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Products Obtained after in Vitro Reaction of 7,12-Dimethylbenz[*a*]anthracene 5,6-Oxide with Nucleic Acids[†]

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ABSTRACT: Several lines of evidence suggest that oxide derivatives of carcinogenic polycyclic hydrocarbons are the reactive intermediates for in vivo binding to cellular nucleic acids. In the present study the covalent binding of 7,12-dimethylbenz[*a*]anthracene 5,6-oxide to synthetic homopolymers and nucleic acids in aqueous-acetone solutions has been investigated. Poly(G) was found to be the most reactive nucleic acid and underwent approximately 7-10% modification. Alkaline hydrolysis of the poly(G)-dimethylbenzanthracene conjugate yielded chromatographically distinct polycyclic hydrocarbon-modified nucleotides which were further characterized by spectral analyses and enzymatic and chemical degradation. When the oxide was allowed to react with GMP or dGMP, at least two products were obtained in about 1% yield. Acid hydrolysis of the dGMP-dimethylbenzanthracene conjugates liberated the corresponding guanine-dimethylbenzanthracene products. Mass spectral analysis of the modified bases provided direct evidence that we had obtained covalent binding of the polycyclic hydrocarbon to guanine. The mass spectral cleavage patterns suggest that one of these products is a hydroxydihydro derivative of dimethylbenzanthracene bound to guanine and the other is a dimethylbenzanthracene-guanine conjugate. Additional structural aspects of these guanine derivatives are discussed.

The covalent binding of carcinogenic polycyclic aromatic hydrocarbons[1] (PAHs) to nucleic acids in the intact animal (Brookes and Lawley, 1964) and in cell culture (Baird et al., 1973; Duncan et al., 1969) is well established and it seems likely that this interaction is essential for the carcinogenic process (Miller, 1970). Since the parent compounds are not themselves chemically reactive, they must undergo metabolic conversion in vivo to activated forms capable of chemical interaction with the target molecules. The aryl hydrocarbon hydroxylase system, associated with the microsomal fraction of cells, is responsible for the conversion of PAHs into a number of oxidized derivatives (Jerina and Daly, 1974; Sims et al., 1973). This group of enzymes is inducible by a variety of substrates and probably plays a key role in both the detoxification and activation of carcinogenic PAHs (Gelboin, 1969a,b; Borden et al., 1973). Several laboratories (Gelboin, 1969a; Grover and Sims, 1969; Borden et al., 1973; Pietropaolo and Weinstein, 1975) have shown that incubation of tritium-labeled PAHs in a subcellular system containing rat liver microsomes and NADPH leads to covalent binding of the PAH to nucleic acids, but the precise reaction mechanism and structures of the derivatives have not been elucidated.

In 1950 Boyland proposed that arene oxides might be the primary intermediates in the oxidative metabolism of PAHs to hydroxylated derivatives and suggested that the transient and chemically reactive oxides may be the proximate carcinogens. Subsequent studies have established arene oxides as intermediates in the microsomal oxidation of PAHs and the K-region oxides of DMBA (Keysell et al., 1973) and other PAHs (Sims et al., 1971; Grover et al., 1972) have indeed been detected after incubation of the parent hydrocar-

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[1] Abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; DMBA oxide, 7,12-dimethylbenz[*a*]anthracene 5,6-oxide; PAH, polycyclic aromatic hydrocarbon; poly(G)-DMBA, GMP-DMBA, and dGMP-DMBA are the nucleic acid products obtained after treating poly(G), GMP, and dGMP, respectively, with DMBA oxide (the DMBA abbreviation in these cases does not necessarily indicate that the parent hydrocarbon itself is present in the product); λ_{max}, wavelength of maximal absorbance in the ultraviolet (uv) spectrum; $A_{310}/A_{\lambda_{max}}$ ratio of absorbance at 310 nm to that at λ_{max}; MS, mass spectrum.

bon with microsomal preparations. For a review of this subject see Jerina and Daly (1974).

It has been demonstrated in cell cultures that K-region oxides of carcinogenic PAHs bind to cellular macromolecules to a greater extent than do the parent hydrocarbons or the related phenols or dihydrodiols (Kuroki et al., 1971). They are also generally more potent in transforming hamster and mouse cell cultures than the parent PAH (Kuroki et al., 1971; Marquardt et al., 1972). In the same studies oxides of noncarcinogenic PAHs such as phenanthrene and chrysene did not transform these cell cultures. Epoxide hydrase inhibitors, which diminish the conversion of oxides to hydroxylated derivatives, have been found to enhance the subcellular microsomal-dependent binding of PAHs to nucleic acids (Pietropaolo and Weinstein, 1975; Burki et al., 1974; Selkirk et al., 1974); they also enhance tumorigenesis by PAHs (Burki et al., 1974). Taken together, these findings support the hypothesis that oxides of PAHs may indeed be the proximate carcinogens responsible for the in vivo macromolecular binding and oncogenic effects of the parent compounds. Although the arene oxides were found to exhibit less carcinogenic activity than the parent hydrocarbon in intact animals (Miller and Miller, 1967; Miller, 1970), this may be due to their rapid dissipation in tissue before they can react with the critical target cells and macromolecules.

