# Fluorescence Anisotropy and Circular Dichroism Study of Conformational Changes in Histone IIb2†

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ABSTRACT: Histone IIb2 (F2b) has been studied by the techniques of fluorescence anisotropy and circular dichroism (CD). Series of measurements have been performed as functions of pH, sodium phosphate and sodium chloride concentration at pH 7.4, and sodium chloride concentration at pH 3.5. There is no evidence of time-dependent changes with histone IIb2 that have been observed for histone IV [Li, H. J., Wickett, R. R., Craig, A. M., and Isenberg, I. (1972), *Biopolymers 11*, 375; Wickett, R. R., Li, H. J., and Isenberg, I. (1972), *Biochemistry 11*, 2952]. Changes in the intrinsic tyrosine fluorescence properties and in the CD, upon addition of salt or change in pH, take place in a period of time faster than the resolution of our techniques. The effects of salt upon histone conformation may be interpreted as resulting from anion binding to histone. In the salt-induced state there are changes in the CD spectra which result, primarily, from random-coil to $\alpha$-helix formation. We estimate 18 $\alpha$-helical residues at pH 7.4 and 33 at pH 3.5. Effective equilibrium constants for inducing conformational changes are calculated from both fluorescence and CD measurements at pH 7.4. The same constant is obtained from both sets of observables. Sodium phosphate is 40 times as efficient as sodium chloride at pH 7.4. The fraction of molecules in the salt-induced state is a sigmoidal function of the sodium chloride concentration at pH 3.5, but not at pH 7.4. Twenty residues of $\alpha$-helix at positions 65–84 are predicted by nearest-neighbor analysis [Wu, T. T., and Kabat, E. A. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1501]. Our results are related to previous studies of histone IIb2 and compared to similar studies of histone IV.

Histones may serve a general function in gene regulation by interaction with DNA (Stellwagen and Cole, 1969). This possibility has stimulated many studies of the interaction of histones with DNA (Adler and Fasman, 1971; Adler *et al.*, 1971; Delange and Smith, 1971; Fasman *et al.*, 1970, 1971; Johns and Forrester, 1969; Li and Bonner, 1971; Li *et al.*, 1971; Olins, 1969; Olins and Olins, 1972; Richards and Pardon, 1970; Richards *et al.*, 1970; Shih and Bonner, 1970; Shih and Fasman, 1971, 1972; Taun and Bonner, 1969; Wagner, 1970). While such studies are, of course, important, our knowledge, to date, of the physical properties of the individual histones themselves is still meager.

This laboratory recently reported results on salt-induced conformational changes of histone IV (Li *et al.*, 1972; Wickett *et al.*, 1972). Fluorescence anisotropy and circular dichroism were used in these studies, not only because of their sensitivity, but also because changes in the observables could be quantitated with respect to the conditions of the measurements.

Addition of salts to histone IV were shown to induce a series of conformational changes in the protein. Those changes could be decomposed into a very fast change involving an increase in tyrosine rigidity and $\alpha$-helix formation of about 17 residues, and a slower change involving $\beta$-sheet formation of 20–30 residues/molecule. The fluorescence anisotropy of the fast step increases with increasing salt and histone concentration. The dependence of the histone concentration satisfied a model for histone dimer formation.

Histone IIb2 is one of the histones whose complete primary structure is now known (Iwai *et al.*, 1970). In addition several studies have been undertaken on histone IIb2.

Boublik *et al.* (1970) investigated conformation changes of IIb2 in sodium chloride solutions at pH 3.2 using optical rotatory dispersion (ORD) and proton magnetic resonance. Working at concentrations of 1–50 mg/ml, these workers found an increased rigidity in certain parts of the IIb2 molecule with increasing pH, sodium chloride concentration, or histone concentration. They concluded that the residues principally affected were located in the peptide chain at positions 60–102. Edwards and Shooter (1969) reported ultracentrifuge studies of histone fractions from calf thymus. Working at pH 3.4 and concentrations of 10 mg/ml, they reported aggregation of histone IIb2 which increased with increasing sodium chloride or histone concentrations. In 0.50 M NaCl, for example, the aggregates contained four or five molecules of histone. Diggle and Peacocke (1971) reached similar conclusions from osmotic pressure and sedimentation equilibrium studies.

In the present paper we report studies on the intensity and polarization of fluorescence of the tyrosines of histone IIb2. We have also measured the circular dichroism (CD) of the peptide bonds of the protein and have used these measurements to follow conformational changes in histone IIb2 as functions of sodium chloride and sodium phosphate concentrations at pH 7.4 and 3.5. The changes in histone IIb2 differ markedly from those found in histone IV; and in later sections of this paper, we shall contrast the changes in the two histones.

## Experimental Section

Calf thymus histone IIb2 was prepared by gradient chromatography on carboxymethylcellulose, according to the method of Senshu and Iwai (1969). As final steps in the preparation, the histone was desalted on a Sephadex G-25 column equilibrated with 0.01 M HCl and then lyophilized. Lyophilized histone was stored at −15° in desiccated containers. Amino acid analysis of constant-boiling HCl hydrol-

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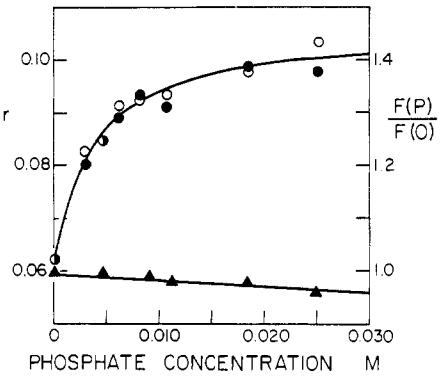


FIGURE 1: Anisotropy, $r$, and relative fluorescence intensity, $F(P)/F(0)$, of histone IIb2 ($c = 1.0 \times 10^{-5}$ M) as functions of sodium phosphate concentration at pH 7.4. Anisotropy values measured within several hours after preparation are denoted by ● and values after refrigeration of the samples overnight are given by ○. $F(P)/F(0)$ is denoted by ▲.

ysates gave excellent agreement with that predicted from the sequence, and there was but a single band in polyacrylamide gel electrophoresis in 2.5 M urea (pH 3.2) (Panyim and Chalkly, 1969).

Fluorescence anisotropy was measured with an instrument described elsewhere (Evett and Isenberg, 1969; Li *et al.*, 1972). Anisotropy is defined as $r = (E - B)/(E + 2B)$ in which $E$ is the fluorescence component polarized parallel to the vertically polarized exciting light and $B$ is the fluorescence component polarized perpendicular to the exciting beam.

