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Physical Studies on Proinsulin. A Comparison of the Titration Behavior of the Tyrosine Residues in Insulin and Proinsulin†

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ABSTRACT: The titration behavior of the tyrosine residues in proinsulin and insulin has been determined. The tyrosines in proinsulin titrate in the same manner as those in insulin. The titration behavior of these residues in both proteins is a function of protein concentration and thus the association state of the protein. In the absence of zinc, the tyrosine ionization is not time dependent, while in the presence of zinc a time-dependent tyrosine ionization is observed. In addition, the presence of zinc, which binds to both proteins and forms metal–protein complexes of each, decreases the amount of tyrosine ionized at equilibrium. The tyrosines affected are tentatively identified based on the X-ray crystal structure (Blundell, T. L., Dodson, G. G., Dodson, E., Hodgkin, D. C., and Vijayan, M. (1971), *Progr. Hormone Res. 27*, 1). The equivalency in the results for the two proteins lends further support to our proposal that the insulin moiety in proinsulin has the same conformation as insulin itself (Frank, B. H., and Veros, A. J. (1968), *Biochem Biophys. Res. Commun. 32*, 155).

We have been examining the physical and chemical properties of proinsulin in order to gain information on its conformation in solution. Among the properties of proinsulin that can be readily examined is the titration behavior of the tyrosine residues in the molecule. Proinsulin contains four tyrosines which are located in the same positions in the insulin portion of proinsulin as in insulin itself (Chance *et al.*, 1968). Therefore, a study of the titration behavior of the tyrosines should increase our knowledge of the state of these residues in the proinsulin molecule.

Upon examining the earlier studies on the titration of the tyrosine residues in insulin (Crammer and Neuberger, 1943; Tanford and Epstein, 1954a,b; Fredericq, 1954; Inada, 1961; Morris *et al.*, 1970), we concluded that the potential effects of factors such as ionic strength, disulfide bond cleavage, protein concentration, and zinc ion interaction had not been defined. Therefore, each of these factors has been carefully

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examined, and each has been found to have a strong effect on the tyrosine titration behavior in both insulin and proinsulin. Most striking are the effects that arise because both proteins self-associate very strongly as well as interact with zinc to form zinc–protein complexes (Frank and Veros, 1968; Frank *et al.*, 1972; Grant *et al.*, 1972).

By referring to the X-ray crystal structure of insulin (Blundell *et al.*, 1971), we can tentatively assign which residues are affected by each of the factors mentioned above. Since the results for both proteins are essentially identical, the state of the tyrosine residues in the proinsulin molecule appears to be the same as in the insulin molecule.

Experimental Section

Highly purified porcine and bovine insulins and proinsulins were kindly provided by Dr. R. E. Chance of the Lilly Research Laboratories. The materials had been prepared according to the procedures of Chance *et al.* (1968). All of the samples used were amorphous zinc-free preparations.

Ultraviolet spectra were recorded on a Cary 14R spectrophotometer equipped with thermostatable cell holders. All titration studies were performed at 24°. A Corning Model 10 expanded scale pH meter equipped with a Thomas 4858-L60 electrode was used for pH determinations.

Stock solutions of zinc chloride were prepared, and their zinc ion concentration was determined by atomic absorption spectroscopy. All other chemicals were analytical or reagent grade.

Protein solutions were prepared by dissolving the protein in the appropriate solvent and then adjusting the pH to neutral pH if necessary with NaOH. The solution was then filtered through a 0.22-μ Millipore filter. Addition of zinc if desired was done after filtering. The concentration of protein was determined from the ultraviolet absorption spectrum. The values of $A^{276}_{0.1\%}$ used in the calculations were 1.05 for bovine and porcine insulin, 0.67 for porcine proinsulin (Frank and Veros, 1968), and 0.70 for bovine proinsulin.[1] The zinc content of zinc–protein solutions determined by atomic absorption spectroscopy on random samples always agreed (to within 5%) with the value calculated from dilution of the stock zinc solution.

