic acids (Phillips and Sims, 1979). The metabolism of BP and the interaction of its metabolites with nucleic acids are the subjects of several extensive reviews (Phillips and Sims, 1979; Gelboin, 1980).

BP is metabolized by the microsomal mixed-function oxidase, aryl-hydrocarbon hydroxylase, which is found predominantly in the liver. Sims et al. (1974) showed that the 7,8-diol-9,10-epoxide is the chemically reactive intermediate which binds to DNA. There are two stereoisomeric diol epoxides formed and will be referred to as BPDE I and BPDE II. Although BPDE I and BPDE II will both covalently bind to DNA, metabolic and binding studies indicate that BPDE I is the major diol epoxide which interacts with DNA

and other cellular macromolecules (Koreeda et al., 1976; Koreeda et al., 1978). The guanine base is the primary target in both DNA and RNA for the diol epoxide. The adduct formed is derived by the trans addition of the N-2 amino group of guanine to the C-10 position of the diol epoxide (Gelboin, 1980). In addition, the diol epoxides of BP have been found to also covalently bind to a lesser extent to the adenine and cytosine bases in nucleic acids (Straub et al., 1977).

Gamper et al. (1978) and Koreeda et al. (1978) have reported a modification of the phosphate backbone in nucleic acids by the BP diol epoxides. The alkylation of the phosphodiester linkage in DNA by various other carcinogenic and mutagenic agents has been quantitated by several investigators (Sun and Singer, 1975; Beranek et al., 1980; Bannon and Verly, 1972). The extent of binding at the phosphodiester site varies greatly depending on the nature of the alkylating agent used. This is shown in Appendix A. Shooter (1978) has reported that there is, as yet, no record of a compound that reacts with the phosphates of nucleic which is not a carcinogen.

In general, DNA phosphotriesters are stable species resulting from the absence of the 2'-hydroxyl group in the pentose sugar (Bannon and Verly, 1972). Recent studies have also suggested that phosphotriesters induced in vivo are not eliminated by cellular DNA-repair systems (Shooter,

1978). The persistence of the phosphotriester adduct, therefore, might be used as a measure of the extent of DNA damage and human exposure to carcinogens. However, the presence of a β-hydroxyl group on the C-9 position of the BP diol epoxides could lead to triester hydrolysis and DNA strand scission (Walles and Ehrenberg, 1968). Gamper et al. (1977) observed a rapid nicking of plasmid DNA by BP diol epoxides in vitro. The kinetics of the nicking supported a phosphotriester mechanism and was not consistent with depurination or depyrimidination strand scission. This led them to postulate a mechanism of DNA strand scission by BP diol epoxides in which each triester hydrolysis gives a nick. The mechanism is as follows:

1. the triester forms between the C-10 position of the hydrocarbon and the DNA phosphate;
2. the C-9 hydroxyl group then displaces one of the sugars breaking the DNA backbone and forming a cyclic triester;
3. the tertiary cyclic phosphate hydrolyzes rapidly to relieve ring strain with the hydrocarbon remaining attached to the phosphate.

The biological implications of the covalent binding of BP diol epoxides to the phosphate backbone in DNA and the resulting strand scission is still unknown. The low frequency of BP-phosphotriester formation in vitro (Gamper et al., 1977; Shooter et al., 1977) does not rule out the

existence of triesters *in vivo*, where they may play a role in the carcinogenic activity of diol epoxides. The study presented here will examine both the *in vivo* and *in vitro* binding of the BP diol epoxides to the phosphodiester linkage in DNA.

The potential worker and general population exposure to polycyclic aromatic hydrocarbons has generated an interest among researchers to quantitate the interaction of these organic compounds with target cells and to obtain estimates of human exposure risks. Since the BP found in the environment is not labelled, it is not possible to use standard radiometric methods of detection. Rahn et al. (1982) have devised a nonradiometric method for quantitating low levels of BP metabolites that are covalently bound to the DNA of BP-treated mice. This procedure consists of acid hydrolyzing DNA to remove the BPDE-adducts in the form of the isomeric tetrols of BP. The tetrols are then quantitated, after HPLC separation, by fluorescence detection. This method is extremely sensitive, allowing femtomole amounts of covalently bound diol epoxide to be measured. In the present study, this approach with some modifications is utilized. Chemical and enzymatic methods are used to selectively release tetrols from the BPDE-phosphate adducts in DNA. The tetrols are then detected and quantitated by fluorometry.

II. METHODS

Sample Preparation

In Vivo. Six male and female C3H mice (9 weeks) were used. To the shaved dorsal skin of the mouse, 200 µg of BP in 100 µl of acetone was topically applied. After 24 hours, the dorsal skin was removed and cut into several pieces.

In Vitro. The method of Rahn et al. (1980) was used to prepare calf thymus BPDE-DNA. Calf thymus DNA in phosphate buffer (0.01 M, pH 7) was treated with BPDE I for 1 hour at 37° C. The sample was then phenol extracted three times and dialyzed three times against phosphate buffer. To further ensure that all noncovalently associated BP derivatives had been removed from the DNA, the sample was extracted two times with CIP, two times with ethyl ether, and one time with ethyl acetate (see below).

Isolation of DNA from Mouse Skin

The DNA isolation procedure used was that of Shugart et al. (1983) with some modifications. Mouse skin was placed in a 15 ml glass scintillation vial containing 10 mg pronase in 5 ml DNA extraction buffer (20 mM Tris-HCl pH 7.8, 20 mM NaCl, 1 mM EDTA, and 0.1 % SDS w/v) and incubated at 50°C for 4 hours with continuous shaking.

To the sample 3 ml of CIP (chloroform:isoamyl alcohol: phenol- 24:1:25- v/v) were added. The contents of the

sample vial were then transferred to a 15 ml screw-cap glass centrifuge tube with 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, and mixed continuously for 30 minutes at room temperature. The two phases were separated by centrifugation for 10 minutes at 1000 rpm (Beckman model TJ-6: 4°C). The aqueous phase was removed and re-extracted with an additional 3 ml of CIP (as above). The re-extracted aqueous phase was saved while the two CIP phases were combined and extracted with 1 ml of 0.1 M Tris-HCl buffer, pH 8.0. The CIP phase was then discarded and the aqueous phases combined. The aqueous phase was further extracted with 3 ml anhydrous ethyl ether, followed by 3 ml water saturated ethyl acetate, and finally with an additional 3 ml anhydrous ethyl ether. The two phases were separated each time by centrifugation (see above) and the organic phase discarded.

