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Interaction of Deoxyribonucleic Acid with Histone f2b and Its Half-Molecules. Circular Dichroism Studies†

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ABSTRACT: The moderately lysine-rich histone, f2b (IIb2), from calf thymus was combined with homologous DNA by means of gradient dialysis to form reconstituted complexes at physiological salt concentration. The circular dichroic spectra of these complexes are characterized by a positive band at 272 nm which is blue-shifted and greatly enhanced relative to the corresponding band for native DNA. This result indicates that the conformational change induced in DNA upon binding of histone f2b is somewhat similar to that caused by combination with f2a1, but is very different from the structure of f1–DNA complexes. f2b was cleaved by cyanogen bromide to form half-molecules of approximately equal size, but with the N-terminal half much richer in basic amino acids than the C-terminal fragment. DNA complexes prepared with the N-terminal polypeptide exhibited circular dichroism changes similar in type to but smaller in magnitude than the distortions caused by intact f2b. The C-terminal half-molecule had a reduced binding affinity for DNA and failed to effect significant change in DNA circular dichroism upon complexation. These findings present additional evidence that the two ends of unevenly charged histone molecules interact differently with DNA. When complexes were reconstituted containing both N- and C-terminal fragments, the C-terminal half-molecule was capable of inhibiting the circular dichroism change caused by the N terminus. This antagonism is not found in the intact f2b molecule, where the C segment appears to be necessary to produce maximum circular dichroism change, indicating that the intact molecule is necessary to produce the significant histone conformation.

The histones are a set of basic proteins which are found combined with DNA in cell nuclei of higher organisms. Histones appear to stabilize the supercoiled, condensed structure of nuclear DNA (Pardon and Wilkins, 1972). They may, in addition, function as gross regulators of transcription (Elgin *et al.*, 1971), perhaps due to their effect upon the conformation of DNA. The interaction between histones and DNA is dominated by electrostatic forces; in chromatin the histones contain sufficient basic amino acid residues to neutralize nearly all the negative charges on the ionized DNA phosphates. However, if any specificity between DNA and histones exists, other interactions must play a controlling role.

This laboratory has been utilizing circular dichroism as a method of assessing conformational variations in calf thymus DNA brought about by interaction of the DNA with homologous histones. Histone–DNA complexes reconstituted with histone fractions f1 (Fasman *et al.*, 1970), f2a1 (Shih and Fasman, 1971), mixtures of f1 and f2a1 (Shih and Fasman, 1972), and f1 fragments (Fasman *et al.*, 1971) have been examined, and show that the type of circular dichroic distortion observed depends upon the nature of the histone. In the present study this series is extended to histone f2b and its half-molecules, with all complexes formed in 0.14 M NaCl.

Histone fraction f2b (IIb2) is slightly lysine-rich and contains much serine. The amino acid sequence of calf thymus f2b has been determined by Iwai *et al.* (1970). [Details of the sequencing procedure can be found in a series of papers by the same group: Ishikawa *et al.* (1972), Hayashi and Iwai (1972), and Iwai *et al.* (1972).] The protein contains 125 amino acid residues, has a mol wt of 13,800 and has a lysine : arginine ratio of 2.5. Tissue and species differences appear to be negligible (Hnilica, 1966). f2b, because of its high positive charge density, is randomly coiled in water, but acquires considerable secondary structure as the ionic strength of the medium is raised (Bradbury and Rattle, 1972; Bradbury *et al.*, 1972; D'Anna and Isenberg, 1972); concurrently there is increased aggregation of the histone (Edwards and Shooter, 1969a, 1970; Bradbury and Rattle, 1972). Histone f2b is also capable of interacting with fractions f2a1 (D'Anna and Isenberg, 1973) and f2a2 (Skandrani *et al.*, 1972; Kelley, 1973).

The distribution of the basic amino acid residues in f2b is very uneven (Iwai *et al.*, 1970). In the first 34 residues from the amino terminus there are 12 lysines and 3 arginines (occurring in clusters of 2–5), in addition to 4 prolines (which would be expected to assist the high charge density in preventing α-helical structure). In contrast, the next segment of 68 residues (35–102) contains only 4 lysines, 5 arginines, and 1 proline (with no basic clusters), and has an amino acid composition like that of a typical enzyme, with many hydrophobic residues.

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The remaining segment (residues 103–125) is fairly basic, containing 4 lysines, no arginine, and 1 proline. This uneven distribution is a general characteristic of all 5 histone types (De Lange and Smith, 1972); in all cases the N-terminal 30–40 residues are very basic and the remainder of the molecule is relatively uncharged, with the exception of a short basic C-terminal tail. (This tail is long for the much larger molecule of f1.) f2b can be cleaved by cyanogen bromide (Iwai *et al.*, 1970) to yield two fragments of nearly equal length but different charge density. The N-terminal half-molecule, N[1], contains residues 1–58, and 32% of its residues carry a positive charge. Half-molecule C has residues 63–125 and is 20% charged. Residues 59–62 are lost in this cleavage. These large fragments can be utilized to examine the effect of histone composition and conformation upon interaction with DNA.