Histone IIb2 has five tyrosines and two phenylalanines (Iwai *et al.*, 1970). Because of the relatively small absorbance and low quantum yield of the phenylalanines, the observed emission is, effectively, that of the tyrosines only. Two Jarrell-Ash 1/4 meter monochromators in series were used in the excitation beam and single monochromators plus Corning CS-054 filters were used in the emission beams. Excitation was at 279 nm and the emission was measured at 325 nm. Samples were at 22 ± 1° in 1-cm² cuvets.

Circular dichroism spectra were measured with a Durrum-Jasco Model J-10 CD recorder and, in some instances with a vacuum CD spectrophotometer built by W. C. Johnson of this department (Johnson, 1971). Cells of various path length were used for the measurements and the effective product of

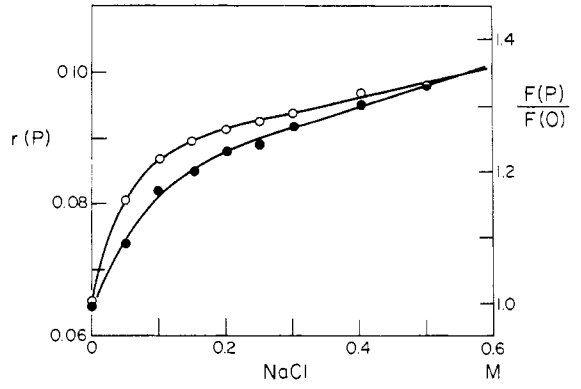


FIGURE 2: Anisotropy (●) and relative fluorescence intensity, $F(P)/F(0)$ (○), of histone IIb2 ($c = 1.0 \times 10^{-5}$ M) as functions of sodium chloride concentration at pH 7.4.

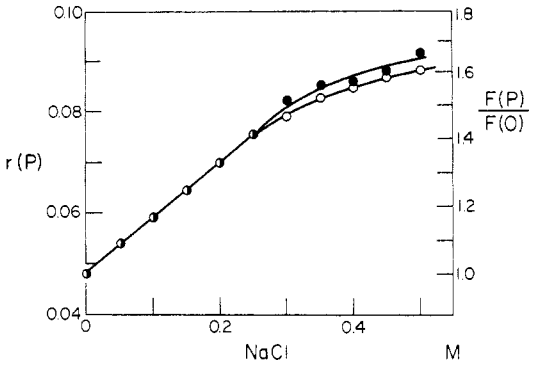


FIGURE 3: Anisotropy (●) and relative fluorescence intensity, $F(P)/F(0)$, of histone IIb2 ($c = 1.0 \times 10^{-5}$ M) as functions of sodium chloride concentration at pH 3.5.

path length and concentration was determined from absorption spectra. CD spectra were measured at 22 ± 1° and are reported as $\Delta\epsilon = \epsilon(\text{left}) - \epsilon(\text{right})$, and measured in units of $cm^{-1}$ l. per mole of residue.

Concentrations of IIb2 were determined by measurement of the optical density at 276 or 230 nm using the respective extinction coefficients of $6.7 \times 10^3$ or $5.43 \times 10^4$ $cm^{-1}$ l. (mole of histone)$^{-1}$. The extinction coefficient at 275 nm was assumed to be the sum of the extinction coefficients of *N*-acetyl ethyl esters of five tyrosine and two phenylalanine molecules (Herskovits and Sorenson, 1968). The extinction coefficient at 230 nm was assigned according to the spectral intensity ratio. Electronic absorption spectra were measured with a Cary 14 spectrophotometer.

Solutions of IIb2 in phosphate buffer (pH 7.4), sodium chloride–0.005 M cacodylate buffer (pH 7.4), or sodium chloride–0.005 M acetic acid buffer (pH 3.5) were prepared by dilution of concentrated aqueous solutions with the desired salt solutions. To achieve a series of solutions of IIb2 in sodium chloride and buffer, aliquots of concentrated sodium chloride in 0.005 M buffer were added to a histone IIb2, buffered, starting solution. A Corning Digital 112 pH meter equipped with a Corning semimicro combination electrode was used for the pH measurements.

## Results

*Salt Effects on Fluorescence Emission Properties.* The fluorescence anisotropy of histone IIb2 rises with increasing concentrations of either sodium chloride or sodium phosphate (Figures 1, 2, and 3). In contrast to histone IV, at least at the concentrations used here, we have not seen a slow component of the changes. The changes in anisotropy and CD are, effectively, instantaneous as determined by our measurement techniques. Even if our IIb2 samples were left for several hours at room temperature, or overnight at 4° there was no change in either CD or anisotropy.

The addition of sodium phosphate or sodium chloride affects the emission intensity differently. While the addition of phosphate results in a small amount of quenching, addition of chloride produces a considerable amount of fluorescence enhancement both at pH 7.4 and 3.5 (Figures 1, 2, and 3).

*Salt Effects on Circular Dichroism.* Figure 4 shows CD spectra of histone IIb2 in several solvents: water, 0.0033 M sodium phosphate, and 0.026 M sodium phosphate. These spectra were measured on the vacuum uv–CD spectrophotometer

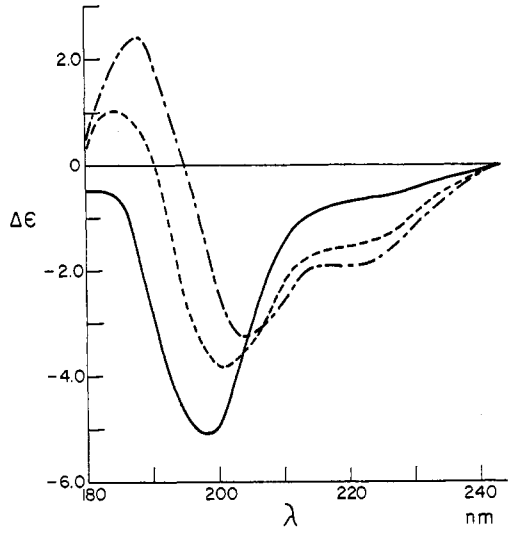


FIGURE 4: CD spectra of histone IIb2 ($1.2 \times 10^{-5}$ M) from 250 to 180 nm as measured on the vacuum CD spectrophotometer; in water (pH 4.3) (——), in 0.0033 M sodium phosphate (pH 7.4) (– – –), and in 0.026 M sodium phosphate (pH 7.4) (–·–·–). Spectra were measured in a 0.50-mm cell.

