Photo-induced Reactions of Benzo(a)Pyrene with DNA in Vitro

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ABSTRACT

The induction of lesions in DNA by combinations of benzo(a)pyrene (B(a)P) and near-ultraviolet light (NUV) with wavelengths of 300 to 480 nm was investigated. Using centrifugal methods or agarose gel electrophoretic techniques to monitor the conversion of the superhelical double-stranded PM2 DNA to the relaxed circular form, it was possible to quantitate the rates of single-strand breakage in the DNA when analyzed in neutral or alkaline conditions. Irradiation of a B(a)P and DNA mixture with NUV resulted in a 12-fold increase in the single-strand breaks observed, compared to breaks induced by radiation alone. In oxygen, there were about 40% more single-strand breaks induced compared to breaks induced in an argon environment. Rates of the single-strand breakage observed under alkaline conditions were 1- to 2-fold greater than those observed in neutral conditions. Using a phenol extraction procedure and molecular sieve column chromatographic methods, it was possible to quantitate the amount of B(a)P that was bound to the DNA as a function of NUV fluence.

In an argon-saturated medium, 12 B(a)P molecules were bound per PM2 genome per single-strand break, whereas about 16 B(a)P molecules were bound per PM2 genome per single-strand break when irradiation was performed in oxygen. From studies concerning the alkaline stability of “bound” B(a)P, it is concluded that as much as 30% of the B(a)P molecules bound in the presence of oxygen may be linked to the DNA through phosphotriester bonds. NUV-induced complexes of B(a)P and DNA that were enzymically digested to nucleoside residues and eluted through a Sephadex LH-20 column revealed several distinct peaks eluting through a 30 to 95% methanol gradient that were indicative of B(a)P-nucleoside adducts.

INTRODUCTION

PAH constitute a class of environmental pollutants. Their capacity to promote neoplastic transformation has been recognized for several decades (23). The mechanism(s) that is responsible for the triggering of neoplastic transformation is unknown; however, it has been determined that there exists a good correlation between the binding capacity of PAH to DNA and their "carcinogenic power" (6). The binding of PAH to cellular nucleophilic targets requires initial activation of the hydrocarbon (13). This activation is achieved by a complex metabolic route involving several enzyme-mediated oxidative steps in which phenolic and epoxide derivatives of the hydrocarbon are produced (9). Various in vitro and in vivo studies have been performed with B(a)P and its metabolized derivatives to determine their reactivity toward cellular nucleophilic components including DNA, RNA, and protein (25, 37, 52). It is currently believed that a diol-epoxide derivative of B(a)P, 7,8-dihydroxy-9,10-oxo-7,8,9,10-tetrahydrobenzo(a)pyrene, is the major reactive intermediate formed in the metabolism of B(a)P (54). This compound can link covalently to 3 of the 4 bases in both DNA and RNA (18, 33, 45) and possibly to the internucleoside phosphate groups (26). In addition to its binding to nucleic acids, metabolically activated B(a)P also induces strand breakage (10, 47) and conformational changes in the structure of DNA (22). Metabolically activated B(a)P and other PAH have been shown to be cytotoxic, mutagenic, and carcinogenic (14, 16, 44, 53).

In addition to enzymatic activation, PAH can be activated chemically to reactive radical intermediates and, via various radiations, to excited and/or oxidative states (11, 12, 15, 17, 29, 34). Gibson et al. (11) activated various PAH with 60Co γ-radiation, producing products that were both cytotoxic and mutagenic to Salmonella typhimurium. Photoexcitation of PAH was recognized over 4 decades ago in the experiments of Lewis (30) who showed an enhancement in killing of cultured chick embryo cells with combinations of PAH and light. Enhancement in skin tumors in mice exposed to B(a)P and NUV has been reported (7, 39). It was recognized by Ts'o et al. (48, 49) that chemical linkage between B(a)P and DNA in vitro could be achieved by exposing the mixture to NUV. The mechanism of activation of PAH by NUV is not well understood; however, there is evidence for the production of phenoxy radicals of B(a)P that was bound to the DNA as a function of NUV fluence. In addition, binding of a photoactivated PAH to DNA in cultured mammalian cells has been reported (5).

