A Complex of Histones IIb2 and IV†

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ABSTRACT: Histones IIb2 and IV have been shown, by their circular dichroism (CD) and fluorescence properties, to interact in the presence of salts. The interaction is faster than the resolution of our techniques and is characterized by instantaneous increases in the observables as compared to ideal solutions. Continuous variation plots of fluorescence and CD properties are consistent with formation of a complex containing one molecule of histone IIb2 and one molecule of histone IV. The association constant of the complex is estimated to be of the order of 10^5–10^6 M^{-1}. Concurrent with complex formation is an increase in the CD. This increase is interpreted as an increase of a minimum of eight residues of α helix in the complex as compared to the noncomplexed histone constituents in their salt-induced states. The complex has a minimum α-helix content of 42 of 227 residues; there is no evidence of the β structure that is observed in the slow step of histone IV [Li, H. J., Wickett, R. R., and Isenberg, I. (1972), *Biopolymers 11*, 375]. In the absence of salts, the CD and fluorescence properties of mixtures of histones IIb2 and IV are characteristic of noninteracting histones. It is also shown that the phosphate-induced CD and anisotropy changes of histone IIb2 and histone IV are functions of histone concentration. A scheme is proposed to indicate the salt-induced conformational changes and the cross interactions of histones IIb2 and IV. The possible significance of the histone IIb2–histone IV complex to chromatin structure is discussed.

Histones are found in association with DNA in the nuclei of higher organisms (Murray, 1964). They are believed to serve as structural proteins and, perhaps, perform a general function in gene regulation (Stellwagen and Cole, 1969; Johns, 1971). Although the physical properties of histones have been of interest for many years (Cruft *et al.*, 1958), it is only recently that reasonably pure histones have become available. Subsequently, several physical studies of the histones have appeared (Barclay and Eason, 1972; Bradbury and Rattle, 1972; Boublik *et al.*, 1970a,b; Bradbury *et al.*, 1972; D'Anna and Isenberg, 1972; Diggle and Peacocke, 1971; Edwards and Shooter, 1969; Li and Isenberg, 1973; Li *et al.*, 1972; Wickett and Isenberg, 1972; Wickett *et al.*, 1972).

A picture of the histones is emerging in which addition of salt or extremes of pH induce conformational change in the histones and in which there is considerable aggregation within histone groups. It has also been noted by some workers that different histones may interact. In 1958, Cruft *et al.* mentioned, in passing, that "subsidiary histones" inhibit aggregation of "β histones." Laurence (1966) noted that the increase of fluorescence intensity of ANS[1] in mixtures of "histone F2a," ANS, and salt was severely reduced upon the addition of "histone F2b." Shih and Bonner (1970), while preparing histone–DNA complexes, noted turbid solutions with mixtures of "histone IIb," histone IV, and DNA. They suggested that the turbidity might reflect some "special mutual interaction" of the two histones.

Johns (1972) has suggested that self-aggregation and co-aggregation of histones, as well as histone–acidic protein interaction, may be important to the functioning of chromatin in the nucleus.

This laboratory has investigated the conformational changes of histones IV and IIb2 upon addition of a variety of salts [Li *et al.*, 1972; Wickett *et al.*, 1972; Wickett and Isenberg, 1972; Li and Isenberg, 1973; D'Anna and Isenberg, 1972]. Intrinsic tyrosine fluorescence properties and circular dichroism (CD) have been utilized in these studies.

Histone IV undergoes conformational changes upon addition of salts. These changes can be decomposed into a fast change followed by a slower aggregation to a species of undetermined size. The fast change is characterized by increased tyrosine rigidity and by α-helix formation of 17–22 residues depending on the salt used. In the slow step there is a time-dependent increase in fluorescence anisotropy and time-dependent β-sheet formation.

Addition of salt to solutions of histone IIb2 results in conformational changes which are instantaneous by our measurement techniques. There is no evidence of time-dependent changes as in histone IV. It is estimated that there are 18 residues of α helix in the salt-altered histone IIb2 molecules.

In this article we demonstrate that histone IIb2 interacts with histone IV. The interaction inhibits the slow change of histone IV. The method of continuous variations is applied to mixtures of histones IIb2 and IV and indicates that the histones form a 1:1 complex. The association constant of the interaction is high, of the order of 10^5–10^6 M^{-1}.

Experimental Section

Histone IIb2 was prepared by the method of Senshu and Iwai (1970), and histone IV was prepared by the method of Mauritzen *et al.* (1967). Samples of histones IIb2 and IV were deemed pure by amino acid analyses and electrophoresis on 15% acrylamide gels in 2.5 M urea, pH 3.2 (Panyim and Chalkley, 1969). In spite of the apparent purity, however, we noticed quantitative variations in different batches of histone IIb2. Nearly all of the histone IIb2 used in this work agreed to within 5% in CD measurements. One batch differed from the others by nearly 12%; it was used only for the measurements in Figure 4b. In spite of the quantitative differences, the different batches of protein showed the same type of interaction with histone IV.

Fluorescence anisotropy was measured with an instrument

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate.

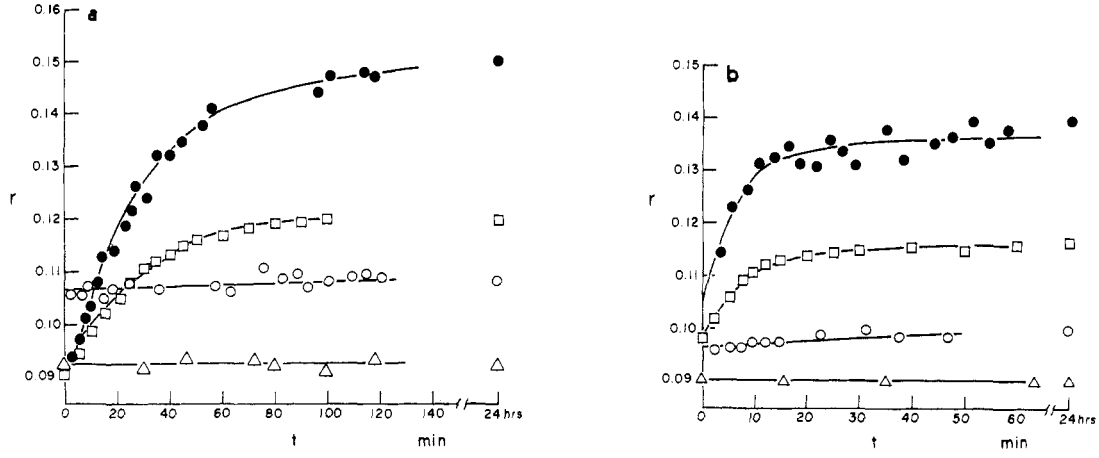

FIGURE 1: Anisotropy of histone solutions as a function of time at (a) 0.016 M phosphate, pH 7.0, and (b) 0.20 M NaCl, pH 7.0: 0.90×10^{-5} M histone IV (●); 0.90×10^{-5} M histone IIb2 (Δ); 0.90×10^{-5} M histone IV plus 0.90 M histone IIb2, measured (○) and calculated (□) for a mixture of noninteracting histones.

