Structural Studies of A-Form Sodium Deoxyribonucleic Acid: Phosphorus-31 Nuclear Magnetic Resonance of Oriented Fibers†

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ABSTRACT: A highly oriented sample of A-form sodium deoxyribonucleic acid (DNA) has been investigated by using proton-enhanced ^{31}P nuclear magnetic resonance (NMR). Proton-decoupled spectra taken with different angles between the magnetic field direction and the fiber direction are compared to theoretical spectra which are calculated by assuming the following: (1) the orientation of the phosphate groups in the fiber is given by the A-form DNA coordinates suggested by Arnott & Hukins [Arnott, S., & Hukins, D. W. L. (1972) *Biochem. Biophys. Res. Commun. 47*, 1504–1509]; (2) the DNA phosphate groups may be considered stationary on the NMR time scale; (3) the relevant features of the spectra are determined solely by chemical shift anisotropy of the phosphorus atoms. The experimental and calculated spectra are in excellent agreement and support the validity of the above assumptions contrary to conclusions drawn in another investigation [Shindo, H., Wooton, J. B., Pheiffer, B. H., & Zimmerman, S. B. (1980) *Biochemistry 19*, 518–526]. In particular, we find no evidence to support the notion of a highly irregular phosphodiester backbone. Comparison of observed and simulated spectra allows the determination of the orientation of the ^{31}P chemical shielding tensor relative to the bonding framework of the phosphodiester group. The orientation agrees with that expected from NMR studies of phosphodiester model compounds [Kohler, S. J., & Klein, M. P. (1976) *Biochemistry 15*, 967–973; Herzfeld, J., Griffin, R. G., & Haberkorn, R. A. (1978) *Biochemistry 17*, 2711–2718] and X-ray diffraction of oriented fibers [Arnott, S., & Hukins, D. W. L. (1972) *Biochem. Biophys. Res. Commun. 47*, 1504–1509].

A wide variety of structures all composed of Watson–Crick base pairs have been proposed which support a polymorphic view of double-helical nucleic acids in solution (Arnott, 1980). The operational definitions of the known conformational states available to double-helical nucleic acids are their respective fiber diffraction patterns (Wang et al., 1979; Arnott et al., 1981; Jack et al., 1976; Arnott & Hukins, 1972; Milman et al., 1967; Fuller et al., 1965; Langridge et al., 1960). Nonetheless, one-to-one correspondence between fiber diffraction data and unique molecular structures does not exist, and from time to time alternative structures are proposed that differ fundamentally from accepted structures but which would give similar X-ray diffraction patterns (Rodley et al., 1976).

It is important, therefore, to test alternatives and to be able to estimate the accuracy of structures by methods other than X-ray diffraction. We believe that nuclear magnetic resonance of oriented fibers can be a particularly powerful approach in this regard. Recently, ^{31}P NMR[1] studies of oriented fibers have been interpreted as demonstrating (1) a nonuniform backbone conformation for DNA, (2) rotational motion about the helical axis (Shindo et al., 1980), and (3) sequence-dependent differences in the orientation of the phosphate groups in DNA (Shindo & Zimmerman, 1980). If verified by further experiments, these results are important in understanding how enzymes and regulatory proteins recognize specific DNA sequences.

In our work, we have similarly exploited the use of NMR of oriented fibers and have, in this case, applied it to the examination of the A form sodium salt of DNA. The method is based on the directional dependence of the NMR spectrum due to the anisotropy of the chemical shift for a nucleus involved in molecular bonding. If the NMR spectrum of a solid material is dominated by the chemical shift interaction, then it will be directionally dependent in the manner shown in Figure 1 where the observed chemical shift, σ_{obsd}, is

$$\sigma_{obsd} = \sigma_{11} \cos^2 \theta_1 + \sigma_{22} \cos^2 \theta_2 + \sigma_{33} \cos^2 \theta_3 \qquad (1)$$

where

$$\cos^2 \theta_1 + \cos^2 \theta_2 + \cos^2 \theta_3 = 1$$

Further, as illustrated by Figure 2, the direction cosines for the principal elements of the chemical shielding tensor relative to the magnetic field direction may be determined by comparing spectra from both ordered and randomly arranged samples. In this way, by obtaining ^{31}P NMR spectra of aligned fibers of DNA, we can determine the orientation of the principal elements of the shielding tensor relative to the fiber direction. Use of the molecular coordinates (Arnott & Hukins, 1972) gives the orientation of the shielding tensor relative to the phosphodiester group. A test for the assumed atomic structure for DNA may be made then by comparing the observed shielding tensor orientation with that obtained from NMR studies of phosphodiester groups in model compounds where the relation between the ^{31}P shielding tensor and the molecular bonding has been determined directly (Herzfeld et al., 1978; Kohler & Klein, 1976).

We report the results of a proton-enhanced ^{31}P NMR study of a highly oriented sample of A-form NaDNA obtained from calf thymus. Our results, in contrast to those of Shindo et al. (1980), indicate that little, if any, motion occurs in the DNA fibers and are consistent with a unique configuration of the

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[1] Abbreviations used: NMR, nuclear magnetic resonance; DNA, deoxyribonucleic acid; MHz, megahertz; kHz, kilohertz; Hz, hertz; ppm, parts per million; PAS, principal axis system; LAB, laboratory axis system; GONIOMETER, fiber axis system.

