Rowan, L. G., Hahn, E. L., and Mims, W. B. (1965), *Phys. Rev. A 137,* 61–71.
Stigbrand, T., Malmström, B. G., and Vänngård, T. (1971), *FEBS Lett. 12,* 260–262.
Sutherland, I. W., and Wilkinson, J. F. (1963), *J. Gen. Microbiol. 30,* 105–112.
Suzuki, H., and Iwasaki, H. (1962), *J. Biochem. (Tokyo) 52,* 193–199.
Walker, F. A., Sigel, H., and McCormick, D. B. (1972), *Inorg. Chem. 11,* 2756–2763.
Yudanov, V. F., Raitsimring, A. M., Tsvetkov, Yu. D. (1968), *Theor. Exp. Chem. 4,* 335–341.
Zhidomirov, G. M., and Salikhov, K. M. (1968) *Theor. Exp. Chem. 4,* 332–334.

Comparison of the Phosphorus Magnetic Resonance and Circular Dichroism Properties of Calf Thymus DNA and Chromatin†

Sue Hanlon,* Thomas Glonek, and Aurelia Chan

ABSTRACT: Dual measurements of the ^{31}P magnetic resonance spectra and the circular dichroism spectra have been made on calf thymus DNA and purified chromatin. The ^{31}P magnetic resonance signals for all samples fell at 1.2 ± 0.1 ppm relative to 85% orthophosphoric acid. The full width at half-height of the signal of samples in which the molecular weight of the native DNA component was in the 2–9 × 10^6 range was ca. 50 Hz. This bandwidth was reduced dramatically to ca. 20 Hz by reducing the molecular weight to 140 000 (by sonication) or by heat denaturation of the high-molecular-weight DNA. The position of the signal and the bandwidth of the chromatin samples did not differ significantly from that of the DNA samples of comparable molecular weight and state of nativity. The intensities of the chromatin signals, however, were all less than those of signals of DNA in companion runs conducted under comparable experimental conditions. The reduction of the intensity of the magnetic resonance signal paralleled the lowering of the intensity of the positive band above 260 nm in the circular dichroism spectrum of the given sample of chromatin relative to the spectrum of protein-free DNA. In fact, the percent reduction of the magnetic resonance signal of chromatin relative to protein-free DNA was, within experimental error, equal to the percentage of nucleotide residues in the Watson–Crick B secondary structure. Since the latter fraction of residues can be correlated with those in the interbead regions of the superstructure of chromatin, we have concluded that the signal of the nucleotide residues in the beads, or υ bodies, has been broadened to the point of extinction by a packing arrangement which maximizes phosphate–protein interactions and structural rigidity.

In preceding papers from this laboratory we have proposed a model for purified calf thymus chromatin in which the DNA constituent is in two discrete secondary structures. As ascertained by circular dichroism (CD)[1] spectral analysis, anywhere from 30 to 50% of the nucleotide residues are in a form resembling the Watson–Crick B conformation, whereas the remainder are in a C conformation, which is a helical variant of the B form. The distribution of residues between the two conformations can be varied by several methods. Exhaustive dialysis against EDTA,[1] high concentrations of denaturants such as urea, proteolytic digestion, and removal of histone proteins with dissociative reagents such as sodium deoxycholate or NaCl will result in substantial increases in the B character at the expense of the C (Hanlon et al., 1974a), whereas nuclease digestion (with subsequent removal of the low-molecular-weight products of digestion) results in a macromolecular product which displays a CD spectrum almost identical to that exhibited by the C form of DNA (Hanlon et al., 1972; Johnson et al., 1972; Rill and Van Holde, 1973; Hanlon et al., 1975). The arguments in favor of discrete conformations in intact chromatin have included the results of this latter experiment, as well as the almost exact correlation between the %B character and the fraction of bases melting out in the lower transition regions of the complex melting profile which is exhibited by purified chromatin (Hanlon et al., 1972, 1974b; Johnson et al., 1972).

Electron microscopic observations on chromatin from interphase nuclei have revealed a structure resembling "beads on a string" (Olins and Olins, 1974). The beads, referred to as υ bodies by Olins and Olins (1974), can be produced by nuclease treatment (Van Holde et al., 1974; Finch et al., 1975) or extensive sonication (Senior et al., 1975). Since the products of digestion by nucleases have a CD spectrum which is almost identical with C spectrum DNA above 250 nm (Johnson et al., 1972; Rill and Van Holde, 1973), it is reasonable to conclude that the DNA in the "beads" or υ bodies is in the C conformation, whereas the DNA in the interbead or string regions of intact chromatin is in the B conformation (Hanlon, S., Berman, S., and Chan, A., in preparation).

A further aspect of our model includes the limited accessibility of the portions of chromatin in which DNA is in the C form. These regions are resistant to nuclease digestion and seem to be very well stabilized to heat denaturation as they melt in the high T_m transitions of the thermal melting profile of chromatin (Johnson et al., 1972; Rill and Van Holde, 1973).

† From the Department of Biological Chemistry, College of Medicine, and the Research Resources Center of the University of Illinois at the Medical Center, Chicago, Illinois (60612). *Received February 19, 1976.* This work was supported by Grant BMS 75-16488 from the National Science Foundation, and Grant PHS-GRSG 615 from the National Institutes of Health.

[1] Abbreviations used are: EDTA, the sodium salt of (ethylenedinitrilo)tetraacetic acid; CD, circular dichroism; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; TNH, purified chromatin.