previously described (Evett and Isenberg, 1969) and with a modification of that instrument in which the photomultiplier circuits have been interfaced to a PDP8/e computer (Ayres *et al.*, 1972[2]). Anisotropy is defined as $r = (E - B)/(E + 2B)$; E is the fluorescence component polarized parallel to the vertically polarized exciting light, and B is the horizontally polarized fluorescence component.

Histone IIb2 has five tyrosines and two phenylalanines of 125 residues/molecule (Iwai *et al.*, 1970). Histone IV has four tyrosines and two phenylalanines of 102 residues/molecule (Ogawa *et al.*, 1969; DeLange *et al.*, 1969). Because of the relatively small absorbance and low quantum yield of phenylalanine, the observed emission from each of the histones is, effectively, that of tyrosine only. Excitation was at 279 nm and the emission was monitored at 325 nm. Samples were maintained at 22.0 ± 0.5° in 1.0-cm² cuvets.

Circular dichroism spectra were measured with a Durrum-Jasco Model J-10 CD recorder and spectrophotometer. Cells of various path lengths were used, and their relative lengths were checked using solutions of *d*-10-camphorsulfonic acid. The various CD ranges and instrument linearity were also checked with *d*-10-camphorsulfonic acid. CD spectra were measured at 22.0 ± 0.5°. CD measurements are reported as $\Delta\epsilon = \epsilon(\text{left}) - \epsilon(\text{right})$ in units of cm^{-1} l./mol of residue or as $\Delta\epsilon' = \Delta\epsilon'(\text{left}) - \Delta\epsilon'(\text{right})$ in units of cm^{-1} l./mol of histone.

Solutions of histone IIb2 or histone IV were prepared by dilution of stock aqueous solutions of histone. Stock aqueous solutions of histone IIb2 and/or histone IV were added to a test tube, and concentrated salt solutions were added to it. The tube was closed and then inverted several times. For measurements as a function of time, the resulting solutions were transferred as quickly as possible to appropriate cells and instruments.

The concentration of histone was determined from the measurement of the optical density of aqueous solutions at 275 or 230 nm. Extinction coefficients of 6.7×10^3 and 5.4×10^4 cm^{-1} l./mol of histone were used for histone IIb2, and extinction coefficients of 5.4×10^3 and 4.2×10^4 cm^{-1} l./mol of histone were used for histone IV. The extinction coefficients at 275 nm were assumed to be the sum of the extinction coefficients of the *N*-ethyl esters of the number of tyrosines and phenylalanines per molecule of histone (Beaven and Holiday, 1952). Shih and Fasman (1971) have determined an extinction coefficient of 5.3×10^3 cm^{-1} l./mol of histone at 275 nm for histone IV from absorption measurements of histone solutions prepared from dried and weighed histone. This is nearly the same extinction coefficient as we calculated using the *N*-ethyl ester extinction coefficients. Absorption spectra were measured on a Cary 14 spectrophotometer.

Measurements were performed at pH 7.0 in sodium phosphate or NaCl–0.005 M cacodylate unless specified otherwise. A Corning Digital 112 pH meter, equipped with a Corning semimicro combination electrode, was used for pH determinations.

Results

Repression of the Slow Change of Histone IV by Histone IIb2. Salt solutions of histone IIb2, histone IV, and mixtures of histones IIb2 and IV in a molar ratio of 1:1 were prepared. Their CD and fluorescence properties were measured as functions of time. Examples of the measurements, in sodium phosphate (pH 7.0) solutions, are given in Figures 1 and 2. In the same figures are shown curves expected for solutions of noninteracting histones IIb2 and IV. The measured curves of the mixed solutions are very different from the calculated curves; clearly, histones IIb2 and IV interact in salt solutions.

At the histone and salt concentrations of the measurements, the anisotropy is characterized by an increase, which is instantaneous by our techniques, and a very small amount of slower change.

Measurements of the CD of the mixed histone salt solutions at 220 nm are also striking. No slow step whatsoever is observed in the CD. Additionally, the CD of the mixtures is significantly greater than curves calculated for mixtures of noninteracting histones.

In contrast to the behavior of the mixed histone solutions in the presence of salt, CD and fluorescence properties of mixed histone solutions in water (pH 4.3) or 0.005 M cacodylate (pH 7.0) are, to within experimental error, characteristic of independent histone fractions (Table I). Hence, it appears that salts are necessary for the interaction of histones IIb2 and IV, at least in the pH range of 4.3–7.0.

[2] Ayres, W. A., Small, E. W., and Isenberg, I. (1972), manuscript in preparation.

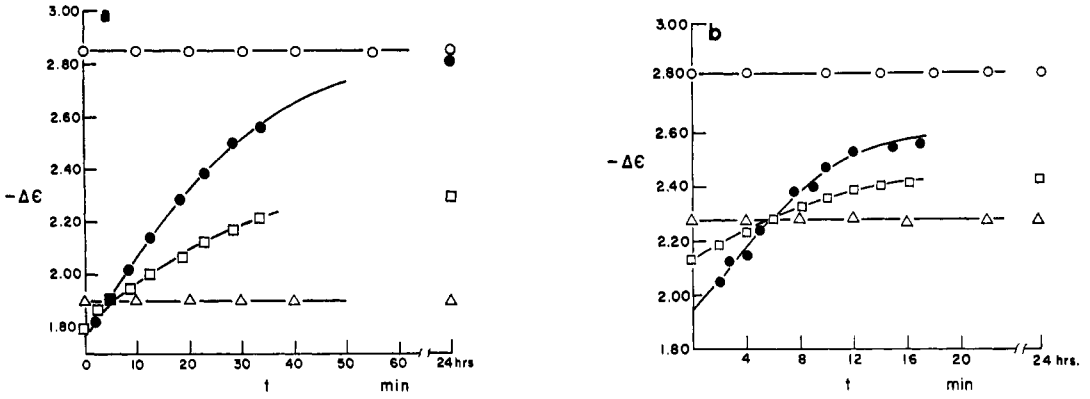

FIGURE 2: CD (220 nm) of histone solutions as a function of time at (a) 0.016 M phosphate, pH 7.0, and (b) 0.24 M NaCl, pH 7.0: 0.90×10^{-5} M histone IV (●); 0.90×10^{-5} M histone IIb2 (Δ); 0.90×10^{-5} M histone IIb2 and histone IV, measured (○) and calculated (□) for a mixture of noninteracting histones.