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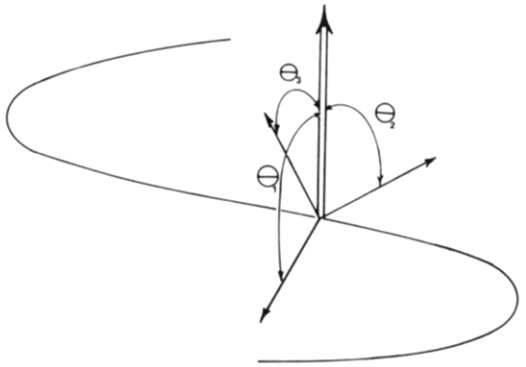

FIGURE 1: Relationship between magnitudes and directions of the principal elements of a chemical shielding tensor, the magnetic field direction and the observed chemical shift, σ_{obsd}. σ_{11}, σ_{22}, and σ_{33} are respectively the most to least shielded elements of the chemical shielding tensor in its principal axis system. θ_1, θ_2, and θ_3 are the angles between the principal axes and the magnetic field direction. The dependence of σ_{obsd} on these parameters is given in eq 1.

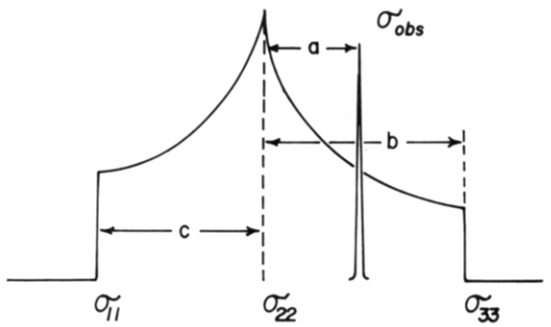

FIGURE 2: Schematic representations of NMR line shapes for a randomly oriented sample with an asymmetric shielding tensor ($\sigma_{11} \neq \sigma_{22} \neq \sigma_{33}$). Note that the magnitudes of the principal elements of the shielding tensor may be read off directly from the discontinuities in the powder pattern line shape. The relation of σ_{obsd} for a particular orientation of the nuclei to the parameters obtained from the powder spectrum (see eq 1) yields the following relation between the direction cosines: $\cos^2\theta_3 = a/b + (c/d)\cos^2\theta_1$. Relations of this type obtained for several orientations of the nuclei relative to the magnetic field allow the determination of the orientation of the shielding tensor in the sample.

backbone phosphate groups in DNA. Further, we find that our results are in good agreement with spectral simulations based on ^{31}P NMR studies of phosphodiester group model compounds and with the phosphate group coordinates proposed by Arnott & Hukins (1972).

Materials and Methods

Sample Preparation and Handling. The methods (Rupprecht, 1963, 1966) and equipment (Rupprecht, 1970a) used to prepare the highly oriented A-form calf thymus DNA samples have been described previously. After their initial preparation, the samples were stored in a desiccator over a saturated solution of $NaClO_3$ in order to maintain a relative humidity of 75%. Samples used for obtaining NMR spectra were trimed to fit a 1.5-cm length of 3-mm NMR tube such that the sample took up about one-third of the volume inside the tube. When spectra were taken, the NMR tubes were sealed with plastic caps and used for measurements which lasted for a period of several hours. Immediately following the acquisition of data, the samples were returned to the desiccator for storage.

NMR Spectra. ^{31}P NMR spectra were obtained on a pulsed spectrometer by using the technique of proton enhancement with proton decoupling during the observation of the ^{31}P free induction decay (Pines et al., 1973). The magnetic field strength was 5.6 T, resulting in a Larmor frequency of 240.1 MHz for protons and 97.2 MHz for the ^{31}P nuclei. The match condition for the proton enhancement was set by adjusting the phosphorus radio-frequency transmitter for a maximum in the free induction decay from a single crystal of $NH_4H_2PO_4$. The contact time for the corresponding experiment on the DNA fibers was adjusted to 5 ms. Under these conditions the spectra were identical with those obtained from 90° pulse experiments. The sample temperature was monitored during data acquisition by means of a thermocouple positioned near the sample and was 23 ± 2 °C. The accumulation time for each spectrum was ~20 min.

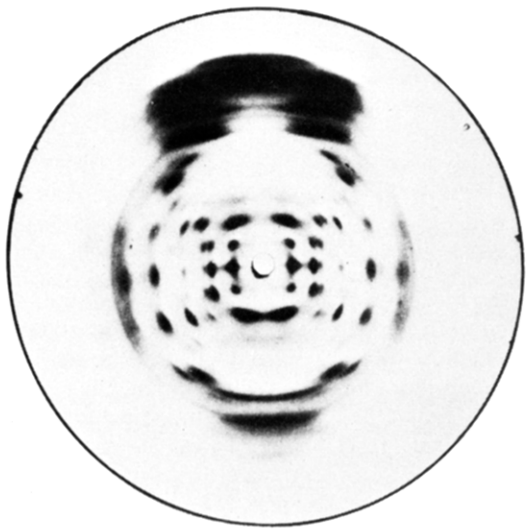

FIGURE 3: X-ray diffraction pattern of the oriented sodium DNA sample used for ^{31}P NMR experiments. The pattern demonstrates that the sample is highly crystalline, A-form sodium DNA.

Reproducible orientation of the sample with respect to the magnetic field was accomplished by mounting the NMR tube on a goniometer head in a manner that allowed rotation through an angle of ~360° about an axis perpendicular to the magnetic field direction (Pausak et al., 1974). In order to find the zero-angle position where the direction of orientation for the fibers is made to coincide with the direction of the magnetic field, we estimated the initial position and took trial spectra in 1° increments on each side of the estimated zero position. The true zero position was assumed to be the angle at which the ^{31}P line width was narrowest. The spectra that were obtained at a given increment on both sides of this zero point were the same, as required by symmetry. The estimated error in setting this angle was ±3°. Spectra taken at other angles are relative to the zero point determined by NMR.

