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Nucleoside Adducts from the in Vitro Reaction of Benzo[*a*]pyrene-7,8-dihydrodiol 9,10-Oxide or Benzo[*a*]pyrene 4,5-Oxide with Nucleic Acids†

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ABSTRACT: The covalent binding of benzo[*a*]pyrene 4,5-oxide and benzo[*a*]pyrene-7,8-dihydrodiol 9,10-oxide isomer I and isomer II to nucleic acids in aqueous acetone solutions has been investigated. Benzo[*a*]pyrene 4,5-oxide reacted preferentially with guanosine residues. On the other hand, benzo[*a*]pyrene-7,8-dihydrodiol 9,10-oxide isomer I and isomer II reacted extensively with guanosine, adenosine, and cytidine residues. Time course studies showed that the reactivity of isomer I or isomer II with homopolyribonucleotides followed the order poly(G) > poly(A) > poly(C). Alkaline or enzymatic hydrolysis of the modified nucleic acids and subsequent chromatography on Sephadex LH-20 columns yielded benzo[*a*]pyrene-nucleotide adducts. These were enzymatically converted to the corresponding nucleosides which were resolved into several distinct components by high-pressure liquid chromatography. Evidence was obtained for the presence of multiple nucleoside adducts of guanosine, adenosine, cytidine, deoxyguanosine, deoxyadenosine, and deoxycytidine. The HPLC profiles of adducts formed with isomer I were different from the corresponding profiles of adducts formed with isomer II. Structural aspects of these nucleoside adducts are discussed.

Many polycyclic aromatic hydrocarbons[1] (PAHs) are potent carcinogens and mutagens which are widely distributed pollutants in the human environment. Following metabolic activation the compounds bind covalently to cellular proteins (Abell and Heidelberger, 1962; Heidelberger and Moldenhauer, 1956; Miller, 1951) and nucleic acids (Brookes and Lawley, 1964; Goshman and Heidelberger, 1967) in the intact animal and in cell culture (Baird et al., 1973; Brookes and Heidelberger, 1969; Duncan et al., 1969). This covalent interaction is probably critical to the carcinogenic process (Miller, 1970; Gelboin et al., 1972). In order to react with these cellular targets the PAHs must be metabolized to reactive intermediates. The microsomal enzyme system, aryl hydrocarbon hydroxylase, oxidizes PAHs to a variety of derivatives (Jerina and Daly, 1974). Covalent binding of PAHs to nucleic acids in vitro has been achieved by incubation in the presence of microsomes and NADPH (Borgen et al., 1973; Gelboin, 1969; Grilli et al., 1975; Grover and Sims, 1968; Pietropaolo and Weinstein, 1975; Thompson et al., 1976).

Boyland (1950) proposed that oxides are the reactive intermediates which form covalent bonds with nucleophilic centers in cellular macromolecules. Incubation of parent PAHs with microsomes, oxygen, and NADPH produces arene oxide intermediates including the K-region oxides of 7,12-dimethylbenz[*a*]anthracene (DMBA) (Keysell et al., 1973) and benzo[*a*]pyrene (BP) (Grover et al., 1972; Wang et al., 1972; Selkirk et al., 1975). These K-region oxides react covalently with nucleic acids in vitro (Baird et al., 1973, 1975; Blobstein et al., 1975, 1976; Swaisland et al., 1974a), are mutagenic in bacterial (McCann et al., 1975) and mammalian (Huberman et al., 1971) cells, and transform various cells in culture (Sims and Grover, 1974; Marquardt et al., 1976). However, there is evidence that the K-region oxides are not the major reactive intermediates in vivo (Baird et al., 1973, 1975). Evidence has been presented that in vivo the major reactive derivatives of benzo[*a*]anthracene (Swaisland et al., 1974b) and benzo[*a*]pyrene (Borgen et al., 1973; Daudel et al., 1975; Ivanovic et al., 1976; Sims et al., 1974) are dihydrodiol oxides.

In the case of BP, two isomers of the dihydrodiol oxide have

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[1] Abbreviations used: BP, benzo[*a*]pyrene; PAH, polycyclic aromatic hydrocarbon; DMBA, 7,12-dimethylbenz[*a*]anthracene; poly(G)-BP-7,8-dihydrodiol 9,10-oxide, poly(G)-BP 4,5-oxide, etc. are the nucleic acid products obtained from reaction with the indicated BP oxide and this does not indicate that the epoxide form remains intact in the product; λ_{max}, wavelength of maximum absorbance in the ultraviolet (UV) spectrum; HPLC, high-pressure liquid chromatography; CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography.

been synthesized, isomers I and II (Figure 1). Hulbert (1975) proposed that isomer II would have enhanced reactivity with DNA in vivo compared with isomer I based on conformational considerations and Yagi et al. (1975) have reported that isomer II reacts much more rapidly with sodium *p*-nitrothiophenolate than isomer I. Both isomers I and II were found to be mutagenic to bacteria (Malaveille et al., 1975; Wislocki et al., 1976a,b); however, isomer I was much more mutagenic to mammalian cells than II (Huberman et al., 1976). Huberman et al. (1976) also showed indirectly that isomer I is formed from microsomal oxidation of 7,8-dihydrodiolbenzo[*a*]pyrene.