Spectra of equimolar mixtures of histones IIb2 and IV in cacodylate (pH 7.0), in 0.016 M phosphate (pH 7.0), as well as the difference spectrum calculated from the spectra in the two media, are given in Figure 3. The difference spectrum is characteristic of CD changes attributable to primarily coil → α-helix formation in proteins [Li *et al.*, 1972; Wickett *et al.*, 1972; D'Anna and Isenberg, 1972). There is no evidence of the considerable β-structure formation observed in the slow step of histone IV alone. Of course, a small fractional content of β structure in the presence of considerable α-helical content would be difficult to distinguish (see Figure 6 of D'Anna and Isenberg, 1972). Similar CD spectra are obtained at other phosphate concentrations as well as in sodium chloride solutions.

Continuous Variations. CIRCULAR DICHROISM. Interaction of histones IIb2 and IV upon addition of salt has been demonstrated for solutions in which the histone IIb2 to histone IV molar ratio is 1.0. Is this, however, the stoichiometry of maximum interaction? To answer this we applied the method of continuous variations (Job, 1928; Vosburgh and Cooper, 1941).

TABLE I: Fluorescence and CD Properties of Histone IV, Histone IIb2, and Histone IV plus Histone IIb2 in Water, pH 4.3, and in 0.005 M Cacodylate Buffer, pH 7.0.

Sample	Solvent	r	F^a	$\Delta\epsilon_{220}$ (cm^{-1} M^{-1})
IIb2	H_2O	0.053	0.296	−0.80
IV	H_2O	0.059	0.337	−0.61
IIb2 + IV (measd)	H_2O	0.055	0.589	−0.71
IIb2 + IV (calcd)	H_2O	0.056	0.597	−0.71
IIb2	Cacodylate	0.065	0.296	−0.97
IV	Cacodylate	0.065	0.326	−0.68
IIb2 + IV (measd)	Cacodylate	0.065	0.593	−0.80
IIb2 + IV (calcd)	Cacodylate	0.065	0.586	−0.83

[a] Arbitrary fluorescence units.

In the following continuous variation measurements, the analytical concentrations of the starting histone solutions were varied so that the sum of the concentrations were equal to C_0. Concentrated salt solutions were added to aqueous protein solutions.

Two principal assumptions are inherent to the method of continuous variations as it is most often utilized (Job, 1928; Rossotti and Rossotti, 1961): (1) a single complex AB_n is

$$A + nB = AB_n \quad (1)$$

formed by interaction of the species A and B and (2) the species A, B, and AB_n are characterized by certain intensive and/or extensive properties. As we shall see, the assumptions are not entirely correct for our systems because of histone self-aggregation (*e.g.*, histone IIb2–histone IIb2 interaction). However, under certain conditions, histone self-aggregation may be shown to have only a small effect on the properties of interest; thus, the continuous variation results may be reasonably interpreted. We shall proceed in this section as if there were no histone self-aggregation and comment upon the results in a later section.

If it is assumed that there exist the intensive properties $\Delta\epsilon_A'$, $\Delta\epsilon_B'$, and $\Delta\epsilon_{AB_n}'$, which are independent of histone concentration, then the concentration of the complex, AB_n, is directly proportional to $\Delta\epsilon' - \Delta\epsilon_I'$.

$$\Delta\epsilon' - \Delta\epsilon_I' = \frac{\Delta\epsilon_{AB_n}' - \Delta\epsilon_A' - n\Delta\epsilon_B'}{C_0}[AB_n] \quad (2)$$

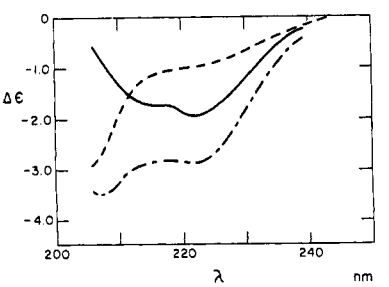

FIGURE 3: CD spectra and difference spectrum of histones IIb2 plus IV: 1.0×10^{-5} M histone IIb2 plus 1.0×10^{-5} M histone IV in 0.005 M cacodylate, pH 7.0 (- - -), and in 0.016 M phosphate, pH 7.0 (- - -); the difference spectrum between the spectra at 0.016 M phosphate and 0.005 M cacodylate (—).

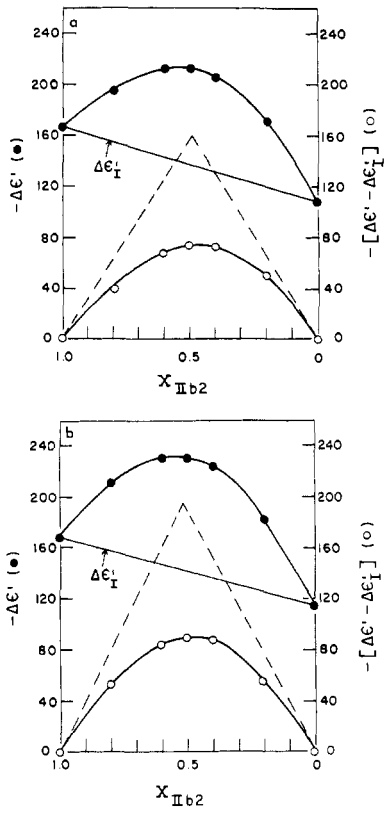

FIGURE 4: CD (220 nm) continuous variation curves, $\Delta\epsilon'$ *vs.* X_{IIb2} (●) and $\Delta\epsilon' - \Delta\epsilon_I'$ *vs.* X_{IIb2} (○), at (a) 0.0031 M phosphate, pH 7.0, $C_0 = 0.5 \times 10^{-5}$ M, and (b) 0.0031 M phosphate, pH 7.0, $C_0 = 0.5 \times 10^{-5}$ M, and (b) 0.0031 M phosphate, pH 7.0, $C_0 = 1.0 \times 10^{-5}$ M. Dotted lines are extrapolations of the slopes of the $\Delta\epsilon' - \Delta\epsilon_I'$ curve at $X_{IIb2} = 0$ and $X_{IIb2} = 1.0$.