The ultraviolet difference titration spectra were done according to the methods described by Wetlaufer (1962). A value of 2300 for $\Delta\epsilon^{295}_{\text{tyrosine}}$ was used to calculate the amount of ionized tyrosine. For the concentration difference spectra and the salt effect studies, buffered solutions were used in order to maintain constant pH. The concentration difference spectra were obtained using the method described by Rupley *et al.* (1967).

Cleavage of disulfide bonds was determined by the method of Ellman (1959). Protein solutions were deoxygenated by bubbling N_2 through the solution and then were kept under a nitrogen atmosphere.

Sedimentation velocity experiments were done using a Beckman Model E analytical ultracentrifuge. Experiments were performed in duplicate at 20 ± 0.2° using a synthetic boundary cell (Frank *et al.*, 1970). All samples were freshly prepared, and the zinc was added to the insulin solution before the sample was placed in the centrifuge cell.

[1] The value of $A^{276}_{0.1\%}$ for bovine proinsulin was calculated from the ratio of its molecular weight to that of bovine insulin.

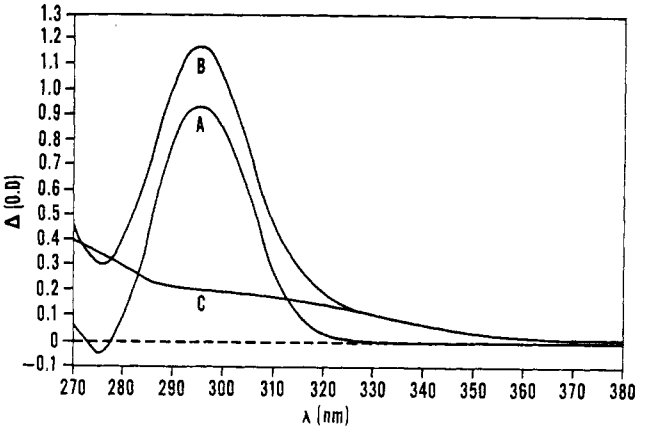

FIGURE 1: Insulin tyrosine difference spectrum: reference cell contains insulin at pH 7.0; sample cell contains insulin at pH 11.5; protein concentration, 0.65 mg/ml; solvent of 0.2 M KCl. Curve A was recorded 30 sec after adjusting sample cell pH to 11.5. Curve B was recorded 2 hr after curve A. Curve C is the difference between curves B and A; cell path length of 1.0 cm.

Results

The ultraviolet difference spectrum generated upon adjusting the pH of an insulin solution to basic pH is that normally observed for phenol group ionization (see Figure 1, curve A). The shape and magnitude of this spectrum do not change over a 1-hr period at room temperature even with the pH of the sample solution as high as 11. The spectrum does, however, change with time when the pH is more basic, *e.g.*, pH 12 (see Figure 1, curve B). The magnitude of the change is both pH and time dependent. This type of time-dependent spectral change has been described previously by Inada (1961) and Garratt and Walton (1967). Cavallini *et al.* (1970) have suggested that this change is a result of disulfide bond cleavage and oxidation. The change (see Figure 1, curve C) certainly does not resemble that expected for phenol group ionization. Insulin solutions exhibiting this type of spectral change react with Ellman's reagent, therefore demonstrating the cleavage of disulfide linkages. We have also found a time-dependent spectral change in zinc–insulin solutions (see below) that is obviously quite different from that seen when disulfide bonds are cleaved.

Proinsulin in basic solutions (pH > 11) exhibits the same spectral changes as insulin solutions. We have not attempted to quantitate the rates of disulfide bond cleavage for proinsulin and insulin under these conditions. Qualitatively the rates appear similar.

Neither the addition of zinc nor the removal of oxygen appeared to change the character of the disulfide bond cleavage in the insulin solutions.