In this paper, we report our findings concerning lesions induced in DNA by B(a)P activated by NUV. In addition to quantitating strand breaks and alkali-labile lesions, we have attempted to quantitate the amount of B(a)P bound to the DNA as a function of NUV exposure. Furthermore, samples of NUV-induced B(a)P-DNA complexes have been isolated, enzymically digested, and eluted through a Sephadex LH-20 column in an attempt to determine the DNA base specificity in NUV-induced B(a)P linkage.

MATERIALS AND METHODS

Reagents. All chemicals were of analytical grade unless otherwise specified. Tris (Ultra Pure), sucrose (Ultra Pure,
density gradient grade), and cesium chloride (biological grade) were obtained from Schwarz/Mann (Orangeburg, N. Y.). [methyl-3H]Thymidine, [2-'4C]thymidine, and [G-3H]B(a)P were purchased from New England Nuclear (Boston, Mass.), and [7,10-'4C]B(a)P was purchased from California Bionuclear Corporation (Sun Valley, Calif.).

**PM2 DNA Isolation.** Propagation of radioisotopically labeled PM2 bacteriophage was as described previously (50). Phage was purified by differential centrifugation including 10 min at 10,000 x g to remove debris, followed by 7 hr at 28,000 x g which pelleted the phage particles. This pellet was resuspended in 0.65 M NaCl:0.025 M Tris:0.025 M CaCl2 (pH 7.0), and solid cesium chloride was added to a final density of 1.28 g/ml. Isopyknic banding of the phage was accomplished in a Beckman type 50 Ti rotor (Beckman Instruments, Inc., Palo Alto, Calif.) centrifuged at 40,000 rpm for 40 hr at 4°. The phage band was removed, dialyzed against 0.1 M NaCl:0.025 M Tris:0.001 M EDTA (pH 7.0), and the DNA was extracted as follows. The phage solution was adjusted to 0.5% sodium lauryl sarcosinate (Chemical Additives Co., Farmingville, N. Y.), and 3 repeated phenol extractions were performed at room temperature. This was followed by 2 repeated extractions with chloroform:isoamyl alcohol (24:1), and the resultant aqueous phase was extracted 3 times with ethyl ether. The extracted DNA solution was dialyzed against 0.001 M Tris:0.0001 M EDTA (pH 7.5) and was stored frozen at –70° after the addition of ethanol to 1 to 2%. We typically obtained 1 to 2 mg of purified PM2 DNA per liter of starting culture. The concentration of DNA was determined spectrophotometrically. The radioactivity of the DNA was determined spectrophotometrically. The radioactivity of the B(a)P initially was dissolved at 1 mg/ml in either absolute ethanol or dimethyl sulfoxide and stored in sealed containers in the dark, removal of B(a)P from the DNA was greater than 99.95% with one phenol extraction. Subsequent phenol extraction followed by ethyl ether extraction removed traces amounts of [A]P such that less than 0.01% of the starting B(a)P remained in the aqueous phase in control experiments.

**Sucrose Gradient Analysis.** Samples (50 to 100 µl) were layered onto preformed neutral sucrose gradients (5 to 20%) containing 0.5 M NaCl, 0.001 M Tris, and 0.0001 M EDTA (pH 7.5). Centrifugation was performed in a Beckman type SW 50.1 swinging bucket rotor at 50,000 rpm for 60 min at 4°. Each gradient was fractionated into 32 aliquots, to which were added 0.9 ml of water and 10 ml of Aquasol II (New England Nuclear, Boston, Mass.) for radioactivity determination by scintillation spectrophotometry. Alkaline sucrose gradients (5 to 20%) contained 0.9 M NaCl and 0.2 M NaOH and were centrifuged as above except for a running time of only 50 min. Twenty equal fractions were collected and neutralized with dilute HCl; after the addition of 15 ml of Aquasol II, they were monitored for radioactivity.

