Zinc Binding, Circular Dichroism, and Equilibrium Sedimentation Studies on Insulin (Bovine) and Several of Its Derivatives†

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ABSTRACT: Physicochemical studies performed on insulin and its derivatives are correlated with the corresponding biological activities and with the three-dimensional structure of the crystalline hormone. Equilibrium dialysis of the binding of ^{65}Zn by insulin at pH 8.0 showed heterogeneous binding which could be attributed to two classes of binding sites characterized by stoichiometries (n in g-atoms/mole of insulin monomer) and apparent association constants (K in M^{-1}) of 0.92 (n_1), 1.86×10^5 (K_1) and 1.76 (n_2), 7.3×10^3 (K_2). At pH 8.0, desAsn^{A21}-desAla^{B30}-insulin and triMet$^{AO,BO,\epsilon\text{-}B29}$-insulin, derivatives with significantly decreased hormonal activities, exhibited the heterogeneous binding characteristic of insulin at this pH. Thus, the ability to bind zinc in a manner similar to insulin is not in itself a sufficient criterion for exhibiting the hormonal activity. This along with other considerations strongly suggests that zinc is not essential for the hormonal effects of the molecule. At pH 8.0, desoctapeptide$^{B23\text{-}30}$-insulin exhibited homogeneous binding with stoichiometry of 1.71 and an association constant of 3.81×10^4 M^{-1}. The circular dichroism spectrum of insulin shows negative minima at 208, 222, and 273 nm and fine structure represented by shoulders at 256, 262, and 283 nm. The first two bands are assigned to α-helical and β-structure contributions while the remaining circular dichroism minima are considered to arise from aromatic side chains. The ellipticity bands at 222 nm and those in the aromatic region of the spectrum show a dependency on protein aggregation. Perturbation by Zn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Pb^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, and Ni^{2+} ions occurs to the 208-nm minimum and to the aromatic bands. Similar studies on desoctapeptide$^{B23\text{-}30}$-insulin, desAsn^{A21}-desAla^{B30}-insulin and triMet$^{AO,BO,\epsilon\text{-}B29}$-insulin showed significant differences from insulin particularly noticeable in the 222-nm band and in the aromatic region. The latter has a smaller magnitude and shows either slight or no perturbation by zinc ions. The data from equilibrium sedimentation experiments was fit to a monomer–dimer–tetramer–hexamer aggregation mechanism for zinc-free insulin in agreement with the structure of the crystallographic unit cell and its symmetry relationships. Apparent association constants for the formation at pH 8 of dimers, tetramers, and hexamers are estimated to be $K_{12} = 2.22 \times 10^5$ M^{-1}, $K_{24} = 40$ M^{-1}, and $K_{46} = 220$ M^{-1}. Desoctapeptide-insulin does not aggregate. Both desAsn-desAla-insulin and triMet-insulin exhibited significantly smaller association constants for dimer formation as compared with that for insulin. Using a scheme of aggregation involving monomer–dimer–trimer–tetramer, the calculated apparent association constants corresponding to desAsn-desAla-insulin and triMet-insulin were $K_{12} = 1.74 \times 10^3$ M^{-1}, $K_{23} = 1.11 \times 10^3$ M^{-1}, and $K_{34} = 0.03 \times 10^3$ M^{-1}, and $K_{12} = 4.83 \times 10^3$ M^{-1}, $K_{23} = 1.23 \times 10^3$ M^{-1}, and $K_{34} = 1.97 \times 10^3$ M^{-1}, respectively. The changes in circular dichroic and sedimentation properties of the insulin derivatives studied here suggest a structural as well as a hormonal role for asparagine-A21 and components of the B23–30 octapeptide.

The correlation of structure and function of insulin depends on a detailed chemical and physical characterization of the native hormone and its cellular receptor(s). Very important contributions in this regard were made by the elucidation of the primary structure of insulin by Sanger and his collaborators (Sanger *et al.*, 1955; Ryle *et al.*, 1955) and more recently, of the secondary, tertiary, and quaternary structure of the crystalline hormone (Adams *et al.*, 1969; Blundell *et al.*, 1971). A review of the relationship between the crystal structure and the chemistry and biology of insulin has recently appeared (Blundell *et al.*, 1972).

This report is concerned with a comparison of the zinc binding and circular dichroic and aggregation behavior of bovine insulin with three of its derivatives: desoctapeptide$^{B23\text{-}30}$-insulin, desAsn^{A21}-desAla^{B30}-insulin, and triMet$^{AO,BO,\epsilon\text{-}B29}$-insulin. The hormonal activities of these derivatives are approximately 1, 5, and 50%, respectively, of that of insulin. These measurements were made to determine relationships between physical properties and hormonal activity of the molecules. Observations on insulin are rationalized in terms of the three-dimensional structure in an attempt to examine the relatedness of the crystalline and dissolved forms of this protein.