In the present paper we have studied the in vitro reaction of isomer I, isomer II, and BP 4,5-oxide with various natural and synthetic nucleic acids to determine reactivities and base specificities. The nucleoside adducts have also been resolved by high-pressure liquid chromatography (HPLC). These studies provide useful BP nucleoside markers for comparative studies on materials obtained from in vivo sources.

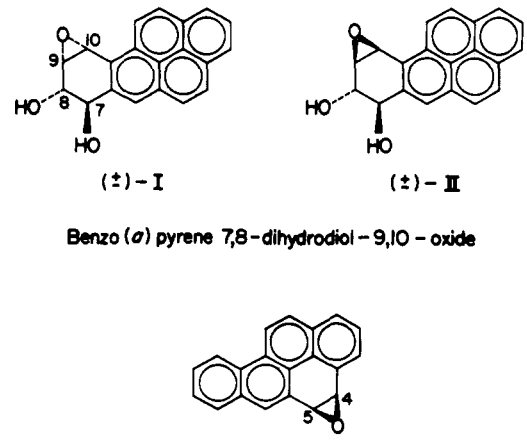

FIGURE 1: Structures of isomers I and II, benzo[*a*]pyrene-7,8-dihydrodiol 9,10-oxide, and benzo[*a*]pyrene 4,5-oxide.

Materials and Methods

BP 4,5-oxide and the two isomers (I and II) of BP-7,8-dihydrodiol 9,10-oxide were prepared as described (Harvey et al., 1975; Beland and Harvey, 1976). [8-^{14}C]-5′-dGMP (specific activity, 36 mCi/mmol), poly(G), and poly(I) were obtained from Schwarz/Mann. Calf thymus DNA, bovine spleen phosphodiesterase, snake venom phosphodiesterase, and *E. coli* alkaline phosphatase were purchased from Worthington Biochemicals. Poly(A), poly(C), poly(U), poly(dC), and poly(dT) were purchased from Miles Laboratories, Inc., and poly(dG) and poly(dA) from P-L Biochemicals. 5′-dGMP and DNase I were obtained from Sigma Chemical Co. P1 nuclease was a gift from Dr. A. Kuninka (Kuninka et al., 1973). Rabbit reticulocyte ribosomal RNA was a gift from Peter Pulkrabek. T1 ribonuclease was from Calbiochem. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals.

Glass-distilled water was used in all cases. Methanol was spectral grade. All other solvents were reagent grade. All reactions were done in the absence of light and subsequent procedures were performed in subdued light.

Spectra. Absorption measurements were determined on a Zeiss spectrophotometer (Model PMQ II) and a Cary 14 recording spectrometer.

Reaction of Nucleic Acids with BP Oxides. Unless specified otherwise, nucleic acids were reacted with the various BP oxides in acetone–water (2:1 or 1:1), pH ~5, according to methods previously described (Blobstein et al., 1975; Weinstein et al., 1976). In some cases the reaction was performed at slightly alkaline conditions (pH 8 reactions) using dilute KOH to adjust the pH. The reaction mixtures were incubated at 37 °C for approximately 24 h (except time course studies). Unbound hydrocarbon derivatives of BP 4,5-oxide reactions were removed by ethyl ether extraction (6–10 times). Ethyl acetate and ethyl acetate–butanol mixtures were used to extract noncovalently bound derivatives of the BP-7,8-dihydrodiol 9,10-oxides fom the pH 5 and pH 8 acetone–water reactions. Nucleic acids were then ethanol precipitated twice. Further solvent extractions or ethanol precipitations did not alter the absorption spectra. Absorption spectra were determined on aliquots of this final material in water and the extent of BP binding was expressed as the $A_{350}/A_{\lambda_{max}}$ ratio with 7,8-dihydrodiol 9,10-oxides or $A_{330}/A_{\lambda_{max}}$ ratio with the 4,5-oxide. The approximate percent modification in the case of BP 4,5-oxide was calculated from the absorption spectra of the modified polymers assuming ϵ_{330} (BP 4,5-oxide) = 1.0×10^4 M^{-1} cm^{-1}, and ϵ_{260}'s of the polymer as follows: DNA (assumed denatured), 8.5×10^3; poly(A), 9.4×10^3; poly(I), 6.25×10^3; poly(G), 9.0×10^3; poly(C), 5.25×10^3; and poly(U), 8.9×10^3; % modification = $100 \times \epsilon_{260} \times A_{330}/\epsilon_{330} \times [A_{260} - 3.7(A_{330})]$. The absorbance at 260 nm was corrected for the contribution from BP 4,5-oxide by the factor $3.7 \times A_{330}$. A_{260} and A_{330} are the absorbances obtained from the spectra of the modified polymer; ϵ_{260} is the extinction coefficient of the nucleic acid and ϵ_{330} is the extinction coefficient of the BP 4,5-oxide. The percent modification of polymers by BP-dihydrodiol oxides was similarly calculated as previously described (Weinstein et al., 1976).