Computer Calculations. Programs used to generate the line shape function for predicted spectra were written in FORTRAN IV and run on a Modcomp II computer. The equations and procedures employed are described under Appendix.

X-ray Diffraction. A pinhole camera with a 250-μm aperture was used to obtain the X-ray diffraction pattern of the oriented DNA sample on a flat photographic film. The specimen chamber was constantly flushed with helium gas previously bubbled through a saturated solution of NaCl to maintain the relative humidity at 75%. The DNA film was tilted 17° from being normal to the incident Cu Kα X-ray beam in order to record the meridional reflection.

Results

X-ray Diffraction. The X-ray diffraction pattern in Figure 3 clearly indicates that the sample is highly crystalline and

Table I: Orientation of ^{31}P Shielding Tensor in A-Form DNA[a]

	$\cos\theta_1$	$\cos\theta_2$	$\cos\theta_3$	θ_1	θ_2	θ_3
exptl[b]	0.4434	−0.8566	−0.2638	64	149	105
calcd[c]						
Arnott & Hukins (1972)	0.1847	−0.9559	−0.2265	79	163	103
S. Arnott and R. Chandrasekaran, unpublished experiments	0.3745	−0.8715	−0.3183	68	151	109

[a] Direction cosines and angles (in degrees) describing the orientation of the shielding tensor relative to the helical axis (see Figure 1). [b] Obtained by comparing observed and simulated spectra (see Appendix and Figure 5). [c] The calculated values are obtained by using the molecular coordinates from the indicated source along with a symmetrical orientation of the shielding tensor relative to the phosphodiester group bonding framework (Figure 6).

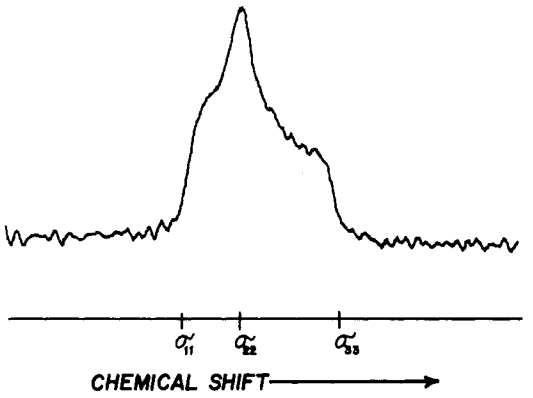

FIGURE 4: Observed ^{31}P NMR spectrum for a randomly oriented sample of sodium DNA at 75% relative humidity. σ_{11}, σ_{22}, and σ_{33} are equal to −83, −18, and +104 ppm, respectively (relative to 85% phosphoric acid).

extremely well oriented. The observed layer lines and the intensity distribution undoubtedly correspond to the familiar classical A-form DNA.

^{31}P NMR of Sodium DNA Fibers. As shown in Figure 4, the proton-decoupled spectrum of a randomly oriented sample of sodium DNA fibers gives an asymmetric powder pattern from which the values of σ_{11}, σ_{22}, and σ_{33} have been determined and are given relative to 85% phosphoric acid as −83, −18, and +103 ppm, respectively.

Figure 5b shows that the ^{31}P NMR spectra of oriented fibers of A-form sodium DNA are dependent on the angle between the fiber axis and the magnetic field direction. This dependence is a direct result of the chemical shift anisotropy described in Figures 1 and 2. Therefore, the expected line shapes for the spectra may be computed by summing spectra given by eq 1 for all ^{31}P nuclei in the sample (see Appendix for details). The orientation of the ^{31}P chemical shielding tensor relative to the helical axis may be determined by comparing the observed spectra to the simulated spectra (Figure 5 and Appendix). If one assumes a particular relationship between the phosphodiester group and the orientation of the chemical shielding tensor, then the orientation of the phosphate group may be inferred. Selection of a particular orientation of the shielding tensor relative to the phosphodiester group is guided by ^{31}P NMR studies of phosphodiester group model compounds (Kohler & Klein, 1976; Herzfeld et al., 1978).

Assignment of Shielding Tensor Principal Axis System. With the help of the atomic coordinates (Arnott & Hukins, 1972), the orientation of the ^{31}P chemical shielding tensor relative to a molecular axis system has been determined for A-form sodium DNA (Table I). The orientation of the phosphodiester group has not been determined, although the small number of model compound studies that have been performed suggests that the directions of the principal elements of the shielding tensor coincide with an axis system for the phosphodiester group based on molecular symmetry (Figure 6) to within 10–20° (Kohler & Klein, 1976; Herzfeld et al., 1978). Our shielding tensor orientation obtained by comparing calculated and observed spectra agrees with the symmetrical axis system referenced to the coordinates of Arnott & Hukins (1972) to within these limits. The agreement is much better when an improved set of A-form DNA coordinates (S. Arnott and R. Chandrasekaran, unpublished experiments) is used to calculate the expected shielding tensor orientation.