TABLE I: Properties of DNA and Chromatin Samples Examined by ^{31}P Magnetic Resonance.

Sample	W (g/g)	$s^0_{20,w}$ DNA (S)	$M_{DNA} \times 10^{-6}$	E_{259} (M^{-1} cm^{-1})	Circular Dichroism: $[\theta]_{275} \times 10^{-3}$ (deg cm^2 $dmol^{-1}$) Before	After	Circular Dichroism: % B ± 2 Before	After
DNA	0.01	21.2	8.6	6600	8.42	8.42	98	98
DNA (denatured)	0.01			9050		9.20		
DNA (sonicated)	0.01	5.4	0.14	6617	8.42	8.42	98	98
TNH (1)	1.44	13.7	2.7	6750	3.73	3.63	43	43
TNH (2)	1.62	20.5	7.9	6750	3.48	3.59	43	43
TNH (3)	1.48	15.5	3.8	6750	3.38	7.90	42	90

This would imply a particularly effective association of the DNA in this conformation with the basic histone proteins maintaining that conformation. This package of histone proteins, furthermore, is particularly well structurally organized, possessing either a high average helix content (Hanlon et al., 1974b) or relatively long stretches of peptide residues in helical secondary structures (Berman and Hanlon, unpublished).

The general picture that emerges from these as well as other current studies on chromatin (for a comprehensive review see Elgin and Weintraub (1975)) is that the DNA component of purified chromatin is in two grossly different structural and chemical environments. With this in mind, we have undertaken an examination of the phosphorus resonance spectral properties of the phosphodiester signal in DNA and purified chromatin in order to ascertain whether this difference in environment affects the magnetic resonance signal and whether this is correlated with the circular dichroism properties of the DNA component. This present paper reports the results of these studies with intact chromatin whose DNA component possesses variable content of B character, as well as samples of protein-free DNA in various states of nativity.

Experimental Procedure

A commercial sample of calf thymus DNA obtained from Calbiochem (lot no. 900007) was employed in the DNA experiments. Its protein content was experimentally determined by the Lowry method (Lowry et al., 1951) to be less than 1%. Its extinction coefficient, E_{259}, at 259 nm had been previously determined as 6600 M^{-1} cm^{-1} (Johnson et al., 1972). A denatured sample was prepared by heating a solution of this preparation at a concentration of 1.73 mM (in phosphorus) in the solvent (3 mM NaCl, 3 mM EDTA, 2 mM Tris, pH 7.3) for 10 min at 90 °C and then quickly cooling to room temperature by plunging the heated solution into an ice bath. Another aliquot of the solution of the native high-molecular-weight sample of DNA at a concentration of 2 mM in 20 mM NaCl, 2 mM EDTA, 2.5 mM Tris, pH 7.4, was sonicated at 1 °C for a total of 150 s in 10-s pulses, separated by 30-s intervals, in a Sonifier cell disruptor (Model W185 D) in order to reduce the molecular weight. The extinction coefficients of the denatured and the lower molecular weight sample were determined by measuring the absorbance of the given solution before and after the experimental manipulation.

Purified chromatin (TNH[1]) was prepared from frozen calf thymuses by Method i of Maurer and Chalkley (1967). The details of the tissue collection as well as the physical and chemical properties of the product, including its extinction coefficient at 259 nm (6750 M^{-1} cm^{-1}), have been previously described (Johnson et al., 1972). One of the samples (Sample 1) was sheared according to the method of Shih and Fasman (1970) in order to reduce its molecular weight.

Absorption spectra were measured over a wavelength range of 400–220 nm in a Cary Model 14 CMR recording spectrophotometer equipped with thermostated adaptors. Temperature was maintained at 25 °C by a Haake circulating water bath and monitored by a Telethermometer Bridge and probe assembly manufactured by Yellow Springs Instrument Co. Minor corrections (ca. 3%) of the absorption maximum in a given spectrum were generally made for the effects of light scattering, using the approach of Oster (1948). Spectra obtained and corrected in this manner were employed for the calculation of concentrations of the macromolecular solutes, DNA and chromatin, using the extinction coefficients reported in Table I. These concentrations are all given in terms of mol of nucleotide/l., or mol of phosphorus/l.

The protein content of the chromatin preparations was also determined spectrally, after correcting for light scattering, by measuring the ratio of the absorbance at 230 and 260 nm and substituting in the equation of Tuan and Bonner (1969) as previously described (Johnson et al., 1972). Protein content is expressed for chromatin as a weight ratio, W, of protein to DNA.

Circular dichroism spectra were determined at 27 °C in a 1-cm, quartz cell with a Cary Model 60 recording spectropolarimeter equipped with a 6001 CD attachment. All spectral data are given in terms of molecular ellipticities, based on the nucleotide, or phosphorus, molar concentration in solution. These spectral data were analyzed for fractional amounts of the A, the B, and the C secondary structures of DNA by methods described in our earlier publications (Hanlon et al., 1972, 1974a, 1975; Johnson et al., 1972). Basically, the CD spectrum of chromatin above 260 nm, which reflects only the DNA contribution, is assumed to consist of a linear combination of the contributions of the various secondary structures. The observed molecular ellipticities, $[\theta]_\lambda^{obsd}$, at selected wavelength positions, λ, in the spectral region may thus be expressed as

$$[\theta]_\lambda^{obsd} = f_A[\theta]_\lambda^A + f_B[\theta]_\lambda^B + f_C[\theta]_\lambda^C \quad (1)$$

where $[\theta]_\lambda^A$, $[\theta]_\lambda^B$, and $[\theta]_\lambda^C$ are the reference ellipticities of the A, the B and the C secondary structures at wavelength λ and f_A, f_B, and f_C are the fractions of the total nucleotide population present in those conformations. By appropriate least mean square averaging procedures, one may solve for the fractions of these several conformations, using the reference spectra in Hanlon et al., 1975. This approach revealed that, within the experimental error of the determination, 96% of the entire nucleotide population in the chromatin complexes could

be accounted for in terms of the B and the C secondary structures.