**Agarose Gel Electrophoresis.** Electrophoretic separation of the closed circular PM2 DNA (Form I) from the nicked forms (II) and linear duplex (Form III) was achieved by a modification of the procedures of Johnson and Grossman (21). Horizontal slab gels containing 0.6% agarose (w/v) (Bio-Rad Laboratories, Richmond, Calif.) and ethidium bromide, 1 µg/ml in 0.04 M Tris:0.005 M sodium acetate:0.001 M disodium EDTA, adjusted to pH 8.2 with acetic acid, were poured to a thickness of approximately 3 mm in 10- x 20-cm bridges of the Bio-Rad Model 1400 electrophoresis cell. Ten wells/bridge (approximately 1.5 x 7 x 2.5 mm) were formed with a comb. Samples containing 1 µg or less of DNA were adjusted to 10% glycerol and to 0.002% bromophenol blue before application to each well in a total volume of 20 µl. Samples subjected to alkaline hydrolysis were neutralized with HCl before application to the gel. Gels were run in 0.04 M Tris:0.005 M sodium acetate: 0.001 M disodium EDTA buffer at 80 V (4 V/cm) for 3 hr, during which time the marker dye migrated about 12 cm. The mobilities relative to the dye of native PM2 Forms I, II, and III (linear duplex) were 0.50, 0.37, and 0.39, respectively, whereas that of the denatured Form I was 0.55, and the denatured components of Forms II and III (single-strand rings and rods) migrated together with a mobility of about 0.58. Each band of DNA, visualized by ethidium bromide fluorescence, was cut from the gel slab, melted at 93° in a scintillation vial containing 1 ml of water, and mixed with 10 ml of Aquasol II scintillation cocktail. The quantity of DNA was determined by liquid scintillation spectrophotometry.

**Molecular Sieve Column Chromatography.** Glass barrel columns [0.7 x 10 cm (analytical) or 0.9 x 35 cm (preparative)] were packed with degassed Bio-Gel A-5m agarose gel beads (Bio-Rad Laboratories) and washed with 0.005 M ammonium acetate. Samples (0.2 to 2.5 ml) were layered carefully, absorbed into the gel, and eluted into fractions of 0.25 or 0.50 ml each with 0.005 M ammonium acetate. Aliquots of each fraction were monitored for radioactivity. Peak fractions to be used in subsequent experiments were pooled, frozen, and lyophilized.

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Enzymic Digestion of DNA. Digestion of DNA to deoxyribonucleosides was accomplished using the procedure of Baird and Brookes (1) with the exception that phosphodiesterase and alkaline phosphatase additions were repeated one time during the noted incubation times. DNase I from bovine pancreas, phosphodiesterase from Crotalus adamanteus venom (type II), and alkaline phosphatase from Escherichia coli (type III) all were obtained from Sigma Chemical Company (St. Louis, Mo.). Digested samples were stored frozen at −70° until used.

Sephadex LH-20 Column Chromatography. A glass barrel column (1.5 x 28 cm) was packed with Sephadex LH-20 dextran gel (Pharmacia, Piscataway, N. J.) that had been swollen overnight in 30% methanol. Enzymically digested DNA samples were layered onto the gel, absorbed, and washed with 30% methanol (about 75 ml total). The column was then subjected to a linear 30 to 95% methanol gradient (about 190 ml total) followed by a 100% methanol wash. The eluted material was fractionated into 2-ml aliquots which were monitored for radioactivity.

RESULTS

It has been reported that metabolically activated B(a)P can induce strand breakage in DNA with low efficiency (10, 47). By monitoring the conversion of the superhelical form of PM2 DNA (I) to the open, relaxed circle (II), we have been able to quantify the rate of single-strand breakage in the DNA by simultaneous exposure to B(a)P and NUV. Neutral or alkaline sucrose gradient analyses were performed on samples of PM2 DNA exposed to NUV with or without the addition of B(a)P at a mass ratio of 10:1 (DNA:B(a)P). From the sedimentation profiles, the percentage of remaining PM2 I was calculated and plotted versus irradiation time. The data for experiments in which the samples were bubbled with either oxygen or argon are shown in Chart 1. The rates of induction of single-strand breaks and alkali-labile lesions were calculated from these data and are presented in Table 1. Single-strand breaks are induced in the DNA in the presence of B(a)P at a rate 12 times the rate observed for DNA exposed to NUV alone. When the irradiation is performed in an oxygen environment, about 40% more single-strand breaks are induced compared to breaks formed in the DNA when the irradiation is performed in argon. An additional alkali-labile lesion is induced by the radiation for each single-strand break formed in oxygen, whereas about 2 alkali-labile lesions are induced per single-strand break formed in argon. However, the sum total of radiation-induced single-strand breaks and alkali-labile lesions in mixtures of DNA and B(a)P is the same whether the irradiation is performed in oxygen or argon.

