Electron Spin Resonance Spin-Label Studies of Intercalation of Ethidium Bromide and Aromatic Amine Carcinogens in DNA

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SUMMARY

A spin-labeled technique has been developed for the study of carcinogen-DNA intercalation mechanisms utilizing a modified ethidium bromide as a model ligand and specifically synthesized spin labels of carcinogenic aromatic amines such as 2-aminofluorene, 2-aminoanthracene, and 6-aminochrysene.

The technique takes advantage of the characteristic electron spin resonance anisotropy of the corresponding spin labels complexes with DNA. Analysis of the orientation-dependent information thus obtained has clearly shown that the ligand moieties in all 4 respective complexes are accommodated in adjacent base-pair layers of the DNA with the nitroxide reporter oriented in a preferential direction. The geometry of the nitroxide rings with respect to their respective constraining ligands in a particular complex appeared to be largely dependent upon the size of the ligand moiety and the position of the reporter attachment on the ligand molecule. A coplanar conformation is most favored for the spin labels in which the ligand moiety per se is a 3-fused ring with the nitroxide substituted at a position on or close to the longitudinal end of the ligand molecule.

Temperature studies in which the heat-induced release of the bound label is monitored by electron spin resonance gives an accurate measure of the complex dissociation characteristics that appear to parallel the helical coil cooperative melting transition monitored by absorbance measurements. The extent of dissociation of the bound spin labels parallels almost exactly the melting, i.e., unzipping of the DNA double helix, suggesting that the spin labels are immobilized by intercalation at the site on the DNA directly involved in the maintenance of the double helical structure.

Analysis of the electron spin resonance spectra of the complexes in terms of the extent of immobilization indicates that, for aromatic amine spin labels, two different binding species exist, one strongly immobilized presumably at a guanine-cytosine base-pair region and the other partially immobilized in a region other than guanine-cytosine base pair.

Competitive binding of the labels with respect to their parent ligand and binding isotherm results show that nitroxide substitution does not affect the type of binding but does affect the extent of binding, i.e., binding constant with the labeled compound having a lower affinity.

INTRODUCTION

The early studies of Lerman (20) in which an intercalation mechanism was postulated for the DNA-acridine dye complex to explain the strong binding of these dye molecules to DNA and their resultant frame-shift mutations, have stimulated considerable interest in applying similar mechanisms to the noncovalent and covalent interactions of carcinogens with DNA (7, 23, 33).

There appears to be convincing evidence that in some systems the initial step in chemical carcinogenesis is the noncovalent or covalent binding of the carcinogen to DNA. In the case of DNA the simplest and probably most attractive mechanism suggested is the somatic mutation hypothesis, wherein a base sequence is altered by the intercalation and subsequent covalent binding of a carcinogen into the DNA helix.

The recent studies of Ames et al. (1) and McCann et al. (27) have clearly shown that many proximate and suggested ultimate carcinogens are in fact mutagenic. They proposed that polycyclic hydrocarbons are carcinogenic because of the mutagenicity of epoxide intermediates formed during metabolism and that the mechanism of action most likely involves intercalation of the activated carcinogen followed by covalent binding. It remains therefore to demonstrate clearly that with these carcinogens intercalation is in fact a mode of binding to DNA.

The physical methods used to date to demonstrate intercalation mechanisms in DNA are varied and have been applied primarily to the acridine dyes and similar molecules with a strong affinity for DNA (3, 47). However, none of them appear to demonstrate unequivocally that intercalation has occurred. The problem becomes even more difficult in demonstrating intercalation of carcinogens because the extent of binding of most carcinogens at saturation is far lower (25) than that of the well-known intercalative dyes such as acridine (20) and EB (45).

The abbreviations used are: EB, ethidium bromide; ESR, electron spin resonance; EBSL, spin-labeled EB; AFSL, spin-labeled 2-aminofluorene; AASL, spin-labeled 2-aminoanthracene; ACSL, spin-labeled 6-aminochrysene; SSC, standard saline citrate (0.15 M NaCl-0.15 M sodium citrate, pH 7.0); AAF, 2-N-acetylaminofluorene.

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We have developed a spin-label method that can possibly detect whether or not intercalation has occurred. The method is based on an earlier demonstration by Ohnishi and McConnel (34) using ESR, that the stable free radical ion of chlorpromazine intercalates into DNA. The spin-label technique as developed by Stone et al. (40) has been widely used in the probing of small molecule-macromolecule interactions. The application of the method by us to demonstrate intercalation in DNA involved the synthesis of spin labels of known intercalative dye molecules such as EB with a paramagnetic nitroxide attached and a series of modified aromatic amine carcinogens with similar nitroxides attached. The anisotropy of the hyperfine interaction of these spin labels is such that their ESR spectra are highly dependent on the orientation of the label with respect to the molecule to which it binds. Intercalation of these molecules into DNA implies a fixed orientation relative to the DNA helical axis. ESR spectra obtained as a result of orientation of the DNA-spin label complex relative to the magnetic field will clearly show this spectral anisotropy if intercalation has occurred. Further analysis of the ESR spectra of these complexes in terms of the extent of immobilization of the spin label and the characteristics of their release from the DNA upon melting yield additional evidence as to whether or not intercalation has taken place.

MATERIALS AND METHODS

Spin-labeled Ligands. EBSL [3-N-(3'-carbonyl-1'-oxyl-2',2',5',5'-tetramethylpyrroline)-3,8-diamino-5-ethyl-6-phenylphenantridinium bromide]:

![EBSL Structure]

was used as a model ligand. For EB there already exists a considerable body of evidence for intercalative binding with DNA (11, 19, 45), and from thence the compound stands as a type-specific example of an intercalating drug. The aromatic amine spin labels used in this study were AFSL [2-N-(3'-carbonyl-1'-oxyl-2',2',5',5'-tetramethylpyrroline)amino-fluorene]:

![AFSL Structure]

and ACSL [6-N-(3'-carbonyl-1'-oxyl-2',2',5',5'-tetramethylpyrroline)aminochrysene].