Any contaminating RNA was removed by adding 100 µg of RNase per ml of aqueous phase and incubating in a 37°C water bath overnight. The DNA was then precipitated by adding 177 µl of 0.1 M spermine tetrahydrochloride per ml of aqueous phase, mixing well, and placing in a 4°C refrigerator overnight.

The BPDE-DNA samples were transferred to polypropylene centrifuge tubes and washed two times, each time with 5 ml of 75 % ethanol in 0.3 M sodium acetate and 10 mM magnesium acetate. The DNA was recovered after each wash

by centrifugation (Beckman model J2-21: 10,000 rpm, 4°C, for 60 minutes). After the second ethanol wash the DNA pellet was resuspended in 5 mM sodium phosphate, pH 7.1, in 50 mM NaCl.

DNA Determination

The method of Vytasek (1982) was used to fluorometrically determine the DNA content by reacting m-diaminobenzoic acid dihydrochloride (DABA) with deoxyribose liberated by perchloric acid (PCA). The DABA reagent was prepared by mixing three volumes of 10 mM Na_2CO_3 in 1 M NaOH and one volume of 20 % (w/v) DABA. The reagent was allowed to incubate at room temperature for atleast 1 hour before use. In a capped glass vial, 100 µl of sample (0.5-2 µg DNA in water) were added to 100 µl of 1 M PCA and heated at 80°C for 1 hour. The fluorescence yielding reaction was then initiated by mixing 200 µl of the DABA reagent with the sample in 0.5 M PCA for 90 minutes at 37°C. The mixture was diluted with 1250 µl 1 M HCl and the fluorescence measured on a Turner model 430 Spectro-Fluorometer. The fluorometer excitation monochrometer was set at 407 nm and the emission monochrometer at 520 nm. A calf thymus DNA solution of known DNA concentration was used as the standard.

Acid Hydrolysis of DNA

To each ml of DNA sample (taken to volume with water) in a capped glass vial, 10 µl of 12 N HCl were added. The

mixture was placed in a dry air oven at 80°C for 6 hours. These conditions have been previously shown by Rahn et al. (1982) to maximize the yield of tetrol from BPDE-DNA adducts and provide the optimal fluorescence signal. The acid hydrolyzed DNA was then passed thru a C_{18} Sep-Pak cartridge (see below).

Alkaline Hydrolysis of DNA

To a BPDE-DNA sample, NH_4OH was added to a final concentration of 1 M. This solution was heated at 80°C for 4 hours in a capped glass vial. The vial contents were then evaporated to dryness and stored at 4°C until C_{18} extraction .

Alkaline hydrolyzed DNA that was to be further treated with alkaline phosphatase was neutralized with 1 N HCl prior to the addition of the enzyme.

Enzyme Hydrolysis of DNA

BPDE-DNA samples were enzymatically hydrolyzed in sodium borate buffer, pH 8.0, and 1 mM $MgCl_2$ at 37°C for 18 hours, using snake venom phosphodiesterase and deoxyribonuclease I. A portion of that sample was further hydrolyzed with alkaline phosphatase at 37°C for 6 hours. All enzymatically hydrolyzed DNA was passed through a C_{18} Sep-Pak cartridge prior to HPLC.

C_{18} Extraction of Tetrols

Shugart et al. (1983) have shown that C_{18} extraction of tetrols after treatment of DNA gives a more stable HPLC profile, with a reduction in nontetrol fluorescent material. DNA samples were diluted with 2 ml of 20 % methanol in water (v/v) and passed through a C_{18} Sep-Pak cartridge (Waters Assoc. Inc., USA). The cartridge was washed three times with 2 ml of 20 % methanol and once with 2 ml of water. The tetrols were recovered in 2 ml of 100 % methanol and taken to dryness. Recovery of tetrols from Sep-Pak treatment is close to 100 % (Rahn et al., 1982).

HPLC

The method of Shugart et al. (1983) was used. Samples (200 µl) in 50 % methanol were injected into a Dupont 850 liquid chromatographic system equipped with a Zorbax ODS column (250 x 4 mm). Elution of the sample was done isocratically with 50 % methanol in water (v/v), using a flow rate of 1 ml/minute and a column temperature at 50°C. Prior to elution oxygen was removed from the 50 % methanol solution by bubbling helium through it. The fluorescence of the elutant was monitored using a Schoeffel FS-970 fluorometer equipped with a deuterium lamp as an excitation source. The fluorometer excitation monochrometer was set at 246 nm and a 370 nm emission filter was used.