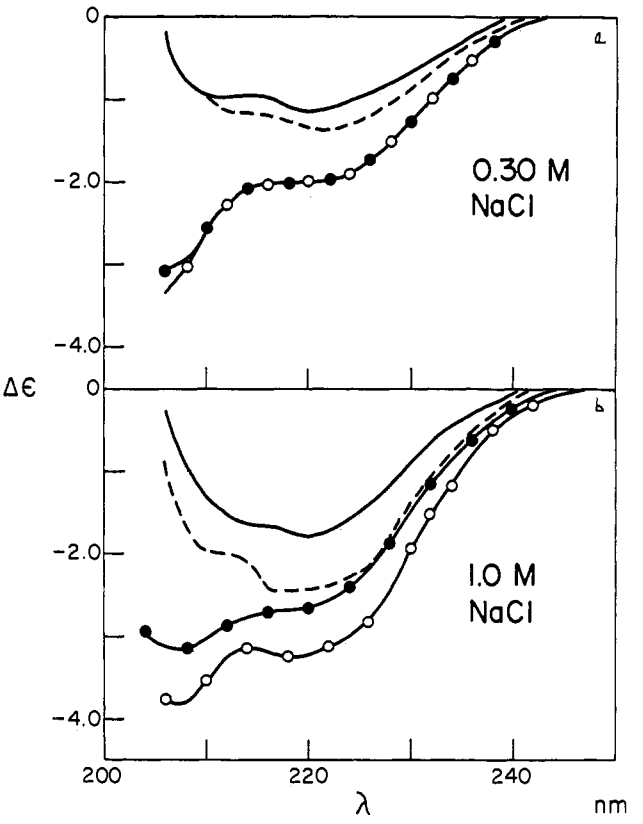


FIGURE 5: CD spectra and difference spectra of histone IIb2. (a) In 0.30 M NaCl at pH 3.5 and 7.4. CD spectrum, pH 7.4 (●); CD spectrum, pH 3.5 (○); CD difference spectrum, $\Delta\epsilon_\lambda$(0.30 M NaCl) − $\Delta\epsilon_\lambda$(0.005 M cacodylate), pH 7.4 (——); CD difference spectrum, $\Delta\epsilon_\lambda$(0.30 M NaCl) − $\Delta\epsilon_\lambda$(0.005 M acetic acid), pH 3.5 (– – –). (b) In 1.0 M NaCl at pH 3.5 and 7.4. CD spectrum, pH 7.4 ●; CD spectrum, pH 3.5 (○); CD difference spectrum, $\Delta\epsilon_\lambda$(1.0 M NaCl) − $\Delta\epsilon_\lambda$(0.006 M cacodylate), pH 7.4 (——); CD difference spectrum, $\Delta\epsilon_\lambda$(1.0 M NaCl) − $\Delta\epsilon_\lambda$(0.005 M acetic acid) ●, pH 3.5 (– – –).

built by Curtis Johnson (Johnson, 1971). Spectra of histone measured in 0.30 M NaCl at pH 7.4 and 3.5 are given in Figure 5a. These spectra were measured on a Durrum-Jasco recorder. It should be noted that the spectra in 0.3 M NaCl at pH 7.4 and 3.5 are nearly superimposable. Spectra of IIb2 in sodium phosphate (pH 7.4) at appropriate concentrations are also of the same shape. Hence, from zero salt to at least 0.050 M phosphate and 0.50 M NaCl, the CD spectra are similar in shape, even at pH 3.5.

With the present state of CD theory, it is necessary to choose standards in order to identify and make quantitative interpretations of CD spectral changes. We have used the CD difference spectra method of Li *et al.* (1972). The CD spectrum of IIb2 in 0.005 M HCl has been assigned as the random coil of IIb2. As models of protein with 100% helicity of 100% $\beta$ structure, the CD spectra of polylysine (Greenfield and Fasman, 1969) were chosen. Subtraction of the values of IIb2 "random coil" from that of polylysine should generate the curve expected for a 100% coil–helix transition or a 100% coil–$\beta$ transition. By properly weighting these curves, we can calculate difference spectra for coil to various admixtures of $\alpha$ helix and $\beta$ sheet (Figure 6a).

Comparison of shapes of difference spectra of IIb2 in 0.015 M phosphate and in water (pH 7.4) (Figure 6b) to that of calculated difference spectra (Figure 6a) suggests that the changes in the spectra are primarily that for random coil to $\alpha$-helix formation. Similar difference spectra were obtained at all of the sodium phosphate concentrations we have used and at sodium chloride concentrations at pH 7.4 up to about 0.50 M.

Examples of difference spectra of histone IIb2 in sodium chloride and in the buffer before salt addition are given in Figure 5a. There are some differences in the difference spectra because of the changes in CD of histone with pH (see later section). However, the shapes are still such that the salt-induced changes in the spectra are primarily that for an $\alpha$ helix.

At very high salt concentrations (1.0 M NaCl) the CD difference spectra are not that for an $\alpha$ helix alone (Figure 5b).

To monitor changes in the CD spectra as a function of salt, we have plotted $\Delta\epsilon$, at 220 nm, as a function of salt for the several titrations (Figure 7). The curves of $\Delta\epsilon_{220}$(P) for sodium phosphate and sodium chloride at pH 7.4 are similar in shape to the classical Langmuir absorption isotherm, while the curve of $\Delta\epsilon_{220}$(P) for sodium chloride at pH 3.5 is sigmoidal.

*Data Analysis and Effective Equilibirum Constants.* A number of questions arise regarding the conformational changes resulting from the interaction of salts with histone, as seen in our measurements. First, do the changes in fluorescence properties and in the CD follow the same functional dependence? Second, can we quantitate the effectiveness of a salt in altering the properties of the histone? Finally, how do the salts differ in their effectiveness in inducing changes in histone IIb2?

In order to obtain a quantitative measure of effectiveness, it is necessary to make a number of simplifying assumptions regarding the interaction of salt with histone. The following assumptions are, essentially, the same as those invoked by Li *et al.* (1972) and Wickett *et al.* (1972) in studies on histone IV.

It is assumed that there exist a set of binding sites on the protein, and that, if ions bind to such sites, conformational changes are induced. It is also assumed that the number of protein molecules undergoing a change is proportional to the amount of salt bound. These assumptions are equivalent to a general two-state system in which the histone molecules that have altered conformation upon interacting with salt are in equilibrium with those that do not (Wickett *et al.*, 1972).

