Properties of Benzopyrene–DNA Complexes Investigated by Fluorescence and Triplet Flash Photolysis Techniques

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Abstract: Properties of benzo(*a*)pyrene (BaP) and benzo(*e*)pyrene (BeP) in physical complexes with native calf thymus DNA dissolved in aqueous solutions at 24 ± 1 °C were studied by means of fluorescence quantum yield, single photon counting fluorescence decay, and triplet flash photolysis techniques. There are three types of binding sites for these polycyclic aromatic hydrocarbons bound to DNA. Between 40 and 60% of the aromatic molecules are located at a type of site where the excited singlet states decay nonradiatively and the other sites are characterized by different fluorescence decay times; for BaP the fluorescence lifetimes are 3.2 and 10 ns, while for BeP the lifetimes are 10 and 33 ns. Addition of silver ions, which in the range of silver ion/nucleotide ratios used (<0.13) bind to GC sites predominantly, results in up to a 70% quenching of the fluorescence of the aromatic hydrocarbons. It is proposed that most of the fluorescence originates from BaP and BeP bound to GC-GC intercalation sites, while the nonfluorescent molecules are bound at AT containing intercalation sites. This conclusion is supported by the relative quenching efficiencies of the fluorescence of BaP by mononucleosides dissolved in aqueous ethanol solutions: 2′-deoxythymidine is more effective (by a factor of >10) as a quencher than cytidine, 2′-deoxyguanosine and -adenosine. The strong quenching of the fluorescence in DNA is partially accompanied by the production of triplet excited states. The triplets are not as susceptible to quenching in DNA as the singlets and the lifetimes of BaP and BeP triplets in DNA are 35 and 155 ms, respectively. The triplet lifetimes are sensitive to the conformation of the DNA in solution, to the ionic strength, and to the oxygen concentration. Employing oxygen quenching techniques, the triplets can be used as probes to determine the accessibility of the polycyclic aromatic molecules in macromolecular complexes. Oxygen quenching constants for triplets of BaP, BeP, benz(*a*)anthracene, 7,12-dimethylbenz(*a*)anthracene, and acridine orange are in the range of $1-2 \times 10^8$ l. mol^{-1} s^{-1}, about 10–20 times smaller than in fluid solution; this decrease is attributed to intercalation.

Polycyclic aromatic hydrocarbon carcinogens and structurally related noncarcinogenic compounds are known to bind to DNA in aqueous solution.[1-5] The solubility of most of these aromatic hydrocarbons increased by factors of ~1000 in the presence of DNA in aqueous solutions. Evidence has been presented that polycyclic aromatic hydrocarbons form physical complexes via intercalation; the planes of the aromatic hydrocarbons are believed to be sandwiched between two adjacent base pairs within the double helix.[6,7]

The covalent binding of carcinogenic polycyclic aromatic hydrocarbons to nucleic acids in vivo (intact animal and cell cultures) is well established; this type of covalent interaction may be essential for the initiation of carcinogenesis.[8] A number of recent papers have described the use of fluorescence techniques to characterize the nature of polycyclic aromatic carcinogens bound covalently to DNA both in vivo and in vitro.[9-12] Little is known about the physicochemical and structural details of the covalent DNA–polycyclic aromatic adducts. Of particular interest is how these properties determine the differences in biological activities displayed by structurally similar polycyclic compounds.

In spite of the widespread use of fluorescence techniques in characterizing both the physical and covalent adducts of polycyclic aromatic hydrocarbons to DNA, the full potential as well as the limitations of these techniques have not been fully realized in this area of research.

In this work, the properties of physically bound complexes of some polycyclic aromatic hydrocarbons and DNA are studied by means of fluorescence photon counting and flash photolysis techniques. Physical complexes rather than covalently bound polycyclic aromatic–DNA adducts have been selected for this study because the samples can be easily prepared in large quantities and because the nature of the physical complexes is better defined than those of the covalent complexes. The aromatic nature of the hydrocarbons is not altered in physical complexes as may be the case in the covalent adducts, and considerable evidence is available that the hydrocarbon molecules bind physically to DNA by the intercalation mechanism. A characterization of the physical complexes where the mode of binding is established should provide valuable guidelines for future characterizations of the covalent complexes.

By determining the quantum yields of fluorescence and from a detailed analysis of the fluorescence decay curves it is shown that there are three types of physical binding sites for benzo(*a*)pyrene and benzo(*e*)pyrene in DNA. The fluorescence of the aromatic molecules is totally quenched at one of these sites. The quenching of the fluorescence emitting singlet excited states is, to a large extent, accompanied by the generation of triplet excited states. The observation of triplet excited states of aromatic molecules complexed to DNA in aqueous solution at room temperature is reported here for the first time. The triplet excited states can be used as probes for the accessibility of the aromatic molecules to quenching molecules such as oxygen, nitroxide derivatives, paramagnetic ions, etc., and thus can provide evidence for or against intercalation. Because of its long lifetime the triplet excited state is sensitive to the local microenvironment and to the conformation of the DNA molecule.

Evidence is presented that the fluorescence of benzo(*a*)pyrene bound to DNA emanates from GC-GC intercalation sites. The fluorescence of benzo(*a*)pyrene bound to sites containing A-T base pairs appears to be quenched. Evidence on which these conclusions are based is twofold: (1) In solutions of ethanol/water, 2′-deoxythymidine is over ten times more efficient in quenching the fluorescence of benzo(*a*)pyrene than the other three nucleosides 2′-deoxyguanosine, cytidine, and 2′-deoxyadenosine. (2) When silver ions are added to aqueous solutions of the benzo(*a*)pyrene–DNA complex, the fluorescence of benzo(*a*)pyrene is strongly quenched. At low values of silver ion/base ratio, Ag^+ ions bind predominantly to guanosine in DNA.[13-16] The strong quenching of the fluorescence is accompanied by a corresponding increase in the production of triplets of benzo(*a*)pyrene. This indicates that Ag^+ ions

exert their quenching action by an enhanced spin-orbit coupling mechanism requiring a close-range exchange type interaction rather than by some induced conformational change in the DNA molecule. Since the Ag^+ ions are located at GC sites, the fluorescence emitting molecules which are quenched by silver ions are also located at these sites.

Experimental Section

Materials. The DNA used was a highly polymerized calf thymus DNA purchased from Sigma Chemical Co. Initial experiments were carried out using type V DNA (protein content 7.5% as determined by the method of Keller).[17] Aromatic hydrocarbon triplet decay times using this type of DNA were erratic. Type I DNA (protein content ~2%) and type I redeproteinized by a modified method of T'so and Lu[18] (protein content ~0.2%) yielded similar results and all of the data reported here pertain to this type of DNA. The hyperchromicity of the aqueous DNA solutions was found to be ~30%. Cytidine was purchased from Calbiochem, 2'-deoxythymidine was purchased from Boehringer Mannheim, and 2'-deoxyadenosine and 2'-deoxyguanosine were purchased from Sigma, and all were used as received.

The aromatic hydrocarbons, benzo(*a*)pyrene and benzo(*e*)pyrene, were purchased from Rütgers Werke, AG.; benz(*a*)anthracene was purchased from Eastman Organic, 7,12-dimethylbenz(*a*)anthracene was purchased from Sigma, acridine orange was purchased from the National Aniline Division of Allied Chemical and Dye Corporation; all compounds were recrystallized from ethanol. The pyrene was zone purified. All solvents were spectral grade and used without further purification. $AgNO_3$ and NaCl were reagent grade.