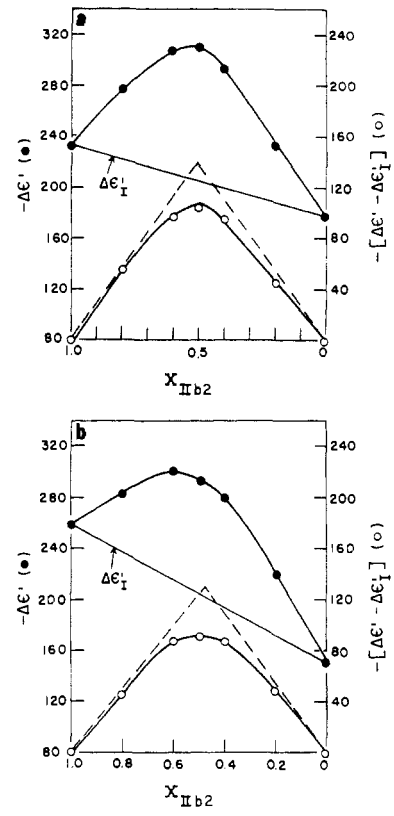

FIGURE 5: CD (220 nm) continuous variation curves, $\Delta\epsilon'$ *vs.* X_{IIb2} (●) and $\Delta\epsilon' - \Delta\epsilon_I'$ *vs.* X_{IIb2} (○), at (a) 0.016 M phosphate, pH 7.0, $C_0 = 1.0 \times 10^{-5}$ M, and (b) 0.15 M NaCl, pH 7.0, $C_0 = 0.5 \times 10^{-5}$ M. Values of $\Delta\epsilon'$ are obtained from extrapolation to $t = 0$ for X_{IIb2} less than 0.5. The dotted lines are extrapolations of the slopes of the curves of $\Delta\epsilon' - \Delta\epsilon_I'$ at $X_{IIb2} = 0$ and $X_{IIb2} = 1.0$.

The term $\Delta\epsilon'$ is calculated from the CD measurements on the basis of C_0, and $\Delta\epsilon_I'$ is calculated assuming noninteracting species A and B in mixed solutions. The mole fraction of component A, X_A, at which a maximum occurs in $\Delta\epsilon' - \Delta\epsilon_I'$ is related to n by

$$n = (1 - X_A)/X_A \tag{3}$$

Histone IV alone will undergo slow changes in the CD or anisotropy at suitable histone and salt concentrations (Li *et al.*, 1972; Wickett *et al.*, 1972). Hence, excess histone IV which is not complexed to histone IIb2 in mixed solutions may undergo slow changes. It is, therefore, desirable to perform the measurements under conditions at which there is no slow step of histone IV; otherwise the physical properties of the initial interaction must be extrapolated to $t = 0$ to allow for possible slow changes of histone IV.

In the present study we observed no slow change in 3.1×10^{-3} M phosphate, pH 7.0, at concentrations of 1.2×10^{-5} M histone IV or less; slow change was observed at higher histone concentrations. Continuous variation measurements of histones IIb2 and IV in 0.0031 M phosphate, pH 7.0, were performed at C_0 equal to 0.50×10^{-5} and 1.0×10^{-5} M (Figure 4). Both curves of $\Delta\epsilon' - \Delta\epsilon_I'$ have maxima at a mole fraction of IIb2 of 0.47 which suggests that the stoichiometry might be 1:1. The curve maximum of $\Delta\epsilon' - \Delta\epsilon_I'$ is not so great at $C_0 = 0.50 \times 10^{-5}$ M as at $C_0 = 1.0 \times 10^{-5}$ M, suggesting that there is a histone concentration effect on the maximum amount of complex formed.

Continuous variation CD curves measured at 0.016 M phosphate and 0.15 M NaCl are given in Figure 5. No slow change whatsoever was observed for mole fractions of histone IIb2 greater than 0.4. At mole fractions of IIb2 less than 0.4 the CD change following salt addition is characterized by an instantaneous "jump" and a slower change. These results are consistent with a very fast interaction of histone IIb2 and histone IV followed by a slower change for excess histone IV. Therefore, $\Delta\epsilon'$ at $t = 0$ was taken as a measure of the histone IIb2–histone IV interaction. Curves of $\Delta\epsilon' - \Delta\epsilon_I'$ again have maxima at X_{IIb2} equal to 0.50 which agrees with the results at 0.0031 M phosphate. Similar results were obtained from measurements at 0.016 M phosphate, $C_0 = 0.50 \times 10^{-5}$ M and at 0.24 M NaCl, $C_0 = 0.5 \times 10^{-5}$ M.

Therefore, over a fivefold phosphate concentration range, and in sodium chloride solutions, the CD continuous variation data indicate a complex with a stoichiometry of 1:1. From extrapolation of the initial slopes at mole fractions 1.0 and 0 and the curve maxima, association constants (Table II) are estimated for the equilibrium expression in eq 4 (Schaeppi and Treadwell, 1948).

$$[AB]/[A][B] = K \tag{4}$$

FLUORESCENCE PROPERTIES. Equations analogous to the CD equations may be developed to relate the fluorescence properties to the concentration of the complex AB_n. If the species A, B, and AB_n are characterized by the intrinsic molar fluorescence, F_i, and anisotropy r_i, which are independent of con-

TABLE II: Apparent Association Constants of Histone IIb2–Histone IV Interaction from Continuous Variation Plots.

