Conclusion

We conclude that there are two pathways for forming cross-links. In one, a monoadduct intermediate must experience a conformational change in its surrounding DNA before it can productively absorb a second photon and form a cross-link. In the other pathway, no such conformational change is necessary, or if one is required, it takes place within the lifetime of a 10-ns laser pulse, instead of requiring the 1.3 μs characteristic of the first pathway. The monophotonic behavior of cross-linking via the second pathway may result from very efficient cross-linking of a few monoadducts ($k'' \gg k'$), from light saturation of the MA' → XL photoreaction, or from the reaction actually requiring only a single photon. The two pathways probably correspond to different populations of intercalated psoralens, distinguished by local base sequence or perhaps by random flexing of the DNA at the site.

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Geometry of the Phosphodiester Backbone in the A Form of Deoxyribonucleic Acid Determined by Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy[†]

Heisaburo Shindo, Jan B. Wooten, and Steven B. Zimmerman*

ABSTRACT: ^{31}P NMR spectra were obtained from poly(dA-dT)·poly(dA-dT) fibers which gave an X-ray diffraction pattern similar to that of the A form of natural deoxyribonucleic acid (DNA). The analysis of the line shape indicated that the A form of poly(dA-dT)·poly(dA-dT) has a single uniform backbone conformation; the orientation of the phosphodiester group relative to the helical axis was determined to be $\beta = 70°$ and $\gamma = 52°$. The ^{31}P NMR spectra of poly(dA-dT)·poly(dA-dT) were in remarkable contrast to the ^{31}P NMR spectra of the A form of natural DNA, which exhibited an unusual line shape. The origins of the abnormalities in the line shape for the A form of natural DNA are discussed in terms of phosphodiester orientations.

I t has been shown that the ^{31}P NMR characteristics of deoxyribonucleic acid (DNA) in solution (Shindo et al., 1979; Simpson & Shindo, 1980) are essentially identical with those of B form DNA fibers at high relative humidity (98%) (Shindo et al., 1980; Shindo & Zimmerman, 1980) and that both can be consistently interpreted in terms of a nonuniform backbone conformation. The nonuniformity of the backbone conformation has several important implications. For example, any single conformation used to characterize natural DNA must be an average conformation, and, further, that irregularity in conformation may well be base-sequence dependent.

We have extended these studies to the A form of DNA, which can generally be induced in fibers containing appropriate

salts when exposed to lower relative humidities. The ^{31}P NMR spectra of such A form fibers from natural DNA were complex. It seemed possible that a number of extraneous factors might be contributing to the observed spectra. For example, at lower relative humidities there is an increased probability of mixture of different conformations, of varying fractions of amorphous materials, and of variation in molecule packing within the crystal (Landridge et al., 1960). All these factors could influence the observed ^{31}P NMR spectra. X-ray fiber diffraction patterns have been routinely collected from the fibers used in our NMR studies (Shindo et al., 1980); the patterns indicate a relative absence of these complications. Nonetheless, it seemed important to contrast the complex NMR results for the A form of natural DNA with those of a more homogeneous polynucleotide. Accordingly, the NMR spectrum of the A form of poly(dA-dT)·poly(dA-dT) has been obtained and has proven much simpler to interpret.

In the present paper, we will show that the A form of poly(dA-dT)·poly(dA-dT) possesses a single backbone conformation which allows us to determine the orientation of the phosphodiester group in the molecule under some restricted conditions, and we also discuss some of the complexities underlying the A form of natural DNA.

Materials and Methods

Poly(dA-dT)·poly(dA-dT) was purchased from P-L Biochemicals, Inc. The polymer was dissolved into 0.1 M NaCl, dialyzed against deionized water by using an ultramembrane filter (Amicon Model 10), and then lyophilized. A small amount of water was added to the lyophilized polymer; the resulting gel was kept at ~70 °C for 1 h and cooled slowly to room temperature. Fibers were formed at 4 °C. Fibers were also prepared from salmon sperm DNA (Calbiochem) for purposes of comparison. Diffraction patterns were collected at 79% relative humidity as described previously (Zimmerman & Pheiffer, 1979).