Molecular weights of DNA and the DNA component of chromatin were estimated from the data from sedimentation velocity experiments. The experiments on protein-free DNA were conducted in 0.10 M NaCl, 0.05 M NaH_2PO_4–Na_2HPO_4 buffer, pH 7. The measurements on the DNA of chromatin were made in 2.5 M NaCl, 0.05 M NaH_2PO_4–Na_2HPO_4 buffer, pH 7, in order to dissociate the protein from the DNA. Ultracentrifuge studies were conducted with a Spinco Model E analytical ultracentrifuge equipped with absorption optics suitable for following the DNA component. Films obtained from this optical system were scanned with an Analytrol densitometer. Top loading cells were employed in order to avoid shear degradation of the higher molecular weight samples. The median sedimentation coefficient was determined by following the half-height position of the boundary as a function of time. Total concentrations of sedimenting species were all less than 1.2×10^{-4} M, at ionic strengths of 0.1 M or higher, and hence the observed data can be assumed to be that appropriate for infinite dilution. All sedimentation coefficients were corrected to standard conditions of a solvent with the viscosity and density of water at 20 °C (Schachman, 1959) and the preferential solvation of DNA in those solvents when this correction was significant (e.g., 2.5 M NaCl, 0.05 M NaH_2PO_4/Na_2HPO_4). For these latter corrections, the data of Cohen and Eisenberg (1968) were employed.

Molecular weights were calculated from the median sedimentation coefficients by using either the equation of Eigner and Doty (1965)

$$s^0_{20,w} = 0.116\ M^{0.325} \quad (2)$$

for the sonicated sample, and the equation of Crothers and Zimm (1965)

$$0.445 \log M = 1.819 + \log [s^0_{20,w} - 2.7] \quad (3)$$

for the higher molecular weight preparations.

Phosphorus (^{31}P) nuclear magnetic resonance spectral analyses were performed in a manner previously described (Glonek et al., 1970, 1974). The spectrometer employed was a Bruker HFX/5 with ^{2}D stabilization, operating at 36.43 MHz for ^{31}P (21 kG magnetic field) and incorporating facilities for all modes of Fourier transform signal-averaging and broad-band and continuous wave heteronuclear ^{1}H decoupling. All spectra were obtained under ^{1}H decoupling conditions. Large bore (10–13 mm) spinning sample tubes were employed with sample volumes of from 3–5 ml at concentrations of 10^{-3} M in phosphorus at 27 °C. Smaller bore capillaries containing various reference compounds were coaxially mounted within the sample tube. These references served the several functions of providing a field frequency ^{2}D locking signal for stabilization and a shift and/or intensity (area) reference for the polynucleotide signals. Initial experiments employed only acetone-d_6 as a locking signal in order to view the polynucleotide signal in the absence of interfering signals from reference compounds containing phosphorus. Relative intensities of the phosphodiester signals in companion experiments were estimated by comparing spectra taken under identical instrumental conditions at comparable signal/noise ratios.

Later experiments employed additional capillaries containing phosphorus compounds as intensity and shift references. A number of these employed a capillary containing inorganic sodium pyrophosphate and its hydrolysis product, inorganic orthophosphate, in D_2O at a total concentration of phosphate of 0.05 M. When freshly prepared at a pD of 9.5, the sodium pyrophosphate signal fell at 8.28 ppm, relative to 85% orthophosphoric acid. With continued use, the pyrophosphate hydrolyzed yielding orthophosphate whose signal fell at ca. −1 ppm. As the pH decreased in this process, the two peaks, corresponding to pyrophosphate and its breakdown product, orthophosphate, shifted upfield and hence this capillary could not be used as a shift reference. The total area under the two peaks, however, remained constant, regardless of the extent of hydrolysis and the shift in signal. Thus, this capillary was employed as an intensity reference in a series of experiments comparing the intensity of DNA and chromatin signals.

The experiments comparing DNA samples of two different molecular weights employed a different phosphorus reference, methylenediphosphonate at 0.05 M in D_2O (5 M NaOD). This compound provided a more stable signal in the form of a single peak at −16.2 ppm, relative to 85% orthophosphoric acid, which did not change appreciably with time. It could thus be used as both an intensity and a shift reference. This signal was sufficiently displaced from the phosphodiester signal such that the integrator function of the instrument could be used. The relative intensity of the sample signal was calculated as the ratio of the magnitude of the integrator signal, h', for the sample and the reference.