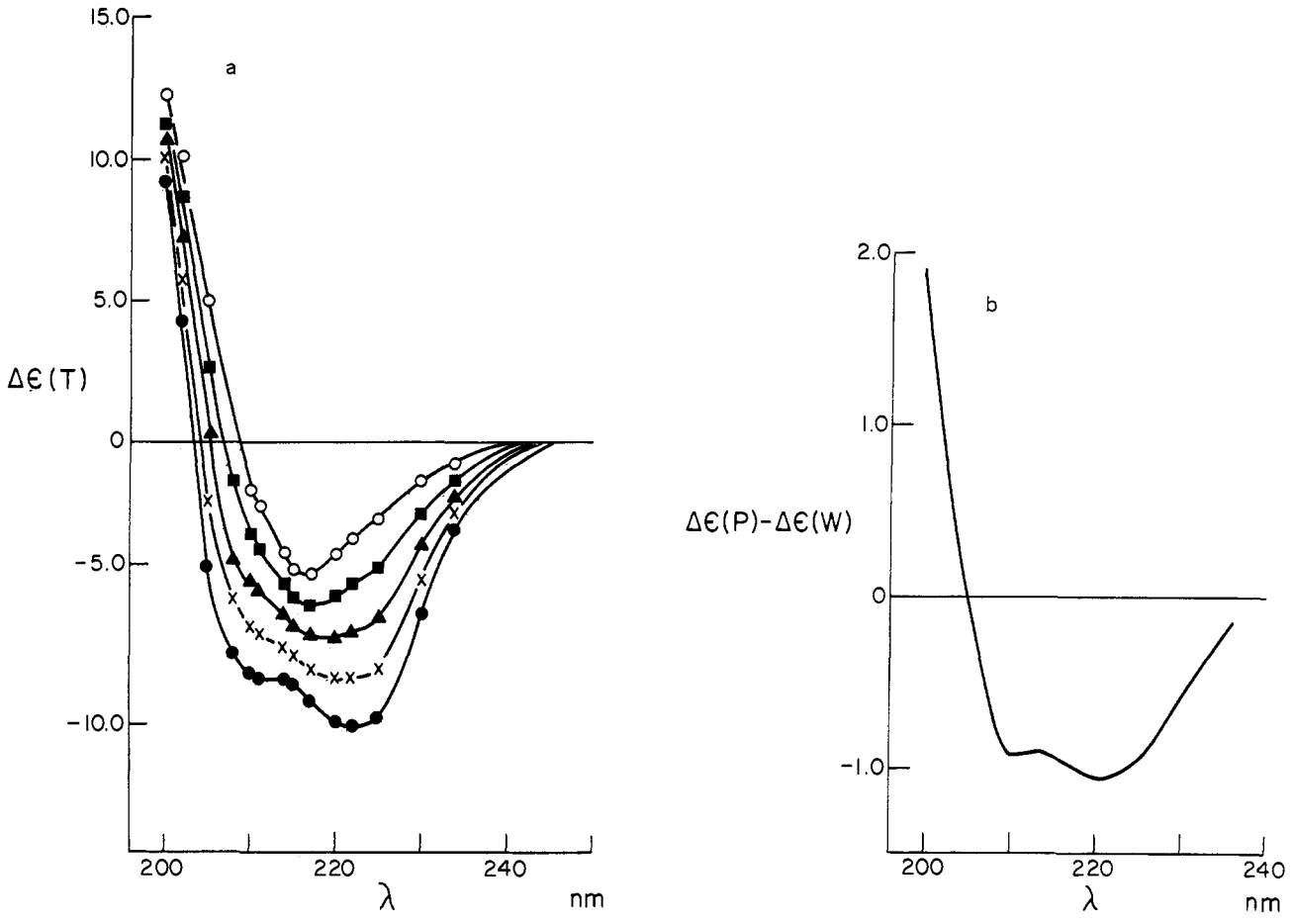


FIGURE 6: (a) Computed difference spectra for coil–100% $\alpha$ helix (●); coil–75% $\alpha$ helix, 25% $\beta$ sheet (×); coil–50% $\alpha$ helix, 50% $\beta$ sheet (▲); coil–25% $\alpha$ helix, 75% $\beta$ sheet (■); and coil–100% $\beta$ sheet (○). (b) Difference spectrum computed from CD spectra of histone IIb2 in 0.014 M phosphate and in water (pH 7.4).

However, it should be emphasized that it is possible to analyze a multistate system by two-state equations provided that appropriate average quantities are used for the various parameters. (See appendix of Wickett *et al.*, 1972.) The use of two-state equations does not imply that there are, in actuality, only two real physical states.

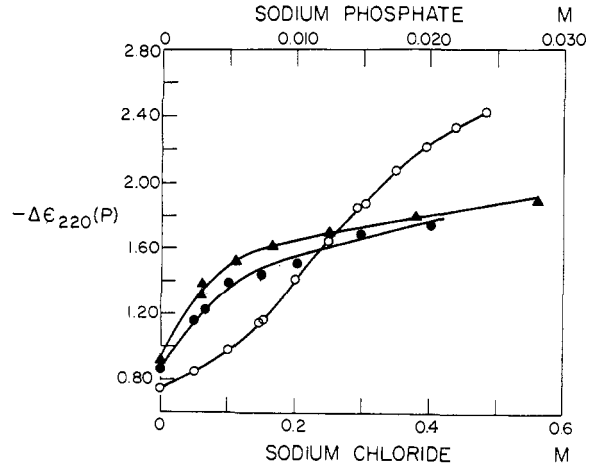


FIGURE 7: Dependence of $\Delta\epsilon_{220}(P)$ of histone IIb2 as a function of salt concentration: phosphate, pH 7.4 (▲); chloride, pH 7.4 (●); chloride, pH 3.5 (○).

As more information is obtained on these conformationa changes, it is likely that one or more, perhaps all, of these assumptions may be found to be wrong. Making them, however, has the virtue of permitting us to give a quantitative answer to many of our questions regarding histone–salt interaction and to make comparisons among different histones.

Two points must be emphasized. The effectiveness of ions in inducing CD changes does not necessarily have to be the same as the effectiveness in inducing anisotropy changes. For histone IV, most salts do indeed show the same effectiveness for the two different changes, but perchlorate does not (Wickett *et al.*, 1972). Secondly, the binding constants obtained from our observables are not necessarily the same as the physical binding constants which govern how much salt actually binds to the protein. It is conceivable, and may even be probable, that there could be many binding sites which, even when filled, will not lead to a change in the protein conformation.

For the simplest case in which the binding sites affecting a given observable are equivalent, we may write

$$B + P \rightleftharpoons BP$$

$$K = \frac{[BP]}{[B][P]} \qquad (1)$$

in which [B] is the concentration of available ion binding

TABLE I: Percentage of $\alpha$ Helix in the Salt-Induced State as Calculated from Eq 4 and/or Extrapolation.

| Salt | $\Delta\epsilon_{220}(\infty) - \Delta\epsilon_{220}(0)$ ($M^{-1}$ $cm^{-1}$) | Random-Helix Residues | $\Delta\epsilon_{220}(\infty) - \Delta\epsilon_{220}$ (0.005 M HCl), $M^{-1}$ $cm^{-1}$ | $\alpha$-Helix Residues |
|---|---|---|---|---|
| Sodium chloride (pH 7.4) | −1.25 | 15.6 | −1.40 | 17.5 |
| Sodium phosphate (pH 7.4) | −1.31 | 16.4 | −1.46 | 18.3 |
| Sodium chloride (pH 3.5) | −2.65 | 33.1 | −2.65 | 33.1 |

sites, [P] the salt concentration, and [BP] the concentration of bound sites.

