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Acid Lability of the Hydrocarbon–Deoxyribonucleoside Linkages in 7,12-Dimethylbenz[*a*]anthracene-Modified Deoxyribonucleic Acid†

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ABSTRACT: DNA containing bound radioactive 7,12-dimethylbenz[*a*]anthracene was isolated from mouse fetal cell cultures exposed to this carcinogen. The carcinogen–deoxyriboside adducts within the DNA were found to be sensitive to acid-catalyzed hydrolysis. Adducts derived from reaction of a *syn*-dihydrodiol epoxide with deoxyadenosine residues in DNA were the most sensitive to acid and were hydrolyzed to yield a 1,2,3,4-tetrahydrotetraol of 7,12-dimethylbenz[*a*]anthracene under mild conditions. The structure of this tetraol was established by synthesis and mass spectrometry. Although definitive structures cannot be assigned at present to the nucleic acid adducts of this potent carcinogen, the present findings confirm and extend earlier work assigning partial structures to the major adducts.

Since 7,12-dimethylbenz[*a*]anthracene is one of the most potent tumor initiators among the polycyclic aromatic hydrocarbon carcinogens, we have been anxious to discover whether there are significant differences in the DNA interactions for this carcinogen and those for less potent, but more extensively studied, carcinogens such as benzo[*a*]pyrene. The metabolic activation of benzo[*a*]pyrene was shown by Sims et al. (1974) to involve formation of a vicinal dihydrodiol epoxide, and this was found to react almost exclusively with the exocyclic amino group of guanine residues in nucleic acids (Jeffrey et al., 1976b; Koreeda et al., 1976; Osborne et al., 1976). Minor products attributed to reaction with adenine residues have also been described (Jeffrey et al., 1979), though in mouse skin, these represent less than 3% of total DNA adducts (Ashurst & Cohen, 1981).

Metabolic activation of 7,12-dimethylbenz[*a*]anthracene is similarly believed to occur through a dihydrodiol epoxide (Baird & Dipple, 1977; Moschel et al., 1977; Vigny et al., 1977; Ivanovic et al., 1978), but in this case, three major adducts are formed in similar amounts, resulting from reaction of an *anti*-dihydrodiol epoxide to similar extents with deoxyguanosine and deoxyadenosine residues and from selective reaction of a *syn*-dihydrodiol epoxide with deoxyadenosine residues in DNA (Sawicki et al., 1983; Dipple et al., 1983). We now report that the 7,12-dimethylbenz[*a*]anthracene–deoxyadenosine adducts in DNA exhibit a remarkable instability in that they are hydrolyzed readily under mildly acidic conditions to yield hydrocarbon tetraols. Such reactions have not been reported for benzo[*a*]pyrene–DNA adducts.

Experimental Procedures

[G-^{3}H]-7,12-Dimethylbenz[*a*]anthracene was purchased from Amersham/Searle Corp. (Arlington Heights, IL) and purified on silicic acid as described earlier (Dipple et al., 1979).

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Servacel DHB was purchased from Accurate Chemical and Scientific Corp., Hicksville, NY. Methods used for growing primary cultures of NIH Swiss mouse fetal cells (Dipple et al., 1979), for isolation of DNA by CsCl gradients (Lieberman & Dipple, 1972), for enzymic hydrolysis of the DNA (Sawicki et al., 1983), for recovery of carcinogen–nucleoside adducts on Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) (Bigger et al., 1983), and for separation of these adducts by high-pressure liquid chromatography or Servacel DHB chromatography (Sawicki et al., 1983; Moschel et al., 1983) have all been described before. Ultraviolet absorption spectra were obtained on a Gilford Model 250 spectrophotometer and fluorescence spectra were measured on solutions in quartz tubes with a Perkin-Elmer MPF-3 spectrofluorometer.

Synthesis of 7,12-Dimethylbenz[a]anthracene 1,2,3,4-Tetrahydrotetraols. 7,12-Dimethylbenz[a]anthracene *trans*-3,4-dihydrodiol (1.16 mg) kindly prepared by our colleague Dr. A. Haber according to Sukumaran & Harvey (1979) was dissolved in tetrahydrofuran (1 mL) and mixed with pyridine (3 mL) and a 2.5% solution of OsO_4 in 2-methyl-2-propanol (0.25 mL). After 10 min at room temperature, 10% mannitol–1% KOH in water (10 mL) together with toluene (10 mL) was added, and the mixture was stirred at room temperature for 1 h. The upper toluene layer was then removed and concentrated to dryness to yield a yellowish solid, which was extracted with methanol and again concentrated to dryness. The residue was then dissolved in 45% methanol and subjected to chromatography on an Altex Ultrasphere ODS column under the conditions described in the legend to Figure 1. A small UV-absorbing peak, eluting with the first 15 mL of eluent, did not exhibit a UV absorbance consistent with a tetraol structure. The major product that eluted in fractions 56–58 and the minor product that eluted in fractions 84–87 both exhibited similar UV-absorption spectra with λ_{max} at 267 nm and long-wavelength absorption maxima of much lower intensity at 360, 380, and 400 nm. The fractions corresponding to the two products were separately pooled, diluted with a large volume of water (50 mL), and passed through Sep-Pak C_{18} cartridges. The tetraols were then recovered by elution of these cartridges with methanol. The solutions of each tetraol were subjected to further purification by elution in methanol from two Whatman Partisil PXS 10/25 ODS-2 columns joined in series, and the UV-absorbing fractions were pooled and evaporated to dryness.

Mass Spectral Analyses of Synthetic Tetraols. Mass spectrometric data were obtained on a reversed geometry VG ZAB-2F (8-kV) and a 70 EQ (6-kV) instrument (VG Analytical, Altrincham, U.K.) using a VG 11/250 data system. One microliter of a *N,N*-dimethylformamide solution of each tetraol was placed on the platinum coil of a commercially available VG direct-exposure DCI probe and allowed to dry for "in-beam" sample introduction. A linearly programmed current from 0 to 1.43 A was passed through the coil at a maximum rate of 160 mA/s, and the thermally desorbed molecules were ionized by electron-impact (50 and 70 eV) and by positive chemical ionization (NH_3). Repetitive scanning was accomplished at a rate of 1 s/decade (0.2-s interscan delay on the 70 EQ and 1 s on the ZAB-2F) with a resolution of 1000. The source temperature was 150 °C. Accurate mass measurements were obtained at a resolution of 5000 (5% crossing-over definition) with perfluorokerosene ions as references and a scanning rate of 3 s/decade (1-s interscan delay).