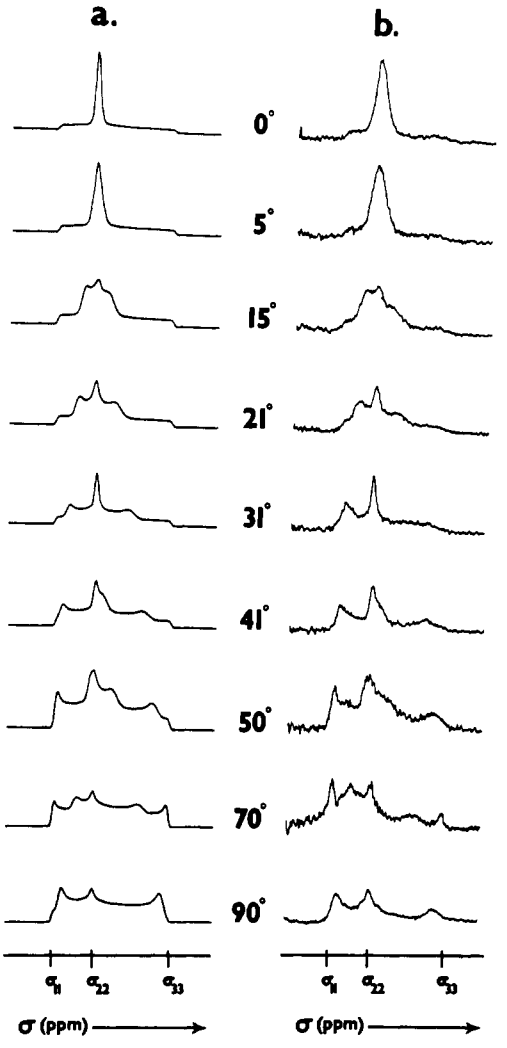

FIGURE 5: (a) Simulated and (b) observed ^{31}P NMR spectra for an aligned sample of A-form sodium DNA at indicated angles between fiber direction and magnetic field direction. The observed spectra have been proton decoupled in order to remove the effects of dipolar interactions with protons. The simulated spectra have been calculated by using standard methods described by Haeberlen (1976) (see Appendix for details).

Discussion

Spectral Simulations. The calculation of expected line shapes for spectra of oriented fibers of DNA is straighforward

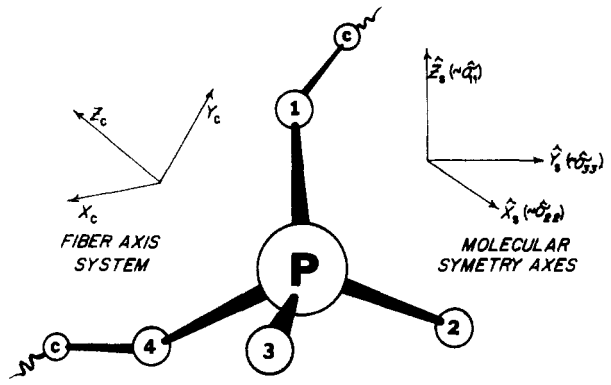

FIGURE 6: Relationship between the symmetrical axis system, the fiber axis system, and the phosphodiester group. The definitions of the unit vectors which define the symmetrical axis system are (1) Z_s bisects the O_2–P–O_3 angle. (2) Y_s lies in the O_2–P–O_3 plane and is perpendicular to Z_s. (3) X_s is perpendicular to both Y_s and Z_s. Our fiber axis system is that of Arnott & Hukins (1972). The origin is at the intersection of the helical axis and the base-pair dyad axis with the Z_c direction along the helical axis.

and in agreement with our results, if one makes the following assumptions.

(1) There Is No Motional Averaging of ^{31}P Chemical Shifts. This restriction limits any motions that might occur to frequencies of <20 kHz (the range of chemical shifts spanned by ^{31}P in DNA), although motions as slow as 1 kHz or less are capable of significantly distorting spectral line shapes of both random and oriented samples (Mehring, 1976). The crucial factor is that the frequency of the motion be considerably slower than the frequency range of the chemical shifts explored by the anisotropically shielded ^{31}P atom as it changes its orientation in the magnetic field.

(2) Orientation of Principal Elements of Chemical Shielding Tensor Is Known Relative to the Molecular Framework Surrounding the ^{31}P Atom. Herzfeld et al. (1978) have reported single-crystal NMR studies of barium diethyl phosphate, a model compound for phosphodiester groups. Their results demonstrate that the shielding tensor in the crystalline solid is oriented as much as 13° away from the molecular symmetry axes. This suggests that either crystal lattice forces, bond angles, or bond lengths are important in bringing about the noncoincidence of the molecular symmetry axes and the chemical shielding tensor. We have used the symmetrical orientation (Kohler & Klein, 1976) as a starting point for our spectral simulations. In the absence of more appropriate model compound studies, any quantitative statements relating to the orientation of the phosphodiester group may contain a systematic error of as much as 10–20°.

(3) Sample of Oriented DNA May Be Considered To Be Composed of Crystallites Which Are Oriented Relative to One Another Such That Their Dispersion about the Experimentally Determined Zero Angle Is Gaussian with Standard Deviation, σ. The intervening areas between the crystalline regions contain amorphous material which is randomly oriented. This model of the microscopic properties of our sample is identical with one discussed by Flory (1953) for crystalline polymers except that we have modified the model so that it applies to oriented polymeric samples by postulating an alignment of the crystallites. The standard deviation of the crystallites from perfect alignment has been estimated from X-ray diffraction of fibers (Rupprecht, 1970b) and our spectral simulations to be between 2 and 10° (see Appendix).