Several workers have noted the charge asymmetry of histone molecules and have suggested that the amino end of histones (plus the carboxyl half of f1) may be the primary sites of interaction with and binding to DNA, whereas the remainder of the histone molecules would be relatively free for other functions, including formation of definite secondary conformations and consequent specific interaction with regulatory protein molecules (Bustin and Cole, 1970; Iwai *et al.*, 1970; De Lange and Smith, 1972). There is now growing evidence to confirm this hypothesis: modification of chromatin (Simpson, 1971, 1972) indicates that the termini of histone molecules are bound to DNA and the central portions are free. In reconstituted histone–DNA complexes formed with fragments of f1, only the more basic part of the molecule induced changes in circular dichroism of the DNA (Fasman *et al.*, 1971). Two studies of great relevance to the present work involve complexes reconstituted with DNA and the N and C half-molecules of f2b. First, thermal denaturation data (Li and Bonner, 1971) show that the combination of DNA with the N fragment protects it from melting more than does complexation with C. Second, nuclear magnetic resonance experiments (Bradbury and Rattle, 1972) on f2b, N, C, and f2b–DNA complexes indicate that the central, uncharged portion of f2b self-interacts, while the basic ends combine with DNA, under proper ionic conditions. Other investigations of synthetic f2b–DNA complexes deal with thermal denaturation (Shih and Bonner, 1970; Ansevin and Brown, 1971), aggregation (Edwards and Shooter, 1969b), surface tension (Chattoraj *et al.*, 1972), and template properties (Shih and Bonner, 1970; Johns, 1972).

In the present investigation, intact f2b histone is seen to affect the DNA circular dichroism spectrum in a manner somewhat similar to f2a1, but very differently from f1. In addition, a comparison has been made between the interaction of DNA with intact f2b and with its two cleaved half-molecules, incorporated separately and together into complexes with DNA. If DNA could bind strongly at many sites along the N-terminal fragment of f2b, as is suggested by its amino acid composition, then N might be expected to bind more tightly to DNA and to produce more dramatic changes in the DNA circular dichroic spectrum than would C. This was found to be the case. Furthermore, when both half-molecules were bound simultaneously to DNA, the C-terminal fragment inhibited the circular dichroic distortion caused by N. This result is in contrast with the role which the C-terminal portion of f2b apparently plays in the intact histone molecule, where it augments the conformational effect of the N segment upon DNA, thus indicating that the conformation of the intact molecule plays a significant role.

[1] Abbreviations used are: N and C denote the N-terminal and C-terminal cleaved halves, respectively, of f2b; Gdn·HCl, guanidine hydrochloride; OD, optical density.

Materials and Methods

Histone Preparation and Cleavage. Histone fraction f2b was isolated from calf thymus by method 2 of Johns (1964) and was further purified by gel filtration chromatography on Sephadex G-75 in 0.01 M HCl. The protein was homogeneous in gel electrophoresis (Bonner *et al.*, 1968a).

The histone was cleaved at methionine residues 59 and 62 as follows (Gross, 1967): 40 mg (0.003 mmol) of f2b was dissolved in 1 ml of 0.1 M HCl and was mixed with 90 mg of cyanogen bromide (Eastman Kodak) (0.9 mmol), in an additional 1 ml of 0.1 M HCl. The reaction proceeded for 24 hr at room temperature, after which the mixture was lyophilized to remove HCl and CNBr, dissolved in 1 ml of 0.1 M acetic acid, dialyzed against 0.1 M acetic acid, and lyophilized again. Similar procedures have been used by others for cleavage of f2b (Iwai *et al.*, 1970; Li and Bonner, 1971; Johns *et al.*, 1972).

Chromatography of Fragments. The products from CNBr cleavage were dissolved in 2 ml of 5% Gdn·HCl[1] (Research Plus absolute grade) in 0.1 M sodium phosphate buffer (pH 6.8) and applied to a Bio-Rex 70 (200–400 mesh, Na^+ form) column (2 × 30 cm) (Bonner *et al.*, 1968a). The column was eluted first with 5% Gdn·HCl (+buffer), then with a linear gradient of 5–27% Gdn·HCl (+buffer), 500-ml total gradient. The flow rate was 1 ml/min; 5-ml fractions were collected. Absorbance of peptides at 230 nm was measured on a Gilford spectrophotometer; the per cent Gdn·HCl in the fractions was determined with a Bausch and Lomb refractometer.

The elution pattern was similar to that shown by Li and Bonner (1971), except that the 5% Gdn·HCl run-off peak (fragment C)[1] was less contaminated. N plus uncleaved f2b were eluted together at 9% Gdn·HCl. Tailing fractions were discarded. Fractions containing each half-molecule were pooled separately, dialyzed *vs.* 0.1 M acetic acid, and lyophilized.

Gel electrophoresis showed that the N fraction was contaminated at this stage with 19% uncleaved f2b and 6% C. Therefore, N was purified by chromatography on G-50 Sephadex (fine) in 0.02 M HCl, 1 × 60 cm column, fraction size 0.8 ml. f2b was eluted close to the void volume and was discarded; fractions containing N plus a small amount of C came through at 1.5 void volumes and were pooled and lyophilized. Final yields were: C, 4 mg, electrophoretically pure; N, 5 mg, contains 6% C.

Gel Electrophoresis. f2b and its half-molecules were examined by polyacrylamide (15%) disc gel electrophoresis in 6 M urea. The method was essentially that of Bonner *et al.* (1968a), except that the gels were longer (9.5 cm) and lower currents were applied (2 mA/gel). Running times were 5.5 hr. Gels were stained with Amido Black, destained electrophoretically, and scanned at 630 nm in a Zeiss microdensitometer. Integrated band areas were obtained with a Du Pont 310 curve resolver. Under the conditions used f2b travelled 2.8 cm, C 3.5 cm, and N 4.7 cm.

Protein Solutions. Stock solutions of f2b and its fragments were prepared in water, at concentrations of 3–5 × 10^{-3} M peptide residue, adjusted to pH 7.0, and stored frozen. Histone concentrations were determined by the Nessler micro-Kjeldahl method (Lang, 1958), or by a modified biuret assay (Adler *et al.*, 1971) utilizing poly(L-lysine) as standard and a color factor (determined by Nessler analysis) of 0.94 for f2b.