Results

Instability of 7,12-Dimethylbenz[a]anthracene–DNA Adducts. When DNA isolated from mouse embryo cells exposed to [^{3}H]-7,12-dimethylbenz[*a*]anthracene is enzymically hydrolyzed to deoxyribonucleosides and the hydrolysate is separated by high-pressure liquid chromatography, the elution of radioactive adducts follows the profile illustrated in Figure 1a (Sawicki et al., 1983; Dipple et al., 1983; Moschel et al., 1983; Bigger et al., 1983). The nature of the major adducts, i.e., II, IV, and V in Figure 1a, has been partially established such that it is known that these arise from the reaction of a bay region *anti*-dihydrodiol epoxide with deoxyguanosine (II), of a bay region *syn*-dihydrodiol epoxide with deoxyadenosine (IV), and of a bay region *anti*-dihydrodiol epoxide with deoxyadenosine (V), respectively (Sawicki et al., 1983; Dipple et al., 1983). These studies also indicate that adducts VI and

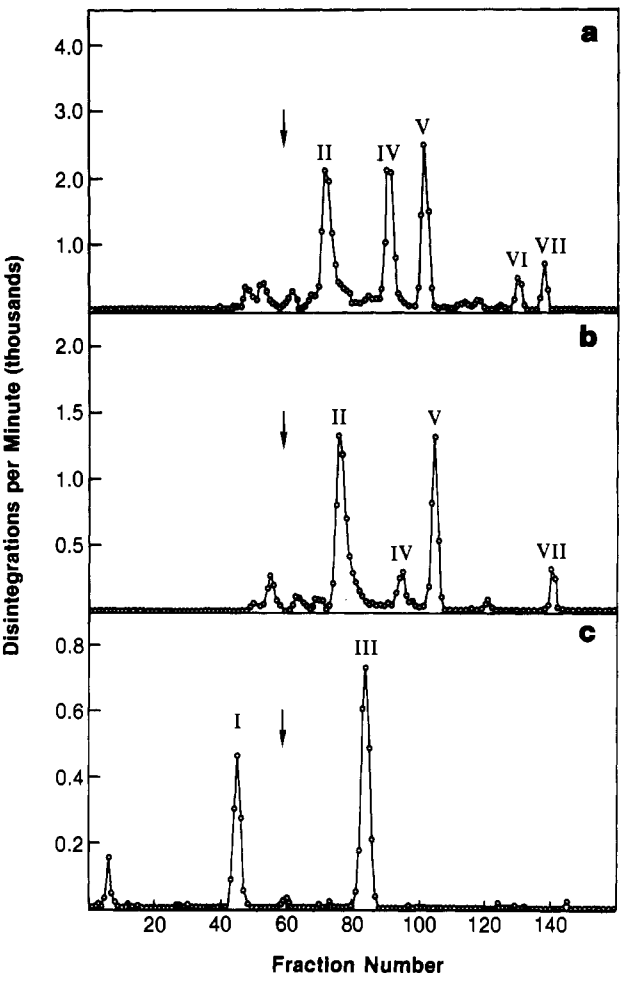


FIGURE 1: High-pressure liquid chromatographic separation of 7,12-dimethylbenz[*a*]anthracene–deoxyribonucleoside adducts [before (a) and after (b) incubation at pH 6.1 for 150 h at 37 °C] and of the 1-butanol-extractable breakdown products after incubation as above (c). A portion of DNA extracted from mouse embryo cells exposed to [^{3}H]-7,12-dimethylbenz[*a*]anthracene for 24 h was enzymically hydrolyzed to deoxyribonucleosides, and the adducts were recovered on a Sep-Pak cartridge and separated on an Altex Ultrasphere ODS (5 μm, 0.46 × 25 cm) column with a concave gradient from 45% to 65% methanol–water (No. 3 gradient on an LDC Gradient Master) over a 150-min period at a flow rate of 1 mL/min and collecting fractions each minute (a). Another portion of this DNA solution in 0.01 M Tris-HCl buffer–0.002 M NaCl, pH 7.5, was treated with 0.1 M KH_2PO_4 to obtain a final pH of 6.1 and was then incubated in the dark at 37 °C for 150 h. This solution was then extracted with an equal volume of water-saturated 1-butanol. The lower aqueous phase was washed with 2 volumes of ether to remove any butanol, and its pH was adjusted to 7.5 prior to dialysis against 0.01 M Tris-HCl–0.002 M NaCl, pH 7.5, for 20 h. It was then enzymically hydrolyzed and chromatographed (b). An aliquot (100 μL) of the butanol extract was loaded directly on to the Altex column and separated under the conditions used above (c). The arrow denotes the position of elution of an added marker of toluene.

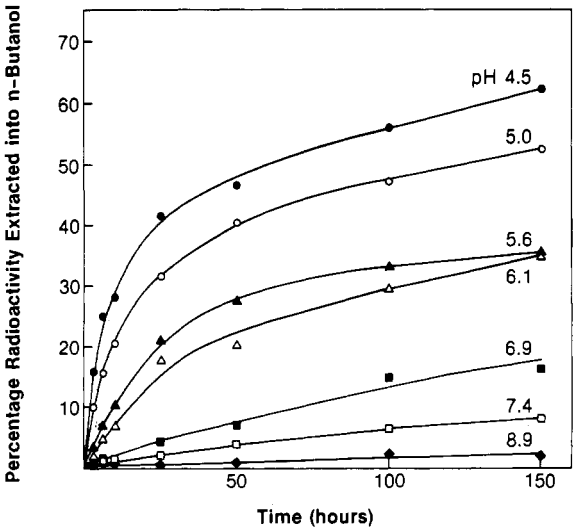


FIGURE 2: Time-dependent conversion of radioactivity in [^{3}H]-7,12-dimethylbenz[*a*]anthracene–DNA into a butanol-extractable form as a function of the pH of the DNA solution. Aliquots of the same DNA solution used for the experiments in Figure 1 were adjusted to the desired pH by diluting 7 volumes of DNA solution with 18 volumes of appropriate buffer as follows: for pH 4.5, 5.0, and 5.6, 0.2 M ammonium acetate was used; for pH 6.1 and 6.9, 0.1 M potassium phosphate was used; for pH 7.4 and 8.9, 0.2 M Tris-HCl buffer was used. Aliquots of solutions at each pH were extracted with water-saturated 1-butanol at the indicated times, and the radioactivity in the upper and lower phases was determined.