Previous investigators have demonstrated that in vitro incubation of tritium-labeled arene oxides with nucleic acids, in the absence of microsomes, leads to the incorporation of radioactivity into the nucleic acid (Grover and Sims, 1970, 1973). This approach did not establish the covalent binding of the PAH to nucleic acid since the result could have been due to tritium exchange. In the present study we have succeeded in modifying nucleic acids with the K-region oxide of DMBA to an extent that has allowed us to monitor binding by spectral means. Subsequently hydrolysis and chemical analyses have established the *covalent* linkage of DMBA oxide to guanine residues when the substrate was poly(G). In addition, we have for the first time demonstrated covalent binding following the reaction of an arene oxide with a mononucleotide, either GMP or dGMP. Evidence has been obtained that these reactions yield at least two guanine–DMBA conjugates and preliminary structural information is presented. Brief reports of our results have appeared elsewhere (Weisgras et al., 1974; Grunberger et al., 1974). During the preparation of this manuscript Swaisland et al. (1974a,b) have also published evidence that PAH oxides bind to poly(G) with the formation of at least two guanine derivatives.

Materials and Methods

Dimethylbenz[*a*]anthracene and cellulose thin-layer chromatography (TLC) sheets (6065) were purchased from Eastman Kodak. DMBA 5,6-oxide and the corresponding K region cis and trans diols were prepared as described (Goh and Harvey, 1973; Harvey et al., 1975). [^{3}H]GMP (specific activity 10 Ci/mmol) and [^{14}C]GMP (specific activity 43.4 mCi/mmol) were obtained from Schwarz/Mann. Guanosine monophosphate, deoxyguanosine monophosphate, and 8-bromoguanosine monophosphate were purchased from Sigma Chemical Co. Nucleic acids were obtained from the following sources: yeast tRNA, Plenum Scientific Research Inc.; calf-thymus DNA, Worthington Biochemical Corp.; poly(G) and poly(I), Schwarz/Mann; poly(A), poly(U), and poly(I), Miles Laboratories, Inc. To prepare denatured DNA, native DNA was heated in H_2O at 100° for 2 min and quickly cooled. Bacterial alkaline phosphatase (36 units/mg) was purchased from Worthington Biochemicals and T_1 ribonuclease from Calbiochem. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals and silica gel GF plates (250 μ) were from Analtech Inc.

Spectra. Absorption measurements were determined on a Zeiss spectrophotometer (Model PMQ II) and a Cary recording spectrophotometer (Model 14). Fluorescence measurements were conducted on an Aminco-Bowman or a Perkin-Elmer (Model MPF-2A) spectrophotofluorometer, fitted with a xenon lamp and 1-cm light path. One A_{260} unit is that amount of material which has an absorbance of 1 when dissolved in 1 ml of water and measured at 260 nm with a 1-cm light path. The absorption spectra in Figures 1 and 4 have been presented to illustrate qualitative differences (and similarities) between the starting materials and the modified products. The spectra presented do not take into account differences in molar extinction coefficients of the materials. The ϵ for GMP at pH 7 is 13.5×10^3 at 252 nm and for DMBA 5,6-oxide in ethanol it is 4.0×10^4 at 275 nm and 4.1×10^4 at 266 nm. Low-resolution mass spectra were recorded on a JOEL (Model JMS-07) mass spectrometer.

Reaction of Nucleic Acids with DMBA 5,6-Oxide. Solutions containing about 1 mg of the different synthetic polyribonucleotide homopolymers or tRNA in 0.20 ml of water, or 1 mg of DNA in 0.4 ml of water, were mixed with an equal volume of acetone. One milligram of DMBA 5,6-oxide in 0.20 ml of acetone was added and the mixture incubated at 37° for 18 hr in the absence of light. Under these solvent conditions the nucleic acids and the oxide remained in solution although a faint turbidity was sometimes observed at the end of the incubation. Unbound hydrocarbon derivatives were removed by ether extraction (5–10 times), the nucleic acid was precipitated from the aqueous phase by addition of 0.1 vol of 2.0 *M* sodium acetate (pH 5.0) and 2.5 vol of ethanol, and the mixture was left at −20° overnight. The precipitate was collected by centrifugation at 5000 rpm and the pellet was dissolved in 0.5 ml of water. Absorption spectra on aliquots of this material were determined in water. Similar reaction conditions were scaled up to prepare larger amounts of DMBA modified nucleic acids.

Alkaline Hydrolysis of Poly(G)-DMBA. About 20 A_{260} units of poly(G)-DMBA was incubated in 2 ml of 0.33 *N* KOH at 37° for 18 hr in the absence of light. The mixture was neutralized and then chromatographed on an LH-20 column (see below).

Reaction of Mononucleotides with DMBA 5,6-Oxide. Twelve milligrams of dGMP or GMP was dissolved in 2 ml of water and then 2 ml of acetone followed by 2 ml of acetone containing 12 mg of DMBA 5,6-oxide were added. This mixture gave a clear solution which was incubated at 37° for 18 hr in the absence of light. The acetone was removed with a stream of nitrogen and the turbid suspension extracted 4–6 times with ether to remove unbound hydrocarbon derivatives. The aqueous solution containing a mixture of modified and unmodified nucleotides was then chromatographed on an LH-20 column.

Alkaline Phosphatase Hydrolysis of GMP-DMBA. Five A_{260} units of modified nucleotide was dissolved in 0.10 ml of 0.02 *M* NH_4HCO_3 (pH 8.2) containing 200 μg of *Escherichia coli* alkaline phosphatase and the mixture was incubated at 37° in the dark for 24 hr. The reaction mixture

Table I: Extent of Binding of DMBA 5,6-Oxide to Different Nucleic Acids.