When mixtures of DNA and B(a)P are exposed to NUV, there is a fluence-dependent increase in the amount of B(a)P that partitions into the aqueous phase upon phenol extraction. Using molecular sieve column chromatography, we have been able to isolate and quantitate the portion of this aqueous-soluble material that is tightly associated with the DNA. These radiation-induced "bound" molecules include those covalently linked to the DNA and perhaps molecules that are associated with the DNA through strong, noncovalent (e.g., hydrophobic) interactions. Chart 2 demonstrates the elution pattern through an agarose column (Bio-Gel A-5m) of unlabeled PM2 DNA that had been NUV-irradiated in the presence of oxygen and a mixture of [G-3H]B(a)P, and [7,10-14C]B(a)P. Only the aqueous phase after phenol extraction of the irradiated solution was applied to the column. The peak at Fraction 20, coincident with the excluded PM2 DNA, contains both 3H and 14C radioactivity,
binding of B(a)P to DNA in the presence and absence of oxygen and verifies the oxygen enhancement. Both curves clearly are linear, and there is a 1.8-fold increase in the rate of binding when oxygen is present during irradiation. A summary of the binding rates and, for comparison, the rates of strand breakage and induction of alkali-labile lesions is found in Table 1. Binding of B(a)P to DNA accounts for the great majority of total DNA damage induced by photoactivated B(a)P. There are about 16 and 12 molecules of B(a)P bound per single-strand break for irradiations performed in oxygen and argon, respectively.

It seemed likely that the NUV-induced bound B(a)P was in the form of adducts with the DNA bases, as has been found with metabolically activated B(a)P. To test this hypothesis, 80% of this B(a)P is covalently linked to the DNA. The trailing, more diffuse, peak which is retained in the molecular sieve (Fractions 30 to 46) contains unbound but aqueous-soluble derivatives of B(a)P. Mass spectra of this material indicate the presence of a variety of phenols, diols, dihydrodiols, and quinones. In the case of [7,10-14C]-B(a)P, this unbound material accounts for about 50% of the aqueous-partitioned radioactivity; the remaining 50% is associated with the DNA. Unique to the 3H radioactivity profile is a third peak (Fractions 47 to 57), which contains more than 90% of the total aqueous-partitioned 3H. Most of this material is lost upon lyophilization, indicating an extensive photo-induced exchange of 3H from the B(a)P to water. To avoid this difficulty, all subsequent experiments were performed using [14C]B(a)P and 3H-labeled DNA. The 3H label in the DNA ([methyl-3H]-thymidine) was stable in the experimental conditions used.

The trailing, unbound peaks of oxidized B(a)P derivatives seen in Chart 2 indicate that photooxidation of the hydrocarbon might play a role in the photoactivation of B(a)P. If this is the case, we might expect a decreased amount of B(a)P bound to the DNA if the irradiation were performed in an anoxic environment. Chart 3 demonstrates that such a decrease did occur. The agarose elution profiles are shown for mixtures of [7,10-14C]-B(a)P and [methyl-3H]thymidine-labeled PM2 DNA through a Bio-Gel A-5m column. Samples were bubbled with either argon or oxygen in the dark prior to and during 60 min of irradiation with NUV.

Chart 4 shows the dose-response curves for photo-induced binding of B(a)P to DNA with NUV. Mixtures of [7,10-14C]-B(a)P and [methyl-3H]thymidine-labeled PM2 DNA were irradiated with NUV for various times in oxygen or argon. The samples were extracted with phenol and eluted through Bio-Gel A-5m columns (0.7 x 10 cm). The amount of B(a)P that eluted with the excluded PM2 DNA was determined from known specific activities from which the number of molecules of B(a)P per PM2 genome [m.w., 6.7 x 10^9 (21)] was calculated. Each point represents the average of a minimum of 3 independent determinations.