Let $[B_0]$ be the total number of available binding sites which affect an observable. Then $f_2$, the fraction of bound sites, is equal to $[BP]/[B_0]$. At salt concentrations such that the initial salt concentration, $[P_0]$, is much greater than $[B_0]$, $[P] \approx [P_0]$, and substitution in eq 1 yields eq 2. In terms of a general

$$\frac{1}{f_2} = \frac{1}{K[P_0]} + 1 \qquad (2)$$

two-state model (Wickett *et al.*, (1972), $f_2$ is the fraction of histone molecules to which salt has been bound.

*Circular Dichroism Results.* For CD we simply assume that

$$f_2 = \frac{\Delta\epsilon_\lambda(P) - \Delta\epsilon_\lambda(0)}{\Delta\epsilon_\lambda(\infty) - \Delta\epsilon_\lambda(0)} \qquad (3)$$

and substitution into eq 2 yields (Li *et al.*, 1972; Wickett *et al.*, 1972)

$$\frac{1}{\Delta\epsilon_\lambda(P) - \Delta\epsilon_\lambda(0)} = \frac{1}{\Delta\epsilon_\lambda(\infty) - \Delta\epsilon_\lambda(0)}\left[\frac{1}{K[P_0]} + 1\right] \qquad (4)$$

In eq 3 and 4 $\Delta\epsilon_\lambda(P)$ is the value of $\Delta\epsilon$ measured at the wavelength $\lambda$ and salt concentration [P].

Data from the titrations of histone with phosphate (pH 7.4) and sodium chloride (pH 7.4) are plotted according to eq 4 in Figures 8 and 9. The plots are linear and, therefore, the data are not obviously inconsistent with the assumptions used in deriving eq 4. From the plots we have obtained $\Delta\epsilon_{220}(\infty) - \Delta\epsilon_{220}(0)$ which is the total CD change inducible by salt. (It may be recalled that the difference spectra of histone IIb2 in the presence and absence of sodium phosphate and sodium chloride at pH 7.4 were indicative of primarily $\alpha$-helix formation.) From this and $\Delta\epsilon_{220}$(helix) − $\Delta\epsilon_{220}$(random) = $-10.0$ $cm^{-1}$ $M^{-1}$ for a 100% coil to helix transition, we may estimate the percentage helical change inducible in histone IIb2 by the salt. We find (Table I) 13% or 16.3 residues changed for sodium phosphate and 12.4% or 15.5 residues changed for sodium chloride.

A value of 16 residues is only the change observed at pH 7.4. It is difficult to pinpoint this number as the actual number of residues in the folded state especially since the CD is pH dependent. It might be more reasonable, for example, to take the CD spectrum at pH 2.3 as random coil. Relative to this form there are 18 residues in the folded state at pH 7.4. In any case the difference between 16 and 18 residues is probably within, or close to, the experimental error in the measurement.

We note that, if any particular protein molecule alters its conformation in an all or none fashion, then 18 residues of $\alpha$ helix are formed per altered protein molecule under any salt condition. If the change is otherwise, then the values give the average change per altered molecule.

A plot of the pH 3.5 CD data from Figure 7 according to eq 4 is nonlinear, indicating that the conformational change is more complex than in the two previous cases. Hence, we have simply calculated $f_2$ by eq 3 (Figure 10) for later comparison with the fluorescence analysis.

Extrapolation of $\Delta\epsilon_{220}(\infty)$ to infinite sodium chloride at pH 3.5 gives a value of $\Delta\epsilon_{220}(\infty) = -3.40$ $M^{-1}$ $cm^{-1}$ and $\Delta\epsilon_{220}(\infty) - \Delta\epsilon_{220}(0) = -2.65$ $M^{-1}$ $cm^{-1}$. Based on 100% $\alpha$ helix, this corresponds to 33 residues of $\alpha$ helix, twice as many as at pH 7.4.

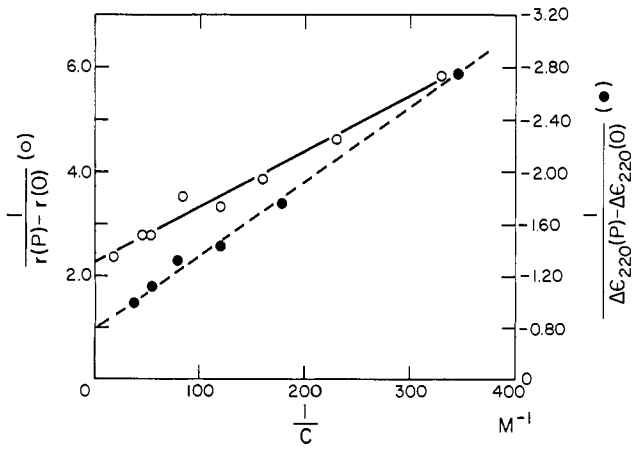


FIGURE 8: Plots of $1/[\Delta\epsilon_{220}(P) - \Delta\epsilon_{220}(0)]$ (●) and $1/[r(P) - r(0)]$ (○) *vs.* the reciprocal of sodium phosphate concentration, at pH 7.4, according to eq 4 and 10.

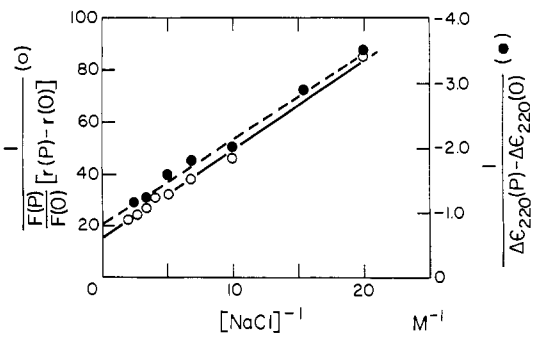


FIGURE 9: Plots of $1/\Delta\epsilon_{220}$ (P)−$\Delta\epsilon_{220}$ (0) (●) and $1/[F(P)/F(0)][r(P) - r(0)]$ (○) *vs.* the reciprocal of sodium chloride concentration, at pH 7.4, according to eq 4 and 10.

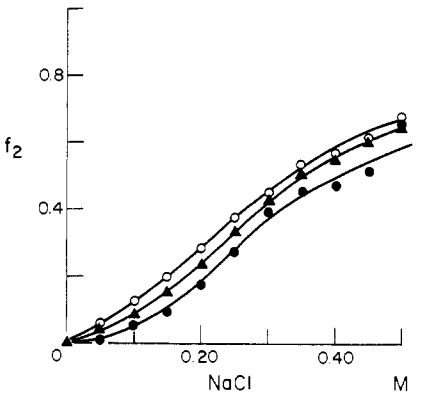


FIGURE 10: A plot of $f_2$ as a function of sodium chloride concentration at pH 3.5 according to: eq 3 (▲), eq 7 (●), and eq 9 (○).

