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Salt Effects on Histone IV Conformation†

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ABSTRACT: The addition of salts to water solutions of histone IV induces conformational changes. These changes have been followed by observing the circular dichroism of the peptide bond, and the fluorescence anisotropy of the tyrosine emission. Both fast and slow conformational changes occur. At low enough salt concentrations there is only a fast change, in which the altered molecules have 17–22 residues of α helix. Critical concentrations of salt exist, however, specific for each anion, above which the fast change is followed by a much slower one. In the slow change there are 20–30 β-sheet residues per altered molecule. At sufficiently high salt concentrations, the amount of slow change diminishes with added salt.

Many workers have noted that the addition of salts to aqueous solutions of histones results in an increase in secondary structure (Bradbury *et al.*, 1965, 1967; Jirgensons and Hnilica, 1965; Tuan and Bonner, 1969; Boublik *et al.*, 1970; Shih and Fasman, 1971; Li *et al.*, 1971, 1972). It has also been long known, and commented upon frequently, that under a variety of conditions histones will aggregate (Davison and Shooter, 1956; Cruft *et al.*, 1957, 1958; Mauritzen and Stedman, 1959; Phillips, 1965, 1967; Johns, 1968, 1971; Fambrough and Bonner, 1968; Edwards and Shooter, 1969; Boublik *et al.*, 1970; Diggle and Peacocke, 1971). Aggregation may, of course, be considered a special type of structural alteration.

Insight into the nature of some of the salt-induced changes in histone IV conformation was obtained by the nuclear magnetic resonance studies of Boublik *et al.* (1970), who found that the addition of NaCl to histone IV induced line broadening in the proton magnetic resonances of certain amino acid residues, particularly the hydrophobic ones which predominate in the C-terminal half of the histone IV molecule (Delange *et al.*, 1969, Ogawa *et al.*, 1969).

Our laboratory recently reported (Li *et al.*, 1972) that the addition of sodium phosphate to histone IV solutions produces a series of conformational changes in the protein. The overall process appears complex, but we showed how it could be decomposed into a set of more elementary steps which could be at least partially understood.

At sufficiently low phosphate concentrations, a rapid conformational change occurs. At higher concentrations, a fast conformational change occurs initially, but this is followed by a considerably slower change. In the fast step approximately 17 residues, out of the 102, go into α-helical conformation. In the slow conformational change, 20–30 residues go into β sheet. The number that go into β sheet is not a monotonic function of salt, but, as salt is added, rises to a maximum and then declines. β-sheet formation is intermolecular. Consequently the slow step also involves the formation of higher order associations.

The circular dichroism (CD) change in the fast step is independent or nearly independent of histone concentration. In contrast, however, the intrinsic fluorescence anisotropy increases with increasing histone IV concentration. By assuming that this concentration dependence indicated an equilibrium between two different states of the system we showed that the data could be fit by a dimer equilibrium equation.

In the present paper we report further work on salt-induced conformational changes of histone IV. We have studied the comparative effects of a set of salts, *viz.*, sodium phosphate, sodium sulfate, sodium fluoride, sodium perchlorate, sodium chloride, and magnesium chloride.

Materials and Methods

Calf thymus histone IV was prepared by the method of Ogawa *et al.* (1969). Our samples were electrophoretically homogeneous, and the amino acid composition agreed with the published sequence (Ogawa *et al.*, 1969; DeLange *et al.*, 1969). Histone IV concentrations were determined spectrophotometrically using $\epsilon_{230} = 4.7 \times 10^2$ cm^{-1}/mole of residue per l. (Ohlenbush *et al.*, 1967; Shih and Bonner, 1970). H_0 will here denote the total histone concentration in moles of protein per liter.[1]

Salt stock solutions were added to histone IV in 5.0 ×

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[1] We note that, in two previous papers from this laboratory (Li *et al.*, 1971, 1972), we measured histone concentrations in moles of residue per liter. Histone IV has 102 residues/molecule.

