Infrared Linear Dichroism Reveals That A-, B-, and C-DNAs in Films Have Bases Highly Inclined from Perpendicular to the Helix Axis†

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ABSTRACT: Infrared linear dichroism has been employed to investigate the inclination of the bases in films of poly[d(AC)]·poly[d(GT)], poly[d(AG)]·poly[d(CT)], and natural DNAs (from *Escherichia coli* and calf thymus). All DNAs investigated assume the B-form at high (>94%) relative humidity. Poly-[d(AC)]·poly[d(GT)], *E.coli* DNA, and calf thymus DNA assume the A-form at low (75%) relative humidity, whereas poly[d(AG)]·poly[d(CT)] assumes the C-form at low (66%) relative humidity. Infrared linear dichroism demonstrates that the bases for DNA in films are highly inclined from perpendicular to the helix axis, even for B-DNA. C-DNA has almost same inclinations as in B-DNA, and the inclinations are slightly increased in A-DNA. These inclination angles confirm our earlier UV linear dichroism results for the orientation of the bases for DNA in solution. Infrared linear dichroism has also been used to obtain conformational angles for the phosphodiester backbone geometry of the A-, B-, and C-forms of DNA.

The recent development of techniques for analyzing solution structures of biopolymers, such as optical spectroscopy, FTIR, and NMR spectroscopy, reveals the structural details of nucleic acids in aqueous solution, which resembles the real environment in the interior of living cells. But despite progress in studying nucleic acid structure in the solution state as well as in solid state, there is still uncertainty about the molecular structure of DNA. This is due mainly to the polymorphic nature of DNA, since the structure depends on the sequence (Bram & Tougard, 1972; Leslie et al., 1980; Dickerson & Drew, 1981; Sarai et al., 1988), solvent (Girod et al., 1973; Ivanov et al., 1973; Pohl, 1976), and salt (Fuller & Wilkins, 1965; Bram & Tougard, 1972; Ivanov et al., 1973; Leslie et al., 1980). One feature of the molecular structure of a nucleic acid is the inclination of the base pairs in the double-helical structure of DNA. This knowledge about base orientation in DNA is required to understand many biological processes such as DNA–protein, DNA–ligand, or DNA–drug interactions.

Linear dichroism (LD) is a useful technique to get information about the inclination of chromophores in biopolymers. Many groups have used electric or flow linear dichroism in the ultraviolet (UV) to measure the inclination angle of the bases in free DNA with respect to the helix axis (Charney & Milstien, 1978; Hogan et al., 1978; Charney & Yamaoka, 1982; Charney et al., 1986; Clack & Gray, 1992). A more detailed review of this work can be found in Chou and Johnson (1993). Measurements have also been made on DNA complexed with ligands or drugs (Sen et al., 1986; Hard, 1987; Schurr & Fujimoto, 1988; van Amerongen et al., 1990; Norden et al., 1992). Our laboratory has used vacuum UV flow LD (UV LD) to extract inclination angles for individual bases in the A-, B-, and Z-forms of synthetic polynucleotides (Causley & Johnson, 1982; Edmondson & Johnson, 1985a, 1986) and natural DNAs (Dougherty et al., 1983; Edmondson & Johnson, 1985b) in solution. Specific inclinations have been determined for the bases in natural and synthetic polynucleotides containing four different types of bases using a sophisticated algorithm recently developed in our laboratory (Chou & Johnson, 1993; Kang & Johnson, 1993). Recently Cheng et al. (1992) and Nibedita et al. (1993) have reported B-DNA structures with bases rather highly tilted from the helical axis using NMR measurements and distance geometry methods. The results obtained from all these studies indicate that the bases are more highly inclined from perpendicular to the helical axis in solution than is found in fibers or crystals, especially for B-DNA. In addition to these experimental reports, many workers (Levitt, 1978; Zhurkin et al., 1978; Singh et al., 1985; Rao et al., 1986; Edmondson, 1987; Ansevin & Wang, 1990; Srinivasan et al., 1990; Swaminathan et al., 1991) have used energy minimization, molecular mechanics, or molecular dynamics for modeling DNA structures and have reported energetically favorable B-DNA models with base pairs highly inclined from perpendicular to the helical axis.

With the development of Fourier transform interferometers and special software that enables rapid and accurate data collection and management, infrared spectroscopy is becoming one of the most useful techniques to probe the molecular structure of nucleic acids in aqueous solution, as well as in gels or in films. Various synthetic polynucleotides and natural DNAs have been investigated to obtain specific marker bands for A-, B-, C-, D-, and Z-forms of DNA [Taillandier and Liquier (1992) and references therein]. By using oriented samples and polarized light, it is also possible to get information about the orientation of the transition dipoles for carbonyl bond stretching and ring stretching vibrational modes in the base plane, which, in turn, is related to the inclination of the bases. Baret et al. (1978) and Flemming et al. (1988) have used infrared LD (IR LD) to extract inclination angles for bases in natural DNA and poly[d(AT)]·poly[d(AT)] in films and reported high inclination angles for both A- and B-DNA.

In this study we apply IR LD measurements to synthetic polynucleotides poly[d(AC)]·poly[d(GT)] and poly-[d(AG)]·poly[d(CT)] and natural DNAs (from *E. coli* and calf thymus) in oriented films to extract inclination angles for the bases in the A, B-, and C-forms. We compare the IR LD results with our earlier UV LD results about base inclination in aqueous solution (Chou & Johnson, 1993; Kang & Johnson, 1993). In addition, since infrared spectroscopy is a very sensitive tool for monitoring the phosphodiester backbone

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geometry of nucleic acids, we also use IR LD to extract conformational angles of the phosphodiester backbone for A-, B-, and C-forms of these DNAs. The inclination of the bases in these DNAs using the LD of vibrational transitions reported here supports our earlier reports using ultraviolet transitions and gives a more solid basis for understanding base inclinations for DNA in the interior of living cells.

MATERIALS AND METHODS

Sample Preparation. Poly[d(AC)]·poly[d(GT)] and poly-[d(AG)]·poly[d(CT)] were purchased from Pharmacia and used without further purification. Natural DNAs (calf thymus DNA from Pharmacia, and *Escherichia coli* DNA from Sigma Co.) were treated with phenol/chloroform to remove residual protein and were ethanol-precipitated. About 1 mg of each DNA was dissolved in doubly distilled water. After 24 h at 4 °C to ensure the formation of a homogeneous solution, the sample was washed several times with doubly distilled water using a Centricon-10 microconcentrator (Amicon Co.), lyophilized, and redissolved in a small amount of doubly distilled water to yield a viscous gel of DNA. Poly[d(AG)]·poly-[d(CT)] was treated similarly, except 1 mM sodium phosphate buffer, pH 8.0, was used to avoid the formation of a triple helix. A representative sample of each DNA was dialyzed against 0.5 M NaCl and 10 mM EDTA (pH 8.0) for 24 h to remove multivalent cations (Devarajan & Shafer, 1986). This special treatment did not affect any spectroscopic results.