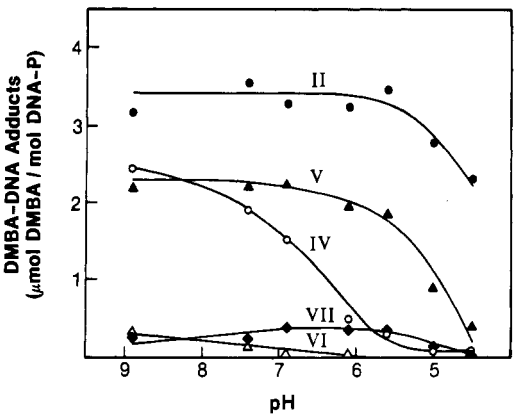


FIGURE 3: Disappearance of individual 7,12-dimethylbenz[*a*]-anthracene–DNA adducts in 150-h incubations of DNA solutions at various pHs at 37 °C. Solutions were extracted with 1 volume of water-saturated 1-butanol, and then, following the procedures described in the legend to Figure 1, the aqueous phase was analyzed as in Figure 1b. On the basis of radioactivity recovered in each chromatographic peak and the initial level of binding to DNA (14.7 μmol/mol of DNA P), the absolute amount (in μmol/mol of DNA P) of each adduct present in the aqueous phase was calculated.

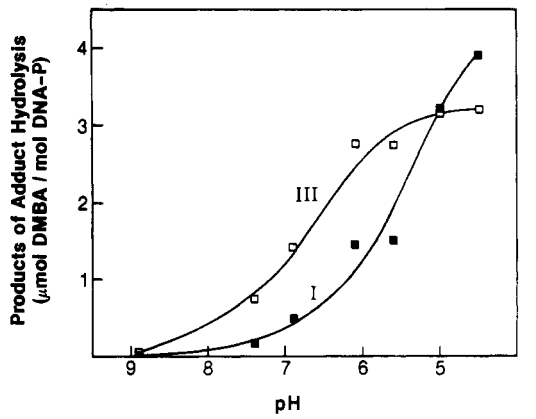
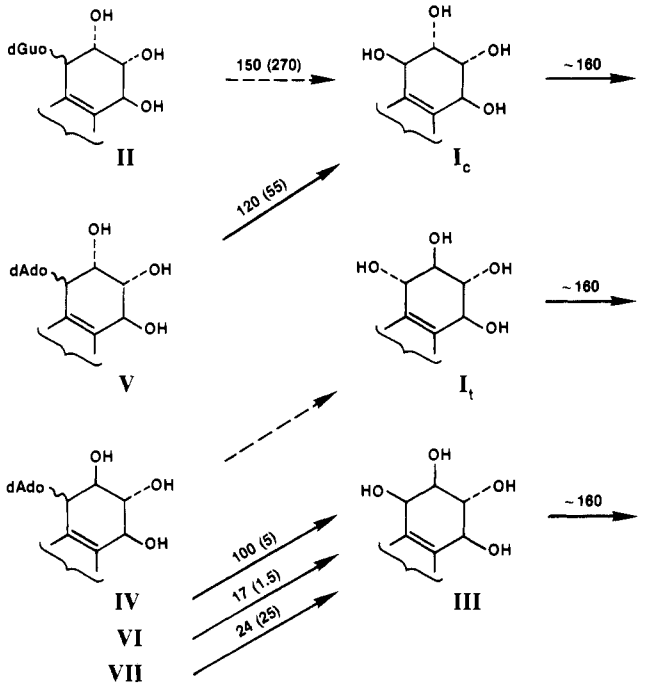


FIGURE 4: Appearance of 7,12-dimethylbenz[*a*]anthracene–DNA decomposition products in 150-h incubations of DNA solutions at various pHs, 37 °C. The butanol extracts referred to in the legend to Figure 3 were analyzed by high-pressure liquid chromatography as described for Figure 1c, and each breakdown product was quantified as described under Figure 3.

VII arise from reaction of some metabolite of 7,12-dimethylbenz[*a*]anthracene with deoxyadenosine residues in DNA, but insufficient material was recovered in these peaks for fuller characterization (Dipple et al., 1983). We now report that all of these 7,12-dimethylbenz[*a*]anthracene–deoxyribonucleoside adducts (i.e., II and IV–VII) are unstable at pH values below neutrality. Thus, when an aliquot of the DNA sample used for the experiment summarized in Figure 1a was incubated at pH 6.1, 37 °C, for 150 h prior to enzymic hydrolysis and chromatography, the radioactive adducts IV and VI (Figure 1a) were almost completely lost and two new radioactive components (I and III) appeared, such that the chromatographic profile obtained was essentially a composite of Figure 1b,c. Some indication of the chemical nature of the changes occurring was obtained when an aliquot of the DNA solution that had been incubated at pH 6.1 was extracted with water-saturated 1-butanol and it was found that some 35% of the total radioactivity was transferred into the organic phase, i.e., this radioactivity was no longer covalently bound to the DNA. Furthermore, following enzymic hydrolysis and chromatography, the DNA retained in the aqueous phase (Figure 1b) showed substantial losses of adducts IV and VI in particular, and the new radioactive products formed from this breakdown (i.e., I and III) were found to have been quantitatively transferred to the 1-butanol phase, which, on chromatography, exhibited the profile shown in Figure 1c. Since only the 7,12-dimethylbenz[*a*]anthracene was originally labeled with tritium, the breakdown products I and III must contain the hydrocarbon residue resulting from cleavage of the hydrocarbon–deoxyribonucleoside linkage or they could result from cleavage of the glycosidic bond in a hydrocarbon-substituted deoxyribonucleoside in DNA.

