Stereoelectronic Aspects of the Intercalative Binding Properties of 7,12-Dimethylbenz[a]anthracene Metabolites with DNA

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Abstract: The physical binding properties of nonreactive metabolites and metabolite model compounds of the potent carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) with DNA have been examined using fluorescence spectroscopy. The molecules studied include *trans*-3,4-dihydroxy-3,4-dihydro-DMBA, 1,2,3,4-tetrahydro-DMBA, 9,10-dimethylanthracene, anthracene, *trans*-5,6-dihydroxy-5,6-dihydro-DMBA, and 5,6-dihydro-DMBA. The first three molecules have steric and π electronic properties similar to the bay region epoxide of DMBA. The latter two molecules have properties similar to the K-region epoxide. The results indicate that intercalation with association constants in the range $(1.5-3.1) \times 10^3$ M^{-1} is the major mode of binding for the bay region epoxide model compounds. These molecules intercalate into DNA much better than model compounds of the less carcinogenic K region epoxide. For example, the intercalative binding constant of the proximate carcinogen *trans*-3,4-dihydroxy-3,4-dihydro-DMBA is 7.7 times greater than that of the noncarcinogenic metabolite *trans*-5,6-dihydroxy-5,6-dihydro-DMBA. Similarly, 1,2,3,4-tetrahydro-DMBA binds 10.8 times better than 5,6-dihydro-DMBA. Equilibrium dialysis studies of the bay region metabolites and metabolite model compounds, *trans*-3,4-dihydroxy-3,4-dihydro-DMBA, 1,2,3,4-tetrahydro-DMBA, and 9,10-dimethylanthracene (DMA), indicate that DNA intercalation is the most important binding mode, accounting for more than 95% of the total binding. Comparison of results from hydrocarbons with and without methyl groups show that, in some cases, methyl substitution can electronically enhance intercalation. For example, the intercalation binding constant of DMA is 6.7 times greater than that of anthracene. The electronic enhancement, due to methyl substitution, arises from an increase in hydrocarbon polarizability. This difference in polarizability is reflected in the UV photoelectron spectra of anthracene and DMA which indicate that the five highest occupied orbitals in DMA are destabilized a total of more than 1.5 eV compared to the corresponding orbitals in anthracene.

Carcinogenic hydrocarbons such as benzo[a]pyrene (BP)[1-3] and DMBA[4,5] are enzymatically activated to metabolites containing reactive epoxide groups. Results of several studies suggest that the carcinogenic and mutagenic activity of different hydrocarbon metabolites depends on the ability of these molecules to form covalent DNA and RNA adducts,[6] and it is likely that the interaction of hydrocarbon metabolites with genetic material is important to hydrocarbon carcinogenesis. The reactions of an ultimate carcinogen derived from benzo[a]pyrene, *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydro-BP (BPDE), with DNA have been widely examined.[7] Several studies suggest that the initial interaction involves a penetration of BPDE between the DNA bases.[7e,i,l,n,u,v,8-10] This is followed by reaction of the epoxide group. The binding of analogous reactive DMBA metabolites with DNA probably occurs by a similar mechanism.

The initial physical binding of epoxide-containing hydrocarbon metabolites to DNA may determine reaction sites on DNA and may influence the stereochemistry of reactive binding.[7e,1,9-11] In preliminary studies it was found that model compounds of different benz[a]anthracene (BA) metabolites exhibit significantly different DNA physical binding properties. For example 1,2,3,4-tetrahydro-BA intercalates into DNA 4.4 times better than 5,6-dihydro-BA or 8,9,10,11-tetrahydro-BA.

Since different metabolites formed from the same hydrocarbon exhibit widely different carcinogenic activities, these results may be of biological significance. For example, two major epoxide-containing metabolites of DMBA are the bay region diol epoxide 3,4-dihydroxy-1,2-epoxy-1,2,3,4-tetrahydro-DMBA and the K region oxide, 5,6-epoxy-5,6-dihydro-DMBA. The former is the principal metabolite of DMBA which binds covalently to DNA in vivo and is considered to be the probable active carcinogenic form of DMBA.[1,4,5,12] The metabolic precursor *trans*-3,4-dihydro-3,4-dihydroxy-DMBA is a more potent carcinogen than the parent hydrocarbon.[13] In contrast, 5,6-epoxy-5,6-dihydro-DMBA is essentially inactive as a carcinogen.[14] The structures of these metabolites are shown in Figure 1.

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Table I. Fluorescence Lifetimes[a,b]

	$[PO_4^-] = 0$		$[PO_4^-] = 4.5 \times 10^{-4}$ M	
	τ	χ^2	τ	χ^2
trans-3,4-dihydroxy-3,4-dihydro-DMBA (**1**)	15	1.07	15	1.10
9,10-dimethylanthracene (**3**)	16	1.09	15	1.02
anthracene (**4**)	4	1.13	4	0.98
trans-5,6-dihydroxy-5,6-dihydro-DMBA (**5**)	21	1.05	20	1.22
5,6-dihydro-DMBA (**6**)	14	1.16	13	1.06

[a] In nanoseconds. [b] The estimated uncertainty in the reported lifetimes is ±2 ns.

Other structural factors such as methyl substitution greatly alter the carcinogenic activity of benz[a]anthracene.[15] While benz-