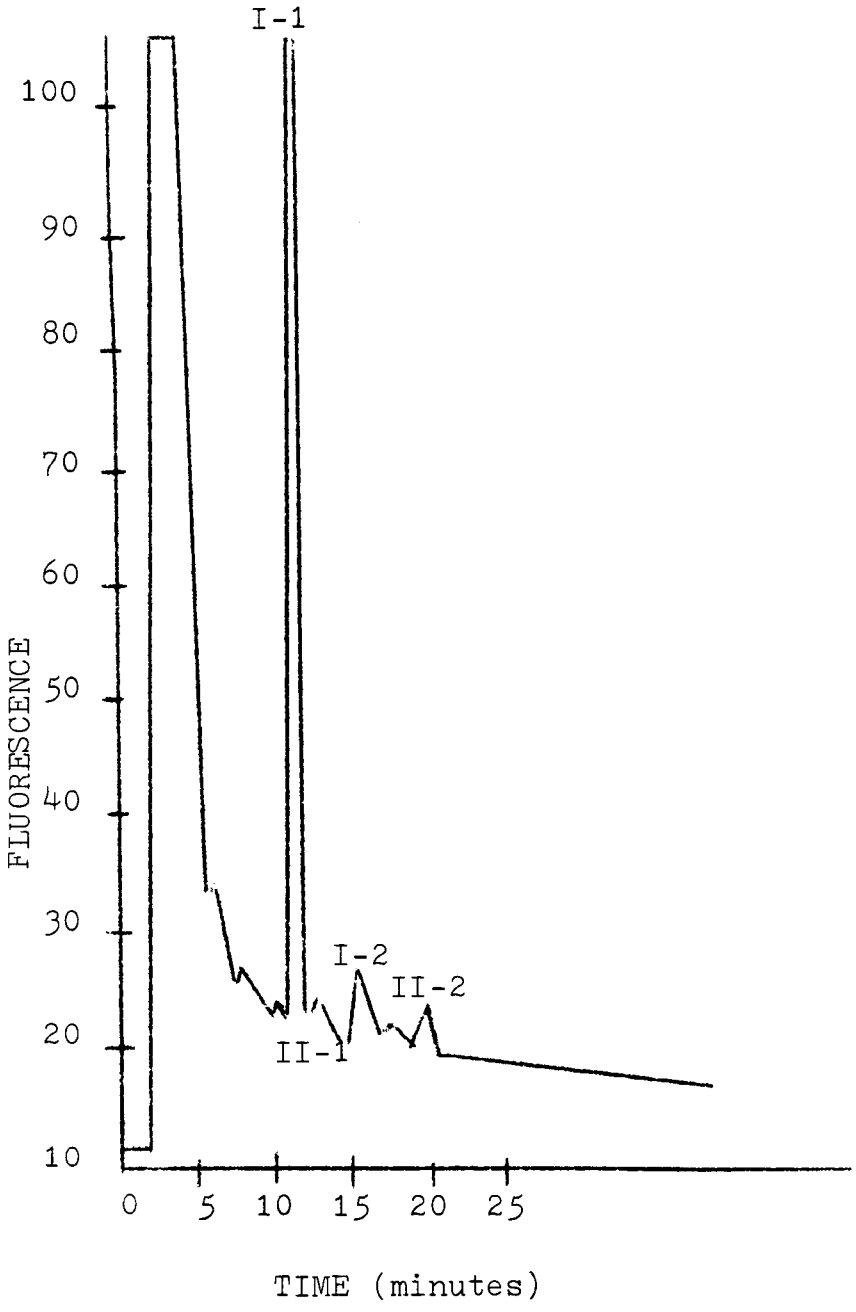
III. RESULTS

Shugart et al. (1983) have previously described a procedure for the isolation and purification of DNA from BP-treated mouse skin. They have shown that the partioning of nucleic acids with CIP will remove non-covalently bound BP and BP metabolites. Furthermore, it has been demonstrated that adduct formation via the diol epoxide can be quantitated by HPLC and the acid induced removal of highly fluorescent pyrenyl moieties in the form of tetrols (Rahn et al., 1982).

Figure 1 shows the fluorescent profile of acid hydrolyzed DNA from the dorsal skins of mice that were each exposed to 200 µg of BP for 24 hours. It can be calculated that for the 97 µg of DNA hydrolyzed, 534 pg of BPDE I (tetrol peak I-1) were covalently bound to the DNA. The presence of acid released tetrol demonstrates that adduct formation occurred via the metabolic activation of BP to the diol epoxide. It should be noted that BPDE I is the major diol epoxide moiety binding to DNA and accounts for approximately 75 % of the covalent interaction with DNA via the diol epoxides (Shugart et al., 1983).

Figure 2 is the fluorescent profile of non-hydrolyzed DNA obtained from the dorsal skins of mice each exposed to 200 ug of BP for 24 hours. As anticipated no significant fluorescent peak, indicating the presence of tetrol, is

Figure 1. Acid hydrolysis of *in vivo* BPDE-DNA. DNA was isolated from the dorsal skin of mice exposed to a single BP treatment (200 µg for 24 h.). The tetrols were liberated by acid hydrolysis (0.12 N HCl at 80° C for 6 hours) and quantitated by HPLC and fluorescent analysis. Peaks I-1 and I-2 represent the tetrols released from BPDE I adducts, while peaks II-1 and II-2 represent tetrols generated from BPDE II adducts.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TIME (minutes)

Figure 1

Figure 2. Non-hydrolyzed *in vivo* BPDE-DNA. DNA was isolated from the dorsal skin of mice each exposed to a single treatment of BP (200 μg for 24 hours). The tetrols were analyzed by HPLC and fluorescent analysis.

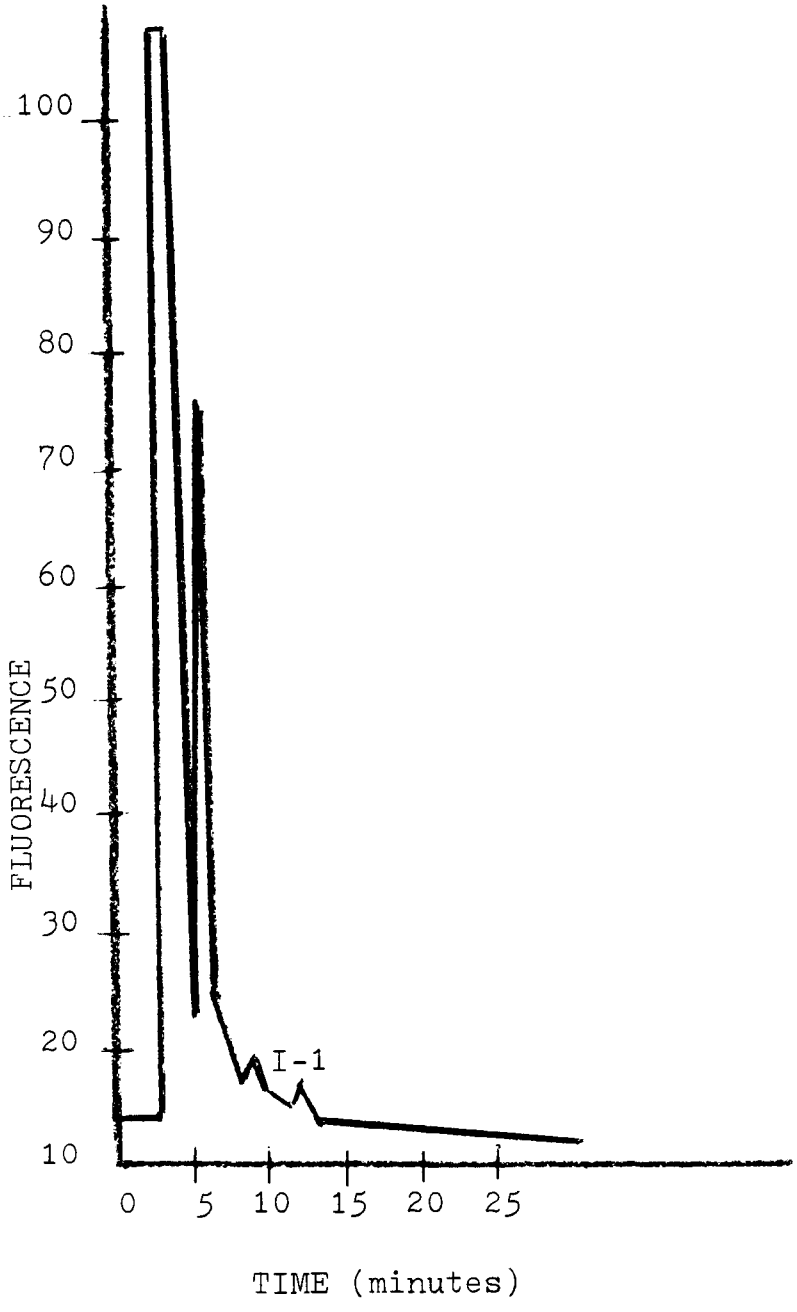
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Figure 2