Time and pH Dependence of Adduct Breakdown in DNA. In order to better understand the nature of this carcinogen–DNA adduct decomposition, the conditions under which it occurs were investigated in some detail. Since only breakdown products are extracted from DNA solutions by 1-butanol, the percentage of total radioactivity extracted into butanol was used to monitor the time dependence and pH dependence of the decomposition. Figure 2 illustrates that the rate of decomposition increases with decreasing pH in the range 8.9–4.5 such that at near and above neutrality less than 10% decomposition is seen in 50 h at 37 °C while these conditions lead to 20%–50% decomposition at pH 6.1–4.5. In order to examine the fate of individual adducts in DNA, an aliquot of each DNA solution that had been incubated at a given pH for 150 h was extracted with butanol, and the aqueous phase, after enzymic hydrolysis, and the butanol extract were separately subjected to high-pressure liquid chromatography as described in parts b and c of Figure 1, respectively. This allowed the amount of each individual adduct (Figure 3) and each individual breakdown product (Figure 4) to be determined after a 150-h incubation at each pH value. Again, it is clear that the *syn*-dihydrodiol epoxide–deoxyadenosine adduct (IV) and adduct VI are the least stable and that these adducts show significant decomposition even at neutral pH (Figure 3). The *anti*-dihydrodiol epoxide–deoxyguanosine and –deoxyadenosine adducts (II and V, respectively) only decompose extensively below pH 7. Breakdown product III begins to appear at higher pH values than does breakdown

Scheme I



product I (Figure 4), indicating that it is produced from adduct IV.

In the same fashion, we have also monitored the rate of disappearance of individual adducts from DNA at a fixed pH of 4.5. Plots of the logarithm of the fraction of total adduct present at various times vs. time were linear, and half-lives in hours, corrected to two significant figures, for each adduct in DNA are given in parentheses in Scheme I. These demonstrate that the sensitivities of the various adducts in DNA to acid-catalyzed decomposition vary widely. The adducts exhibit half-lives for decomposition ranging from 1.5 and 5 h for VI and IV, the least stable adducts, to ~11 days for the *anti*-dihydrodiol epoxide–deoxyguanosine adduct, II, which is the most stable of the adducts. It was not possible to obtain accurate estimates of the rates of appearance of the breakdown products I and III because they were not totally stable themselves (Figure 5) and eventually gave rise to radioactive material eluting at the beginning of the elution gradient used for chromatographic analysis. A small peak was already present here in the experiment shown in Figure 1c. It is apparent in Figure 5, however, that product III is formed faster than product I but that after 50 h the amount of III present begins to decline, presumably because the rate at which it is being formed is then lower than its rate of decomposition.

Kinetics of Adduct Hydrolysis after Isolation from DNA. A remarkable aspect of our findings is that, once the adducts have been isolated from DNA, most of them exhibit labilities that are quite different from those exhibited within the DNA structure. It should be emphasized that the data presented to this point (Figures 1–5 and the half-lives in parentheses in Scheme I) are derived from monitoring the decomposition of 7,12-dimethylbenz[*a*]anthracene–DNA samples. In this study, however, the instability at pH 4.5, 37 °C, of individual adducts after enzymic hydrolysis of the DNA to deoxyribonucleosides was monitored. Again, linear semilogarithmic plots of adduct disappearance vs. time were obtained, and the half-lives (two significant figures) in hours are given in Scheme I. Comparison of these values with those in parentheses indicates that, of the two minor adducts, VII exhibits about the same half-life for decomposition at the nucleoside and DNA levels but VI is much more labile within the DNA structure. Similarly, the

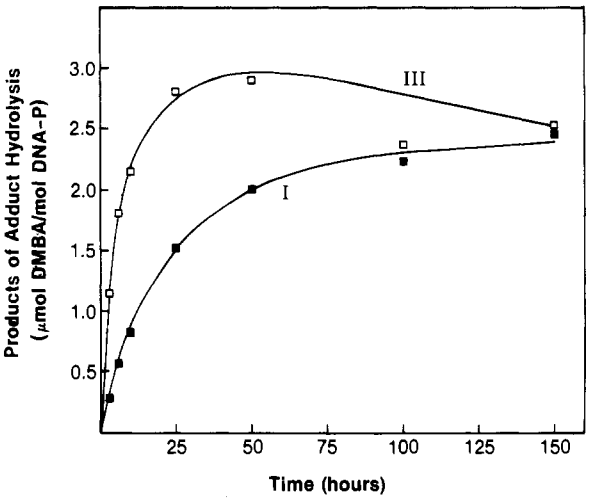


FIGURE 5: Time-dependent release of products I and III from 7,12-dimethylbenz[*a*]anthracene–DNA at pH 4.5, 37 °C. The data were obtained in the same experiment described in the legend to Figure 4.

major deoxyadenosine adducts, V and particularly IV, are both labilized in the DNA structure while, the major deoxyguanosine adduct, II, is stabilized by its environment in DNA.

Characterization of Breakdown Products. In order to understand these interesting decompositions, the nature of the breakdown products from 7,12-dimethylbenz[*a*]anthracene-modified DNA was investigated. DNA obtained from a large-scale incubation of 7,12-dimethylbenz[*a*]anthracene with mouse embryo cells was incubated at pH 4.5, 37 °C, for 46 h, and then the combined products, I and III, were isolated, and the fluorescence spectrum of these products was recorded on an aliquot of this mixture (Figure 6a). The remainder of the mixture was subjected to chromatography as in Figure 1c, and the fractions containing products I and III were separately pooled so that the fluorescence spectra of these separated materials could be examined (parts b and c of Figure 6, respectively). The long-wavelength maxima for I appear at 2–3 nm longer wavelength than those for III, but in both cases these maxima are at shorter wavelength than those reported previously for the isolated deoxyribonucleoside adducts, II, IV, and V (Moschel et al., 1983). Since there are no dramatic differences between the spectra of I and III, since both arise similarly through acid hydrolysis of 7,12-dimethylbenz[*a*]-anthracene-modified DNA and since both are similarly partitioned between water and 1-butanol, it is likely that they are structurally similar.