(1) Harvey, R. G. *Acc. Chem. Res.* **1981**, *14*, 218.
(2) (a) Sims, P.; Grover, P. L. In "Polycyclic Hydrocarbons and Cancer"; Gelboin, H. V., Ts'o, P. O. P., Eds.; Academic Press: New York, 1981; Vol III, p 117. (b) Jerina, D. M.; Lehr, R. E.; Yagi, H.; Hernandez, O.; Dansette, P. M.; Wislocki, P. G.; Wood A. W.; Chang, R. L.; Levin, W.; Conney, A. H. In "In Vitro Metabolic Activation in Mutagenesis Testing"; DeSerres, F. J., Fouts, J. R., Bend, J. R., Philpot, R. M., Eds.; Elsevier: Amsterdam, 1976; pp 159-177. (c) Jerina, D. M.; Lehr, R. E. In "Microsomes and Drug Oxidation"; Ulrich, V., Roots, I., Hildebrandt, A., Estabrook, R. W., Conney, A. H., Eds.; Pergamon: Oxford, 1977; pp 709-720. (d) Nordquist, M.; Thakker, D. R.; Yagi, H.; Lehr R. E.; Wood, A. W.; Levin, W.; Conney, A. H.; Jerina, D. M. In "Molecular Basis of Environmental Toxicity"; Bhatnagar, R. S., Ed.; Ann Arbor Science Publishers: Ann Arbor, MI, 1980; pp 329-357.
(3) Yang, S. K.; Roller, P. P.; Fu, P. P.; Harvey, R. G.; Gelboin, H. V. *Biochem. Biophys. Res. Commun.* **1977**, *77*, 1176.
(4) Ivanovic, V.; Geacintov, N. E.; Jeffrey, A. M.; Fu, P. P.; Harvey, R. G.; Weinstein, I. B. *Cancer Lett.* **1978**, *4*, 131.
(5) Chou, M. W.; Yang, S. K. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5466.
(6) (a) Brookes, P.; Lawley, P. D. *Nature (London)* **1964**, *202*, 781. (b) Duncan, M. E.; Brookes, P.; Dipple, A. Int. J. *Cancer* **1969**, *4*, 813. (c) Ashurst, S. W.; Cohen, G. M.; Nesnow, S.; DiGiovanni, J.; Slaga, T. J. *Cancer Res.* **1983**, *43*, 1024. (d) Brookes, P.; Osborne, M. R. *Carcinogenesis (London)* **1982**, *3*, 1223.

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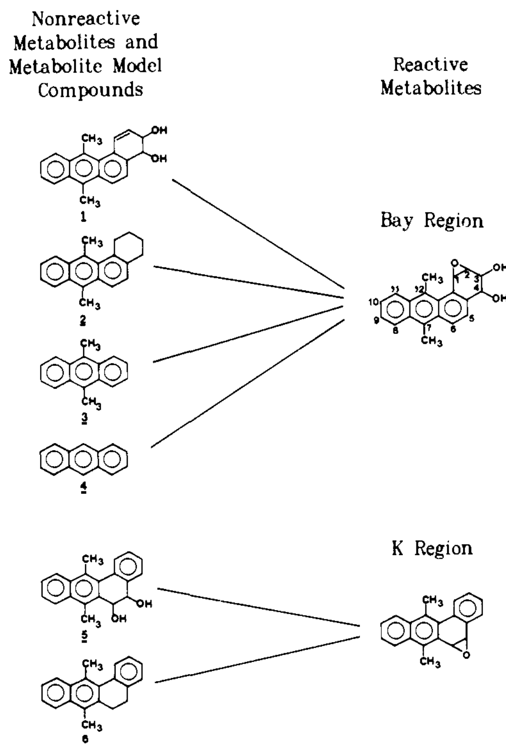

Figure 1. Bay and K region epoxides of DMBA and nonreactive metabolites and metabolites model compounds.

[a]anthracene is only marginally active, DMBA is among the most potent carcinogenic hydrocarbons known.

Earlier investigations of the variation in carcinogenic activity exhibited by different epoxides derived from a given parent hydrocarbon have focused on differences in epoxide reactivity.[2b-d] This reactivity, which changes markedly depending upon where epoxidation of the parent occurs, depends strongly on the π electronic structure of the metabolite. The reversible binding of hydrocarbon metabolites to DNA is also expected to be strongly influenced by π electronic structure.

A major goal of the present investigation is to provide a quantitative comparison of how changes in π structure which occur in different DMBA metabolites and metabolite model compounds influence reversible binding to DNA. Another is to demonstrate how differences in reversible binding properties associated with different π electronic structures are modulated by the presence of polarizing oxygen atoms. In hydroxyl- and epoxide-containing metabolites oxygen plays an important role in determining physical binding properties.

Previous studies of the physical binding of the bay region diol epoxide of benzo[a]pyrene to DNA have depended partly on kinetic and mechanistic studies of DNA-catalyzed epoxide hydrolysis.[7n,8] From the epoxide hydrolysis studies varying values have been reported for the association constant of the bay region diol epoxide of benzo[a]pyrene to DNA.[7n,8a] More reliable binding constants for the nonfluorescing benzo[a]pyrene diol epoxide have been obtained from UV absorption studies.[8] Because there is a strong overlap in the UV absorption spectra of nonfluorescing DMBA epoxides and the absorption spectrum of DNA, and because there is no current information about the hydrolysis of DMBA epoxides, different approaches are needed to study the reversible binding of DMBA metabolites. In the present study, DMBA metabolites and metabolite model compounds which have high fluorescence quantum yields were employed and the effects of hydrocarbon binding to DNA on the hydrocarbon emission properties were monitored. The DMBA metabolites and metabolite model compounds were chosen so that they possess similar π electronic structure and many of the steric features of the bay and K region epoxides of DMBA. They include *trans*-3,4-dihydroxy-3,4-dihydro-DMBA (**1**), 1,2,3,4-tetrahydro-DMBA (**2**), DMA (**3**), anthracene (**4**), *trans*-5,6-dihydroxy-5,6-dihydro-DMBA (**5**), and 5,6-dihydro-DMBA (**6**). Their structures are given in Figure 1.

Experimental Section

A Perkin-Elmer 650-10 Fluorescence Spectrometer was used in fluorescence quenching experiments. Fluorescence lifetimes were measured with a Photochemical Research Associates Model 2000 nanosecond fluorescence spectrometer equipped with a cooled photomultiplier. The photoelectron spectrum of DMA was measured with a Perkin-Elmer PS-18 photoelectron spectrometer.

Samples of **1**, **2**, **5**, and **6** were prepared using previously described methods.[16] Calf thymus DNA, anthracene, and DMA were purchased from Sigma Chemical Co. Reagent grade $MgCl_2$ from Aldrich was employed in experiments involving Mg^{2+}. Unless otherwise indicated all binding studies were carried out at a temperature of 23 ± 2 °C.