Determination of the Stability of the BP-7,8-dihydrodiol 9,10-Oxides during Reaction with Nucleic Acids. After either a 1 h or a 17 h incubation at 37 °C, aliquots of the above described acetone–water reaction mixtures containing poly(G), poly(U), or no nucleic acid (control) were extracted twice with ethyl acetate. The ethyl acetate phase was quickly evaporated to dryness, and the residue was dissolved in acetone and added to a solution of poly(G) so that the final conditions were 2:1 acetone–water and 1 mg/mL poly(G). This mixture was incubated at 37 °C for 20 h and then extracted and the nucleic acid isolated as described above. The percentage of reactive BP oxide remaining after the first incubation was based on a comparison of the $A_{350}/A_{\lambda_{max}}$ ratio observed with the modified poly(G) obtained from the second incubation to the ratio obtained in the usual one step 20 h incubation with poly(G).

Alkaline Hydrolysis of Polyribonucleotide–BP-7,8-Dihydrodiol 9,10-Oxide Product. The "modified" RNA, poly(A), poly(C), or poly(G), obtained as described above, was hydrolyzed at 37 °C for 18 h with 0.3 N NaOH. The nucleotide mixtures were neutralized with acetic acid and chromatographed on Sephadex LH-20 columns (see below). The poly(G)-BP 4,5-oxide product was unstable when subjected to alkaline hydrolysis and was, therefore, hydrolyzed enzymatically.

Enzymatic Hydrolysis of Poly(G)-BP 4,5-Oxide Product. About 100 A_{260} units of poly(G)-BP 4,5-oxide product was treated with 2500 units of *T*1 ribonuclease and 4 units spleen phosphodiesterase in 2.5 mL of 20 mM Tris-HCl, pH 7.5, at 37 °C for 7 days. The nucleotide mixture was then chromatographed on Sephadex LH-20.

Enzymatic Hydrolysis of Polydeoxyribonucleotide BP-Oxide Products. Approximately 50 A_{260} units of the modified

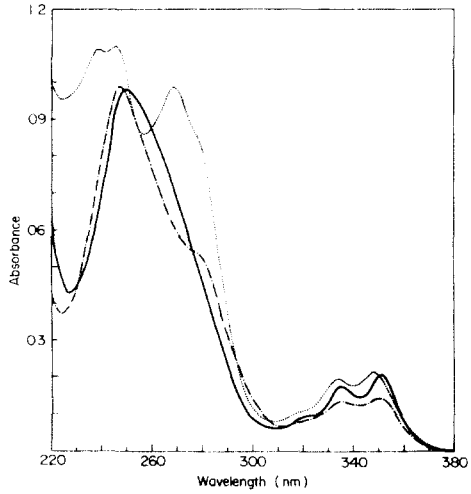

FIGURE 2: Absorption spectra of polynucleotides modified with isomer I at pH 5, measured in water. (—) Poly(A); (· · ·) poly(C); and (- · - · -) poly(G).

DNA in 3.5 mL of 10 mM Tris-HCl(pH 7.8)–10 mM $MgCl_2$ was incubated with 1000 units of DNase I and 0.25 unit of venom phosphodiesterase at 37 °C for 5 days, after which 10 units of alkaline phosphatase was added and incubation continued for 2 more days. Subsequent studies have shown that equal digestion is obtained after 5 h at pH 7.0 with DNase I, followed by overnight incubation at pH 8.5 with alkaline phosphatase and venom phosphodiesterase. The resulting nucleoside mixtures were chromatographed on Sephadex LH-20. In the case of the homopolydeoxyribonucleotide–BP-7,8-dihydrodiol 9,10-oxide products, approximately 5 A_{260} units in 0.6 mL of 10 mM Tris-HCl (pH 7.0)–10 mM $MgCl_2$ was incubated with 200 units of DNase I, 0.003 unit of venom phosphodiesterase, 0.435 unit of spleen phosphodiesterase, and 40 μg of P1 nuclease at 37 °C for 24 h, after which 10 units of alkaline phosphatase was added and incubation continued for 4 more days. These nucleoside mixtures were evaporated to dryness and the methanol soluble products analyzed directly by HPLC.

Alkaline Phosphatase Hydrolysis of Nucleotide–BP Oxide Products. One unit of alkaline phosphatase was added per A_{260} unit of nucleotide adduct in 20 mM NH_4HCO_3 (pH 8.5), and the mixture was incubated at 37 °C for 24 h. The resulting nucleoside mixture was then chromatographed on Sephadex LH-20.