In other investigations of the nature of I and III, each product, recovered as outlined above, was subjected to chromatography on *m*-phenylboronic acid containing Servacel DHB columns, which can distinguish between compounds containing or not containing vicinal cis-hydroxyl groups. We previously used this system to distinguish deoxyribonucleoside adducts derived from the syn or anti bay region dihydrodiol epoxides of 7,12-dimethylbenz[*a*]anthracene (Sawicki et al., 1983). As shown in Figure 7a, breakdown product III binds to Servacel DHB, indicating the presence of a vicinal cis-hydroxyl grouping in III.

We noted earlier that III originates largely from IV, the *syn*-dihydrodiol epoxide–deoxyadenosine adduct that does not contain vicinal hydroxyl groups (Sawicki et al., 1983; Dipple et al., 1983). Thus, the formation of III from IV requires the acquisition of vicinal cis hydroxyl groups. In previous studies where deoxyadenosine residues in DNA were labeled with carbon-14 and the bound hydrocarbon was labeled with tritium

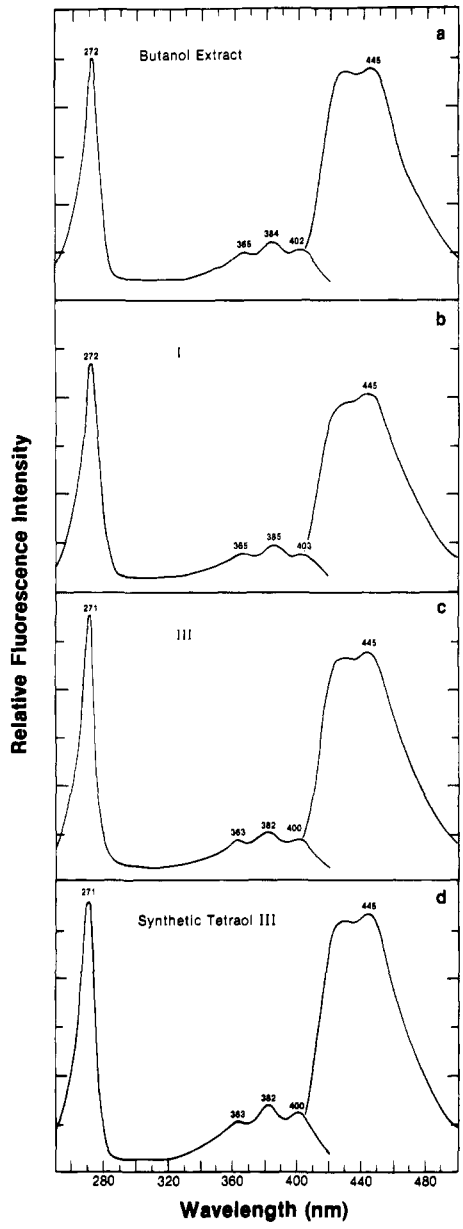


FIGURE 6: Uncorrected fluorescence excitation and emission spectra in methanol solution of products liberated in the acid hydrolysis of 7,12-dimethylbenz[*a*]anthracene-modified DNA. Mouse embryo cells grown in 19 roller bottles were exposed to [^{3}H]-7,12-dimethylbenz-[*a*]anthracene (0.2 μg/mL) (0.92 Ci/mmol) for 24 h. DNA was isolated and purified (see Experimental Procedures) and was found to be modified to the extent of 133 μmol of carcinogen/mol of DNA P. Approximately 3 mg of this DNA in 12 mL of H_2O was mixed with 10 mL of 0.2 M sodium acetate buffer, pH 4.5. After incubation for 46 h, 37 °C, the DNA solution was extracted with 40 mL of H_2O-saturated 1-butanol. The butanol solution was concentrated to 2 mL under reduced pressure and diluted with 25 mL of H_2O. This was concentrated to approximately 10 mL, and the mixture of extracted materials in H_2O was adsorbed on a Sep-Pak cartridge as described (see Experimental Procedures). The mixture of products I and III was eluted from the Sep-Pak cartridge with methanol (2.2 mL). The fluorescence spectrum for an aliquot (0.2 mL) of this mixture is shown in panel a. The remainder of this solution was evaporated to 0.45 mL under N_2 and diluted with 0.55 mL of H_2O and chromatographed under conditions described in the legend to Figure 1. Radioactive fractions corresponding to products I and III were pooled and recovered from Sep-Pak cartridges with methanol. The spectra of recovered products I and III are shown in panels b and c, respectively. Panel d shows the fluorescence spectrum in methanol of a synthetic 7,12-dimethylbenz[*a*]anthracene 1,2,3,4-tetrahydrotetraol (see Experimental Procedures), which is chromatographically inseparable from hydrolysis product III. In all cases, emission spectra were determined with λ_{ex} at 272 nm, and excitation spectra were determined with λ_{em} at 445 nm.

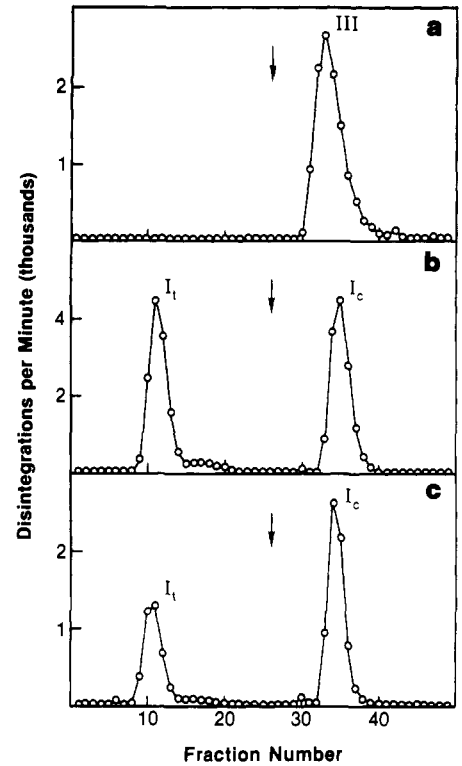


FIGURE 7: Examination of hydrolysis products, I and III, by boronate (Servacel DHB) chromatography. Separate DNA solutions were adjusted to pH 4.5 with ammonium acetate buffer and were then incubated at 37 °C for 17, 50, or 67 h. After incubation, each sample was extracted with 1-butanol, and the butanol layer was removed, diluted with water, and concentrated to about 0.1 mL. This was adjusted to be 20% in methanol and loaded onto the Altex Ultrasphere chromatography column and eluted, as described for Figure 1. From the DNA sample incubated for 50 h, hydrolysis product III was pooled, and this was applied to the boronate (Servacel DHB) column and eluted with 30 mL of morpholine buffer followed by sorbitol-containing buffer as described by Sawicki et al. (1983), to yield the data summarized in panel a. Panels b and c summarize the data obtained when hydrolysis product I was pooled from the 17- and 67-h incubations, respectively, and analyzed on the Servacel DHB column, as above. The arrows indicate the point at which the elution buffer was changed.