Methods. The aromatic hydrocarbon-DNA complexes were made by mixing fine crystals of hydrocarbon with DNA in glass distilled water and shaken a minimum of 17 h at room temperature. The suspensions were filtered through Whatman No. 1 filter paper and centrifuged in a Sorvall RC2-B centrifuge in Corex tubes for at least 0.5 h at 10 000 rpm. This technique was found to be sufficient to remove any crystals remaining in the solution. The effective concentration of hydrocarbons complexed with DNA was in the range 10^{-6} to 5×10^{-6} M. The bound hydrocarbons showed the characteristic 10 nm red shift in the absorption spectrum upon solubilization by DNA. The absorption and fluorescence maxima exhibited by benzo(*a*)pyrene-DNA complexes were in excellent agreement with those previously published[1,3,7] (absorption, 357, 374, 394 (main peak), 405 nm (shoulder); fluorescence, 414 and 437 nm). The fluorescence polarization for a typical complex was found to be $+0.18 \pm 0.01$ which compares well with the value (+0.183) obtained by Green[19] and shows that the hydrocarbons are indeed bound to the DNA. With benzo(*e*)-pyrene-DNA complexes, absorption bands were observed at 327 and 340 nm while the fluorescence bands were at 385, 396, 408, and 415 nm.

All absorption measurements were made on a Cary Model 14 recording spectrophotometer and all fluorescence measurements were made on a Perkin-Elmer Hitachi MPF-2A fluorimeter. Fluorescence decay times were measured by the single photon counting technique. The system is composed of Ortec components (9352-ns lamp pulser (nitrogen filled), 9290-lamp power supply, 437A time to amplitude converter, 403A time pick off control, 271 constant fraction timing discriminator) and an Amperex 56 DVP.03 photomultiplier. For determination of singlet lifetimes a Corning 7-37, 7-54 filter combination and a 310-nm interference filter were used to select the excitation wavelengths while a Corning 3-73 and 436 nm interference filter and a Corning 3-75 and 405 nm interference filter were used to view the fluorescence emission for benzo(*a*)pyrene and benzo(*e*)pyrene, respectively. Fluorescence decay curves of the complexed hydrocarbons were the same in both air saturated and deaerated solutions.

In the flash photolysis technique a strong flash of light excites the ground state singlets (S_0) to the first excited singlet state (S_1) of the aromatic hydrocarbons. S_1 may decay to S_0 by fluorescence ($S_1 \rightarrow S_0$) or by nonradiative intersystem crossover ($S_1 \rightsquigarrow T_1$) to the lowest triplet state T_1. A monitoring beam of light (100 W 12 V tungsten lamp) is used to excite T_1 to upper triplet states T_n ($T_n \leftarrow T_1$), resulting in an attenuation of the monitoring beam. As the triplets decay to the ground state ($T_1 \rightsquigarrow S_0$), the transient absorption decreases in time. The decay time of this transient absorption yields the triplet lifetime. In this work the decay was monitored photoelectrically either by photographing an oscilloscope trace (for microsecond time domains) or with a multichannel analyzer in the signal averaging mode (millisecond time domains). Two types of flash photolysis systems were used, a conventional xenon-flash lamp-pumped system (Northern Precision No. FP-2R, 50 J, 20 μs lamp width at half-height) and a frequency doubled ruby laser system (50–100 mJ at 347.1 nm). The laser system had a sample path length of 10 cm and was used when the triplet lifetime was in the microsecond range, whereas the conventional system has a 20 cm path length and was used when the lifetimes were in the millisecond range.

Oxygen was removed from the solutions by two techniques. Complexes were subjected to 7–10 freeze–pump–thaw cycles on a mercury free vacuum system and sealed off with an O-ring high vacuum stopcock at a pressure of $\sim 5 \times 10^{-5}$ mm. These solutions gave the longest observed triplet lifetimes. Other samples were deoxygenated by vigorously bubbling oxygen-free nitrogen gas (less than 0.5 ppm O_2, purchased from the Linde Division of Union Carbide Corp.) through the solution. This was accomplished by constructing a cell with two side arms, both sealed with septum caps. Nitrogen was admitted to the solution through a needle and allowed to escape through another needle. Solutions were bubbled for at least 0.5 h until a limiting lifetime was observed. The longest lifetime attained using the N_2 bubbling technique was 79 ms for benzo(*e*)pyrene in DNA, whereas the freeze–pump–thaw method gave 155 ms. Measurements showing the effects of various salts on the triplets were made by using the N_2 bubbled solutions and admitting a small amount of salt from a stock solution to the cell with a Hamilton syringe. The solution was then rebubbled until no further change in lifetime was observed. Using this technique had the advantage of being able to perform the measurements sequentially on a single sample.

All measurements were made at room temperature (24 ± 1 °C). The hydrocarbon-DNA complexes showed a high degree of stability. A long-lived benzo(*e*)pyrene-DNA complex (vacuum deaerated) was left in the photolysis cell at room temperature for 25 days with a decrease of only 24% in the lifetime of the triplet. The triplet lifetime of benzo(*a*)pyrene in DNA is independent of DNA concentration in the range of 0.4–3 mM DNA (based on phosphate residues). After repeated flashing of a benzo(*a*)pyrene or a benzo(*e*)pyrene-DNA complex the solution was extracted with benzene five times. There was no detectable hydrocarbon in the aqueous phase showing that no covalently linked hydrocarbon-DNA complex was formed as a result of repeated flash illumination. However, it is known that covalent aromatic hydrocarbon-DNA complexes are formed upon more prolonged irradiation.[20,21]

Results and Discussion

Fluorescence Study of the Heterogeneity of Binding Sites. Polycyclic aromatic hydrocarbons are only sparingly soluble in water[22] (typically in the range of 10^{-7}–10^{-9} M). The extent of binding of these aromatic molecules in DNA in aqueous solutions is about one to ten aromatic molecules per 10^4 base pairs. The extent of binding appears to be related to the size of the aromatic hydrocarbons, being smaller for the larger molecules.[23] Little is known about the specific modes of binding of the polycyclic molecules to DNA.

Fluorescence decay curves for benzo(*a*)pyrene and benzo(*e*)pyrene are shown in Figures and 1 and 2, respectively. The decay profile of the lamp and the photon counts picked up with a DNA solution without the aromatic hydrocarbons is also shown. The latter signal is 15–110 times smaller than the fluorescence and can thus be neglected.

The decay of the fluorescence is clearly nonexponential. The solid line in Figures 1 and 2 represents a convoluted computer fit of the decay using two exponentials according to the equation:

$$I(t) = I(0)x_1e^{-t/\tau_1} + I(0)x_2e^{-t/\tau_2} \quad (1)$$

$I(t)$ is the fluorescence intensity as a function of time t, and $I(0)$ is the fluorescence intensity at $t = 0$. x_1 and x_2 are the fractions of binding sites where the decay time is τ_1 and τ_2, respectively ($x_1 + x_2 = 1.0$).

A good fit is obtained if one neglects the tail of the fluorescence decay in Figure 1; however, less than 1% of the total emission occurs in this tail so that it can be safely neglected.