Proton dipolar decoupled and cross-polarized ^{31}P NMR spectra at 24.3 MHz were obtained on a TT-14 spectrometer (Nicolet, Inc.) which was extensively modified for solid-state NMR. Orientation of the DNA fibers relative to the magnetic field and control of relative humidity were carried out as described previously (Shindo et al., 1980). Computer analyses were performed with an interactive modeling system (MLAB) on the Digital Equipment Corp. PDP-10 at the Computer Center of the National Institutes of Health.

Results and Discussion

Comparison of ^{31}P NMR Spectra of the A Form. An X-ray diffraction pattern from a fiber of the sodium salt of poly-(dA-dT)·poly(dA-dT) at 79% relative humidity is shown in Figure 1. This pattern is similar to the diffraction pattern of the A form of DNA (Fuller et al., 1965) and is distinct from that of the D form (Davis & Baldwin, 1963; Arnott et al., 1974). The pitch was measured to be 27.8 Å, which is nearly identical with the value of typical A-form DNA (28.1 Å) (Fuller et al., 1965). The A form of this polymer has been reported to be metastable and to eventually change into the D form at 79% humidity (Davies & Baldwin, 1963). However, we have taken pictures from such fibers at intervals for several days after preparation as well as for several weeks before and after the NMR measurements. In all cases, the fibers gave patterns essentially identical with that shown in Figure 1. Therefore, we conclude that the A form of poly(dA-dT)·poly(dA-dT) is stable under our conditions.

Figure 2B shows the ^{31}P NMR spectra (24.3 MHz) from poly(dA-dT)·poly(dA-dT) fibers oriented either perpendicular
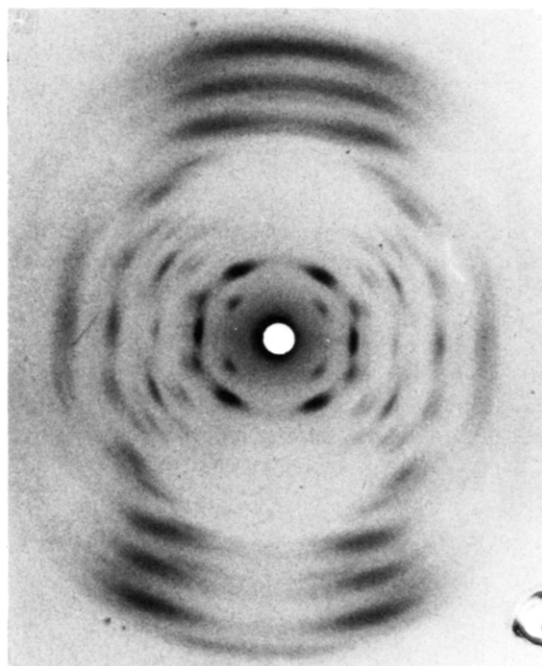

FIGURE 1: X-ray diffraction pattern obtained from a poly(dA-dT)·poly(dA-dT) fiber at 79% relative humidity at room temperature.

or parallel to the magnetic field. The parallel spectrum of this synthetic copolymer has a narrow single resonance with a width of 39 ppm and displays a maximum intensity at −25.7 ppm. These values may be compared with those of the parallel spectrum of natural DNA, namely 54 ppm in line width and −18 ppm in chemical shift. We have previously reported a doublet in the parallel spectrum of the B form of poly(dA-dT)·poly(dA-dT) fibers (Shindo & Zimmerman, 1980); the average individual line widths were estimated to be 13 ppm. The parallel spectrum observed here for the A form of poly-(dA-dT)·poly(dA-dT) fibers is much broader than that for the B form of the same polymer. This difference in line widths may result from the fact that DNA in the A form is much more restricted in motion than is DNA in the B form (Shindo et al., 1980).

The perpendicular spectrum of the A form of poly(dA-dT)·poly(dA-dT) exhibits a trapezoidal spectral pattern in remarkable contrast to that of natural DNA. If the orientation of the phosphodiester group is symmetric about the helical axis and molecules are immobile in the fiber, the perpendicular spectrum should appear as a doublet (Shindo et al., 1980). Two causes will be considered which might result in the deformation of a doublet to the observed trapezoidal line shape: the presence of rotational motion about the helical axis of the molecules and imperfect orientation of the molecules along the fiber axis; dipolar interactions ([31]P–[31]P and [31]P–[23]Na) were considered to be negligible.