(Dipple et al., 1983), adduct IV was found to contain both radioactive labels, while product III contained only the tritium label indicating that the hydrocarbon–purine linkage is cleaved in the formation of III from IV. Together, these observations indicate that the conversion of IV to III probably involves the replacement of deoxyadenosine in IV by a hydroxyl group such that III is a 7,12-dimethylbenz[*a*]anthracene 1,2,3,4-tetrahydrotetraol bearing cis hydroxyl groups at the 1- and 2-positions. As shown in Scheme I, the relative stereochemistry of this tetraol (III) is then defined by the knowledge of its origin from a *syn*-dihydrodiol epoxide–deoxyadenosine adduct (Sawicki et al., 1983) because this latter can only have the relative stereochemistry shown for IV in Scheme I.

The similarities between I and III, noted above, suggest that product I could be one of the other three diastereomeric 7,12-dimethylbenz[*a*]anthracene 1,2,3,4-tetrahydrotetraols that are possible based on earlier work with benzo[*a*]pyrene (Yang et al., 1976; Thakker et al., 1976). However, chromatography of I on Servacel DHB resolved this product into two components, one that bound to the phenylboronic acid residues of the stationary phase and therefore contained a cis hydroxyl grouping, I_c, and one component that did not interact strongly with the Servacel DHB and, therefore, did not contain a vicinal cis hydroxyl grouping, I_t (Figure 7b). Since only one of the four possible tetraols exhibits trans stereochemistry for all

vicinal hydroxyl groups, the structure of I_t can be presumed to be that shown in Scheme I. The structure of I_c shown in Scheme I is consistent with its binding to Servacel DHB (Figure 7b) and is consistent with the origin of I_c and synthetic studies that are discussed below.

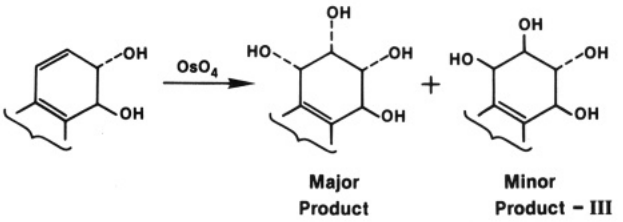
The ratio of I_c to I_t in product I varies with the time of incubation of the 7,12-dimethylbenz[*a*]anthracene-modified DNA in acid media (compare parts b and c of Figure 7). This presumably results either from differences in their rates of formation, from differences in the amounts of their respective precursors present, or from differences in their rates of decomposition.

To test the latter possibility, the rate of decomposition of each of these products, i.e., I_c and I_t as well as III (recovered by high-pressure liquid chromatography and Servacel DHB chromatography of a butanol extract), was monitored at pH 4.5, 37 °C, by analyzing aliquots at various times. The half-lives for disappearance of each of these tetraols are all very similar (~160 h) and are listed in Scheme I, where precursor–product relationships are also indicated. The half-lives of decomposition of the deoxyribonucleoside adducts after isolation from DNA are also given in Scheme I, and in parentheses are given the half-lives for decomposition of these deoxyribonucleoside adducts within the DNA structure. All the half-lives listed in Scheme I refer to pH 4.5, 37 °C.

The product–precursor relationships in Scheme I were established by isolating the individual adducts II and IV–VII by high-pressure liquid chromatography, incubating them at pH 4.5, 37 °C, for various times, and determining which decomposition products were formed by further chromatography. A solid arrow in Scheme I indicates that at least 10% of a given adduct was converted to the designated tetraol. Where conversion was less than 10%, a dashed arrow is used. Thus, incubation of adduct II produced only 5% of total radioactivity eluting as I on chromatography. This low yield is not surprising in view of the similar rates of decomposition for II and for its product, I_c (Scheme I). In contrast, the more rapid decomposition of adduct V produced a more convincing 10% of initial radioactivity eluting as I. Since both II and V are known to be derived from an *anti*-dihydrodiol epoxide (Sawicki et al., 1983), they are unlikely to yield I_t, and the I produced would be, therefore, I_c, as shown in Scheme I. For the even more unstable deoxyadenosine adducts IV, VI, and VII, incubation at pH 4.5 led to conversion of about 3%–7% radioactivity to material eluting as I. Since none of these initial adducts is derived from an *anti*-dihydrodiol epoxide, the hydrolysis products from these would be expected to be I_t rather than I_c, but only small amounts of I_t were found in all these cases. This is not because of slow rates of decomposition of these adducts (IV, VI, and VII) but because the major product from these adducts is III. Under the conditions studied, 22% of IV, 57% of VI, and 51% of VII were converted to III, making it clear that the hydrocarbon residue in IV, VI, and VII is the same. While Sawicki et al. (1983) earlier reported findings indicating that VI and VII did not contain vicinal cis hydroxyl groups, the present findings represent the first substantial evidence to indicate that VI and VII are derived from the bay region *syn*-dihydrodiol epoxide, as is the case for IV (Sawicki et al., 1983).