These spin labels were chosen because (a) the parent non-spin labeled compounds are aromatic amines consistent with the model ligand, EB and their intercalative nature with DNA is most likely (1, 17, 27); (b) they were known to be carcinogenic in humans or animals (1, 18, 28); and (c) they were shown to be activated by human liver homogenates to form potent frame-shift mutagens (1). All the spin labels used in this experiment were prepared by G. Bartolini of this laboratory (the synthetic procedures will be published elsewhere). The labels were demonstrated to be pure by thin-layer chromatography in which only a single spot was observed for each label.

Spin-labeled Ligand-DNA Complex. Most of the spin label solutions were prepared by dissolving the respective label in acetonitrile. The concentration of spin labels was computed by comparing a 1st-moment calculation of the respective nitroxide sample with a standard solution of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl. DNA solutions were prepared by dissolving commercial calf thymus DNA (No. D-1501, Lot 73C-9500; Sigma Chemical Co., St. Louis, Mo.) in SSC, or in 0.1 x SSC. The concentration, expressed in terms of the nucleotide as a unit of DNA, was determined spectrophotometrically at 260 nm, using the molar extinction coefficient of 6600 (26). The preparation of a spin label-DNA complex in solution was carried out by allowing direct contact of the spin label with the DNA as follows. An excess amount of spin label initially in acetonitrile was deposited as a film by blowing nitrogen gas on the inner wall of a tube containing the spin-label solution. The DNA solution was then poured over the film and shaken (9), using a Veri-Whirl shaker for 5 to 12 hr at room temperature. In some cases the acetonitrile spin-label solution was added directly to the DNA solution and stirred. In these cases the acetonitrile was never greater than 1% by volume. The complex solution prepared in either case was filtered using an Amicon 52 ultrafilter under 50 psi nitrogen gas pressure until no more filtrate was expelled. By this procedure, a paste-like, sticky, condensed spin label-DNA complex, excluding most of the free spin label, was obtained.

A spin label-DNA complex precipitate was prepared by adding to the complex solution 2 volumes of ethanol plus 0.1 volume of 2 M sodium acetate (24) followed by centrifuging, decanting, and washing of the precipitate.

Methods. The ESR spectra of all the spin labels were recorded on a 9.5-GHz Varian E-4 EPR spectrometer. For the melting and low-temperature experiments, a Varian-4540 variable temperature accessory was used with dry ice-acetone as coolant and dry nitrogen. The temperatures were calibrated with a copper-versus-constantan thermo-
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A number of studies (19, 45) suggested that EB intercalates into DNA between base pairs with a binding constant of 2.35 x 10\(^{-6}\) in SSC and a ratio of dye to nucleotide, n = 0.25 (2). It has also been shown that such intercalation stabilizes the DNA, increasing its melting temperature (19). Attachment by an amide linkage of a nitrooxide to the amino group in position 3 of EB provides a suitable spin-label model to test the intercalation hypothesis by ESR. In Chart 2a the characteristic free spin or unbound ESR spectrum of the spin label is shown. In Chart 2, b, c, and d, ESR spectra of the label in the presence of DNA is shown first simply as a viscous DNA
dividing point with within ±0.1°. 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, \(A_e = 17.1\) gauss in aqueous media (10), was used as the standard in the determination of the hyperfine constant of both the isotropic and anisotropic ESR spectra of the spin labels. The extent of melting of the spin label-DNA complexes using ESR was estimated by comparing the relative change in the signal intensity of the high-field line (\(l_{31}\)) of the 3-line free label ESR spectrum. The intensity of this line is directly proportional to the amount of unbound label. As melting occurs bound label is dissociated from the complex, thus increasing the free label spectrum. By plotting the change in unbound or free label as a function of temperature, the melting curve was obtained. The melting curve based upon the hyperchromicity of DNA at 260 nm was made on a Gilford Model 2400 spectrophotometer, equipped with a variable temperature programmer.

The ESR anisotropy data was obtained using a device that allowed the oriented spin-label-DNA complex to be rotated relative to the magnetic field. The device consists of a rectangular quartz plate on both sides of which the DNA fiber complexed with spin label, which is a highly viscous solution, was stretch-oriented by drawing the solution with a brush across the plate in a direction perpendicular to the long axis of the plate, and the plate was then placed into a specially constructed goniometer attached to the ESR cavity. Rotations were made around the long axis of the plate (see Chart 1a). After each stepwise rotation of 10°, from 0 to 180° to the applied magnetic field, the ESR spectrum was recorded. Orientation of the complexes was also achieved by flowing solutions of complex through a capillary tube at a shear rate of approximately 3000/sec in a direction either parallel or perpendicular to the magnetic field. This method of complex orientation required a specially designed cavity with sample access ports parallel and perpendicular to the field modulation coils. This latter technique gave the same results as the former but was limited in our hands to only 2 field orientations due to the fact that we cannot rotate our magnet. In general, this method was not as convenient to use. Finally, a 3rd method was used in which condensed complexes are deposited as thin films onto quartz plates and the plates were then placed into the goniometer.

The competitive binding or blocking by nonlabeled ligands of the binding sites to spin labels on DNA was estimated by the relative decrease of immobilized ESR signal intensity, which is a measure of the concentration of label bound to the complex. This quantity was plotted versus the concentration of the blocker. To determine whether or not the spin label binds noncovalently or covalently, the ESR spectra of the complex was monitored after repeated precipitation of the complex followed by repeated extractions of the residue with an ethanol-sodium acetate solvent.