	$A_{310}/A_{\lambda_{max}}$	
Polymer	Polymer Itself	DMBA Oxide Reaction Product[a]
tRNA	0.003	0.059
DNA	0.006	0.042
Poly(U)	0.000	0.003
Poly(C)	0.001	0.020
Poly(A)	0.000	0.067
Poly(G)	0.016	0.143
Poly(I)	0.000	0.020
Poly[d(G)]	0.013	0.097

[a] Nucleic acids were incubated with DMBA oxide for 18 hr, extracted with ether, and ethanol precipitated as described under Materials and Methods. When poly(G) was incubated under similar conditions with DMBA rather than DMBA oxide the reisolated material had a $A_{310}/A_{\lambda_{max}}$ ratio of 0.024.

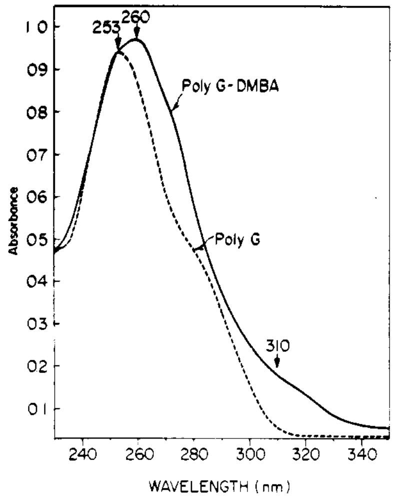

FIGURE 1: Absorption spectra of poly(G)-DMBA (—) and unmodified poly(G) (- - -) measured in water.

was then either chromatographed on an LH-20 column or analyzed by TLC.

Acid Hydrolysis of dGMP-DMBA Derivatives. The two products obtained after reaction of dGMP with DMBA 5,6-oxide (peaks I and II of the LH-20 column, Figure 5) were pooled separately, neutralized with 2.0 *N* HCl, and taken to dryness. The resulting powders were dissolved in 1 ml of 0.1 *N* HCl and incubated at 37° for 16 hr in the absence of light. The solutions were neutralized with 2.0 *N* NaOH and extracted with ethyl acetate. The ethyl acetate phase, which contained the DMBA-modified guanine products, was concentrated by evaporation and the modified bases were further purified by silica gel TLC.

LH-20 Column Chromatography and Thin-Layer Chromatography. Glass columns were packed with Sephadex LH-20 and equilibrated with 0.02 *M* NH_4HCO_3 (pH 8.2) and eluted as described in legends to Figures 3 and 5. When nucleoside products obtained from poly(G)-DMBA were chromatographed, the columns were equilibrated with 40% methanol-60% 0.02 *M* NH_4HCO_3 prior to elution. Glass distilled water was used in preparing the buffer solutions, and reagent grade methanol was distilled prior to use.

GMP-DMBA obtained either from reactions of the monomer or alkaline hydrolysis of poly(G)-DMBA had the following R_f values on cellulose TLC: 0.63 (2-propanol-concentrated ammonia-water, 7:1:2); 0.75 (isoamyl alcohol-water-acetic acid, 3:1:2); 0.88 (2-propanol-concentrated hydrochloric acid-water, 7:1:2). In the respective solvents GMP had the following R_f values: 0.12, 0.30, 0.30.

Results

Binding of DMBA 5,6-Oxide to Nucleic Acids. DMBA and related derivatives show significant uv absorbance at 310 nm whereas unmodified nucleic acids have negligible absorbance in this region. The extent of DMBA 5,6-oxide binding to nucleic acids was therefore assessed from the ratios of absorbance at 310 nm to the absorbance at maximum (absorption maximum) of the modified polymer. The results of binding studies with a variety of nucleic acids are summarized in Table I. It is apparent that DMBA oxide reacted best with poly(G), while significant binding to poly(A) was also observed. Poly(I) and poly(C) reacted to a slight extent and virtually no binding could be detected with poly(U). Under our conditions the oxide reacted equally with native and heat-denatured calf-thymus DNA. This aspect requires further study since the reaction conditions themselves may be associated with denaturation of the native DNA sample. Significant binding was also observed with tRNA.

When the reaction was run with DMBA rather than DMBA oxide only a trace amount of binding to poly(G) was observed (A_{310}/A_{253} = 0.024). Further studies are required to determine whether this represents physically bound (intercalated) material that resists extraction or material which is covalently bound as a result of a small amount of chemical or photooxidation. Since the greatest extent of modification was obtained with poly(G) most of our subsequent studies were conducted with this homopolymer as a substrate.

Characterization of the Reaction and Evidence for the Covalent Nature of the Poly(G)-DMBA Conjugate. A time course for the reaction of DMBA oxide with poly(G) indicated that 85% of the maximal binding had taken place after 6 hr. Only after 10 hr did the increase in amount of binding level off indicating that the oxide has a significant half-life under these conditions. Maximal binding was obtained at an approximately neutral pH; binding was inhibited at alkaline conditions, i.e. greater than pH 8–9.

The absorption spectrum of the poly(G)-DMBA product (Figure 1) revealed trailing in the 290–340-nm region which is characteristic of DMBA derivatives and is not present in the unreacted poly(G). In addition a shift in the λ_{max} from 253 nm for poly(G) to 260 nm for the modified poly(G) and broadening of the absorption peak were observed.