(4) Intrinsic Line Width for Fibrous A-Form DNA Is about 0.5–1.0 kHz (5–10 ppm). This is the line width that would be observed for a hypothetical perfectly oriented sample when it is aligned with the magnetic field. Since our ^{31}P spectra are obtained while protons are being decoupled, the major source of broadening for the ^{31}P atoms has been removed, leaving chemical shift anisotropy as the dominant factor in determining spectral line shape. Some spectral broadening does remain which is not reduced by increasing the proton-decoupling power.

The residual broadening may be attributed to a variety of factors, but two of the most likely are phosphorus–phosphorus and phosphorus–sodium dipolar interactions. Using a nearest-neighbor phosphorus–phosphorus distance of 5.6 Å from the crystal structure of guanylyl-3′,5′-cytidine nonahydrate (GpC) (Rosenberg et al., 1976), we estimate an average line width due to the phosphorus–phosphorus dipolar interaction to be ~250 Hz (2.6 ppm) (Abragam, 1961). Correspondingly, a line width estimate from sodium–phosphorus dipolar contributions (3.5 Å apart) could be ~890 Hz (9.2 ppm).

Phosphate Backbone of Fibrous A-Form DNA Forms a Rigid Lattice. The agreement between the simulated and observed spectra demonstrates the lack of significant motion within the fibers. This conclusion is substantiated by the fact that the powder spectrum of sodium DNA exhibits the usual features characteristic of phosphates known to be in a rigid lattice (Kohler & Klein, 1976; Herzfeld et al., 1978). Although the details depend on the particular type of motion involved, a good rule of thumb is that motion will be evident from the powder spectrum when the rate (in hertz) of that motion exceeds one-tenth of the total shift anisotropy [see Mehring (1976)]. The observed breadth and normal features of the powder spectrum thus limit any motions to frequencies less than ~2.0 kHz. In particular, fast motions (>20 kHz) about a unique axis (for example, the helical axis) are definitely ruled out since such motion would bring about the collapse of the observed axially asymmetric powder pattern for ^{31}P in DNA to an axially symmetric pattern. Motions of <2.0 kHz or of small amplitude are not ruled out, but there is no spectral evidence for their existence.

Regularity of the Phosphodiester Backbone. It is not necessary to invoke an irregular phosphodiester backbone in order to explain our results. The line width of the spectrum taken with the fiber direction and the magnetic field direction aligned is 21 ppm. The line width is adequately accounted for by the intrinsic line width of a spin isochromat (2–10 ppm) in combination with the estimated dispersion in the alignment of the crystallites relative to one another (2–10°). In particular, our zero-angle spectrum with its 21-ppm line width can be compared to that reported by Shindo et al. (1980), who attribute the 53.5-ppm line width of their zero-angle spectrum to a "nonuniform backbone conformation" which results in a dispersion of chemical shifts. Although our spectrum is obtained at ~4 times the magnetic field strength, line widths due to chemical shift dispersion when measured in parts per million are independent of field strength. On the other hand, dipolar broadening at the lower field strength employed by Shindo et al. (1980) would be 4 times larger in parts per million than at our field strength and could account for the major portion of their reported line widths.

While the spectral data can be accounted for in terms of a regular backbone, we do not wish to suggest that the phosphodiester backbone is perfectly regular. The line width of the spectrum with the fiber direction aligned with the magnetic field is ~21 ppm. This line width can be accounted for by three factors: (1) dipolar broadening (3–10 ppm); (2) chemical shift dispersion due to a corresponding dispersion in the relative orientation of the crystallites in the sample (4–20

ppm); (3) chemical shift dispersion due to an irregular phosphodiester backbone (0–15 ppm). On the basis of the above estimates of the extent to which each factor might contribute to the total line width, it is estimated that the phosphodiester group shielding tensors in A-form DNA fibers are oriented in the same manner to within ±5°.[2]

Our spectrum obtained with the fiber direction perpendicular to the magnetic field direction differs drastically from that reported by Shindo et al. (1980). Their spectrum gave a single broad resonance rather than the expected bimodal pattern. This inconsistency was attributed to motion about the helical axis rapid enough to collapse the expected bimodal pattern into the observed single broad resonance. However, our results at 90° (Figure 5b) can be accounted for in terms of a model that assumes no motion at all (rigid lattice). Our observed trimodal spectrum is accounted for if one assumes the presence of a small amount of randomly oriented DNA. This assumption is supported by (1) X-ray diffraction analysis of the sample indicating some disordered material, (2) accepted physical models of highly crystalline polymers (Flory, 1953), and (3) coincidence of the center peak position in our 90° spectrum of the oriented sample with σ_{22} from the ^{31}P NMR powder spectrum of sodium DNA (compare Figures 4 and 5b).

Conclusions. Shindo et al. (1980) have reported ^{31}P NMR studies of oriented fibers of A-, B-, and C-form DNA, while our studies are limited to A-form sodium DNA. For the A-form samples our spectra are qualitatively different from those of Shindo et al. (1980). Their spectra are inconsistent with their spectral simulations, a fact which they attribute to (1) motion of the phosphodiester group about the helical axis and (2) significant variation of the orientation of the phosphodiester group along the DNA molecule. Our spectra are in excellent agreement with spectral simulations (Figure 5). Therefore, we find no evidence for either motion about the helical axis (faster than ~2 kHz) or an irregular phosphodiester backbone (to within ±5°). The qualitative discrepancy between our results and those of Shindo et al. (1980) can be accounted for by (a) a major difference in the degree of sample orientation and (b) the higher magnetic field strength at which our spectra were obtained (5.6 vs. 1.4 *T*).[3]

The orientation of the ^{31}P shielding tensor relative to the helical axis has been determined for A-form sodium DNA. The orientation is consistent with the atomic coordinates given by Arnott & Hukins (1972) and NMR studies of phosphodiester group model compounds (Herzfeld et al., 1978; Kohler & Klein, 1976). When an improved set of A-form DNA coordinates (S. Arnott and R. Chandrasekaran, unpublished experiments) and the symmetrical axis system are combined to calculate the expected orientation of the shielding tensor (Table I), the agreement with the experiment is excellent (within 4°).