**RESULTS**

The usefulness of the spin-label technique to obtain information about the presence or lack of an orientation in binding of small molecules such as chemical carcinogens to large macromolecules such as DNA, derives from the dependence of the ESR spectra of the reporter group (a nitrooxide attached to the small molecule) and its orientation in space. Thus when a spin label such as a modified carcinogen is included in a highly ordered structure such as is found with DNA and is constrained to reflect the structural arrangement wherein it is bound, the splitting of the hyperfine lines of the ESR spectrum will depend upon the angle between the helical axis of the DNA and the applied magnetic field (34). In order that the spin label reflect its orientation dependence it must be rigidly held in the DNA and possess a well-defined geometrical relationship between the nitrooxide group and the rest of the molecule. It is this latter requirement that has been difficult to establish in the labels used here. The linkage between the nitrooxide group and the planar aromatic rings of the intercalating molecule is through an amide bond. This linkage would allow rotation of the nitrooxide around this bond; however, from space-filling molecular models it would be a highly hindered rotation; thus it is assumed that, for all the labels used here except the aminochrysene derivative, the nitrooxide is approximately coplanar and nonrotating in some cases or partially rotating in others with respect to the aromatic ring when binding occurs. The angular dependence of the hyperfine splittings of the spin label is directly related to the orientation and motion of the principal axes of the hyperfine and g tensors of the nitrooxide moiety with respect to the plane of the DNA fiber through the spin Hamiltonian, \(\hat{H}\)

\[\hat{H} = |\beta| H \cdot \hat{S} + hS \cdot \hat{T} - \frac{I}{2}\]

where g and T are tensors for the spectroscopic splitting factor and hyperfine interactions, respectively. The electron and nuclear spin operators are designated as \(\hat{S}\) and \(\hat{I}\), |\(\beta|\) is the Bohr magneton, and \(H\) is the magnetic field vector. For the nitroxides used here the hyperfine tensor is axially symmetrical, \(T_{xx} = T_{yy}\), and one describes the hyperfine splitting in the plane perpendicular to the p-orbital containing the nitrooxide unpaired electron by \(T_z\) and that parallel by \(T_x\). In Chart 1 a scheme is drawn showing a possible orientation of the DNA fibers relative to the magnetic field and the orientation of the p-orbital of the nitroxide if it is intercalated between base pairs and thus perpendicular to the helical axis. In this representation \(T_{xx}\) is aligned parallel to the field, and \(T_{xx}\) and \(T_{xy}\) would be perpendicular if perfect orientation of the DNA was achieved. In single crystal studies of nitroxides, \(T_x\) is 30.8 gauss and \(T_y\) is 5.8 gauss with the isotropic coupling constant 14.1 gauss. These values vary slightly depending upon the nitrooxide and its environment.

**EBSL-DNA Complex.** EBSL was synthesized as a model compound to test the technique for detecting intercalation. A number of studies (19, 45) suggested that EB intercalates into DNA between base pairs with a binding constant of 2.35 x 10\(^{10}\) in SSC and a ratio of dye to nucleotide, n = 0.25 (2). It has also been shown that such intercalation stabilizes the DNA, increasing its melting temperature (19). Attachment by an amide linkage of a nitrooxide to the amino group in position 3 of EB provides a suitable spin-label model to test the intercalation hypothesis by ESR. In Chart 2a the characteristic free spin or unbound ESR spectrum of the spin label is shown. In Chart 2, b, c, and d, ESR spectra of the label in the presence of DNA is shown first simply as a viscous DNA
Chart 1. Schematic model for the intercalation of spin labels into DNA and the relative geometrical orientation of the spin label in the complex: a, sketch illustrating the complex fiber oriented on a quartz plate in the ESR resonance cavity and the direction to which this fiber can be rotated around the x-axis with respect to the applied magnetic field; b, the secondary structure of normal DNA whose helical axis is oriented parallel to the magnetic field; and c, the spin label-DNA complex fiber, in the same orientation, in which the nitroxide ring plane is coplanar with respect to its constraining ligand moiety (in black). The x-axis is taken as parallel to the NO bond, with z parallel to the nitrogen 2π-orbital that holds the odd electron and y is perpendicular to the zz-plane. The helix is drawn as viewed from a remote point, so that the base pairs and the intercalated spin labels appear only in an edgewise projection, and the phosphate-deoxyribose backbone appears as a smooth coil [after Lerman (20)].

Chart 2. ESR spectra of EBSL in different environments: a, spectrum of 1 x 10^{-6} M spin label in 0.1 x SSC at room temperature; b, spectrum of the solution obtained after mixing 5 x 10^{-3} M EBSL in acetonitrile with 5 ml of 8.7 x 10^{-5} M DNA in 0.1 x SSC; c, the same mixture as b after condensing the complex by ultrafiltering most of the unbound spin away; d, the precipitate obtained by adding 2 volumes of ethanol plus 0.1 volume of 2 M sodium acetate to the condensed mixture of c, followed by collection and drying; e, the spin label alone dissolved in 98% glycerol (saturated) and spectrum recorded at -30°.

Complex solution, second as a condensed residue after most of the unbound spin was filtered away, and then as a dried precipitate. In Chart 2a, ESR of the label alone in glycerol at -30° is shown, and it typifies a spin label powder spectrum. The maximum splitting between the low- and high-field peaks is characteristic of the motion of the label and the maximum T_{zz} splitting of the anisotropic hyperfine tensor was found to be 62.2 gauss (see Chart 2b). As can be seen from Chart 2b, even in solution the labels are highly immobilized and constrained in the structure of the DNA. There is a slight increase in immobilization as measured by the increase in separation of the high- and low-field peaks in going from the solution (Chart 2b) to the precipitate (Chart 2d; 67.2 gauss), indicating some mobility of the label relative to the macromolecule when in solution. Chart 2e, however, yields an even greater separation (71.2 gauss) in these peaks and is interpreted not as increased immobilization but as a change in the polarity of the environment surrounding the label. It is well known that the hyperfine constant for these nitroxides increases with polarity of the surrounding environment (15). We would conclude from this that the binding region of the DNA is more hydrophobic than 98% glycerol.