TABLE I: Circular Dichroism Spectra of f2b and Its Fragments.[a]

Histone	Solvent	Band 1 λ (nm)	Band 1 [θ]	Band 2 λ (nm)	Band 2 [θ]
f2b	0.01 M NaF	220	−4300	202	−15,100
N	0.01 M NaF	220	−1600	202	−18,000
C	0.01 M NaF	221	−3600	203	−12,300
f2b	0.14 M NaF	219	−7400	205	−12,800
N	0.14 M NaF	220	−1500	200	−18,100
C	0.14 M NaF	218	−4100	203	−9,300

[a] Ellipticity values, [θ], are reported per mole of peptide residues. Average deviations in $[\theta]_{220}$ are ±200 and in $[\theta]_{202}$ they are ±800 for duplicate measurements.

DNA. Calf thymus DNA was prepared as described by Adler *et al.* (1971). Its median mol wt was 9.6×10^6 determined by sedimentation velocity. DNA concentrations were determined from OD_{258}, using ϵ_{258} (per mol of nucleotide residues) = 6.8×10^3.

Histone–DNA Complexes. Complexes of DNA with f2b or with its fragments (N and/or C) were reconstituted by mixing these components, at the desired concentration and ratios, under dissociating conditions (in 2 ml of 5 M Gdn·HCl + 0.002 M Tris (pH 7.0)), and then gradually lowering the guanidine concentration by means of a continuous-flow linear-gradient dialysis (Carroll, 1971) in order to anneal the complexes. In this procedure the mixtures of DNA and histone were first dialyzed against 200 ml of 1 M Gdn·HCl + 0.002 M Tris for 2 hr, and then set in 500 ml of 1 M Gdn·HCl and dialyzed against a continuous guanidine gradient from 1 M Gdn·HCl down to 0.14 M Gdn·HCl (all containing 0.002 M Tris). The gradient utilized 1536 ml each of 0.86 M Gdn·HCl + Tris and of 0.002 M Tris (with no Gdn·HCl) in the gradient reservoirs (Carroll, 1971). All dialysis solvents were filtered through a medium porosity sintered glass filter. All dialyses were run at 4°. The flow rate of the linear gradient used for dialysis was 4 ml/min. The Gdn·HCl used for dialysis was Eastman reagent grade. After the gradient was completed the complexes were allowed to dialyze against 0.14 M Gdn·HCl + 0.002 M Tris for 2–5 hr. Then the guanidine was removed by dialysis against three changes of 0.14 M NaCl + 0.002 M Tris (pH 7.0) (1 l. each); this was the final solvent for circular dichroism measurements. Complexes were not centrifuged to remove turbidity. This dialysis method ensures reproducible reconstitution of histone–DNA complexes.

Concentrations of DNA in the complexes were determined by ultraviolet spectroscopy following dissociation of the complexes by 0.1% sodium dodecyl sulfate (Gallard Schlesinger Co.) (Fasman *et al.*, 1971). Concentrations of complexes were usually $1.2–1.4 \times 10^{-4}$ M DNA nucleotide residues. Histone: DNA ratios (*r*) are reported as moles of histone (f2b, N, or C) peptide residues per mole of DNA nucleotide residues, and were determined from the input concentrations.

Optical Measurements. Circular dichroism spectra were recorded at 23° on a Cary 60 recording spectropolarimeter with a Model 6001 CD attachment as reported previously (Fasman *et al.*, 1971). In addition the Cary 6003 modification was employed, which modulates the Pockels cell at 327 Hz for a better signal: noise ratio. The light source was a Xenon arc lamp with suprasil envelope. The path length for histone–DNA complexes was 1 cm. Mean residue ellipticity values, [θ], are given per mole of nucleotide residue in the complexes.

Circular dichroism spectra of histone solutions alone (f2b, N, and C) were measured at a 1.5×10^{-3} M peptide concentration in a 1-mm cell and at 4×10^{-3} M in a 0.5-mm cell. Ultraviolet spectra were obtained on a Cary 14.

Centrifugation of Complexes. The extent of binding of f2b and its fragments to DNA was determined by high-speed centrifugation of the complexes. Complexes of f2b–DNA, N–DNA, and C–DNA were prepared by linear gradient Gdn·HCl dialysis. Each had a concentration of 1.0×10^{-3} M nucleotide residue, $r = 1.5$, in 0.14 M NaF. The complexes were spun in a Spinco Model L centrifuge, SW 50.1 rotor equipped with 0.8-ml tube adapters at 4° for 7 hr at 40,000 rpm. Under these conditions, DNA and DNA–histone complexes were pelleted while unbound histone remained in the supernatant. The supernatants were aspirated off, and their ultraviolet spectra (185–400 nm) were scanned in a 1-mm path length cell. Histone concentrations in the supernatants were determined by utilizing extinction coefficients which had been measured at several wavelengths with f2b, N, and C solutions whose concentrations were known from biuret assays. For example, ϵ_{195} (M^{-1} peptide, cm^{-1}) values were 11,900 for f2b, 7500 for N, and 6500 for C.

Results

Circular Dichroism of f2b and Its Half-Molecules. The conformations of f2b, N, and C were examined, at low and moderate salt concentrations, by means of circular dichroism. The peak ellipticity values are given in Table I. In 0.01 M NaF f2b and both of its fragments yield spectra characterized by a negative band at 202 nm and a negative shoulder at 220 nm, indicating randomly coiled protein structures (Adler *et al.*, 1973). At 0.14 M NaF (which was chosen as the final salt molarity for complexes), half-molecule N remains devoid of secondary structure, presumably because of its high charge density. However, sufficient shielding has occurred at 0.14 M NaF to allow both f2b and C to acquire considerable structure; the shapes of the circular dichroism curves indicate approximately 30% α helix and very little, if any, β structure in either polypeptide (Greenfield and Fasman, 1969). The salt effects observed here are consistent with the optical rotatory dispersion results on f2b of Bradbury *et al.* (1972), although they found a greater induction of secondary structure in C than in f2b. At each salt concentration the low-wavelength peak ellipticity value for f2b is the average of those for its N and C fragments; this simple additivity of parts, observed for the proteins alone, does not apply to the histone–DNA complexes reconstituted from these polypeptides, as will be seen later.