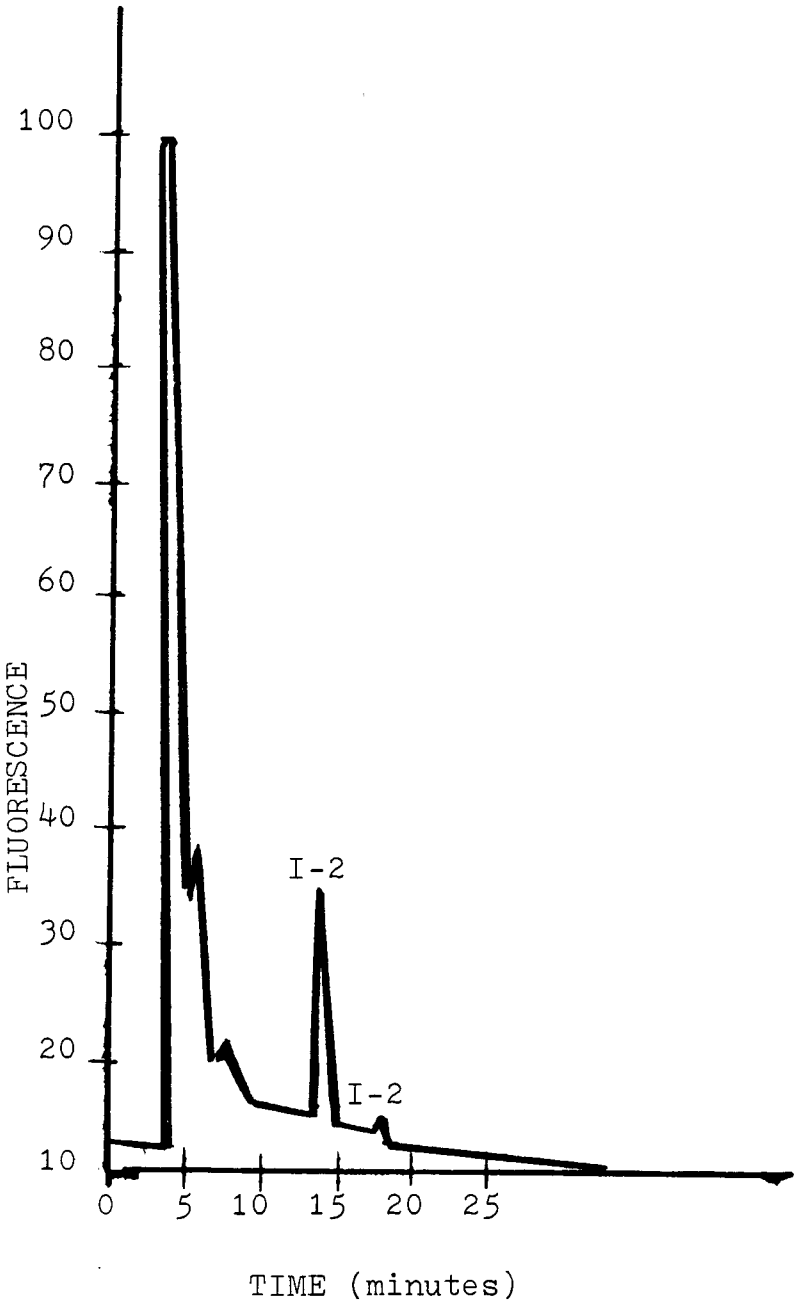
observed in the absence of acid hydrolysis. This demonstrates that the DNA isolation procedure will adequately remove non-covalently bound BP metabolites.

Figure 3 is the fluorescent profile of acid hydrolyzed calf thymus DNA previously treated in vitro with BPDE I. From the peak heights it can be calculated that 5,560 ng of BPDE I became covalently bound per gram of DNA isolated. In the absence of acid hydrolysis, there is again no significant amount of fluorescent tetrol present. The profile is not presented here but is similar to Figure 2.

In the quantitation of the amount of tetrol represented by the peak heights, a mixture of known amounts of tetrols I-1 and II-2 were applied to the HPLC column. The peak heights shown in Appendix B were used as a means of calibration. Since many factors (i.e. temperature, flow rate, and column) can influence the retention times of tetrols, tetrol standards were chromatographed at the beginning of each series of HPLC runs.