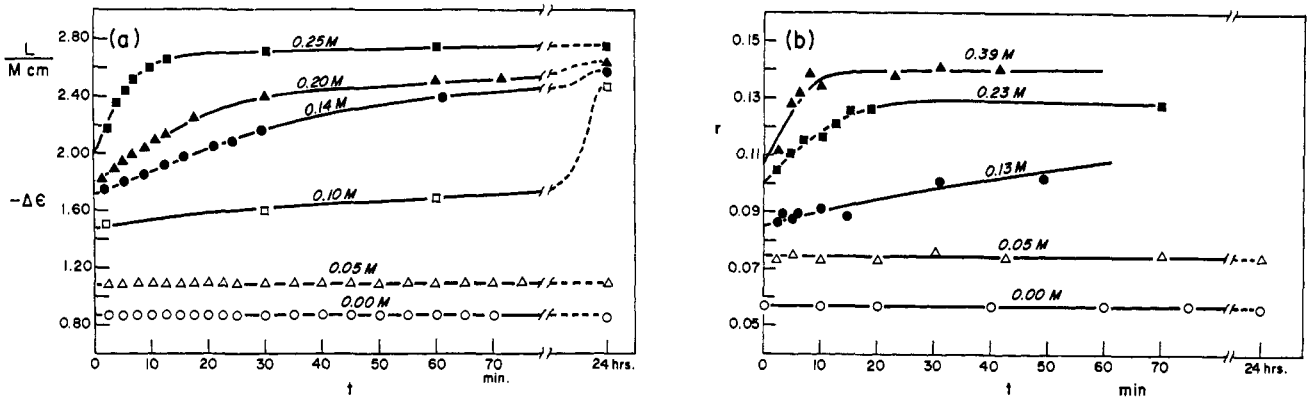

FIGURE 1: Time dependence of NaCl effects on histone IV by CD (a) and fluorescence anisotropy (b), 5.0×10^{-3} M cacodylate buffer (pH 6.5), $H_0 = 0.8 \times 10^{-5}$ mole of histone/l. of NaCl concentrations as marked on the figure.

10^{-3} M cacodylate buffer with constant mixing. The time of salt addition is called zero time. The temperature was maintained at 22.0 ± 0.5°.

The fluorescence anisotropy of the histone IV tyrosine emission was measured on a previously described instrument (Evett and Isenberg, 1969; Li *et al.*, 1972). Samples were excited by plane-polarized light. I_E will denote the intensity of light emitted with a polarization parallel to that of the excitation beam, and I_B the intensity polarized perpendicularly to the excitation beam. The anisotropy of emission is defined as $r = (I_E - I_B)/(I_E + 2I_B)$. Excitation was at 280 nm and emission was measured at 325 nm.

We note that, for histone IV samples, it is important to test for, and eliminate, possible scattering artifacts (Weber, 1956). In our work, to obtain spectral purity, two Jarrell-Ash $^1/_4$ M Ebert monochromators were used in series in the exciting beam and similar, but single, monochromators, plus CS-054 Corning filters, were used in the emission beams. We tested for possible scattering artifacts by using additional filters, and different path-length cuvets, as described by Weber (1956). With none of the samples described here, or in our previous work (Li *et al.*, 1972), did we find such scattering artifacts.

We denote the anisotropy in water by r_w and let $r(t)$ be the anisotropy at time t in the presence of salt. $r(t)$ was measured by averaging $E - B$ for 30 sec, and then $E + 2B$ for 30 sec. Since $E - B$ changes much more rapidly than $E + 2B$, t denotes the midpoint of the $E - B$ measurement. However the changes reported here were slow enough so that any error due to this procedure was less than one-third of the experimental error due to other causes.

CD spectra, and CD changes as a function of time, were recorded on a Model CD-SP Durrum-Jasco CD recorder. $\Delta\epsilon_w(\lambda)$ will denote the CD of histone IV in water at a wavelength λ. $\Delta\epsilon(t,\lambda)$ will be the CD in salt, at time t, and wavelength λ. CD results are reported as $\Delta\epsilon = \epsilon_l - \epsilon_r$, where ϵ_l and ϵ_r are extinction coefficients for left- and right-handed circularly polarized light. The units of $\Delta\epsilon$ will be cm^{-1} l. per mole of residue.