Circular Dichroism of f2b–DNA Complexes. When complexes are formed from intact calf thymus f2b and DNA by means of a continuous Gdn·HCl gradient, striking changes are produced in the circular dichroic spectrum of DNA. In Figure 1 it can be seen that, at physiological salt concentration and a [peptide]: [nucleotide] ratio, *r*, of 1.5, the positive band of DNA ($[\theta]_{278} = 8400$) is blue-shifted and enhanced to $[\theta]_{273} = 22{,}800$. The negative DNA band ($[\theta]_{246} = -10{,}100$) is decreased in magnitude to −6100, and is blue-shifted and broadened. Since the protein itself does not contribute to the circular dichroism spectrum at $\lambda > 244$ nm (as can be seen from the calculated curves for isolated histone plus DNA mixtures), the distortions in DNA circular dichroism are brought about through changes in DNA conformation or coiling.

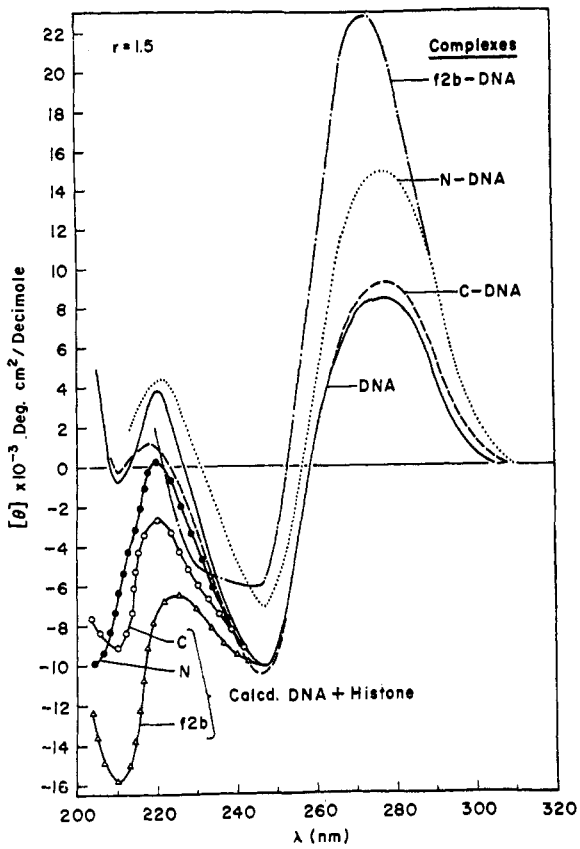

FIGURE 1: Circular dichroism spectra of complexes reconstituted from DNA and f2b (or its fragments) at $r = 1.5$. The concentration ratio of histone (moles of peptide residues) to DNA (moles of nucleotide residues) in the complexes, r, equals 1.5 (except for the control of DNA). N and C indicate the N-terminal and C-terminal f2b half-molecules, respectively. Complex concentration = 1.2–1.4 $\times 10^{-4}$ M nucleotide residue; path length, 1 cm; temperature, 23°; solvent, 0.14 M NaCl + 0.002 M Tris (pH 7.0). The circular dichroism of DNA alone (—) is given for comparison. The curves "calculated DNA + histone" are the calculated sums of isolated DNA and f2b (or N or C) contributions (at $r = 1.5$) in the solvent used; these ellipticities are calculated per mole of nucleotide, and all of these curves merge with that for DNA at $\lambda > 245$ nm.

Data are presented in Figure 2A which show the progressive changes in circular dichroism as the f2b:DNA ratio is increased from zero to three. (At $r > 3$ the complexes precipitated.) The type of spectral changes brought about by f2b are somewhat similar to those caused by f2a1 (Shih and Fasman, 1971). However, in the case of f2a1–DNA complexes there was a small negative band at 305 nm, and the circular dichroism changes reached a maximum at $r = 1.5$ ($[\theta]_{272} = 16{,}000$) and then diminished at higher r ratios. An entirely different type of variation was found for f1–DNA complexes (Fasman *et al.*, 1970); the only similarity of f1–DNA to f2b–DNA complexes is that, in both cases, the changes continue as r is increased. In native calf thymus chromatin the f2b:DNA residue ratio, r, is about 0.75 ± 0.15; at that ratio the positive DNA band has approximately doubled in magnitude. This physiological value for r was calculated from the weight ratio of histone to DNA, 1.05 ± 0.15 g/g (Bonner *et al.*, 1968b), the estimate that f2b comprises 24.3 ± 1.2% of this histone mixture (Panyim and Chalkley, 1969), and mean residue weights of 105 for f2b and 309 for DNA.