Ionic Strength Effects. Because of charge effects, changes in the ionic strength of the medium would be expected to affect the titration behavior of tyrosine residues (Tanford, 1962). Thus, the ionization of the tyrosine residues in insulin is depressed at low ionic strengths (μ = 0.01) as compared to their ionization at higher ionic strengths (μ = 0.1)—see Figure 2A. No discernible change is observed in the ionization curve obtained with μ = 0.6 as compared to μ = 0.1, if the data are obtained using conventional pH-difference spectral techniques (see Figure 2A). However, by comparing buffered insulin solutions at the same pH, but at different ionic strengths, a difference spectrum can be observed (see Figures 3A and 3B). The difference spectrum found for insulin at

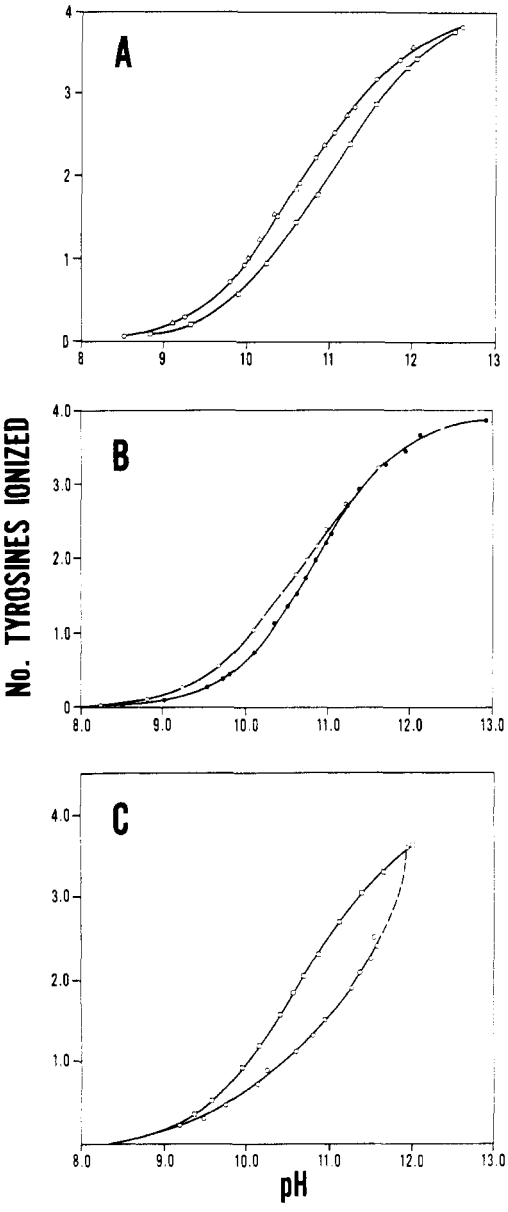

FIGURE 2: Titration curves of insulin. (A) At different ionic strengths: insulin concentration of 0.53 mg/ml; concentration of KCl in solvent, ⊙, 0.1 M; Δ, 0.6 M; □, 0.01 M; 25°. (B) At different protein concentrations: insulin concentrations, ○, 0.86 mg/ml; ●, 8.4 mg/ml; solvent of 0.2 M KCl; 25°. (C) In the presence and absence of zinc: □, insulin with no zinc; ○, insulin with 1 zinc per 2 insulin; solvent of 0.1 M KCl. The values of the number of tyrosines ionized in the presence of zinc were obtained by extrapolation of the OD^{296} to zero time.

