Cox, A. D., & McMillan, P. W. (1981) *J. Non-Cryst. Solids 44*, 257-264.
Cramer, S. P., & Hodgson, K. O. (1979) *Prog. Inorg. Chem. 25*, 1-39.
Doniach, S., Eisenberger, P., & Hodgson, K. O. (1980) in *Synchrotron Radiation Research* (Winick, H., & Doniach, S., Eds.) Plenum Press, New York and London.
Dubin, S. B., & Cannell, D. S. (1975) *Biochemistry 14*, 192-195.
Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., Söderberg, B.-O., Tapia, O., Brändén, C.-I., & Åkeson, A. (1976) *J. Mol. Biol. 102*, 27-59.
Gerhart, J. C., & Schachman, H. K. (1965) *Biochemistry 4*, 1054-1062.
Gerhart, J. C., & Holoubek, H. (1967) *J. Biol. Chem. 242*, 2886-2892.
Gerhart, J. C., & Schachman, H. K. (1968) *Biochemistry 7*, 538-552.
Gibbons, I., Ritchey, J. M., & Schachman, H. K. (1976) *Biochemistry 15*, 1324-1330.
Griffin, J. H., Rosenbusch, J. P., Blout, E. R., & Weber, K. K. (1973) *J. Biol. Chem. 248*, 5057-5062.
Hart, M., & Rodrigues, A. R. D. (1978) *J. Appl. Crystallogr. 11*, 248-253.
Jacobson, G. R., & Stark, G. R. (1973) *Enzymes, 3rd Ed. 9* (Part B), 225-308.
Johnson, R. S., & Schachman, H. K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1995-1999.
Klug, H. P. (1966) *Acta Crystallogr. 21*, 536-546.
Lee, P. A., & Pendry, J. B. (1975) *Phys. Rev. B: Solid State 11*, 2795-2811.
Macgillavry, C. H., & Rieck, G. D., Eds. (1968) *International Tables for X-ray Crystallography*, Vol. 3, The Kynoch Press, Birmingham, United Kingdom.
Monaco, H. L. (1978) Ph.D. Thesis, Harvard University.
Monaco, H. L., Crawford, J. L., & Lipscomb, W. N. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5276-5280.
Moody, M. F., Vachette, P., & Foote, A. M. (1979) *J. Mol. Biol. 133*, 517-532.
Nelbach, M. E., Pigiet, V. P., Gerhart, J. C., & Schachman, H. K. (1972) *Biochemistry 11*, 315-327.
Pendry, J. B. (1974) *Low Energy Electron Diffraction*, Academic Press, New York.
Phillips, J. C. (1981) *J. Phys. E 14*, 1425-1428.
Prescott, L. M., & Jones, M. E. (1969) *Anal. Biochem. 32*, 408-419.
Rosenbusch, J. P., & Weber, K. K. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1019-1023.
Savage, C. R., Schumer, J. M., & Weinfeld, H. (1973) *Anal. Biochem. 53*, 431-440.
Shulman, R. G., Eisenberger, P., & Kincaid, B. M. (1978a) *Annu. Rev. Biophys. Bioeng. 7*, 559-578.
Shulman, R. G., Eisenberger, P., Teo, B. K., Kincaid, B. M., & Brown, G. S. (1978b) *J. Mol. Biol. 124*, 305-321.
Stern, E. A. (1978) *Contemp. Phys. 19*, 289-310.
Weber, K. K. (1968) *Nature (London) 218*, 1116-1119.
Yang, Y. R., & Schachman, H. K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5187-5191.

Electric Dichroism of Deoxyribonucleic Acid in Aqueous Solutions: Electric Field Dependence†

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ABSTRACT: The ionic strength and molecular weight dependence of the electric field dichroism of DNA, using native and sonicated calf thymus DNA, has been examined in the range of 0.09-1.0 mM NaCl solutions for molecular weights ranging from 1.24×10^5 to 44×10^5. The application of a classical theoretical analysis implies that the orientation results from an induced moment which appears to saturate at moderate fields of several killivolts per centimeter and that the induced moment is at least partly characteristic of that expected of a polyion whose charge is partially counterion compensated. The orientation leads to dichroism values which are in accord with other observations and are lower than expected for a B-form rod of DNA. These values, however, are not uniquely interpretable as resulting from base tilting.

Structural and conformational studies of DNA have long been pursued by various physical methods under vastly different conditions because of the vital importance of DNA in biological activity. Nevertheless, there remain a number of serious questions to be resolved with respect to the polyelectrolyte properties and the structure of DNA in low salt containing aqueous solutions. We report here a series of measurements made on sonicated fragments and "native" (i.e., unsonicated) calf thymus DNA to help resolve some of these questions. The experimental part of the present work was completed before restriction- and endonuclease-digested monodispersed fragments of DNA became available. The sonicated DNA samples used were very carefully treated and characterized. The polydispersity presents some problems of data interpretation but is by no means wholly restrictive. Firm semiquantitative results and conclusions can be drawn.

In an early exploration of the electric dichroism and birefringence of DNA (Yamaoka & Charney, 1973), we demonstrated that native calf thymus DNA did not appear to obey the classical theoretical prescription (Langevin, 1910; Yamaoka & Charney, 1972) that the orientation be quadratic in field strength. This anomaly was attributed to the flexibility of DNA partly on the basis that an earlier though preliminary report (Charney & Yamaoka, 1971) proved that the orien-

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tation of a sonicated, small molecular weight DNA (sDNA) is proportional to E^2 in the low field strength region. The molecular weight of the native DNA of this early work was approximately 5×10^6 while that of the sonicated DNA was approximately 2.4×10^5. Although it is generally accepted that there must be a quadratic component, recent measurements (Hogan et al., 1978) appear to show that the orientation of a monodispersed sample of low molecular weight DNA (ca. 1.6×10^5) is linearly dependent on the field strength, leading to further questions regarding the nature of the orientational energy and the flexibility of this polyion.

The objectives of the present paper are to (1) verify that a molecular weight dependent quadratic field component exists at measurable orientations over a large range of molecular weights, (2) to establish the effect of the concentration of added salt on the orientation of low molecular weight rodlike DNA and to evaluate quantitatively the apparent permanent and induced electric moments by comparing the results with the classical theory for the field orientation, and (3) to estimate the angle between the axis of the orientation of sDNA and the direction of the transition moment of the 260-nm absorption band of the DNA bases. A discussion of the ionic strength dependence (objective 2), based in part on experiments reported here, has recently been presented (Charney et al., 1980).

Materials and Methods

Materials. A calf thymus DNA preparation (DNA 3CA REFR18) was purchased from Worthington Biochemical Corp.

Sample Preparation. DNA (0.25 g) was slowly dissolved to a gel at 0 °C in 20 mL of distilled cold water. To the softened gel containing 0.25 g of DNA was added 0.2 M NaCl solution (20 mL) in small portions and then in larger portions (total 60 mL), and finally, the 0.4 M NaCl solution (25 mL) was added to make a DNA stock solution (ca, 2 mg of DNA/mL in ca. 0.2 M NaCl).