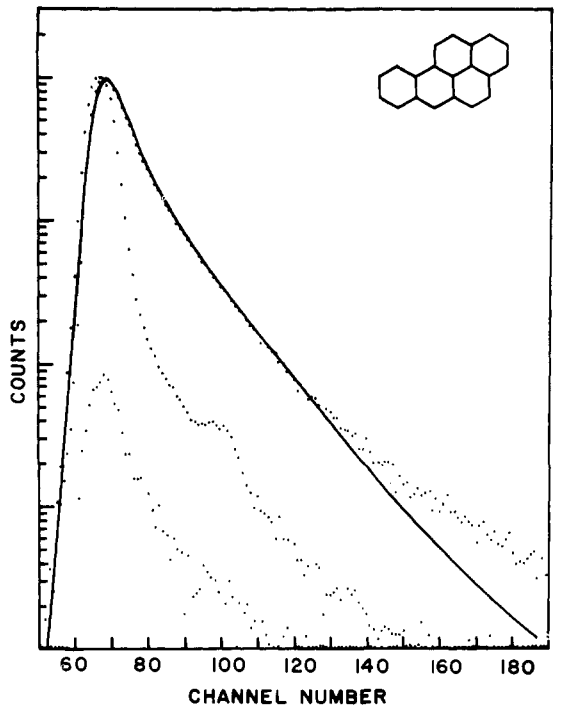

Figure 1. Upper data points: fluorescence decay curve of benzo(*a*)pyrene–DNA complex (1.4×10^{-3} M phosphate residues in aqueous solution). Solid line: computer fit of decay curve according to eq 1 with $f_1/f_2 = 4.38$, $\tau_1 = 3.2$ ns, and $\tau_2 = 10$ ns. Middle set of data points: decay profile of the excitation flash (normalized to upper data points at the peak). Lowest set of data points: blank DNA solution (no benzo(*a*)pyrene); same counting time as for upper decay curve. The counting time for both DNA solutions was 2000 s. Time scale for all of the data: 0.781 ns/channel.

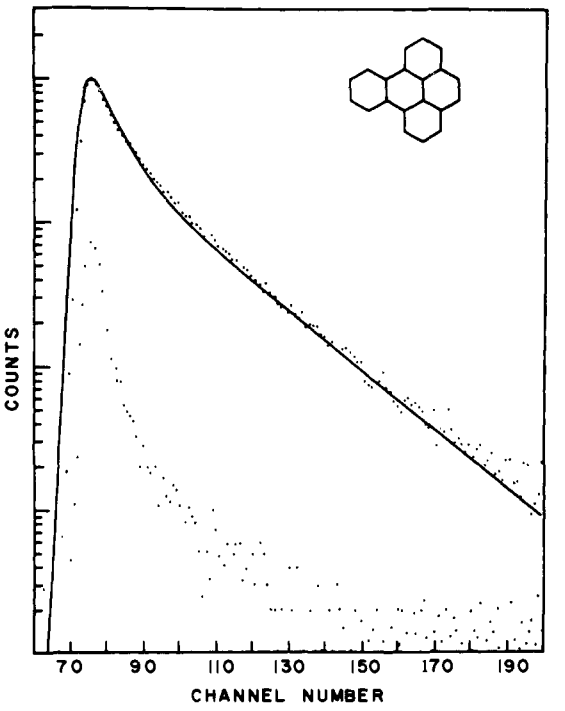

Figure 2. Upper data points: fluorescence decay curve of benzo(*e*)pyrene–DNA complex (1.4×10^{-3} M phosphate residues in aqueous solution). Solid line: computer fit of decay curve according to eq 1 with $f_1/f_2 = 3.62$, $\tau_1 = 9$ ns, and $\tau_2 = 33$ ns. Lower data points: blank DNA solution (no benzo(*e*)pyrene); counting time for both sets of data was 2000 s. Time scale: 1.56 ns/channel.

The values of x, τ_1, and τ_2 for both benzo(*a*)pyrene and benzo(*e*)pyrene are shown in Table I. In degassed benzene the fluorescence decays exponentially and is 30 ns for benzo(*a*)pyrene and 61 ns for benzo(*e*)pyrene (in degassed cyclohexane these values are 47 and 61 ns, respectively). It is therefore evident from the shorter lifetimes in the DNA complexes that strong quenching of the fluorescence occurs. There are two types of fluorescence emitting sites for these polycyclic hydrocarbons as is evidenced by the two decay times. By taking into account the quantum yield of fluorescence of the DNA complexes relative to the fluorescence of the aromatic hydrocarbons dissolved in benzene (Table I) it can be shown that there is a third binding site (or type of sites). At this site, or type of sites, static quenching[24] of the aromatic hydrocarbon singlet excited states takes place, i.e., none of the excited molecules decay by fluorescence. At the other two sites where the molecules decay with a shortened lifetime, the quenching is of a dynamic[24] nature.

The quantum yield of fluorescence of the aromatic hydrocarbons in DNA was measured relative to their quantum yields in degassed benzene. The aromatic hydrocarbons were excited in a wavelength range where there is no absorbance due to the DNA. If the quantum yield of the fluorescence of the polycyclic molecules in benzene is denoted by Q_{BENZ}, and if the fluorescence yield in the DNA complex is denoted by Q_{DNA}, then it can be shown that the relative quantum yield Q is:

$$Q = \frac{Q_{DNA}}{Q_{BENZ}} = \frac{I(0)}{I_B(0)}\left[\frac{x_1\tau_1 + x_2\tau_2}{\tau_B}\right] \qquad (2)$$

$I_B(0)$ and $I(0)$ are the fluorescence intensities at time $t = 0$ in the degassed benzene and DNA solutions, respectively, and are the intensities of fluorescence extrapolated to the time corresponding to the beginning of the fluorescence decay curve. τ_B is the fluorescence decay time of the aromatics in degassed benzene. The quantities $I(0)x_1\tau_1$ and $I(0)x_2\tau_2$ in the numerator are proportional to the number of molecules at the two different binding sites in DNA from which fluorescence can occur. These two sites will be denoted sites 1 and 2, while the third type of site from which fluorescence does not occur is termed site 3. The fraction of molecules at each type of site will correspondingly be denoted by f_1, f_2, and f_3 with $f_1 + f_2 + f_3 = 1.0$.

Table I. DNA Complexes of Benzo(*a*)pyrene (BaP) and Benzo(*e*)pyrene (BeP) in Aqueous Solutions at 24 °C[a]

f_1	~0.35	~0.47
f_2	~0.08	~0.13
f_3	0.57 ± 0.02	0.40 ± 0.05
x_1	0.82	0.78
x_2	0.18	0.22
τ_1	3.2 ns	9.0 ns
τ_2	10 ns	33 ns
τ_B(in C_6H_6)	30.1 ns	61.0
Q	0.063 ± 0.004	0.14 ± 0.02

[a] Fluorescence decay time (τ) and fractions of molecules located at fluorescence emitting sites, f_1 and f_2, and at nonemitting sites (f_3). Q is the quantum yield of fluorescence of the aromatic molecules in the DNA complexes relative to the quantum yield of the same aromatic molecules dissolved in degassed benzene. x_1 and x_2 are defined in eq 1.

Using eq 2 and the measured values of Q, x_1, x_2, τ_1, and τ_2, we can calculate $I(0)/I_B(0)$. This is the fraction of molecules bound at fluorescence emitting sites in DNA. The term in the brackets in eq 2 would be equal to Q ($I(0) = I_B(0)$) if the quenching of the hydrocarbons was of a totally dynamic nature. The fraction of molecules at static quenching sites in DNA is therefore equal to

$$f_3 = 1 - I(0)/I_B(0) \qquad (3)$$

Knowing the values of f_3, we can then calculate f_1 and f_2 using $f_1 = (1 - f_3)x_1$ and $f_2 = (1 - f_3)x_2$. The results for f_1,

f_2, and f_3 and the data from which these were calculated are summarized in Table I. The results of this analysis indicate that about 40–60% of all the aromatic hydrocarbon molecules are located at binding sites in DNA where the decay of the singlet excited states is nonradiative in nature.