Poly(G)-DMBA also revealed intense fluorescence with an excitation maximum (uncorrected) of 310 nm and an emission maximum of 380 nm (Figure 2). These fluorescence maxima are similar to those obtained with free DMBA. The unreacted poly(G) exhibited a low intensity of fluorescence (approximately one-twentieth that of the modified material) with excitation and emission maxima at 293 and 343 nm, respectively. Repeated ether extraction of aqueous solutions of poly(G)-DMBA followed by ethanol precipitation did not change the absorption or fluorescence spectra of the product, indicating that we were probably dealing with a covalently bound material.

To further establish the covalent nature of the conjugate

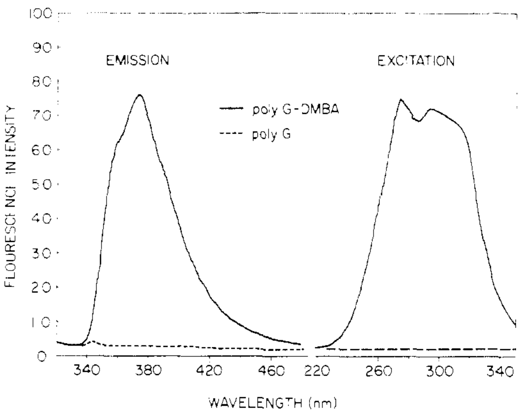

FIGURE 2: Uncorrected fluorescence emission (excitation at 310 nm) and fluorescence excitation (emission at 380 nm) spectra of poly(G)-DMBA (2 A_{260} units/ml; A_{310}/A_{260} = 0.13). As background the fluorescence emission (excitation at 295) and fluorescence excitation (emission at 343) of an equivalent amount of unmodified poly(G) were recorded on the same scale. The spectra were measured in water.

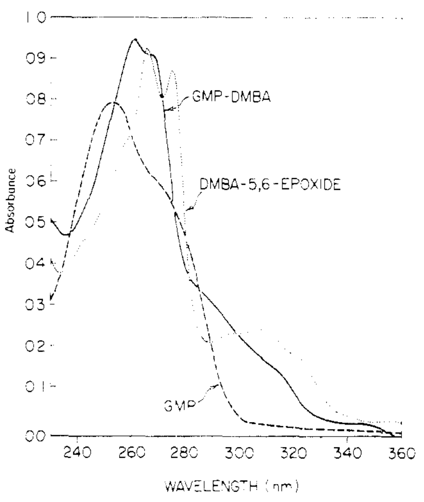

FIGURE 4: Absorption spectra of GMP in water (- - -), DMBA oxide in absolute ethanol (· · ·), and GMP-DMBA in water (—). The latter was obtained from an alkaline hydrolysate of poly(G)-DMBA as described in Figure 3.

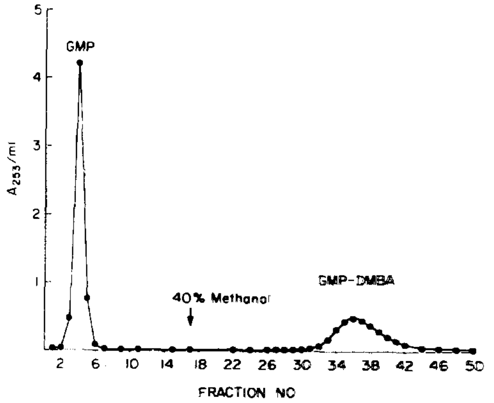

FIGURE 3: Chromatography of an alkaline hydrolysate of poly(G)-DMBA (18 A_{260} units; A_{310}/A_{260} = 0.13) on a Sephadex LH-20 column. The column (1.3 × 19 cm) was equilibrated with 0.02 *M* NH_4HCO_3 (pH 8.2) and the neutralized hydrolysate (1 ml) was applied. The unmodified GMP residues were eluted with the NH_4HCO_3 buffer at a flow rate of 0.5 ml/min. GMP-DMBA was then eluted with 40% methanol-60% 0.02 *M* NH_4HCO_3 (v/v) at a flow rate of 0.44 ml/min. Fractions of 2.5 ml were collected and assayed for absorbance at 253 nm.

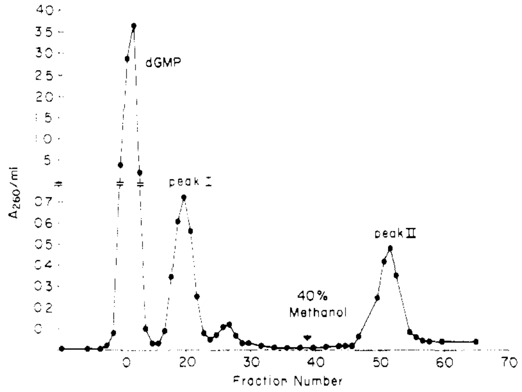

FIGURE 5: LH-20 column chromatography of dGMP (8 mg) previously treated with DMBA oxide (8 mg) in acetone-water and then ether extracted to remove unbound hydrocarbon derivatives. The column (1.1 × 50 cm) was equilibrated with 0.02 *M* NH_4HCO_3 (pH 8.2) and the sample applied in 1 ml of water. The column was first eluted with 95 ml of 0.02 *M* NH_4HCO_3 at a flow rate of 0.5 ml/min, and then 40% methanol-60% 0.02 *M* NH_4HCO_3 (v/v) at a flow rate of 0.4 ml/min. Fractions of 2.5 ml were collected and assayed for absorbance at 260 nm. Peaks I and II represent dGMP-DMBA complexes (see text).