Sonication of Calf Thymus DNA. The sonicator employed was a Branson Model S125 sonifier. A rosette vessel for the sonication was cleansed with a 0.2 M NaCl solution for 2 min at the no. 8 setting (ca. 9 A). The sonicator probe was immersed 0.5 in. below the liquid level. The DNA stock solution was transferred into the vessel in an ice bath, and helium gas was introduced for 15 min to remove the dissolved air. A total of 20 min of sonication in 40 bursts (30 s for each burst) was applied to the solution for 5 min at each intermission. The power setting of the sonifier, to which a large solid horn was attached, was at no. 6 (ca. 7.5 A). This sonicated DNA sample was designated as sDNA(T). Another DNA stock solution (60 mL)e was sonicated in the same manner as above at a power setting of no. 2 (ca. 2.3 A). In this case, the initial deaeration by helium gas was done for 30 min). This sonicated DNA sample was designated as sDNA(X). A third DNA stock solution containing 1.5 mg of DNA/mL in 0.2 M NaCl (36 mL) was sonicated in a plastic centrifugation tube at a power setting of no. 4 (4 A). A total of 3 min of sonication by 13 bursts (10 s for each burst) was applied to the solution. Helium gas was bubbled through this solution for 1 min at each intermission. This sonicated DNA sample was designated as sDNA(L).

Dialysis of DNA Preparations. Dialysis tubes, purchased from Union Carbide Corp. (20 mm in diameter), were thoroughly rinsed both inside and outside in a warm 1% $NaHCO_3$ solution (1.4 L) for 20 min, washed with warm distilled water (total of 6 L) for 2 h, and then treated in anhydrous ethanol (1 L) at room temperature for 5 min. Finally, the tubes were washed with distilled water both inside and outside. These dialysis tubes were soaked in a 0.01 M Na_2EDTA solution (P-H Tamm Laboratories AB) just prior to dialysis. We described this preparation because tubing treated rigorously was found to elute, upon agitation, measurable quantities of a UV-absorbing contaminant. The 0.1 M Na_2EDTA solution (3 mL) was added to each preparation (60 mL) just before dialysis. Each DNA preparation (35 mL) was dialyzed against 0.01 M NaCl (1 L) for 9 h at ca. 0 °C and then against seven batches of 1 mM NaCl solution (total of 7 L) for about 72 h. The dialyzed stock DNA in 1 mM NaCl solution was kept frozen.

Hyperchromicity of Dialyzed DNA Solutions. The hyperchromicity of the four solutions in millimolar NaCl was determined conventionally (Yamaoka & Resnik, 1966). The hyperchromicity is 35.0 ± 1.5% which indicates that all three sDNA preparations are native, albeit the sonication, as compared with the native DNA.

Molecular Weight of DNA Samples. The weight-average molecular weight of four DNA samples was determined by ultracentrifugation in a 1 M NaCl solution buffered with the 0.01 M Na_2HPO_4–NaH_2PO_4 solution. The relation between the sedimentation coefficient, s_{20}, and the molecular weight, M_w, given by $\log s_{20} = \log 0.0882 + 0.346 \log M_w$ was utilized (Studier, 1965). The mean values ($\times 10^5$) are 1.24, 1.74, 5.0, and 44.0, respectively, for samples labeled T, X, and L and the native DNA. The polydispersity as measured by the half-width of the distribution is fractionally about the same for all the samples.

Preparation of DNA Solution in Different Ionic Strengths. Each of the dialyzed stock DNA solutions (ca. 17 mL) was diluted and/or dialyzed to final NaCl concentrations at low temperature. Samples were retained at 1 mM NaCl for the determination of hyperchromicity.

Methods and Measurements of Electric Dichroism. Methods of measuring and processing the electric dichroism signal have been described together with the instrumentation (Fredericq & Houssier, 1973; Yamaoka & Charney, 1973; Charney et al., 1980). At a fixed optical wavelength in an applied electric field, the dichroisms parallel, $\Delta A_\parallel$ and perpendicular, $\Delta A_\perp$, to the field of an assembly of like ionized, prolate macroions in aqueous solution are related to the product of the optical term and the orientation function, $\Phi,(\beta,\gamma,\sigma)$, as

$$\frac{\Delta A_\parallel}{A} = \frac{A_\parallel - A}{A} = (3\cos^2\theta - 1)\Phi \qquad (1)$$

and

$$\frac{\Delta A_\perp}{A} = \frac{A_\perp - A}{A} = \frac{1}{2}(1 - 3\cos^2\theta)\Phi \qquad (2)$$

where A is the isotropic absorbance of the sample solution in the absence of an electric field and θ is the angle between the direction of the transition moment vector of a particular absorption band and the orientation axis of the molecule to which the chromophoric group is tightly attached. Φ represents the average degree of orientation of the molecule at a given external electric field strength, E (volts per centimeter), and is generally a complicated function of the dielectric properties of the molecule; the permanent dipole moment μ, the polarizability anisotropy $\Delta\alpha$, and the induced dipole moment due to the ionic atmosphere polarization respectively determine β, γ, and σ.

When the molecule is represented by an uncharged long cylinder ($\sigma = 0$) whose axis of symmetry (the 3 axis) coincides

with the orientation axis, the orientation function, $\Phi(\beta,\gamma)$ (O'Konski et al., 1959), has its orientation energy parameters in the field given by

$$\beta = \frac{\mu_3 E}{kT} \qquad \gamma = \frac{(\alpha_{33} - \alpha_{11})E^2}{2kT} > 0 \tag{3}$$

where μ_3 is the apparent permanent dipole moment along the o axis, α_{33} and α_{11} ($=\alpha_{22}$) are the atomic and electronic (covalent) polarizabilities, and k is the Boltzmann constant. For a charged polyelectrolyte, moments may be induced by the polarization of its ionic atmosphere. The interaction term between the electric field and the counterions of the macroion, the σ term, has lately been under investigation [see Discussion and Kikuchi & Yoshioka (1976), Sokorov & Weill (1979), Charney et al. (1980), Charney (1980), and Rau & Charney (1981)]. Two approaches are possible in describing the orientation energy: one is to treat the ionic atmosphere polarization separately, and the other is to sum the covalent and ionic atmosphere polarizations so that γ becomes the orientational energy arising from all sources of field-induced moments; i.e., γ and σ are incorporated in a single term. The latter approach will be strictly valid only if all the induced moments have the same dependcne on E. At low field strengths, all the published theoretical treatments agree that this is a good approximation. While these parameters may vary with the nature of the molecular moiety, eq 2 remains valid in all circumstances where the structure, and therefore the angle, is independent of the field. If no distortion of the molecular structure by the electric field occurs, the isotropic absorption, A^E, calculated from measurements of the parallel and perpendicular dichroism, is given by

$$A^E = \tfrac{1}{3}(A_\parallel + 2A_\perp) \tag{4}$$

Under these conditions, A^E should be identical with the absorbance A measured isotropically in an ordinary zero-field unpolarized absorption measurement; i.e., no hyper-or hypochromsim arises from an electric field induced distortion. The absence of such effects on native DNA in 1 mM NaCl up to fields of 4.75 kV/cm has been shown previously (Yamaoka & Charney, 1973). The dichroism can be affected in one of several ways which would result in an inequality between $\Delta A_\parallel$ and $-2\Delta A_\perp$: first, a distortion of the unaxial property of the system by the field; second, electrochromism (Yamaoka & Charney, 1972), a field-induced wavelength shift in the dichroic spectrum, may appear; third, the value of the dichroism reached at high fields can be different (higher in the case of the straightening discussed above) than expected from the orientation of the molecules without any field-induced distortion. In the present measurements, no electrochroism was observed. Equation 4 was observed to hold within experimental error for all the sonicated DNA samples.