(7) (a) Pulkrabek, P.; Leffler S.; Weinstein, I. B.; Grunberger, D. *Biochemistry* **1977**, *16*, 3127. (b) Geacintov, N. E.; Gagliano, A. G.; Ivanovic, V.; Weinstein, I. B. *Biochemistry* **1978**, *17*, 5256. (c) Drinkwater, N. R.; Miller, J. A.; Miller, E. C.; Yang, N. C. *Cancer Res.* **1978**, *38*, 3247. (d) Yang, N. C.; Ng, L. K.; Neoh, S. B.; Leonov, D. *Biochem. Biophys. Res. Commun.* **1978**, *82*, 929. (e) Meehan, T.; Straub, K. *Nature (London)* **1979**, *277*, 410. (f) Prusik, T.; Geacintov, N. E.; Tobiasz, C.; Ivanovic, V.; Weinstein I. B. *Photochem. Photobiol.* **1979**, *29*, 223. (g) Prusik, T.; Geacintov, N. E. *Biochem. Biophys. Res. Commun.* **1979**, *88*, 782. (h) Lefkowitz, S. M.; Brenner, H. C.; Astorian, D. G.; Clarke, R. H. *FEBS Lett.* **1979**, *105*, 77. (i) Gamper, H. B.; Straub, K.; Calvin, M.; Bartholomew, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2000. (j) Geacintov, N. E.; Ibanez, V.; Gagliano, A. G.; Yoshida, H.; Harvey, R. G. *Biochem. Biophys. Res. Commun.* **1980**, *92*, 1335. (k) Undeman, O.; Sahlin, M.; Gräslund, A.; Ehrenberg, A. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 458. (l) Meehan, T.; Becker, J. F.; Gamper, H. *Proc. Am. Assoc. Cancer Res.* **1981**, *22*, 92. (m) Lefkowitz, S. M.; Brenner, H. C. *J. Am. Chem. Soc.* **1981**, *103*, 5257. (n) Geacintov, N. E.; Yoshida, H.; Ibanez, V.; Harvey, R. G. *Biochem. Biophys. Res. Commun.* **1981**, *100*, 1569. (o) Hogan, M. E.; Dattagupta, N.; Whitlock, J. P. *J. Biol. Chem.* **1981**, *256*, 4504. (p) Geacintov, N. E.; Gagliano, A. G.; Ibanez, V.; Harvey, R. G. *Carcinogenesis (London)* **1982**, *3*, 247. (q) Poulos, A. T.; Kuzmin, V.; Geacintov, N. E. *J. Biochem. Biophys. Methods* **1982**, *6*, 269. (r) Lefkowitz, S. M.; Brenner, H. C. *Biochemistry* **1982**, *21*, 3735. (s) MacLeod, M. C.; Mansfield, B. K.; Selkirk, J. K. *Carcinogenesis (London)* **1982**, *3*, 1031. (t) Undeman, O.; Lycksell, P. O.; Graslund, A.; Astlind, T.; Ehrenberg, A.; Jernstrom, B.; Tjerneld, F.; Norden, B. *Cancer Res.* **1983**, *43*, 1851. (u) Agarwal, K. L.; Hrinyo, T. P.; Yang, N. C. *Biochem. Biophys. Res. Commun.* **1983**, *114*, 14. (v) Geacintov, N. E.; Yoshida, H.; Ibanez, V.; Jacobs, S. A.; Harvey, R. G. *Biochem. Biophys. Res. Commun.* **1984**, *122*, 33. (w) Geacintov, N. E.; Ibanez, V.; Gagliano, A. G.; Jacobs, S. A.; Harvey, R. G. *J. Biomol. Struct. Dyn.* **1984**, *1*, 1473. (x) MacLeod, M. C.; Tang, M. *Cancer Res.* **1985**, *45*, 51.

(8) (a) Geacintov, N. E.; Yoshida, H.; Ibanez, V.; Harvey, R. G. *Biochemistry* **1982**, *21*, 1864. (b) MacLeod, M. C.; Selkirk, J. K. *Carcinogenesis (London)* **1982**, *3*, 287. (c) Meehan, T.; Gamper, H.; Becker, J. F. *J. Biol. Chem.* **1982**, *257*, 10479.

(9) Lin, J. H.; LeBreton, P. R.; Shipman, L. L. *J. Phys. Chem.* **1980**, *84*, 642.

(10) (a) Subbia, A.; Islam, S. A.; Neidle, S. *Carcinogenesis (London)* **1983**, *4*, 211. (b) Miller, K. J.; Burbaum, J. J.; Dommen, J. In "Polynuclear Aromatic Hydrocarbons: Sixth International Symposium on Physical and Biological Chemistry"; Cooke, M., Dennis, A.; Eds; Battelle Press: Columbus, OH, 1981; pp 515–528. (c) Taylor, E. R.; Miller K. J.; Bleyer, A. J. *J. Biomol. Struct. Dyn.* **1983**, *1*, 883.

(11) Yang, N. C.; Hrinyo, T. P.; Petrich, J. W.; Yang, D. H. *Biochem. Biophys. Res. Commun.* **1983**, *114*, 8.

(12) (a) Dipple, A.; Piqott, M.; Moschel, R. C.; Constantio, N. *Cancer Res.* **1983**, *43*, 4132. (b) Vigny, P.; Kindts, M.; Cooper, C. S.; Grover, P. L.; Sims, P. *Carcinogenesis (London)* **1981**, *2*, 115. (c) Slaga, T. J.; Gleason, G. L.; DiGiovanni, J.; Sukumaran, K. B.; Harvey, R. G. *Cancer Res.* **1979**, *39*, 1934.

(13) Slaga, T. J.; Gleason, G. L.; DiGiovanni, J.; Berry, D. L.; Juchal, M. R.; Fu, P. P.; Sukumaran, K. B.; Harvey, R. G. In "Polycyclic Aromatic Hydrocarbons"; Jones, P. W., Leber, P., Eds.; Ann Arbor Science Publishers: Ann Arbor, MI, 1979; p 703.

(14) Flesher, J. W.; Harvey, R. G.; Snydor, K. L. *Int. J. Cancer* **1976**, *18*, 351.

(15) (a) Huggins, C. B.; Pataki, J.; Harvey, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 2253. (b) Harvey, R. G. In "Safe Handling of Chemical Carcinogens, Mutagens, Tetratogens, & Highly Toxic Substances"; Walters, D. B., Ed.; Ann Arbor Science Publishers: Ann Arbor, MI, 1980; Vol. II, pp 43–44.