*Fluorescence Results.* For a two-state system in dilute solution, the fluorescence intensity $F$, observed at a salt concentration [P], is given by

$$F(\mathrm{P}) = K[\epsilon_1(\mathrm{P})q_1(\mathrm{P})f_1 + \epsilon_2(\mathrm{P})q_2(\mathrm{P})f_2] \quad (5)$$

$\epsilon_i(\mathrm{P})$ and $q_i(\mathrm{P})$ are the extinction coefficient and quantum yield of state $i$ at concentration [P]. $K$ is a constant. The anisotropy and the fractional fluorescence intensities are related by the addition law (Weber, 1952), where the fraction

$$r = \varphi_1(\mathrm{P})r_1(\mathrm{P}) + \varphi_2(\mathrm{P})r_2(\mathrm{P}) \quad (6)$$

of light emitted by state $i$ is $\phi_i$.

In the most general formulation, using eq 5 and 6, one would like to be able to allow for possible changes in $\epsilon_1(\mathrm{P})q_1(\mathrm{P})$ and $\epsilon_2(\mathrm{P})q_2(\mathrm{P})$ as functions of salt. However, a determination of both of these dependencies is, in general, not calculable from measurements of anisotropy and fluorescence intensity alone (Evett and Isenberg, 1969). For a given calculation it is therefore necessary to make an additional assumption. It is sometimes reasonable to take one, or the other of $\epsilon_1(\mathrm{P})q_1(\mathrm{P})$ or $\epsilon_2(\mathrm{P})q_2(\mathrm{P})$ as constant, and to test if the one not assumed to be constant, varies with salt. As described elsewhere (Evett and Isenberg, 1969; Ellerton and Isenberg, 1969), we may derive the following equations. If $\epsilon_1 q_1$ is constant, that is, if $\epsilon_1(\mathrm{P}) \cdot q_1(\mathrm{P}) = \epsilon_1(0)q_1(0)$, we obtain

$$f_2 = 1 - \frac{F(\mathrm{P})}{F(0)}\frac{r(\infty) - r(\mathrm{P})}{r(\infty) - r(0)} \quad (7)$$

$$\frac{1}{\frac{F(\mathrm{P})}{F(0)}[r(\infty) - r(\mathrm{P})]} = \frac{1}{[r(\infty) - r(0)]}[K[\mathrm{P}_0] + 1] \quad (8)$$

On the other hand, if $\epsilon_2(\mathrm{P})q_2(\mathrm{P}) = \epsilon_2(\infty)q_2(\infty)$, then we get

$$f_2 = \frac{F(\mathrm{P})}{F(\infty)}\frac{[r(\mathrm{P}) - r(0)]}{[r(\infty) - r(0)]} \quad (9)$$

$$\frac{1}{\frac{F(\mathrm{P})}{F(0)}[r(\mathrm{P}) - r(0)]} = \frac{1}{\frac{F(\infty)}{F(0)}[r(\infty) - r(0)]}\left[\frac{1}{K[\mathrm{P}]} + 1\right] \quad (10)$$

TABLE II: Effective Equilibrium Constants of Salt Binding to Histone IIb2.

| Salt (pH 7.4) | $K_{CD}$ ($M^{-1}$) | $K_r$ ($M^{-1}$) |
|---|---|---|
| Sodium phosphate | 150[a] | 220[b] |
| | 260[b] | |
| Sodium chloride | 5.4[a] | 4.3[b] |

[a] Histone IIb2 concentration is $0.5 \times 10^{-5}$ M. [b] Histone IIb2 concentration is $1.0 \times 10^{-5}$ M.

We have applied eq 10 to the fluorescence data of histone IIb2 as a function of phosphate concentrations (Figure 8). Since, when phosphate is added, there is only a very small change in fluorescence intensity (Figure 1), we have set $F(\mathrm{P})/F(0) = 1$ and $F(\infty)/F(0) = 1$ in eq 10. The plot in Figure 8 is a straight line, from which $k$ is calculated to be $2.4 \times 10^2$ $M^{-1}$ and $r(\infty) - r(0) = 0.045$.

For the sodium chloride data (Figure 5), in which there is a significant dependence of fluorescence intensity on salt concentration, there is no *a priori* reason for choosing either eq 8 or 10. However, eq 10 gives better linearity (Figure 9). Using it we obtain an effective equilibrium constant of 4.3 $M^{-1}$.

Equilibrium constants at pH 7.4 obtained from the various equations are compiled in Table II. Values obtained from CD and fluorescence anisotropy for each salt are sufficiently similar so that the differences must be considered to be within experimental error and are not significant. Therefore, the changes in the CD and fluorescence anisotropy at pH 7.4 are interpreted to arise from the same salt–histone interaction. Since the CD and fluorescence anisotropy measure quite different properties, it seems reasonable to infer that in the salt-induced state there has been a conformational change involving large parts, and perhaps the entire molecule.

It is clear from the data in Table II that at pH 7.4, sodium phosphate is 40–50 times more efficient than sodium chloride at inducing conformational changes in histone IIb2. Since both salts contain the sodium cation, the anion must be primarily responsible for inducing the structural changes in histone IIb2.

As was observed with the CD results, at pH 3.5, the fluorescence properties of IIb2 titration with sodium chloride are not consistent with a simple model of equivalent binding sites. Plots by either eq 8 or 10 do not yield straight lines. Therefore, $f_2$ was calculated by both eq 7 and 9 for comparison to plots of $f_2$ from the CD results. The three plots are given in Figure 9. There is close agreement between the plots and the agreement indicates that the fluorescence and CD data follow the same functional dependence with regard to salt concentration. Hence, at pH 3.5, as at pH 7.4, the CD and fluorescence properties appear to be sensitive to the same conformational changes.

*pH Effects.* Figure 11a,b shows the pH dependence of the anisotropy and relative fluorescence intensity of histone IIb2 solutions in three different media of low ionic strength: 0.005 M HCl, 0.005 M cacodylic acid, and water. In all three media there was essentially the same anisotropy and fluorescence intensity.