Results

As the analysis of the experimental results obtained under different conditions has some bearing on each of the objectives, this section is organized according to the range of experimental parameters studied. First, both the low-field orientation of DNA fragments of different molecular weights and the effect of the ionic strength are considered. Next the range of field strengths is extended, and then these results are used to discuss the polyelectrolyte properties of DNA. The dividing line between low and high field strengths is essentially the region where the dichroism begins to deviate from a quadratic dependence on the field. Finally, we consider how these results affect the expectation values of the optical factors which should

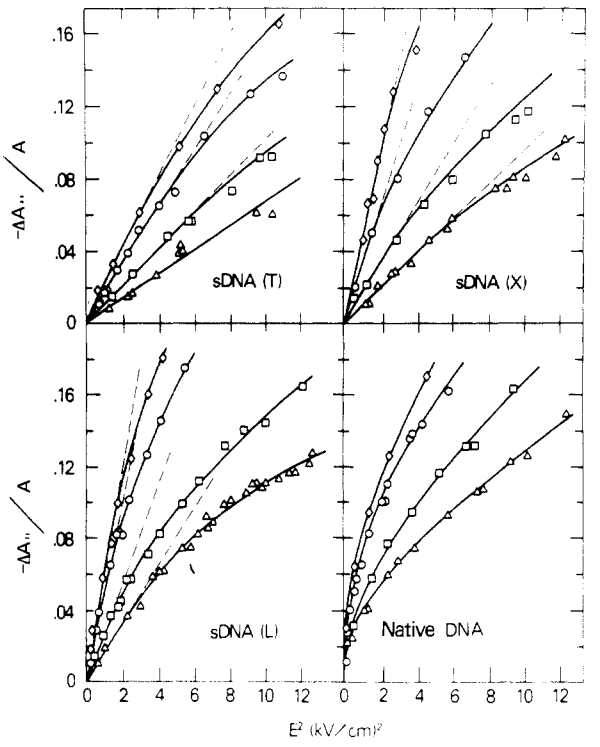

FIGURE 1: Parallel dichroism, $\Delta A_\parallel/A$, at 270 nm of four DNA samples in four different NaCl-containing solutions at 7 °C without buffer salts. The concentration of added NaCl is (◇) 0.09, (○) 0.52, (□) 0.6, and (△) 1 mM. The field strength is expressed in kilovolts per centimeter.

be observed if the molecules could be fully oriented.

Low Electric Fields and the Ionic Environment. Figure 1 shows the electric field dependence of the reduced parallel dichroism, $\Delta A_\parallel/A$, at 270 nm of four samples, three sonicated sDNAs (T, X, and L) and one native DNA, each in four different NaCl solutions in a (low) field strength range of 0–3.5 kV/cm. Note that since the system is well-behaved ($\Delta A_\parallel = -2\Delta A_\perp$), either ΔA, $\Delta A_\parallel$, or $\Delta A_\perp$ may be used to monitor the parametric variations. Four significant results are apparent from Figure 1. First, the $\Delta A_\parallel/A$ values are proportional to the square of field strengths at low field strengths as the dashed lines indicate; thus, at low enough fields, the Kerr law holds for all sDNA samples in spite of a wide difference in the molecular weights. This result is a clear confirmation of our early report (Charney & Yamaoka, 1971) and suggests the failure to observe the region of quadratic dependence cannot be interpreted in terms of the inapplicability of the Kerr law to semiflexible molecules. Neither is the DNA source a factor. We note, for example, the recent reports by Rau & Bloomfield (1979) and by Marion & Roux (1980) on T7 DNA and rat liver nucleosomal DNA, respectively, in which an E^2 dependence is observed at low fields. Undoubtedly, as the molecular weight increases, the backbone flexibility must become a factor; the mean molecular size of the sDNA(T) sample is only about 630 Å, while that of the sDNA(L) sample is over 2500 Å. Recent investigations by Stellwagon (1980), Hagerman (1981), Elias & Eden (1981), and Charney & Milstien (1978), using the electric field of orientation and the light scattering studies of Borochov et al. (1981), clearly show that DNA molecules larger than 1500 Å are not rigid rods even at these low salt concentrations. Recent theoretical treatments give similar results for the onset of flexibility (Olson, 1979; Schellman, 1980; Hagerman & Zimm, 1981). On the basis of these investigations, our lowest molecular weight sample, DNA(T), must be nearly rodlike as the persistent lengths are

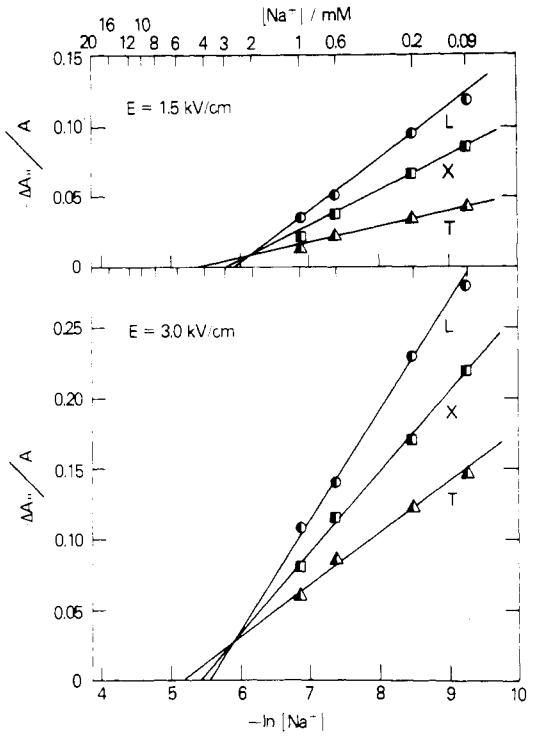

FIGURE 2: Dependence of the parallel dichroism at 270 nm and at 7 °C of four DNA samples on the concentration of added NaCl. The electric field strength is 1.5 kV/cm in the upper figure, while it is 3.0 kV/cm in the lower figure. The DNA samples are (▲) T, and (□) X, and (●) L.

now generally assumed to be larger than 600 Å at a Na^+ concentration of 1 mM except in the recent work of Borochov et al. (1981) where the application of an excluded volume correction to the data reduced the value to about 600 Å. Second, and at least partially related to this factor, the smaller the molecular weight of the sDNA, the wider is the range of field strengths for which the Kerr law persists; e.g., the $\Delta A_\parallel/A$ values of sDNA(T) (in a 1 mM NaCl solution) whose molecular weight is 1.24×10^5 vary linearly with E^2 up to 3 kV/cm and greater. The magnitude of this factor is affected by the polydispersity of the sample. At the relatively low polydispersities of these samples (as will be shown in a forthcoming paper by Yamaoka and Fukudome), this effect is small at low field strengths but increases in the region above deviation from Kerr behavior. Third, with a decrease in ionic strength, the dichroism increases but tends to deviate from the Kerr law at much lower electric fields in each sDNA solution. Fourth, although the dichroism behavior of the high molecular weight DNA(H) is not observably quadratic in E even at the lowest field strengths at which these measurements were made, the Kerr law is undoubtedly followed in the limit of very low fields. Since the molecular weight of DNA(H) is about 35 times higher than that of sDNA(T), with a mean end-to-end distance much shorter than its contour length, the field strength dependence of its dichroism must be much more complex. A quadratic dependence at very low field has been reported for the electric birefringence of nucleosomal DNA molecular weights in the range of $(4–40) \times 10^5$ (Elias & Eden, 1981) for which the field dependence should be the same as that of the electric dichroism. Plots of the ionic strength dependence at fixed values of the field strength are shown in Figure 2. The $\Delta A_\parallel/A$ values of sDNA decrease linearly with the logarithm of the reciprocal of Na^+ concentration. Other recent experiments on the ionic strength dependence of the reduced dichroism $\Delta A_\parallel/A$ have confirmed and extended the ionic strength dependence observed here (Charney, 1980; Hogan et al., 1978). The slope for the linear low-field portion of the $\Delta A_\parallel/A$ vs. E^2 curve of each DNA sample solution of different ionic strength may be obtained from Figure 1. The magnitude of the slope, $(-\Delta A_\parallel/A)E^{-2}$, is generally larger for the higher molecular weight sDNA at a given ionic strength and also larger for the lower ionic strength in a given sDNA sample. However, the classical expression for the dichroism of electrically neutral molecules (Yamaoka & Charney, 1972)