If the DNA complex in Chart 2c is oriented relative to the applied magnetic field either by flowing through a capillary tube or by stretch-orienting the fibers on a thin plate (see "Materials and Methods"), the orientation dependence of EBSL can be determined as illustrated in Chart 3, which illustrates the spectral changes observed as a function of orientation of the EBSL-DNA with the magnetic field. The 1st spectrum shows the fibers oriented parallel to the field, and in the subsequent spectra the fibers are rotated through 90° from this orientation in 10° intervals to the perpendicular orientation. In the parallel configuration the spectrum clearly delineates T_{zz} but is broad and resembles a powder spectrum, suggesting that motion of the label is restricted and that perfect orientation has not been achieved. With rotation of the sample about the x-axis (Chart 1a) the 2 outer extreme lines characteristic of the maximum anisotropic hyperfine splitting where T_{zz} is parallel to the field, slowly move toward the center. Chart 4 illustrates the angular dependence of these outer lines. In the perpendicular configuration T_{xx} is readily observed; however, the broad peak at Chart 3a, about 18 gauss from the center line which is at the minimum of the curve in Chart 4, and the lack of a peak at Chart 3b is suggestive of a distribution of fibers in a static condition not all oriented perpendicular to the field but distributed across a rather wide angle (38). The majority of the fibers are reasonably oriented in the plane of the plate as suggested in Chart 1a, and they give a 2T_{xx} of 64.3 gauss and 2T_{zz} of 17.3 gauss.

This orientation dependence of the spectrum, assuming the nitroxide to be coplanar with the EB ring, can only be interpreted as evidence of a fixed orientation of the EB, namely intercalation between base pairs in the DNA helix.
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Chart 3. Angular dependence of the ESR spectrum of oriented EBSL-DNA complex fibers with respect to the laboratory magnetic field: a, observed position of the low-field peak with a fiber orientation perpendicular to the magnetic field; b, expected position of the peak that would be observed when the fibers are ideally oriented; c, those complexes that are nonoriented and therefore field independent, and probably the result of an edge effect in the sample cell; d, those labels unbound and in equilibrium with the bound spin. Dotted line, angular dependence of the outer extreme of the spectra. The separation between the low field and center peak is taken as a measure of the angular dependence of the hyperfine splitting of the spectrum.

The anisotropy of the spectra clearly demonstrates that for most of the complexes the EBSL is oriented in the DNA such that the z-axis of the nitroxide is parallel to the fiber axis and that the plane of the nitroxide ring is parallel to the base-pair layers of the DNA and perpendicular to the DNA helical axis. It is also apparent from the perpendicular spectrum that in addition to this fixed orientation there are either a number of DNA molecules to which the EB is randomly bound, or that there are a number of complexes randomly distributed relative to the field whose spectra given by Chart 3c in effect show no orientation dependence. This lack of orientation could be attributed to edge effects in the sample cell.

Chart 5, a and b, illustrates a similar spectral orientation dependence for the complex oriented either by flowing through a capillary tube or rotating a thin film relative to the field. In the case of flowing, only 2 orientations are possible, parallel and perpendicular. A comparison of Chart 5 with Chart 3 suggests that the flowing method is less efficient in orienting the complexes than the stretching or thin-film method.

Further evidence for label intercalation and that non-orientation of the complex rather than random binding of EBSL is responsible for that family of complexes showing no field dependence (Peak c in Chart 3) is given by the melting profiles for the EBSL-DNA complex. In monitoring the changes in the ESR spectra of EBSL-DNA complex as a function of dissociation of the complex, one would expect a transition from the broad-bound anisotropic spectrum of Chart 2c to the sharp isotropic spectrum of Chart 2a, as the DNA helix began to unfold and release the spin label (see Chart 6). If the label was intercalated this release, as monitored by the growth of the isotropic spectrum (intensity of high-field peak, I), should increase with temperature following the typical sigmoidal-type melting profile (16). In Chart 7 we plot the intensity of the unbound label (I) as a function of temperature (see solid lines). The results clearly show that as the complex melts the label is dissociated in a cooperative fashion and at approximately 90° only unbound label is present in the solution. Upon attempted renaturation, the label is partially associated into complex again but the bound spectra of the complex are completely different from the starting complex (see dotted line in Chart 6), suggesting nonspecific binding and incomplete renaturation of the DNA.

In Chart 7 we have shown for comparison the melting profiles for DNA alone, Curve 1, and DNA with EB, Curve 5, as measured by the absorbence at 260 nm where melting point Tm is defined as the midpoint of helix coil transition.
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Carcinogen Aromatic-Amine Spin Label-DNA Complexes. A series of aromatic amine carcinogens, 2-aminofluorene, 2-aminoanthracene, and 6-aminochrysene were modified to produce spin labels by adding the nitroxide to the amine through an amide linkage. Carcinogenicity of the modified compounds was determined for each using the bacterial system of Ames, Salmonella typhimurium TA98 (see Ref. 27), and by implants of wax-impregnated carcino-

and the dissociation profiles of the varying D/P dye to DNA ratios of EBSL-DNA complexes, Curves 2, 3, and 4. We define $T_d$ for the EBSL complexes as the midpoint of dissociation transition of the spin label from the DNA as measured by ESR. It is suggested that $T_d$ and $T_m$ are the same (35), but these experiments cannot be directly compared.