Medium, pH 7.0 (M)	C_0 (M)	$K \times 10^{-6}$ (M^{-1})
Phosphate (0.0031)	0.5×10^{-5}	0.6
Phosphate (0.0031)	1.0×10^{-5}	0.3
Phosphate (0.016)	0.5×10^{-5}	2.7
Phosphate (0.016)	1.0×10^{-5}	2.4
NaCl (0.15)	0.5×10^{-5}	3.0
NaCl (0.24)	0.5×10^{-5}	3.0

centration, then one may derive eq 5 and 6. F and r are the

$$F - F_I = k(F_{AB_n} - F_A - nF_B)[AB_n] \quad (5)$$

$$Fr - F_I r_I = k(F_{AB_n} r_{AB_n} - F_A r_A - nF_B r_B)[AB_n] \quad (6)$$

measured values of the mixed solutions, and F_I and r_I are the calculated values assuming noninteracting species A and B. k is a constant characteristic of the cell and spectrometer.

Continuous variation results at 0.0031 and 0.016 M phosphate, $C_0 = 1.0 \times 10^{-5}$ M, are given in Figure 6. At 0.016 M phosphate the anisotropy and fluorescence intensity were functions of time for X_{IIb2} less than 0.5. As with the CD measurements, the values obtained from extrapolation to $t = 0$ were taken as indicators of histone IIb2–histone IV interaction. Both sets of data indicate a maximum at X_{IIb2} of slightly greater than 0.5, but independent runs have given maximum values of X_{IIb2} shaded to less than 0.5. We conclude that the fluorescence continuous variation curves, like the CD results, indicate a 1:1 complex of histones IIb2 and IV.

Histone Self-Aggregation. Several groups have reported aggregation within a pure histone fraction (Edwards and Shooter, 1969; Boublik *et al.*, 1970a,b; Barclay and Eason, 1972; Diggle and Peacocke, 1971; Li *et al.*, 1972). Li *et al.* (1972) reported changes in the fluorescence anisotropy of the fast step of histone IV in 0.0067 M phosphate, pH 7.4, as well as the slow step. It was also concluded that the CD of the fast and slow steps was rather insensitive to histone concentration over the range studied. Because of this accumulation of data, it is prudent that we examine the histone concentration effects upon the histone CD and fluorescence properties with respect to their effects on the continuous variation results.

The CD curves (220 nm) as functions of histone IIb2 concentration at 0.0031 and 0.016 M phosphate (pH 7.0) have been measured (Figure 7a). Similar data are shown for the fast and slow steps of histone IV in 0.0067 M phosphate, pH 7.4, and for the fast step in 0.0031 M phosphate (Figure 7b). Clearly $\Delta\epsilon$ is dependent upon histone concentration for histone IIb2 in 0.016 M phosphate, pH 7.0, and for both the fast and slow steps of histone IV in 0.0067 M phosphate, pH 7.4. The concentration effects in 0.0031 M phosphate, pH 7.0, are much less pronounced for both histones.

These results of histone IV are not at variance with the CD results of Li *et al.*, as may be seen from Figure 7b in which the earlier results are also plotted. The data of Li *et al.* are within experimental error of the present data. However, here we measure to a slightly lower concentration and show clearly the concentration dependence of the CD. We also note that

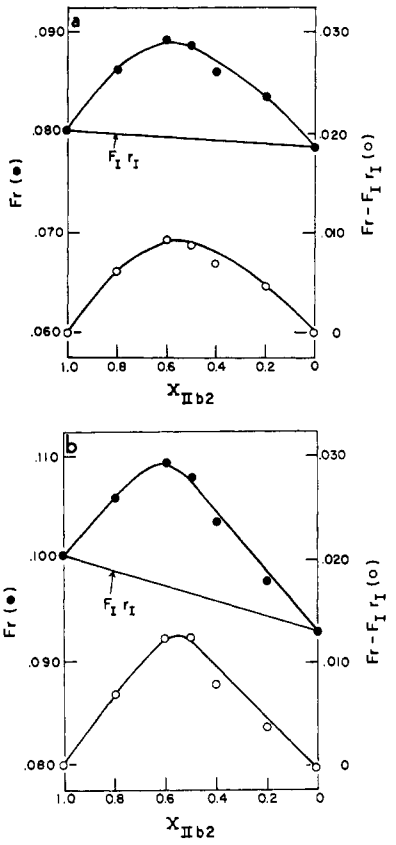

FIGURE 6: Fluorescence continuous variation curves, Fr (●) and $Fr - F_I r_I$ (○) *vs.* X_{IIb2}, at (a) 0.0031 M phosphate, pH 7.0, and (b) 0.016 M phosphate, pH 7.0. At 0.016 M phosphate, the values of F and r were obtained from extrapolation to $t = 0$ for X_{IIb2} less than 0.5.

the CD of the fast and slow steps extrapolate to similar values of $\Delta\epsilon$ at zero concentration of histone lending confidence to our measurements.

Fluorescence intensity (not shown) and fluorescence anisotropy (Figure 8) were measured under the same conditions as was the CD. To within experimental error, the quantum yield, as monitored at 325 nm, was independent of concentration for both histones IIb2 and IV. The observed fluorescence was proportional to the light absorbed by the sample at 280 nm. This latter quantity was estimated by $k(1 - 10^A)$ in which A is the absorbance of the sample and k is an instrumental constant. As an example, the calculated and measured relative fluorescence intensities of histone IV in 0.0067 M phosphate, pH 7.4, are given in Table III.

Evaluation of Continuous Variations Results. In assessing the effects of histone self-aggregation upon the validity of the continuous variation results, we must consider the relative effects of self-aggregation and complex formation upon the observables of interest.

In 0.0031 M phosphate, pH 7.0, the changes in $\Delta\epsilon'$ in going from $X_{IIb2} = 1.0$ to $X_{IIb2} = 0.5$ are ~15 times greater than those measured for histone IIb2 or histone IV self-aggregation. In the fluorescence measurements the factor is about 10.

At 0.016 M phosphate the factor is reduced to ~7 in the CD. The most unsatisfactory results would be expected for the fluorescence continuous variation curves at 0.016 M phosphate, pH 7.0. Under these conditions changes induced by cross-dimerization are larger by only a factor of 3.