Small but predictable changes in the perpendicular spectrum such as some decrease in intensity at the center of the spectrum and a sharper drop at the upfield side in the spectrum were observed when the temperature was lowered from 25 to 2 °C (Figure 3). The small influence of this temperature change is consistent with the fact that the fibers of the A form of DNA are largely composed of crystalline regions in which molecular motion would presumably be greatly diminished. Therefore, motion of the molecules in fibers of the A form at low relative humidity (79%) is much more restricted than in fibers of the B-form DNA at high relative humidity (98%), where rapid rotational motion about the helical axis is taking place (Shindo et al., 1980; Shindo & Zimmerman, 1980).
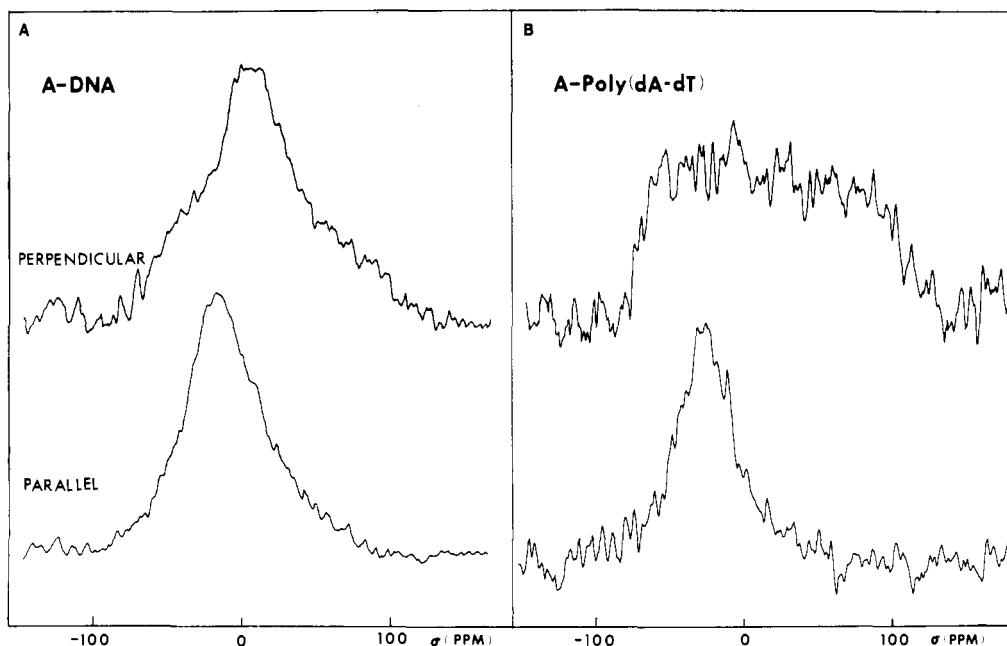
FIGURE 2: Proton dipolar decoupled and cross-polarized ^{31}P NMR spectra (24.3 MHz) from DNA (A) and poly(dA-dT)·poly(dA-dT) fibers (B) oriented parallel or perpendicular to the magnetic field. The spectra of natural (salmon sperm) DNA are from Shindo et al. (1980). Temperature is ~25 °C and relative humidity is 79%. Spectral conditions were the same as in Shindo et al. (1980). Chemical shift measured upfield is positive relative to trimethylphosphate as an external reference.
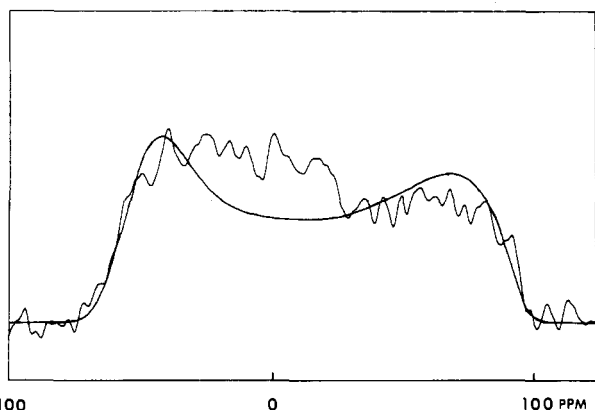