* W. D. Spall and G. F. Strnisk, Manuscript in preparation.
NUV-induced B(a)P-DNA complexes were phenol extracted, eluted through a Bio-Gel A-5m column, enzymically digested to nucleosides, and applied to a Sephadex LH-20 column. The column was washed with 30% methanol and subjected to a linear 30 to 95% methanol gradient. The results are shown in Chart 5A. About 50% of the B(a)P label elutes in the 30% methanol wash preceding the elution of free nucleosides (note that only thymidine is labeled with $^{3}H$). The composition of this material remains undetermined but may include unextracted B(a)P photoproducts or B(a)P bound to DNA oligomers rendered resistant to enzymic digestion due to the nature of the adduct(s). In the 30 to 95% methanol gradient, the region where B(a)P-nucleoside adducts should elute (25, 36, 43), there are several resolvable peaks containing $^{14}C$. One peak elutes prior to the marker 4-(p-nitrobenzyl)pyridine, and 2 major and perhaps several minor B(a)P-containing products elute after the marker. The elution pattern of a mixture of unirradiated [G-$^{3}H$]B(a)P and the aqueous fraction after phenol extraction of [7,10-$^{14}C$]B(a)P irradiated with NUV in the absence of DNA is found in Chart 5B. Both samples contain 2 peaks of material in the 30% methanol wash that are similar, although in much smaller quantity, to those present in the digestion of B(a)P-DNA complexes. These early-eluting peaks account for greater than 95% of the applied material in the case of NUV-irradiated B(a)P and for a minor component of the unirradiated B(a)P, further indicating that B(a)P photoproducts probably elute in this wash region. Unaltered B(a)P binds tenaciously to the column and elutes only upon washing with 100% methanol. It is clear that neither the unirradiated B(a)P nor the B(a)P irradiated in the absence of DNA contains material that corresponds to the putative B(a)P-nucleoside adducts seen in Chart 5A from Fractions 70 to 135.

Another possible site of B(a)P binding to DNA is the internucleoside phosphate group. Alkylating agents are known to react with DNA, resulting in the formation of phosphotriesters (2, 27). Koreeda et al. (26) have reported that a 7,8-diol-9,10-epoxide of B(a)P reacts with polyguanylic acid mostly at the N-2 position of guanine but that 10 to 15% of the B(a)P was released by treatment with alkali, a characteristic property of alkyl phosphotriesters (40, 41). This finding, together with the apparent formation of a hydrocarbon-phosphate adduct upon mixing the diol-epoxide of B(a)P with phosphate buffer, led them to speculate that aryl phosphotriesters accounted for a significant portion of the hydrocarbon-polymerucleotide adducts. If such triesters are formed in the present study, they should be evident upon alkali treatment not only by strand breakage but also by hydrolytic release of some fraction of the bound hydrocarbon (40, 41). The lability of the NUV-induced B(a)P-DNA complex to hydrolysis in 0.4 M NaOH is shown in Chart 6. Within 4 hr at $37^\circ$, about 25% of the B(a)P originally bound to the DNA is no longer excluded with the polymer through Bio-Gel A-5m but is found in the trailing "unbound" peak. Beyond 4-hr incubation in alkali, there is a gradual release of B(a)P from the DNA that parallels the release from the control sample incubated at neutral pH. The nature of this loss of bound B(a)P is not known but could result from the slow release of unstable, aryalted bases in the DNA. In any event, the data suggest that about 25% of the bound B(a)P as detected by column analysis may be linked to DNA as phosphotriesters.

It is tempting to correlate the alkali-induced strand breaks reported above with the observed release of B(a)P from the DNA, since both observations are consistent with the existence of aryl phosphotriesters. However, the possibility must be considered that the 2 effects of alkali treatment are unrelated. This caution is especially warranted, since diol-epoxides of B(a)P have been reported to form adducts with DNA at the N-7 position of guanine that depurinate readily at neutral pH, giving...
rise to alkali-labile apurinic sites (24). Shooter and Merrifield (40, 41) have reported that, for DNA containing both apurinic sites and alkyl phosphotriesters, the 2 lesions can be distinguished on the basis of their different degrees of sensitivity to alkali, the apurinic sites being significantly more sensitive. We examined the time kinetics of alkali-induced strand breakage in NUV-induced B(a)P:DNA complexes. Chart 7 shows that 2 classes of alkali-labile sites are distinguishable, similar to that found for alkylated DNA. A very alkali-sensitive component, completely hydrolyzed in 10 min, accounts for about one-half of the strand breaks induced over the course of the experiment. The slower component appears still incompletely hydrolyzed after 3 hr at 37°C in 0.2 M NaOH. For comparison, DNA modified by previously published chemical methods to produce apurinic sites and alkyl phosphotriesters was treated with alkali in parallel experiments. As seen in Chart 7, the rapidly hydrolyzed component is very similar to apurinic sites produced in mild acid, whereas the slowly hydrolyzed component shows kinetics similar to DNA containing ethyl phosphotriesters; the major alkali-labile lesion in DNA reacted with ethyl nitrosourea (46). NUV alone clearly produces few, if any, alkali-labile lesions of either class, indicating the involvement of B(a)P in both photoinduced processes.