The net effects of histone self-aggregation appear to be:

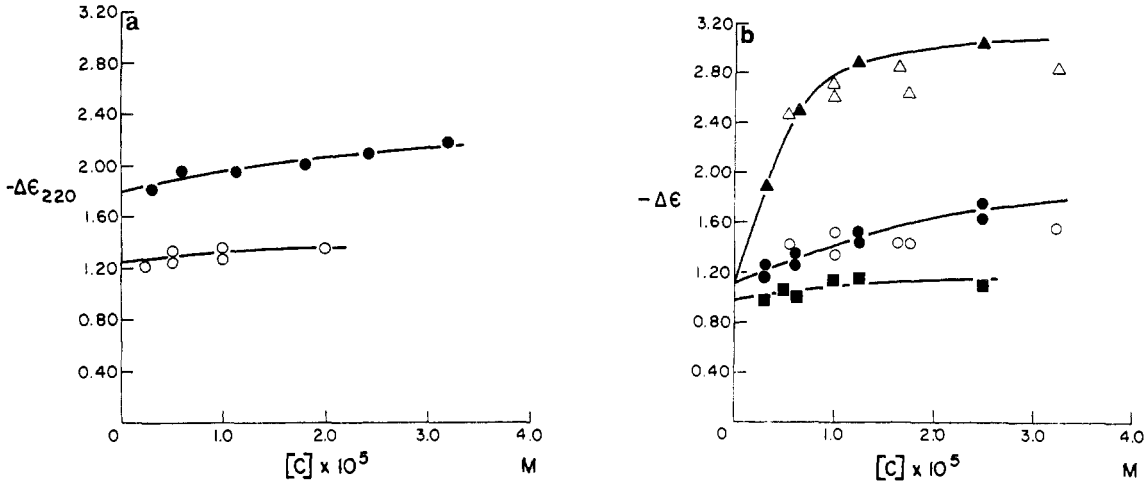

FIGURE 7: CD (220 nm) curves as functions of histone concentration: (a) histone IIb2 in 0.0031 M phosphate, pH 7.0 (○), and 0.016 M phosphate, pH 7.0 (●); (b) histone IV in 0.0031 M phosphate, pH 7.0, fast step (■); in 0.0067 M phosphate, pH 7.4, fast step (●); slow step (▲); fast step from Li *et al.* (1972) (○); slow step from Li *et al.* (1972) (△). The $\Delta\epsilon$ values of the fast step of histone IV were obtained from extrapolation to $t = 0$.

(1) to complete with cross-complexing and (2) to reduce the height of the continuous variation curves relative to the ideal case. In the ideal case, non-cross-complexed histone would be totally in the monomeric forms.

It is fortunate that the magnitudes of the CD and anisotropy changes due to self-aggregation are the same for both histones. This similarity should tend to round the continuous variation curves somewhat equally, rather than distort one side much more than the other. Therefore, we conclude that the continuous variation plots, especially at 0.0031 M phosphate, pH 7.0, are reasonable indicators of the cross-complex stoichiometry. On the other hand, the equilibrium constants should, because of rounding effects, be considered as order of magnitude estimates.

CD of the Histone IIb2–Histone IV Complex as a Function of Phosphate Concentration. CD at 220 nm of solutions 0.25 × 10^{-5} M in histone IV plus 0.25 × 10^{-5} M in histone IV, 0.50 × 10^{-5} M histone in IIb2, and 0.50 × 10^{-5} M in histone IV were measured as functions of phosphate concentration, pH 7.0. The same histone and phosphate stock solutions were used for the pure and mixed histone samples. Plots of the experimental data and a calculated plot for ideal noninteracting solutions of histones IIb2 and IV are given in Figure 9.

At all phosphate concentrations greater than zero, and even at infinite phosphate (values obtained by extrapolation), the experimental CD changes of the mixed solutions are greater than those calculated for noninteracting mixtures. These results demonstrate that there is a real increase in structure of the 1:1 histone IIb2–histone IV mixtures as compared to the individual histones. Thus, the increase in structure does not result simply from possible shifts in equilibrium due to histone IIb2–histone IV interaction (see equilibrium scheme in Figure 10).

Earlier it was concluded that the CD change accompanying histone IIb2–histone IV complexing was characteristic of α-helical formation. Now, we should like to estimate the α-helical content of the mixed complex as compared to the noncomplexed constituent IIb2 and IV molecules.

In making estimates of the helical content of histones IIb2 and IV, Li *et al.* (1972), Wickett *et al.* (1972), and D'Anna

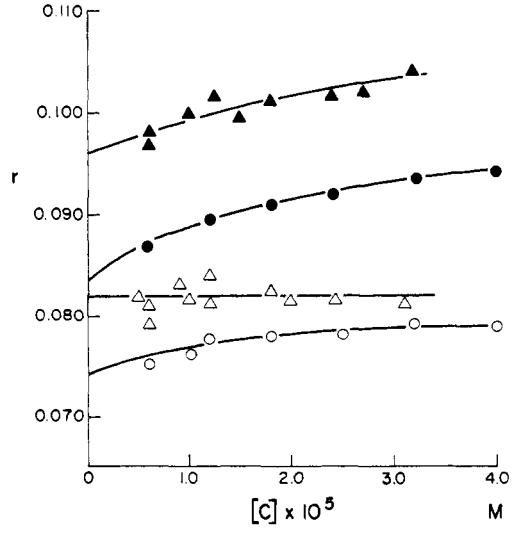

FIGURE 8: Anisotropy as a function of histone concentration: histone IIb2 in 0.016 M phosphate, pH 7.0 (▲), and 0.0031 M phosphate, pH 7.0 (△); histone IV fast step in 0.0067 M phosphate, pH 7.4 (●), and at 0.0031 M phosphate, pH 7.0 (○). The values of r for the fast step of histone IV were obtained from extrapolation to $t = 0$.

TABLE III: Comparison of Calculated and Measured Relative Fluorescence Intensity for Samples of Histone IV in 0.067 M Phosphate, pH 7.4.

	Rel Fluorescence	
[Histone IV] × 10^5 (M)	Calcd	Measd
4.0	1.00	1.00
3.2	0.83	0.80
2.4	0.65	0.65
1.8	0.51	0.51
1.2	0.35	0.35
0.6	0.18	0.18

TABLE IV: Percentage of α Helix Induced by Phosphate, pH 7.0, or by Phosphate and Cross-Complex Formation.

Sample	$-\Delta\epsilon(\infty)$ (cm^{-1} M^{-1})[a]	$-[\Delta\epsilon(\infty) - \Delta\epsilon(0)]$ (cm^{-1} M^{-1})	% of Residues Coil → α Helix	Residues Coil → α Helix	Helix Content[b]
IIb2	2.24	1.26	12.6	15.8 of 125	17.7
IV	2.22	1.45	14.5	14.8 of 102	15.8
IIb2 + IV (measd)	2.65	1.72	17.2	38.7 of 227	41.8
IIb2 + IV (ideal)	2.23	1.35	13.5	30.6 of 227	33.5

[a] Obtained from extrapolation of $\Delta\epsilon$ *vs.* the reciprocal of phosphate concentration. [b] Relative to the coil form at pH 4.3.