FIGURE 3: ^{31}P spectrum of poly(dA-dT)·poly(dA-dT) at 2 °C. The smooth solid line represents the theoretical line shape with a distribution of molecular disorder about the fiber axis; a standard deviation of $\langle \theta \rangle$ = 10° and 5 ppm in line broadening were assumed. Abscissa, chemical shift.
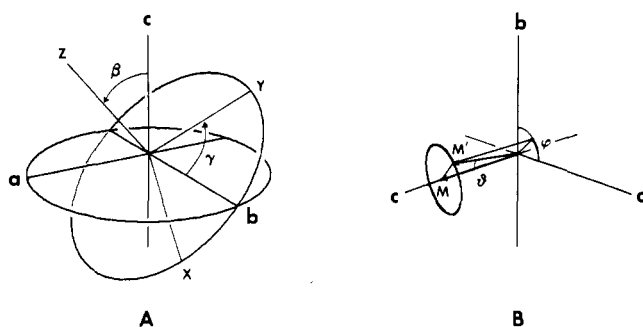

FIGURE 4: (A) Principal axis system of the chemical shift tensor relative to the helical axis system; the relationship between the two systems is described by the Euler angles β and γ. (B) Representation of disorientation of the DNA helical axis, M', about the fiber axis, M.
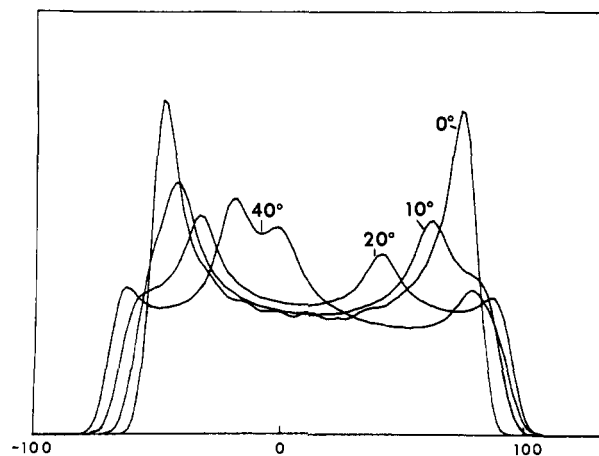

FIGURE 5: Calculated changes in the spectral patterns due to molecular disorientation about the fiber axis. The angle (θ) shows the disorientation assumed for each curve and is defined in Figure 4B. Abscissa, chemical shift in ppm.

The effect on line broadening due to imperfect molecular orientation along the fiber axis has been discussed previously for the parallel spectrum (Shindo et al., 1980). As before, the principal axis system is related to the molecular coordinate system by two Euler angles β and γ (Figure 4A), and disorientation of the helical axis from the fiber axis is defined by angles θ and ϕ (Figure 4B). Calculated changes in the perpendicular spectrum due to molecular disorientation are drawn at different θ values in Figure 5 by using Euler angles β = 70° and γ = 55° and chemical shift tensor values (Terao et al., 1977) σ_{11} = -82, σ_{22} = -22, and σ_{33} = 113 ppm. The characteristic changes in the spectrum due to disorientation are as follows: the total anisotropy obviously increases and one of the two peaks shifts towards higher field strengths while the other moves downfield to a greater extent; the peaks overlap at θ values >40°.