From pH 4.0 to 7.4 there is a monotonic 20% increase in anisotropy and a 5% increase in fluorescence intensity. Above

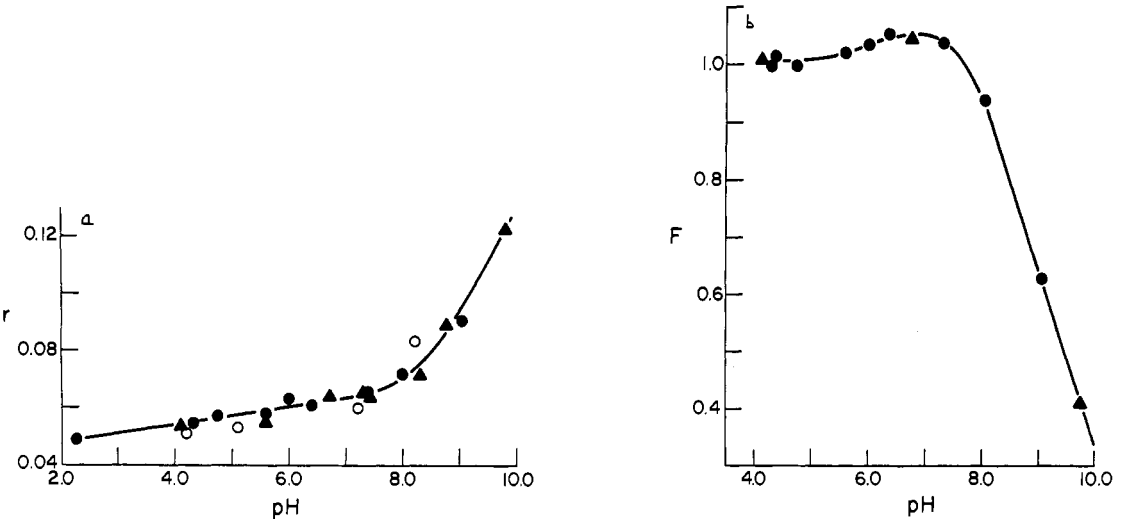


FIGURE 11: (a) Anisotropy of histone IIb2 ($1 \times 0 \times 10^{-5}$ M) as a function of pH in 0.006 M chloride (●), 0.0005 M cacodylate (▲), and water (○). (b) Relative fluorescence intensity, $F$, of histone IIb2 as a function of pH in 0.005 M chloride (●) and cacodylate (▲).

7.5 the anisotropy increases rapidly with pH and the fluorescence intensity shows a sharp decrease.

CD spectra of histone IIb2 in 0.005 M NaCl at pH values of 2.3, 7.4, and 9.2 are given in Figure 12. There is little change in the CD spectra in the range of 2.3–7.4, but there is considerable difference in going to pH 9.2. The change in the spectrum in going from pH 7.4 to 9.2 is typical of $\alpha$-helix formation and represents an approximate increase of 9% (11 residues) in the helical content. The small difference in the CD spectra between pH 2.3 and 7.4 indicates that, at pH 7.4, the IIb2 molecules are still essentially in a random-coil conformation.

The groups which would be expected to titrate in the pH range of 2.3–7.4 are the carboxyl groups of aspartic and glutamic acids and the imidazole ring of histidine. At higher pH, tyrosine, lysine, and arginine titrate. Tyrosinate ion is known to have a very low fluorescence quantum yield (Eisinger *et al.*, 1969; Truong *et al.*, 1967).

## Discussion

The most striking observation in the present work is the contrast between the changes seen in histone IIb2 and histone IV. All of the changes in fluorescence properties and CD upon addition of salt to histone IIb2 were instantaneous in contrast to those of histone IV which show both a fast step and a slower conformational change.

It is possible, of course, that, at higher concentrations, histone IIb2 would also show a slow change. However, at the concentrations we have used we have seen no evidence of it; and, even if one exists at high concentrations, we must still conclude that the overall changes in histones IV and IIb2 are quite different.

The changes induced by salt addition are highly pH dependent. At pH 7.4, sodium phosphate and sodium chloride induce CD spectral changes showing $\alpha$-helix formation of about 16 residues. At pH 7.4 the change in CD has the same functional dependence as the fluorescence changes, and these were consistent with a model of salt interaction with equivalent histone anion binding sites.

At pH 3.5 the functional dependence of the salt-induced state with sodium chloride concentration is very different from that at pH 7.4. The fraction of histone in the salt induced state as a function of sodium chloride concentration becomes sigmoidal suggesting that there is cooperative interaction of salt with histone. However, despite the difference in salt functional dependence at pH 3.5 and pH 7.4, the CD spectra are qualitatively the same, *viz.*, the change is that for $\alpha$-helical formation.

It should be noted that at pH 3.5 as well as at pH 7.4 both the CD and polarization of fluorescence data yield the same fraction of salt altered protein. This occurs in spite of the great differences in the functional dependence with pH and the difference in the magnitude of the CD. CD and polarization of fluorescence measure fundamentally different physical properties and tyrosines are located at positions 35, 40, 42, 83, and 121 in the 125 residue peptide chain (Iwai *et al.*, 1970). We therefore conclude that the conformational change observed in the CD and fluorescence data envelops a large portion, if not the entire molecule. Such an analysis does not preclude conformational changes in the histone random-coil or the existence of intermediate conformations such as

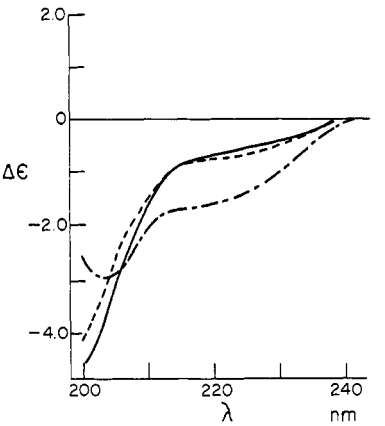


FIGURE 12: CD spectra of histone IIb2 in 0.006 M chloride at pH 2.3 (——), pH 7.4 (– – –), and pH 9.2 (–·–·–). Spectra were measured in a 1.0-mm cell at $1.0 \times 10^{-5}$ M histone IIb2 on the Durrum-Jasco recorder.

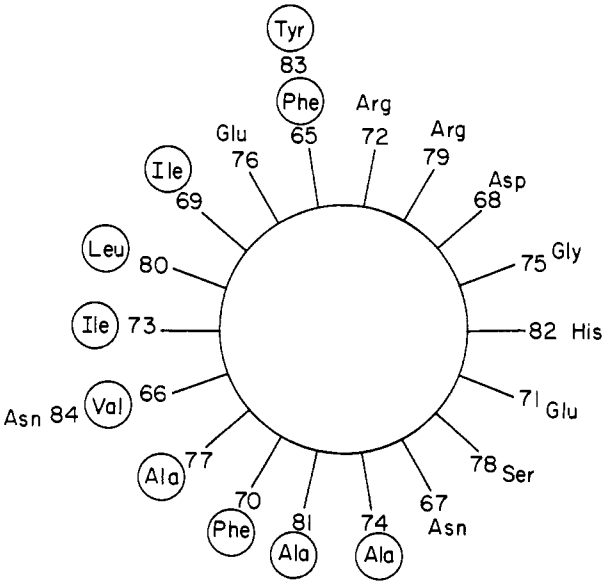


FIGURE 13: Helical wheel of residues 65–84.

those observed in the thermal folding of ribonuclease A (McPhie, 1972). Indeed, we find that eq 10, but not eq 8, fits the data, and this implies that the quantum yield of the random coil depends on the salt concentration.