Results

Fast and Slow Changes Resulting from Salt Addition. All of the salts studied induce both fast and slow conformational changes. At sufficiently low salt concentrations only the fast change occurs but at higher concentrations this change is followed by the slower one, which takes minutes to hours depending on solution conditions. We have studied these changes by two techniques—CD in the region of 190–250 nm, and the polarization of fluorescence of the sample when excited at 280 nm, which is within the absorbance band of the tyrosines of histone IV.

In the region of 190–250 nm, the CD is principally that of the peptide linkage (Holzworth and Doty, 1965). The fluorescence anisotropy, on the other hand, depends on the rotational mobility of the transition moments of the tyrosine residues. This, in turn, is a function of the local structural rigidity and the size and shape of the molecule. It also is a function of dimerization or the formation of higher order units or aggregates.

Figure 1a shows $\Delta\epsilon(t, 220)$ of histone IV as a function of time, at a number of NaCl concentrations. Figure 1b is a similar plot of fluorescence anisotropy, $r(t)$. As can be seen, both quantities increase immediately upon salt addition, that is $r(0)$ varies with salt concentration. At sufficiently high salt concentrations, a slow change also occurs. This change becomes more rapid as the salt concentration is raised. However, at sufficiently low concentrations, there is only a fast, but no slow, change.

CD Difference Spectra of the Fast and Slow Steps. We have taken fast and slow change CD difference spectra for all of the salts used in this study, and compared them to the theoretical spectra that were calculated previously (Li *et al.*, 1972). The theoretical spectra were calculated for a change from the structure giving the spectrum of histone IV in water, assumed to be random coil to either 100% α helix or 100% β sheet. These latter spectra were taken to be that for polylysine as given by Greenfield and Fasman (1969).

As discussed by Shih and Fasman (1971), histone IV in water is essentially in a random coil configuration. Consistent with their work, we note, in addition, that the CD of histone IV in water does not change as the pH is lowered from 7.4 to 2.0.

Operationally, we obtain the experimental difference spectrum for the fast step by subtracting the spectrum of histone IV in water from that of histone IV in salt, at zero time. We note that this is very close in shape to the theoretical difference spectrum for an α helix. This similarity was obtained for all of the salts tested (Figure 2).

Experimental difference spectra for the slow step were calculated by subtracting the spectrum at zero time from that at infinite time. Again, for all of the salts tested, these spectra

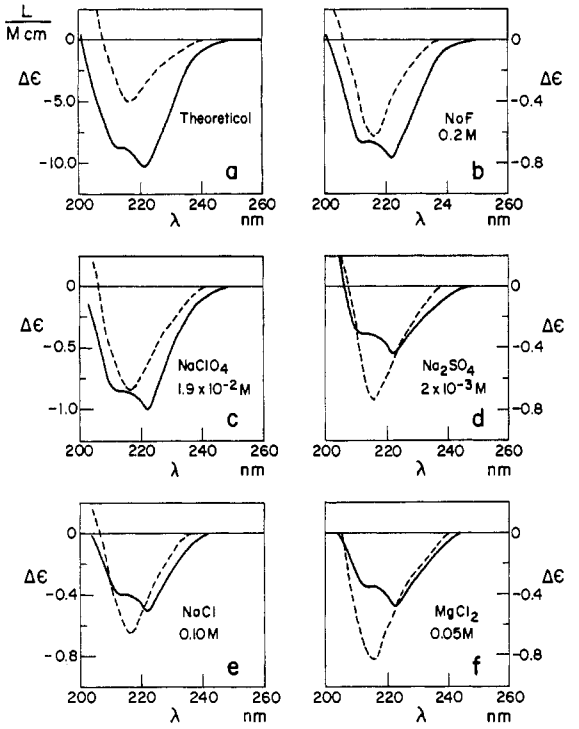

FIGURE 2: Theoretical and experimental CD difference spectra for histone IV, 5.0×10^{-3} M cacodylate buffer (pH 6.5), $H_0 = 0.8 \times 10^{-5}$ M. Spectra are $\Delta\epsilon\,(0,\lambda) - \Delta\epsilon_w(\lambda)$ (—) and $\Delta\epsilon(\infty,\lambda) - \Delta\epsilon(0,\lambda)$ (- - -) Salts and salt concentrations as shown in the figure.

agreed in shape with the theoretical β sheet spectrum (Figure 2).