**DISCUSSION**

PAH can be activated with light. In this report, we have examined the interactions of NUV-activated B(a)P with DNA *in vitro*. The mechanism(s) of photoactivation of PAH is not well understood; however, it has been suggested that electronic excited states may be involved (34). There are also data supportive of the suggestion that activation of PAH by light may proceed via an oxidative process resulting in reactive radical intermediates (5, 17). For example, Inomata and Nagata (17) have shown that the 6-oxybenzo(a)pyrene radical is produced upon light irradiation of B(a)P in benzene. This oxygenated radical also is produced in the metabolism of B(a)P from the autooxidation of 6-hydroxybenzo(a)pyrene, a major component of the PAH metabolites formed. The 6-oxybenzo(a)pyrene radical has been shown to react with various cellular macromolecules (35, 48), or it can be further oxidized, resulting in the production of 3 B(a)P diol epoxides (28, 32, 35, 48), with the simultaneous production of reactive oxygen intermediates including the superoxide anion radical, hydrogen peroxide, and hydroxyl radicals. We have found that when B(a)P in aqueous solution is exposed to NUV in the presence of oxygen, oxidized derivatives (phenols, diols, dihydrodiols, quinones) of the PAH are produced which partition into the aqueous phase during phenol extraction. Some of the B(a)P derivatives are separated from the B(a)P:DNA complexes in Bio-Gel A-5m columns (see Chart 3); however, they are absent if the irradiation is performed in an argon environment. This result suggests that possibly 2 different activation processes are involved in the photoactivation of B(a)P. When the irradiation is performed in argon, the activation of B(a)P may proceed via an electronic excited state, and with the addition of oxygen to the irradiation medium, further reactive species may be generated via a photo-induced oxidative process. This could explain the enhancement seen in the number of B(a)P molecules bound to DNA when the irradiation is performed in oxygen. Furthermore, the photoactivation of B(a)P may result in the production of reactive oxygen intermediates (free radicals) that could enhance the rate of strand breakage in the DNA when the irradiation is performed in oxygen. For example, hydroxyl radicals are known to induce single-strand breaks in DNA (38). A mechanism has been proposed in the metabolism of B(a)P that results in the indirect formation of base damage in DNA by reactive oxygen radical species produced as a consequence of B(a)P metabolism (8). Similar damage in the DNA could result indirectly as a consequence of NUV activation of B(a)P. Modifications to the base and sugar moieties could result in an increase in the alkali sensitivity of the polymer, resulting in additional strand scissions (51). In any case, the NUV is inducing structural, oxidative changes within the B(a)P molecules, resulting in lesion induction in the DNA molecule through either direct or indirect modes of action.

Exposure of B(a)P to NUV also causes extensive hydrogen exchange, as was evident from the data presented in Chart 2. Exchange of the 3H label from [G-3H]B(a)P to water is induced by the light. This effect was more pronounced (4-fold enhancement in exchange) if the irradiation was conducted in oxygen compared to argon (data not shown). In early experiments utilizing [3H]B(a)P, values obtained for binding of the PAH to DNA as a function of light exposure were consistently 40 to 50% lower than were values obtained from using [14C]B(a)P. Therefore, due caution must be exercised in analyzing data in which [3H]B(a)P is photoactivated, since the specific activity of the PAH will be substantially reduced due to 3H exchange.

Metabolically activated PAH can induce strand breaks in DNA (10, 47); however, it was found that breaks in the DNA...
constituted only a minor fraction of the modifications seen in the DNA (about 1%). For photoactivated B(a)P, the ratios of strand breaks to bound B(a)P were 0.06 and 0.08 for irradiations in oxygen and argon, respectively. No strand breaks or alkali-labile sites are induced, and no B(a)P molecules are bound if the mixtures of PAH and DNA are stored in the dark for 24 hr (with or without gas introduction).