The covalent binding of the diol epoxide to the phosphodiester linkage in DNA was examined by attempting to selectively remove the diol epoxide by alkaline hydrolysis. Figure 4 shows the fluorescent profile obtained by alkaline hydrolysis and HPLC for DNA isolated from mouse skins each exposed to 200 μg of BP for 24 hours. There is no increase in the fluorescence of tetrol peak I-1 or any of the other possible tetrol products, when compared to the control

Figure 3. Acid hydrolysis of *in vitro* BPDE-DNA. Calf thymus DNA was treated with BPDE I for 1 hour at 37°C. BPDE I adducts were released by acid hydrolysis (0.12 N HCl at 80°C for 6 hours) and quantitated by HPLC and fluorescent analysis. Peaks I-1 and I-2 represent the isomeric tetrols released from BPDE I-DNA adducts upon acid hydrolysis.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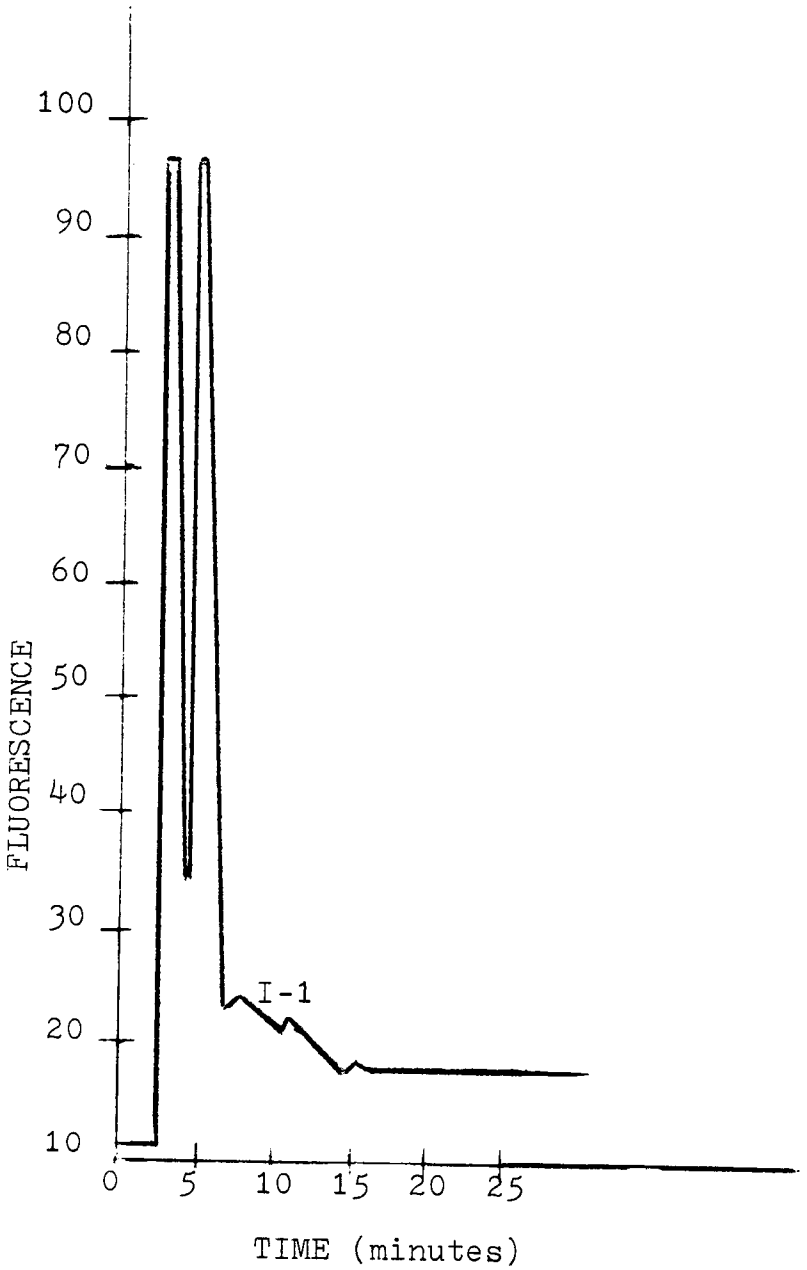
TIME (minutes)

Figure 3

Figure 4. Alkaline hydrolysis of *in vivo* BPDE-DNA. DNA was isolated from the dorsal skin of mice each exposed to a single BP treatment (200 μg for 24 hours). A sample was then alkaline hydrolyzed (1 M NH_4OH at 80°C for 4 hours) and the tetrols quantitated by HPLC and fluorescent analysis.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TIME (minutes)