There are chemically ten distinct sites in DNA for intercalation[25] of the polycyclic molecules between adjacent base pairs of DNA. Using the nearest neighbor frequencies of base sequence[26] and considering intercalation sites to be only of the type GC-GC, GC-AT, and AT-AT, we calculate that there are 29% AT-AT sites, 17.5% GC-GC sites, and 53.6% AT-GC sites in calf thymus DNA. Thus, about 71% of all the sites contain at least one GC base pair, and 82% contain at least one AT pair. Of interest is what is the distribution of polycyclic aromatic molecules among these sites and what is the nature of the fluorescence emitting binding sites. Evidence will be presented below that most of the fluorescence is emitted from molecules bound at GC containing sites and that the nonemitting binding sites are probably AT containing sites.

Quenching of Fluorescence by Nucleosides in Solution. In an attempt to explain the quenching of the fluorescence of aromatic hydrocarbons bound to DNA and in order to gain an insight into the mechanism of quenching, the effect of the different purines and pyrimidines on the fluorescence of BaP dissolved in ethanol/water mixtures was investigated. The selection of the ethanol-water solvent system had a twofold purpose. First, the solubility of the aromatic hydrocarbon in the mixture of ethanol and water is reasonably high, permitting the accurate determination of singlet decay times. Second, the effect of solvent polarity on the quenching efficiency of the different nucleosides can be investigated by varying the water content of the mixtures.

The experiments consisted of determining the fluorescence lifetimes of BaP solutions ($\sim 10^{-6}$ M) in the presence of the different nucleosides (concentration $\sim 10^{-2}$ M). The fluorescence quenching follows the Stern-Volmer equation

$$\frac{1}{\tau} = \frac{1}{\tau_0} + K[Q] \tag{4}$$

where τ is the fluorescence lifetime in the presence of the quencher whose concentration is [Q], τ_0 is the lifetime when [Q] = 0, and K is the quenching constant. We have studied the quenching of the fluorescence by 2′-deoxythymidine (T), cytidine (C), 2′-deoxyadenosine (A), and 2′-deoxyguanosine (G). The values of τ_0 and K for these nucleosides are given in Table II. It is evident that T is the most potent quencher of the fluorescence by at least one order of magnitude. Similar results were obtained with pyrene.

The quenching ability of the nucleosides increases with solvent polarity. The differences between τ and τ_0 for C, G, and A in the 50/50 and 30/70 water/ethanol mixtures were of the order of 1 ns only. Therefore, for these particular combinations only the upper limits of the quenching constants K are listed.

Neither the fluorescence nor the absorption spectra of the aromatic hydrocarbons was altered by the presence of the nucleosides in the solvent mixtures listed in Table II. This behavior, together with an increasing quenching efficiency with increasing polarity of the solvent, is indicative of a charge-transfer exciplex quenching mechanism.[27]

In this quenching mechanism, there is no association between the quencher and aromatic hydrocarbon molecules in the ground state. However, a short-lived complex can be formed when the aromatic hydrocarbon molecule in the excited state collides with a nucleoside. This process is diffusion controlled and the complex has a charge-transfer character. It has a tendency to dissociate into a radical cation and a radical anion, particularly in polar solvents or solutions. In polar solutions, the solvation of the ions by the solvent molecules lowers the energy to such an extent that upon recombination of the cation and anion the total energy is below that of the singlet excited state and either triplet or ground state molecules are formed, and the fluorescence is thus quenched. The initial dissociation rate constant of the complex increases with increasing polarity of the solvent thus giving rise to a higher quenching efficiency at higher polarities.[27]

Table II. Quenching of the Fluorescence of Benzo(*a*)pyrene by Different Nucleosides in Water/Ethanol Mixtures[a]

		$K \times 10^{-8}$ l. mol^{-1} s^{-1}			
Solution (% vol)	τ_0[b]	T	C	G	A
70% H_2O/30% ethanol	35.6 ± 0.1	15.9	2.2	1.9	1.3
50% H_2O/50% ethanol	39.1 ± 0.1	3.8	≲0.2[c]	≲0.2[c]	≲0.2[c]
30% H_2O/70% ethanol	41.3 ± 0.1	0.89	≲0.2[c]	≲0.2[c]	≲0.2[c]

[a] K is the quenching constant defined in eq 4. T is 2′-deoxythymidine, C is cytidine, A is 2′-deoxyadenosine, G is 2′-deoxyguanosine. [b] Lifetime in nanoseconds, oxygen-free solutions, without any nucleosides added. [c] The differences in the lifetimes (between τ and τ_0 in eq 4) were too small to permit accurate calculations of K.

Of interest is, which molecule, the aromatic hydrocarbon or the nucleoside, is the electron acceptor and which is the donor in the charge-transfer complex. This is determined by the relative ionization potentials and electron affinities of the two molecules in the complex. The ionization potentials of the aromatic hydrocarbons[28] are generally lower than those of the bases.[29] The electron affinities are not that well known, but calculations[30-32] indicate that pyrimidines are better electron acceptors than purines. Furthermore, guanine has the lowest ionization potential and is the best electron donor while thymine has the highest ionization potential. Since thymidine is the best quencher of fluorescence, it may well be that it is the acceptor rather than the donor in the quenching reactions taking place in the solvent mixtures used in this work.

The significantly higher quenching efficiency of T in solvent mixtures probably indicates that the fluorescence of BaP is also quenched strongly at intercalation sites in DNA containing at least one A-T base pair. The fluorescence emitting sites are thus probably G-C sites. This conclusion is further supported by silver ion quenching experiments described below.

Quenching of Excited States by Other Molecules or Ions. In the studies of the nature of the covalent binding of carcinogens to DNA, the question can arise whether the carcinogen is intercalated or bound on the outside of the DNA helix. Indirect evidence for intercalation are the magnitude of the red shift in the absorption and fluorescence of the bound hydrocarbon and changes in the circular dichroism spectra. Additional diagnostic experiments which have a bearing on this question are desirable. Quenching of the excited states by molecules (or ions) other than DNA is a bimolecular process which can provide information about the accessibility of the carcinogens when they are bound to DNA.

The excited singlet and triplet states of polycyclic aromatic hydrocarbons dissolved in fluid solutions are readily quenched by paramagnetic molecules and ions,[29-34] as well as by diamagnetic atoms or ions of relatively high atomic number[34-36] (the heavy-atom effect). The lifetimes τ of the excited states in the presence of a quencher whose concentration is denoted by [Q] are described by the well-known Strn-Volmer relationship in eq 4. In this equation K is the dynamic quenching constant and is a measure of the frequency of the bimolecular encounters and the probability of quenching per encounter. K

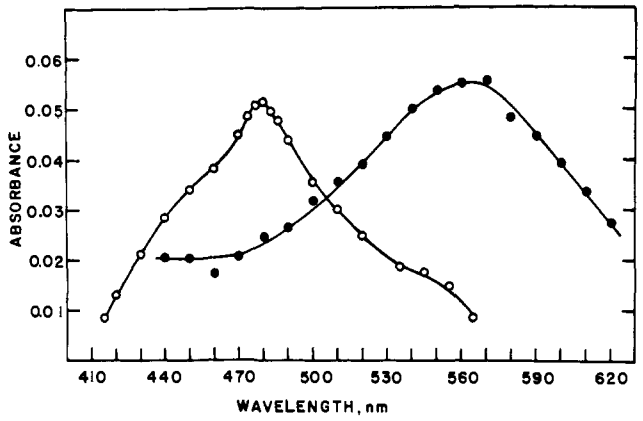

Figure 3. Typical triplet-triplet absorption spectra of benzo(*a*)pyrene-DNA complex (○) and benzo(*e*)pyrene-DNA complex (●) in aqueous solution (1×10^{-3} *M* phosphate residues).