The detailed structural information obtainable from NMR studies of oriented polymers is limited by the problem of properly assigning the shielding tensor to the molecular bonding framework. Improvement on this front must await the results of NMR studies of single crystals of numerous model compounds in order to evaluate the relative contributions

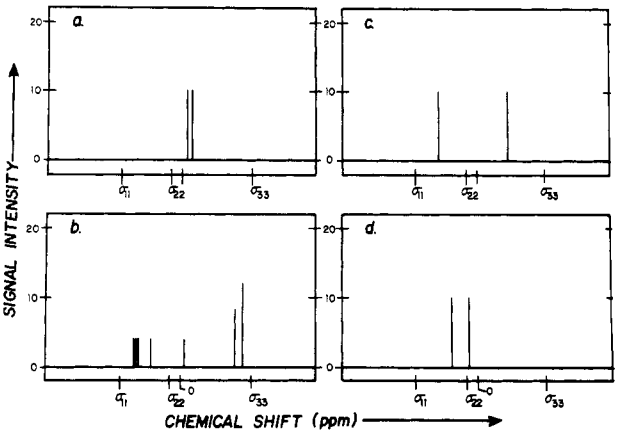

FIGURE 7: Predicted ^{31}P NMR spectra of oriented (a) Z- (Wang et al., 1979; A. Rich, unpublished experiments), (b) "side by side" (Rodley et al., 1976), (c) S- (Arnott et al., 1980; S. Arnott and R. Chandrasekaran, unpublished experiments), and (d) "alternating B"-form DNA (Klug et al., 1979) for the case where the fiber axis and the magnetic fields are aligned. The spectra were calculated by using eq 1, the phosphodiester group coordinates, and the symmetrical orientation of the ^{31}P chemical shielding tensor (Kohler & Klein, 1976).

of bond lengths, molecular configuration, and crystal lattice forces in bending the shielding tensor away from the approximate symmetry axes for the molecule. However, one can take the position that *changes* in the orientation of the phosphate group can be determined accurately (perhaps to within 3–4°) when comparing structurally different DNA fibers. To a first approximation, the orientation of the shielding tensor relative to the bonding framework should remain invariant for small changes in the orientation of the phosphate group, thus allowing changes to be determined with considerably greater accuracy than absolute orientations. In this manner, the determination of the shielding tensor orientation made in the present work may provide a bench mark for determining changes in phosphodiester orientations in DNA fibers which have been altered by the introduction of intercalating dyes, proteins, or metal ions or which are simply in alternative structural forms.

Even if such a comparative approach is not taken, the NMR method would be of great utility in distinguishing among structural possibilities which require large differences in the orientation of particular groups. To illustrate this point, in Figure 7 we present the idealized ^{31}P NMR spectra of several proposed structures of DNA for the case where the fiber is aligned along the direction of the magnetic field. It is clear that these structures may be easily distinguished on the basis of a single NMR spectrum. As we have shown, off-angle spectra are also extremely sensitive to structural differences (Figure 5) and can be used to confirm or reject preliminary structural assignments made on the basis of a single spectrum. The possibility of molecular motion in DNA may be investigated by the present method for oriented fibers, by traditional relaxation measurements on unoriented samples, or by some more recently developed techniques for studying molecular motions in solids (Rothwell & Waugh, 1981; Suwelack et al., 1980; Pines et al., 1976).

Of course, there is no reason to limit the NMR studies to phosphorus since ^{13}C, ^{1}H, and ^{15}N nuclei can also be used to probe the DNA structure in a similar manner. Selective labeling of particular sites along the double helix would be especially useful in this regard. In fact, ^{13}C NMR should be of greater value than ^{31}P magnetic resonance studies, since more model compound data are available on ^{13}C and since carbon plays a more central role in the structural aspects of biopolymers.

[2] The direction cosines reported in Table I are weighted averages if one takes the position that the shielding tensor orientation (or, equivalently, the phosphate group orientation) is dependent on nucleotide sequence. From the line width of the zero-angle spectrum, we estimate that variation in the angles reported in Table I due to nucleotide sequence can be no more than ±5°.

[3] Our higher field strength will decrease the relative effect of phosphorus–phosphorus and sodium–phosphorus dipolar broadening by a factor of 4 (see Discussion).

Acknowledgments

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Appendix

Simulation of Spectra. The procedures for calculating the chemical shift spectra of single crystals and randomly oriented samples have been discussed thoroughly in Haeberlen (1976). We have applied these methods in the simulation of spectra for oriented samples. The procedure is briefly outlined below and is for the most part equivalent to that of Shindo et al. (1980).