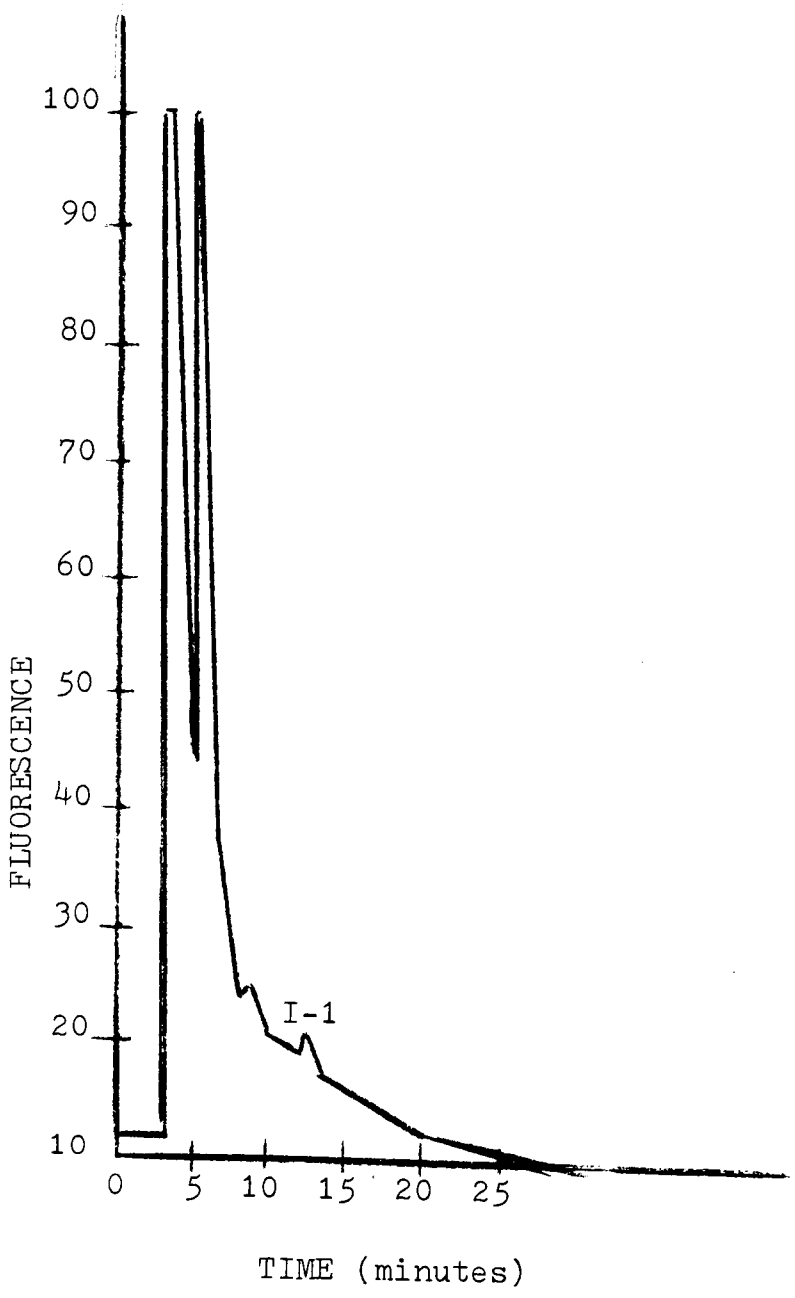
Figure 4

(see Figure 2, page 14). Essentially the same quantitative data was obtained from the in vitro calf thymus DNA reacted with BPDE I (data not shown).

In the event that the diol epoxide remained attached to a phosphate moiety after alkaline hydrolysis, alkaline hydrolyzed DNA was further treated with alkaline phosphatase (Figure 5). No increase in the fluorescence of the tetrol peaks, indicating tetrol release, was observed.

Enzyme hydrolysis of the BPDE-DNA samples was also used to attempt to selectively remove the bound diol epoxides from the phosphodiester backbone. DNA was hydrolyzed to the nucleoside level with deoxyribonuclease I, snake venom phosphodiesterase, and bacterial alkaline phosphatase. The fluorescent profile of the enzyme hydrolyzed, BP-treated mouse skin DNA is shown in Figure 6. No increase in tetrol release nor any new fluorescent peak was observed. In agreement with others (Ashurst et al., 1983; Koreeda et al., 1978) the major hydrocarbon-deoxyribonucleoside adduct observed is BPDE-deoxyguanosine. This adduct made up approximately 70 % of the total diol epoxide bound to DNA and is consistent with the values found by other investigators. This suggests that the enzymes were functional and the hydrolysis was complete. Similar results were obtained with the in vitro calf thymus BPDE-DNA.

Figure 5. Alkaline hydrolysis and alkaline phosphatase treatment of *in vivo* BPDE-DNA. DNA was isolated from the dorsal skin of mice each exposed to a single BP treatment (200 μg for 24 hours). After alkaline hydrolysis (1 M NH_4OH at 80°C for 4 hours), the sample was neutralized with HCl and treated with bacterial alkaline phosphatase for 6 hours at 37°C. Tetrol release was then measured by HPLC and fluorescence analysis.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TIME (minutes)

Figure 5

Figure 6. Enzyme hydrolysis of *in vivo* BPDE-DNA. DNA was isolated from the dorsal skin of mice each exposed to a single treatment of BP (200 μg for 24 hours). A BPDE-DNA sample was then hydrolyzed with snake venom phosphodiesterase and deoxyribonuclease I for 18 hours, followed by treatment with bacterial alkaline phosphatase for an additional 6 hours. The release of tetrol was detected by HPLC and fluorescence analysis. Peak A represents the BPDE-deoxyguanosine adduct.