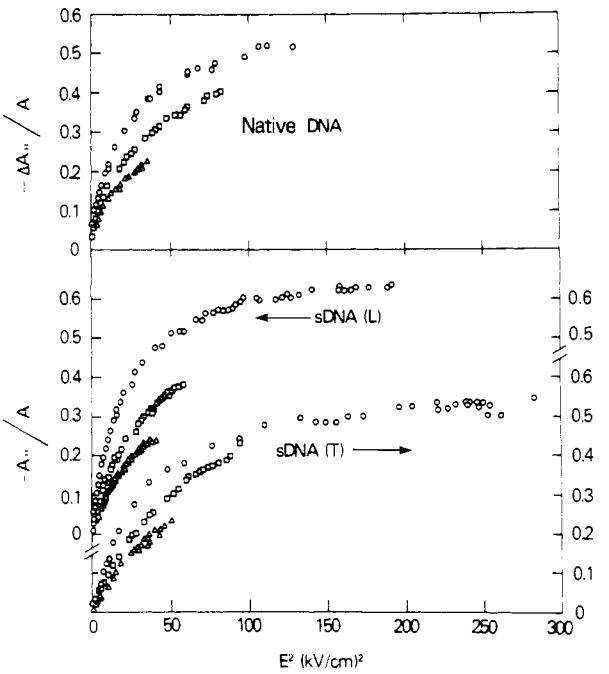

FIGURE 3: Electric field dependence of the parallel dichroism at 270 nm of sDNA and native DNA in four solutions of different NaCl concentrations. The NaCl concentrations are (△) 1, (□) 0.6, and (○) 0.2 mM. The data are plotted against the square of field strength.

$$\frac{\Delta A_\parallel}{A}\left(\frac{1}{E^2}\right)_{E^2\to\infty} = \frac{1}{15}(3\cos^2\theta - 1)\left[\left(\frac{\mu_3}{kT}\right)^2 + \frac{\alpha_{33} - \alpha_{11}}{kT}\right] \quad (5)$$

contains no parameter explicitly dependent on the ionic strength of the solvent because neither the classical polarizability, α, nor the dipole moment, μ, is dependent on the solvent ionicity. The data in Figures 1 and 2, therefore, require a more detailed examination of the orientation mechanism for DNA than is suggested by the classical approach. Two analytical approaches are possible. One is to attempt to fit the data over the entire range of field strengths to the classical orientation function and thus to obtain apparent values of μ_3 and $\Delta\alpha$. The other is to examine only the low-field results and make use of the reasonable assumption that DNA has no significant permanent dipole moment. In this case, DNA is considered as a polyelectrolyte whose orientational energy at low-field strengths is entirely dependent on an induced moment that may have both ionic strength dependent and independent components. The latter approach has been initiated in some of our recent work [see, e.g., Charney & Milstien (1978) and Charney et al. (1980)] while an earlier development has been discussed by Sokorov & Weill (1979). We consider first the former approach and examine the electric field strength dependence over the entire range of available data.

Electric Field Dependence. The experimental data for the sonicated samples (T and L) and the native sample are shown in Figure 3. A similar result was also obtained for sDNA(X). For both sDNA(L) and sDNA(T) in low salt concentrations

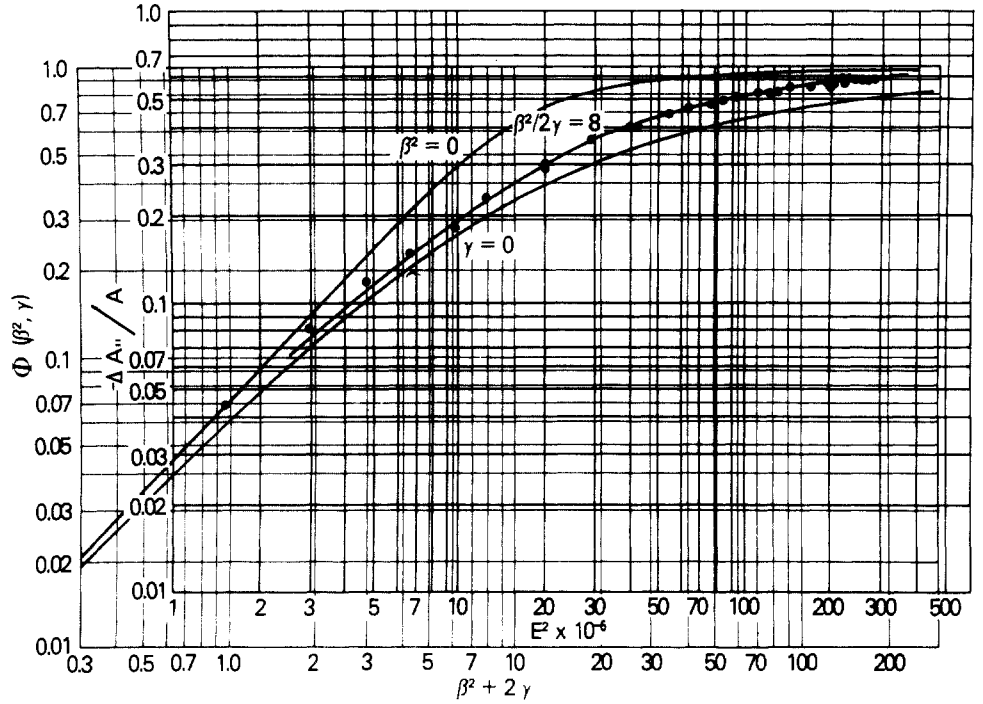

FIGURE 4: Example of the matching procedure. The $\Delta A_{\parallel}/A$ values of sDNA(X) in 0.2 mM NaCl solution (circles) were plotted against E^2 on full logarithmic graph paper which was matched to one of the master curves, the parameter of which was $\beta^2/(2\gamma) = 8$ (solid curve). Note that $\Delta A_{\parallel}/A = -0.64$ at $\Phi = 1$ and $E^2 = 158$ (kV/cm)^2 at $\beta^2 + 2\gamma = 100$.