When the pyrophosphate capillary was used as a intensity reference, however, the signal due to the orthophosphate overlapped significantly with the sample signal. This necessitated measuring the areas under the two signals after resolving them into their respective curves. We approximated the latter by Gaussians whose maxima were located at the observed peak positions of the signals. The areas were measured by tracing the resolved peaks and that peak due to pyrophosphate on graph paper and weighing the cutouts. The relative intensity of the sample signal was calculated as the ratio of the area under the resolved sample signal to the sum of the areas of the resolved ortho- and the pyrophosphate peaks.

For purposes of comparing the intensities of the DNA and the TNH signals, the relative intensities obtained as described above were divided by the concentration of the sample to yield normalized intensities which were independent of instrumental conditions and concentration of sample. In the one set of experiments in which an intensity reference was not employed (TNH sample 1 and its companion DNA run) the normalized intensity was calculated by simply dividing the area of the sample peak by the concentration of sample. In order to compare the normalized intensities of the TNH sample with that of the DNA in a companion experiment, spectra were selected that were obtained under the same instrumental operating conditions.

Because the ^{31}P signals obtained in these experiments were very broad and the concentration of sample quite low, extensive signal averaging of the samples of 2–4 days duration was required to reduce the level of the background spectral noise for accurate signal area and signal amplitude measurements. In a typical determination on native high-molecular-weight DNA, for instance, 270 563 repetitive measurements were averaged over a period of 2.6 days to yield a signal with a root mean square signal to noise ratio of 57.5. (Instrumental conditions for this averaging were as follows: total sweep width examined, 2500 Hz (200 μs/data point), pulse width, 4.5 μs, cycling time, 832 ms, 4K data points/free induction decay.) In the above case, a filter time constant introducing a 9.3 Hz broadening of the bandwidth at half-height was employed, although filter time constants introducing as little as 1.2 Hz broadening were

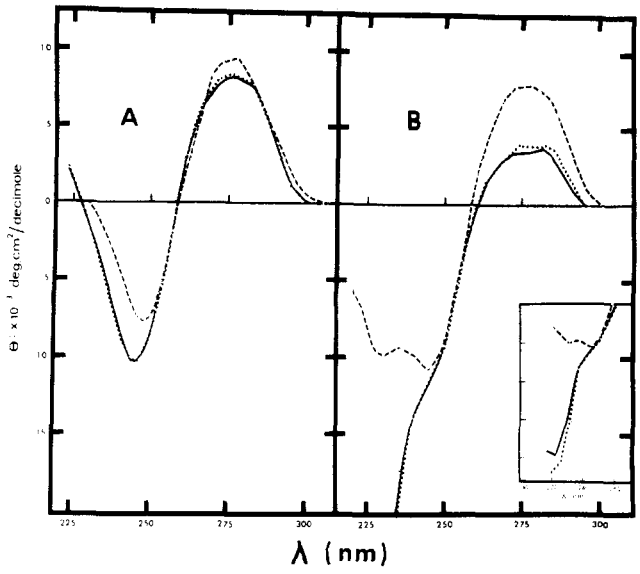

FIGURE 1: Circular dichroism spectra of samples of DNA and chromatin recovered from the ^{31}P magnetic resonance experiments. These spectra were obtained at 27 °C and plotted in terms of molecular ellipticity, $[\theta]$ (in deg cm^2 dmol^{-1} of nucleotide) vs. wavelength, λ, in nm. (A) Spectra of DNA: native DNA of 8.6×10^6 daltons (—) and 240 000 daltons (····) in the solvent (20 mM NaCl, 2 mM EDTA, 2.5 mM Tris, pH 7.3). Heat denatured DNA (- - - -) in the solvent (3 mM NaCl, 3 mM EDTA, 2 mM Tris, pH 7.3). (B) Spectra of chromatin: (····) sample (1) in the solvent (0.7 mM EDTA, 2.0 mM Tris, pH 7.5), (—), sample (2) in the solvent (2.0 mM EDTA, 2.5 mM Tris, pH 7.8), (- - - -) sample (3) in the solvent (2 mM EDTA, 2.5 mM Tris, pH 7.8). The inset in the lower right hand corner of this panel displays the spectral properties of these three samples below 260 nm in the region in which the protein constituents make significant contributions.

successfully employed for the higher molecular weight material as well. Spectral analyses of peak position, bandwidth (full width at half-height), and intensity always employed those spectra taken under conditions of minimum broadening by filter time constants.

Results and Discussion

Since we anticipated that protein content, molecular weight, state of nativity, and secondary structure might be important variables in these magnetic resonance studies, these properties were examined in the manner described under the Experimental Section of this paper. Table I presents the results of these analyses. Column 2 gives the protein/DNA weight ratio (W) (an important variable for TNH), column 3 gives the sedimentation coefficients, $s^0_{20,w}$, of the *DNA component,* and column 4 gives the molecular weight calculated from these values of $s^0_{20,w}$. The fifth column gives the extinction coefficients at 259 nm on a mol of nucleotide/liter basis. The high value for the denatured DNA reflects almost complete base unpairing, as well as some degradation which ensued during the heating process. The slight elevation of the extinction coefficient for TNH compared to protein free DNA reflects the small protein contribution at this wavelength.