formed following reaction of DMBA oxide with poly(G), hydrolysis studies were performed. Poly(G)-DMBA was treated with 0.33 *N* KOH and the hydrolysate chromatographed on a Sephadex LH-20 column as described in Figure 3. The large peak of material which eluted at the beginning of the column was guanine mononucleotide, as confirmed by its uv spectrum and chromatographic behavior on TLC. The material which eluted after addition of 40% methanol was a DMBA-GMP conjugate. This conclusion is based on the following evidence. The presence of a DMBA moiety was established by uv absorbance (Figure 4) and fluorescence spectra. The material gave a positive orcinol test, indicating the presence of ribose, and a less polar compound (the corresponding nucleoside) was obtained after treatment with alkaline phosphatase, indicating that we were dealing with a nucleotide derivative. The material was not just a simple mixture of the unmodified nucleotide and a DMBA derivative since it moved as one spot in three different solvent systems on TLC (see Materials and Methods) and the R_f values were identical with those found with a more extensively characterized product obtained by reacting GMP itself with DMBA oxide (see below). Studies in progress suggest that the GMP-DMBA product obtained from alkaline hydrolysates of modified poly(G) is not a single homogeneous substance.

In additional studies poly(G)-DMBA (14 A_{260} units) was hydrolyzed enzymatically, rather than with alkali, using T_1 ribonuclease (300 units in 600 µl of 0.02 *M* Tris-HCl (pH 7.5) at 37° for 23 hr) followed by incubation with alkaline phosphatase (4.3 units in 700 µl of 0.2 *M* NH_4HCO_3 (pH 8.2)) for 18 hr at 37°, and the digest placed on an LH-20 column. In addition to free guanosine, which eluted at the beginning of the column, a product that eluted with 80% methanol and had spectral characteristics expected for a guanosine-DMBA conjugate was obtained. Due to its high degree of secondary structure it was not possible, however, to obtain complete enzymatic digestion of the modified poly(G) and the yield was, therefore, low. The GMP-DMBA peak in Figure 3 was obtained after hydrolysis of poly(G)-DMBA with alkali. When this peak was subjected

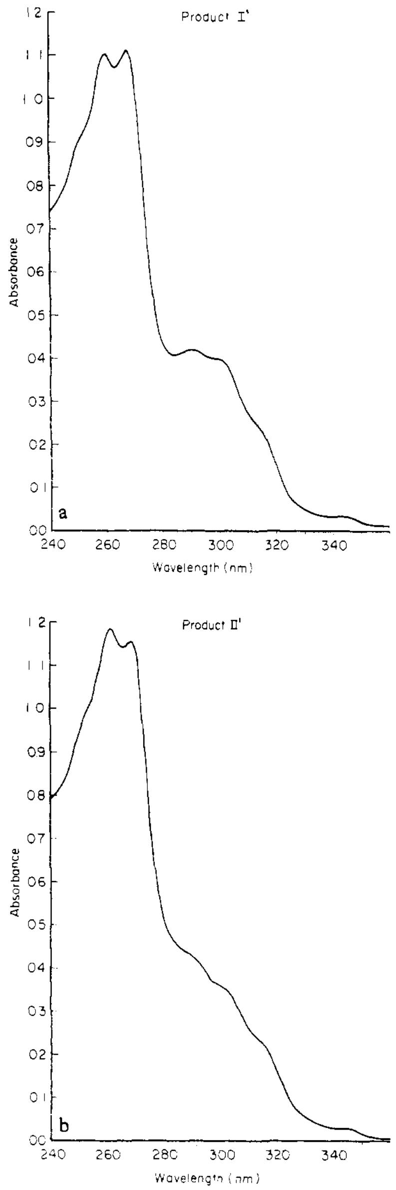

FIGURE 6: Absorption spectra of guanine–DMBA products obtained after acid hydrolysis of peaks I and II of Figure 5. I′ is the spectrum of the material obtained from peak I and II′ that obtained from peak II. Spectra were recorded in 90% ethanol–10% water (v/v).

to alkaline phosphatase to convert it to guanosine–DMBA it also eluted in the 80% methanol region of the LH 20 column suggesting that alkaline and enzymatic hydrolyses of poly(G)-DMBA liberate similar if not identical guanosine–DMBA derivatives. Attempts to liberate a guanine–DMBA residue directly from poly(G)-DMBA or from GMP-DMBA by strong acid hydrolysis (1 *N* HCl at 100° for 1 hr) have thus far been unsuccessful, perhaps due to the lability of the derivative under the harsh conditions required to hydrolyze the glycosidic bond.

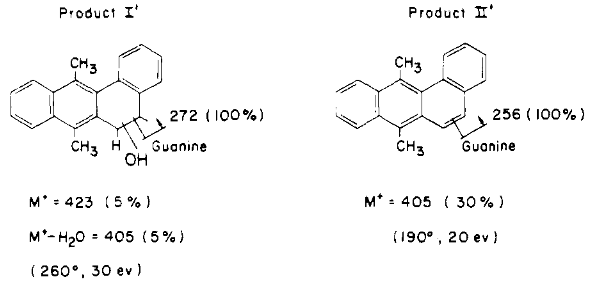

FIGURE 7: Pertinent ions observed in the low-resolution mass spectra of guanine–DMBA products I′ and II′. I′ and II′ correspond to the materials described in Figure 6.