Gels of the DNA samples were then oriented on a calcium fluoride or a KRS-5 window by manual unidirectional stroking until dry, as described previously (Bradbury et al., 1961; Pilet & Brahms, 1973; Pilet et al., 1975; Kursar & Holzwarth, 1976; Baret et al., 1978). The salt content of the sample was controlled by previously described methods (Rupprecht & Forslind, 1970; Pilet & Brahms, 1973; Pilet et al., 1975) that equilibrate an oriented film in a 73% ethanol–water solution containing 0.1 M NaCl for 2–3 days at 4 °C. This procedure gives samples with a salt content ranging from 3% to 4% NaCl (w/w).

Infrared Measurements. The windows were placed in a sealed chamber under controlled relative humidity (r.h.), which was governed by a saturated salt solution in either H_2O or D_2O (Bradbury et al., 1961; Pilet & Brahms, 1973; Pilet et al., 1975; Kursar & Holzwarth, 1976; Baret et al., 1978). All salts used were reagent grade, and D_2O (99.9%) was from Aldrich. The hydration of the film was determined by monitoring the 3420-cm^{-1} band, which is due to the water in the film. To ensure that each film is equilibrated for sufficient time at each hydration level required to cause the desired transition, a slow, stepwise dehydration–rehydration protocol was used as described by Keller and Hartman (1986). Each r.h. value was equilibrated for 24 h before spectra were recorded. The B-, A-, and C-forms of DNA were obtained at 94% r.h. (K_2SO_4), 75% r.h. (NaCl), and 66% r.h. ($NaNO_2$), respectively. Sometimes heating the film at 45 °C for 3 h with the humidity controlled by a saturated NaCl solution was required to induce the B- to A-form transition. This treatment introduces no denaturation as measured by the intensity of the 1710-cm^{-1} band.

Infrared spectra were measured on a Nicolet 510P Fourier transform infrared spectrometer equipped with a triglycine sulfate (TGS) detector. About 100–200 scans were collected at a 4 cm^{-1} resolution (with 2-cm^{-1} intervals), and the resulting interferogram was Fourier transformed using a Happ-Genzel apodization function. The dichroic spectra were recorded for the oriented samples with the electric vector of light polarized parallel and perpendicular to the orientation axis, which was achieved by rotating a wire-grid silver bromide polarizer (Cambridge Physical Sciences) through 90°. Nonoriented samples showed no dichroic ratio. The dichroic ratio of the oriented film was independent of how the film was rotated in the spectrometer, indicating that we were not measuring artifacts.

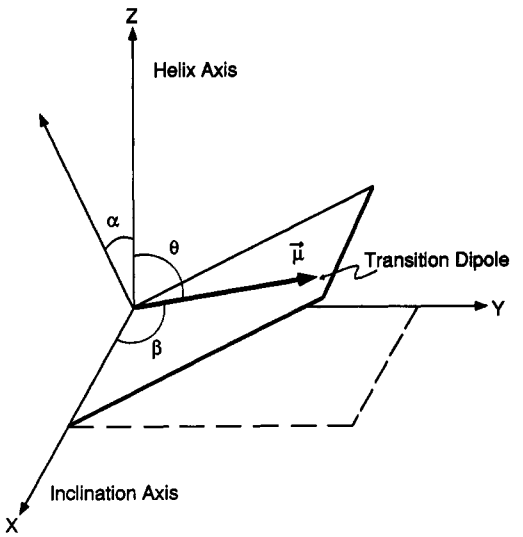

FIGURE 1: Diagram showing definitions of angles θ, α, and β. α is the angle between the base normal and the helix axis, β is the angle between the inclination axis and the transition dipole, and θ is the angle between the transition dipole and the helix axis.

Theoretical. For a partially oriented sample, which may be regarded as containing a fraction S of perfectly oriented material and a fraction $(1-S)$ of randomly oriented material, the dichroic ratio (R) of each band is related by Fraser's formula (Fraser, 1953):

$$R = A_\perp/A_\parallel = \frac{(1/2)S\sin^2\theta + (1/3)(1-S)}{S\cos^2\theta + (1/3)(1-S)} \quad (1)$$

where $A_\perp$ and $A_\parallel$ are the absorption of polarized radiation with the electric vector perpendicular and parallel to the orientation axis, and θ is the angle between the transition dipole of the vibrational mode and the orientation axis of the sample. This θ angle depends both on the direction of transition dipole within the base plane relative to the axis around which the base inclines (β) and on the angle of inclination for the base with respect to the helix axis (α) (Figure 1). The factor S depends on the macroscopic orientation of the nucleic acid, and since this is separable from the microscopic orientation of the bases defined by θ, the effect of any tertiary structure will reside in S (Norden, 1978; Charney, 1988).

In order to determine θ from the measured R value, the orientation parameter of the sample, S, has to be estimated. It is generally assumed that the θ of the vibrational band that has the largest dichroic ratio is 90°, and S is then estimated from the measured R value of that vibrational band. If the θ of that band is less than 90°, then the α values will be underestimated. The band at about 1710 cm^{-1} or the band at about 1526 cm^{-1} for the films hydrated with H_2O (Pilet & Brahms, 1973; Pilet et al., 1975; Flemming et al., 1988) and the band at about 1696 or 1672 cm^{-1} for the films hydrated with D_2O (Bradbury et al., 1961; Baret et al., 1978) were used to estimate S. Once S is known for a particular sample, we can calculate θ for other absorption bands using eq 1. If the bases are inclined, θ will be 90° only if the transition dipole of the most dichroic band is lying on the axis around which the base is inclined. The axis of inclination in the base plane, deduced from our earlier vacuum UV LD measurements, is

only about 10° different from the dipole directions expected for these transitions, indicating that the assumption is reasonable. Although we correct for this difference, the correction is very small.

Data Analysis. Measured spectra were smoothed using a Savitzky–Golay algorithm with 9 points, and the base line due to the calcium fluoride or KRS-5 windows was subtracted. All numerical manipulations and data treatment used the software developed by Nicolet Instrument Co. For the phosphodiester backbone orientation, the absorbances of the antisymmetric and symmetric stretching bands of the phosphate (PO_2^-) group at about 1230 and 1090 cm^{-1}, respectively, are well separated, and their strength was simply determined by integrating each band.

The in-plane stretching bands of the DNA bases in the range of 1750–1500 cm^{-1} exhibit a rather complex band contour, especially around 1650 cm^{-1}, which includes the water absorption. Therefore, we collected the spectra of the DNA samples under D_2O vapor saturated with the appropriate salt to avoid the interference of the water absorption. These overlapping bands should be separated into individual bands to get a reasonable band intensity and a reliable dichroic ratio for each band. Several band-narrowing procedures, such as the Fourier self-deconvolution (FSD), the derivative method, and curve fitting, can be used to enhance the resolution of the observed spectrum. For quantitative results, curve fitting of the original band contour has been suggested by many workers (Maddams, 1980; Arrondo et al., 1993; Lamba et al., 1993; Surewicz et al., 1993). Here, we decomposed the overlapping bands by means of a least-squares curve fitting program based on the Levenberg–Marquardt algorithm (Brown & Dennis, 1972). We used a sum of the Lorentzian and the Gaussian band shapes to represent the observed spectrum. The fitting program was written to vary the fraction of both band shapes to give the best fit. To get the number of bands and their positions for the initial input into the fitting program, the second derivative and FSD algorithms (Nicolet Instrument Co.) with a half-width of 18 cm^{-1} and k values between 1.2 and 1.5 were applied to the original spectrum. The fraction of the two band shapes was set to 0.5, and the width and the intensity of each band were estimated visually from the Fourier self-deconvoluted spectra for the initial input parameters. From these initial estimates for band parameters, the fitting algorithm iteratively generates a sequence of approximations toward the minimum sum of squares error. The fitting was stopped at the point where the variances of each parameter were stable, and the resulting integrated intensity was used to calculate the dichroic ratio (R) of individual vibrational bands. This curve fitting procedure was repeated several times with the slightly modified initial input parameters to make sure our band decomposition was reproducible.