(16) (a) Goh, S. H.; Harvey, R. G. *J. Am. Chem. Soc.* **1973**, *95*, 242. (b) Harvey, R. G.; Goh, S. H. *J. Am. Chem. Soc.* **1975**, *97*, 3468. (c) Sukumaran, K.; Harvey, R. G. *J. Am. Chem. Soc.* **1979**, *101*, 1353. (d) Sukumaran, K.; Harvey, R. G. *J. Org. Chem.* **1980**, *45*, 4407. (e) Fu, P. P.; Lee, H. M.; Harvey, R. G. *J. Org. Chem.* **1980**, *45*, 2797. (f) Inbasekaran, M. N.; Witiak, D. T.; Barone, D.; Loper, J. C. *J. Med. Chem.* **1980**, *23*, 278.

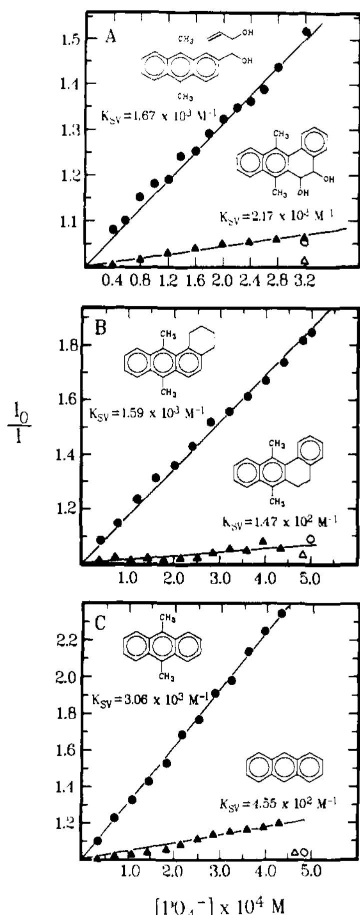

Figure 2. Stern–Volmer plots and quenching constants derived from the fluorescence quenching of *trans*-3,4-dihydroxy-3,4-dihydro-DMBA (**1**) and *trans*-5,6-dihydroxy-5,6-dihydro-DMBA (**5**) (A), 1,2,3,4-tetrahydro-DMBA (**2**) and 5,6-dihydro-DMBA (**6**) (B), and DMA (**3**) and anthracene (**4**) (C). Closed symbols give data obtained with native DNA. Open symbols give data obtained with denatured DNA.

Binding studies of molecules **2**, **3**, **4**, and **6** were carried out in a solvent system of double-distilled water and methanol (15% by volume). The more soluble diols, molecules **1** and **5**, were studied without methanol. All solutions were maintained at a pH of 7.1 with 10^{-3} M sodium cacodylate. The hydrocarbon concentrations were in the range 10^{-6}–10^{-7} M. DNA concentrations are reported in terms of PO_4^- molarity calculated from an average base-pair molecular weight of 617.8. This is based on a calf thymus DNA composition which is 60% A-T base pairs.[17] DNA concentrations have been corrected for the amounts of H_2O and Na^+ reported by the supplier for each DNA batch. From the DNA absorption spectrum it is found that one A_{260} unit equals 1.5×10^{-4} M [PO_4^-].

In control experiments DNA obtained from the supplier was repurified by using a phenol-chloroform extraction procedure[18] which yielded an A_{260}/A_{280} ratio of 2.0. When the Stern–Volmer quenching constants for DMA were compared in experiments using purified DNA and DNA directly from the supplier, the difference was less than 10%. Subsequent experiments were carried out without repurification of the DNA.

Denatured DNA was prepared by heating native DNA to 95 °C for 5 min. The denatured DNA exhibited a hyperchromicity of 30% at 260 nm. For molecules **1–6**, Stern–Volmer plots were measured at excitation wavelengths of 388, 300, 358, 358, 308, and 305 nm, respectively. The corresponding emission wavelengths were 445, 410, 398, 403, 372, and 375 nm.

Quenching constants, K_{SV}, were obtained from the Stern–Volmer plots and are equal to the slopes of least-squares fits to the quenching data of Figure 2. The symbol I_0 corresponds to the emission intensity of hydrocarbon without DNA. The symbol I corresponds to the intensity with DNA. Values of I_0 and I were measured at the maxima in the uncorrected emission spectra.

Lifetime studies of molecules **1–6** were carried out at excitation wavelengths of 385, 270, 256, 250, 314, and 268 nm. The emission wavelengths were 444, 415, 406, 382, 372, and 375 nm. For 1,2,3,4-tetrahydro-DMBA (**2**), low solubility required that a subtraction procedure be employed to correct for scattered light from DNA. In this case the decay profile of a DNA blank was measured and subtracted from profiles measured for molecule **2** with DNA. A similar procedure was used for molecule **1** because of the small separation between the emission and excitation wavelengths. Lifetime studies with DNA were carried out at a PO_4^- concentration of 4.5×10^{-4} M. Analysis of the lifetime data was carried out using a least-squares deconvolution method.[19]

In the equilibrium dialysis experiments, dialysis bags (no. 250-9u) were purchased from Sigma Chemical Co. The bags were pretreated by previously described procedures.[20,21] The hydrocarbon concentrations in the dialysis experiments were in the range 10^{-6}–10^{-7} M. The DNA concentration inside the dialysis bags was 5.0×10^{-4} M. In the dialysis experiments the hydrocarbon fluorescence intensities were compared inside and outside the dialysis bag after equilibrium was reached, using the emission and excitation wavelengths used to measure the Stern–Volmer plots. Control experiments were carried out to verify that equilibrium was reached under the conditions used. In the control experiments bags initially containing only solvent were stirred in a hydrocarbon solution. After 4 days the emission intensities measured inside and outside the dialysis bags were equal. The DNA binding experiments were carried out by stirring the solution for 4 days at 4 °C. The fluorescence measurements were also made at 4 °C.

In order to examine effects due to changes in DNA concentration dialysis experiments with molecules **1** and **3** were carried out at a PO_4^- concentration of 2.4×10^{-4} M as well as at 5.0×10^{-4} M. Temperature effects were examined by comparing dialysis results for molecules **1** and **3** obtained at 23 °C with results obtained at 4 °C.