The large difference between $T_m$ for the EB-DNA complex and $T_d$ for the EBSL-DNA complex is due to the difference in the extent of binding of the 2 ligands. The binding constant for EBSL-DNA in SSC is 2 orders of magnitude smaller than that of EB alone (2). It is because of this large difference in binding constants that absorption methods could not be used to study the melting profiles for the EBSL-DNA complexes. In order to compare directly using the same D/P ratios would require absorbances beyond the range of our spectrophotometer. Nevertheless, the smooth cooperative dissociation of the labels from the complex as detected by an increase in the number of unbound or released labels by ESR strongly suggests that most, if not all of the labels were in fact intercalated (16). Random nonspecific binding would not be expected to yield a cooperative dissociation of the label upon thermal denaturation of the DNA. Such nonspecific binding is not dependent on the double-stranded structure of the DNA, whereas intercalative bind-

Chart 6. Temperature dependence of the ESR spectrum of the EBSL-DNA complex. The spectra represent a composite absorption consisting of bound labels designated by $a$, and unbound label, $b$, at different temperatures. No significant spectral changes are observed between 30 and 65°. Dissociation of label occurs above 65°, and $b$ increases at the expense of $a$. An abrupt change occurs upon increasing the temperature by 1° from 73 to 74°. The spectrum at 78° is close to the $T_d$, dissociation transition temperature, at temperatures above 90° all the spin labels in the system are believed to be dissociated and free. The peaks at $c$ are due to $^{13}$C peaks resulting from a natural abundance of this isotope on the carbon adjacent to the NO moiety. Dotted line, spectrum obtained after subsequent cooling from the state of complete dissociation at 90° to the original temperature, 30°. Final concentration of EBSL, $1.33 \times 10^{-4}$ M, and DNA, $8.8 \times 10^{-4}$ M: D/P ratio approximately $1.5 \times 10^{-4}$ in 0.1 x SSC. Since the high-field peak, $b$, of the unbound spin resonance in the composite spectrum is distinctly separated from the bound resonance, Peak $a$, its intensity, i.e., $I_a$, was taken to estimate the relative amount of the label dissociated from the complex in the course of the helix coil transition upon heat treatment.
orientation dependent, but in addition there is a 3rd complex in low concentration similar to that found in the EBSL spectra that is strongly immobilized and field orientation independent. Again, it is felt that this 3rd species results from random orientation of the complexes and not non-oriented binding of the amines. As with EBSL, the results for the carcinogens suggest that the nitroxide moiety is approximately coplanar with the aromatic amine for AFSL and AASL, with the nitroxide x-axis aligned parallel to the fiber axis, and is immobilized upon insertion of the amines by intercalation between base pairs of the DNA. Orientation of the complexes is not as complete for the aromatic amines as it was for EBSL-DNA complexes. In the perpendicular configuration the minimum splitting $T_s$ is not observed. The peak at 17 gauss similar to Point a in Chart 3 to the low-field side of the center line in the perpendicular configuration (Chart 9a) suggests a wide angular distribution of fibers in a static condition relative to the perpendicular orientation. The fact that 2 species of complexes with different degrees of immobilization are observed that are field orientation dependent suggests 2 distinct binding modes for these amines, one in which the nitroxide is strongly immobilized and the other has freedom of motion about the nitroxide.

Chart 7. Plot of the heat-induced helix coil transition of DNA and EB-DNA complex and dissociation transition of EBSL-DNA complexes. Lines 1 and 5, helix coil transition curves of the DNA and EB-DNA complex, respectively, obtained by plotting hyperchromicity at $A_{260nm}$ versus temperature; Lines 2, 3, and 4, dissociation transition curves obtained by plotting $T_d$ versus temperature for EBSL-DNA complexes with varying $D/P$ ratios. Final concentration of DNA for the former system, $7.6 \times 10^{-4}$ M, and the latter, $8.8 \times 10^{-4}$ M, respectively, and that of EB, $1.58 \times 10^{-4}$ M. All solutions were made using 0.1 M SSC.

Chart 8. ESR spectra of AFSL in different environments; a, spectrum of $1 \times 10^{-4}$ M free label in 0.1 M SSC; b, spectrum of condensed mixture obtained by 3-h ultrafiltering mixed solution of 0.35 ml of 1 mg of AFSL per ml in acetonitrile and 10 ml of $1.8 \times 10^{-4}$ M DNA; c, spectrum of precipitation obtained by adding to the condensed mixture 2 volumes of ethanol plus 0.1 volume of 2 M sodium acetate followed by collection and drying; d, spectrum of the spin label alone dissolved in 98% glycerol (saturated) recorded at $-30^\circ$. Point a, outer extrema of the strongly immobilized component; b, that of the partially immobilized component of the bound spin species, and c) unbound spin component, respectively.
Aromatic rings are indeed oriented within the DNA structure and most likely intercalated, but that the nitroxide is rotated almost 90° to the plane of the rings and restricted in its motion.

All 3 aromatic amines showed cooperative heat-denaturation curves with increased melting temperatures, $T_d$, as measured by ESR (see Chart 10). The melting temperature increases for all 3 were very similar to that of EB alone, even though the binding constants were again considerably less than that for EB. An additional feature of the aromatic amine spin label-DNA complexes is their instability. Particularly, the spectrum of the AFSL-DNA complex in solution, as well as the oriented fiber, slowly changed with time. This instability was not observed with the other complexes such as EBSL-DNA, and is possibly due to the denaturing effect of the fluorene molecule on the DNA.

Competitive binding studies of native EB and AAF with the associated spin labels was made to check whether the binding sites for the 2 classes of molecules were identical. Chart 11 illustrates how the free spin on unbound spectrum of AFSL increases when native AAF in varying concentrations is incubated with the label and DNA. The $B/C$ ratio of the intensity of the broad anisotropic peaks of the immobilized label to the sharp isotropic peaks of the unbound label is a direct measure of the extent of binding of the label. Chart 12 shows a typical competitive binding plot for AAF and AFSL. It is quite clear from these results that the binding site of the modified molecule, i.e., spin label, is identical to the parent compound.

**DISCUSSION**

The results presented here strongly suggest that the spin labels, EBSL, AFSL, AASL, and ACSL, bind to DNA by intercalation. Such a result is not unexpected, considering the structure of the molecules and assuming no large perturbation of this structure by the attachment of the nitroxide label.

Chart 9. Angular dependence of ESR spectra of the oriented AFSL-DNA, a, AASL-DNA, b, and ACSL-DNA, c, complex fibers. Solid and dashed spectra, obtained with the complex fiber orientation parallel and perpendicular to the applied magnetic field, respectively. Point a, the outer extrema of the strongly immobilized component; Point b, partially immobilized component of the bound spin species; Point c, trace of unbound spin component, respectively. $A_i$ and $A_r$ measure the splitting between outer extrema of the corresponding resonance components when their fibers are oriented parallel and perpendicular to the magnetic field, respectively.