RESULTS AND DISCUSSION

Characterization of the A-, B-, and C-Forms. Figure 2 gives representive normal FTIR spectra of poly[d(AC)]·poly[d(GT)] and poly[d(AG)]·poly[d(CT)] in films hydrated with H_2O at high and low relative humidity (r.h.). The characteristic marker bands for each form of DNA are indicated in the figure and are summarized in Table 1 with their proposed assignments. The spectra of *E. coli* and calf thymus DNA are quite similar to those of natural DNA reported previously (Tsuboi, 1969; Taillandier et al., 1985; Keller & Hartman, 1986; Liquier et al., 1990) (data not shown), and the characteristic marker bands are summarized in Table 1. The spectra of poly[d(AC)]·poly[d(GT)] at high and low r.h. are quite similar to those reported previously (Taillandier et al., 1984; Taboury & Taillandier, 1985). The spectra of all polynucleotides tested at high (>94%) r.h. have absorption bands at 1422, 1329, and 1281 cm^{-1}, characteristic of C2′-endo sugar pucker for B-DNA (Taillandier et al., 1984; Adam et al., 1986; Taillandier & Liquier, 1992). Several marker bands for B-DNA are also observed at 968, 893, and 833 cm^{-1}. The position of strong absorption bands at 1223 cm^{-1} due to the antisymmetric stretching mode of the phosphate (PO_2^-) groups also suggests these DNAs assume the B-form at high (>94%) r.h.

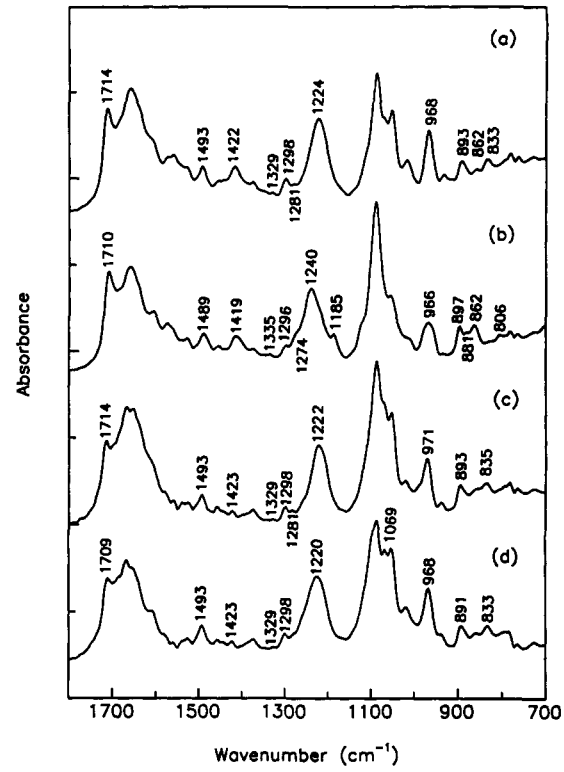

FIGURE 2: Infrared spectra of (a) poly[d(AC)]·poly[d(GT)] in the B form at 94% r.h., (b) poly[d(AC)]·poly[d(GT)] in the A-form at 75% r.h., (c) poly[d(AG)]·poly[d(CT)] in the B-form at 94% r.h., and (d) poly[d(AG)]·poly[d(CT)] in the C-form at 66% r.h. Characteristic marker bands are indicated by their wavenumbers.

A slow, stepwise dehydration–rehydration protocol was used to induce the B- to A-form transition for each DNA, as suggested by Keller and Hartman (1986). For this purpose, K_2SO_4, KCl, $(NH_4)_2SO_4$, NaCl, $NaNO_2$, and NaBr were used to achieve 94%, 86%, 80%, 75%, 66%, and 56% r.h., respectively. It is evident from Figure 2 and Table 1 that poly[d(AC)]·poly[d(GT)], *E. coli* DNA, and calf thymus DNA equilibrated at 75% r.h. have several A-DNA marker bands at 1489, 1335, and 1274 cm^{-1}, characteristic of C3′-endo sugar pucker (Taillandier et al., 1984; Adam et al., 1986; Taillandier & Liquier, 1992). Several A-DNA marker bands are also observed at 966, 897, 881, 862, and 806 cm^{-1}. The shift of the strong absorption band due to the antisymmetric stretching mode of the PO_2^- groups from 1223 to 1239 cm^{-1} and the appearance of a new band at 1185 cm^{-1} also indicate that these DNAs assume the A-form at 75% r.h. In the spectral region due to in-plane double-bond stretching of the bases, characteristic bands for the B- and A-forms are observed at 1714 and 1710 cm^{-1}, respectively, for all DNAs hydrated with H_2O.

In addition to these specific marker bands for each form of DNA, the B- and A-forms of DNA can be characterized by measuring dichroic spectra of an oriented sample. B-DNA is characterized by an almost zero dichroic ratio for the band at 1223 cm^{-1} and a highly perpendicular dichroic ratio for the

Table 1: IR Absorption Bands (cm^{-1}) Observed for Different DNA Conformations in H_2O[a]

poly[d(AC)]·poly[d(GT)]		poly[d(AG)]·poly[d(CT)]		natural DNA		
B-form	A-form	B-form	C-form	B-form	A-form	assignment
1714	1710	1714	1709	1714	1710	in-plane
1493	1489	1493	1493	1493	1490	
1456	1455	1458	1458	1457	1457	
1422		1423	1423	1422		C2′-endo/anti
	1419				1419	C3′-endo/anti
1375	1373	1374	1374	1374	1374	purine/anti
	1335				1335	dA,dT C3-endo/anti
1329		1329	1329	1329		dT C2′-endo/anti
1298	1296	1298	1298	1298	1296	
1281		1281		1281		dT C2′-endo/anti
	1274				1274	dT C3′-endo/anti
1224	1240	1222	1220	1224	1238	antisymmetric PO_2^-
	1185				1185	
1088	1088	1087	1088	1086	1086	symmetric PO_2^-
			1069			
1052	1053	1052	1053	1053	1053	
968	966	971	968	968	966	deoxyribose
893	897	893	891	893	897	deoxyribose
	881				881	
862	862	862		862	862	deoxyribose, backbone
833		835	833	837		backbone
	806				805	backbone

[a] B-, A-, and C-DNA were obtained at 94%, 75%, and 66%, r.h., respectively.