Modification of Guanine Mononucleotides with DMBA Oxide. The data presented in Table I suggested that DMBA oxide preferentially reacts with guanine residues in nucleic acids. It was therefore of interest to see if we could obtain a reaction between DMBA oxide and guanine mononucleotide. Eight milligrams of dGMP was allowed to react with DMBA oxide and the water-soluble material remaining after ether extraction was fractionated on an LH-20 column (Figure 5). The first peak to elute from the column was unmodified dGMP, as indicated by its uv spectrum and R_f on TLC. Immediately following the dGMP peak there was a product (peak I) which from its uv spectrum corresponded to a dGMP-DMBA conjugate. The small peak following peak I was identified as free guanosine by its uv spectrum and R_f on TLC, reflecting a contaminant in the dGMP stock. Subsequent elution of the column with 40% methanol led to the elution of another product (peak II) whose absorption spectrum was also characteristic of a dGMP-DMBA derivative. Qualitatively similar results were obtained when GMP was allowed to react with DMBA oxide. Again two major products were obtained from the LH-20 column although there was relatively less of the product which eluted in the 40% methanol region and more of the product eluting in the buffer region than with dGMP.

The materials in peaks I and II (Figure 5) obtained from the reaction with dGMP were collected separately and subjected to acid hydrolysis in 0.1 *N* HCl to liberate the corresponding guanine–DMBA products. These were then extracted into ethyl acetate and purified by silica gel TLC. The R_f values of the modified bases obtained from each peak were identical (0.55) in the solvent system used (ethyl acetate–1-propanol–water, 4:1:2, upper phase). Peak I yielded 6 A_{260} units of product and peak II 1.6 A_{260} units. In addition, 0.7 A_{260} unit of a material having the same R_f (0.7), uv, and MS as an authentic sample of DMBA-5,6-diol was isolated as a contaminant in the hydrolysate of peak II.

The absorption spectra of the corresponding guanine–DMBA products, I′ and II′, are shown in Figure 6. Although they are quite similar, the distinct differences in the 280-nm region suggested that they have different structures and this was confirmed by their mass spectral cleavage patterns (Figure 7).

Low-resolution mass spectra of the guanine–DMBA product obtained after acid hydrolysis of peak I (Figure 5) indicated a parent ion (M^+) at *m/e* 423 corresponding to the sum of the elements of guanine–DMBA oxide. The base peak at *m/e* 276 (M^+ − guanine) was further evidence for a simple guanine–DMBA oxide adduct. A peak at *m/e* 405 (M − 18) was attributed to elimination of the elements of H_2O in the mass spectrometer. The latter was also observed in the MS of DMBA-5,6-diol, the vicinal glycols of benzopyrene (Selkirk et al., 1974), and is known to be a common cleavage pattern of alcohols. These results strongly suggest that the material in peak I is the anticipated product of nucleophilic attack of guanine on DMBA oxide, i.e., a hydroxydihydro-DMBA–guanine adduct. The modified base obtained from acid hydrolysis of the material eluting in the 40% methanol region, peak II of Figure 5, had a parent ion at *m/e* 405 corresponding to the mass of guanine plus

DMBA, while its base peak (*m/e* 256) corresponded to the mass of DMBA alone. This material was presumably formed by elimination of water from the guanine-DMBA oxide adduct during the initial reaction. With both of these materials there were no peaks in the mass spectra between the parent ion and the base peak suggesting that fragmentation occurred at the DMBA to guanine bond. Mass spectra have thus far, therefore, failed to reveal the site on guanine to which DMBA is bound.

Mild acid hydrolysis (0.1 *N* HCl, 37°, for 40 hr) of poly[d(G)] or DNA previously treated with DMBA oxide yielded materials with the same R_f values on silica gel TLC as the guanine-DMBA product obtained by acid hydrolysis of dGMP-DMBA. The precise structures of the DMBA adduct(s) derived from the nucleic acids by this approach remain to be established.

Additional Characterization of the Products. By treating DMBA oxide with [8-^{14}C]GMP and subsequently isolating and purifying the product we have been able to determine the molar extinction coefficient of the conjugate. In the case of peak I, Figure 5, this is 3.72×10^4 at 260 nm and 9.61×10^3 at 310 nm. Assuming a similar value for the products obtained with poly(G) we estimate that with our poly(G) product ($A_{310}/A_{260} = 0.13$) about 7-10% of the residues were modified by DMBA oxide. Utilizing GMP as a substrate, there is approximately a 1% conversion of the GMP to a GMP-DMBA complex.

When [8-^{3}H]GMP was allowed to react with DMBA oxide we observed a quantitative loss of tritium label in the modified product which eluted in the peak I region of an LH-20 column, whereas no loss of tritium was observed when the unmodified fraction of [8-^{3}H]GMP was reisolated from the reaction system. These results suggest that the hydrocarbon is bound either at the C-8 position or, since alkylation at N-7 of guanine is also known to labilize the C-8 proton (Tomaz, 1970a,b) of guanosine, at the N-7 position of GMP. The material in peak II of the column was not analyzed for tritium loss, since in this experiment a very low yield of this product was obtained. We have also found that 8-bromoguanosine 5′-phosphate reacted to about the same extent as GMP or dGMP with DMBA oxide indicating that a free C-8 proton on guanosine is not an absolute requirement for the oxide binding reaction.