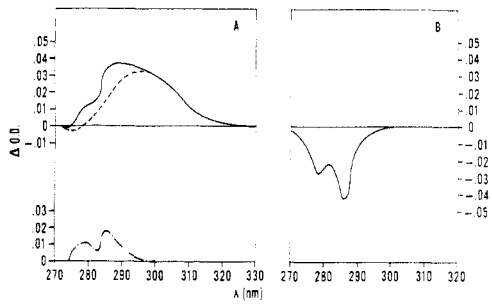

FIGURE 3: Difference spectra for insulin as a function of ionic strength: insulin concentration of 0.83 mg/ml; path length 2 cm; 25°. Solid curve A: sample cell, insulin in 0.11 M KCl–0.01 M borate; reference cell, insulin in 0.01 M borate. Solid curve B: sample cell, insulin in 0.11 M KCl–0.01 M borate; reference cell, insulin in 0.68 M KCl–0.01 M borate. All protein solutions at pH 9.88; (----) calculated tyrosine ionization contribution; (—·—) difference between solid curve A and calculated curve (---).

TABLE I: Observed Sedimentation Coefficients for Insulin in the Presence and Absence of Zinc at Basic pH.[a]

Insulin Concn (mg/ml)	Moles of Zn: Mole of Ins	S_{obsd} pH 9.5	S_{obsd} pH 10.5
0.52	0	1.3	1.1
0.52	1:2	2.5	1.2
0.52	3:1	3.6	1.4
5.8	0	2.7	2.0
5.8	1:2	3.2	2.6
5.8	3:1	3.4	3.0

[a] All studies done in a solvent of 0.2 M NaCl–0.01 M borate buffer at the appropriate pH and at 20 ± 0.2°.

μ = 0.01 *vs.* insulin at μ = 0.12 is not simply a tyrosine ionization difference spectrum but is a more complex combination of spectral effects. The difference spectrum (Figure 3B) found for insulin at μ = 0.12 *vs.* insulin at μ = 0.69 is not at all like a tyrosine ionization difference spectrum, and in fact is essentially identical with the spectrum found for differences in the association state of the insulin molecule (see the section on concentration effects for the explanation of this type of difference spectrum). At the higher ionic strength (μ = 0.69) the insulin molecule would be expected to aggregate more strongly due to the decrease in intermolecular charge repulsion effects.

In analyzing the difference spectrum shown in Figure 3A, we have assumed that the difference spectrum at 300 nm is due to more ionized tyrosine residues in the higher ionic strength solution (μ = 0.12). The spectrum for ionized tyrosines has been generated over the wavelength range of interest and subtracted from the observed difference spectrum. Shown in the lower portion of Figure 3A is the resultant spectrum—a curve which is qualitatively an inverted form of Figure 3B. Thus, spectrum 3A reflects both a difference in the amount of tyrosine ionized and a difference in the association state of the insulin at the two ionic strengths. Proinsulin solutions examined under identical conditions yield similar results.

Protein Concentration Effects. The tyrosine titration curve of insulin is also dependent on the protein concentration used (see Figure 2B). The effect on the titration curve is most readily detected at protein concentrations above 1 mg/ml. This occurs because insulin self-associates to a significant degree even in basic pH solutions. The strong self-association of insulin in basic solutions is evident from the results of sedimentation velocity experiments summarized in Table I. The observed sedimentation coefficient of the 5.8 mg/ml insulin solutions increases over that measured for the 0.52 mg/ml insulin solutions at both pH 9.5 and 10.5.

Concentration difference spectra demonstrate vividly the different degree of ionization of tyrosines as a function of insulin concentration (see Figure 4). This difference spectrum can be shown to be a composite of spectral effects by generat-