Reaction of 5′-dGMP with BP-7,8-Dihydrodiol 9,10-Oxides. [8-^{14}C]-5′-dGMP was diluted with 5′-dGMP to a specific activity of 16.6 μCi/mmol. Solutions containing 1.5 mg of [8-^{14}C]-5′-dGMP in 0.7 mL of water were mixed with 1.4 mL of acetone containing 1.5 mg of BP-7,8-dihydrodiol 9,10-oxide isomer I or II. The mixture was incubated at 37 °C for 22 h. The samples were extracted with ethyl acetate and ethyl acetate–butanol mixtures (six to nine times) to remove unbound hydrocarbon. The aqueous solution containing a mixture of modified and unmodified nucleotides was then chromatographed on a Sephadex LH-20 column.

Sephadex LH-20 Column Chromatography. Sephadex LH-20 was washed extensively with methanol and then water before use. Glass columns were packed with Sephadex LH-20 to give a column approximately 1.1 × 55 cm. When nucleotide products obtained from alkaline hydrolysis of the polymers were chromatographed, the columns were equilibrated with 20 mM NH_4HCO_3 (pH 8.5). The sample was applied and the column washed with 20 mM NH_4HCO_3 to remove unmodified nucleotides. The modified nucleotides were then eluted with a linear gradient (450 mL) of 20% methanol–20 mM NH_4HCO_3 (v/v) to 80% methanol–20 mM NH_4HCO_3 at a flow rate of 0.7 mL/min, and 2.8-mL fractions were collected. When nucleoside products obtained from enzymatic hydrolysis of the polymers were chromatographed, the columns were equilibrated with 5 mM NH_4HCO_3 (pH 8.5). The sample was applied and the column washed with this buffer to remove unmodified nucleosides. The modified nucleosides were then eluted with a linear gradient (500 mL) of 30% methanol–5 mM NH_4HCO_3 to 100% methanol. Modified nucleosides obtained from alkaline phosphatase treatment of the corresponding nucleotides were chromatographed on small (0.8 × 15 cm) columns of Sephadex LH-20 equilibrated with water. The sample was applied, the column washed with water to remove salts and enzyme, and the modified nucleoside was eluted with 50% methanol–water.

HPLC Chromatography. The modified nucleosides were analyzed as described (Jeffrey et al., 1976b) by HPLC on a Du Pont 830 instrument with reverse phase Zorbax ODS columns (2.1 mm i.d.) operated at 50 °C, 3000 psi, and eluted with methanol–water gradients.

Results

Binding of BP 4,5-Oxide to Nucleic Acids. BP 4,5-oxide shows three major absorption peaks in the 300–340-nm region of the UV spectrum. Since nucleic acids have negligible absorbance in this regions, the extent of BP 4,5-oxide binding to nucleic acids was estimated from the ratio of absorbance at 330 nm to the absorbance at the maximum wavelength of the modified polymer. The results of binding studies with different nucleic acids indicated the following extents of modification expressed as percent of total bases modified: poly(G), 6.1%; DNA, 2.2%; poly(A), 0.5%; poly(I), 0.3%; poly(C), 0.1%; and poly(U), less than 0.1%. The absorption spectrum of poly(G) modified with BP 4,5-oxide showed bands in the 300–350-nm region, a shift of λ_{max} from 252 nm for poly(G) to 256 nm and broadening of the major absorption peak.

Binding of BP-7,8-Dihydrodiol 9,10-Oxide I and II to Nucleic Acids. Separate studies (Weinstein et al., 1976) showed that both isomers I and II of BP-7,8-dihydrodiol 9,10-oxide react significantly with poly(G), poly(A), and poly(C) at pH 5. At pH 8 only poly(G) reacted significantly and the extent of binding was lower than at pH 5. The absorption spectra of poly(A), poly(C), and poly(G) modified with isomer I (Figure 2) at pH 5.0 show absorption bands in the 300–360-nm region which are characteristic of BP-7,8-dihydrodiol 9,10-oxide and BP-7,8,9,10-tetrahydrotetraol. Similar spectra were seen with polynucleotides modified with isomer II, although the relative intensities of the maxima differed from those of isomer I, because of the lower binding. The extent of binding of isomers I and II to nucleic acids was estimated from the ratio of absorbance at 350 nm to the absorbance at the wavelength of maximum absorbance of the modified polymer.