Although the identification of all the major breakdown products of 7,12-dimethylbenz[*a*]anthracene–deoxyribonucleoside adducts as tetraols should be considered to be tentative, the identification of III was firmly established by synthesis. Thus, oxidation of *trans*-3,4-dihydro-3,4-dihydroxy-7,12-dimethylbenz[*a*]anthracene (Sukumaran & Harvey, 1979) with osmium tetroxide, a *cis*-diol-generating reagent (Criegee et al., 1942), produced two products, separable by the chromatography system used in these studies (Scheme II). Both exhibited similar UV-absorbance spectra that were reminiscent of that of 9,10-dimethylanthracene, indicating that saturation of the 1,2,3,4 ring had occurred. The minor product exhibited the same retention time as III in the chromatography system of Figure 1 while the major product eluted between I and III in this system, approximately in correspondence with the very small peak that can be seen in Figure 1c. Each of these tetraol products was characterized by mass spectrometry, and the spectra are shown in Figure 8. The top panel (a) shows the low-resolution electron-impact spectrum for the minor product (III) while the lower panel (b) shows similar data for the major product and also includes high-resolution data. Both spectra are clearly consistent with the proposed tetraol structures (Schemes I and II) showing molecular ions at m/z 324 and major fragments, due to the loss of one (m/z 306) and two (m/z 288) molecules of water from the parent ion. The base peak (m/z 246) for the major tetraol (Figure 8b) was shown to have the elemental compo-

Scheme II



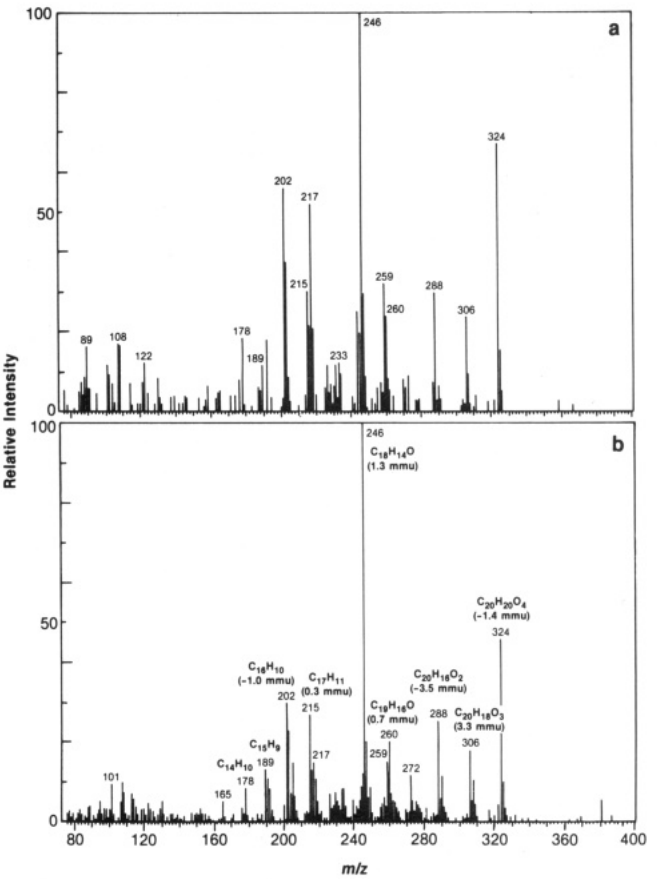


FIGURE 8: Mass spectra of the minor (a) and major (b) synthetic tetraols. In the lower panel the empirical formulas for the various ions are indicated, and the difference (in millimass units) between the observed and calculated mass for these ions is included in parentheses.

sition $C_{18}H_{14}O$, presumably resulting from loss of ketene from the doubly dehydrated ion. The identity of the molecular ion at m/z 324 was confirmed by observation of a $[M + H]^+$ ion at m/z 325 in the desorption chemical ionization (NH_4^+) spectrum. These mass spectral data along with the synthetic route followed in their preparation establish these synthetic compounds as tetraols.

The minor synthetic tetraol coeluted with tritium from decomposition product III in the chromatography system of Figure 1; these materials also coeluted on Whatman Partisil PXS ODS-2 columns eluted with methanol or with 80% methanol; and the fluorescence spectra of these two materials (Figure 6c,d) are very similar. It is reasonable to conclude, therefore, that the minor synthetic tetraol and III are identical. Since III is generated by acid hydrolysis of *syn*-dihydrodiol epoxide–deoxyribonucleoside adducts, III can be assigned a structure where the hydroxyl groups at positions 2 and 3 are trans to one another and, since the OsO_4 reagent generates cis hydroxyl groups, the cis configuration for the 1- and 2-hydroxy groups defines the relative stereochemistry of III as that shown in the schemes. The major synthetic tetraol then has to have the structure shown in Scheme II resulting from OsO_4 attack on the face of the dihydrodiol opposite to that involved in the generation of III. Evidence for the structures of the other products I_c and I_t is less compelling, but if these are assumed to be tetraols (because of the similarities in fluorescence spectra and other properties with those of III), they would have the structures assigned in Scheme I. The structure of I_t follows because this is the only tetraol structure without vicinal *cis*-hydroxyl groups, in concert with the findings summarized in Figure 7. The structure of I_c is then assigned because this is the only tetraol structure that has not already been assigned to the other products.

Discussion

The findings described above indicate that the adducts formed between the active metabolites of 7,12-dimethylbenz[*a*]anthracene and the deoxyribonucleoside constituents of DNA can be hydrolyzed at mildly acidic pH to release tetraols. These reactions can occur both in the isolated carcinogen–deoxyribonucleoside adducts and while these adducts are present within the DNA structure. The rates of all these hydrolyses are measureable at pH 4.5, but at pH 6.1, even in DNA where most rates are faster (Scheme I), only two *syn*-dihydrodiol epoxide–deoxyadenosine adducts, IV and VI in Figure 1, are notably unstable. The hydrolyses of these particular adducts are markedly accelerated by their environments in DNA, suggesting that either DNA phosphate groups or DNA secondary structure plays an active catalytic role. For adducts derived from the *anti*-dihydrodiol epoxide, the major and possibly exclusive tetraol formed, I_c, has trans hydroxyls at the 1,2-positions. In direct contrast, the major product from *syn*-dihydrodiol epoxide adducts, III, has cis hydroxyls at the 1,2-positions. Thus, the hydrolysis products of these adducts are analogous to the products formed upon hydrolysis of the *anti*- and *syn*-dihydrodiol epoxides of benzo[*a*]pyrene (Thakker et al., 1976).