As discussed previously (Shindo et al., 1980), if the molecular disorder is randomly distributed with respect to θ, then the spectrum from the fiber can be calculated by summing all the composite spectra for each given θ value after weighting by a Gaussian factor with a standard deviation $\langle \theta \rangle$. The resulting spectral pattern having a standard deviation $\langle \theta \rangle$ = 10° is shown in Figure 3. Clearly, this spectrum is closer to the observed trapezoidal shape than is the spectrum for the fiber in which molecules are perfectly oriented along the fiber
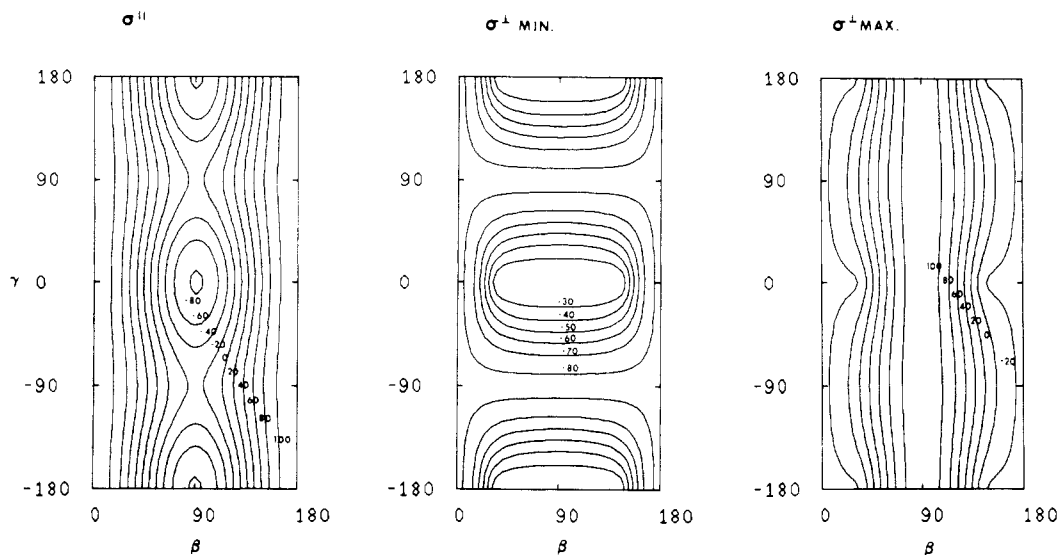
FIGURE 6: Contour maps of chemical shifts (ppm) for parallel and perpendicular orientations of the fiber relative to the magnetic field. $\sigma^{\parallel}$ represents the chemical shift of the parallel spectrum, and $\sigma_{min}^{\perp}$ and $\sigma_{max}^{\perp}$ correspond to the peak positions of a doublet in the perpendicular spectrum.

axis (i.e., $\theta = 0°$ in Figure 5). If some amorphous regions and/or reorientational motion about the helical axis are present, then the spectrum would become even more trapezoidal in shape, because both factors bring spectral components from the near edges to the isotrophic chemical shift region (i.e., 3 ppm). On the basis of the above analysis and the single resonance observed for the parallel orientation, we can conclude that the A form of poly(dA-dT)·poly(dA-dT) has effectively a single backbone conformation with respect to the helical axis; this is an interesting contrast to the two distinct conformations observed for the B form of this polymer (Shindo & Zimmerman, 1980).

For the A form of natural DNA, the limits of the chemical shift values for a perpendicular orientation of the fibers are poorly defined because of their spectral shape (Figure 2). The perpendicular spectrum is characterized as follows: it has a maximum peak at ~ 0 chemical shift and long tails both in the upfield and downfield directions, unlike the sharp drops observed for poly(dA-dT)·poly(dA-dT). Such a spectral pattern may be interpreted in terms of disorder in orientation of the phosphodiester groups with respect to the fiber axis. For example, a large deviation of the molecules from the fiber axis deforms a simple doublet pattern into a nonsymmetric pattern with maximum peaks near 0 chemical shift (compare the spectra for $\theta = 0°$ and $40°$ in Figure 5). Judging from the X-ray patterns, however, the average molecular disorientation $\langle\theta\rangle$ in crystalline regions must be $<10°$. Another potential source of variation in phosphodiester environment in A-DNA fibers is the lattice fields associated with the crystalline form. However, such fields seem unlikely to be responsible for the differences between the NMR patterns of natural DNA and poly(dA-dT)·poly(dA-dT), since the X-ray patterns indicate that they share the same space group and have very similar unit cell dimensions. Another possible source of the difference in the spectral patterns between natural DNA and poly(dA-dT)·poly(dA-dT) fibers may be attributed to the difference in their molecular motions. However, only slow molecular motion was estimated for the A form of DNA, judging from the total chemical shift anisotropy (Shindo et al., 1980). Furthermore, only small changes in spectral shape were observed for the A form of poly(dA-dT)·poly(dA-dT) fibers when the temperature was lowered from 25 to 2 °C. These facts indicate that it is unlikely that molecular motion is responsible

for the broad asymmetric single resonance of the perpendicular spectrum for the A form of natural DNA. Certainly, the most obvious difference between these polymers is in the difference in their sequence heterogeneity. It is tempting to correlate the simpler NMR pattern for poly(dA-dT)·poly(dA-dT) with its simple repeating sequence and the more complex NMR pattern for natural DNA with its heterogeneous sequences. This implies that a sequence dependence of backbone conformation in the A form of natural DNA is likely, as was earlier concluded for the B form of natural DNA (Shindo et al., 1980; Shindo & Zimmerman, 1980). It is difficult, however, to see how a large variation in phosphodiester orientation would be accommodated in the regular crystalline lattice of the A form of natural DNA. Thus, it may be possible that the A-DNA fibers contained a significant amount of noncrystalline material which was not detected by the X-ray method and which resulted in an abnormal line shape in the NMR spectra.