Time Course for the Reaction of BP-7,8-Dihydrodiol 9,10-Oxide I and II with Nucleic Acids. The time dependence of the reaction of isomer I and II with various nucleic acids is shown in Figure 3. The reaction with poly(G) was virtually complete within 1 h for both isomers I and II. With isomer I the reaction was completed with DNA and poly(A) within 6 h, whereas reaction with poly(C) was much slower and less than half complete after 6 h. With isomer II all reactions were virtually complete within 6 h, except for poly(C) which again

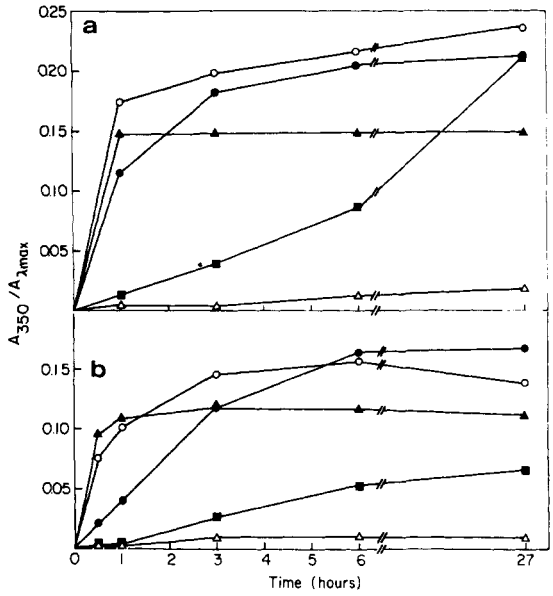

FIGURE 3: Time dependence of the reaction of BP-7,8-dihydrodiol 9,10-oxide with polynucleotides and DNA. (a) Reaction of isomer I with (●) poly(A), (▲) poly(G), (■) poly(C), (○) DNA, and (△) poly(U). (b) Reaction of isomer II with (●) poly(A), (▲) poly(G), (■) poly(C), (○) DNA, and (△) poly(U).

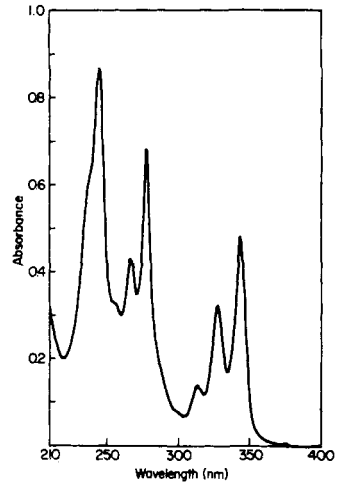

FIGURE 4: Absorption spectrum of GMP-BP-7,8-dihydrodiol 9,10-oxide I product in 50% methanol–water.

reacted more slowly. A general trend in reactivity with both isomers was poly(G) > DNA > poly(A) > poly(C).

It is possible that the above results related to differences in the stabilities of isomers I and II in the incubation system. Yagi et al. (1975) reported that under their conditions the reaction rate of isomer II with sodium *p*-nitrothiophenolate was 100-fold faster than that of isomer I. Therefore, we studied the effects of various polynucleotides and pH of incubation on the loss of reactive material from incubation systems containing either isomer I or II. The procedure is described in Materials and Methods and results are shown in Table I. Incubation of either isomer I or II with poly(G) in acetone–water (2:1) at pH 5 resulted in complete loss of residual reactive polycyclic hydrocarbon after only a 1-h incubation. In the presence of poly(U), however, about 25% of the reactive material remained and in the absence of polymer (acetone–water mixture) over 50% of the reactivity remained, with both isomers I and II. Incubation at pH 8 tended to stabilize both isomers as shown by a much greater percentage of reactive material remaining after a 1-h incubation with poly(G), poly(U), or in the absence of polymer. Evidence was obtained for a greater instability of isomer II since even at pH 8 and in the absence of polymer very little residual reactive material was observed at 17 h (Table I).

Isolation of Covalent Nucleotide–BP Conjugates. Nucleic acids isolated after reaction with BP-7,8-dihydrodiol 9,10-oxide or BP 4,5-oxide were hydrolyzed to mononucleotides with either 0.3 M NaOH or enzymatically, and the hydrolysates were chromatographed on Sephadex LH-20 columns, as described in Materials and Methods. The large peak eluted early with buffer alone (fractions 5–15) was guanine mononucleotide, as confirmed by its UV spectrum and by TLC. GMP-BP-7,8-dihydrodiol 9,10-oxide I adducts eluted as two peaks with the methanol–buffer gradient at approximately 40% methanol (fractions 66–86). The UV spectrum (Figure 4) of the latter material established the presence of a BP-7,8,9,10-tetrahydro-type chromophore. Less polar products were obtained after treatment of this material with alkaline phosphatase, indicating conversion of nucleotide adducts to the corresponding nucleoside derivative. The material moved as one spot on TLC (R_f 0.34 on silica gel in 1-butanol–1-propanol–2.5 M NH_4OH, 2:1:1) and, therefore, was not simply a mixture of unmodified nucleotide (R_f 0.28) and BP-7,8-dihydrodiol 9,10-oxide derivatives (R_f 0.54, 0.74). Similar results were obtained with poly(A) and poly(C). The nucleoside adducts obtained after alkaline phosphatase treatment of the corresponding nucleotides were subsequently analyzed by HPLC (see below).