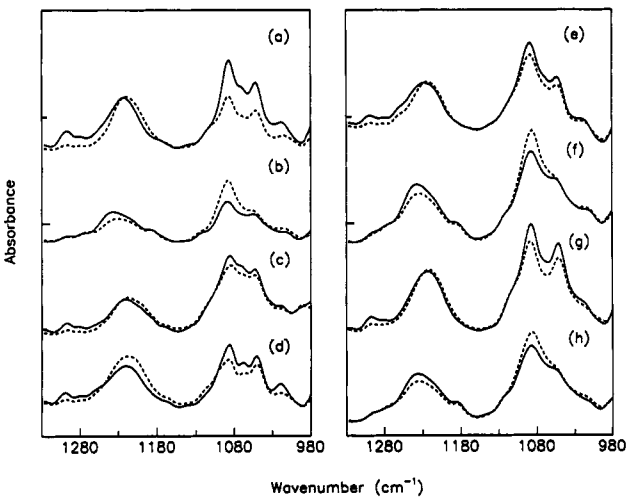

FIGURE 3: Dichroic spectra in the PO_2^- stretching vibration region for the oriented DNA samples measured with the electric vector of the polarized light perpendicular (—) and parallel (···) to the helix axis. (a) B-form of poly[d(AC)]·poly[d(GT)], (b) A-form of poly-[d(AC)]·poly[d(GT)], (c) B-form of poly[d(AG)]·poly[d(CT)], (d) C-form of poly[d(AG)]·poly[d(CT)], (e) B-form of calf thymus DNA, (f) A-form of calf thymus DNA, (g) B-form of *E. coli* DNA, and (h) A-form of *E. coli* DNA. B-, A-, and C-DNAs are obtained at 94%, 75%, and 66% r.h., respectively.

band at 1087 cm^{-1}. In contrast, A-DNA shows a perpendicular dichroic ratio at 1239 cm^{-1} and a parallel dichroic ratio at 1086 cm^{-1} (Bradbury et al., 1961; Pilet & Brahms, 1973; Pilet et al., 1975; Kursar & Holzwarth, 1976; Baret et al., 1978). Figure 3 shows the dichroic spectra of oriented DNA samples hydrated with H_2O in the spectral region of the PO_2^- vibration. Poly[d(AC)]·poly[d(GT)], poly[d(AG)]·poly[d(CT)], and natural DNAs at 94% r.h. show an almost zero dichroic ratio for the antisymmetric stretching band of PO_2^- at 1223 cm^{-1} and a strong perpendicular dichroic ratio for the symmetric stretching band of PO_2^- at 1087 cm^{-1}. These dichroic ratios are reversed for poly[d(AC)]·poly[d(GT)] and natural DNAs at 75% r.h., but the spectrum of poly[d(AG)]·poly[d(CT)] at low r.h. has a parallel dichroic ratio for the 1223 cm^{-1} band and a strong perpendicular dichroic ratio for the 1087-cm^{-1} band of the PO_2^- groups.

Spectra of oriented DNA with a parallel dichroic ratio for the antisymmetric stretching band and a strong perpendicular dichroic ratio for the symmetric stretching band for PO_2^- groups are believed to characterize C-DNA (Brahms et al., 1973; Fritzsche et al., 1976; Taillandier & Liquier, 1992). Usually, the C-form of DNA is produced under specific conditions such as high Li^+ salt and low relative humidity (Marvin et al., 1961; Brahms et al., 1973; Loprete & Hartman, 1989). To decide whether our parallel dichroic ratio observed for the antisymmetric phosphate stretching band in the oriented film of poly[d(AG)]·poly[d(CT)] at 66% r.h. indicates the C-form, we carefully investigated other regions of the infrared spectra. Our FTIR spectra for poly[d(AC)]·poly[d(GT)] and natural DNA hydrated with D_2O vapor at high and low r.h. have a peak at 1675 and 1682 cm^{-1}, respectively, characteristic of B- and A-DNA, but do not have a band at 1660 cm^{-1} characteristic of C-DNA (Loprete & Hartman, 1989). In contrast, for poly[d(AG)]·poly[d(CT)] hydrated with D_2O the peak at 1673 cm^{-1} is shifted to 1663 cm^{-1} by lowering the r.h., indicating a B to C transition (data not shown). The shifts of bands at 1714 to 1709 cm^{-1}, 1222 to 1220 cm^{-1}, 971 to 968 cm^{-1}, 893 to 891 cm^{-1}, and 835 to 833 cm^{-1} for poly-[d(AG)]·poly[d(CT)] hydrated with H_2O (Figure 2) also indicate that this polynucleotide undergoes B → C transition by decreasing r.h. (Loprete & Hartman, 1989). We also observed the shape of the band due to the symmetric phosphate stretching that represents the C-form; that is, it does not show a weak shoulder at 1071 cm^{-1} but has a sharp, well-separated band at 1069 cm^{-1} indicative of C-DNA (Loprete & Hartman, 1989). We tried extensively to produce the A-form of poly-[d(AG)]·poly[d(CT)] by adjusting salt and r.h., but failed. This is consistent with Leslie and Arnott (1980), who have reported that poly[d(AG)]·poly[d(CT)] in fibers cannot undergo a B to A transition, but instead undergoes a B to C transition by lowering the r.h.

Clearly, poly[d(AC)]·poly[d(GT)], *E. coli* DNA, and calf thymus DNA assume the B- and A-forms at high (>94%) and low (75%) r.h., respectively, and poly[d(AG)]·poly[d(CT)] assumes the B- and C-forms at high (>94%) and low (66%) r.h., respectively. All of these transitions were fully reversible upon rehydration.

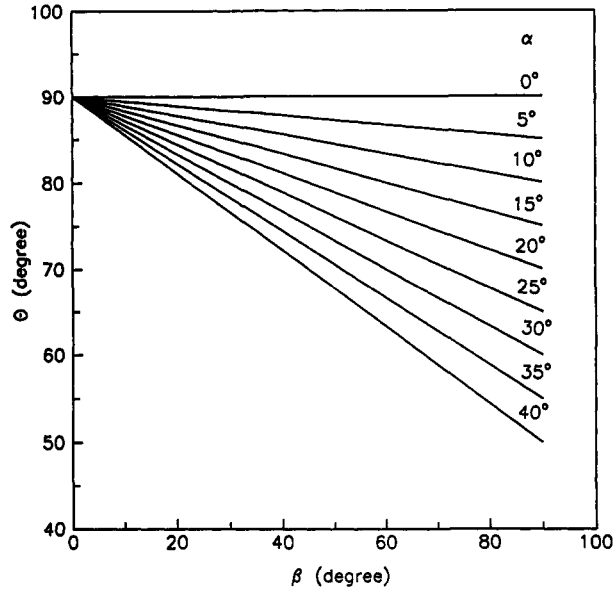

FIGURE 4: Dependence of the θ angle on both the base inclination (α) and the relative angle between the transition dipole and the axis of inclination (β).