is a function of the diffusion coefficients of Q and the excited molecule. According to our measurements using molecular oxygen as the quencher and the polycyclic aromatic compounds pyrene[37] and benzo(*a*)pyrene dissolved in water (the solubility is about 10^{-6} and 10^{-9} M, respectively), K is equal to $1\text{-}2 \times 10^{10}$ l. mol^{-1} sec^{-1}. This is close to or equal to the diffusion controlled limit.[38] When aromatic hydrocarbons are buried within the hydrophobic regions of a globular protein dissolved in aqueous solutions, K decreases by a factor of 10-100.[37,38] Measurements of this type are indicative of the accessibility of a relatively small aromatic polycyclic molecule to oxygen when it is complexed to a large biomacromolecule. The two different modes of binding of aromatic hydrocarbons to DNA, intercalation and outside binding, should give characteristically different values of K. For ethidium bromide intercalated in DNA, Lakowicz and Weber[38] found that K is 1.06×10^8 l. mol^{-1} s^{-1}, which is considerably smaller than values of K obtained in solutions without DNA. However, in order to determine K for ethidium bromide bound to DNA, Lakowicz and Weber had to resort to high (up to 100 atm) partial pressures of oxygen. At 1 atm partial pressure of oxygen, the concentration of oxygen in water is only 1.3×10^{-3} mol l.$^{-1}$, which is not sufficient to obtain a measurable change in the singlet lifetime from which K could be calculated using eq 4. Accordingly, there is no observable change in the fluorescence decay curves of BaP and BeP bound to DNA at partial pressures of oxygen of up to 1 atm. In order to measure K for these aromatic hydrocarbons bound to DNA, the use of high-pressure cells might give rise to sufficient decreases in the singlet lifetimes. However, analyses might be somewhat complicated because of the nonexponentiality of the decay and the fact that the lifetimes are low even in the absence of oxygen.

In order to determine accessibilities we studied the quenching by oxygen of the triplet excited states of the polycyclic compounds bound to DNA. Because transitions from the triplet to the singlet ground state are spin forbidden, the triplet excited states, in the absence of strong quenchers, have lifetimes which are orders of magnitude longer than those of the singlets. The triplet excited states are thus sensitive to even low concentrations of oxygen in solution,[37] since there is a larger probability of an oxygen molecule diffusing toward and colliding with the excited molecule during the latter's longer lifetime.

We have observed, using flash photolysis techniques, the excited triplet states of polycyclic aromatic hydrocarbons complexed with DNA in aqueos solutions at room temperature. Before we proceed to a discussion of the quenching by molecular oxygen, the properties of the triplets of aromatic hydrocarbons bound to DNA will be described.

Triplet States of Polycyclic Aromatic Compounds Bound to DNA. Using laser flash photolysis or conventional flash photolysis techiques[39] a transient absorption is observed following the brief exciting flash. The spectra of this transient absorption for benzo(*a*)pyrene- and benzo(*e*)pyrene-DNA complexes dissolved in aqueous solutions at room temperature are shown in Figure 3. The spectra for benzo(*a*)pyrene and benzo(*e*)-pyrene agree well with the triplet-triplet absorption spectra of these compounds in other solvents.[37,40,41] In the case of benzo(*a*)pyrene in DNA the maximum occurs at 480 rather than at ~470 nm (470 nm is typical in organic solvents[40] and bovine serum albumin).[37] The maximum is at 550-570 nm for benzo(*e*)pyrene in DNA and is considerably broader and less well structured than the analogous maximum at 559 nm in a solution of alcohol and tetrahydro-2-methylfuran.[41]

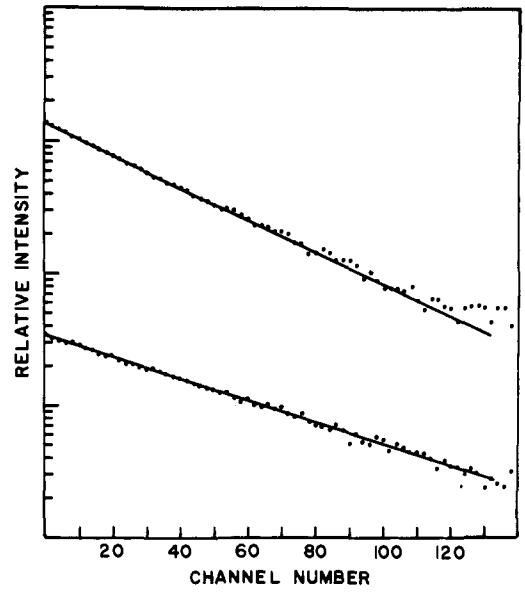

Figure 4. Decay curves of triplet-triplet absorption of benzo(*a*)pyrene-DNA complex (upper curve, 1 ms/channel, decay time 35 ms) and benzo(*e*)pyrene-DNA complex (lower curve, 3 ms/channel, decay time 155 ms) in aqueous solutions (1×10^{-3} M phosphate residues). The solutions were degassed by ten or more repeated freeze-pump-thaw cycles.

The red shift in the $T_1 \rightarrow T_n$ absorption in benzo(*a*)pyrene is similar to the 10-nm bathochromic shift observed in the case of the absorption and fluorescence of this compound. This shift is due to an increase in the polarizability[42] in the excited states T_n and their increased interaction with the base pairs of DNA. As a result of this interaction the upper excited triplet T_n appears to be more stabilized than the energetically lower T_1 state, thus accounting for the red shift.

The decay times for rigorously degassed solutions were in the range of 30-150 ms. Two examples of the time dependence of the $T_1 \rightarrow T_n$ absorption for benzo(*a*)pyrene and benzo(*e*)-pyrene complexes in DNA are shown in Figure 4. The maximum triplet decay time observed with benzo(*e*)pyrene was 155 ms and that with benzo(*a*)pyrene was 35 ms. More typically, the decay times were about 110-120 and 25-30 ms, respectively. The longest triplet decay times are obtained after repeated (7-10) freeze-pump-thaw degassing cycles. Nitrogen bubbling can also produce lifetimes in the range of 10-30 ms for benzo(*a*)pyrene and 40-80 ms for benzo(*e*)pyrene. Thus, nitrogen bubbling tends to give lower lifetimes than the more rigorous freeze-pump-thaw degassing procedure. It should be further emphasized that exact reproducibility of triplet lifetimes is difficult to achieve. Due to their long lifetimes, the triplets can be easily quenched by adventitious impurities, particularly traces of dissolved oxygen. Also, if the DNA is not carefully deproteinized, shorter lifetimes are generally obtained. However, the significant difference between the triplet lifetime of benzo(*a*)pyrene and benzo(*e*)pyrene is real. If these

lifetimes are limited by adventitious impurities, then the quenching probability for benzo(*e*)pyrene must be lower by a factor of 4–5. Traces of oxygen cannot account for the limiting triplet lifetimes measured, since in that case, the lifetimes for both compounds would be the same (the oxygen quenching constants are nearly the same, see below). It is therefore concluded that a possible reason for the differences in the highest values of the triplet lifetimes in degassed solutions is a specific quenching interaction of the triplets with DNA. We note that for BeP both the singlet and triplet decay times are longer than for BaP, indicating that the interactions of the excited states of BaP with DNA are stronger.

The triplet lifetime of benzo(*a*)pyrene in a rigid inert matrix is ~80 ms,[37] whereas it is 35 ms in the DNA complex. Since the lifetime of the triplet of this molecule in DNA approaches the intrinsic unimolecular value in an inert matrix, the triplet quenching processes which are operative in DNA are much less effective than the singlet quenching for these same molecules. For benzo(*e*)pyrene, the triplet lifetime in a rigid plastic matrix is 2.0 s at room temperature, which is about 13 times larger than the longest triplet lifetime observed in DNA. Comparing singlet and triplet lifetimes for these molecules it is evident that the dynamic quenching constant for quenching of the excited states in DNA complexes is 10^6 times *smaller* for triplets than for singlets.