Modification of [8-^{14}C]-5′-dGMP with BP-7,8-Dihydrodiol 9,10-Oxide I and II. [8-^{14}C]-5′-dGMP was reacted with BP-7,8-dihydrodiol 9,10-oxide I or II in acetone–water (2:1), and water soluble products remaining after ethyl acetate and butanol–ethyl acetate extraction were chromatographed on Sephadex LH-20. Unreacted 5′-dGMP and deoxyguanosine (a contaminant) eluted with buffer. A linear gradient (300 mL) of 20% methanol–20 mM NH_4HCO_3 to 90% methanol–20 mM NH_4HCO_3 then eluted the adducts at ~45% methanol. The nucleotide adducts were converted to nucleosides by alkaline phosphatase treatment. From the known specific activity of the [8-^{14}C]-5′-dGMP and the UV absorption of the product, it was possible to calculate the molar extinction coefficient of the conjugate, $\epsilon_{344} = 2.9 \pm 1.1 \times 10^4$ M^{-1} cm^{-1}. Assuming a similar value for the products obtained with nucleic acids, we estimate that ~6% of the residues of poly(G), ~9% with DNA, ~8% with poly(A), and ~5% with poly(C) are modified with isomer I at pH 5, after a 27-h incubation. Isomer II modified poly(G) ~5%, DNA ~4%, poly(A) ~6%, and poly(C) ~1%, under similar conditions. We must emphasize that these are approximate values since they do not take into account possible differences in absorption when the chromophore is present in the polymer structure.

Characterization of Nucleoside–BP Adducts by HPLC. HPLC analysis of the nucleoside adducts obtained from the reaction at pH 5 of poly(G) with BP-7,8-dihydrodiol 9,10-oxide I showed two major and one minor products (Figure 5). The analogous compounds from isomer II were resolved into four peaks. Reaction of poly(C) with isomer I gave rise to six derivatives which eluted in approximately the same region as the guanosine derivatives. The adenosine derivatives obtained with isomer I were more hydrophobic (eluted later) than the C or G adducts and were resolved into at least four components. In separate studies, a sample of RNA modified by reaction with either isomer I or II revealed the presence of gua-

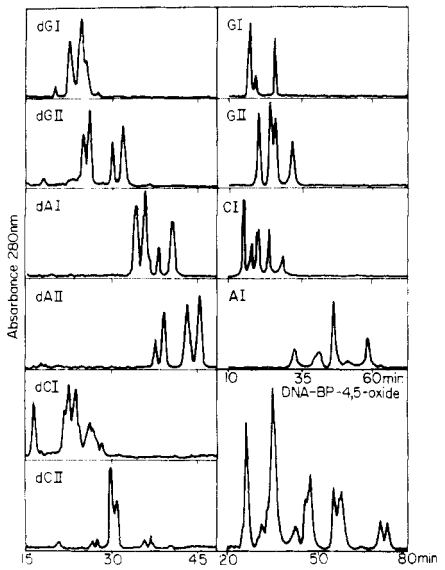

FIGURE 5: High-pressure liquid chromatographic separation of BP-mononucleoside derivatives using Zorbax ODS columns (2.1 mm i.d.) at 50 °C and 3000 psi. G, C, and A indicate the base; prefix d is derived from a deoxyhomopolymer; and I and II are the particular isomers of BP-7,8-dihydrodiol 9,10-oxide. Samples in the dGI-dCII column were eluted with linear gradients from 30 to 55% methanol in water in 50 min and GI-AI with a concave (Du Pont No. 2) gradient from 30 to 80% methanol in water over 100 min. The DNA-BP-4,5-oxide adducts were eluted with a 100-min concave (Du Pont No. 3) gradient from 45 to 80% methanol in water.

nosine, adenosine, and cytidine adducts, with a predominance of the guanosine adducts, when analyzed by HPLC.

Similar studies of the nucleoside derivatives obtained from reaction of deoxynucleotide homopolymers with isomer I or II also revealed multiple derivatives (Figure 5). Products obtained with isomer I again had a different elution profile from those with isomer II and the deoxyadenosine adducts were more hydrophobic (eluted later) than the deoxyguanosine or deoxycytidine adducts. Hydrolysates of samples of DNA modified by reaction with isomer I or II gave a very complex profile by HPLC with evidence of the presence of dG, dA, and dC adducts.

The HPLC profile (Zorbax ODS, 7.9-mm i.d., 45% methanol in water, 3000 psi) of the guanosine adducts obtained from a sample of poly(G) modified by reaction with BP 4,5-oxide and then hydrolyzed enzymatically (see Materials and Methods) showed three major peaks which eluted at 31, 47, and 50 min and a number of minor components. Under the same conditions the four guanosine adducts obtained from the reaction of poly(G) with another K-region oxide, DMBA 5,6-oxide, eluted between 20 and 27 min (cf. Jeffrey et al., 1976b). The deoxynucleoside products obtained from the reaction of DNA with BP 4,5-oxide are shown in Figure 5. Again multiple products were found. Although we have not yet separately characterized the individual components, it is likely that the derivatives eluted between 20 and 50 min represent dG and dC adducts and those eluted from 50 to 80 min represent dA adducts.