The circular dichroism spectra of the samples of DNA and chromatin were examined both before and after having stood at room temperature for 4 days, the maximum length of time required for the magnetic resonance experiments. In some cases, spectra were run on solutions recovered from the magnetic resonance experiments. In all but one instance, these "before" and "after" spectra were identical, although the CD characteristics of the individual preparations varied among themselves. The after spectra are shown in Figure 1. The values of $[\theta]$ at 275 nm, as well as the results of the analysis of the secondary structure of the DNA constituent, are presented in Table I. The characteristics of the native protein-free DNA samples, both high- and low-molecular-weight preparations, and the TNH samples (1) and (2) are identical to those previously obtained in this laboratory (Hanlon et al., 1975; Johnson et al., 1972) under comparable experimental conditions. (It might be noted that TNH preparations in the presence of EDTA typically exhibit a fractional B content higher than the usual value of 35% obtained with preparations isolated by the Maurer and Chalkley method.)

In contrast to the stability of the spectral properties of TNH samples 1 and 2, the spectral properties of sample 3 altered dramatically upon standing at room temperature for the length of time required for the magnetic resonance experiments. The CD spectrum of this sample after 4 days at room temperature is shown as the dashed curve in Figure 1B. This preparation appeared to have possessed a highly active endogenous protease whose action on the histone proteins resulted in a disruption of the tertiary structure of the preparation.[2] The unfolding of the υ bodies results in the loss of the C secondary structure with concommitant increases in the B character of the DNA constituent. The positive band of this spectrum is almost identical with that of protein-free DNA. There are also corresponding marked changes in the intensity of the negative band at 220 nm, reflecting changes in the secondary structure and extensive degradation of the protein constituents as well.

Some typical ^{31}P magnetic resonance spectra of DNA are shown in Figure 2A,B and the spectral characteristics of these as well as other samples are reported in Table II. Figure 2A compares the spectra of DNA of 9×10^6 daltons in its native and its denatured states. Figure 2B shows the spectra of the same native sample before and after sonication that reduced its molecular weight to 140 000. The minor peak in this latter figure at ca. −16 ppm is due to the intensity and shift reference, methylenediphosphonate. The position of the major peak in both Figures 2A,B at ca. 1.0 ppm coincides with that previously reported for the phosphodiester signal of the polynucleotide backbone of tRNA (Guéron, 1971; Guéron and Shulman, 1975). (At these molecular weights, the contributions of the terminal 5′ and 3′ phosphates would not be significantly above the background noise, and hence are undetectable in the present experiments. They would normally be expected to fall anywhere from −1 to −4 ppm downfield (Guéron and Shulman, 1975) from the main cluster of phosphate resonances.) The average position for the signal of the native samples of DNA is 1.18. Because of the breadth of the peaks and the low signal/noise ratios, the experimental precision is on the order of ±0.1 ppm. Hence we do not feel that the signal position of denaturated DNA at 1.1 ppm is significantly different from that of the native samples.

As might be anticipated, the phosphodiester signal for the intact high-molecular-weight sample of DNA is quite broad,

[2] The evidence for an enhanced endogeneous protease activity in sample 3 is indirect. If the CD spectra of dilutions of sample 3 are followed as a function of time of standing at room temperature, a pattern of changes is observed similar to that which is obtained when chromatin is digested by pronase, with the only difference being that of the time scale. These changes in the CD spectra are paralleled by a substantial reduction in the frequency of appearance and increases in the average center to center distance of intact υ bodies of the sample, as observed by electron microscopy. This slow transformation of the CD spectral properties and the disappearance of the υ bodies can be significantly retarded by the addition of $NaHSO_3$, a potent protease inhibitor, at concentrations of 0.001-0.005 M to the chromatin sample.

TABLE II: ^{31}P Magnetic Resonance Characteristics of Calf Thymus DNA and Chromatin.

Sample	$M_{DNA} \times 10^{-6}$	Concn (mM in P)	Solvent (pH 7.3–7.8)	$\Delta\nu_{1/2}$[a] (Hz ± 5)	μ[b] (ppm)	% Intensity ±5%
DNA	8.6	1.73	3 mM NaCl 3 mM EDTA 2 mM Tris	41	1.2	100
DNA (denaturated)		1.73	Same as above	19	1.1	85–95
DNA	8.6	2.20	20 mM NaCl 2 mM EDTA 2.5 mM Tris	46	1.15	100
DNA	8.6	4.46	20 mM NaCl 2 mM EDTA 2.5 mM Tris	53	1.15	100
DNA	8.6	1.24	Same as above	56	1.20	100
DNA (sonicated)	0.14	1.41	Same as above	17 Av:	1.20 1.18	100
TNH (1) % B = 43	2.7	2.83	2 mM Tris 0.7 mM EDTA	50	1.15	47
TNH (2) % B = 43	7.9	1.90	2.5 mM Tris 2 mM EDTA	55	1.20	43
TNH (3) % B = 90	3.8	4.12	2.5 mM Tris 2 mM EDTA	55 Av:	1.30 1.22	94

[a] Full width at half-height, corrected for broadening by filters. [b] Relative to 85% orthophosphoric acid.

the full width at half-height, $\Delta\nu_{1/2}$, being about 40–50 Hz. This broadness reflects the relatively long half-life of all phosphate conformations in the polynucleotide structure. This long lifetime is, in part, attributable to the relative rigidity of the organized secondary structure of the nucleic acid and, in part, due to the excessively high micro- and macroviscosity of the solution. At these DNA concentrations, the solution viscosity is little affected by changes in polynucleotide concentration and ionic strength less than an order of magnitude. Hence, it is not surprising that the data in Table II reveal little change in the line width as these factors are varied by two- to threefold.