If our method of estimating the $\alpha$-helical content is permissible, then there are 33 residues of $\alpha$ helix in the salt bound state at pH 3.5 and only 18 residues at pH 7.4. The larger number of residues at pH 3.5 is, at first glance, somewhat puzzling, but it may simply be the result of neutralization of aspartic and glutamic acid residues.

We have seen that the fluorescence intensity of histone IIb2 is affected differently by the chloride and phosphate ions. At pH 7.4, phosphate leads to a slight decrease in emission, while the addition of chloride leads to a considerable fluorescence enhancement at both pH 7.4 and 3.5. It has been reported by Feitelson (1964) and by Chen and Cohen (1966) that phosphate is an effective quencher of tyrosine fluorescence. On the other hand, chloride does not affect the quantum yield of tyrosine (Chen and Cohen, 1966). The similarity of the functional behavior of CD and fluorescence properties and the extent of helix formation by histone and phosphate or chloride at pH 7.4 would suggest that the conformational changes are the same, and independent of salt. Thus, it appears that the conformational change induced by the salts leads to an enhancement of the tyrosine fluorescence. However, when phosphate is used to induce the change, there is both an enhancement because of the protein folding and a decrease of emission intensity because of quenching by the phosphate. If this is so, it follows that in the folded condition at least one, and possibly a number, of the tyrosines are still exposed to the medium. They are, therefore, on the outside of the folded protein form.

The quenching due to phosphate that we suggest here is larger than that found for the phosphate quenching of free tyrosine (Chen and Cohen, 1966). However, binding of phosphate to histone may aid the interaction of phosphate and tyrosine.

Boublik *et al.* (1970) reported changes in the ORD of histone IIb2 (10 mg/ml) at pH 3.2 for several sodium chloride concentrations. If their $b_0$ values are plotted as a function of sodium chloride concentration, the curve has the Langmuir absorption isotherm shape like those we obtained at pH 7.4 rather than at pH 3.5. Plotted according to eq 4, we get an effective binding constant of 8 $M^{-1}$ and an extrapolated $b_0$ value of $-192°$ from their ORD data. Based on $b_0 = -635°$ for 100% $\alpha$ helix, we calculate 38 residues of $\alpha$ helix in the salt-induced state, which compares well to our value of 33. However, the Langmuir isotherm shape is not obtained by us. The difference may be due to the differences in histone concentrations used.

In their 1970 paper Boublik *et al.* constructed helical wheels and applied the Schiffer–Edmundson (1967) rules to predict helical regions in histone IIb2. Alone, the rules are not particularly successful for histone IIb2, and it was predicted by Boublik *et al.* that helical regions might be formed for residues 40–47, 74–81, 96–102, and 105–113. From their nuclear magnetic resonance data at pH 3.2, Boublik *et al.* concluded that the helical regions were most likely to be located from residues 60 to 102.

Recently, Wu and Kabat (1971) published a nearest-neighbor method for predicting conditionally helical regions. Only to these regions are the Schiffer–Edmundson rules then applied. We have applied the neraest neighbor analysis to histone IIb2 and predict conditionally helical regions at residues 65–84, 91–94, and 100–117. Of these regions, residues 65–84, 20 residues, seem the most likely to be helical by the helical wheel method (Figure 13). A value of twenty residues is in good agreement with the number of 18 residues we obtained from CD measurements at pH 7.4. The 20 residues predicted by the Wu and Kabat method do not, of course, agree with the greater helical content observed for pH 3.5, but this does not invalidate the method. The Wu–Kabat statistical analysis used proteins with charged glutamic and aspartic acids, conditions that are met at pH 7.4 but not at pH 3.5.

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# Low-Temperature Absorption and Circular Dichroism Studies of Phytochrome†

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ABSTRACT: At temperatures between +4° and −70°, phytochrome in 66% glycerol can exist in four forms: $P_R$, $P_{BL}$, $P_{FR}$, and A. These forms are interconverted *via* the thermal and photochemical reactions depicted in eq 1–5. The absorption spectra of $P_R$ and $P_{FR}$ between +4° and −70° are quite similar to those at room temperature, although the circular dichroism (CD) spectra show a more significant temperature dependence. $P_{BL}$ has a weak absorption band (relative to $P_R$) and a large negative CD band centered at about 660 nm. Intermediate A has an absorption band and a negative CD band centered at 658 nm. Theoretical calculations have been made of the variation in absorption intensities as a function of molecular geometry, and the results compared with experimental data. Such comparisons suggest that the phytochrome chromophore has an extended conformation in $P_R$, $P_{FR}$ and A, and a folded conformation in $P_{BL}$. The transformations from extended to folded conformations require cis–trans isomerizations.

The plant pigment phytochrome exerts a controlling influence over important aspects of plant growth and development, such as flowering, seed germination, and stem elongation (Hendricks and Borthwick, 1965). It is a chromoprotein which has an absorption maximum at approximately 660 nm after exposure to far-red light, and at 730 nm after exposure to red light. The phytochrome form that absorbs at 730 nm ($P_{FR}$) is more susceptible to standard denaturants than the form absorbing at 660 nm ($P_R$), thus indicating substantially different protein conformations in $P_R$ and $P_{FR}$ (Butler *et al.*, 1964). Chromatographic and spectral properties of the isolated prosthetic group indicate that it is a bilatriene similar to certain bile pigments and algal chromophores (Siegelman *et al.*, 1966); however, its exact structure and conformation have not been established. The photoconversion of $P_R$ to $P_{FR}$ at low temperatures has been studied by Spruit (1966a, 1966b), Cross *et al.* (1968), Pratt and Butler (1968), and Anderson *et al.* (1969). Their results indicate that the absorption of a photon chemically changes the bilatriene prosthetic group, and a sequence of thermal reactions ensues. By varying the temperature, one can stop the sequence at a number of intermediate stages. In this paper we extend the study of the thermal reactions occurring above −70°. In addition, we report the low-temperature absorption and circular dichroism (CD) spectra of $P_R$, $P_{FR}$, and two intermediates. An analysis which correlates the absorption spectra to the prosthetic group conformation is included.

## Materials and Methods

The procedure for phytochrome isolation is patterned after one described by Siegelman and Firer (1964). Phytochrome is extracted from the coleoptiles of 5-day-old etiolated oat (*Avena sativa*, Clintland variety). Harvested tissue (1.5 kg) is added to 1 l. of aqueous 0.1 M Tris buffer (pH 8) containing 0.1 M thioglycol and 0.002 M EDTA, and ground

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