Estimation of the Orientation Parameter. In eq 1 there are two unknowns, θ and S. In order to determine θ, it is necessary to estimate S. This is possible by using an appropriate intense and well-separated band for which the direction of the transition dipole of that vibrational mode is known from the results of other methods. The in-plane stretching bands of the DNA bases are spread over the wavenumber range of 1750–1500 cm^{-1}. The band with maximum value of dichroic ratio (R) is usually assumed to have $\theta = 90°$ and used to estimate S. For this purpose, a band at about 1710 or 1526 cm^{-1}, and in the deuterated form at about 1696 or 1672 cm^{-1}, is chosen (Bradbury et al., 1961; Pilet & Brahms, 1973; Pilet et al., 1975; Baret et al., 1978; Flemming et al., 1988).

In dichroic spectra for our oriented DNA samples, the most dichroic band was observed at about 1710 cm^{-1} in H_2O and at about 1696 cm^{-1} in D_2O. We assign this band to the C4=O bond stretching of thymine. The band at about 1710 cm^{-1} in H_2O or at about 1696 cm^{-1} in D_2O was previously assigned to the C2=O bond stretching vibration of thymine (Tsuboi et al., 1973; Kursar & Holzwarth, 1976; Baret et al, 1978). However, Ovaska et al. (1984) used a fixed partial charge model to calculate the transition dipole directions for the double-bond region vibrations of pyrimidine bases and reported that the band at about 1670 cm^{-1} is mainly the C2=O stretching, and the band at about 1710 cm^{-1} is mainly due to C4=O stretching of thymine. In addition, our earlier UV LD results (Kang & Johnson, 1993; Chou & Johnson, 1993) showed that the axis of inclination for the thymine base lies in the same direction as the transition dipole of the C4=O bond stretching vibration. Since this band has the largest dichroic ratio we expect its transition dipole to be in the same direction as the axis of inclination, confirming the assignment of the band at about 1710 cm^{-1} to the C4=O bond stretching vibration rather than C2=O bond stretching vibration of thymine.

Many authors have assumed the θ angle for the most dichroic IR band to be 90° when estimating the orientation parameter S. However, since θ depends on both the inclination of base and the direction of transition dipole in the base plane (Flemming et al., 1988), this assumption is really valid only if the transition dipole direction of the most dichroic band is collinear with the axis around which the bases are inclining. Figure 4 represents the dependence of θ on the inclination of the base, α, and direction of transition dipole relative to the axis of inclination in the base plane, β. The directions of vibrational transition dipoles for the pyrimidine bases were calculated by Ovaska et al. (1984), and the axes of inclination for each base are known from our UV LD measurements (Kang & Johnson, 1993; Chou & Johnson, 1993). It is obvious from Figure 4 that θ is more affected by the relative direction of the transition dipole with respect to the axis of inclination for more highly inclined bases. Our UV LD results indicate that the average angles of inclination for thymine bases are about 29 ± 4° and 37 ± 2° for B- and A-form, respectively, of synthetic polynucleotides and natural DNAs in solution, and the inclination axis relative to the vector N1–C4 are about 41 ± 10° for both the B- and A-forms of DNA. Since the direction of the transition dipole for the thymine C4=O bond stretching at about 1710 cm^{-1} was estimated to be about 245° (Flemming et al., 1988), based on the fixed partial charge model approximation (Ovaska et al., 1984), the relative angle β between these two vectors is about 24°. This corresponds to a θ of 82° and 79° for B- and A-forms of DNA, respectively (Figure 4).

From these considerations, we use 82° and 79° for the θ of the most dichroic band in B- and A-DNA, respectively, to estimate S. It is interesting to note that the θ values for the most dichroic band used to estimate S are comparable with those values, 84° for B-DNA and 76° for A-DNA, calculated from the energetically favored tilt and propeller twist angles in the several conformations of DNA for the θ of transition dipole of the C2=O bond stretching of thymine (Pohle et al., 1984). However, these small differences do not significantly affect the final inclination angles, since only 1–2° changes in the final inclination angles result from the variation of 90–80° for the θ of the most dichroic band.

Decomposition of Overlapping Bands. The infrared spectrum of a DNA film in the range of 1750–1500 cm^{-1} contains several overlapping bands due to in-plane stretching of carbonyl bonds and ring stretching of the bases, and the transition dipole direction for each vibrational mode gives an individual measure of the inclination angle of bases. These overlapping bands should be decomposed into individual bands to obtain quantitative intensities and reliable dichroic ratios for each band. Figures 5 and 6 represent the examples of band decomposition for a poly[d(AC)]·poly[d(GT)] film hydrated with D_2O at high (94%) r.h. and a poly[d(AG)]·poly[d(CT)] film hydrated with D_2O at low (66%) r.h. in the range of 1750–1500 cm^{-1}. We used a sum of the Lorentzian and the Gaussian band shape to represent each band. Usually, either the Lorentzian or the Gaussian band shape has been used to decompose the overlapping bands of infrared spectrum (Maddams, 1980; Arrondo et al., 1993; Lamba et al., 1993; Surewicz et al., 1993). However, reports by Maddams (1980) and Pitha and Jones (1966, 1967) showed that combined use of the Lorentzian and the Gaussian band shape gave better results to curve fitting of infrared data. Therefore, we tried to decompose the observed spectra using either the Lorentzian or the Gaussian band shape, or using both band shapes. We confirmed that combined use of both band shapes resulted in better fit to the observed spectrum (data not shown).

To estimate the most important values for the input parameters to the curve fitting routine (the number of bands and their positions), second derivative and FSD algorithms with a half-width of 18 cm^{-1} and k values between 1.2 and 1.5 were applied to the measured spectrum. The horizontal base line was set with the absorption value at the highest wavenumber. None of the input parameters (position, width, intensity, and fraction of band shape) were kept constant during the curve fitting procedure.

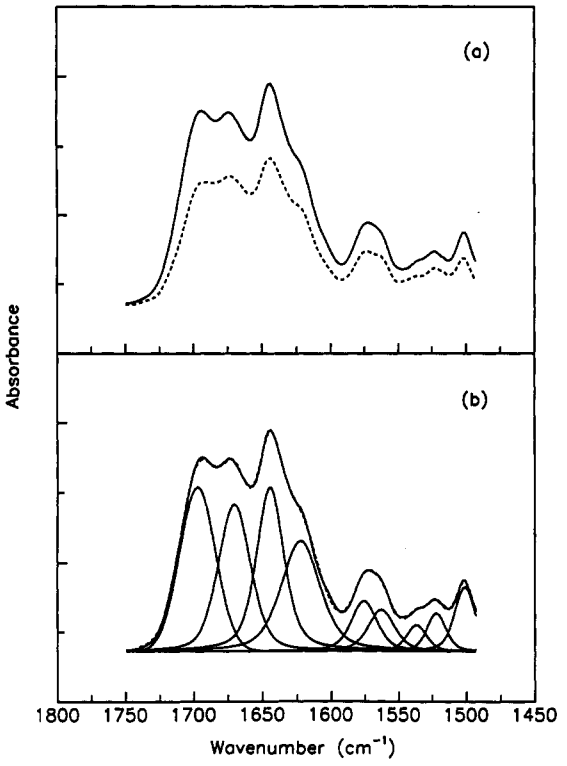

FIGURE 5: (a) Dichroic spectra for the in-plane double-bond stretching region for an oriented poly[d(AC)]·poly[d(GT)] film hydrated with D_2O at 94% r.h. The electric vector of the light polarized is perpendicular (—) and parallel (···) to the helical axis. (b) Decomposition of overlapping absorption bands (—) with sum of individual bands (···). A sum of the Lorentzian and the Gaussian band contour is used to represent the observed band shape.