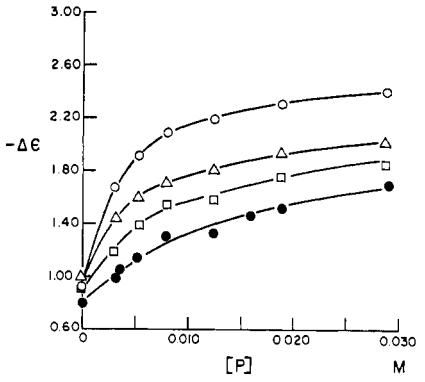

FIGURE 9: CD (220 nm) of histone IIb2, the fast step of histone IV, and histones IIb2 plus IV as functions of phosphate concentration, [P], pH 7.0: 0.50×10^{-5} M histone IIb2 (△); 0.50×10^{-5} M histone IV (●); 0.25×10^{-5} M IIb2 plus 0.25×10^{-5} M histone IV, measured (○) and calculated (□).

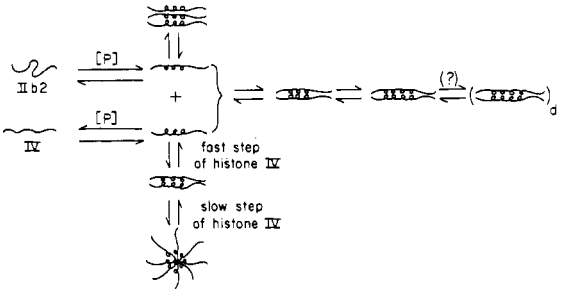

FIGURE 10: Proposed scheme of the conformational changes and interactions of histones IIb2 and IV; [P] represents the concentration of phosphate or chloride ions.

and Isenberg (1972) performed extrapolations to infinite salt concentration. The CD change in going from water to infinite phosphate was taken as a limiting increase in helical content of an all or none histone conformational change induced by salt.

Our results have shown that salt is necessary to cross-complex formation. If we extrapolate our data to infinite salt concentration, we obtain maximum amounts of histones IIb2 and IV available for complex formation. However, we do not know that all of the molecules in the extrapolated state are complexed. Thus, the change in the CD of the mixed solutions in going from zero to infinite phosphate at pH 7.0 must be regarded as the minimum helical change induced by salt *and* by formation of the cross complex. Knowing this CD change and that $\Delta\epsilon_{220}$(helix) $-$ $\Delta\epsilon_{220}$(random) ≈ -10.0 cm^{-1} M for a 100% coil to helix transition (Li *et al.*, 1972; D'Anna and Isenberg, 1972), we may estimate the percentage of helical change induced by salt and cross-complex formation. Values calculated from the data of Figure 10 are given in Table IV.

If we consider the changes in $\Delta\epsilon$ at 220 nm with pH (see Table I), we may estimate the total increase in helical content of histone IIb2, histone IV, and the cross complex (D'Anna and Isenberg, 1972) induced by pH and salt. The estimated total helical content is also given in Table IV.

The number of 39 residues of α helix per 227 induced by salt and complex formation is significantly greater than 31 residues per 227 as calculated from the sum of residues of α helix induced by salt for the pure histones. Hence, we conclude that an increase of a minimum of eight residues of α helix accompanies histone IIb2–histone IV interadtion.

Discussion

Histones IIb2 and IV have been shown, from CD and fluorescence properties, to interact in the presence of salt. The interaction is very fast as measured by our techniques and appears as instantaneous changes in our measurements. Continuous variation results by both CD and fluorescence techniques are consistent with a complex containing a single molecule each of histones IIb2 and IV.[8]

Accompanying formation of the complex is an increase in the CD relative to the noninteracting component histones IIb2 and IV at all salt concentrations measured. The increase in CD has been interpreted as a net increase in the helical content relative to histone IIb2 and to the fast step of histone IV at a given salt concentration. From extrapolation of $\Delta\epsilon$ as a function of phosphate it is estimated that a minimum of 42 residues of α helix are contained in the dimer at infinite phosphate as contrasted to 34 residues calculated for the sum of ideal mixtures of histones IIb2 and IV.

Continuous variation fluorescence measurements are characterized by a small increase in fluorescence (5%) and increased anisotropy centered about a mole fraction of 0.5 in IIb2 and IV. The anisotropy increase for the IIb2–IV cross interaction is of the same order of magnitude as observed for the aggregation of pure histone IIb2 or the fast step of histone IV as functions of histone concentration. Fluorescence quantum yields at 325 nm are independent of concentration for

[8] It should be noted that an equilibrium scheme of the type

$$n_1A + n_2B = A_{n1}B_{n2}$$

will also give a maximum in the continuous variation plots, so long as $n_1/n_2 = 1$, Therefore, while we cannot definitely rule out a complex with $n_1 = n_2 > 1$, we feel the following discussion favors the dimer assignment.

self-aggregation of histones IIb2 and the fast step of histone IV. This insensitivity of the fluorescence quantum yield in the homo and hetero complexes suggests that the average tyrosine environment is not significantly altered by complex formation. Based on this premise, the similar increases of anisotropy accompanying homo and hetero complexing imply that both types of complexes have similar rotary diffusion and size.

The apparent equilibrium constant for the histone IIb2–histone IV complex increases with phosphate concentration (Table II) and is of the order of 10^5–10^6 M^{-1}. This equilibrium constant is at least an order of magnitude larger than that of histone IV–histone IV dimerization (Li *et al.*, 1972). Hence, in salt solutions, the histone IV–histone IIb2 interaction will be favored.

Formation of the cross dimer eliminates the slow step characteristic of histone IV in both CD and fluorescence properties. For mixtures of histones, 0.9×10^{-5} M each in histones IIb2 and IV, there was a little slow step at 0.016 M phosphate and 0.24 M NaCl following an initial jump (Figure 1). However, in the continuous variation measurements at $C_0 = 1.0 \times 10^{-5}$ M histone, there was no slow process observed for mixtures at a mole fraction of IIb2 = 0.5. It is not clear if further aggregation of the histone cross dimer occurs at increased histone concentration or if the slow step arises from small amounts of residual histone IV.