As was found by Olins and Olins (1971) for other histone species, when f2b–DNA complexes were reconstituted in the presence of urea instead of Gdn·HCl (required to prevent

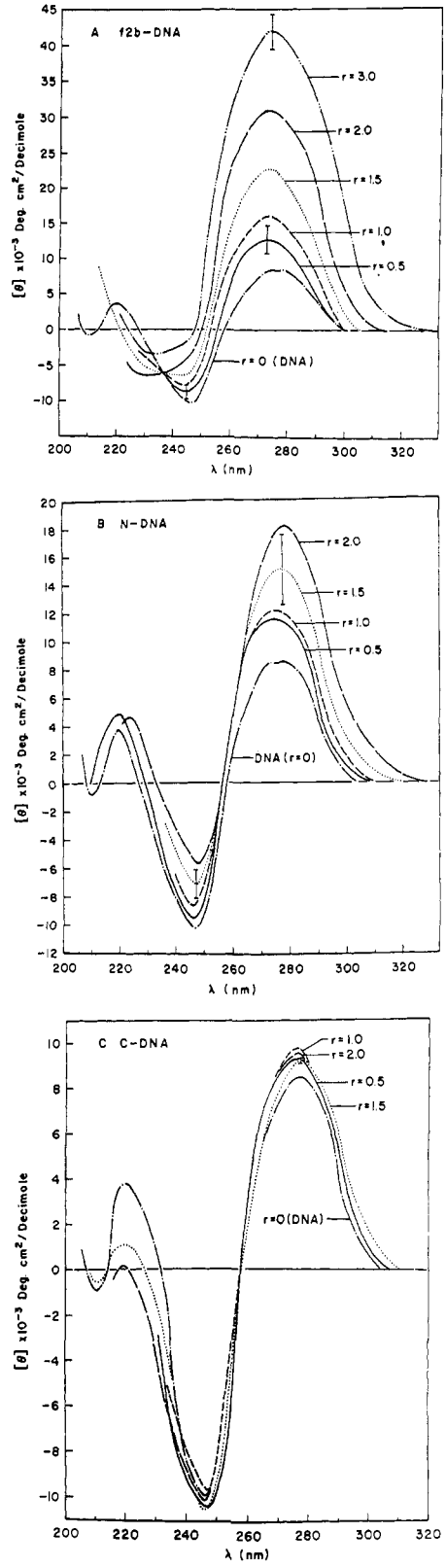

FIGURE 2: Circular dichroism of DNA complexes with f2b or its half-molecules at various values of r. Same conditions as in Figure 1. Error bars indicate average deviation of ellipticity data in duplicate experiments using different complexes; reproducibility of wavelengths at extrema is typically ±1 nm. Note changes in ordinate scales: (A) intact f2b–DNA complexes, $r = 0$ (DNA alone)–3.0; (B) fragment N–DNA complexes, $r = 0$–2.0; (C) fragment C–DNA complexes, $r = 0$–2.0.

histone aggregation during complex formation), smaller changes in circular dichroism were observed. When a stepwise

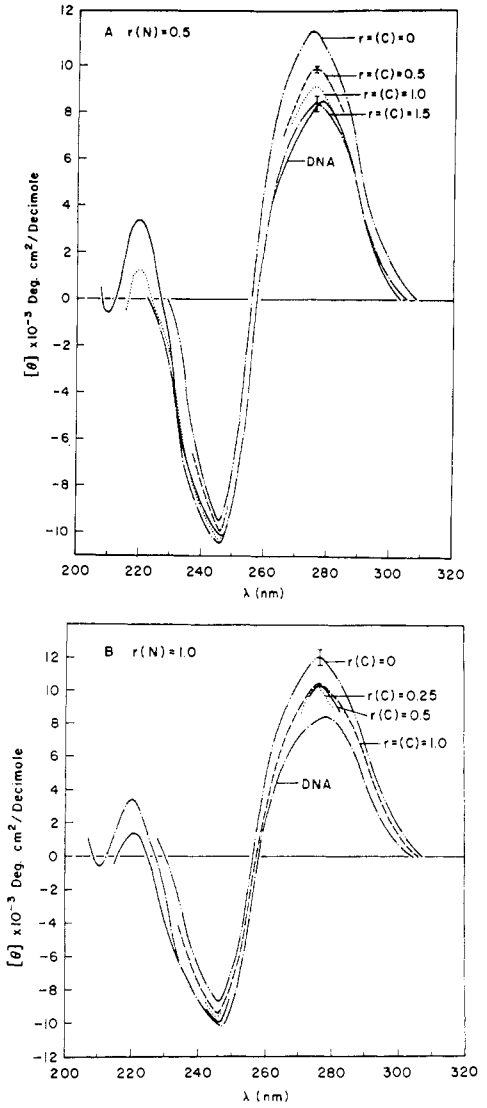

FIGURE 3: Circular dichroism of complexes reconstituted with DNA and mixtures of f2b half-molecules (N + C). In each set of curves the peptide:nucleotide ratio, r(N), is held constant for fragment N, and r(C) for fragment C is varied. Same experimental conditions as in Figure 1: (A) r(N) = 0.5, r(C) = 0–1.5; (B) r(N) = 1.0, r(C) = 0–1.0.

NaCl gradient containing 5 M urea (Shih and Fasman, 1971) was employed to anneal f2b–DNA at r = 1.5, the resultant circular dichroic spectrum showed the same type of changes as seen in Figure 2A but of smaller magnitude and, in addition, showed the appearance of a small negative long-wavelength band ($[\theta]_{302}$ = −1400, $[\theta]_{272}$ = 9000, $[\theta]_{243}$ = −10,400). On the other hand, when a complex of f2b–DNA, r = 1.5, was annealed by means of a continuous linear gradient of NaCl (2–0.14 M) in the presence of 1 M Gdn·HCl (until the removal of Gdn·HCl at the end of dialysis), even larger ellipticity changes were observed ($[\theta]_{302}$ = −4400, $[\theta]_{267}$ = 27,200, $[\theta]_{234}$ = −2000) than with the usual Gdn·HCl gradient (1–0.14 M) without NaCl. [The final solvent was 0.14 M NaCl + 0.002 M Tris in all cases.] This strong dependence upon the type and concentration of denaturing agent present during reconstitution of complexes underlines the complexity of histone–DNA interactions. In particular, a balance between hydrophobic protein–protein and protein–DNA forces must be achieved during complex formation, as well as electrostatic binding. Bartley and Chalkley (1972, 1973) have discussed this problem as it affects the influence of urea upon chromatin dissociation and conformation.