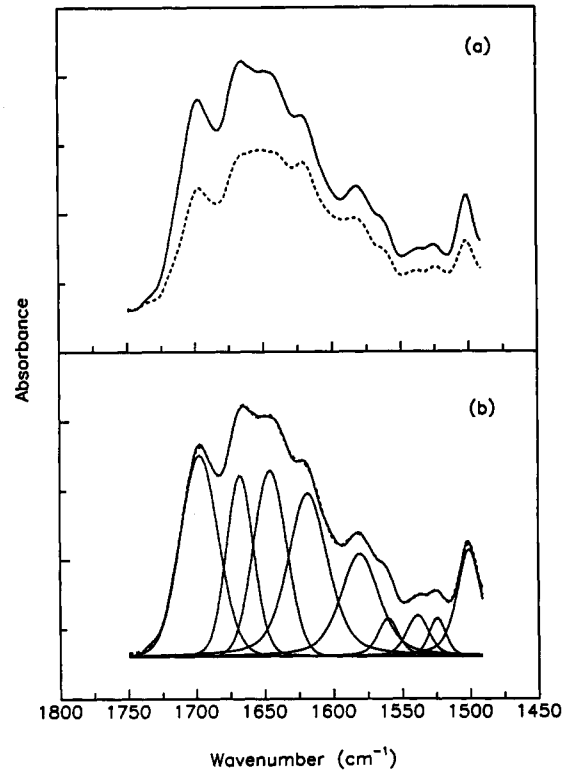

FIGURE 6: (a) Dichroic spectra and (b) decomposition of overlapping bands for an orientedpoly[d(AG)]·poly[d(CT)] film hydrated with D_2O at 66% r.h. The conditions of measurements and decomposition are the same as those in Figure 5.

As represented in Figures 5 and 6, in all cases the infrared spectra in the range of 1750–1500 cm^{-1} for DNA samples hydrated with D_2O are decomposed into 9 bands, each of which shows a definite inflection in the measured spectrum. The positions of each band after separate curve fitting for each spectrum were almost the same as the values of the parameters estimated from the original spectra using the second derivative and FSD algorithms with the fraction of each band shape varied randomly between 0 and 1. In all cases, the fittings were extremely good as judged by the very low sum of square error.

Although it is not necessary to assign each vibrational mode to the specific transition dipoles, we try to assign the decomposed bands of IR spectra for synthetic polynucleotides and natural DNAs to known vibrational modes in the base plane. However, because of the proximity of the peak positions for the in-plane stretching modes of DNA bases in the DNA samples with four different types of bases, it is not easy to assign individual absorption bands to specific C=O bond stretching or ring stretching vibrations. We used the reported assignments of vibrational modes for purine and pyrimidine bases (Tsuboi et al., 1962; Howard & Miles, 1965; Kyogoku et al., 1967; Ovaska et al., 1984), and for simpler polynucleotides such as poly[d(A)], poly[d(G)], poly[d(A)]·poly[d(T)], and poly[d(G)]·poly[d(C)] (Miles, 1964; Miles & Frazier, 1964; Howard et al., 1969; Baret et al., 1978). We tentatively assign the 1696-cm^{-1} band to the C4=O thymine stretching mode and the band at 1671 cm^{-1} to both the C2=O thymine and C6=O guanine stretching modes. The band at 1644 cm^{-1} is assigned to both the C2=O cytosine and C=C thymine stretching modes, and the band at 1622 cm^{-1} to the C=C and C=N double-bond stretching modes for adenine and cytosine. Finally, the bands at 1575 and 1561 cm^{-1} are assigned to the ring stretching vibration of adenine, guanine, and cytosine. Assignments of the bands below 1550 cm^{-1} are very difficult, as it is believed that the base and sugar units give rise to many bands here; the peaks arise from the superposition of many bands (Bradbury et al., 1961).

Inclination of the Bases. The θ angle depends on both the direction of the transition dipole of the vibrational mode within the base plane and the inclination angle of base. If the transition dipole is perpendicular to the axis around which the base is inclined, then $\theta = 90° - \alpha$, where α represents the inclination of the base normal with respect to the helical axis. Otherwise, $\theta > 90° - \alpha$ and determines the minimum α. However, we do not have enough information to determine both the specific angles of inclination for individual bases and their axes from IR LD measurements. Thus the θ angle calculated from eq 1 will correspond to minimum angle of base inclination.

The infrared spectra used to determine the inclination of the bases were measured in a D_2O atmosphere, because the deuteration of DNA films not only frees the 1750–1500 cm^{-1} region from interfering H_2O absorption but also replaces N–H in the bases with N–D, which simplifies the vibrational modes of the DNA bases in this spectral region. We use the absorption band showing the largest dichroic ratio at about 1695 cm^{-1} as a reference to estimate orientation parameter, S. The dichroic ratios and their corresponding θ angles for the other in-plane stretching vibrational bands of DNA films with different degrees of orientation are calculated and summarized in Tables 2–4 for poly[d(AC)]·poly[d(GT)], poly[d(AG)]·poly[d(CT)], and natural DNAs (from *E. coli* and calf thymus). Since the bands below 1550 cm^{-1} arise from a combination of the base and sugar units, we consider only 6 bands in the range of 1750–1550 cm^{-1} to deduce the inclination of bases. To compensate for any possible errors in estimating orientation parameter, S, DNA films with different degrees of orientation were investigated, and the

Table 2: Dichroic Ratios from Various Experiments and Corresponding θ Angles (deg) for Poly[d(AC)]·Poly[d(GT)] in Films Hydrated with D_2O

	1696	1671	1644	1622	1575	1561
			B-Form at High r.h. (>94%)			
R	2.47	2.38	2.04	1.96	2.21	1.72
	2.61	2.61	2.15	1.99	2.40	2.20
	1.62	1.50	1.41	1.61	1.51	1.48
	1.60	1.50	1.54	1.50	1.40	1.50
	1.35	1.31	1.28	1.17	1.28	1.28
	1.40	1.28	1.28	1.21	1.20	1.28
θ	82.0[a]	80.1	74.3	73.0	77.1	69.1
	82.0	82.0	74.6	72.3	78.4	75.4
	82.0	75.2	71.3	81.3	75.7	74.3
	82.0	76.0	78.1	76.0	71.4	76.0
	82.0	77.2	74.4	66.1	74.4	74.4
	82.0	71.7	71.7	67.2	66.6	71.7
av	82.0	77.0 ± 3.3	74.1 ± 2.2	72.7 ± 5.1	73.9 ± 3.9	73.5 ± 2.4
			A-form at Low r.h. (75%)			
R	3.20	3.00	2.25	2.48	2.25	1.81
	1.78	1.67	1.48	1.51	1.30	1.50
	1.44	1.34	1.34	1.20	1.15	1.24
	1.46	1.21	1.17	1.19	1.24	1.29
θ	79.0[a]	77.4	70.9	73.0	70.9	66.6
	79.0	75.2	69.5	70.3	64.3	70.1
	79.0	72.4	72.4	64.9	62.4	67.0
	79.0	65.1	63.1	64.1	66.5	69.0
av	79.0	72.5 ± 4.6	68.9 ± 3.5	68.1 ± 3.7	66.0 ± 3.1	68.1 ± 1.4

[a] The θ angles were set at this value to calculate orientation parameter (*S*).