In contrast to their behavior in salt solutions, the mixed histones in water or 0.005 M cacodylate have the properties of noninteracting histones. These results suggest that cross complexing occurs between histones in their salt-induced states. Our results are consistent with those of Boublik *et al.*, (1970b) who reported no change in the optical rotary dispersion of histone IIb2 in water with histone concentration. It appears that salts are necessary for any type of intermolecular interaction over a large range of histone concentration and pH (~3.0–7.0). This is perhaps not surprising in view of the high positive charge on the histone molecules.

A scheme is proposed in Figure 10 to illustrate the interactions of histones consistent with these results and those in the literature.

Bradbury and Rattle (1972) and Hayashi and Iwai (1971) have independently proposed that highly basic regions of histone interact with DNA, and that other regions are available for histone–histone interaction. In the case of histone IIb2, Bradbury and Rattle (1972) have proposed, from computer analyses of proton magnetic resonance data, that DNA interacts with histone IIb2 primarily at regions 1–30 and 102–125 in the IIb2 peptide chain. It was suggested that the middle portion of histone IIb2 molecules would be available for histone–histone interactions. Hayashi and Iwai (1971) proposed that the basic ends of histone IIb2 bind to DNA, and the remaining portions interact to effect what is believed to be superhelicity in chromatin (Richards and Pardon, 1970). Still more recently Simpson (1972) has shown that tryptic digestion of chromatin leads to proteolysis of 30–55% of the histone residues, the rest being apparently unaltered and bound to DNA. The physical properties of the altered chromatin were consistent with the bridging models of Bradbury and Rattle (1972) and of Hayashi and Iwai (1971).

We feel that it is premature to postulate a biological role for the histone IIb2–histone IV interaction. However, it can be said that the interaction is strong and specific. With regard to the proposed model of Bradbury and Rattle (1972), the histone IIb2–histone IV complex—if it exists in chromatin—offers an interaction that is at least one, even two, orders of magnitude stronger than histone IIb2 or histone IV self-aggregation.

Acknowledgment

We wish to thank Miss Roswitha Blohm for preparation of histones IIb2 and IV. We also thank Mr. Bob Howard for amino acid analyses and Dr. K. E. Van Holde for use of his JASCO CD recorder.

Appendix

Assume we are dealing with the equilibrium $A + B \leftrightarrows AB_n$ and the species A, B, and AB, and AB_n have the concentration-independent molar fluorescence intensities F_A, F_B, and F_{AB_n} and the concentration-independent anisotropies r_A, r_B, and r_{AB_n}. If $[A_0]$ and $[B]_0$ represent the initial analytical concentrations of A and B, then for dilute solutions

$$F = k\{F_{AB_n}[AB_n] + F_A([A]_0 - [AB_n]) + F_B([B]_0 - n[AB_n])\} \quad (7)$$

and from the addition law (Weber, 1952)

$$r = \frac{k}{F}\{F_A([A]_0 - [AB_n])r_A + F_B([B]_0 - n[AB_n])r_B + F_{AB_n}[AB_n]\} \quad (8)$$

k is a spectrometer constant. On the other hand, if there were no interaction between A and B, expressions 7 and 8 would be replaced by eq 9 and 10. Subtraction of eq 9 from eq 7

$$F_I = k(F_A[A]_0 + F_B[B]_0) \quad (9)$$

$$r_I = \frac{k}{F_I}(F_A[A]_0 r_A + F_B[B]_0 r_B) \quad (10)$$

yields eq 5. Rearrangement of eq 8 and 10 followed by subtraction yields eq 6.

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Carboxymethylation of the Histidyl Residues of Insulin†

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ABSTRACT: The reaction of [^{14}C]iodoacetate with insulin at pH 5.6 and 30° yields a carboxymethylated derivative in 13.8% yield provided zinc is first removed from the insulin. Zinc-insulin reacts very slowly. A pure product can be obtained in which the sole modification, as shown by amino acid analysis, is the carboxymethylation of the two histidyl residues of the B chain. This modification fails to alter the immunologic reactivity of insulin in standard radioimmunoassays but markedly reduces the biological activity of the derivative as measured by glucose oxidation or glycogen synthesis in the isolated rat diaphragm.

During studies on iodohistidine formation in simple proteins it became apparent that many histidine residues were difficult to iodinate, but that special cases existed in which electrostatic facilitation appeared to yield iodination rates that were comparable to those of exposed tyrosyl residues (Covelli and Wolff, 1966a,b; Wolff and Covelli, 1969). In insulin, iodination of one histidyl residue appeared to be controlled by the presence of zinc in the molecule (Covelli and Wolff, 1967). Since alkylation reactions proceed by similar electrophilic displacement and are also subject to factors such as electrostatic facilitation (Heinrikson *et al.*, 1965), we have investigated the reactivity of the two histidyl residues of the B chain (B^5 and B^{10}) toward iodoacetate at pH 5.6.

In the present work we describe the preparation and the purification of an insulin derivative, in which the sole modification was the N-carboxymethylation of the two histidyl residues.

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Materials and Methods

Crystalline bovine zinc insulin (25.4 IU/mg, Mann) with a zinc content of 0.48% and a moisture content of 5.2% was used. Zinc-free insulin was prepared according to a modification of the method of Sluyterman (1955; Covelli and Wolff, 1967). It contained less than 0.04% zinc, as calculated by the Versene titration method of Flaschka (1952), and a moisture content of 7.9%. Iodoacetic acid (Eastman) was recrystallized twice from petroleum ether (bp 30–60°) and the colorless crystals were dried *in vacuo* and stored at −20°. Iodoacetic acid-*1*-^{14}*C* (The Radiochemical Centre, Amersham, England) had a specific activity of 1.3 mCi/mmole. Glucose-*1*-^{14}*C* (The Radiochemical Centre, Amersham, England) and the Insulin Radioimmunoassay Kit (Wellcome, England, or Società Ricerche Nucleari, Italy) were also used. Cyanogum-41 was purchased from British Drug Houses. Ultra Pure urea was a product of Mann; it was stored at +4° and solutions were made immediately before use to minimize the formation of cyanate (Stark *et al.*, 1960). All other reagents were of analytical grade and triple-distilled water was used throughout.

Time Course of Alkylation. In a standard experiment the reaction mixture contained 6–8 μmol of insulin dissolved in 8