Results

Fluorescence Quenching Experiments. Figure 2 contains Stern–Volmer plots and quenching constants for the fluorescence quenching of molecules **1–6**. Panel A compares quenching data for the proximate carcinogen *trans*-3,4-dihydroxy-3,4-dihydro-DMBA (**1**) with that for the K region diol *trans*-5,6-dihydroxy-5,6-dihydro-DMBA (**5**). The results indicate that molecule **1** is much more strongly quenched by DNA than molecule **5**.

The difference between the DNA fluorescence quenching of bay and K region metabolites of DMBA is also observed in the Stern–Volmer plots for the model compounds 1,2,3,4-tetrahydro-DMBA (**2**) and 5,6-dihydro-DMBA (**6**), which are shown in panel B. Here the model compound of the bay region metabolite, molecule **2**, is 10.8 times more strongly quenched than that of the model of the K region metabolite, molecule **6**.

Figure 2C compares Stern–Volmer plots for DMA and anthracene. These results point out that the quenching constant of the methylated derivative is 6.7 times greater than that of anthracene.

Figure 2 also contains data obtained with denatured DNA. The results indicate that the strong fluorescence quenching of molecules **1–3** by native DNA is dependent upon DNA secondary structure. The quenching is greatly reduced in denatured DNA.

The reduction in the fluorescence quenching of molecules **1–3** which occurs when DNA is denatured is similar to the reduction in quenching observed for standard intercalating hydrocarbons such as pyrene. For pyrene, intercalation into native DNA leads to a red shift in the UV absorption spectrum and to fluorescence quenching.[22] Both of these spectral effects depend on the sandwiching of pyrene into stacked nucleotide bases and are greatly diminished when DNA is denatured. Unlike pyrene, molecules **1–6** do not lend themselves to UV absorption studies of DNA binding. For these molecules the absorption spectra strongly overlap that of DNA.

For molecules **1–6** fluorescence data are more useful. In cases where the fluorescence quantum yield of the intercalated complex

(17) Adams, R. L. P.; Burdon, R. H.; Campbell, A. M.; Leader, D. P.; Smellie, R. M. S. "The Biochemistry of the Nucleic Acids"; 9th ed.; Chapin and Hall: London, 1981; p 36.

(18) Maniatis, T.; Fritsch, E. F.; Sambrook, J. "Molecular Cloning A Laboratory Manual"; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1982; pp 458–460.

(19) Hui, M. H.; Ware, W. R. *J. Am. Chem. Soc.* **1976**, *98*, 4718.

(20) McPhie, P. *Methods Enzymol.* **1971**, *12*, 25–28.

(21) Ibanez, V.; Geacintov, N. E.; Gagliano, A. G.; Brandimarte, S.; Harvey, R. G. *J. Am. Chem. Soc.* **1980**, *102*, 5661.

(22) (a) Boyland, E.; Green, B. *Br. J. Cancer* **1962**, *16*, 347, 507. (b) Boyland, E.; Green, B.; Liu, S. L. *Biochim. Biophys. Acta* **1964**, *87*, 653.

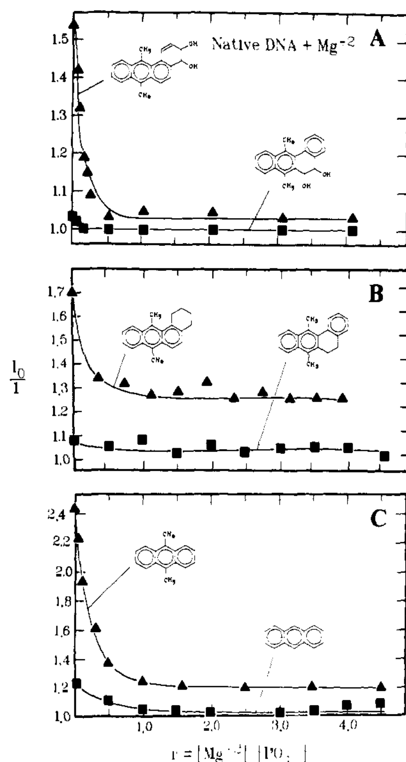

Figure 3. Effects of Mg^{2+} upon the fluorescence quenching of *trans*-3,4-dihydroxy-3,4-dihydro-DMBA (**1**) and *trans*-5,6-dihydroxy-5,6-dihydro-DMBA (**5**) (A), 1,2,3,4-tetrahydro-DMBA (**2**) and 5,6-dihydro-DMBA (**6**) (B), and DMA (**3**) and anthracene (**4**) (C). The data in (A) were obtained at $[PO_4^-] = 2.9 \times 10^{-4}$ M; the data in B and C were obtained at $[PO_4^-] = 5.0 \times 10^{-4}$ M.

is low compared to that of the free hydrocarbon, an association constant for intercalation can be derived from the Stern–Volmer plots. Here fluorescence quenching obeys the following equation:[23]

$$I_0/I = (1 + k_d\tau_F[PO_4^-])(1 + K_A[PO_4^-]) \quad (1)$$

In eq 1, k_d is the bimolecular rate constant for collisional quenching, τ_F is the mean radiative lifetime of the excited state of the free hydrocarbon in the absence of collisional quenching, and K_A is the association constant for intercalation.

In previous studies of phenanthrene derivatives with longer lifetimes ($\tau_F > 35$ ns) than the hydrocarbons studied here,[24] it was found that at the present DNA concentrations dynamic quenching, even if diffusion-controlled, influences hydrocarbon fluorescence in only a minor way. At the DNA concentrations used here $[PO_4^-]k_d\tau_F << 1$. In these experiments the association constant for intercalation, K_A, is closely approximated by the Stern–Volmer quenching constant K_{SV} and is given by

$$K_A = K_{SV} = [PO_4^-]^{-1}[I_0/I - 1] \quad (2)$$

If eq 2 is valid, it is expected that K_{SV} will remain constant when measured at varying excitation wavelengths. For the strongly quenching molecules it was found that when the excitation wavelengths were varied by ±10 nm, K_{SV} changes less than 2%.