(0.2–0.09 mM), values of $\Delta A_{\parallel}/A$ appear to approach a limiting value asymptotically at high field strengths. At higher salt concentrations, the ability of the field pulser to deliver sufficient charge limited the range of E. Because no experimentally verified complete form of the orientation function Φ has been developed for polyelectrolytes as yet (Charney et al., 1980; Kikuchi & Yoshioka, 1976), a physically meaningful quantitative description of the electric dichroism of DNA in still beyond reach. It is informative, nevertheless, to examine the data by using the function $\Phi(\beta,\gamma)$ developed for the nonionic cylindrical model (O'Konski et al., 1959). When this is done, the assumption is made (vide supra) that contributions of the distortion of condensed counterions and the diffuse ion atmosphere to the induced moment have the same field dependence as that of the covalent polarizability, at least until the strong ionic effects become independent of further increases of the field strength. Under these conditions, the orientation may continue to be dependent on the field strength, with the "saturated" induced moment acting like a permanent dipole moment.

Simple Graphical Method. A set of "master curves" is drawn on translucent 2 × 3 cycle double-logarithmic graph paper by plotting the values of $\Phi(\beta)$ (Yamaoka & Charney, 1972; Matsumoto et al., 1967), on the ordinate, against $\beta^2 + 2$, on the abscissa, with $\beta^2/2$ as the parameter. The experimentally obtained $\Delta A_{\parallel}/A$ values are plotted against E^2 on another piece of translucent graph paper of the same scale, which is then placed on the master curves sheet and moved both laterally and vertically until the experimental curve coincides with one of the master curves. By use of this matching procedure, illustrated in Figure 4, three critically important quantities, β^2, 2γ, and the asymptototic value at high fields, $(\Delta A_{\parallel}/A)_s$ and $(\Delta A/A)_s$, can be determined. Values of μ_3 and $\Delta\alpha$ obtained from β and γ by this procedure are given in Table I.

It should be emphasized that the values of the dielectric parameters β and γ (and μ_3 and $\Delta\alpha$) so obtained must be considered only "apparent" values, because of the classical

Table I: Apparent Dipole Moment μ_3 and Polarizability Anisotropy $\Delta\alpha$ ($=\alpha_{33} - \alpha_{11}$) of DNA Samples in Solutions of Different Added Salt Concentrations

		[NaCl] (mM)			
DNA		0.09	0.2	0.6	1
T	μ_3[a]	8170	7050	5100	4200
	$\Delta\alpha$[a]	2.16	2.04	1.92	0.89
X	μ_3	11100	8700	6500	5100
	$\Delta\alpha$	4.03	2.45	1.38	0.85
L	μ_3	11300	9900–~9100	7400	6900–~6700
	$\Delta\alpha$	5.46	2.43	1.67	0.12
native	μ_3		10100	7500	10100
	$\Delta\alpha$		1.31	1.22	1.31

[a] μ_3 is in D and $\Delta\alpha$ is in 10^{16} cm^{-3}.

nature of the theoretical orientation functions. The result of matching between the experimental points and the master curves can be summarized as follows: The best fits were obtained for the shorter sDNA sample at the lower salt concentrations. When both the salt concentration and the molecular weight are higher, the data points show a tendency to fit to the master curve for which only γ is finite ($\beta = 0$) in very low fields, and then at higher fields to transfer to one of the lower orientation curves [$\beta^2/(2\gamma) \neq 0$]. The results are summarized in Table I. Using this procedure, it appears that an initially induced moment does indeed saturate at some value of the field strength and then contributes to the orientation at higher fields as if it were a permanent moment μ_3. We note that the Kikuchi & Yoshioka model (1976) of the orientation of a polyelectrolyte, although quantitatively at variance with the observations (Charney & Milstien, 1978; Sokorov & Weill, 1979), predicted just such a behavior.

DNA as a Polyelectrolyte. The foregoing results may be compared with an analysis of the data based on the fact that at or near neutral pH, where these experiments are performed, DNA is a highly charged molecule carying one negatively charged phosphate group every 1.7 Å (in projection on the helix axis) compensated in part by condensed positive coun-

Table II: Number of Bound Na^+ Counterions (n) in Solutions of Different Added NaCl Concentrations

DNA		[NaCl] (mM) 0.09	0.2	0.6	1.0
T	n[a]	86 (4.4)[b]	64 (5.9)	34 (11)	23 (17)
X	n	82 (6.4)	50 (11)	28 (19)	17 (31)
L	n	10 (151)	7.8-~6.6 (195-~230)	4.3 (348)	3.8-~3.6 (400-~425)

[a] n was calculated from eq 7. [b] The values in parentheses are the number of bases per bound counterion.

Table III: Parallel Dichroism at Infinite Field Strength at 270 nm $(\Delta A_\parallel/A)_s$ and the Apparent Angle θ_{270} of DNA Samples in Solutions of Different Added Salt Concentrations

DNA		[NaCl] (mM) 0.09	0.2	0.6	1
T	$(\Delta A_\parallel/A)_s$	−0.58	−0.60	−0.60	−0.60
	θ_{270}	68	69	69	69
X	$(\Delta A_\parallel/A)_s$	−0.58	−0.64	−0.64	−0.60
	θ_{270}	68	70	70	69
L	$(\Delta A_\parallel/A)_s$	−0.67	−0.70	−0.67	−0.60
	θ_{270}	71	72	71	69

terions. The question is how the polarization of this moiety in an electric field results in an orientational torque. There are good reasons for the assumption that DNA does not possess a classical permanent dipole in unbuffered aqueous solutions. First is the stereoregular antiparallel structure of the double helix, the essential features of which are probaby retained in solutions. Second, the rise curve of the transient dichroism signal, $\Delta A_\parallel$, is always much faster than the decay curve at higher fields, but both rise and decay curves approach a symmetric pattern at low fields, which can be taken as proof that the orientation at low fields results entirely from polarizability anisotropy of the orientating molecule (Benoit, 1951; Tinoco & Yamaoka, 1959). The initial slope of the rise curve is neither zero nor nearly zero, which otherwise would be indicative of a major permanent dipole contribution to orientation (Nishinari & Yoshioka, 1969). Hence, although the "classical" analysis shows that at intermediate field strengths a field-induced moment appears to saturate and then behave like a permanent moment, the intrinsic permanent dipole moment parameter, β, is null and should be deleted from eq 1 and 2.

We are thus confronted with a major problem of explaining the observed salt-dependent high orientability of sDNA on the one hand and a lack of a permanent dipole on the other. The salt-dependent orientation of sDNA requires that the field-induced polarization depend at least in part on the polyionic charge and its interaction with the ionic atmosphere of the solvent. Based in part on the results of these experiments, an expression for the orientation has recently been proposed for rigid rodlike sDNA in the low-field range (Charney et al., 1980):

$$\Delta A_\parallel = \frac{1}{15}(3\cos^2\theta - 1)\left(2 + \frac{nK^2}{3}\right) \qquad (6)$$