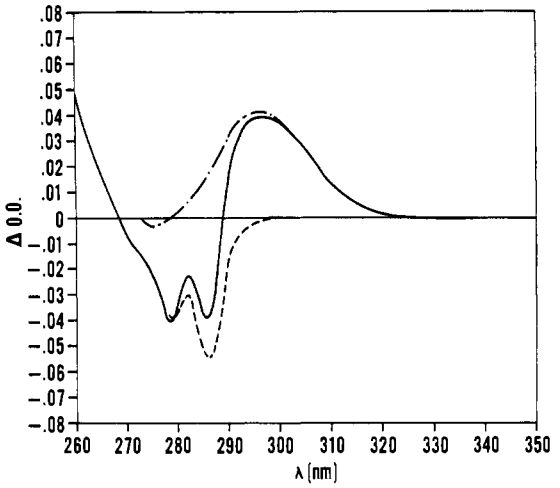

FIGURE 4: Insulin concentration difference spectrum at basic pH. Sample cell contained insulin at 0.9 mg/ml while reference cell contained insulin at 9 mg/ml; solvent, 0.1 M KCl–0.01 M borate; pH 9.51; (—·—) calculated tyrosine ionization contribution; (---) the difference between the observed spectrum and calculated tyrosine ionization contribution; cell paths, sample 1.0 cm, reference 1.0 mm.

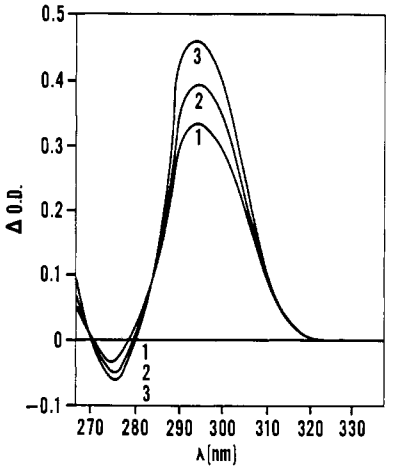

FIGURE 5: Insulin tyrosine ionization spectrum in the presence of zinc. Reference cell contains zinc–insulin (1 zinc per 2 insulin) at pH 7.3. Sample cell contains zinc–insulin at pH 10.7. Curve 1 recorded 30 sec after adjusting pH to 10.7. Curve 2 recorded 5 min after curve 1. Curve 3 recorded 2 hr after curve 1.

ing the appropriate tyrosine ionization spectrum (see Figure 4) based on the optical density above 300 nm. The remaining portion of the observed spectrum (Figure 4) appears to be quite similar to Figure 3B with the maximum at 286 and 278 nm. Rupley *et al.* (1967) have shown that at acid pH this type of spectral difference is observed when insulin monomers associate to form dimers. Our results indicate that there are differences both in the degree of association and in the amount of tyrosine ionized at the two protein concentrations.

Due to the large quantities of material needed for this type of study, we have not undertaken a similar detailed study with proinsulin. However, since we have shown previously that proinsulin self-association is the same as that of insulin (Frank and Veros, 1968; Frank *et al.*, 1972), we would expect the same tyrosine titration effects in proinsulin solutions as discussed above for insulin.

Zinc Effects. The presence of zinc in insulin solutions results in marked changes in the titration behavior of the insulin tyrosine residues. When the pH of a zinc–insulin solution (containing 1 g-atom of zinc per 2 mol of protein) is adjusted to basic pH, the pH–difference spectrum changes with time (see Figure 5). We have ruled out the possibility that this change is due to disulfide bond cleavage on the following basis. First, the spectral changes seen are not similar to those seen when S–S bonds are cleaved (see Figure 1 for comparison), but are identical with those found when increasing amounts of tyrosine ionize. Second, analysis of the zinc–insulin solutions (after the spectral change has occurred) with Ellman's reagent gives no indication of cleavage of disulfide bonds. Finally, returning the pH of the zinc–insulin solution to 7 restores the original ultraviolet spectrum of the insulin molecule.

The amount of tyrosine *initially* ionized when the pH of the zinc–insulin solutions was adjusted to basic pH was determined by recording the change in optical density at 295 nm as a function of time. This curve was then extrapolated to $t = 0$. In order to exclude the possibility that these changes were due to pH drifts, these studies were performed both in the presence and absence of a buffer in the pH 10–11 range and the same results were found under both conditions.