In contrast to the decay of the singlets, the decay of the triplets is exponential over at least 2–3 lifetimes (Figure 4). We have compared the relative yield of triplets produced in the DNA complexes and in benzene solution. This was done by measuring the relative absorbance $A_T(0)$ at time $t = 0$, i.e., just following the exciting flash, for solutions of different optical densities. This value is proportional to the number of triplet excited states $T(0)$ produced initially. $T(0)$ is proportional to the concentration of aromatic molecules present either in benzene or in the DNA complex. A plot of $A_T(0)$ as a function of the optical densities of the solutions (kept below a value of 0.1 with a 1 cm path length and measured in a Cary Spectrophotometer) gave straight lines. The relative quantum yield of triplets is given by the ratio of the slopes S of these lines multiplied by the ratio of molar extinction coefficients ϵ:

$$Q' = \frac{Q'_{DNA}}{Q'_{BEN}} = \frac{S_{DNA}}{S_{BEN}} \frac{\epsilon_{DNA}}{\epsilon_{BEN}} \qquad (5)$$

The ratios $\epsilon_{DNA}/\epsilon_{BEN}$ are not known for $T_1 \rightarrow T_n$ absorption. Analogous ratios for singlet–singlet absorption spectra for benzo(*a*)pyrene and benzo(*e*)pyrene have been determined by Mayevskii et al.[21] After taking into account the red shift in the absorption spectra, the $S_0 \rightarrow S_1$ absorption coefficient appears to be roughly constant in the case of benzo(*a*)pyrene and to increase by about 33% for benzo(*e*)pyrene upon changing the environment from ethanol to DNA.[21] The changes in ϵ for $T_1 \rightarrow T_n$ are not likely to be much larger. The values of Q' obtained (ignoring any variation in ϵ) are $Q' = 0.4$ for benzo(*a*)pyrene and $Q' = 1$ for benzo(*e*)pyrene. These numbers are accurate to within about a factor of 2 only due to the neglect of the possible variation in ϵ. However, it is evident by comparison with the values of Q in Table I that the overall static quenching of triplets in DNA is also less effective than the static quenching of the singlet excited states in the DNA complexes.

Transient absorption attributable to triplets was also observed with other compounds: benz(*a*)anthracene, 7,12-dimethylbenz(*a*)anthracene, and acridine orange. No signal was observed with pyrene unless small amounts of silver ions were added (see below).

Effects of Salt Concentration on Triplet Lifetimes. At low ionic strengths ($\mu < 10^{-4}$ M) DNA tends to be partially denatured.[43] The extent of binding of benzo(*a*)pyrene tends to be highest at low ionic strengths and to decrease with increasing salt concentration.[19,44] Based on this type of behavior, it is believed that polycyclic aromatic hydrocarbons bind to DNA preferentially at disordered regions of the double helix. As the salt concentration is increased, the electrostatic repulsion between adjacent phosphate groups is decreased due to the shielding effect by the counterions and the helical structure is stabilized.

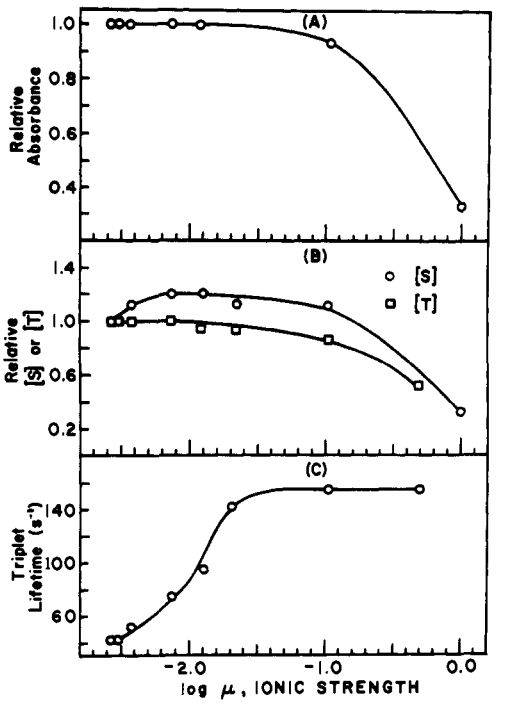

Figure 5. Effect of salt (NaCl) concentration on properties of benzo(*a*)pyrene–DNA complexes; μ is the total ionic strength of the solution. The DNA concentration is constant (2.7×10^{-3} M phosphate residues). (A) Relative absorbance of benzo(*a*)pyrene at 394 nm. (B) (○) Relative concentration of singlet excited states [S] upon steady state excitation as determined by fluorescence intensity. (□) Relative concentration of triplets [T] determined from initial triplet–triplet absorbance immediately (within <0.1 ms) after the excitation flash. (C) Triplet decay rates (the decays were exponential at all salt concentrations). All of the solutions were degassed by nitrogen bubbling.

The effect of increasing the ionic strength on the relative number of benzo(*a*)pyrene molecules complexed, the relative yield of triplets per flash [T], the fluorescence intensity, and the triplet lifetimes are shown in Figure 5. There is little change in the amount of aromatic hydrocarbons bound for $\mu \lesssim 0.1$; in this range [T] is fairly constant, whereas the fluorescence tends to increase somewhat with increasing μ. The triplet lifetime, on the other hand, suffers the sharpest decrease which tends to level out at values of $\mu \gtrsim 2 \times 10^{-2}$.

The quenching of the triplet excited states in DNA is a spin-forbidden process and is most likely due to a short range exchange interaction with the quencher. With increasing salt concentration the contact between base pairs and the aromatic hydrocarbons increases since the electrostatic repulsion between adjacent phosphate groups is decreased. This is a likely explanation for the salt concentration effect shown in Figure 5. The singlet excited states are probably less affected by these effects since the interaction with neighboring base pairs can occur via a longer range Coulombic interaction.

Quenching of Triplets by Oxygen. In the presence of oxygen the lifetime of the triplets is significantly shortened. In air saturated aqueous solutions the decay is exponential and lifetimes are typically in the range of 15–40 μs. It has been shown elsewhere[45] that τ^{-1}, where τ is the triplet lifetime, is proportional to the oxygen concentration, and thus follows the Stern–Volmer quenching law, eq 4. The quenching constant

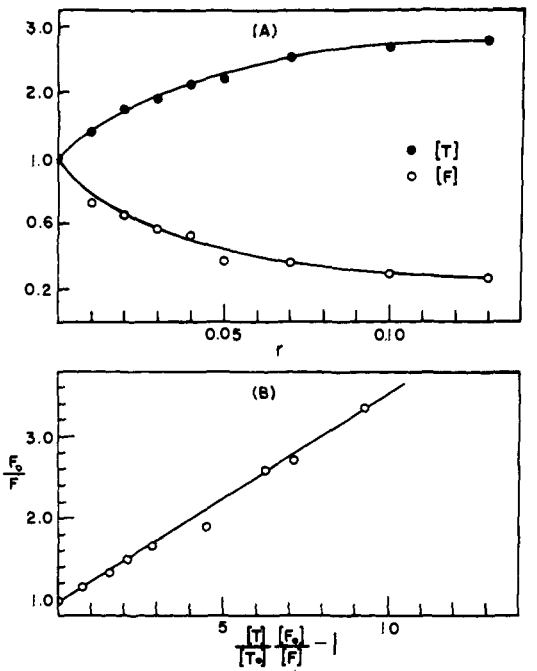

Figure 6. Effect of silver ions. (A) The relative fluorescence intensity [F] and initial concentration of triplets [T] just following the excitation flash in benzo(*a*)pyrene–DNA complexes in aqueous solutions (DNA concentration: 1×10^{-3} M phosphate residues). The silver ion concentration is expressed in terms of r (moles of $AgNO_3$/mole of DNA phosphate residue). (B) Data in (A) plotted according to eq 6, see text.