The transformation to the principal axis system (PAS) of the shielding tensor from the laboratory frame is effected in the manner

$$\text{LAB} \xrightarrow{D^2(0,\theta,0)} \text{GONIOMETER} \xrightarrow{D^2(\alpha,\beta,\gamma)} \text{PAS} \qquad (1A)$$

where the $D^2(a,b,c)$ are elements of the $l = 2$ Wigner rotation matrix. The Euler angles α, β, and γ specify the orientation of the PAS for the shielding tensor with respect to the fiber axis while the Euler angle θ is the direction of the alignment axis of the fibers with respect to the magnetic field. The two-part transformation in (1A) may be expressed mathematically as

$$\sigma_{2,0}^{\text{LAB}} = \sum_{m,m'} D_{m,0}^2(\theta) D_{m',m}^2(\alpha,\beta,\gamma) \rho_{2,m'} \qquad (2A)$$

where the $\rho_{2,m'}$ are the components of the $l = 2$ irreducible spherical tensor operators in their principal axis system. For a particular choice of the angles θ, α, β, and γ, a single NMR line will be observed at a chemical shift given by

$$\sigma_{\text{obsd}} = \sigma_{\text{iso}} + (2/3)^{1/2} \sigma_{2,0}^{\text{LAB}} \qquad (3A)$$

or

$$\sigma_{\text{obsd}} = \sigma_{\text{iso}} + \frac{1}{2}\sin^2\theta(A\cos 2\alpha + B\sin 2\alpha) - \frac{1}{2}\sin 2\theta(C\cos\alpha - D\sin\alpha) + \frac{(3\cos^2\theta - 1)}{2}E \qquad (4A)$$

where

$$A = \frac{3}{2}\sin^2\beta(\sigma_{33} - \sigma_{\text{iso}}) - \frac{1 + \cos^2\beta}{2}\cos 2\gamma(\sigma_{22} - \sigma_{11})$$

where

$$B = \cos\beta \sin 2\gamma(\sigma_{22} - \sigma_{11})$$

$$C = \cos\beta \sin\beta \cos 2\gamma(\sigma_{22} - \sigma_{11}) + \frac{3}{2}\sin 2\beta(\sigma_{33} - \sigma_{\text{iso}})$$

$$D = +\sin\beta \sin 2\gamma(\sigma_{22} - \sigma_{11}) \qquad (5A)$$

and

$$E = \frac{3\cos^2\beta - 1}{2}(\sigma_{33} - \sigma_{\text{iso}}) - \frac{1}{2}\sin^2\beta \cos 2\gamma(\sigma_{22} - \sigma_{11})$$

In the above formulas, σ_{11}, σ_{22}, and σ_{33} are the principal elements of the shift tensor and σ_{iso} is given as

$$\sigma_{\text{iso}} = \frac{1}{3}(\sigma_{11} + \sigma_{22} + \sigma_{33}) \qquad (6A)$$

A spectrum at angle θ is simulated on the basis of eq 4A by adding together subspectra for values of α from 0 to 360°. The values of β and γ are assumed from the crystal structure. In this manner, the one dimensionally oriented nature of the fibers is taken into account.

The implementation of the above procedure produces a spectrum for a hypothetical perfectly oriented fiber. In order to take into account the dispersion in orientation of the crystallites about the alignment direction of the fiber, we weight and add spectra in the neighborhood of θ_0 by the Gaussian function

$$G(\theta_0 - \theta) = (e^{-(\theta-\theta_0)^2/(2\sigma^2)})/(\sigma\pi^{1/2}) \qquad (7A)$$

where σ is the standard deviation of misalignment as estimated by X-ray diffraction. The random-coil regions in the fiber are simulated as a Bloembergen–Rowland-type powder pattern by using the experimentally determined principal shielding elements. The resulting spectra were smoothed in order to account for the 1–2-ppm magnetic field inhomogeneity.

References

Abragam, A. (1961) *The Pinciples of Nuclear Magnetism*, pp 103–124, Oxford University Press, London.

Arnott, S. (1980) *Trends Biochem. Sci. (Pers. Ed.) 5*, 231–234.

Arnott, S., & Hukins, D. W. L. (1972) *Biochem. Biophys. Res. Commun. 47*, 1504–1509.

Arnott, S., Chandrasekaran, R., Birdsall, D. L., Leslie, A. G. W., & Ratliff, R. L. (1980) *Nature (London) 283*, 743–745.

Arnott, S., Chandrasekaran, R., Bond, P. J., Birdsall, D. L., Leslie, A. G. W., & Puigjaner, L. C. (1981) *Struct. Aspects Recognition Assem. Biol. Macromol., Proc. Aharon Katzir-Katchalsky Conf., 7th 2*, 231–234.

Flory, P. J. (1953) *Pinciples of Polymer Chemistry*, pp 48–50, Cornell University Press, Ithaca, New York.

Fuller, W., Wilkins, M. H. F., Wilson, H. R., Hamilton, L. D., &Arnott, S. (1965) *J. Mol. Biol. 12*, 60–80.

Haeberlen, U. (1976) *High Resolution NMR in Solids, Selective Averaging*, Academic Press, New York.

Herzfeld, J., Griffin, R. G., & Haberkorn, R. A. (1978) *Biochemistry 17*, 2711–2718.

Jack, A., Ladner, J. E., & Klug, A. (1976) *J. Mol. Biol. 108*, 619–649.

Klug, A., Jack, A., Viswamitra, M. A., Kennard, O., Shakked, A., & Steitz, T. A. (1979) *J. Mol. Biol. 131*, 669–680.

Kohler, S. J., & Klein, M. P. (1976) *Biochemistry 15*, 967–973.

Langridge, R., Marvin, D. A., Seeds, W. E., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F., & Hamilton, L. D. (1960) *J. Mol. Biol. 2*, 38.

Mehring, M. (1976) *High Resolution NMR Spectroscopy in Solids*, p 38, Springer-Verlag, New York.