Circular Dichroism of N–DNA and C–DNA Complexes. The circular dichroism spectra of complexes reconstituted with DNA and each half-molecule of f2b independently are given in Figures 1 and 2. In Figure 1 the curves for f2b–DNA, N–DNA, and C–DNA, each at r = 1.5, are compared. When the highly basic amino-terminal half-molecule, N, is complexed to DNA it causes about half the amount of change in DNA ellipticity as does intact f2b at the same residue ratio; the type of distortion is qualitatively the same. However, the less basic carboxy-terminal fragment has very little effect upon the circular dichroism of DNA. (The calculated curves for protein–DNA mixtures are different, at low wavelength, for each polypeptide, because of the ellipticity differences indicated in Table I.)

The progression of circular dichroism change as r(N) is increased is shown in Figure 2B. At each ratio the spectrum is qualitatively like the corresponding f2b–DNA curve (Figure 2A), but the difference from the DNA (r = 0) value is only about half as large.

On the other hand, the bands for C–DNA complexes at r = 0.5–2.0 (Figure 2C) are only slightly different ($[\theta]_{277}$ = 9400 ± 200) from free DNA, and do not vary significantly with r. It appears that a small amount of C fragment can saturate DNA and cause a limited conformational change. As will be shown later, the binding affinity of C for DNA is lower than that of N or f2b; at r = 1.5, C is only 60% bound to DNA under the conditions studied. However, since C induces much less than 60% of the distortion in DNA brought about by intact f2b, factors other than reduced binding must contribute to this lack of sensitivity of DNA toward C.

Complexes Formed with DNA and Mixtures of N and C. Circular dichroism spectra for reconstituted complexes containing both cleaved half-molecules of f2b (present together throughout dialysis) are given in Figure 3. In the data of Figure 3A the residue ratio, r, of fragment N incorporated into the complexes is held constant at 0.5, while that of C is varied from 0 (N–DNA alone) to 1.5. As more C is incorporated into the complexes, the ellipticity changes caused by N decrease until, at r(C) = 1.5 (C:N = 3), they are eliminated and the spectrum is nearly identical with that of free DNA. The data can be fitted quite well in terms of competition of N and C for binding sites on DNA.

In Figure 3B is seen the effect of incorporation of various amounts of C into complexes which also contain a constant amount of N (r = 1). In this case, with the larger amount of N in the complexes, the presence of C still inhibits the effect of N, but not to the extent seen at r(N) = 0.5 (Figure 3A). At r(N) = 1 the antagonistic effect of the C fragment appears to level off with only a small amount of C, r(C) = 0.25, or C:N = 0.25. Unlike the case of r(N) = 0.5, the curves at r(N) = 1 cannot be brought into agreement with a simple binding competition. (The 6% of C present as a contaminant in this preparation of half-molecule N is not sufficient to seriously reduce the effect of N in DNA complexes.)

The power of fragment C to inhibit the conformational changes induced by the more highly charged N half of f2b is very different from the situation with histone f1 (Fasman *et al.*, 1971). In the case of f1 the less charged fragment (the amino-terminal third of the f1 molecule), although incapable of inducing significant circular dichroic change in DNA, acts synergistically with the more basic f1 fragment to produce even greater distortion than that for the latter alone. An additional difference between the two histones is that for

intact f2b the less basic carboxyl half aids the charged amino-terminal segment in producing maximum effect on DNA circular dichroism (see Figure 1), whereas for whole f1 inclusion of the less basic segment into the intact molecule hinders the full effect of the more basic part.

Turbidity of Complexes. A general property of histone–DNA complexes is that, like chromatin, they form aggregates at moderate salt concentration, and therefore, scatter light (Fasman *et al.*, 1970; Adler *et al.*, 1971; Shih and Fasman, 1971). Complexes of f2b–DNA and N–DNA are typical in this respect. Their turbidity rises linearly as r is increased and, at any given value of r, the amount of turbidity is identical for f2b–DNA and for N–DNA. For example, at $r = 1.5$, the value of OD_{400}/OD_{258}, an empirical measure of light scattering, is equal to 0.11 ± 0.01 for both complexes. Therefore, the greater effectiveness of intact f2b in promoting circular dichroic change in the DNA cannot be attributed to artifacts caused by light scattering.

Complexes of C–DNA are unusual in that they are not at all turbid; $OD_{400}/OD_{258} < 0.005$ for these complexes for $r = 0$–2.0. Furthermore, when the C fragment is incorporated into complexes which also contain N, C does not contribute at all to the turbidity caused by N, even at high r(C); the light scattering is a function only of r(N). These findings may indicate that C binds to DNA in a manner much less conducive to specific aggregation of complexes.

Centrifugation of Complexes. The extent of binding of f2b and its half-molecules to DNA was determined by high-speed centrifugation. f2b–DNA, N–DNA, and C–DNA complexes were prepared, each of which had a DNA residue concentration of 10^{-3} M and a protein:DNA ratio of 1.5. Ultraviolet spectroscopy of the supernatants after centrifugation showed that, for f2b–DNA and N–DNA, very little of the histone remained unbound in the supernatant; maximum unbound amounts of protein were 2.4 and 5.8%, respectively. However, in the case of C–DNA, 40.4% of the protein remained in the supernatant. All of the DNA was pelleted in each complex, along with the bound histone.