Mg^{2+} Ion Experiments. Evidence supporting the conclusion that the binding of molecules **1–3** involves intercalation is provided by studies of the effects of DNA stabilizers such as Mg^{2+} on fluorescence quenching by native DNA. The results of these experiments are given in Figure 3. In Figure 3, I_0/I, for $[PO_4^-]$ equal to 5.0×10^{-4} M, is plotted vs. r, the ratio of the Mg^{2+} to PO_4^- concentrations. For all molecules it was found that DNA stabilization[8,24c,25] through the addition of Mg^{2+} reduces the ability of native DNA to quench hydrocarbon fluorescence. In this regard the binding of molecules **1–3** which gives rise to fluorescence quenching behaves like that of the strongly intercalating ultimate carcinogen BPDE. The DNA intercalation of this molecule[8] is also inhibited by the addition of Mg^{2+}.

For weakly quenched molecules **4–6**, the low initial values of I_0/I are further reduced by adding Mg^{2+} and reach a value of 1.0 at $r = 1.0$. At this value of r virtually all binding giving rise to hydrocarbon quenching is inhibited. For the strongly quenched molecules **1–3**, the ratio I_0/I is significantly greater than 1.0, even at high Mg^{2+} concentrations ($r = 2.0$).

The results of Figure 3 show that for the more strongly quenched molecules residual binding persists over the entire range of Mg^{2+} concentrations examined.

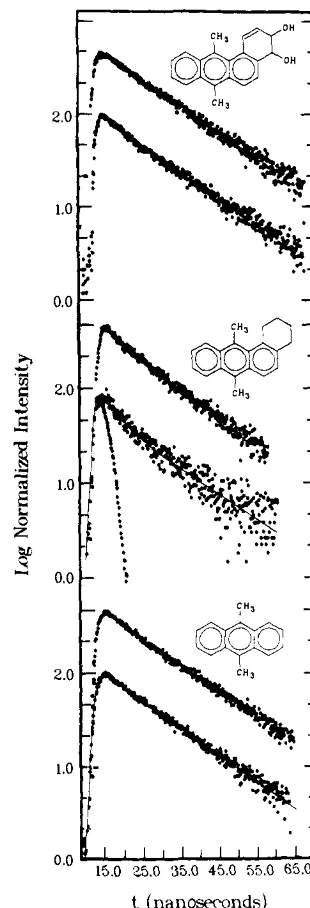

Figure 4. Fluorescence decay profiles for *trans*-3,4-dihydroxy-3,4-dihydro-DMBA (**1**), 1,2,3,4-tetrahydro-DMBA (**2**), and 9,10-dimethylanthracene (**3**). For each molecule the upper decay profile shows data obtained without DNA. The lower decay profile shows data obtained with DNA ($[PO_4^-] = 4.5 \times 10^{-4}$ M). An instrument response curve is shown along with data for 1,2,3,4-tetrahydro-DMBA with DNA. The solid lines show typical fits of experimental data obtained from the data given in Table II.

Fluorescence Lifetime Studies. Further information about the DNA binding properties of molecules **1–6** is provided by fluorescence lifetime measurements. Figure 4 shows fluorescence decay profiles of *trans*-3,4-dihydroxy-3,4-dihydro-DMBA (**1**), 1,2,3,4-tetrahydro-DMBA (**2**), and DMA (**3**) measured with and without DNA. For all three molecules Figure 4 indicates that the decay profiles remain qualitatively similar when DNA is added. The same result is obtained for molecules **4–6**.

The present data suggest that both with and without DNA the observed emission arises primarily from unquenced hydrocarbons. This result is expected if the fluorescence quantum yield of the

(23) Moon, A. Y.; Poland, D. C.; Scheraga, H. A. *J. Phys. Chem.* **1965**, *69*, 2960.

(24) (a) Shahbaz, M.; Harvey, R. G.; Prakash, A. S.; Boal, T. R.; Zegar, I. S.; LeBreton, P. R. *Biochem. Biophys. Res. Commun.* **1983**, *112*, 1. (b) Prakash, A. S.; Zegar, I. S.; Shahbaz, M.; LeBreton, P. R. *Int. J. Quantum Chem., Quantum Biol. Symp.* **1983**, *No. 10*, 349. (c) Zegar, I. S.; Prakash, A. S.; LeBreton, P. R. *J. Biomol. Struct. Dyn.* **1984**, *2*, 531.

(25) Gilbert, M.; Claverie, P. *J. Theor. Biol.* **1968**, *18*, 330.

Table II. Summary of Dialysis Experiments[a]

	I_{in}/I_{out}
trans-3,4-dihydroxy-3,4-dihydro-DMBA (**1**)	1.01
1,2,3,4-tetrahydro-DMBA (**2**)[b]	1.03
9,10-dimethylanthracene (**3**)[b]	1.04
anthracene (**4**)[b]	1.01
trans-5,6-dihydroxy-5,6-dihydro-DMBA (**5**)	1.01
5,6-dihydro-DMBA (**6**)[b]	1.01

[a] Carried out at 4 °C with $[PO_4^-]$ = 5.0 × 10^{-4} M. [b] Measured in 15% methanol.

quenched hydrocarbons is small and if eq 2 validly yields the intercalation binding constants.

For molecules **1** and **3–6** the decay profiles measured with and without DNA were fitted with a single-exponential decay law. The results of the lifetime studies for these molecules are summarized in Table I, which lists lifetimes and values of χ^2 obtained in the data analysis. The lifetime data for 1,2,3,4-tetrahydro-DMBA (**2**) was of poorer quality than that for the other molecules. For molecule **2** the analysis of the lifetime data with a single-exponential decay law gave a lifetime of 14 ns, with and without DNA, with χ^2 values of 1.43 and 1.34, respectively. Use of a double-exponential decay law led to χ^2 values of 1.08 and 1.06. Results from the double-exponential analysis indicate that both with and without DNA the short-lived component had a lifetime of 5 ± 1 ns and made up 20 ± 3% of the total emission intensity. The long-lived component had a lifetime of 16 ns. Both with and without DNA it is likely that the short-lived component is due to errors introduced when background scatter is subtracted. Of the molecules studied, **2** had the weakest emission intensity.