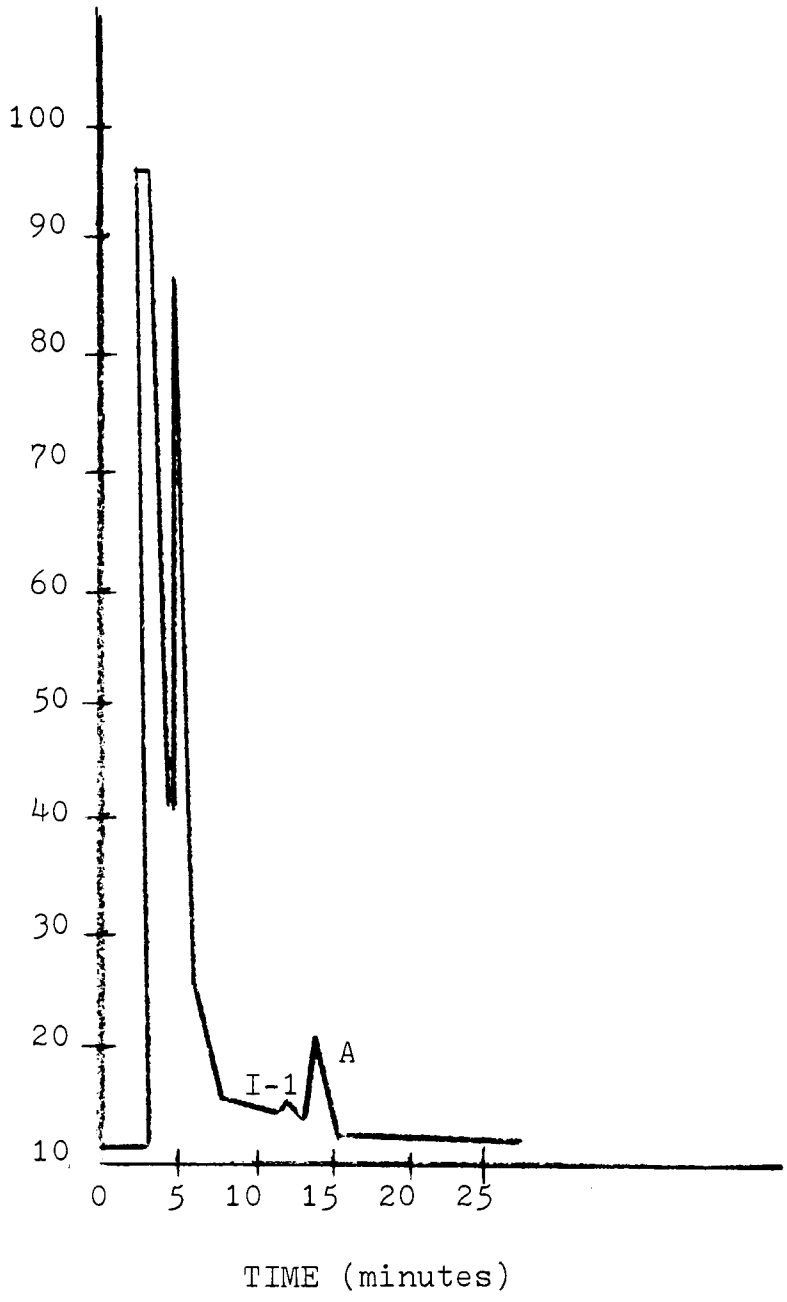

Figure 6

IV. DISCUSSION

The room temperature fluorometric quantitation of tetrols released from BP adducts has been shown to be highly sensitive (Rahn et al., 1982). They have shown that a lower limit of 10 pg of tetrol per 100 µg of BP-modified DNA appears to be detectable. This translates to approximately 1 BP residue/10^7 bases/100 µg DNA and is comparable to the sensitivity obtained by radiometric and immunological techniques (Montesano et al., 1982). In the study presented here, sufficient amounts of BP-modified DNA (100 µg DNA, 500 pg bound BPDE I) were analyzed to allow one to detect a lower limit of 2 % of the total BPDE I binding.

Gamper et al. (1977) have shown that the binding of BP diol epoxides to DNA results in strand scission. The formation of phosphate backbone breakages with the diol epoxide still attached to the phosphate (as a phosphodiester) was the basis of the chemical and enzymatic methods employed in this study. When heated at alkaline pH the phosphodiester could hydrolyze in the following two different ways: to give free tetrol and; to give the diol epoxide attached to a phosphate but liberated from the DNA backbone. Upon alkaline phosphatase treatment the BPDE-phosphate moiety should yield free tetrol. Figure 4 (page 18) shows that no tetrol is released after heating at 80°C for 4 hours in 1 N NH_4OH. Alkaline hydrolysis

followed by alkaline phosphatase treatment also yielded no increase in tetrol release as shown in Figure 5 (page 21). Similar results were found in both the in vivo and in vitro BPDE-DNA samples studied.

There is some question as to whether the 3'- or 5'-phosphotriester bond is hydrolyzed when the diol epoxide interacts with the phosphate backbone. Both possible situations will be considered in the enzyme hydrolysis studies:

1. The 3'-phosphoester bond has been hydrolyzed:

$$p\text{-}X\text{-}(p\text{-}X)_n\text{-}p\text{-}X \quad \overset{R}{\underset{|}{p}}\text{-}X\text{-}(p\text{-}X)_n\text{-}p\text{-}X$$

The snake venom phosphodiesterase should yield compounds of the type $\overset{R}{\underset{|}{p}}\text{-}X$ which should be resistant to further treatment with alkaline phosphatase. The diol epoxide is represented by R.

2. The 5'-phosphoester bond has been hydrolyzed:

$$p\text{-}X\text{-}(p\text{-}X)_n\text{-}p\text{-}X\text{-}\overset{R}{\underset{|}{p}} \quad X\text{-}(p\text{-}X)_n\text{-}p\text{-}X$$

The left fragment is no substrate for the snake venom phosphodiesterase requiring a 3'-OH end. However, the endonucleolytic action of the deoxyribonuclease could release either $p\text{-}X\text{-}\overset{R}{\underset{|}{p}}$ or $\overset{R}{\underset{|}{p}}$. Treatment of those compounds with alkaline phosphatase would yield either $X\text{-}\overset{R}{\underset{|}{p}}$ or R (free tetrol), respectively.

Figure 6 (page 23) shows that the enzyme mixture used produced no increase in tetrol release nor any new

fluorescent peak, corresponding to the X-$\overset{R}{\underset{|}{p}}$ hydrolysis product. As mentioned earlier, the presence of the deoxyquanosine adduct demonstrates that the enzymes were functional.

The failure to detect tetrols, selectively released from the BPDE-phosphate adducts, is consistent with the results obtained by other investigators. Gamper et al. (1977) found that DNA strand scission via diol epoxide hydrolysis of unstable phosphotriesters represented less than 1 % (0.33 %) of the DNA modifications by the diol epoxides. In their system Escherichia coli plasmid Col E1 was reacted in vitro with either diastereoisomeric diol epoxides. Nicking of the superhelical plasmid was monitored by agarose gel electrophoresis and electron microscopy. Shooter et al. (1977) used the technique of differential alkaline hydrolysis of DNA to detect phosphotriester formation in vitro. They found no alkali-labile sites in bacteriophage T7 DNA after treatment with the diol epoxides. This led them to suggest that no significant amounts of phosphotriesters were formed; or that its hydrolysis led to the loss of the hydrocarbon group from the nucleic acid and that this reaction has no biological effect.

The study presented here is significant in that it is the first reported attempt to measure the in vivo binding of the ultimate carcinogen BPDE to the phosphate backbone

of DNA. BP, a model skin carcinogen, was topically applied to the skins of intact animals. Following the metabolism of BP to its "active" intermediate, binding of the diol epoxides to target tissue DNA was shown to occur. This approach was also novel in that it utilized the high sensitivity of fluorescence to measure the release of tetrol from the BPDE-phosphate adduct in isolated DNA from the target tissue (the epidermis).