When the solution viscosity is dramatically reduced and polymeric restraints are relaxed, by either sonication or heat denaturation of the DNA sample, however, the phosphodiester signal sharpens considerably, exhibiting a half-width of ca. 20 Hz. There are corresponding increases in the signal intensity at the maximum, but the integrated area under the peaks remain essentially constant, compared to intact native high-molecular-weight DNA. Within experimental error, the position of the signal also remains constant, relative to the high-molecular-weight DNA.

Figure 3A,B,C show spectra of chromatin samples 1, 2, and 3, respectively. In these figures, we have also reproduced the spectra of DNA solutions obtained in companion runs under equivalent instrumental conditions. In Figure 3A, we have reproduced the spectra of chromatin sample (1) and DNA, obtained without an added external phosphorus reference, over the 2500-Hz scan (corresponding to a total chemical shift of 69 ppm) in order to demonstrate the absence of any signal, other than the ones falling at 1 ppm, significantly greater than the noise in these spectra. The additional peaks seen in Figure 3B,C represent the intensity reference, pyrophosphate (8 ppm)

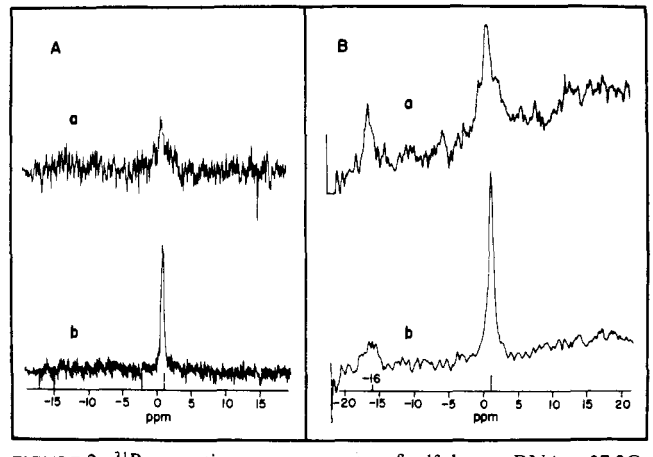

FIGURE 2: ^{31}P magnetic resonance spectra of calf thymus DNA at 27 °C. The 0 ppm position is that for 85% orthophosphoric acid. (A) Intact and heat denatured DNA of 8.6 × 10^6 daltons. Curve a is the spectrum of native DNA, while curve b is the spectrum of the same sample after heat denaturation. Both solutions are at a concentration of 1.7 mM in nucleotide residues in a solvent of 3 mM NaCl, 3 mM EDTA, 2 mM Tris, pH 7.3. Filter broadening is 2 Hz. (B) Effects of variations in molecular weight. Curve a in the spectrum of DNA of 8.6 × 10^6 daltons at a concentration of 1.2 mM. Curve b in the spectrum of the sample at 1.4 mM after sonication to a molecular weight of 140 000 daltons. The peak at −16 ppm in both spectra is due to methylenediphosphonate that served as a shift and intensity reference in these experiments. The solvent in both cases was 20 mM NaCl, 2 mM EDTA, 2.5 mM Tris, pH 7.4. Signal broadening due to filters was 9.3 Hz.

and its hydrolysis product, orthophosphate (−1 ppm). The spectra shown in Figure 3B were obtained at a time where the pyrophosphate had hydrolyzed almost entirely to orthophosphate.