ACSCL showed a completely opposite field orientation dependence compared to the other aromatic amines. The chrysene derivative was also different in that the amine is in position 6 compared to position 2 of the others, and the conjugated aromatic ring system is larger. As can be seen in Chart 8c, changes in the spectra in going from the parallel configuration to the perpendicular one are just opposite of what was observed for the other 2 amines. The characteristics of the spectral changes are similar in that there appear to be 2 field-dependent species, a strongly immobilized form ($A_{\text{max}}$, 63.5 gauss) and a partially immobilized form ($A_{\text{max}}$, 44.0 gauss), except that this maximum splitting or $2T_i^*$ as well as $A_{\text{max}}$ or $2T_i^*$ of 19.9 gauss are observed when the plane of the plate on which the fibers are stretched is perpendicular to the field. This result suggests that the long axis independent of the DNA, giving rise to a partially immobilized spectrum.

Chart 10. Heat-induced dissociation transition curves of AFSL-, AASL-, and ACSL-DNA complexes. All 3 broken lines were obtained by plotting $I_i$ versus temperature of the corresponding composite ESR spectrum for each spin label-DNA complex: AFSL-DNA with $D/P$ ratio of approximately $1 \times 10^{-4}$; AASL-DNA with $D/P$ ratio of approximately $3 \times 10^{-4}$; ACSL-DNA with $D/P$ ratio of approximately $3 \times 10^{-4}$. Solid line, melting curve for $7.6 \times 10^{-4}$ M DNA obtained by plotting hyperchromicity at $A_{260}$ nm DNA versus temperature. All solutions were made in $0.1 \times$ SSC.
Spin-Label Studies of Carcinogen-DNA Intercalation

Ian result with spin-labeled aniline, 1-aminonaphthalene, and 2-aminonaphthalene, have been unsuccessful.

A 2nd requirement for intercalation is the relative orientation of the ligand with respect to base pairs. Recent data (9, 14, 32, 39, 42) strongly indicate that the only hydrocarbons that bind to DNA in appreciable amounts are those that intercalate and orient in such a way that they are protected from contact with the aqueous medium. Those hydrocarbons that must protrude into the medium, even though intercalated into DNA, will bind only in relatively small amounts. This implies, then, that there is at least 1 preferential geometrical orientation which aromatic ligands take with respect to the DNA in a particular complex that results in overlap with the corresponding base pair.

Nitroxides, per se, are not reactive with DNA, and this portion of the spin label will always trail the constraining ligand upon binding with DNA. Thus, the nitroxide can be accommodated in DNA only as a result of the initial interaction between the ligand and the DNA. Accordingly, since the ESR data is obtainable only through the nitroxide as the reporter, the information reflecting the geometry of a spin label-DNA complex is, in fact, entirely dependent upon the molecular parameters that define the structural relationship between the reporter and the ligand moiety. For example, a nitroxide reporter attached to a ligand at a position such that the molecular dimensions and geometrical orientation of the reporter ligand is not that much different from the parent ligand will result in a strongly immobilized reporter; however, if the reporter is attached in a less favorable position such that optimum geometrical orientation is not achieved, a partially immobilized reporter results.

In the currently used spin labels, the substitution of a bulky nitroxide, 3-carbonyl-1-oxyl-2,2,5,5-tetramethylpyrroline residue, certainly does modify some, if not all, of the molecular parameters of the parent ligand molecule and, lard result with spin-labeled aniline, 1-aminonaphthalene, and 2-aminonaphthalene, have been unsuccessful.

A considerable amount of experimental and theoretical work has been done concerning the size requirement for ligands to intercalate into DNA. Requirements such as stéric fit and geometrical similarity of the ligands and the nucleotide base pairs must in general be met (3). The viscometric studies of Lerman (21) suggest that only certain polynuclear hydrocarbons and nitrogen-containing polycyclic carcinogens can, in fact, intercalate into DNA, and that in general compounds structurally lacking 3 fused aromatic rings, which they suggest is a structural requirement, do not intercalate. Thus, in order to fit into the narrow but flexible and stretchable space between base pairs in DNA, the polycyclic compound must have an adequate molecular shape and size similar to the purine-pyrimidine base pairs (6).

The results reported here are in agreement with this prediction. The spin labels, EBSL, AFSL, AASL, and ACSL, each possess at least 3 fused rings in their ligand moieties and are clearly shown to intercalate into DNA by our ESR interpretation, whereas repeated attempts to obtain a simi-
therefore, does influence its binding characteristics. However, it can be shown, using space-filling atomic models, that the molecular size as far as length along the long axis of ethidium, 2-aminofluorene, and 2-aminoanthracene with a nitroxide attached to the 3-, 2-, and 2-amino groups of the respective rings, results in a molecular dimension still able to be accommodated between base pairs, as is the case with the parent ligand.

Based upon these findings and the considerations developed above, the possible geometry of the EBSL-, AFSL-, and AASL-DNA complexes may be illustrated as follows. The planar ligand moieties containing 3 fused rings are accommodated completely between the hydrophobic base-pair layers, whereas the trailing nitroxide that is attached at a position very close to the longitudinal end of the ligand is wedged tightly into only a part of this hydrophobic region and is forced into a coplanar configuration relative to the ligand moiety. This coplanar configuration is favored by the nature of the intermolecular linkage between the ligand and the nitroxide moiety, namely, the amide bond, which because of its double-bond character will have restricted rotation. Space-filling atomic models of EBSL show that there is also considerable steric hindrance as a result of the bulky methyl groups on the nitroxide which further restricts rotation; thus the net intermolecular steric factors in the spin label favor a coplanar orientation among all possible relative configurations. This configuration, of course, is most favorable for the spin label to slide between the base-pair layer. The shape of the nitroxide moiety roughly resembles a wedge; the thickness increases as it goes from the ligand-constraining group, the 3-carbonyl, through the 3- and 4-vinyl, and finally to the 2- and 5-gem-dimethyl groups. The maximum thickness measuring these 2 gem-dimethyl regions approaches a dimension that is almost twice as thick as the planar aromatic ligand. Because of this large thickness of the nitroxide moiety, its complete accommodation between base-pair layers is unlikely. It is quite plausible to assume, therefore, that the nitroxide moiety, despite its partial accommodation, is rigidly fixed in an exclusively preferential geometrical orientation, most likely a coplanar configuration with respect to the ligand moiety and between the base-pair layers.