Discussion

In the present in vitro studies we have found that BP-7,8-dihydrodiol 9,10-oxide isomers I and II form covalent adducts with poly(G), poly(A), and poly(C). The rates of reaction with the different polymers follow the order poly(G) > poly(A) ≫ poly(C).

TABLE I: The Percentage of Reactive Material Remaining after Incubation of BP-7,8-Dihydrodiol 9,10-Oxides with Poly(G) or Poly(U).[a]

		% Reactive Material Remaining			
		Isomer I		Isomer II	
Polymer	pH	1 h	17 h	1 h	17 h
Poly(G)	5	6	4	9	10
	8	75	42	40	11
Poly(U)	5	32	0	20	11
	8	94	83	80	11
None	5	77	71	55	15
	8	85	78	71	10

[a] The percentage of reactive material was determined by extracting the polycyclic hydrocarbon from the incubation systems after either 1 or 17 h and rereacting this material with poly(G). For details, see Materials and Methods.

Isomer II is less stable than isomer I under a variety of conditions and the presence of nucleic acids has a marked effect on the stability of both isomers (Table I). In the presence of poly(G) there is a rapid disappearance of reactive oxide with almost complete loss after 1 h. The disappearance was less rapid in the presence of poly(U) and even less in the absence of any polymer. These results may relate to recent results reported by Murray et al. (1976), who found that BP 4,5-oxide was converted to the phenol during incubation with poly(G), whereas no phenol was formed during incubation in the presence of poly(A), poly(C), or poly(U). They suggested that these results could be explained by the formation of an unstable complex of the BP 4,5-oxide with guanine which spontaneously decomposed to the phenol and guanine. All of the nucleic acids tested, i.e., poly(G), poly(A), poly(C), and DNA, were modified by isomer II to a lower extent than by isomer I. The instability of isomer II may account for this lower degree of modification, especially in the case of poly(C), where the reaction proceeds very slowly. Since the nucleic acids are presumably denatured in the acetone–water mixture used for the reactions, the conformations of the nucleic acids should exert little effect on their reactivities. The extent of modification is modulated by a combination of factors, the stability of the isomer, the effect of the nucleic acid on this stability, and the rate of the reaction with the nucleic acid. The extents of nucleic acid modification by isomers I and II at pH 8 were much less than those at pH 5. The lower extent of nucleic acid modification at pH 8 suggests that the reaction is acid catalyzed. With isomer I after a 17-h reaction at pH 8, there was still over 40% reactive material remaining (Table I). In the case of isomer II, however, no reactive material remained at pH 8 or pH 5 after a 17-h incubation with poly(G), poly(U), or in the absence of polymer. Our studies on stability contrast with those of Wislocki et al. (1976). Under the acetone–water (pH 5) conditions used in our experiments, both isomers I and II retained over 50% of their reactivity after 1 h in the absence of any polymer. Wislocki et al. (1976), however, reported that the half-life of isomer II in a 2% agar medium was less than 30 s. These differences presumably reflect differences in incubation systems. Thus predictions of half-lives in vivo can not be made with certainty except that isomer I could have appreciable stability. Our studies predict that the in vivo stability and reactivity of BP-7,8-dihydrodiol 9,10-oxides is markedly

influenced by pH, concentration of various cellular nucleophiles, and the base composition of cellular nucleic acids.

In the present study we found that the 4,5-oxide of BP, when reacted with homopolymers, showed almost an exclusive preference for reaction with poly(G). It is of interest that this mimics the microsome-mediated binding of BP to nucleic acids (Pietropaolo and Weinstein, 1975) since the microsomal system produces a large number of metabolites including BP 4,5-oxide (Selkirk et al., 1975). Another PAH K-region oxide, DMBA 5,6-oxide, also reacted to a greater extent with poly(G) than with other homopolymers in vitro (Blobstein et al., 1975).

Although some separation of BP nucleoside adducts can be achieved by Sephadex LH-20 column chromatography, our HPLC analyses gave a much higher degree of resolution. HPLC analyses (Figure 5) revealed multiple products even with homopolymers. Both isomers I and II are themselves racemic. Previous studies with DMBA 5,6-oxide and BP isomers I and II (Jeffrey et al., 1976a,c) have shown that pairs of diastereoisomeric nucleoside adducts are formed and can easily be resolved by HPLC; they have circular dichroism spectra of approximately the same shape but opposite sign. The number of products obtained can, therefore, essentially be halved to account for the racemic mixture. In the case of DMBA 5,6-oxide (Jeffrey et al., 1976c), it was found that reaction with guanine residues in poly(G) occurred approximately equally at either the 5 or 6 positions. In separate studies we have established that the reaction of BP-7,8-dihydrodiol 9,10-oxide isomer I with poly(G) (Jeffrey et al., 1976a) involves mainly linkage of the two amino group of guanine to the 10 position of BP. Presumably the 10 position is favored due to electronic factors. We have also established that DMBA 5,6-oxide binds, by trans addition, to the two amino group of guanine (Jeffrey et al., 1976c).