For molecules **1** and **3–6** it was found that no improvement in the fits to the lifetime data were obtained when a double-exponential decay law was employed. For DMA and anthracene the lifetimes obtained here agree with those previously reported (17.4 ns for DMA in heptane and 4 ns for anthracene in ethanol).[26]

The results of the lifetime studies indicate that the observed decay profiles of molecules **1–6** are not significantly altered when DNA is added. This observation is similar to that previously reported in studies of the effect of DNA intercalation on the observed fluorescence decay profiles of pyrene and of the partially saturated BP derivatives *trans*-7,8-dihydroxy-7,8-dihydro-BP, *trans*-4,5-dihydroxy-4,5-dihydro-BP, 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydro-BP (BPT), and *trans*-7,8-dihydroxy-7,8,9,10-tetrahydro-BP.[11,21,27-30]

Equilibrium Dialysis Experiments. In previous equilibrium dialysis studies of BPT it was found that in addition to intercalating sites, which yield complexes with negligible quantum yield, secondary binding sites on DNA occur.[21] At the secondary binding sites the emission properties of BPT were reported to be similar to those of the free molecule.

Equilibrium dialysis was used to study the DNA binding of molecules **1–6**. The results are summarized in Table II which gives the ratio of I_{in}, the fluorescence intensity measured for a sample taken from inside the dialysis bag, to I_{out}, the intensity measured for a sample taken from outside the bag. In all cases the ratio is nearly unity. The results for molecules **1** and **3** remained constant when the equilibrium was reached at 23 °C and when the emission spectra were also measured at 23 °C. The results for these molecules also remained unchanged when the DNA concentration was reduced to 2.4 × 10^{-4} M.

If the small increase in emission measured inside the bag compared to that measured outside the bag is due to secondary site complexes with quantum yields equal to those of unbound molecules, then the secondary binding constants are all less than 80 M^{-1}.

The results of the equilibrium dialysis experiments support the conclusion that intercalated complexes of molecules **1–6** have very small quantum yields. They also show that for the better intercalating agents, molecules **1–3**, binding at nonquenched sites constitutes a very small portion (<5%) of the total binding.

Discussion

In many respects the DNA binding of the DMBA metabolites and metabolite model compounds studied here is similiar to the intercalative binding of pyrene and of the partially saturated benzo[*a*]pyrene derivatives *trans*-7,8-dihydroxy-7,8-dihydro-BP, *trans*-7,8-dihydroxy-7,8,9,10-tetrahydro-BP, 7,8,9,10-tetrahydro-BP, BPT, and *trans*-4,5-dihydroxy-4,5-dihydro-BP.[11,21,27-30] In all cases binding gives rise to quenching of the hydrocarbon fluorescence. In all cases studied to date fluorescence quenching by native DNA is much greater than that by denatured DNA, and quenching by native DNA is inhibited by DNA stabilizers such as Mg^{2+} and spermine.[29,30]

Results of lifetime studies indicate that the addition of DNA has little effect on the observed decay profiles of molecules **1–6** even under conditions where DNA significantly reduces the hydrocarbon emission intensity. In this regard molecules **1–6** are again like pyrene and partially saturated benzo[*a*]pyrenes. For these molecules, observed fluorescence lifetimes measured with and without DNA were also found to be very similar. Moreover, in the previous absorption and fluorescence studies of the binding of pyrene and partially saturated benzo[*a*]pyrenes it was demonstrated that when the observed fluorescence decay profiles measured with and without DNA are similar, eq 2 provides an accurate value of the association constant for intercalation.[11,28,29]

The similar effects that DNA binding has on the fluorescence properties of the molecules studied here compared to those of well-established intercalating agents such as pyrene and saturated benzo[*a*]pyrenes provide strong evidence that the fluorescence quenching of molecules **1–6** by DNA arises from intercalation. Furthermore, the parallel between the emission properties of the molecules studied here and those of pyrene and the previously studied saturated benzo[*a*]pyrenes supports the conclusion that eq 2 validly describes the DNA binding of molecules **1–6**.

The present studies indicate how saturation of different aromatic rings in DMBA influences the DNA binding properties of DMBA metabolites. These results also demonstrate how the electronic effects of methyl substitution influence the ability of a hydrocarbon to intercalate into DNA. It might be expected that addition of a methyl group that is ~1.8 Å thick[31] will inhibit intercalation between nucleotide bases which in the B conformation of DNA are spaced 3.4-Å apart.[32] However, methyl substitution causes the intercalative binding of 9,10-dimethylanthracene to be 6.7 times greater than that of anthracene. Earlier studies[24a] showed that the presence of an alicyclic ring causes the binding of 1,2,3,4-tetrahydro-BA to be 5.2 times greater than that of anthracene.

The greater binding of DMA and 1,2,3,4-tetrahydro-BA compared to anthracene is related to the increased polarizability of the anthracene π system which accompanies substitution of aliphatic groups.[24a,33,34] This is demonstrated by the photoelectron

(26) (a) Ware, W. R.; Baldwin, B. A. *J. Chem. Phys.* **1965**, *43*, 1194. (b) Jung, C.; Heckner, K. H. *Chem. Phys.* **1977**, *21*, 227.
(27) Geacintov, N. E.; Prusik, T.; Khosrovfian, J. M. *J. Am. Chem. Soc.* **1976**, *98*, 6444.
(28) Abramovich, M.; Zegar, I. S.; Prakash, A. S.; Harvey, R. G.; LeBreton, P. R. "The Molecular Basis of Cancer, Part A; Macromolecular Structure, Carcinogens, and Oncogenes"; Rein, R., Ed.; Alan R. Liss: New York, 1985; pp 217–225.
(29) Abramovich, M.; Prakash, A. S.; Harvey, R. G.; Zegar, I. S.; LeBreton, P. R. *Chem.-Biol. Interact.*, in press.
(30) Paulius, D. E.; Prakash, A. S.; Harvey, R. G.; Abramovich, M.; LeBreton, P. R. "Polynuclear Aromatic Hydrocarbons: Chemistry and Carcinogenesis"; Cooke, M., Dennis, A. G., Eds.; Battelle Press: Columbus, OH, 1986; in press.
(31) March, J. "Advanced Organic Chemistry"; Summergill, R. H., Vinnicombe, A. T., Eds.; McGraw Hill: New York, 1977; pp 22–24.
(32) Arnott, S.; Chandrasekaran, R.; Selsing, E. In "Structure and Conformation of Nucleic Acids and Protein–Nucleic Acid Interactions"; Sundaralingam, M., Rao, S. T., Eds.; University Park Press: Baltimore, 1975; pp 577–597.
(33) (a) Yu, C.; O'Donnell, T. J.; LeBreton, P. R. *J. Phys. Chem.* **1981**, *85*, 3851. (b) Peng, S.; Padva, A.; LeBreton, P. R. Proc. *Natl. Acad. Sci. U.S.A.* **1976**, *73*, 2969.
(34) Ts'o, P. O. P. In "Basic Principles in Nucleic Acid Chemistry"; V. I. Ts'o, P. O. P., Ed.; Academic Press: New York, 1974; pp 537–562.