The ESR results for the ACSL-DNA complex suggest that although there is steric hindrance to rotation of the nitroxide relative to the ligand, that coplanarity of the nitroxide is dependent upon the geometrical position of the nitroxide relative to the ligand and how the ligand can fit between base-pair layers. The ACSL-DNA-ESR results clearly show that the nitroxide is not coplanar with the chrysene ring system but instead is perpendicular to the rings. The most plausible explanation of the result is that the ligand moiety, which is actually 4 fused aromatic rings, is accommodated in the base-pair layer such that the longitudinal axis is parallel to the base pairs as with 3 fused rings, but with a small part of the structure not within the layer. Using space-filling models the planes of the chrysene and purine-pyrimidine base pair are superimposed longitudinally along the long axis such that the chrysene ring is readily enclosed in the base-pair region along this axis, but that position 6 is jutting out of the layer, thus forcing the nitroxide out, probably into the small groove of the helix in a transverse direction relative to the longitudinal axis of either chrysene and the base pair. As a result of the binding the net steric hindrance in the region of position 6 could force the plane of the nitroxide moiety to orient itself perpendicular to the base-pair layer, yielding the ESR result observed. A similar orientation is also apparent for the 6-substituted phenyl group in the phenanthridinium ring of EB-DNA complex (11, 42).

Thus, it appears quite clear that an analysis of the ESR anisotropy data of these ligand spin labels can yield definitive information on intercalation, as well as the geometrical relationship between the ligand and reporter group after binding has occurred. The latter relationship, however, assumes little or no rotation of the reporter relative to the ligand upon binding, and no significant modification of the geometry between the ligand and the DNA in the complex.

So far, we have devoted our discussion to only 2 alternative binding mechanisms to interpret our ESR data, intercalation, or random external binding, with the latter being ruled out because it would not yield magnetic field orientation-dependent spectra. There is, however, another binding mechanism that could be field orientation dependent, namely, specific binding to the repetitive grooves apparent in the Watson-Crick DNA model. Binding in the grooves would require stacking on the part of the ligand groups, with concomitant fixed orientation of the reporter relative to the ligand in the groove that would be coplanar in EBSL, AFSL, and AASL but perpendicular in ACSL. In the case of the coplanar arrangement, stacking in the groove would also place the nitroxide spins in close proximity to one another and should result in dipolar broadening. No such broadening has been observed. One would also assume that if stacking was a dominant mechanism it should occur at high concentrations in solutions of the labels independent of the DNA. A further argument against stacking in the groove stems from a consideration of the difference in the 3 dimensional structure between the reporter, which is large and bulky, and the ligand, which is flat and planar; the difference in thickness is nearly 2-fold.

It still remains to be shown if the ESR results can establish whether or not there is more than 1 preferential binding site for a ligand with DNA. As was observed in the ESR spectra of the series of spin-labeled aromatic amine-DNA complexes, AFSL-, AASL-, and ACSL-DNA, there appear to be 2 different species of complexes for each label with varying degrees of immobilization. This is quite different from those complexes formed with the noncarcinogenic ligand, EBSL, in which no more than 1 species of immobilized spin was observed. One explanation for the 2 different ESR spectra is simply that the label is binding in 2 different regions or sites. However, it is not yet possible to give an unequivocal answer on this point because the evidence is conflicting also in cases of well-known complexes such as acridine-DNA (4, 8, 16) or EB-DNA (12, 36, 42, 46, 48). The ESR data presented here for EBSL-DNA complex, in which only a single species of bound spin resonance is observed, however, could support the existence of only 1 specific binding site, or that all the sites in the DNA are equivalent.

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carcinogens in vivo are, on the other hand, very diverse (13, 17, 29). However, for some polycyclic hydrocarbons, particularly AAF, it has been suggested by several authors (13, 17, 22, 29) that C-8 of guanine is a specifically reactive site in DNA. Furthermore, Summers and Szybalski (41) suggest that clusters of G-5 pairs exist in DNA and that those clusters may be different from other parts of the DNA molecule. There is also good evidence that intercalation precedes covalent binding of 3,4-benzpyrene to a guanine residue (22). One might speculate, therefore, that differing orientations of drug molecules dictated by the steric demands and intercalation sites might determine the covalent reaction such as with the C-8 of guanine for polycyclic aromatic amines (43, 47).

Thus, as observed with the spin-labeled polycyclics-DNA binding studied here, it appears that there is a preference for binding. One of the most plausible interpretations for the 2 different resonance components in the ESR spectra of AFSL-, AASL- and ACCL-DNA complexes would then be that there are 2 distinct binding modes for these aromatic amines, one in which the nitroxide is strongly immobilized in a specific binding site, presumably a G-C base-pair region, and the other in which the nitroxide is partially immobilized in a base-pair region other than G-C.

No simple mechanism to explain the difference in the nature of preferential binding between EBLSL and the aromatic amine spin labels that has been observed in this work is presently available. One possible explanation which may be given, however, is that the difference arises from the fact that the molecules differ in both their ability to induce bond polarizability and their steric interaction. The ethidium molecule is a heterocyclic containing a polar quaternary 5-nitrogen and has a phenyl substituent in position 6, whereas the amines do not. This possibility is strongly supported by the recent work of Müller et al. (30) and Müller and Crothers (31), in which they conclude that the binding specificity of intercalative aromatic compounds with DNA arises mainly from electronic factors that are strongly controlled through steric constraints on the possible complex geometries.