is more easily measured using the laser flash photolysis apparatus with air-saturated solutions of the polycyclic aromatic–DNA complexes. The characteristic $T_1 \rightarrow T_n$ absorption spectra are obtained indicating that two-photon ionization is negligible in these experiments. Under these circumstances $\tau^{-1} \approx K_T[O_2]$, and the quenching constant K_T for triplets can be calculated readily using the handbook[46] value of the Henry's law constant. (The oxygen concentration in water at 24 °C is 2.76×10^{-4} M at 760 mm air pressure.) The quenching constant K_T obtained in this manner was $(2.0 \pm 0.2) \times 10^8$ l. mol^{-1} s^{-1} for benzo(*a*)pyrene and $(1.6 \pm 0.2) \times 10^8$ l. mol^{-1} s^{-1} for benzo(*e*)pyrene, benz(*a*)anthracene, and 7,12-dimethylbenz(*a*)anthracene; the quenching constant for acridine orange was the lowest, namely $(0.80 \pm 0.1) \times 10^8$ l. mol^{-1} s^{-1}.

Oxygen quenching constants determined for some of the same aromatic hydrocarbons dissolved in fluid solutions (hexane and benzene) are in the range[47] of $(2\text{-}3) \times 10^9$ l. mol^{-1} s^{-1}. Thus, in the DNA complexes the availability of the polycyclic aromatic molecules to oxygen is reduced by a factor of 10–20. This is to be expected for intercalation of the aromatic compounds between the base pairs of DNA since physical contact between oxygen and the polycyclic molecules, which is necessary in exchange type quenching interactions, is reduced. Triplet quenching studies may thus prove useful in determining the properties of the binding sites in covalent polycyclic aromatic–DNA complexes.

Effect of Conformation of DNA on Triplet Lifetimes. Upon heat denaturation of DNA–benzo(*a*)pyrene complexes in aqueous solution in a sealed, degassed container, the triplet decay times decrease (the samples are heated at 95 °C for 0.5 h and then rapidly cooled in an ice–water bath). Thus, for a 10^{-3} M DNA solution in distilled water the triplet lifetime of benzo(*a*)pyrene is 35 ms, which decreases to 11 ms upon denaturation. Similar results were obtained in a 10^{-2} M HMP buffer solution (pH 6.8), the lifetime decreasing to 20 ms upon denaturation. The oxygen quenching constant, K_T, on the other hand, increases from 2.0 to 2.5×10^8 l. mol^{-1} s^{-1}. At low salt concentrations the extent of binding of benzo(*a*)pyrene to denatured DNA is lower than to native DNA.[44] It is likely that even in denatured DNA there are residual helical regions[48] to which the remaining benzo(*a*)pyrene molecules can bind. Thus, the quenching constant is increased by only 25% upon denaturation. The degassed value of the triplet lifetime is decreased to a larger extent than the quenching constants and is probably due to the overall change in the conformation of the DNA molecule upon denaturation.

Effect of Silver I Ions on the Fluorescence. The binding of silver ions to DNA and polynucleotides has been studied extensively.[13-16] At low concentration of Ag^+ ions such that the extent of binding is $r \lesssim 0.20$ (r = moles of Ag^+ ions bound/mole of DNA base), the type I complex is formed by binding preferentially to GC base pairs without the release of protons; the silver ion appears to be bound predominantly to guanine. The formation of a type II complex for higher values of r is accompanied by the release of protons. In this work fluorescence quenching studies in the range of $0 \lesssim r \lesssim 0.13$ are described in detail.

The quenching of the fluorescence of BaP and BeP in DNA with increasing r is shown in Figure 6A. The decrease in the fluorescence intensity with increasing r is similar to the results obtained by Green[19] for BaP. We have also observed the decay profiles of the fluorescence at these different values of r. While the overall number of photon counts per unit time interval decreased uniformly with increasing r, there was no discernible change in the shape of the decay curves. Therefore, the quenching effect due to the silver ions is a case of static quenching. Upon binding of the silver ion in the vicinity of an intercalated aromatic hydrocarbon molecule, that particular molecule is removed from the pool of potentially fluorescence emitting molecules.

Since there are no energy levels of Ag^+ ions[49] near or below the excited singlet state of the hydrocarbon, energy transfer from the excited molecule to the ion is not possible. A recent study[36] shows that diamagnetic positive ions can quench the fluorescence of aromatic hydrocarbons via an increase in the rate of intersystem crossing $S_1 \rightsquigarrow T_1$. In Figure 6A it is shown that the quenching of the fluorescence of benzo(*a*)pyrene in DNA is accompanied by an increase in the production of triplets. This effect arises because of the increase in the spin-orbit coupling induced by the heavy atom (or ion) and is due to an exchange type interaction requiring a close approach between the heavy ion and the polycyclic aromatic molecule.[34]

Using experimental data of the type shown in Figure 6A, the triplet quantum yield Q'_{DNA} in the absence of the quencher can be calculated. The fluorescence yields in the absence (F_0) and presence of quencher (F) and the relative number of triplet states produced [T_0] and [T] in the absence and presence of the quencher, respectively, are related by the equation:[34]

$$\frac{F_0}{F} = Q'_{DNA}\left[\frac{[T][F_0]}{[T_0][F]} - 1\right] + 1 \qquad (6)$$

The data in Figure 6(A) have been plotted according to eq 6 in Figure 6B and it is evident that the data are consistent with this equation. The slope of this line is equal to the triplet quantum yield in the absence of Ag^+ ions at the fluorescence emitting sites 1 and 2 and is calculated to be $Q'_{DNA} = 0.26$. In solution, the quantum yield of triplets is reported to be equal to 0.58,[50] thus higher than in the DNA complexes. Since the overall production of triplets at all binding sites is ~40% of the benzene value (neglecting any significant differences between ϵ_{DNA} and ϵ_B), the absolute quantum yield of triplets in DNA is calculated to be $0.58 \times 0.40 \approx 0.23$. The measured triplet quantum yield of ~0.26 at sites 1 and 2 only calculated from eq 6 does not produce enough triplets to account for the total

absolute yield of triplets of 0.23 observed at all three sites. It is concluded that triplet states are also produced at the non-fluorescent sites characterized by f_3; thus the singlet excited states are completely quenched at these sites, but this quenching, at least in part, leads to the production of triplet states.

The quenching of the fluorescence by the addition of Ag^+ ions is similar for benzo(*a*)pyrene and for benzo(*e*)pyrene as shown in Figure 7. The quenching of the fluorescence decay times of these molecules by Ag^+ ions was also investigated in a 50/50 (v/v) water/ethanol mixture. The quenching constants were found to be $\sim 10^9$ l. mol^{-1} s^{-1} in this solvent mixture and to be nearly the same for both compounds. The quenching of the fluorescence of benzo(*a*)pyrene and benzo(*e*)pyrene in DNA complexes is a static quenching effect (since the fluorescence decay times are unchanged) and depends on a nearest neighbor contact between the aromatic hydrocarbons and the Ag^+ ion. This can be inferred from the fact that there is an enhanced production of triplets which accompanies the quenching of the fluorescence; this type of interaction is a close-range exchange interaction.