In conclusion, although the alternating sequence in poly(dA-dT)·poly(dA-dT) happened to give rise to a single backbone conformation in the A form, possibly other sequences could utilize other conformations. We are therefore going to investigate various other synthetic DNAs with known sequences in order to attempt to test these implications.

Orientation of Phosphodiester Group. In the case where a molecule is immobile and has a uniform backbone conformation, the orientation of the principal axis system of the chemical shift tensor can be determined from NMR data. Since the principal axis system is closely related to the atomic coordinates of the phosphodiester groups (Kohler & Klein, 1976; Herzfeld et al., 1978), the orientation of the diester group can be estimated.

It is interesting to see how the chemical shift behaves as a function of Euler angles β and γ. Figure 6 shows contour maps of the chemical shifts, where $\sigma^{\parallel}$ represents the chemical shift for the fiber oriented parallel to the magnetic field and $\sigma_{min}^{\perp}$ and $\sigma_{max}^{\perp}$ correspond to the chemical shifts of the doublet in the perpendicular spectrum for a given set of β and γ. All the contour maps have 2-fold symmetry with respect to $\beta = 90°$ and $\gamma = 0°$ and $90°$, reflecting the helical and dyad symmetries of the molecule. Calculated values of $\sigma^{\parallel}$ vary from -82 to 113 ppm, while $\sigma_{min}^{\perp}$ varies from -82 to -22 ppm and $\sigma_{max}^{\perp}$ from -22 to 113 ppm. Other features of these maps
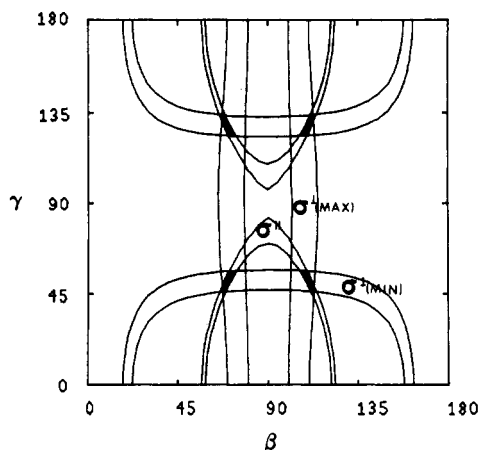
FIGURE 7: Contour lines of chemical shift values observed for the A form of poly(dA-dT)·poly(dA-dT) fibers. Each band corresponds to the error range of the measurements. The intersections of the three contour bands give the solutions for angles β and γ.

Table I: Orientation of the Phosphodiester Group in the A Form of DNA or of Poly(dA-dT)·Poly(dA-dT) As Obtained from ^{31}P NMR and Other Methods

material	method	β^a	γ^a	ref
Na-Poly(dA-dT)· poly(dA-dT)	^{31}P NMR	70 ± 5	52 ± 5	present work
NaDNA	X-ray	65	60	Fuller et al. (1965)
NaDNA	X-ray	77^b	79	Arnott & Hukins (1972)
NaDNA	IR dichroism	65	51	Pilet & Brahms (1972)

^{a} β and γ represents the Euler angles relative to the helical axis when the y axis is the bisector of the O=P-O⁻ bonds and the z axis is the vector between the nonesterified oxygen atoms of the phosphate group. ^{b} The value of β was recalculated on the basis of the A-form DNA model of Arnott & Hukins (1972). We obtain a value of γ_2 or $\beta = 77°$ rather than the value of 69° listed in Table 3 of Arnott & Hukins (1972).

can be described as follows: $\sigma_{max}^{\perp}$ strongly depends on angle β but not on γ; $\sigma_{min}^{\perp}$ is nearly independent of γ when β is near 0 but depends only on angle γ when β is near 90°. These characteristics are useful for understanding the shape of the shoulder in the spectrum when some variation in the orientation of the principal axis (i.e., of the phosphodiester) occurs.