Here $nK^2/3$ is the polyelectrolyte orientation energy parameter, where K is given by $ZeLE/(2KT)$, and n is the number of counterions of charge Z (Na^+ ions in this case) bound to the DNA [Mandel, 1961; see also Weill & Hornick (1974); Kikuchi & Yoshioka, 1976]. With this formulation, the total orientation energy at low or moderate fields is given by

$$\beta_m^2 \equiv \beta^2 = \frac{nK^2}{3} \qquad (7)$$

where the subscript m is used to emphasize that the moment deduced from K becomes independent of further increases in the field strength at some intermediate value of the field strength and thus mimicks a permanent dipole moment. On this basis, it is possible to calculate the number of bound or "condensed" counterions from the dichroism, provided that the length L of each sDNA is assumed to be equal to the contour length of a B-form DNA. Needless to say, this assumption is reasonably good only for low molecular weight fragments and will require critical review because the length should depend on the conformation of DNA. The results with this assumption are given in Table II. Two major observations may be made about these results: First, the number of bound counterions, n, is smaller than the number of bases in each sDNA, a fact which is consistent with counterion condensation theories (Manning, 1978a,b, 1979). This means that the charged DNA phosphate groups are not completely neutralized by the condensed or bound counterions. Second, the number of bound counterions appears to decrease with the increase of ionic strengths; this result is in contrast with a simple chemical equilibrium law but is qualitatively consistent with theoretical developments (Manning, 1978b). The quantitative relationship given in Table II results from the application of the Kikuchi–Yoshioka model. Unfortunately, the model does not account for counterion–counterion repulsion and thus cannot be expected to be an accurate description. However, qualitatively, the results do support an orientation model dependent on the polarization of the polyion with its condensed counterions. Finally, the result that the value of n for sDNA(L) differs absurdly from that of the lower molecular weight samples means that the use of these equations to describe even qualitatively the field orientation of high charge-density polyions much longer than the persistence length is open to serious question.

Dichroism at the Limiting High Field Strength. Because Φ approaches unity at infinitely high fields regardless of the orientation mechanism, the intrinsic (specific) parallel dichroism of a cylindrical molecule with axial symmetry is defined

$$\left(\frac{\Delta A_\parallel}{A}\right)_{E\to\infty} \equiv \left(\frac{\Delta A_\parallel}{A}\right)_s = 3\cos^2\theta - 1 \qquad (8)$$

The values of $(\Delta A_\parallel/A)_s$ at 270 nm and the corresponding apparent orientation angles of the transitions obtained by the matching procedure are given in Table III. It is interesting to note that the $(\Delta A_\parallel/A)_s$ values of sDNA are independent of ionic strengths and nearly but not quite independent of molecular weights in the range of $(1.24–5) \times 10^5$ and that they are all smaller than −1. The two following conclusions may be drawn: (1) the limiting secondary and/or tertiary structures of linear DNA at infinite orientation are independent of the solvent ionicity in the range from 0.09 to 1.0 mM NaCl; (2) the structure appears to be one in which the optical transition moment of the 260-nm band is not perpendicular to the principal axis of orientation. The corresponding angles range from 69° to 72° (Yamaoka & Charney, 1973). Under the assumption of a rigid cylindrical structure for B-form DNA, they would imply that the transition moment direction at 270 nm is not exactly perpendicular to, but rather inclined to the orientation axis of the short sonicated DNA in an orientating electric field, and it has been so interpreted (Hogan et al., 1978). This result, obtained by extrapolation from the matched curves, does not demonstrate unequivocally, however, that the *DNA base pairs* are tilted from the helix axis by 70°

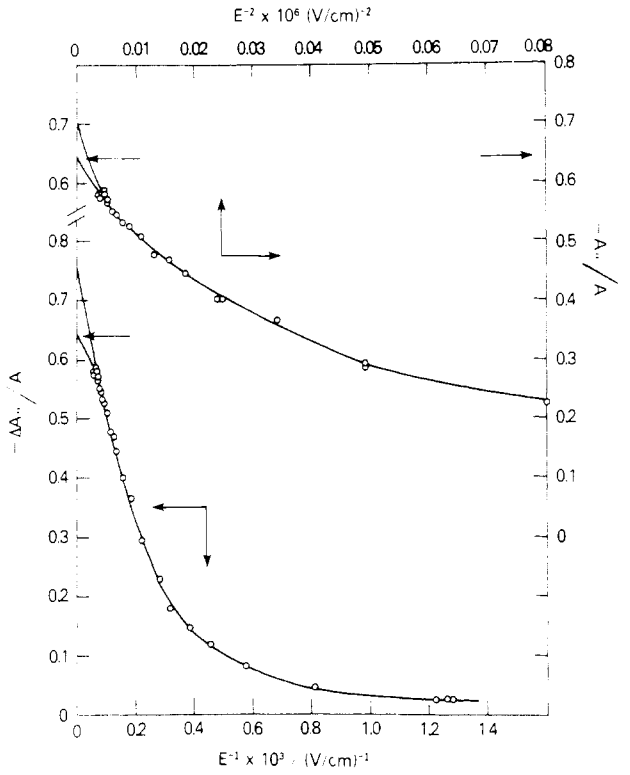

FIGURE 5: Illustration of the extrapolation method for obtaining the intrinsic parallel dichroism $(\Delta A_\parallel/A)_s$. The $\Delta A_\parallel/A$ values of sDNA(X) of Figure 4 are plotted against E^{-2} in the upper curve and against E^{-1} in the lower curve. A value of −0.64 was obtained by the matching procedure and is indicated with a horizontal arrow.

rather than the classical 84 ± 1°. Only if the molecule is a straight rod in the electric field and the principal axis of the orientation ellipsoid coincides with the structural axis of the double helix can the dichroism result be attributed to a tilt of the nucleoside bases in the double helix. It is just, if not more likely, that the orientation axis and double helix axis are not precisely coincident because of a bent or superhelical static structure, or because of a dynamic equilibrium between such structures (C. H. Lee and E. Charney, unpublished experiments). Furthermore, the angle θ is a composite angle unless the absorption band near 270 nm consists of a single transition free from the effect of other neighboring transitions thay may be directed at different angles. These are open questions yet to be verified in the double helix of DNA. Still another reasonable but as yet unproved explanation may be proffered for the smaller-than-expected value of the angle θ: a significant amount of the intensity of the band may be polarized parallel to the DNA double helix axis as a result of intensity borrowing from higher energy transitions. This explanation has been tentatively advanced by us earlier (Charney & Yamaoka, 1971) and is further discussed in a paper on the electric dichroism of poly(rA) (Charney & Milstien, 1978) and in another context by Rizzo and Schellman (personal communication). We are unable to definitively discriminate between these various structural and optical possibilities on the basis of the data reported here.

An alternative method of obtaining the value of $(\Delta A_\parallel/A)_s$ is based on the extrapolation of the experimental dichroism against $1/E$ or $1/E^2$ to infinite field strength (Ding et al., 1972). A comparison between the $(\Delta A_\parallel/A)_s$ values obtained by these techniques for one set of data on a sonicated DNA sample is shown in Figure 5. While the difference between the extrapolated values is structurally significant (the extrapolated value for the E extrapolation in Figure 5 corresponds to an angle of 74° as compared to 69° obtained by the matching procedure for the same DNA sample), the relative insensitivity of $\cos^2\theta$ to the values of $(\Delta A_\parallel/A)_s$ is this region makes it difficult to use this as a criterion for choosing between the methods. Still another extrapolation procedure has recently been suggested by Sokorov & Weill (1979). It requires that an estimate be made of the value of the nominally "permanent" moment produced when the ion-induced moment saturates at some value of the field strength. Further extrapolation of the data to higher field strengths, as a function of $1/\beta = kT/(\mu E)$, where μ is the value of the saturated induced moment, would lead to values of $(\Delta A_\parallel/A)_s$ for the lowest molecular weight DNA higher than those obtained by either of the first two methods. It is difficult to assess the validity of the last procedure, as the saturated moment obtained is so highly dependent on the ionic strength (see Table I).