Centrifugation of the protein solutions (f2b, N, and C) alone, under identical conditions and in the same solvent (0.14 M NaF), showed that about 98% of each protein remained in the supernatant. This result agrees with the centrifugal clearing time calculated from sedimentation constants for partially aggregated f2b at moderate salt concentration (Edwards and Shooter, 1969a). Less than 2% of the DNA, when centrifuged alone, was not included in the pellet. Therefore, f2b histone can be spun out of solution, under these conditions, only when bound to DNA.

The finding that half-molecule C is only 60% bound to DNA at $r = 1.5$ is probably a reflection of the low positive charge density of C, relative to N or f2b. However, since most of the C fragment is incorporated into the complex, this reduced binding affinity of C for DNA is not sufficient to account for the very small amount of circular dichroism change and the lack of turbidity of C–DNA in C–DNA complexes. Additional differences may reside in the detailed mode of C binding to DNA and in the internal structure of the complexes formed, compared with N or intact f2b.

Discussion

Reconstitution of complexes between f2b (moderately lysine-rich) histone and DNA by means of gradient dialysis annealing causes progressive distortion of the DNA circular dichroism spectrum as increasing amounts of f2b are incorporated into the complex. This distortion can be interpreted as an indication of alterations in the asymmetric environment of the DNA nucleotide chromophores. It is not yet resolved whether these changes are conformational in nature or whether they result from supercoiling of the DNA (Pardon and Wilkins, 1972).

Although f2b can be considered a typical histone with respect to its size and its nonuniform charge distribution (De Lange and Smith, 1972), the type of circular dichroism pattern shown by its f2b–DNA complexes is unique. The mode of change is certainly more similar to that exhibited by f2a1–DNA complexes (Shih and Fasman, 1971) than to that caused by the much larger f1 molecule (Fasman *et al.*, 1970). However, there are significant differences: with f2b there is a greater enhancement of the positive DNA band and the distortion does not become reversed at high histone:DNA ratios.

The finding that each histone so far examined induces a different type of conformational distortion in the DNA at physiological ionic strength indicates that histone–DNA binding is not simply a nonspecific charge neutralization. Additional evidence for specificity in histone–DNA interaction is the dependence of the final f2b–DNA complexes upon the type and amount of disaggregating reagent present during various stages of complex formation. Furthermore, the intact f2b molecule is required for proper binding; this point will be discussed later. Since f2b tends to aggregate with itself (Edwards and Shooter, 1969a, 1970) in a manner involving portions of the molecule containing ordered secondary structure (D'Anna and Isenberg, 1972; Bradbury and Rattle, 1972), and since f2b also forms fairly specific 1:1 complexes with f2a1 (D'Anna and Isenberg, 1973) and with f2a2 (Skandrani *et al.*, 1972; Kelley, 1973), a possible role for f2b in chromatin may be to organize clusters of histones for specific binding to DNA.

The f2b molecule was bisected with cyanogen bromide, and complexes of DNA with each half-molecule were examined. One major conclusion to be drawn from this study of f2b cleavage products is that the N-terminal fragment, very rich in basic amino acid residues, binds more strongly to DNA and with a much greater capacity to distort the DNA circular dichroism spectrum than does the C-terminal half. Therefore, in intact f2b the N-terminal part of the molecule is probably the chief site of electrostatic binding to DNA, and is primarily responsible for the structural changes observed in DNA upon combination with this histone. This situation with f2b is similar to that of histone f1, where the very lysine-enriched part of the molecule, clustered in the carboxy-terminal segment in that case, has a stronger binding affinity for DNA and causes more circular dichroism distortion than does the more neutral part of f1 (Fasman *et al.*, 1971).

In an analogous study with f2b half-molecules, Li and Bonner (1971) found that DNA complexes formed with f2b–N half-molecules have melting temperatures 13° higher than those of f2b–C–DNA complexes. Furthermore, these two T_m values could be correlated with biphasic melting curves of native nucleohistone (Li and Bonner, 1971) and of complexes reconstituted with various histones (Ansevin and Brown, 1971). By examining the nuclear magnetic resonance of f2b, its N and C half-molecules, and f2b–DNA complexes at various salt concentrations, Bradbury and Rattle (1972) were able to show that the very basic portions of f2b (residues 1–30 and 103–125) have resonance bands broadened by interaction with DNA; on the other hand, the central, nearly neutral segment of f2b became involved in secondary structure and self-aggregation under conducive salt conditions. Thus,

evidence is accumulating that different portions of histone molecules interact to varying extents with DNA. The very basic sections bind to DNA and the more neutral parts are free to interact with themselves, different histones, or possibly other nuclear proteins. Experiments on chromatin modification (Simpson, 1971, 1972) indicate that the different regions of histone molecules may well have different roles in native chromatin, such that the histones can form bridges between DNA segments.

The intact f2b molecule is required for the specific mode of binding to DNA which results in maximum circular dichroism distortion. In Figure 1 it can be seen that at $r = 1.5$, for example, the ellipticity of the f2b–DNA complex cannot be represented as the average of N–DNA and C–DNA; the changes for whole f2b–DNA are much larger. The C segment aids in inducing conformational change in DNA, although C may not be actively involved in binding to DNA. This lack of additivity of parts does not apply to the chiroptical properties of the free histone fragments, where the optical rotary dispersion curve for f2b is the average of those for N and C (Bradbury *et al.*, 1972). However, the nuclear magnetic resonance characteristics of the half-molecules are not strictly additive either (Bradbury and Rattle, 1972). In intact f2b, residues 35–102 are restricted at physiological salt concentration. In C, residues 70–102 are similarly broadened. However, in N, residues 30–50 are restricted only at extremely high NaCl concentrations. Therefore, the intact f2b molecule is needed in order that residues 31–70 acquire secondary structure at moderate ionic strength. The presence of C attached to N in the intact f2b molecule both alters the conformational properties of N (Bradbury *et al.*, 1972) and helps the N segment to cause characteristic deformation in DNA complexes.