By use of the three observed chemical shifts, $\sigma^{\|} = -25.7 \pm 3$, $\sigma_{min}^{\perp} = -59 \pm 5$ and $\sigma_{max}^{\perp} = 97 \pm 7$ ppm for poly(dA-dT)·poly(dA-dT), the corresponding contour lines within the corresponding error bands can be drawn as in Figure 7. The region in which all three bands intersect must yield the Euler angles β and γ. As is seen from Figure 7, there are four solutions between 0° and 180° for β and γ, of which only one (e.g., 70 ± 5° and 52 ± 5° for β and γ, respectively) is an independent solution due to the helical and dyad symmetries.

The principal axis system of the chemical shift tensor can be related to the atomic coordinates of the phosphodiester group as given by Kohler & Klein (1976). In this system [PAS I of Shindo et al. (1980)], the x axis (σ_{11}) is perpendicular to the plane made by the O-P-O⁻ bonds, the y axis (σ_{22}) is the bisector of the O=P-O⁻ bonds, and the z axis (σ_{33}) is perpendicular to the above two. Thus, the orientation of the phosphodiester group corresponds to that of the chemical shift tensor with the above principal axis system. Another possible principal axis system [PAS II of Shindo et al. (1980)] will be considered later.

It is interesting to compare the observed values with those obtained by other methods. Pilet & Brahms (1972) determined the orientation of the phosphodiester group in DNA from infrared dichroism by using the bands at 1240 and 1090 cm⁻¹ for the A form of DNA. They found that the orientations relative to the helical axis of the O···O vector and of the bisector of the O=P-O⁻ bonds were 65° and 45° which are equivalent to 65° and 51°, respectively, for β and γ in our notation. By use of the A-form atomic coordinates of DNA determined from the X-ray diffraction study of Fuller et al. (1965), Euler angles β and γ were calculated to be 65° and 60°, whereas the later revised model by Arnott & Hukins (1972) gives 77° and 79°, respectively, for these angles. All the orientation angles are listed in Table I.

The values from the NMR method are based on the data from poly(dA-dT)·poly(dA-dT) but not from natural DNA (which exhibited an unusual NMR pattern). The excellent agreement between these values and the values from infrared data indicates that the average conformation of the phosphodiester orientation in the A form of natural DNA may be

similar to that in the A form of poly(dA-dT)·poly(dA-dT).

Of the two structural models proposed for the A form of DNA, that of Fuller et al. (1965), gives the better agreement with the NMR data for the A form of poly(dA-dT)·poly(dA-dT). The influence of another, less likely, principal axis system on this conclusion was tested. The principal axis system which was determined from ^{31}P NMR data for a single crystal of barium diethyl phosphate by Herzfeld et al. (1978) was considered. This system (PAS II) results from a rotation of PAS I by 11° about an axis in the x–z plane (Shindo et al., 1980). By use of this principal axis system, the DNA model of Arnott and Hukins exhibits even further deviation from the NMR data (88.5° and 72.6° for β and γ), whereas the model of Fuller et al. (1965) gives better agreement (71.8° and 54.2°) than when PAS I is assumed.

In conclusion, the orientation of the phosphodiester group in DNA can be determined from ^{31}P NMR of DNA fibers with a high degree of molecular orientation, if the molecules are immobile and contain a limited number of discrete backbone conformations such that their chemical shifts can be resolved in the NMR spectra.

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Identification of the *cis*-Thymine Glycol Moiety in Oxidized Deoxyribonucleic Acid[†]

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ABSTRACT: 5,6-Dihydroxy-5,6-dihydrothymine (thymine glycol) is formed in DNA by reaction with oxidizing agents and as a result of ionizing and near-ultraviolet radiation. We describe a rapid purification of *cis*-5,6-dihydroxy-5,6-dihydrothymine and *cis*-5,6-dihydroxy-5,6-dihydrothymidine (*cis*-thymidine glycol) and their use as markers in identifying the thymine glycol moiety in oxidized DNA. Both glycols were prepared by oxidation of [^{14}C]thymine and -thymidine with $KMnO_4$ followed by purification on Sephadex LH-20 (LH-20). [^{3}H]DNA was oxidized with $KMnO_4$ and the thymidine glycol in DNA identified by enzymatic digestion of the DNA followed by cochromatography of the digest with marker [^{14}C]thymidine glycol on LH-20. The cis conformation of the glycol was confirmed by the change in the elution pattern when borate rather than water was used as eluent. Alkaline hydrolysis of a mixture of [^{14}C]thymine glycol and oxidized [^{3}H]DNA followed by trichloroacetic acid precipitation and LH-20 chromatographic analysis of the neutralized supernatant yielded a complex pattern of radioactive degradation products with coincidence of one ^{14}C marker- and one [^{3}H]-DNA-derived peak. All applied radioactivity was recovered. This methodology should be useful in determining thymine glycol content of irradiated DNA and in elucidating the mechanism by which these altered residues are removed from cellular DNA by repair enzymes.