Table 3: Dichroic Ratios from Various Experiments and Corresponding θ Angles (deg) for Poly[d(AG)]·Poly[d(CT)] in Films Hydrated with D_2O

	1699	1673	1650	1630	1581	1561
			B-Form at High r.h. (>94%)			
R	1.90	1.62	1.49	1.30	1.26	1.52
	1.90	1.64	1.54	1.17	1.26	1.59
	2.10	1.80	1.75	1.60	1.65	1.55
	1.24	1.18	1.20	1.14	1.11	1.20
	1.24	1.20	1.20	1.18	1.19	1.21
θ	82.0[a]	72.7	69.1	63.9	62.3	69.9
	82.0	73.3	70.5	60.1	62.8	71.9
	82.0	75.9	74.6	70.8	72.0	69.6
	82.0	72.6	75.2	68.1	65.1	75.2
	82.0	75.2	75.2	72.6	73.8	76.6
av	82.0	73.9 ± 1.4	72.8 ± 2.6	67.1 ± 4.2	67.2 ± 4.4	72.5 ± 2.8
			C-Form at Low r.h. (66%)			
R	1.89	1.79	1.63	1.50	1.33	1.32
	1.80	1.62	1.53	1.37	1.25	1.30
	1.50	1.40	1.35	1.25	1.20	1.22
θ	82.0[a]	78.1	73.2	69.5	64.8	64.5
	82.0	74.7	71.8	66.8	63.1	64.7
	82.0	74.8	72.0	66.9	64.5	65.4
av	82.0	75.9 ± 1.6	72.3 ± 0.6	67.7 ± 1.2	64.1 ± 0.7	64.9 ± 0.4

[a] The θ angles were set at this value to calculate orientation parameter (*S*).

Table 4: Dichroic Ratios from Various Experiments and Corresponding θ Angles (deg) for Natural DNA Films Hydrated with D_2O

	1695	1671	1647	1620	1577	1561
			E. coli DNA at High r.h. (>94%)			
R	1.34	1.32	1.19	1.27	1.30	1.15
	1.28	1.17	1.11	1.18	1.10	1.10
	1.31	1.25	1.15	1.23	1.20	1.13
θ	82.0[a]	79.3	67.8	74.2	68.6	65.0
	82.0	68.9	63.7	69.8	62.8	62.8
	82.0	74.5	65.9	72.6	69.9	64.4
av	82.0	74.2 ± 4.2	65.8 ± 1.7	72.2 ± 1.8	67.1 ± 3.1	64.1 ± 0.9
			E. coli DNA at Low r.h. (75%)			
R	1.22	1.21	1.09	1.07	1.06	1.04
	1.31	1.15	1.09	1.10	1.11	1.11
	1.19	1.09	1.03	1.12	1.10	1.13
θ	79.0[a]	77.3	63.3	61.4	60.4	58.5
	79.0	65.2	61.0	61.7	62.4	62.4
	79.0	64.6	58.0	68.2	65.8	69.5
av	79.0	69.0 ± 5.9	60.8 ± 2.2	63.7 ± 3.1	62.9 ± 2.2	63.5 ± 4.6
			Calf Thymus DNA at High r.h. (>94%)			
R	2.01	1.71	1.20	1.75	1.27	1.60
	1.60	1.59	1.48	1.32	1.50	1.47
	1.80	1.65	1.34	1.51	1.38	1.53
θ	82.0[a]	73.3	60.6	74.3	62.4	70.6
	82.0	81.3	75.0	68.0	76.0	74.5
	82.0	75.8	65.9	71.2	67.1	71.8
av	82.0	76.8 ± 3.3	67.2 ± 5.9	71.2 ± 2.6	68.5 ± 5.6	72.3 ± 1.6
			Calf Thymus DNA at Low r.h. (75%)			
R	1.78	1.53	1.22	1.25	1.41	1.30
	1.33	1.27	1.13	1.12	1.16	1.21
	1.54	1.40	1.17	1.18	1.30	1.26
θ	79.0[a]	70.9	61.9	62.8	67.5	64.3
	79.0	73.3	63.3	62.6	65.3	68.8
	79.0	71.8	62.1	62.5	67.5	65.8
av	79.0	72.0 ± 0.9	62.4 ± 0.6	62.6 ± 0.1	66.8 ± 1.0	66.3 ± 1.9

[a] The θ angles were set at this value to calculate orientation parameter (*S*).

average θ angles were calculated for each vibrational mode. A glance at the Tables 2–4 tells us that the dichroic ratios and the corresponding θ angles vary according to wavenumber. This wavenumber dependence of θ demonstrates that the bases are not perpendicular to the helix axis. These tables also show that for all synthetic polynucleotides and natural DNAs investigated, the bases in the B- and C-forms are inclined at least 15–25° from perpendicular to the helical axis. The inclinations increased slightly for the A-form of the same DNA samples.

The θ angles obtained by IR LD represent a minimum inclination of the bases and assume that the vibrational modes for C=O stretching and ring stretching are located in the base plane. If there is some out-of-plane portion of the in-plane vibrational modes, the differences in the corresponding θ angles of the bases can no longer be explained simply by base inclination. Any so-called protrusion angle, the angle between the transition dipole of the vibrational mode and the base plane, is believed to be due to the out-of-plane portions of the in-plane vibrational modes created by electronic interaction of overlapping bases or by interaction of the dipoles with the surrounding electrostatic environment (Baret et al., 1978). However, there is no direct evidence that such effects exist. In addition, out-of-plane vibrations generally take place far away from the 1750–1500-cm^{-1} region, where the in-plane stretching vibrations of C=O groups and ring stretching vibrations take place. Flemming et al. (1988) have estimated the protrusion angle for the transition moment of a C=O vibrational band at 1710 cm^{-1} by using tilt and twist angles obtained from X-ray diffraction analysis and discussed why the calculated high protrusion angle could not explain the large inclination angle of DNA bases in films compared with fiber diffraction data. All evidence indicates that protrusion angles are not large, and θ angles deduced from IR LD measurements are due to the inclination of bases in the double-helical structure of DNA.