Complexes reconstituted with mixtures of N and C cleaved fragments indicate an additional complexity in f2b interaction with DNA. C antagonizes the circular dichroic effectiveness of N in complexes, partially but not entirely through binding competition. In the case of f1 the fragments act synergistically in their effect upon DNA circular dichrosim (Fasman *et al.*, 1971), thus presenting more evidence that each histone acts uniquely. A resemblance to the f2b fragment competition is found in complexes reconstituted with mixtures of f1 and f2a1 histones (Shih and Fasman, 1972). Each of these histones antagonizes the effect of the other; f1 is particularly potent, in small amounts, at blocking the characteristic circular dichroic changes induced in DNA by f2a1.

In conclusion, the interaction between histones and DNA is not a simple charge neutralization. Each histone studied yields a different, characteristic circular dichroism pattern when complexed with DNA. The highly positive amino-terminal half-molecule of f2b binds more strongly, and with greater conformational effectiveness, to DNA than does the more nearly neutral carboxyl half. However, the integrity of the intact f2b molecule is necessary for proper function, assayed in this situation, by maximal circular dichroic distortion upon DNA binding. A specific conformation of f2b is likely to be required for correct incorporation into chromatin, such that both its DNA binding regions and the segments involved in other functions are available. Thus, histones may well become included in the growing list of proteins (Anfinsen, 1973) which are fully active only when folded into unique and specific conformations.

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Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy of Phospholipids†

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance studies of a number of phospholipids and related phosphate mono- and diesters indicate that hydrogen bonding occurs in organic solutions of phospholipids. This interpretation is based primarily on the observation that phosphatidylethanolamine (PE), lysophosphatidylethanolamine (lyso-PE), phosphatidylserine (PS), lysophosphatidylserine (lyso-PS), sphingomyelin (SPH), and lysophosphatidylcholine (lyso-PC) all give rise to resonances in the same region of the ^{31}P spectrum; this region is downfield of the ^{31}P chemical shift of phosphalidylcholine (PC) by *ca.* 30 Hz. The chemical shifts of PE, lyso-PE, PS, lyso-PS, SPH, and lyso-PC are consistent with deshielding of the phosphorus nuclide by hydrogen bonding interactions of amine, amide, or hydroxyl protons with a phosphate oxygen. In the case of PC, however, the opportunity for hydrogen-bond-induced deshielding of the phosphorus is minimized due to the absence of the requisite dissociable proton in the molecule. The chemical shift of PC was displaced downfield by 25 Hz when methanol was added to chloroform solutions; the chemical shifts of PE and lyso-PC which contain a dissociable proton were altered to a lesser extent. The contribution of the quaternary nitrogen function of choline to the chemical shifts of PC was assessed by determining the chemical shifts of appropriate phosphate mono- and diesters in aqueous solutions and lyso-PC and lyso-PE in organic solution. This shift contribution was found to be *ca.* 15 Hz.

Phosphorus-31 nuclear magnetic resonance (^{31}P nmr) is proving particularly useful in studies on biological systems for a number of reasons, numbered among which are the relative simplicity of the spectra, the relatively large range of chemical shifts, the sensitivity of the phosphate shift to the presence of metal ions, and the relatively high sensitivity of this 100% naturally abundant nuclide.

Phosphorus data of the type obtained in the present work are already finding application in the study of lipid–lipid and lipid–protein interactions in biology. For example, Michaelson *et al.* (1973) used the differences in ^{31}P chemical shifts of phosphatidylglycerol and phosphatidylcholine to determine the sidedness of cosonicated vesicles. In addition we have applied the differences in ^{31}P chemical shifts of phosphatidylcholine and other phospholipids in studies on the structure of human serum lipoproteins (Glonek *et al.*, 1973a).

We recently reported the application of ^{31}P nmr to the detection, estimation, and identification of alkylphosphonic acid derivatives in biological materials (Glonek *et al.*, 1970; Hilderbrand *et al.*, 1971; Henderson *et al.*, 1972). During the course of these and subsequent studies, we consistently found that lipid fractions from a wide variety of sources in either organic or aqueous solvents gave rise to two absorption bands in the orthophosphate region of the ^{31}P nmr spectrum (*cf.* Figure 1, spectrum A for the spectrum of a bovine liver lipid extract). Similar spectra were obtained from *Tetrahymena pyriformis* (Glonek *et al.*, 1970), *Bunadosoma*, sp. (Glonek *et al.*, 1970; Henderson *et al.*, 1972), *Metridium dianthus* (Glonek *et al.*, 1970), swine brain lipids (T. O. Henderson and M. Dahl, unpublished observations), and intact human serum lipoproteins (Glonek *et al.*, 1973a). These two different resonance bands could arise from either of two sources: (1) two different phospholipids, or (2) the same species of phospholipid in two different molecular environments. In an effort to resolve this question we determined the chemical shifts of a number of purified phospholipids and related phosphorylated compounds as well as bovine liver lipids and fractions thereof.

The data indicate that the resonance bands are derived from different phospholipid species and the position of resonance is interpreted to be dependent, in part, on the ability of the phospholipid to form hydrogen-bonded rings involving the phosphate subunits of the individual lipids.

Materials and Methods

^{31}P Nmr Spectrometry. The instrumentation used for these studies was a Bruker HFX-5 spectrometer with heteronuclear ^{1}H field stabilization and operating at 36.4 MHz for ^{31}P. Detailed descriptions of the instrumentation and the techniques employed have been published (Glonek *et al.*, 1971; Henderson *et al.*, 1972).

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