The amplitude of an experimental difference spectrum differs, of course, from that for the theoretical, and, by a comparison, we obtain an estimate for the α-helical or β-sheet content of the protein. It seems reasonable to conclude that, for all of the salts tested, the fast step involves α-helix formation, and the slow step β-sheet formation. This agrees with the previous report (Li *et al.*, 1972) on the changes induced by the addition of phosphate.

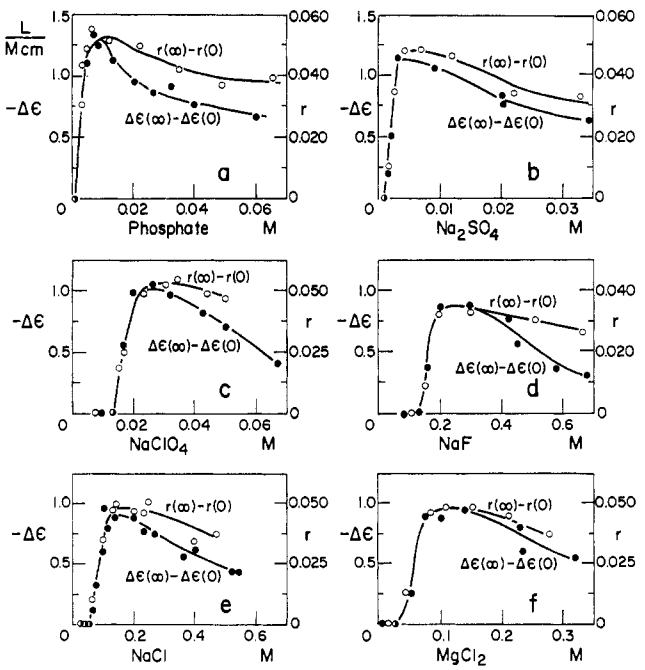

FIGURE 3: Magnitude of the slow changes by CD (●) and fluorescence anisotropy (○). Sodium phosphate (pH 7.4), was used as its own buffer (Li *et al.*, 1972) all others in 5.0×10^{-3} M cacodylate (pH 6.5), $H_0 = 0.8 \times 10^{-5}$ M.

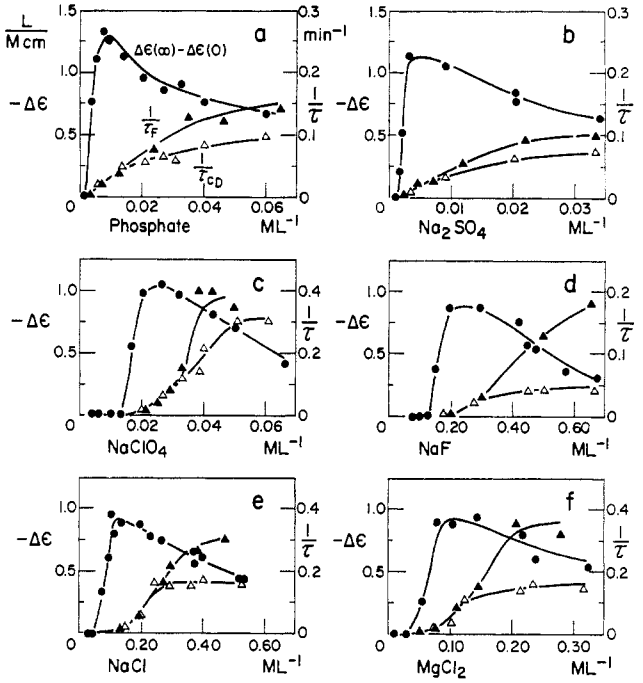

FIGURE 4: Magnitude of the slow change by CD (●) and the experimental rate constants, $1/\tau_{CD}$ (Δ) and $1/\tau_F$ (▲), 5.0×10^{-3} M cacodylate buffer (pH 6.5), $H_0 = 0.8 \times 10^{-5}$ M.