Benzo[*a*]pyrene dihydrodiol epoxide–deoxyribonucleoside adducts have been reported to be cleaved by acid. However, since the conditions used were rather drastic, i.e., 2 N HCl, 100 °C (Osborne et al., 1976), no hydrocarbon tetraol was recovered. The predominant DNA adduct formed with benzo[*a*]pyrene in most systems is an *anti*-dihydrodiol epoxide–deoxyguanosine adduct, i.e., an analogue of our adduct II, which is the most stable adduct in our studies. Therefore, it is possible that a greater instability in the very minor benzo[*a*]pyrene–deoxyadenosine adducts could have been overlooked, and Osborne et al. (1976) did report that their benzo[*a*]pyrene–DNA samples were frequently contaminated with what they believed to be a tetraol. Clearly, such a contaminant could have arisen by hydrolysis of some adducts as described herein for 7,12-dimethylbenz[*a*]anthracene.

The loss of 7,12-dimethylbenz[*a*]anthracene residues from DNA can occur under physiological conditions (Figure 3) and this could serve to some extent as a repair mechanism in vivo. However, our previous studies (Dipple & Hayes, 1979) have shown that, in cultured mouse embryo cells, the loss of 7,12-dimethylbenz[*a*]anthracene–DNA adducts is very slow, so that chemical hydrolysis cannot represent a major repair pathway. The discovery that tetraols are produced from this hydrolytic cleavage of the adducts is supportive of our earlier partial identification of the major 7,12-dimethylbenz[*a*]anthracene–DNA adducts as *anti*-dihydrodiol epoxide–deoxyguanosine (II) and –deoxyadenosine (V) adducts and a *syn*-dihydrodiol epoxide–deoxyadenosine adduct (IV) (Sawicki et al., 1983; Dipple et al., 1983). Furthermore, finding III as a product of both VI and VII establishes that these minor adducts must also arise from a bay region *syn*-dihydrodiol epoxide. Since VI and VII probably involve the same nucleoside, deoxyadenosine, as does IV (Dipple et al., 1983), they presumably differ from IV and from one another either in their site of attachment to deoxyadenosine residues in DNA or in the stereochemistry of this attachment. The major adducts (II, IV, and V) probably involve the amino groups of the bases as the linkage to this hydrocarbon since Dipple et al. (1971) initially showed that polycyclic arylalkylating agents select this site for reaction, and it has been subsequently found that the benzo[*a*]pyrene dihydrodiol epoxides also react with the amino groups of the bases in DNA (Jeffrey et al., 1976b; Koreeda et al., 1976; Osborne et al., 1976). The site of attachment for the minor adducts could only involve speculation at this point.

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Photoaffinity Labeling of Chromatin Using a Tritiated (Azidoaryl)bis(acridinyl)spermidine Photoprobe†

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ABSTRACT: The synthesis of a tritium-labeled photoactive bis(acridine), [1,8-^{3}H]-*N,N'*-bis(9-acridinyl)-4-aza-4-(4-azidobenzoyl)-1,8-diaminooctane, is described. This reagent may be used as a photoprobe for the study of chromatin structure. Photolabeling of nuclease-solubilized chromatin resulted in labeling of both the DNA and the proteins (1% and 5–10% yield, respectively, in terms of added reagent). Equal labeling of the histones, H_1, H_{2A}, and H_3, was observed while no significant labeling of H_{2B} and H_4 was detected. However, in the presence of 6 M urea a drastic increase in the labeling of the core histones including H_{2B} was seen. In the absence of DNA only H_3 was labeled in both cases. It is concluded that the reagent binds preferentially to the internucleosomal linker at low reagent to base pair ratios (~0.01). It is furthermore shown that qualitatively similar histone labeling is obtained with whole cells as well as with isolated nuclei and solubilized chromatin. These results indicate that the reagent may be useful as a probe for chromatin structure both in vitro and in situ.

The structure and function of eukaryotic chromatin are the subject of intense study [recently reviewed by Igo-Kemenes et al. (1982) and Weisbrod (1982)] and detailed knowledge of the architecture of the nucleosome core with regard to interrelationships between the individual histones as well as between the histones and the DNA has been obtained (Mirzabekov, 1980; Klug et al., 1980). However, our understanding of the mechanisms governing the function, e.g., the turning off and on of individual genes, is still fragmentary.

Recently, photoaffinity labeling was introduced as an additional method for studying protein–nucleic acid interactions by development of photosensitive derivatives of 9-(alkylamino)acridines (Nielsen, 1981, 1982, Nielsen et al., 1983). These compounds contain an aminoacridine moiety, which binds to double-stranded DNA by intercalation (Hansen et al., 1983), and in most cases an arylazido moiety which upon irradiation with long wavelength ultraviolet light is able to bind covalently to both proteins and nucleic acids with high efficiency (Nielsen, 1982; Nielsen et al., 1983). This photoreaction most probably takes place with nucleophilic groups of the macromolecules (Nielsen & Buchardt, 1982; Nielsen, 1982). Preliminary results indicated that the labeling pattern of the histones reflects the condition of the chromatin even though a detailed interpretation was not possible (Nielsen, 1981, 1982; Nielsen et al., 1983).

Initially a fluorescence detection system was employed for analysis of the photoaffinity labeling of chromatin (Nielsen, 1981, 1982). However, this technique prevented the use of sodium dodecyl sulfate (SDS)[1] containing gel systems, thereby resulting in a limited application for the technique, and furthermore, a higher sensitivity was warranted.

These problems have now been overcome by the synthesis of a tritium-labeled photoprobe: [1,8-^{3}H]-*N,N'*-bis(9-acridinyl)-4-aza-4-(4-azidobenzoyl)-1,8-diaminooctane ([^{3}H]ABA) (Figure 1). This paper describes the synthesis of this compound as well as results for a further evaluation of the potentials of photoaffinity labeling as a method for analyzing chromatin structure and function both in vitro and in situ.

Materials and Methods

Synthesis of [1,8-^{3}H]-N,N'-Bis(9-acridinyl)-4-aza-4-(4-azidobenzoyl)-1,8-diaminooctane. A total of 400 μCi of [1,8-^{3}H]-4-aza-1,8-diaminooctane ([^{3}H]spermidine) (22 Ci/mmol, 1 Ci/mL; New England Nuclear) was mixed with

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[1] Abbreviations: SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; bp, base pairs.

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