Although our IR LD results do not have the information content to give specific inclination angles for individual bases, the θ angles do provide other important information about base orientation in the double-helical DNA structure. We

have already reported quite high base inclinations for the B- and A-forms of poly[d(AC)]·poly[d(GT)], poly[d(AG)]·poly[d(CT)], and several natural DNAs in aqueous solution using flow UV LD measurements (20–25° for purines and 30–35° for pyrimidines in B-DNA) (Kang & Johnson, 1993; Chou & Johnson, 1993). We also reported that A-DNA has slightly increased inclinations for both the purine and the pyrimidine bases. However, the differences of the inclination angles between B- and A-DNA in solution are not as large as the values deduced from fiber diffraction data (6° for B-DNA vs 20° for A-DNA). IR LD confirms a substantial inclination for the bases of the B- and A-form of synthetic polynucleotides and natural DNAs in films. The inclination angles for the bases of natural DNA in films reported here are quite similar as those published elsewhere (Flemming et al., 1988). Poly[d(AC)]·poly[d(GT)] and poly[d(AG)]·poly[d(CT)] show inclinations comparable with those for poly(dA)·poly(dT) and poly[d(AT)]·poly[d(AT)] in films (Baret et al., 1978). Tables 2–4 also show that A-DNA has a slightly increased base inclination compared with B-DNA, but the difference in the base inclination is not large enough to discriminate between B- and A-DNA in films. These findings are consistent with Flemming et al. (1988), who point out that base inclination is not a good criterion to discriminate between B- and A-DNA. In addition to A- and B-DNA, we report for the first time the base inclination for the C-form of poly[d(AG)]·poly[d(CT)] in a film.

Our results about base inclinations of DNA in films deduced from IR LD measurements using vibrational transitions support our earlier UV LD results using electronic transitions, that the bases are highly inclined, even in the B-form. They also provide other useful information about the orientation of bases in the double-helical structure of DNA. The information about the molecular structure of DNAs in gels or hydrated films provides valuable structural parameters to connect the molecular structure of DNA in solid state (e.g., crystal) with those in solution state. Considering the relatively high condensed environment of nuclei where the DNA molecules are found, the structural information about DNA molecules in films or gels provides important clues to understand the real nature of molecular structure of DNA in living cells.

Conformation of Phosphodiester Backbone Geometry. The torsional angles for the sugar–phosphodiester backbone and glycosidic bond of DNA are a determining factor in the relative stability of specific forms of DNA. X-ray fiber or crystal diffraction analysis, NMR measurements, and theoretical calculations provide useful information about such torsional angles [Pohle et al. (1986) and references therein]. IR LD spectroscopy is another useful technique to yield quantitative information about the geometrical arrangement of the phosphate groups in the backbone of DNA in solution, condensed gel, or hydrated film. Many workers have measured the conformational angles of the phosphate groups in A-, B-, C-, and D-forms of synthetic polynucleotides and natural DNAs by IR LD (Brahms et al., 1973; Pilet & Brahms, 1973; Pilet et al., 1975; Fritzsche et al., 1976; Kursar & Holzwarth, 1976; Baret et al., 1978; Flemming et al., 1988;). The phosphate arrangement is determined by the two angles, θ_{oo} and θ_{opo}, which are formed between the O–O line and the bisector of the OPO and the DNA helix axis, respectively. As discussed in the papers by Pohle et al. (1984, 1986), the conformational angles of the PO_2^- groups for the A- and B-forms of DNA obtained by different methods show a broad range of the angles, rather than having one specific value. For example, the angles for the O–O line and OPO bisector obtained from NMR, IR LD, or theoretical calculations are in the range of 52–56° and 62–70°, respectively, in the B-DNA, and in the range of 59–64° and 46–50°, respectively, in the A-DNA. In contrast, these angles deduced from fiber diffraction data spread over a wider range than those presented above (48–66° and 49–71° for θ_{oo} and θ_{opo}, respectively, in the B-DNA, and 65–79° and 15–49° for θ_{oo} and θ_{opo}, respectively, in the A-DNA). Pohle et al. (1984) compared the angles deduced from IR LD with those from X-ray data and showed that the discrepancies in the conformational angles originated from inaccurate modeling of DNA structure to fit fiber diffraction data, causing these angles to spread over a wide range of θ values, depending on the model used to fit the X-ray data. However, by selecting proper modeling for the X-ray data, values fairly close to IR LD results were obtained for the A- , B-, and Z-forms of DNA. We measured the dichroic spectra of oriented films of DNA samples hydrated with H_2O under different r.h. for A-, B-, and C-form (Figure 3). The orientation parameter of the sample, S, which must be known to calculate θ in eq 1, was determined from the largest dichroic ratio of a band near 1710 cm^{-1} assigned to the C4=O stretching band for the thymine base (see above). The integrated intensities of individual bands arising from the antisymmetric and symmetric stretching vibrations of the PO_2^- group at about 1230 and 1087 cm^{-1} were calculated, and the orientation angles of the O–O line and the OPO bisector relative to the helix axis were determined from the measured dichroic ratio of corresponding bands (Table 5). The dichroic ratios were also checked by measuring the peak intensity of each band from the base line as described by Pohle et al. (1984), and the differences resulting from these two methods were within the experimental error. For B-DNA, the angle for the OPO bisector (θ_{opo}) is about 67° for poly[d(AC)]·poly[d(GT)] and poly[d(AG)]·poly[d(CT)] and is about 63° for natural DNAs. The angle for the O–O line (θ_{oo}) is about 54° for both synthetic polynucleotides and natural DNAs from different sources. For the A-form of natural DNA and poly[d(AC)]·poly[d(GT)], the θ_{opo} and θ_{oo} are about 46° and 64°, respectively. These conformational angles are comparable to angles reported by other workers [Pohle et al. (1986) and references therein]. The orientation angles of the PO_2^- group for the C-form of poly[d(AG)]·poly[d(CT)] are about 66° and 48° for θ_{opo} and θ_{oo}, respectively. These angles are comparable with those reported values for C-DNA (Brahms et al., 1973; Pohle et al., 1984). The agreement of these conformational angles for PO_2^- groups with values reported by other groups indicates that we indeed observed B → A or B → C transitions for our synthetic polynucleotides and natural DNAs by decreasing the r.h.

Table 5: Conformational Angles for Phosphodiester Backbone of DNA in Films Hydrated with H_2O[a]

DNA[b]	form	θ_{oo} (deg)	θ_{opo} (deg)
poly[d(AC)]·poly[d(GT)]	B	53.7 ± 0.8	68.9 ± 2.3
poly[d(AG)]·poly[d(CT)]	B	54.5 ± 1.6	63.2 ± 1.7
E. coli DNA	B	54.5 ± 1.5	64.1 ± 1.1
calf thymus DNA	B	54.9 ± 1.8	60.0 ± 1.6
poly[d(AG)]·poly[d(CT)]	C	47.9 ± 0.7	65.5 ± 2.9
poly[d(AC)]·poly[d(GT)]	A	63.2 ± 2.8	45.2 ± 2.6
E. coli DNA	A	64.5 ± 2.1	44.6 ± 3.5
calf thymus DNA	A	64.2 ± 1.7	48.2 ± 0.5

[a] The angles are averaged values calculated from the dichroic ratio of IR bands from various experiments for DNA films with different degrees of orientation. [b] The B-, A-, and C-forms of DNA were obtained at 94%, 75%, and 66% r.h., respectively.

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