Considerable evidence has been presented that Ag^+ ions bind preferentially to guanosine.[13-16] At an r value of 0.13, it can be calculated using the known composition of DNA (42% G-C) that 55% of all G-C sites contain a silver ion at $r = 0.13$; in this calculation it is assumed that the silver ions bind to guanosine in a random manner, independent of the nature of the binding site (i.e., GC···AT or GC···GC intercalation site). If the Ag^+ ions prefer G-C···G-C sites to G-C···A-T type sites, than these G-C···G-C sites are completely titrated at an r value of 0.09. The fluorescence of benzo(*a*)pyrene is quenched by 75% at $r = 0.13$ and by 70% at $r = 0.09$ and is thus intermediate between these two limiting cases. This strong quenching of the fluorescence suggests that both the Ag^+ ions and the fluorescence emitting benzo(*a*)pyrene molecules are, to a large extent, located at the same sites in DNA, namely at sites which contain at least one G-C base pair. Thus, each Ag^+ ion for $r < 0.1$ appears to quench one benzo(*a*)pyrene molecule. If the fluorescence emitting molecules were located at sites to which the Ag^+ ions do not bind, the quenching efect would be less strong than is observed.

Assuming a single type of binding site whose concentration is given by G_0, the equilibrium can by described by

$$Ag^+ + G \underset{}{\overset{K}{\rightleftharpoons}} C \qquad (7)$$

where [C] is the concentration of the complex (binding sites containing silver ions) [G] is the concentration of sites still available ([G] = [G$_0$] − [C]), and K is the equilibrium constant. The fraction of sites containing Ag^+ is equal to [C]/[G$_0$] and the ratio of the fluorescence intensity at a given Ag^+ ion concentration, F, to the fluorescence intensity in the absence of Ag^+, F_0, is given by

$$\frac{F}{F_0} = \frac{1}{1 + K[Ag^+]} \qquad (8)$$

In eq 8, [Ag^+] denotes the concentration of unbound silver ions, which is proportional to r, the number of Ag^+ ions bound per base pair. The free Ag^+ ion concentration is about 100 times smaller than the concentration of bound silver ions;[16] using this fact, a value of the binding constant K can be calculated from the benzo(*a*)pyrene data shown in Figure 7. This value can then be compared to the values obtained by Yamane and Davidson.[14] The solid line fitting the BaP points in Figure 7 was calculated using this value of $K = 1 \times 10^6$ and using [Ag^+] = $(r/100)P$, where P is the DNA concentration expressed in moles of phosphate groups ($P = 1.5 \times 10^{-3}$ M). The solid line passing through the experimental points for BeP was also calculated using eq 8 and $K = 0.74 \times 10^6$ M^{-1} ($P = 1.74 \times 10^{-3}$M). These values of K compare favorably with the binding constants of (2.1-2.2) $\times 10^6$ M^{-1} obtained by Yamane and Davidson also for calf thymus DNA.[14] Since in this range of r values silver ions bind to guanosine, the strong quenching of Ag^+ ions shown in Figure 7 indicates that fluorescence of BaP and BeP originates at G-C containing sites in DNA. If the fluorescence at all A-T containing sites is indeed quenched completely as the nucleoside fluorescence quenching experiments suggest, then the two different fluorescence emitting sites 1 and 2 could correspond to GC···GC or GC···CG sites in which the guanosine are on the same and on opposite strands, respectively.

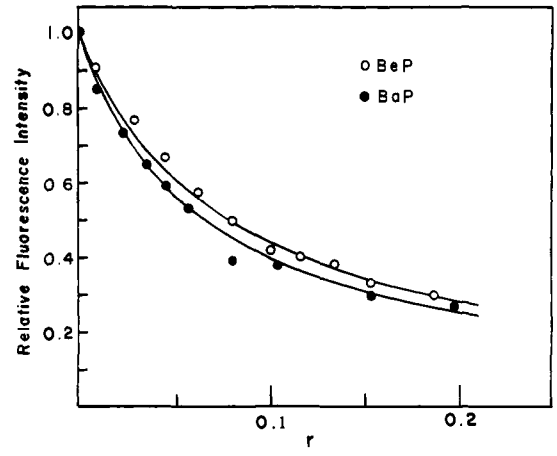

Figure 7. Relative quenching of the fluorescence of benzo(*a*)pyrene (BaP) and benzo(*e*)pyrene (BeP) in DNA complexes in aqueous solutions (1.5 and 1.7×10^{-3} M phosphate residues, respectively) as a function of Ag^+ ion concentration expressed in units of r (moles of $AgNO_3$/mole of DNA phosphate residue).

Pyrene–DNA Complexes. When pyrene is solubilized by DNA in aqueous solutions, the characteristic 10-nm shift is observed in the absorption spectrum of pyrene (main peak at 345 nm, smaller band at 329 nm). The fluorescence of pyrene in DNA is highly quenched and the fluorescence from the DNA pyrene complex is about the same as that of the pyrene present in the aqueous phase.[7] The excitation and emission spectra of the fluorescence of pyrene–DNA solutions is characteristic of the spectra for pyrene dissolved in water (excitation spectrum maxima at 335 (main peak) and 320 nm; emission at 373 (main peak) and 393 nm). Fluorescence decay times of such solutions are also characteristic of pyrene in wter: the lifetime is 221 ns in the absence of dissolved oxygen and 137 ns in air-saturated solutions. We thus conclude that most of the fluorescence of aqueous DNA solutions and pyrene is due to the pyrene dispersed in the aqueous phase, even though the strong red shifts in the absorption spectra indicate that substantial binding of pyrene to DNA has occurred.

Upon addition of silver ions ($r \approx 0.1$) there is a ~15% decrease in the fluorescence intensity with no discernible change in the decay profile.

Of all of the aromatic hydrocarbons tested so far (benzo(*a*)pyrene, benzo(*e*)pyrene, benz(*a*)anthracene, 7,12-dimethylbenz(*a*)anthracene, pyrene), only pyrene failed to show a triplet-triplet absorption. This is a surprising observation since pyrene is solubilized by DNA ~10 times more than the other hydrocarbons tested. However, upon the addition of Ag^+ ions with $r \lesssim 0.1$, a triplet-triplet absorption can be readily observed. Intersystem crossover from the excited singlet S_1 to T_1 is strongly temperature dependent in pyrene.[51] The efficiency of intersystem crossover depends on the energy gap $\Delta E(T_1\text{-}S_1)$, being smaller the larger this gap. In DNA complexes, there is a 10-nm shift of S_1 to lower energies, while the energy of the T_1 level is probably not affected because of its forbidden character. This effect could account for a decrease

in the production of triplets but probably not to its complete disappearance. In addition, in the presence of Ag^+ ions at that site, this quenching process is suppressed and it becomes possible to observe the triplet-triplet absorption of pyrene.

Conclusions

The physical binding of polycyclic aromatic hydrocarbons such as benzo(*a*)pyrene and benzo(*e*)pyrene to DNA in aqueous solutions is heterogeneous in nature. Up to 40–60% of the bound molecules do not exhibit fluorescence and are most probably located at intercalation sites which contain at least one AT base pair. The benzopyrene molecules capable of emitting fluorescence appear to be located at GC-GC intercalation sites. These effects must be considered when fluorescence is used as a diagnostic tool in characterizing the covalent polycyclic carcinogen–DNA complexes obtained in vivo. The absence of fluorescence of the polycyclic aromatic hydrocarbons does not preclude the possibility that binding has occurred at AT rich sites. Furthermore it is conceivable that the aromatic residues which remain after the covalent binding are different at fluorescent and nonfluorescent sites.

The long-lived triplet excited states are highly sensitive to their environment, particularly the conformation of the DNA molecule and the presence of low concentrations of paramagnetic molecules. It is shown here that intercalated polycyclic aromatic molecules are 10–20 times less accessible to even a small quenching molecule such as oxygen. The triplet excited state could be useful in characterizing whether covalently bound carcinogens are intercalated or bound to the outside of DNA by using bimolecular quenching as a probe of accessibility. The lifetimes of singlet excited states are generally too short to be employed conveniently for this purpose.

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