Analogous studies for the binding of the spin labels with varying substituents with a series of synthetic DNA-like polynucleotides of known base composition and sequence would provide valuable information in this regard.

In the synthesis of the spin labels used here, substitution of an amine on the ligand followed by an amide linkage to the nitroxide group yields the representative ligand-spin label. This substitution does not affect the type of binding but does affect the extent of binding, i.e., binding constant. The results given in Chart 12 on blocking of label binding by the parent amine supports the fact that the binding sites are identical for the parent amine and label. The binding-constant differences, especially in SSC, however, can be as much as 2 orders of magnitude with the labeled compound having much lower affinities. This result is consistent with the work of Wakelin and Waring (44), where they showed with substituted phenanthridium only substitution in both positions 3 and 8 simultaneously, reduced binding extensively, whereas substitution at either 3 or 8 individually, reduced the binding only slightly.

Competitive binding studies of a general nature are planned for the future in which each carcinogen label will be compared with both its parent molecules and other nonlabeled carcinogens to see whether any difference in binding sites can be detected in this way.

The mechanism explaining an increase in the $T_m$ of DNA produced by intercalating agents is not completely understood. A number of mechanisms have been suggested to explain this phenomenon, which range from contributions to changes in polarization bonding by the intercalated ligands, to simple hydrophobic bonding the latter effect, which might result in a shielding of sites in the DNA molecule at which fluctuations in the rigid hydrogen-bonded structure occur more readily against the surrounding water structure (3). Such a shielding of these regions by the intercalated ligands would act to stabilize the DNA structure, resulting then in an increase of the temperature necessary for "unzipping" the double helix of the DNA. As a consequence, $T_m$ increases have been regarded as supporting evidence for intercalation as the "physical" binding mechanism for polymeric aromatic amines (3). Assuming that this interpretation is correct, it is reasonable to infer that decreased intercalative binding would result in a smaller $T_m$ increase.

As was shown in Chart 1, for the melting profile obtained by $A_{260 nm}$ measurement of the solutions containing EB-DNA, even at a very low concentration of EB, resulted in a $T_m$ increase of DNA, whereas no detectable increase in the $T_m$ of the same DNA solution containing similar or slightly higher concentrations of EBLSL was detected. This result can only be explained by the fact that the equilibrium concentration of bound EBLSL is an order of magnitude lower than that of EB under the same conditions. If one monitors, however, not the direct uncoiling as measured by $A_{260 nm}$, but instead the loss of binding of the label by ESR, then the thermal stabilization effect of postspin-labeled EB can be detected as demonstrated in Chart 7. The ESR method measures simultaneously the bound and unbound ligand as a function of temperature and is not limited to detecting gross changes in the total DNA structure as is the case using absorbance of the DNA at $A_{260 nm}$. Although because of differences in binding constant and experimental limitations in the optical methods, no direct comparison of $T_d$ obtained by the ESR method for EBLSL-DNA complexes and $T_m$ obtained by $A_{260 nm}$ measurement for EB-DNA could be made, the similarity of the plots in Chart 7 suggest that the 2 methods are equivalent. Pan and Bobst (35) have also presented similar evidence, in their studies on 2-stranded spin-labeled polyadenylic-polyuridylic helical complexes in which the label is covalently linked, that there is an agreement between $T_m$ determined by absorbance measurements and that by ESR for the same double helix. This finding strongly supports the conclusion that the same temperature-dependent structural changes of the DNA, observing hyperchromicity on one hand and mobility of the spin label on the other hand, can be monitored by both techniques. Assuming the same generalization for the system used here of spin label-DNA complexes, the present data of the melting profiles, then, can provide unequivocal evidence that the rate of dissociation of the bound spin labels parallels almost exactly the melting, i.e., unzipping of the DNA double helix. This, in turn, is also strong evidence that the spin
labels are immobilized by intercalation at the site on the DNA directly involved in the maintenance of the double helical structure, namely, between adjacent base-pair layers. In addition, it is entirely consistent with the view that an intact double helical structure of the polynucleotide is a prerequisite for intercalation of the ligands with DNA, as suggested in Lerman’s original model. The “modified intercalation model” (5) in which the dye is inserted between adjacent nucleic acid bases on the same strand is not consistent with the present data.

The most salient feature of this study may be that the spin-label technique will prove a useful tool in studying the sequence of molecular events, i.e., initiation and development in a complex process such as a carcinogenesis, the end result of which is the onset of a cancer.

While a large amount of data is being gathered on the interaction of carcinogens with various cell components, and in particular with the fundamental macromolecules, and while it is obvious that somewhere there must be the decisive step leading to the generation and appearance of tumors, the precise significance of these reactions for carcinogenesis is unclear. The problem of the interaction between the carcinogen and the cellular receptor has 2 facets. The 1st facet concerns the nature of the principal receptor, and this question reduces to: nucleic acid or protein? The 2nd facet concerns the mechanism of the interaction, particularly the predominance of the physical or chemical interaction (37).

These studies for the interaction of carcinogen and DNA have strongly demonstrated the feasibility of the spin-label technique as a powerful tool for providing not only clear-cut evidence of physical binding, i.e., intercalation of the carcinogen with DNA, but also other information such as base-preferential binding and the postbinding structural changes of the host DNA molecule. Moreover, carcinogenic and mutagenic activity of most of the carcinogens used were found to persist even after attachment of the nitrooxide reporter on the respective ligand molecule.

We believe, therefore, that the technique is a highly promising tool in studying these very important aspects of the problem.

REFERENCES

Spin-Label Studies of Carcinogen-DNA Intercalation

Electron Spin Resonance Spin-Label Studies of Intercalation of Ethidium Bromide and Aromatic Amine Carcinogens in DNA

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