Discussion

In addition to these results from our own laboratories and earlier related work summarized in the monograph by Fredericq & Houssier (1973) and in a review by Stellwagon (1976), several recent investigations of the electric field orientation of DNA, already referred to above, have addressed themselves in whole or in part to the same questions posed in this work, principally those of Sokorov & Weill (1979) and of Hogan et al. (1978). We consider first the results of Sokorov and Weill, noting that their experiments were done in the absence of added salt and at concentrations of DNA (on a nucleotide basis) from about 30% up to 5 times higher than ours and that the magnitude of the dichroism has been shown (Yamaoka & Charney, 1973) to be depressed by over 30% at concentrations twice the highest concentration used by those authors. This may account for quantitative difference between their work and ours. For example, their result for the apparent dipole moments of DNA with a contour length of about 1500 Å is 5×10^3 D whereas ours in the same range measured in 0.09 mM NaCl solution is about 11×10^3 D. In contrast, the moment calculated by Hogan et al. on the basis of an entirely different (ion-flow) ad hoc mechanism is about 13×10^3 D for a DNA nuclear digestion fragment only 900 Å long measured in 2.8 mM sodium phosphate buffer in contrast to ours of 11×10^3 D in 0.09 mM Na^+ and 5×10^3 D in 1.0 mM NaCl (nTable I). The ion-flow mechanism thus results in still larger calculated apparent moments, but as it does not distinguish between the polarization produced by an induced moment, which can saturate at modest field strength, and a nonsaturable polarization, it is not surprising that the total apparent moment will be larger than that calculated for a mechanism that does. Hogan et al. indeed argue that saturation would have to occur at very low field strengths in order to satisfy a saturable induced dipole moment and thus dismiss any such mechanism. On the other hand, they also argue that a pure induced nonsaturable moment should produce cubic dependence of the moment on the molecular length while they measure a quadratic dependence. A more recent work (Rau & Charney, 1981) has shown that the ionic atmosphere polarization *at low fields* is a variable function of both the ionic strength (in the form of the Debye shielding parameter) and the molecular length but that in the range of these experimental conditions an approximate quadratic dependence on the length is expected. The polydispersity of the sonicated samples makes it difficult to make a quantitative comparison of the length dependence. The results are nevertheless interesting; for this purpose, we consider only the two smallest sonicated samples whose mean molecular contour lengths, 630 and 880 Å, are smaller than the persistence length in 1 mM

Table IV: Dependence of the Orientation on the Molecular Contour Length[a]

part A			part B	
$\frac{\bar{L}_X}{\bar{L}_T}$	$\left(\frac{\bar{L}_X}{\bar{L}_T}\right)^2$	$\left(\frac{\bar{L}_X}{\bar{L}_T}\right)^3$	$\frac{(\Delta A_\parallel/E^2)_X}{(\Delta A_\parallel/E^2)_T}$	$[Na^+]$ (mM)
1.40	1.96	2.75	2.39	0.09
			2.10	0.20
			1.71	0.60
			1.45	1.00

[a] $\bar{L}_X$ and $\bar{L}_T$ are for sDNA(X) and sDNA(T), respectively, the mean contour lengths calculated on the basis of the sedimentation data and an assumed base pair repeat distance of 3.38 Å.

Na^+ (Borochov et al., 1981). In Table IV, we compare the ratio of the first, second, and third powers of their lengths (part A) with the ratio of the initial slope of the dichroism orientation curves (part B), the latter of which is proportional to the total orientation energy and thus to the moments in the sense of Hogan et al. We see first of all a strong dependence on ionic strength which can only in part be due to polydispersity as the half-width of the molecular weight distribution of these samples was substantially the same. In 0.6 mM Na^+ and more closely in 0.2 mM Na^+, the length dependence is nearly quadratic, but in 1 mM Na, it is linear within the accuracy of the measurement. Hogan has suggested reserve in interpreting dichroism measurements made below 2 mM Na^+ concentration. We are more inclined to believe that considerable structural and behavioral information is available in the low Na^+ concentration range. Moreover, although there is a dependence of the field-free relaxation time of polydispersed samples on ionic strength (K. Yamaoka, Fukudome, and E. Charney, unpublished experiments), there is no dramatic anomaly in this region as observed by Hogan in the rapidly rising field of the pulses produced in their temperature jump apparatus. Further exploration of the length dependence is under way in our laboratories, and a number of investigations of the relaxation phenomena have recently been published (Hagerman, 1981; Elias & Eden, 1981).

One of the principal objectives of this work was to establish the field strength dependence (at low fields) of the orientation of linear polyelectrolyte DNA. The result as expected is unequivocal in that the dependence is classically quadratic. Both the Sokorov and Hogan reports address this question with somewhat different results. Sokorov and Weill observe the quadratic dependence in their own measurement but place emphasis in their theoretical analysis on the nearly linear dependence at intermediate field strengths. Their plot of our earlier data (Figure 10 of their paper) on a linear E scale shows a marked deviation (it is in fact quadratic) for fields less than 6000 V/cm. Hogan et al. utilize the apparent lack of a quadratic dependence in their data to justify the ad hoc ion-flow model. However, a replot of their data on a quadratic scale shows that, within the precision of their measurements at low fields, a quadratic fit is entirely possible although the reported data are insufficient to uniquely demonstrate it. The polarization energy on which the degree of orientation of a molecular dipole is dependent has been known to be quadratic in E since 1910 (Langevin, 1910). It is difficult to conceive of an orientation mechanism which does not depend on the second power of E at low fields and which is not due to some hydrodynamic perculiarity. It is, of course, this latter possibility which led us to emphasize this point. The variation of the range of field strengths at which the quadratic dependence vanishes undoubtedly contains hydrodynamic information in the case of the longer flexible molecules.

Another objective of this work was to estimate the angle between the principal molecular axis of DNA (assumed to be rodlike) and the direction of the 260-nm transition moment. It is clear that neither our measurements nor those of other investigators can do this unequivocally. The data are in reasonable agreement with extrapolated values by various methods, indicating in this molecular weight range that a transition moment exists which is about 70° more or less to the axis of the orienting moiety, but the interpretation of this result in terms of DNA structure is still an unresolved question addressed in more detail in recent work from this laboratory (C. H. Lee and E. Charney, unpublished experiments).