Based on the above considerations of racemic starting PAH oxides, trans additions, number of sites of substitution on the PAH, and binding to the exocyclic amino groups of the bases, four products would be expected from the reaction of BP 4,5-oxide with poly(G) and two major products from the reaction of BP-7,8-dihydrodiol 9,10-oxides with poly(G), poly(A), or poly(C). More than the predicted number of products was seen in each case. This suggests that sites other than the amino groups on the bases are involved in these reactions, that substitution occurs also at position 9 of the BP-7,8-dihydrodiol 9,10-oxides, and/or that both cis and trans additions occur. Further studies including mass, nuclear magnetic resonance, and circular dichroism spectra are required to elucidate the structures of these nucleoside adducts.

We have already obtained definite structural characterization of two of the BP guanine adducts formed from the reaction of isomer I with poly(G) (Jeffrey et al., 1976a). We have also obtained evidence that the in vivo binding of BP to RNA in cultured bovine bronchial mucosa occurs in part through the intermediate BP-7,8-dihydrodiol 9,10-oxide in the form of isomer I and not isomer II. This in vivo reaction is apparently more specific than that which we describe in the present study since only one BP-guanine derivative of this type was found (Weinstein et al., 1976). We are hopeful that the products formed in vitro and resolved by HPLC in the present study will provide additional markers which can be used for comparison with in vivo radioactive labeled BP nucleoside adducts.

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Isolation of the Amino-Terminal Fragment of Lactose Repressor Necessary for DNA Binding†

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ABSTRACT: *lac* repressor can be dissected by trypsin into a homogeneous tetrameric core (accounting for residues 60 to 347), carrying inducer binding activity, and the monomeric amino-terminal peptides ("headpieces") accounting for residues 1 to 59 and 1 to 51, respectively. This restriction of the action of trypsin on *lac* repressor is obtained in 1 M Tris-HCl (pH 7.5)–30% in glycerol at 25 °C since only the peptide bonds at lysine-59 and to a lesser extent at arginine-51 are cleaved under these conditions. The headpieces can be purified by gel filtration. They have ordered secondary structure as revealed by circular dichroism studies. The monomeric headpieces show the relatively weak binding to nonoperator DNA but not the highly specific and strong binding to operator DNA typical for tetrameric *lac* repressor.

The lactose repressor is probably the best characterized protein regulating gene expression in *E. coli*. Specific binding of repressor to *lac* operator DNA controls the lactose genes. Binding of an inducer such as isopropyl β-D-thiogalactoside to repressor reduces the affinity to the operator greatly and allows expression of the lactose genes (for a review, see Müller-Hill, 1975).

There have been two main approaches—genetics and biochemistry—to locate the region(s) within the *lac* repressor polypeptide chain which are responsible for DNA binding. Genetic analysis has revealed that the majority of the mutations which lead to a defective operator binding site but do not disturb either the binding of inducer or the tetrameric structure of repressor are located within the amino-terminal 60 residues of the repressor polypeptide chain (Adler et al., 1972). The same result has been obtained by an extensive study on the suppression of nonsense mutants in the *lac* i gene (Miller et al., 1975). The biochemical approach has used the fact that the action of trypsin on *lac* repressor is very restricted (Platt et al., 1973). Thus it was possible to isolate a homogeneous tryptic core which has lost the amino-terminal 59 residues of the polypeptide chain. This core had full inducer binding activity and tetrameric structure but was inactive in binding to nonoperator or to operator DNA (Platt et al., 1973; Files and Weber, 1976). Repressor derivatives with similar structural and functional properties could also be isolated from some repressor mutant strains due to translational reinitiation past a nonsense codon (Platt et al., 1972; Ganem et al., 1973; Files et al., 1974). The combined genetic and biochemical evidence makes it very likely that the amino-terminal region of the *lac* repressor is directly involved in the strong and specific binding to *lac* operator DNA as well as the weak binding to nonoperator DNA (Lin and Riggs, 1975). Thus far, however, it has not been possible to isolate the amino-terminal part of the repressor and to see if it retains DNA binding properties related to those of the intact repressor.

Here we report the conditions for limited tryptic digestion which allow the dissection of native repressor into a homogeneous tetrameric core and monomeric amino-terminal headpieces. The headpieces have ordered secondary structure and show the weak binding to nonoperator DNA typical for *lac* repressor (Lin and Riggs, 1972, 1974; von Hippel et al., 1974) but not the specific and strong binding to operator DNA.

Materials and Methods

Tryptic Digestion of lac Repressor under Native Conditions. Two main digestion conditions were used: (a) digestion at 25

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