The characteristics of these TNH spectra are also given in

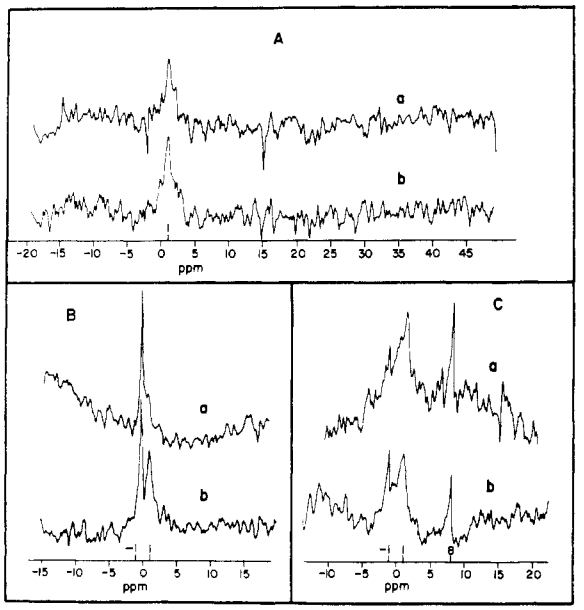

FIGURE 3: ^{31}P magnetic resonance spectra of calf thymus chromatin compared to DNA. The following spectra were taken with a filter that introduced a signal broadening of 9.3 Hz. The 0 ppm position is that for 85% orthophosphoric acid. (A) The spectrum of chromatin between −20 and 45 ppm is shown in curve a, while that of DNA, obtained under equivalent instrumental conditions, is shown in curve b. The chromatin was at a concentration of 2.83 mM in 0.7 mM EDTA, 2.5 mM Tris, pH 7.4. DNA was at a concentration of 1.73 mM in 3 mM NaCl, 3 mM EDTA, 2 mM Tris, pH 7.3. (B) The spectrum of chromatin sample 2 is shown in curve a, while that of DNA obtained under equivalent instrumental conditions is displayed as curve b. The peak at ca. 0 ppm represents the orthophosphate breakdown product of the pyrophosphate that the reference capillary originally contained. The chromatin was at a concentration of 1.9 mM (in P) in 2 mM EDTA, 2.5 mM Tris, pH 7.6, while the DNA spectrum was obtained at a concentration of 2.2 mM (in P) in 20 mM NaCl, 2 mM EDTA, 2.5 mM Tris, pH 7.4. (C) The spectrum of chromatin sample 3, possessing an active endogeneous protease, is shown in curve a and the result of the companion experiment with protein-free DNA is displayed as curve b. The chromatin solution was at a concentration of 4.1 mM (in P) in 2 mM EDTA, 2.5 mM Tris, pH 7.5, while the DNA was at a concentration of 4.5 mM (in P) in 20 mM NaCl, 2 mM EDTA, 2.5 mM Tris, pH 7.4. The peaks at ca. 8 and −1 ppm represent the components in the intensity reference capillary, pyrophosphate, and its hydrolysis product, orthophosphate.

Table II. The average position of the TNH signal is 1.22 ± 0.1 ppm, a value which does not vary significantly from the average position of the DNA signal. The bandwidths of these TNH spectra were also approximately those observed for native DNA of comparable molecular weight.

The magnitudes of the phosphodiester signals of the TNH samples are, however, significantly different from those of DNA. The integrated intensities as well as the intensity at the peak maximum, normalized for differences in concentration, were always less than that of the signals for DNA run under comparable experimental conditions. This reduction was particularly significant for samples 1 and 2, although in the case of sample 3, the normalized intensity came very close to that observed in the companion DNA experiment. The extent of the intensity reduction is shown in Table II as a percentage reduction of the DNA signal obtained in companion experiments. It might be noted that in all three samples, the reduction of the intensity of the TNH signal corresponds, within experimental error, to the reduction in the fraction of the nucleotide residues in the B conformation of the DNA constituent of TNH, determined at the end of the magnetic resonance experiment. (This latter property is reproduced in this table in the sample column.)

A search over a wider magnetic field for the missing intensity in the form of an additional signal was unsuccessful, as is demonstrated by the absence of an appreciable signal in Figure 3A. It might be noted in passing that the concentration of the chromatin sample (1) in the experiment shown in this figure (Figure 3A) was twice that of the DNA control shown in the lower curve b. Thus, the conditions for detecting the missing intensity, if due to a shifted signal, were adequate for bands as broad as the phosphodiester signal at 1 ppm.

Conclusion

There are several explanations for the origin of the pronounced loss of intensity of the ^{31}P signal in TNH samples 1 and 2, compared to 3 and native DNA. The presence of protein per se is not a factor, since sample 3 still had a considerable amount of associated protein. Rather it is more likely attributable to the state of integrity of the "beads", or υ bodies, and the relative proportion of interbead regions. Using the %B character as a reflection of the fraction of nucleotide residues in the latter regions, one would conclude that only the DNA in these interbead portions of the chromatin exhibit a ^{31}P signal. The structural packaging of the DNA in the beads is such that the phosphate signal has been broadened to the point of extinction. This could arise because of one or both factors: (1) the packaging of the DNA in the beads is so rigid that essentially no mobility is permitted a given nucleotide residue and/or (2) the phosphates of the DNA constituent of the υ bodies are interacting strongly with appropriate functional groups of the protein constituents (i.e., the positively charged side chains of arginine, lysine and histidine).

Whatever the origin of this effect, the reduction of the intensity of the ^{31}P signal without marked changes in the position or width of the band adds further support to the conclusion that the DNA of purified chromatin exists in two discrete environments. The striking correlation between these results and the CD data also supports the conclusion that the secondary structure of the DNA in these two regions is different.

References

Cohen, G., and Eisenberg H. (1968), *Biopolymers 6,* 1077–1100.

Crothers, D. M., and Zimm, B. H. (1965), *J. Mol. Biol. 12,* 525–536.

Eigner, J., and Doty, P. (1965), *J. Mol. Biol. 12,* 549–580.

Elgin, S., and Weintraub, H. (1975), *Annu. Rev. Biochem. 44,* 725–774.

Finch, J., Noll, M., and Kornberg, R. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3320–3322.

Glonek, T., Henderson, T. O., Hilderbrand, R. L., and Myers, T. C. (1970), *Science 169,* 192–194.

Glonek, T., Henderson, T. O., Kruski, A. W., and Scanu, D. M. (1974), *Biochim. Biophys. Acta. 348,* 155–161.

Guéron, M. (1971), *FEBS Lett. 19,* 264–266.

Guéron, M., and Shulman, R. G. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3482–3485.

Hanlon, S., Brudno, S., Wu, T. T., and Wolf, B. (1975), *Biochemistry 14,* 1648–1660.

Hanlon, S., Johnson, R. S., and Chan, A. (1974a), *Biochemistry 13,* 3963–3971.

Hanlon, S., Johnson, R. S., and Chan, A. (1974b), *Biochemistry 13,* 3972–3981.

Hanlon, S., Johnson, R. S., Wolf, B., and Chan, A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3263–3267.

Johnson, R. S., Chan, A., and Hanlon, S. (1972), *Biochemistry 11,* 4347–4358.