Upon Sephadex LH-20 column analysis of nuclease-digested, NUV-induced B(a)P-DNA complexes, several resolvable peaks eluted in the methanol gradient similar to the elution patterns for B(a)P-nucleoside adducts metabolically formed. To determine the exact nature of the elution characteristics through Sephadex LH-20 of B(a)P-nucleoside adducts induced by NUV, we have performed experiments with the synthetic polymer, poly deoxyadenylate, cytidylate, guanylate, thymidylate, in which one of the 4 nucleosides was labeled with 3H to a high specific activity. Each polymer was irradiated separately in the presence of [7,10-14C]B(a)P with NUV, eluted through a molecular sieve column, digested enzymically to nucleosides, and chromatographed on Sephadex LH-20. For the polymer in which the 2'-deoxyguanosine was labeled, 2 peaks were evident, chromatographing between Fractions 100 and 120 which contained both 3H- and 14C-labeled material. The 2'-deoxyadenosine-B(a)P adducts eluted as a single peak between Fractions 130 and 140, and the 2'-deoxyctydosine-B(a)P adducts eluted between Fractions 145 and 150. No detectable adduct formation was seen between B(a)P and 2'-deoxythymidylate. From these data,5 we have assigned as B(a)P-2'-deoxyguanosine adds the 2 major peaks in Chart 5A eluting between fractions 100 and 120. Furthermore, 2'-deoxyadenosine adducts (Fractions 130 to 140), if formed, are few in number. Detection of 2'-deoxyctydylate adducts, if present, is beyond the sensitivity of our assay. The structure of the remaining material eluting through the methanol gradient is unknown to date. Experiments in which B(a)P-DNA complexes were formed by NUV in argon showed similar peak patterns on Sephadex LH-20, but the peaks were substantially reduced in magnitude (data not shown).

Although chemical characterization of the specific lesions currently is incomplete, some accounting of the lesion classes measured here, including strand breaks, alkali-labile breaks, and B(a)P-DNA adducts, is possible. For a mixture of DNA and B(a)P irradiated in the presence of oxygen, there are about 16 molecules of B(a)P bound to the DNA per single-strand break. Upon Sephadex LH-20 column analysis, about 50% of the applied "bound" B(a)P elutes where B(a)P-nucleoside adducts would chromatograph, and thus at least 8 of the 16 B(a)P molecules are bound to the nucleosides. Alkaline hydrolysis releases 25%, or 4 molecules, of the bound B(a)P and induces 1.1 additional strand breaks. Both aspects of alkali lability are consistent with the presence of aryl phosphotriesters and, assuming complete hydrolysis, predict that there are 5 such lesions.

There are conflicting reports on the presence of triesters in DNA reacted with the active B(a)P metabolite, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene. In such DNA, Shooter et al. (42) found no alkali-labile bonds that they would classify as triesters, while Koreeda et al. (26) observed an adduct in polyguanylic acid [10 to 15% of the total bound B(a)P] that was released as a tetraol by either heat or alkali, indicative of triesters in polyribonucleotides. Neither group simultaneously measured strand breakage and release of bound hydrocarbon.

Shooter et al. (40, 41) have reported that with phage DNA one-third of methyl and ethylphosphotriesters hydrolyzed in alkali result in strand breakage, whereas the majority revert to stable diesters upon loss of the alkyl group. If we assume that all of the alkali lability observed in this study is due to triesters, we conclude that one-fifth (1.1 of 5.1) of the ary triesters yield a strand break, perhaps indicating that the aryl phosphate linkage is even more labile than the alkyl phosphate. With ethyl triesters of dinucleosides, the proportion of hydrolysis leading to cleavage of the nucleotidyl linkage (analogous to strand breakage) is reportedly much higher than are either of these figures (averaging to 73%) (19, 20). Unlike the polymer work, these experiments dealt with a relatively homogeneous preparation of triesters and accounted for all the products of alkaline hydrolysis. The reason for the apparent discrepancy might be a genuine difference between the hydrolysis of a dinucleotide and a polynucleotide or, in the case of the present work, between alkylation and arylation. Nevertheless, it remains possible that a portion of the relatively large amount of alkali-induced release of B(a)P in the current work originates from some lesion other than phosphotriesters. The possibility suggested in Chart 7 that some of the alkali-induced strand breaks arise from apurinic sites further complicates correlation of these results. These uncertainties await more detailed chemical characterization of the lesions involved.

Companion experiments designed to investigate the photocarcinogenicity of PAH in cultured human fibroblasts are in progress to compare the effects seen in vitro to those induced in vivo.

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