The conformational change in the slow step is highly cooperative. We see that there is a breakpoint in the salt concentration below which there is no slow change. Above the breakpoint, however, there is appreciable change. This breakpoint varies markedly with the salt used, and ranges from 1.0×10^{-3} M for Na_2SO_4 to 0.13 M for NaF.

It should not be concluded that the existence of a breakpoint means that the salt binding is in itself necessarily cooperative although this may, of course, be true. However, the cooperativity may be entirely in the structural change of the protein.[2]

Cooperativity and Polymorphism of the Slow Step. Figure 3 shows CD and anisotropy data for the total change in the slow step. Three regions of these curves may be distinguished. At low salt there is no slow change. Then, at higher concentrations, there is a steep rise to a maximum. At still higher concentrations there is a gradual decline.

Whether or not the anisotropy and CD data coincide in any portion of a curve shown in Figure 3 depends, of course, on the scales used to plot $\Delta\epsilon$ and r. The coincidence that appears in the region of sharp rise is due therefore only to the scales chosen. However, the fact that both parameters rise sharply in the same range of salt concentrations is physically meaningful.

[2] An analogy to the change of polylysine from a random coil to an α helix may be appropriate. Upon raising the pH to neutralize the ϵ-amino groups, there is a cooperative change. This occurs because solution conditions favor ordered polylysine, rather than random coil, as the equilibrium state. Similarly, in the histone studies reported here, upon raising the salt concentration above the breakpoint, solution conditions favor a new state for histone IV.

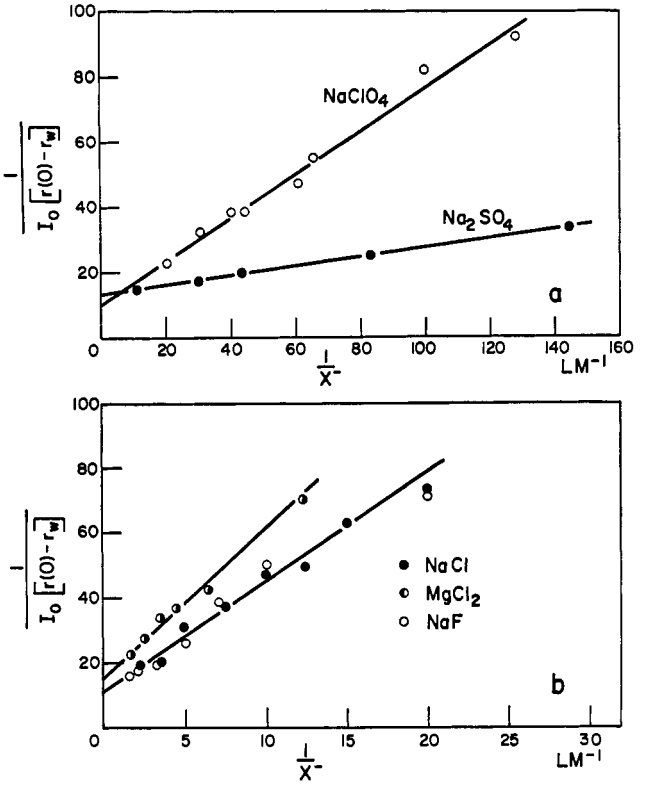

FIGURE 5: Plots of eq 6 for the fast CD change at 220 nm. 5.0×10^{-3} M cacodylate buffer (pH 6.5), $H_0 = 0.8 \times 10^{-5}$ M. Salts are marked on figure.