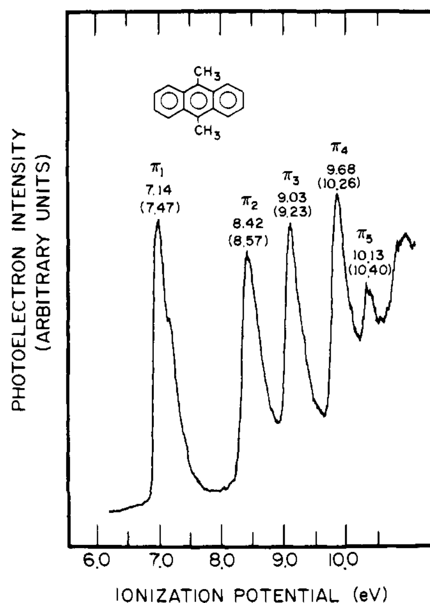

Figure 5. He(I) photoelectron spectrum of 9,10-dimethylanthracene. Assignments are given along with vertical ionization potentials. Numbers in parentheses are ionization potentials taken from ref 35 for corresponding orbitals in anthracene.

data for 9,10-dimethylanthracene and anthracene shown in Figure 5. The data indicate that all five of the highest occupied π orbitals of DMA are destabilized compared to corresponding orbitals in anthracene[35] and that the net destabilization of these orbitals due to methyl substitution is over 1.5 eV. A similar effect is observed in 1,2,3,4-tetrahydro-BA.[24a] In **DMA** and in 1,2,3,4-tetrahydro-BA the electronic influence of aliphatic groups that enhance intercalation are more important than steric effects that inhibit intercalation.

The present results point out that the binding constants of the bay region metabolites and metabolite model compounds of DMBA are significantly greater than those of corresponding K region metabolites and metabolite model compounds. The intercalation binding constant of 1,2,3,4-tetrahydro-DMBA (**2**) is 10.8 times greater than that of 5,6-dihydro-DMBA (**6**). The addition of hydroxyl groups in the bay and K regions modulates this difference. Nevertheless the trend is the same. The binding constant of *trans*-3,4-dihydroxy-3,4-dihydro-DMBA (**1**) is 7.7 times greater than that of *trans*-5,6-dihydroxy-5,6-dihydro-DMBA (**5**).

These differences in the binding properties of molecules **1** and **2** vs. molecules **5** and **6** are probably due to steric effects that arise because molecules **5** and **6** have nonplanar π systems.

Crystallographic data[36] on the K region epoxide, 5,6-epoxy-5,6-dihydro-DMBA, incidate that the angular ring tilts 35° compared to the ring containing the carbon atoms at the 8–11-positions. The tilt of the angular ring causes the overall molecular thickness of 5,6-epoxy-5,6-dihydro-DMBA to be 2.4–2.7 Å. This dimension approaches that of the space between base pairs, and it is likely that the thickness of the K region epoxide inhibits intercalation. On the other hand, the present results for *trans*-3,4-dihydroxy-3,4-dihydro-DMBA and 1,2,3,4-tetrahydro-DMBA suggest that saturation of the angular ring in the bay region diol epoxide, *trans*-3,4-dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetrahydro-DMBA, less seriously hinders DNA intercalation of the methylated anthracene moiety.

Future studies with reactive metabolites are required to directly compare the reversible physical binding of bay and K region epoxides of DMBA to DNA. However, from results of previous binding studies of bay region benzo[*a*]pyrene metabolites and from the present data it is possible to obtain an estimates of the ratio of association constants of the bay region diol epoxide and the K region epoxide of DMBA. A comparison of binding data from earlier studies[7n,8,37] of the bay region diol epoxide and the bay region *trans*-7,8-dihydrodiol of benzo[*a*]pyrene indicate that under identical conditions the dihydrodiol has an association constant that is 1.3–1.7 times greater than that of the diol epoxide. The ratio of the binding constants of the bay region diol epoxide and dihydro diol of DMBA is expected to lie in the same range.

A second comparison of the binding constants of *trans*-3,4-dihydroxy-3,4-dihydro-DMBA (**1**) and 5,6-dihydro-DMBA (**6**) measured under the same conditions indicates that the binding constant of molecule **6** is 6.2 times lower than that of molecule **1**. The association constant of 5,6-dihydro-DMBA (**6**) should be higher than that of the DMBA K region epoxide with a polar oxygen atom which increases water solubility and decreases binding. The oxygen atom, which has bonds that lie at an angle of approximately 90° with the least-squares plane containing the naphthalene moiety also provides a greater steric barrier to intercalation than the H atoms in 5,6-dihydro-DMBA. These considerations of the ratios of the binding constants of the bay region dihydrodiol vs. the diol epoxide of benzo[*a*]pyrene and of *trans*-3,4-dihydroxy-3,4-dihydro-DMBA vs. 5,6-dihydro-DMBA provide evidence that the bay region diol epoxide of DMBA has a binding constant that is 3.5 to 4.6 times greater than that of the K region epoxide.

In summary, the present results demonstrate that differences in the deletion of the π system of DMBA, which arises when metabolism occurs via different pathways, have strong influence on reversible physical binding to DNA. These differences may be related to the varying biological activity of different DMBA metabolites.

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(35) Boschi, R.; Clar, E.; Schmidt, W. *J. Chem. Phys.* **1974**, *60*, 4406.
(36) Glusker, J. P.; Carrell, H. L.; Zacharias, D. E.; Harvey, R. G. *Cancer Biochem. Biophys.* **1974**, *1*, 43.
(37) Prakash, A. S.; Zegar, I. S.; LeBreton, P. R., unpublished results.