The titration curve for the number of tyrosines initially ionized in the zinc–insulin solutions is greatly depressed in comparison to the curve for zinc-free insulin solutions (see Figure 2C). Moreover, at pH values below 10.6, the final degree of ionization of the tyrosines in the zinc–insulin solutions is less than the degree of ionization in zinc-free insulin solutions. These differences are much smaller than the initial differences in the degree of tyrosine ionization. Figure 6 shows the difference spectrum generated when zinc is added to an insulin solution previously adjusted to basic pH (sample and reference solutions buffered with 0.3 M borate). Comparison of Figure 6 to Figure 4 shows they are quite similar and leads us to conclude that differences exist in the degree of tyrosine ionization and association state of the insulin molecule in the presence and absence of the zinc. Further evidence for the zinc interaction with insulin in basic solutions was obtained in a series of sedimentation velocity experiments whose results are summarized in Table I. The presence of zinc in the insulin solutions resulted in significant increases in the observed sedimentation coefficients. Further, the sedimentation boundaries observed in the presence of zinc were

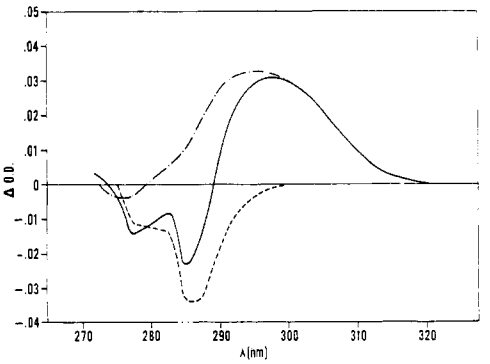

FIGURE 6: Difference spectrum for insulin in the presence and absence of zinc. Sample cell contains insulin with no zinc. Reference cell contains insulin with 1 zinc per 2 insulins. Insulin concentration of 0.76 mg/ml; solvent of 0.3 M borate, pH 9.5; (——) observed difference spectrum; (—·—) calculated tyrosine ionization spectrum based on OD at 300 nm; (---) difference between the observed spectrum and the calculated tyrosine ionization spectrum.

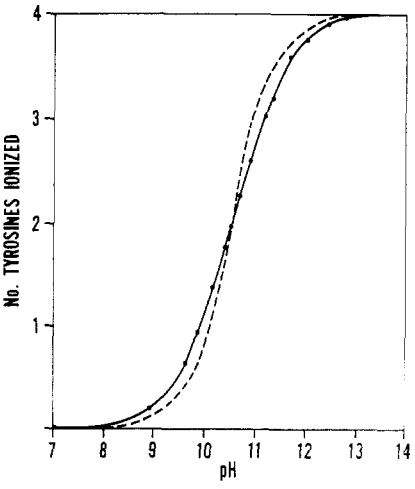

FIGURE 7: Titration curve of proinsulin. Porcine proinsulin (0.085 mg/ml) in 0.2 M KCl, 25°; (-●-●-●-) observed titration curve; (----) theoretical curve for all four tyrosines with $pK_a = 10.5$.

sharper and diffused more slowly than with zinc-free insulin solutions.

We had previously shown that proinsulin interacts with zinc to form a hexamer complex in the same manner as insulin (Frank and Veros, 1970; Grant *et al.*, 1972). Thus, we expected and found the same effect of zinc on the ionization of the tyrosine residues in proinsulin as described above for insulin.

Proinsulin. The insulin titration studies clearly demonstrated the factors that affect the tyrosine titration curve of this molecule. We have noted in each section that these factors also affect the titration behavior of the tyrosine residues in proinsulin. Thus, the following choice was made for a "standard" condition for determining the titration curve of proinsulin: dilute solution (<0.1 mg/ml), ionic strength of 0.2, no zinc present, difference spectrum recorded immediately after addition of base. In order to increase the accuracy of these studies in the dilute solutions, the difference spectrum was recorded from 350 to 230 nm. A peak at ~244 nm is also generated by ionized tyrosine (Wetlaufer, 1962) and is about five times more intense than the 295-nm difference peak. The resultant proinsulin titration is shown in Figure 7. This same experiment was performed with porcine and bovine proinsulins and insulins. Within experimental error, the titration curves for all four proteins were identical with that shown in Figure 7.