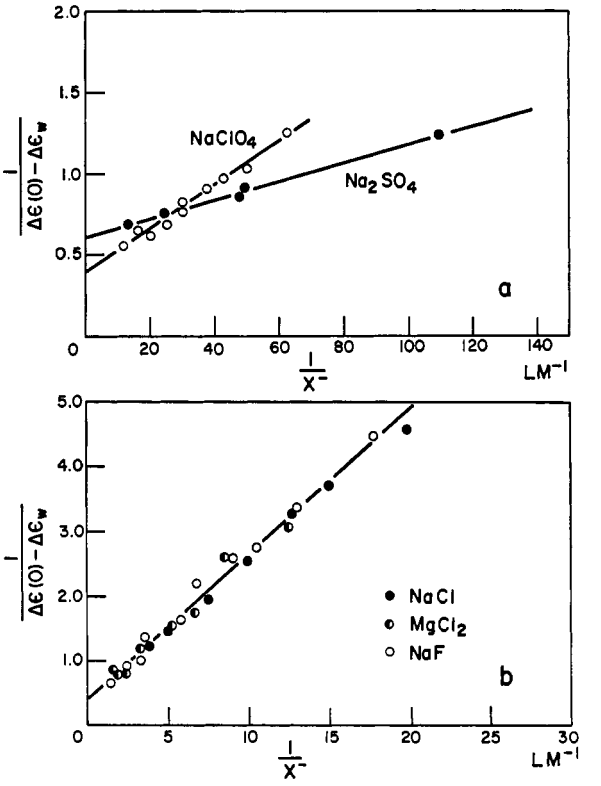

FIGURE 6: Plots of eq 7 for the fast change in fluorescence anisotropy. 5.0×10^{-3} M cacodylate buffer (pH 6.5), $H_0 = 0.8 \times 10^{-5}$ M, excitation wavelength = 280 nm, emission wavelength = 325 nm.

In previous work (Li *et al.*, 1972) we showed that, upon phosphate addition, the slow step could be approximated by a single exponential decay. We find this to be true also for the salts used in the present study. We therefore write

$$\frac{\Delta\epsilon(\infty,220) - \Delta\epsilon(t,220)}{\Delta\epsilon(\infty,220) - \Delta\epsilon(0,220)} = e^{-t/\tau_{CD}} \quad (1)$$

$$\frac{r(\infty) - r(t)}{r(\infty) - r(0)} = e^{-t/\tau_F} \quad (2)$$

Figure 4 shows that the rate constants, as measured by both CD and polarization of fluorescence, are the same for low salt concentrations, but diverge at high concentrations. The divergence begins slightly after the point where the net change is a maximum. It should be noted that the rate constants plotted in Figure 4 may be directly compared to each other, although the CD and polarization comparisons of Figure 3 depend, as discussed, on the scales used to draw the figures. These data suggest that there are at least two different processes occurring in the slow change, one that dominates at concentrations just above the breakpoint, and another that occurs at higher concentrations.

Histone–Salt Equilibrium for the Fast Step. In our earlier work (Li *et al.*, 1972) we noted that, if we assumed that there existed a set of equivalent, independent, anion binding sites on histone IV, we could then describe the effectiveness of the phosphate ions in inducing the fast step in quantitative terms. The effectiveness of the salts studied here will be measured in the same way. Let [A] be the concentration of anions, [B] the concentration of binding sites, and $[A_0]$ and $[B_0]$ the total anion and binding site concentrations.

The following equations then hold (Li *et al.*, 1972):

$$\frac{1}{\Delta\epsilon_{A_0}(0) - \Delta\epsilon_w} = \frac{1}{(\Delta\epsilon_{A_0=\infty}(0) - \Delta\epsilon_w)}\left(1 + \frac{1}{K[A_0]}\right) \quad (3)$$

$$\frac{1}{I_{A_0}(0)(r_A(0) - r_w)} = \frac{1}{I_{A_0=\infty}(0)(r_{A_0=\infty}(0) - r_w)}\left(1 + \frac{1}{K[A_0]}\right) \quad (4)$$

We add two comments to our prior discussion (Li *et al.*, 1972). First we note that eq 4 is one for a two-state system. However (Li *et al.*, 1972), physically, there are clearly at least three states—a random coil monomer, an ordered monomer, and a dimer. The apparent contradiction is removed by the work in the appendix, which shows that any system may be considered a two-state system provided that appropriate, but natural, averages, are used for the various fluorescence parameters.