Discussion

The binding of polycyclic hydrocarbon oxides to nucleic acids has previously been investigated (Grover and Sims, 1970, 1973; Kuroki et al., 1971) in vitro and in cell culture (see introductory statement), mainly by the use of tritium-labeled material. In the present study we have examined the reaction of DMBA 5,6-oxide with nucleic acids utilizing more direct spectral methods and chemical analyses. This approach is essential since labeled polycyclic hydrocarbons may readily undergo tritium exchange (Baird and Brookes, 1973; unpublished data from this laboratory) and eventually one wants to obtain quantitative amounts of products for structural analyses. The data which we have obtained extend the results of Grover and Sims (1973) and Swaisland et al. (1974a,b) indicating that guanine is the preferred target in nucleic acids when they react in vitro with PAH oxides. There is a lower but appreciable reaction with adenine residues, but we have not detected a significant reaction with inosine, uracil, or cytidine. A similar order of reactivity has been observed for the microsomal dependent binding of BP to nucleic acids (Pietropaolo and Weinstein, 1975), demonstrating a parallelism between the base specificity of the enzyme-catalyzed reaction of the parent PAH and the nonenzymatic reaction of the oxide derivative of the PAH.

In the present study we have provided direct evidence for *covalent linkage* of DMBA oxide to GMP residues in poly(G), rather than just physical entrapment in the polymer, by demonstrating that after hydrolysis of the modified nucleic acid we could detect a stable GMP-DMBA conjugate. We have also succeeded in demonstrating a direct reaction between a PAH oxide and either GMP or dGMP, although the product is obtained in low yields. Previous attempts to demonstrate this type of reaction with mononucleotides were unsuccessful (Grover and Sims, 1973). Under similar conditions we have thus far been unable to detect a reaction between DMBA oxide and AMP, CMP, or UMP.

At least two derivatives have been obtained from the reaction of dGMP with DMBA oxide. The first eluted from a Sephadex LH-20 column with aqueous buffer (peak I, Figure 5) and the second required 40% methanol for elution (peak II, Figure 5). Hydrolysates of the poly(G)-DMBA product yielded mainly peak II type material. Studies in progress indicate that the latter material may itself be heterogeneous. Mass spectral analyses suggest that peak I is a hydroxydihydro derivative of DMBA attached to guanine whereas the material in peak II is a simple DMBA-guanine conjugate. These results are consistent with the fact that peak I elutes earlier from the LH-20 column than peak II, since the hydroxyl group would make it less hydrophobic. We have not, however, excluded the possibility that the elements of H_2O were lost from the peak II material during the analysis. The absorption spectrum of the major GMP-DMBA product obtained from an alkaline hydrolysate of poly(G)-DMBA and its elution position from an LH-20 column are similar to those of the peak II product. Alkaline treatment of the peak I product did not convert it to peak II type material. These results suggest that in the reaction between poly(G) and DMBA oxide the only major product formed is the peak II derivative.

Final determination of the sites of attachment between the DMBA residue and guanine awaits nuclear magnetic resonance (NMR) and other structural analyses. We have, however, obtained indirect evidence on this question. Methylation of the N-7 position of guanosine is known to labilize the glycoside bond to acid hydrolysis (Jones and Robins, 1963) and enhances opening of the imidazole ring under alkaline conditions. The methyl-oxygen bond of O^6-methylguanosine is also extremely sensitive to hydrolysis (Friedman et al., 1963). We have found, however, that the stability of the DMBA-guanosine conjugates is similar to that of the unmodified nucleoside. In addition, the DMBA-guanine bond was not cleaved by mild acid treatment. To our knowledge, however, the stabilities of an aryl rather than an alkyl substituent on the N-7 or O-6 position of guanosine have not been previously studied. We cannot, therefore, rule out the possibility that the DMBA residue is attached to either the N-7 or O-6 position of guanine. The results obtained when [8-^{3}H]GMP was used as a substrate, however, suggest that DMBA is attached to either the N-7 or C-8 position of guanine. We have not detected significant shifts in the absorption spectra of the DMBA-guanosine derivatives as a function of pH, presumably due to masking by the DMBA chromophore, and therefore cannot make conclusions about p*K* values of the complex.

Arguments as to the position and stereochemistry of covalent binding of the DMBA moiety to guanine are also tentative. X-Ray crystallographic studies (Glusker et al., 1974) of DMBA oxide show a twisted ring structure with the oxide function oriented above the major ring system. Nucleophilic attack by guanine may be expected to occur axially from the opposite face at the most electrophilic center of DMBA 5,6-oxide which, on the basis of relative bond distances about the asymmetric oxide function, would be the 6 position. Studies on the direction of ring opening in unsymmetrical oxides (Jerina et al., 1973; Jerina and Daly, 1974) similarly predict preferential attack at the 6 rather than the 5 position of DMBA 5,6-oxide. Although steric crowding at the 6 position by the 7-methyl group may exert some influence on the direction of ring opening, it is likely to be minimal since the direction of attack is expected to be axial rather than equatorial. Assays of the carcinogenicity of the 5- and 6-methyl analogs of DMBA and the 5- and 6-fluoro-7-methylbenz[*a*]anthracenes have shown that the 6-substituted compounds were carcinogenic, whereas 5-fluorobenz[*a*]anthracene was inactive and 5-methyl-DMBA was only weakly active (Newman, 1974; Pataki et al., 1971; Miller and Miller, 1960, 1963). Although it is tempting to conclude for these results that the site of attachment to guanine is via the 5 position of DMBA, it is possible that substitution at the 5, but not at the 6 position may simply prevent in vivo activation by the microsomal oxidase system. The reactions of arene oxides with simple nucleophiles are often quite complex (Jerina et al., 1973) and it is conceivable that nucleophilic attack occurs elsewhere in the DMBA 5,6-oxide ring system. There is reasonably good correspondence between the uv spectra of DMBA-5,6-dihydrodiol, which has absorption maxima at 260, 270, and 300 nm (unpublished data), and the modified base obtained from peak I (Figure 5), which suggests that this product is a 5,6 adduct. The stereochemistry of opening the oxide ring is likely to be trans on the basis of preliminary work with smaller polycyclic compounds (Jeffrey et al., 1974), although this remains to be established with certainty. Clearly none of the above arguments are decisive and further studies are required to elucidate these structural features of the DMBA–guanine complexes.