Discussion

The essentially identical titration behavior of the tyrosine residues in proinsulin and insulin has been clearly demonstrated in the present study. Even under a variety of conditions that affect the association state and thus the titration behavior of the tyrosines, both proteins yielded the same results. Thus, we have concluded that the tyrosines must be essentially in the same environments in both proteins. This is quite consistent with the results of previous studies on the properties of these proteins (Frank and Veros, 1970; Grant *et al.*, 1972), as well as with our proposal that the insulin portion of proinsulin is in the same conformation as insulin itself (Frank and Veros, 1968).

The present results together with the detailed studies of Cavellini (1970) and Garratt and Walton (1967) show conclusively that Inada (1961) was not observing the slow ionization of a tyrosine residue but the cleavage of disulfide bonds in the insulin molecule. Therefore, Inada's conclusion that three of the four tyrosine residues in insulin ionize rapidly while the fourth ionizes slowly is incorrect. Morris *et al.* (1970) have also reported observing a slowly ionizing tyrosine residue in insulin which behaved differently than that described by Inada. In addition, Morris *et al.* state that their titration studies done at an ionic strength of 0.1 agree with the results of Crammer and Neuberger (1943) which were obtained at an ionic strength of 0.01. Our results (see Figure 2A) indicate that there are significant differences in the titration behavior at these two ionic strengths. Further, in the absence of zinc, no time-dependent ionization of tyrosine has been observed in the present study. We feel that both of the observations made by Morris *et al.* are most probably the result of their use of a zinc–insulin preparation. As we have shown, the presence of zinc causes the appearance of slowly ionizing tyrosine(s) as well as an overall shift in the observed titration curve toward a lower degree of ionization at a given pH (see Figure 6).

Our results clearly indicate a marked effect of the association state of insulin (and proinsulin) on the titration results. However, even under conditions in which these effects are minimized (see Figure 7), the titration curve cannot be fit using a single ionization constant for all four tyrosines. Since the aggregation effects are negligible under the conditions that these data were obtained, there must be some differences in the state of the four tyrosine residues. However, the titration curve does not exhibit any "breaks," which would be indicative of marked differences ($\Delta pK_a = 1.5$) in the ionization constants such as suggested by Morris *et al.* (1970) for two of the four tyrosine residues.[2]

The information available from the X-ray crystallography studies of insulin (Blundell *et al.*, 1971) indicates that the environments of most of the tyrosine residues are dependent upon the aggregation state of the insulin molecule. Only in the case of tyrosine A-19 does its environment remain unchanged in various aggregation states—the hydroxyl group is exposed to solvent even in the zinc–insulin complex. Both of the B-chain tyrosines (B-16 and B-26) are buried when the zinc hexamer complex is formed. Moreover the B-26 tyrosine continues to be buried between monomer units even in the insulin dimer. Finally, while the A-14 tyrosine is close to the surface in the hexamer complex, it still is in a contact region between monomer units. By assuming that the conformation of insulin in solution is quite similar to that found in the crystal, we have attempted to relate the aggregation state effects seen in the present investigation to particular tyrosine residues in both insulin and proinsulin in solution. The effect of decreasing the amount of ionized tyrosine by causing the protein to associate to a greater degree can be demonstrated by either increasing ionic strength or by using higher protein concentration. This effect is probably related to burying the B-26 tyrosine residue within the dimer, since the ionization of this residue would interfere strongly with dimer formation. The results of the concentration difference spectral studies (see Figure 3) are in agreement with this assignment, since a decrease in the amount of tyrosine ionized is observed along with an increase in the amount of buried tyrosine. The spectral effect observed parallels what has been observed for insulin dimer formation without ionization (Rupley *et al.*, 1967).

[2] Because of the complex nature of the tyrosine titration behavior of insulin and proinsulin, we have chosen not to calculate pK values (apparent or intrinsic) such as was done by Tanford and Epstein (1954a,b).