Second we observe that, while the fluorescence intensity does not change appreciably with added phosphate (Li *et al.*, 1972), it does alter appreciably when other salts are used. Therefore in the present paper, the intensity factor $I_{A_0}/I_{A_0=\infty}$ has not been dropped from eq 5 of Li *et al.* (1972).

Figure 5 shows data plotted according to eq 3 for the salts used. Figure 6 shows similar plots according to eq 4. It may be seen that all plots are good straight lines.

Table I summarizes the pertinent parameters obtained from the plots of Figures 5 and 6. Several features may be noted.

With the exception of $NaClO_4$, for all of the salts, the effective binding constants are the same for CD, and for polariza-

TABLE I

Salt	$\frac{1}{(\Delta\epsilon_A(0\ 220) - \Delta\epsilon_w(220))_{A_0=\infty}}$ (M cm)	K_{CD} (M)	$\frac{1}{I(0)[r(0) - r_w]_{A_0=\infty}}$	K_F (M)
Na_2SO_4	0.61	105	13	95
$NaClO_4$	0.40	32	10	16
NaCl	0.45	2.2	11	2.6
$MgCl_2$	0.45	2.5	15	3.3
NaF	0.45	2.0	11	2.2
NaH_2PO_4[a]		10		14
Na_2HPO_4[a]		120		120
Sodium phosphate (pH 7.4)	0.60	85	14	80

[a] Calculated as discussed in Li *et al.* (1971).

tion of fluorescence measurements. In addition, we note that the effective binding constants for NaCl and $MgCl_2$ are the same, within experimental error. We see, therefore, that there is little or no change upon replacing sodium by magnesium.

The constants for $NaClO_4$ and NaH_2PO_4 are an order of magnitude larger than those of the halides. The constants for the divalent anions are still another order of magnitude larger.

We have previously reported (Li *et al.*, 1972) that in the fast step, as induced by phosphate, about 17 out of 102 residues formed α helix. Our result for Na_2SO_4 yields the same value; our parameters for the other salts yield the slightly higher value of about 22 residues. The values of 17 and 22 residues are reproducible to within one or two residues. Although the difference between 17 and 22 is outside of experimental error, it is difficult to evaluate the significance of this difference. These numbers result from an analysis which includes assumptions concerning the physical processes involved (Li *et al.*, 1972) and, consequently, it is difficult to judge how much confidence one should have in the absolute values of these numbers, although the actual α-helical content cannot vary greatly from our estimate.

Discussion

The salts studied here have effects on the conformation of histone IV which are qualitatively similar to those of phosphate (Li *et al.*, 1972). At sufficiently low salt concentrations there is a fast change, but no slow change. There is a breakpoint above which there is an appreciable slow change which rises steeply to a maximum. At relatively high salt concentrations additional effects occur.

The conformational change in the fast step includes α helical formation of 17–22 residues. In addition, there is a marked increase in the anisotropy of the tyrosine emission. An interpretation of the increase is aided by a comparison to that found when a random copolymer, Trp_5Glu_{95}, goes from random coil to 100% α helix (Pesce *et al.*, 1964). For the random copolymer an increase in r of only 0.017 was reported. This small increase for the copolymer is exceeded in the histone IV change even at relatively low salt concentrations (Figure 1b) where only a small fraction of the histone molecules have undergone a conformational change. If, in addition, we estimate the magnitude of the anisotropy increase when all of the molecules undergo the fast change, we find changes of the order of 0.10, an order of magnitude larger than that which is found for the random copolymer.

For the random copolymer studied by Pesce *et al.* (1964), tyrosine, in the α-helical conformation, projects into the medium. It can then rotate about the side-chain-saturated bonds, and such Brownian rotation leads to an appreciable depolarization.