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References

Benoit, H. (1951) *Ann. Phys. (Leipzig) 6*, 561–609.
Borochov, N., Eisenberg, H., & Kam, Z. (1981) *Biopolymers 20*, 231–235.
Charney, E. (1980) *Biophys. Chem. 11*, 157–166.
Charney, E., & Yamaoka, K. (1971) 23rd International Congress of Pure and Applied Chemistry, Preprint Vol. 1, pp 252–254, Boston, MA.
Charney, E., & Milstien, J. B. (1978) *Biopolymers 17*, 1629–1655.
Charney, E., Yamaoka, K., & Manning, G. S. (1980) *Biophys. Chem. 11*, 167–172.
Ding, D.-W., Rill, R., & Van Holde, K. E. (1972) *Biopolymers 11*, 2109–2124.
Elias, J., & Eden, D. (1981) *Macromolecules 14*, 410–419.
Fredericq, E., & Houssier, C. (1973) *Electric Dichroism and Birefringence*, Clarendon Press, Oxford.
Hagerman, P. (1981) *Biopolymers 20*, 1503–1536.
Hagerman, P., & Zimm, B. H. (1981) *Biopolymers 20*, 1537.
Hogan, M., Dattagupta, N., & Crothers, D. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 195–199.
Kikuchi, K., & Yoshioka, K. (1976) *Biopolymers 15*, 583–587.
Langevin, P. (1910) *Radium (Paris) 7*, 249.
Mandel, M. (1961) *Mol. Phys. 4*, 489–496.
Manning, G. S. (1978a) *Biophys. Chem. 9*, 65–90.
Manning, G. S. (1978b) *Q. Rev. Biophys. 11*, 179–246.
Manning, G. S. (1979) *Acc. Chem. Res. 12*, 443–449.
Marion, C., & Roux, B. (1980) *Biochem. Biophys. Res. Commun. 95*, 27–33.
Matsumoto, M., Watanabe, H., & Yoshioka, K. (1967) *Sci. Pap. Coll. Gen. Educ. Univ. Tokyo 17*, 173–202.
Nishinari, K., & Yoshioka, K. (1969) *Kolloid Z. Z. Polym. 235*, 1189–1192.
O'Konski, C. T., Yoshioka, K., & Orttung, W. H. (1959) *J. Phys. Chem. 63*, 1558–1565.
Olson, W. (1979) *Biopolymers 18*, 1213–1233.
Rau, D. C., & Bloomfield, V. A. (1979) *Biopolymers 18*, 2783–2805.
Rau, D. C., & Charney, E. (1982) *Biophys. Chem. 14*, 129–138.
Schellman, J. A. (1980) *Biophys. Chem. 11*, 321–328.
Sokorov, S., & Weill, G. (1979) *Biophys. Chem. 10*, 168–171.
Stellwagon, N. (1976) in *Molecular Electro-Optics* (O'Konski, C. T., Ed.) Vol. II, pp 645–683, Marcel Dekker, New York.
Stellwagon, N. (1981) *Biopolymers 20*, 399–434.

Studier, F. W. (1965) J. Mol. Biol. 11, 373-390.
Tinoco, I., Jr., & Yamaoka, K. (1959) J. Phys. Chem. 63, 423-427.
Weill, G., & Hornick, C. (1974) in Polyelectrolytes (Selegny, E., Mandel, M., & Strauss, V. P., Eds.) D. Reidel Publishing Co., Dordrecht, The Netherlands.
Yamaoka, K., & Resnik, R. A. (1966) J. Phys. Chem. 70, 4051-4066.
Yamaoka, K., & Charney, E. (1972) J. Am. Chem. Soc. 94, 8963-8974.
Yamaoka, K., & Charney, E. (1973) Macromolecules 6, 66-76.

Proton Magnetic Resonance Investigation of the Influence of Quaternary Structure on Iron-Histidine Bonding in Deoxyhemoglobins†

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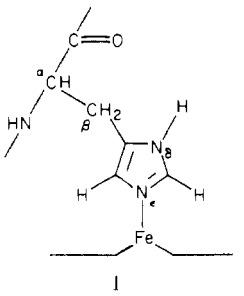
ABSTRACT: The proton nuclear magnetic resonance (NMR) spectra of the separated deoxy chains of normal adult hemoglobin (Hb A), the deoxy form of chemically modified hemoglobins, and Hb Kempsey [Asp-G1(99)β→Asn] have been investigated in H_2O solution with emphasis on the hyperfine-shifted proximal histidyl imidazole exchangeable $N_\delta H$ as indicators of the iron-histidine interaction. While the $N_\delta H$ hyperfine shift was found to be sensitive to chain origin and subunit assembly, the likelihood of change in both electronic effects and various steric strains precludes interpretation of the NMR data for different proteins at this time. However, upon introduction of conformational perturbations for any given protein, correlations between changes in NMR spectral parameters and altered function are interpretable in terms of changes in iron-imidazole bonding. For the individual chains, the α $N_\delta H$ shift, as well as the oxygen affinity, is insensitive to forming the *p*-(hydroxymercuri)benzoate (*p*MB) adduct, while the β-chain $N_\delta H$ shift decreases by 10 ppm and its oxygen affinity is reduced 6-fold by the same reaction. Since the *p*MB binding site is much closer to the proximal histidine of the β chain than the α chain, the β $N_\delta H$ upfield shift reflects some strain in the iron-histidine interaction. In the R quaternary structure, the deoxy subunits of NES-des-Arg(141α)-Hb, des-His(146β)-des-Arg(141α)-Hb, and des-Arg(141α)-Hb exhibit $N_\delta H$ resonances at approximately 77 ppm for both α and β subunits. The R → T conversion is accompanied by a 10-14-ppm decrease in the hyperfine shift of the α subunit; the β subunit is essentially unaffected. The decreased NH hyperfine shift is consistent with the introduction of some strain into the iron-histidine bond of the α subunit during the R → T transition. The observed $N_\delta H$ shift change for the α subunit is comparable to that observed upon binding *p*-(hydroxymercuri)benzoate to the isolated β chain and hence can account for only a small change in the oxygen affinity. In light of the resonance Raman data on $\nu(Fe-N_\epsilon)$, this study supports a dominant steric influence on iron-histidine bonding when Hb undergoes an R → T transition and argues against a major contribution of any electronic effect created by altered hydrogen bonding of $N_\delta H$ to a backbone carbonyl [Stein, P., Mitchell, M., & Spiro, T. G. (1980) J. Am. Chem. Soc. 102, 7795-7797]. In agreement also with the resonance Raman data [Nagai, K., Kitagawa, T., & Morimoto, H. (1980) J. Mol. Biol. 136, 271-289], the strain we observe is localized in the α subunit.

Central to the various hypotheses for the control of the iron reactivity in oxygen-binding hemoproteins in general, and hemoglobins in particular, is the state of the ubiquitous trans-proximal histidyl imidazole (I). Although the two



I

quaternary structures of hemoglobin (Hb)[1] (Monod et al., 1965; Perutz, 1970) have been characterized in some detail by X-ray crystallography, resolution is still insufficient to pinpoint the specific structural differences at the heme which characterize the contrasting O_2 affinities (Perutz, 1976; Baldwin & Chothia, 1979). A difference in steric effects has been postulated by Perutz, where the protein produces strain in the Fe-N_ϵ bonds in the low-affinity T structure which is relaxed in the high-affinity R structure (Perutz, 1979, 1980). Some support for weaker iron-imidazole bonding in the T rather than the R state has been provided by resonance Raman studies (Nagai et al., 1980; Nagai & Kitagawa, 1980). A prominent electronic influence on iron reactivity has been proposed on the basis of variable hydrogen bonding of the proximal histidyl imidazole $N_\delta H$ to a backbone carbonyl (Valentine et al., 1979) which would modulate the imidazole

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[1] Abbreviations: NMR, nuclear magnetic resonance; Hb, hemoglobin; Hb A, normal adult hemoglobin; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane; Tris, tris(hydroxymethyl)aminomethane; *p*MB, *p*-(hydroxymercuri)benzoate; IHP, inositol hexaphosphate; NES, 5-(*N*-ethylsuccinamido)cysteinyl; ppm, parts per million; EXAFS, extended X-ray absorption fine structure.

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