During the course of these studies evidence has appeared suggesting that K-region oxides of PAHs may not be the important intermediates for DNA binding in vivo (Baird et al., 1973, 1975). Swaisland et al. (1974a,b) have made the specific suggestion that the 8,9-dihydroxy-10,11-oxide of benzanthracene is the reactive intermediate in vivo. In view of the multiplicity of products being obtained in our own and other laboratories, their relatively poor state of characterization, and the likelihood that even in vivo there will be tissue and/or species variations in patterns of PAH metabolism (Gentil et al., 1974), and therefore nucleic acid binding, we feel that it is too early to conclude that K-region oxides are unimportant in terms of carcinogenesis. In addition, we are hopeful that the techniques being developed, and the structural information obtained with the DMBA 5,6-oxide nucleoside derivatives, will be useful in isolating and characterizing other nucleoside complexes formed as a result of reaction with various derivatives of PAHs both in vivo and in vitro.

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Characterization and Properties of *Pholas* Luciferase as a Metalloglycoprotein[†]

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ABSTRACT: The luciferase of the bioluminescent boring mollusc, *Pholas dactylus,* has been purified by a new method which includes centrifugation in cesium chloride gradients. Homogeneous preparations have been obtained and molecular weight determinations and subunit analysis support the idea that this preparation is an oxyluciferin-luciferase complex. The preparation catalyzes oxidation of ascorbic acid in presence of H_2O_2, and this peroxidase activity has been used for characterization (thermal and pH stabilities, activity as a function of pH, isoelectric point, turnover number). The existence of two atoms of copper has been established and their involvement in the peroxidase activity indicated. Chemical analyses have shown that *Pholad* luciferase is a glycoprotein and the existence of glucosamine, fucose, mannose, and galactose residues has been demonstrated. The apparent buoyant density (1.340), the sedimentation coefficient (10.7 S), the Stoke's radius (83 Å), the partial specific volume (0.707), and the molecular weight (350,000) have been determined. The frictional ratio (f/f_0 = 1.8) derived from the Stoke's radius indicates that the molecule is asymmetric. The quaternary structure has been examined. Subunits of molecular weight 150,000 and 46,000 have been observed. The latter has electrophoretic properties identical with luciferin or oxyluciferin.

In many bioluminescent systems, it has been possible to characterize the enzyme (called luciferase, but different in each case) and the specific substrate (luciferin) which are responsible for the light emission in aerobic conditions. The luciferins are usually small molecules (mol wt <1000) which are oxidized to oxyluciferins which are often (in the excited state) the actual light emitters. In the boring mollusc *Pholas dactylus,* however, it has been shown that luciferin is itself a protein (mol wt ~45,000) with an oxidizable prosthetic group not yet identified (Henry et al., 1970, 1973). The *Pholas* system differs from most other bioluminescent systems in other important ways. Whereas in the absence of the corresponding luciferase, most luciferins can at best be induced to emit light under a very limited set of reaction conditions, *Pholas* luciferin is luminescent when treated with a striking variety of reagents (Henry and Michelson, 1970, 1973). With several of these reagents, it has been verified that the quantum yield and the spectral distribution of the emission thus obtained were not different from those of the enzymatic reaction. Moreover, the integrity of the protein is needed and in no case could light emission be induced in a tryptic digest of luciferin or from thermally denatured protein. We have also shown that the superoxide ion $O_2\cdot^-$ is a reactive intermediate common to the enzymatic as well as the nonenzymatic luminescence of *Pholas* (Henry and Michelson, 1973; Michelson and Isambert, 1973, Michelson, 1973a,b).

We have earlier described the limited activity of luciferase from *Pholas dactylus* as a peroxidase capable of oxidizing ascorbic acid in presence of H_2O_2 (Henry et al., 1973). In this respect luciferase shows a behavior very similar to that of horseradish peroxidase, one molecule of H_2O_2 oxidizing one molecule of ascorbic acid. Kinetic characteristics of this reaction have been described previously (Henry et al., 1973). The present communication describes further studies of this luciferase as a peroxidase and characterization of the enzyme as a metalloglycoprotein.

Experimental Section

Luciferase Assays. Peroxidase Activity. The absorption at 265 nm of ascorbic acid was used to measure its oxidation by luciferase, in presence of hydrogen peroxide. The standard assay mixture contained 0.15 μmol of H_2O_2, 0.1

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