For the tyrosine emission from histone IV, the larger anisotropy change implies that, in addition to α-helical formation, there is secondary structure to which the CD is insensitive. We are thus led to a picture of salt effects completely analogous to renaturation of denatured enzymes. Upon the addition of salt to histone IV, there is a general conformational change in the fast step. It is highly unlikely that all of the tyrosines are in the region of α helix formation but, even if they were, the evidence indicates that additional structural changes exist.

It is interesting that, for $NaClO_4$, the equilibrium constant, as measured by CD, is clearly different from that measured by fluorescence polarization, although they are equal for the other salts. We conclude that, for most salts, one overall conformational change occurs. We simply measure one or another aspect of the change. However, once we conclude this, it also follows that perchlorate induces a different type of alteration, one having different effects on the CD and the rigidity of the tyrosines.

In our previous study on conformational changes induced by adding phosphate (Li *et al.*, 1972) we showed that our data were consistent with dimer formation in the fast step. We mention here that preliminary observations on NaCl effects show the same thing.

Though the effects of most of the salts are qualitatively similar, they are very different quantitatively. The breakpoint for the slow step, for example, is markedly different for each anion studied, ranging from 10^{-3} M for sulfate to 0.13 M for fluoride (Figure 3). The order of effectiveness in sulfate > phosphate > perchlorate > chloride > fluoride. It is important to note that this order is not that of the Hofmeister series (Von Hipple and Schleich, 1969), nor is the order of magnitude of the salt concentrations typical of that for Hofmeister effects.

The β-sheet formation of the slow step is intermolecular (Li *et al.*, 1972). The slow step therefore involves the association of histone molecules which may explain the extensive aggregation observed at higher histone concentrations (Edwards and Shooter, 1969; Diggle and Peacocke, 1971).

It is quite clear that many different conformational forms of histone IV exist. It is tempting to try to relate such a polymorphism to the functional significances of histones. In a sense, of course, it is very premature to do so, since no simple extrapolation from histone IV to chromatin can exist. Chromatin is a structure involving different histones, as well as DNA and nonhistone protein. Nevertheless, in general terms, the alterations of chromatin in cell division, differentiation, and dedifferentiation must involve histone rearrangement, and conformational alterations, regardless of what specific role, or roles, the histone is playing. It is possible, therefore that the polymorphism that we report is related to the general polymorphisms displayed by chromatin.

Acknowledgments

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Appendix

This appendix shows that any multicomponent system may be considered as a two state system, provided that the proper averages are used for the anisotropy, light emitted, and fraction of molecules in each state.

Let $i = 1, 2, \ldots n, n+1, \ldots N$ label the components. Let the components 1, 2, ... n be in state a, and $n+1$, $n+2, \ldots N$ be in state b. Let

f_i = fraction of molecules of species i
f_a, f_b = fraction of molecules in state a or b
I_i = light emitted by species i
I_a, I_b = light emitted by state a or b
r_i = anisotropy of emission from i
$I = \sum_{i=1}^{N} I_i$
r = anisotropy of the total emission

Then

$$\phi_i = \frac{I_i}{I}$$

and

$$r = \sum_{i=1}^{N} r_i \phi_i$$

$$= \frac{1}{I} \sum_{i=1}^{N} r_i I_i$$

$$= \frac{1}{I} \left\{ \sum_{i=1}^{n} r_i I_i + \sum_{i=n+1}^{N} r_i I_i \right\}$$

Then

$$r = \frac{I_a}{I_a + I_b} r_a + \frac{I_b}{I_a + I_b} r_b$$

$$= \phi_a r_a + \phi_b r_b$$

We therefore have the set of equations

$$I_a + I_b = 1$$

$$f_a + f_b = 1$$

$$r = r_a \phi_a + r_b \phi_b$$

This set is identical to the set for a true two state system. From these equations one may then derive the expressions used in the present paper by methods published elsewhere (Evett and Isenberg, 1969; Ellerton and Isenberg, 1969).

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