Lowry, O. H., Rosebrough, N. J., Farr, A. J., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.
Maurer, H. R., and Chalkley, G. R. (1967), *J. Mol. Biol. 27,* 431–441.
Olins, A. L., and Olins, D. E. (1974), *Science 183,* 330–332.
Oster, G. (1948), *Chem. Rev. 43,* 319–365.
Rill, R., and Van Holde, K. E. (1973), *J. Biol. Chem. 248,* 1080–1083.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N.Y., and London, Academic Press.
Senior M. B., Olins, A. L., and Olins, D. E. (1975), *Science 187,* 173–175.
Shih, T. Y., and Fasman, G D. (1970), *J. Mol. Biol. 52,* 125–128.
Tuan, D. Y. H., and Bonner, J. (1969), *J. Mol. Biol. 45,* 59–76.
Van Holde, K. E., Sahasrabuddhe, C., Shaw, B. R., Van Bruggen, E. F. J., and Arnberg, A. (1974), *Biochem. Biophys. Res. Commun. 60,* 1365–1370.

Near-Ultraviolet Tyrosyl Circular Dichroism of Pig Insulin Monomers, Dimers, and Hexamers. Dipole–Dipole Coupling Calculations in the Monopole Approximation†

E. Hardin Strickland and Dan Mercola*,‡

ABSTRACT: The tyrosyl circular dichroism (CD) has been calculated using the conformation of pig insulin observed in rhombohedral crystals containing 2 zinc atoms per hexamer. These calculations predict that the tyrosyl CD at 275 nm will be enhanced disproportionally as monomers interact to form dimers and as dimers interact to form hexamers. This enhanced tyrosyl CD ($\Delta\epsilon$ per 5800 molecular weight) results from new coupling interactions generated in the regions of contact between monomers and between dimers. These calculations illustrate that a large CD enhancement may accompany aggregation even in the absence of a conformation change in either monomer. The tyrosyl CD intensities calculated for monomers, dimers, and hexamers of 2-zinc pig insulin are compatible with the experimentally observed CD spectra which are enhanced about fourfold in the hexamer compared with the monomer. Zinc ions and other metals do not contribute directly to the tyrosyl CD but only influence the optical properties by promoting the hexameric state. The relation of the integrity of the molecule to dimer formation and the biological activity of the molecules are discussed. The largest calculated contributions to tyrosyl CD arise from interactions with far-ultraviolet transitions of neighboring aromatic groups. In the hexamer, about half of the tyrosyl CD intensity is calculated to arise from Tyr-A14.

Insulin has several advantages as a model system for a theoretical study of the circular dichroism (CD)[1] arising from tyrosyl side chains. Insulin contains a high proportion of tyrosine (4 mol/mol) and phenylalanine (3 mol/mol) but no tryptophan (Dayhoff, 1975). Detailed x-ray analyses have been carried out on insulin crystals containing either of two or four zinc atoms per hexamer (Blundell et al., 1971, 1972; Hodgkin and Mercola, 1972; Bentley et al., 1976). These studies have revealed that the tyrosyl side chains are distributed over three surfaces of the molecule. One of these surfaces is involved in the formation of the dimer and a second surface is involved in contacts between dimers. The dimer contacts are repeated by the crystallographic threefold symmetry to produce a hexamer of insulin. Each aggregation state of insulin as seen in the crystal structure involves tyrosyl side chains in close contact with neighboring molecules (Figure 1). Therefore, the insulin structure provides several possibilities for comparing the calculated and observed states.

The aggregation properties of insulin in solution have been studied extensively (for reviews, cf. Hodgkin and Mercola, 1972; Blundell et al., 1972). The process is best known for zinc-free insulin at pH 2.0 (Jeffrey and Coates, 1966) and at pH 8.0 (Goldman and Carpenter, 1974). In each case the weight-average, molecular weight has been determined as a function of concentration. A least-squares analysis of the data at each pH value has shown that the scheme, 6 monomers $\rightleftharpoons$ 3 dimers $\rightleftharpoons$ 1 hexamer, best explains the data and agrees completely with the organization found in various crystal forms of insulin (Hodgkin and Mercola, 1972; Blundell et al., 1972). The distribution of species in solution can be manipulated by the addition of zinc or other divalent cations (cf. Hodgkin and Mercola, 1972; Blundell et al., 1972; Goldman and Carpenter, 1974). Under neutral and moderately alkaline conditions, zinc added in amounts of 2 g-atoms per 6 mol of insulin is bound

† From the Laboratory of Nuclear Medicine and Radiation Biology, University of California, Los Angeles, California 90024 (E.H.S.), and the Laboratory of Molecular Biophysics, Department of Zoology, Oxford University, Oxford, England (D.A.M.). *Received December 31, 1975.* This work was supported by Contract AT(04-1) GEN-12 between the Atomic Energy Commission and the University of California, by National Science Foundation Grant GB-43450 (E.H.S.), and by The Royal Society, United Kingdom (D.A.M.).

‡ Present address: Department of Zoology, University of Oxford, Oxford 0X1 3PS, England.

[1] Abbreviations used are: CD, circular dichroism; $\Delta\epsilon$ for left circularly polarized light minus that for right circularly polarized light; r_{min}, minimum separation between monopoles of two different side chains; r_{21}, center-to-center distance separating ring groups; uv, ultraviolet; Tris, tris(hydroxymethyl)aminomethane. The insulin residues are labeled by chain (A or B), position, and, where appropriate, by monomer structure (I or II).