Ionizing and near-ultraviolet irradiation of DNA causes modification of thymine with the formation of saturated ring compounds (Hariharan & Cerutti, 1972, 1977). These modified thymines may be the major form of DNA base damage caused by ionizing radiation, and data exist which indicate that such modified thymines are removed from cellular DNA by an active enzymatic repair process (Hariharan & Cerutti, 1971; Mattern et al., 1973; Mattern & Welch, 1979; Painter & Young, 1972). Support for the latter hypothesis stems from reports that DNA which was exposed to ionizing radiation in vitro was attacked by DNA endonuclease activities which did not attack undamaged DNA (Bacchetti & Benne, 1975; Brent, 1973; Strniste & Wallace, 1975). Ring-saturated modified thymines can also be introduced into DNA by oxidation with osmium tetroxide (OsO_4) and potassium permanganate ($KMnO_4$) (Iida & Hayatsu, 1971; Hariharan & Cerutti, 1974; Hariharan et al., 1977), and poly(dA-dT) and DNA oxidized by OsO_4 were attacked by the same endonuclease activities which acted upon irradiated DNA (Gates & Linn, 1977; Hariharan & Cerutti, 1974; Nes & Nissen-Meyer, 1978). Therefore, it is possible that some, if not all, of the ring-saturated thymines are substrates for the enzymatic initiation of DNA excision–repair. Oxidation of DNA by OsO_4 and $KMnO_4$ is reported to yield only *cis*-5,6-dihydroxy-5,6-dihydrothymine (*cis*-thymine glycol, TG)[1] (Burton & Riley, 1966; Iida & Hayatsu, 1971; Hariharan et al., 1977). Thus, it is most probable that this particular modified thymine in DNA is acted upon by a repair *N*-glycosylase and/or DNA endonuclease.

The existing method for identifying TG in DNA is an alkali–acid treatment which recovers only about 20% of the ring-saturated modified thymines and does not distinguish between TG and other ring-saturated thymines (Hariharan & Cerutti, 1974). We have developed a method for the rapid purification of both *cis*-thymine and -thymidine glycols and have used them as markers in the analysis of chemical and enzymatic hydrolysates of oxidized DNA.

Experimental Procedures

Materials

[*methyl*-^{14}C]Thymine (49.6 mCi/mmol), [*methyl*-^{14}C]-thymidine (48.2 mCi/mmol), and [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Sephadex LH-20 and Sephadex G-50 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. *E. coli* W 3110 (*thy*–) J. Cairns strain was obtained from the *E. coli* Genetic Stock Center at Yale University School of Medicine, New Haven, CT.

Methods

Chromatography on Sephadex LH-20 (LH-20). All assays, unless otherwise specified, requiring chromatography on LH-20 were performed on 1.5 × 25 cm columns with either water or 0.05 M sodium borate titrated with boric acid to pH 8.6 as eluents. These columns will be referred to as water and borate LH-20 columns. Fractions of 1 mL (1 mL/min) were collected in liquid scintillation vials, unless absorbance of the samples was to be determined. Samples were counted in 20%

[1] Abbreviations used: thymine glycol (TG), 5,6-dihydroxy-5,6-di-hydrothymine; thymidine glycol, 5,6-dihydroxy-5,6-dihydrothymidine; BSA, bovine serum albumin; Cl_3CCOOH, trichloroacetic acid; NMR, nuclear magnetic resonance; IR, infrared; UV, ultraviolet; TLC, thin-layer chromatography; high-pressure LC, high-pressure liquid chromatography; LH-20, Sephadex LH-20.