The lower limit of detection in this study was approximately 2 % of the total BPDE modification (or 10 pg of released tetrol). The failure to detect binding of the diol epoxide to the phosphate backbone does not entirely rule out the possibility that the metabolites of BP do bind to this phosphate linkage in DNA. It may suggest that the proportion of phosphotriesters formed in vivo is below the sensitivity of the procedure. Since this study is based on the dogma that the BP diol epoxide is the ultimate carcinogen, there is also the possibility that other metabolites (other than BPDE) might form adducts with the phosphate backbone. Other BP metabolites have been shown to bind to DNA (Alexandrov et al., 1982). Further research is needed to determine whether the low level of binding of BP metabolites to the phosphate backbone is of any biological importance, if it occurs at all.

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APPENDIXES

APPENDIX A

Percent of Total DNA Alkylation at the Phosphodiester Site for Various Alkylating Agents

Alkylating Agent	System	Percent of Total DNA Alkylation as Phosphotriester
Benzo(a)pyrene	T7 DNA[a] (in vitro)	-
Benzo(a)pyrene	Col E1 DNA[b] (in vitro)	0.3
Methylmethanesulfonate	salmon sperm DNA[c] (in vitro)	1
Methylnitrosourea	salmon sperm DNA[d] (in vitro)	12.1
Ethylmethanesulfonate	HeLa cell DNA[e] (in vivo)	7
Ethylmethanesulfonate	HeLa cell DNA[e] (in vitro)	13
Ethylmethanesulfonate	salmon sperm DNA[c] (in vitro)	15.2
Diethylsulfate	HeLa cell DNA[e] (in vitro)	16
Diethylsulfate	HeLa cell DNA[e] (in vivo)	20
Ethylnitrosourea	salmon sperm DNA[d] (in vitro)	55.4
Ethylnitrosourea	HeLa cell DNA[e] (in vivo)	66
Ethylnitrosourea	HeLa cell DNA[e] (in vitro)	70

[a]Shooter, K.V., Osborne, M.R., and Harvey, R.G. The interaction of the 7,8-dihydrodiol-9,10-oxides of benzo(a)pyrene with bacteriophages R17 and T7. Chem.-Biol. Interactions 19, 215-223 (1977).

[b]Gamper, H.B., Tung,A.S., Straub, K., Bartholomew, J.C., and Calvin, M. DNA strand scission by benzo(a)-pyrene diol epoxides. Science 197, 671-674 (1977).

[c]Bannon, P., and Verly, W. Alkylation of phosphates and stability of phosphate triesters in DNA. Eur. J. Biochem. 31, 103-111 (1972).

[d]Beranek, D.T., Weis, C.C., and Swenson, D.H. A comprehensive quantitative analysis of methylated and ethylated DNA using high pressure liquid chromatography. Carcinogenesis 1, 595-606 (1980).

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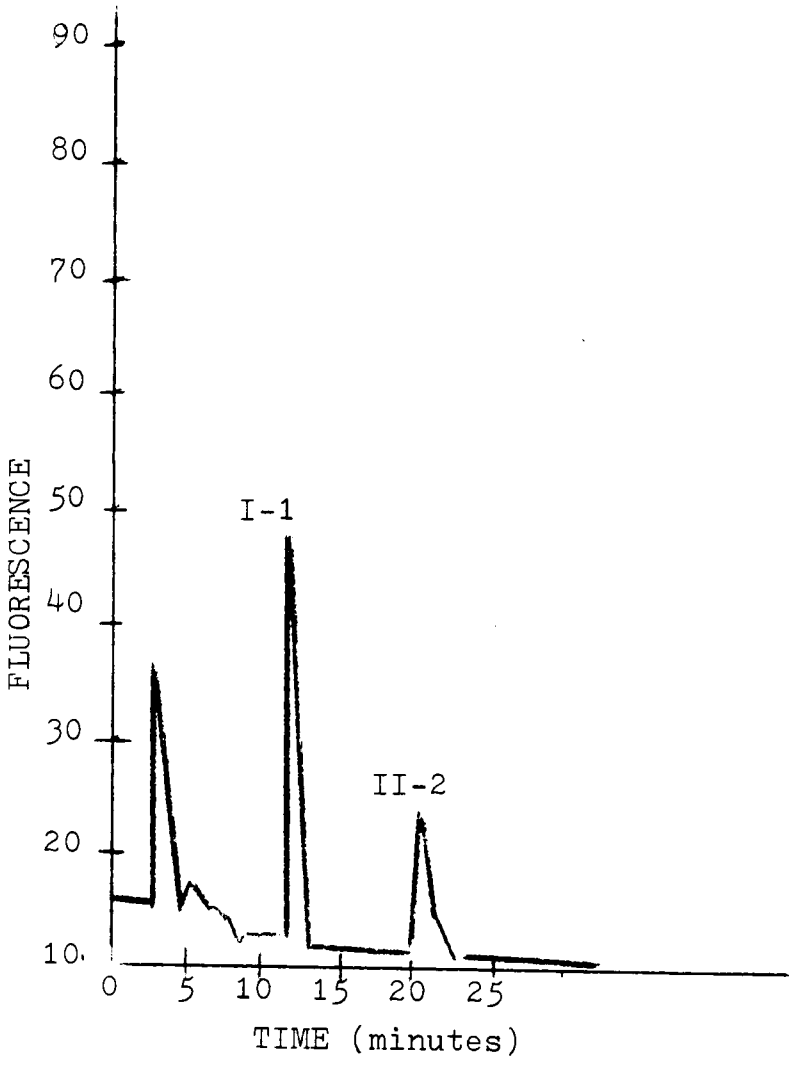
- indicates the absence of any detectable phosphotriester

APPENDIX B

HPLC of Tetrol Standards



HPLC and fluorescent profile of tetrol standards I-1 (188.6 pg) and II-2 (86 pg).

Figure A-1

VITA

Gregory N. Tjossem was born in Decorah, Iowa on June 7, 1958. In the fall of 1976 he entered Earlham College in Richmond, Indiana, and in June 1980 he received a Bachelor of Arts degree with a major in biochemistry. The following year was spent teaching science at Olney Friends School in Barnesville, Ohio. The author entered the Graduate School of Biomedical Sciences of the University of Tennessee in the fall of 1981 in pursuit of a Master of Science degree with a major in biomedical sciences.