The effects observed when the zinc–insulin complex is titrated are complex. The depression of the ionization has both a kinetic and an equilibrium aspect. For example, at pH 11, a total of one less tyrosine ionizes *initially* in the zinc–insulin sample as compared to the zinc-free sample. In all likelihood this is a reflection that more than one particular tyrosine residue is affected. Based on our knowledge of the X-ray crystal structure of the zinc–insulin complex, residues A-14, B-16, and B-26 could all be affected to varying degrees. After the time-dependent ionization has occurred, a decrease in the total amount of tyrosine ionized in the zinc *vs.* zinc-free system is found (see Figure 6). The data from the centrifuge studies indicate that some complex still remains in zinc–insulin solutions at basic pH. This decrease in the number of tyrosines ionized at equilibrium would, therefore, be expected. The tyrosine affected is probably tyrosine B-26, although some effect on B-16 and/or A-14 cannot be excluded.

Other investigators (Morris *et al.*, 1970) have attempted to gain information on the state of the tyrosines in insulin by chemically modifying specific tyrosines and subsequently determining the effect on the titration curve of the modified insulin. The present study, however, demonstrates the need to determine first the effect of chemical modification on insulin self-association and interaction with metal ion before attempting to gain information on the state of the tyrosine residues in the molecule.

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A Novel Procedure for the Synthesis of 2′-*O*-Alkyl Nucleotides†

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ABSTRACT: A novel method is presented for the synthesis of 2′-*O*-alkyl 3′- (or 5′-) nucleotides with a 3′:5-cyclic nucleotide as starting material. This material is methylated or ethylated at the 2′-OH position using an alkyl iodide at alkaline pH. The 2′-*O*-alkylated cyclic phosphate is cleaved with a 3′:5′-cyclic phosphodiesterase or by chemical procedures to yield the 2′-*O*-alkyl 3′(5′)-nucleotide. The 2′-*O*-alkyl 5′-nucleotide is then chemically phosphorylated to become the 2′-*O*-alkyl nucleoside 5′-diphosphate. These compounds are substrates for polynucleotide phosphorylase in the synthesis of polynucleotides. Substantial quantities of poly(2′-*O*-methyladenylic acid), poly(2′-*O*-ethyladenylic acid), poly(2′-*O*-methylinosinic acid), and poly(2′-*O*-methylcytidylic acid) were prepared by this procedure.

Various 2′-*O*-methyl nucleotides have been found in tRNAs (Smith and Dunn, 1959; Hall, 1964, 1971; Zachau, 1969) and rRNAs (Hall, 1964, 1971; Brown and Attardi, 1965; Nichols and Lane, 1966) from many sources. Since these compounds can be regarded as analogs of both ribonucleo- and deoxyribonucleotides, various homopolymers (Rottman and Heinlein, 1968; Janion *et al.*, 1970; Zmudzka and Shugar, 1970; Tazawa *et al.*, 1971) and copolymers (Rottman and Johnson, 1969; Simuth *et al.*, 1971) containing 2′-*O*-methyl nucleotides have been prepared and their physicochemical (Zmudzka and Shugar, 1970; Bobst *et al.*, 1969b; Zmudzka *et al.*, 1969; Alderfer *et al.*, 1971, 1972) and biochemical properties (Dunlap *et al.*, 1971; Gerard *et al.*, 1972) have been studied. In these cases, the 2′-*O* methyl nucleosides,

† From the Department of Radiological Sciences, The Johns Hopkins University, Baltimore, Maryland 21205. *Received June 12, 1972.* This work was supported in part by a grant from the National Institutes of Health (GM-16066-04), a grant from the National Science Foundation (GB-30725X), and a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research (J. L. A.). This paper was presented in part at the 162nd National Meeting of the American Chemical Society, Washington, D. C., Aug 1971. This paper is the first of a series entitled: Studies on 2′-*O*-Alkyl Polynucleotides.

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