Milman, G., Langridge, R., & Chamberlain, M. J. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 1804–1810.

Pausak, S., Tegenfeldt, J., & Waugh, J. S. (1974) *J. Chem. Phys. 61*, 1338–1344.

Pines, A., Gibby, M. G., & Waugh, J. S. (1973) *J. Chem. Phys. 59*, 569–590.

Pines, A., Vega, S., Rubin, D. J., Shattuck, T. W., & Wemmer, D. E. (1976) *Proceedings of the 4th Ampère International Summer School*, Pula, Yugoslavia.
Rodley, G. A., Scobie, R. S., Bates, R. H. T., & Lewitt, R. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2959–2963.
Rosenberg, J., Seeman, N. C., Day, R. O., & Rich, A. (1976) *Biochem. Biophys. Res. Commun. 69*, 979–987.
Rothwell, W. P., & Waugh, J. S. (1981) *J. Chem. Phys.* (in press).
Rupprecht, A. (1963) *Biochem. Biophys. Res. Commun. 12*, 163–168.
Rupprecht, A. (1966) *Acta Chem. Scand. 20*, 494–504.
Rupprecht, A. (1970a) *Biotechnol. Bioeng. 12*, 93.
Rupprecht, A. (1970b) Ph.D. Thesis, Karolinska Institutet, Stockholm 60, Sweden.
Shindo, H., & Zimmerman, S. B. (1980) *Nature (London) 283*, 690–691.
Shindo, H., Wooten, J. B., Pheffer, B. H., & Zimmerman, S. B. (1980) *Biochemistry 19*, 518–526.
Suwelack, D., Rothwell, W. P., & Waugh, J. S. (1980) *J. Chem. Phys. 73*, 2559–2569.
Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680–686.

Ethidium Bromide and Its Photoreactive Analogues: Spectroscopic Analysis of Deoxyribonucleic Acid Binding Properties†

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ABSTRACT: In an effort to determine the in vivo targets for ethidium bromide, the promising new technique of photoaffinity labeling has been applied in the development of two photosensitive ethidium azido analogues. One of these, ethidium monoazide, 3-amino-8-azido-5-ethyl-6-phenylphenanthridinium chloride, has been shown previously to have biological properties similar to those of the parent ethidium prior to photolytic activation. After photolysis, which results in the covalent attachment of the ethidium moiety, the monoazide demonstrates enhanced biological activity. The diazide, 3,8-diazido-5-ethyl-6-phenylphenanthridinium chloride, is much less active. Since nucleic acids are presumed to be one of the targets of ethidium, both the noncovalent and the covalent interactions of ethidium and these azides with calf thymus DNA were analyzed at several salt concentrations by using spectrophotometric and dialysis techniques. The results presented in this paper show that the noncovalent interaction of the monoazide with deoxyribonucleic acid (DNA) is essentially identical with that of the parent ethidium and is primarily intercalative in nature. The DNA interaction with the diazide, apparently a stacking interaction, is quite different as seen by the greater decrease in the apparent association constant at elevated salt concentrations. Furthermore, the covalent interaction of the monoazide with DNA formed with ~40% photolytic efficiency resembled that of the noncovalent complex which suggests that no reorientation of the noncovalently bound ligand is required for covalent attachment. These results demonstrate that the monoazido analogue of ethidium bromide may be useful in determining directly the targets responsible for biological activity.

The mechanisms responsible for ethidium bromide's diverse biological actions have not been elucidated despite extensive efforts devoted to the characterization of the interactions with nucleic acids. A major obstacle is the reversible nature of the drug interaction which precludes isolation of the in vivo and in vitro complexes. In an effort to solve the problem of reversible binding, ethidium was modified to the photosensitive monoazide (3-amino-8-azido-5-ethyl-6-phenylphenanthridinium chloride) (Hixon et al., 1975; Graves et al., 1977) and the diazide (3,8-diazido-5-ethyl-6-phenylphenanthridinium chloride) (Bastos, 1975) shown in Figure 1 in order to provide a means of covalently attaching the parent ethidium to its target sites.

Azide photoaffinity labeling is a powerful technique which will hopefully provide the means of identifying and characterizing the biological targets responsible for biological activity. In the case of drugs interacting with macromolecules, the azido analogue should simulate the parent compound. Once the photoaffinity probe is bound to the target, the azido substituent is activated by light to a nitrene (DeTraglia et al., 1978; Bercovici & Gitler, 1978) which reacts instantaneously to

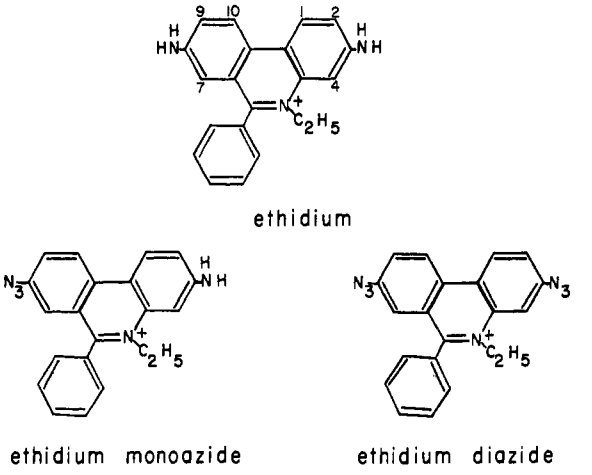

FIGURE 1: Chemical structures and numbering scheme of ethidium bromide and the photoreactive mono- and diazido analogues.

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