Experimental Section

Materials and Methods. Crystalline zinc insulin of bovine origin was a gift from Eli Lilly and Co. (lots OLVOO and PJ-7462). Insulin hydrochloride was made according to Carpenter (1958). Desoctapeptide$^{B23\text{-}30}$-insulin was prepared using a modification of the procedure of Young and Carpenter (1961) which involved hydrolyzing zinc-free insulin (1 mg/ml) with 1-tosylamidophenyl chloromethyl ketone treated-trypsin (Carpenter, 1967) at an insulin:trypsin ratio of 50:1 in 0.001 M $CaCl_2$ on a pH-Stat at pH 9.5 for 4 hr at 25°. The procedure of Slobin and Carpenter (1963a)

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was used to make desAsn^{A21}-desAla^{B30}-insulin. The latter two compounds were purified by DEAE-Sephadex chromatography using the conditions of Bromer and Chance (1967) with a change in pH to 8.1 and, in the case of desAsn-desAla-insulin, using a 0.02–0.15 M NaCl gradient. The eluates from chromatography were freed of urea and salts by extensive dialysis against deionized, distilled water and the products were isolated by lyophilization. Tri-Met$^{A0,B0,\leftarrow B29}$-insulin (Levy and Carpenter, 1966, 1967) was a preparation of Mr. Stanley Shiigi of this laboratory. All of these derivatives were characterized by their amino acid composition which was found consistent with the theoretical values (Goldman, 1971). The zinc content was measured by atomic absorption spectrophotometry. Crystalline zinc insulin (lot no. OLVOO) contained 0.57 g-atom of zinc/monomer. The insulin hydrochloride and other insulin derivatives contained less than 0.003 g-atom of zinc/monomer. Radioactive zinc was obtained as a solution of [^{65}Zn]Cl_2 in 1 N HCl from International Chemical and Nuclear Corp.

Measurement of Zinc Binding. Equilibrium dialysis experiments were performed with cylindrical lucite cells designed similarly to those described by Myer and Schellman (1962). Cellophane membranes were cut from Visking dialysis tubing (18/100) and handled prior to use according to Changeux *et al.*, (1968). Trace metal contamination was avoided as recommended by Thiers (1957). Preliminary experiments intended to determine the time necessary to reach equilibrium were carried out by incubating paired dialysis cells in which identical amounts of ligand had been added to either the protein-containing or the protein-free compartments. Equilibrium was considered to be attained when the free zinc concentration was identical in both cells of a pair. This was found (Goldman, 1971) to take a minimum of 12 hr at 30°. Incubations were performed at 30 ± 1° and radioactivity counting was carried out in a well scintillation counter (Model 810A) attached to a scalar Model 135 of Baird Associates Atomic Instruments Co., Cambridge, Mass.

Calculations on binding data were made according to the equation (Klotz, 1953)

$$1/r = (1/nK)(1/[\mathrm{Zn}]_{\mathrm{free}}) + 1/n$$

where r stands for the number of g-atoms of zinc bound/mole of protein, $[\mathrm{Zn}]_{\mathrm{free}}$ is the molar concentration of free zinc, K is the apparent association constant in M^{-1}, and n represents the apparent stoichiometry of binding in g-atoms of zinc/monomer. Electrostatic effects on the binding of the metal ions were not corrected for in the calculations. Breaks in the double reciprocal plots indicated the existence of multiple classes of binding sites with different affinities for zinc. Therefore, calculations on the lowest affinity sites were performed after subtracting the contribution of the highest affinity sites to the overall metal binding. Pertinent portions of the double reciprocal plots were fitted with a straight line function using the least-squares method and a Fortran IV computer program. A report by Klotz and Hunston (1971) on graphical representation of multiple binding sites, which appeared after our calculations were completed, indicates that the significance of the parameters calculated by the above procedure should be viewed with caution. However, when there is over a 100-fold difference in the apparent binding constants, as was indicated in these studies, the parameters approach the expected significance.

Circular Dichroism (CD) Studies. CD measurements were performed on a Cary Model 60 spectropolarimeter fitted with a Model 6002 CD accessory. Cylindrical quartz cells of optical path lengths varying from 0.01 to 5.00 cm were used. Mean residue ellipticities ($[\theta]_\lambda$) were calculated from the expression

$$[\theta]_\lambda = \theta_\lambda \mathrm{Mo}/\mathrm{Cl} \quad (1)$$

where θ_λ is the observed ellipticity at wavelength λ, Mo is the mean residue molecular weight which for insulin and its derivatives was calculated to be 112, C is the protein concentration in g/ml, and l is the optical path length in cm. All measurements were made at 27°.

Solutions were prepared by dissolving the protein in CO_2-free, distilled, deionized water and adjusting the pH and total volume to the desired values. Insulin concentrations were determined spectrophotometrically using an extinction coefficient of 10.4 at 278 nm and pH 8.0 for solutions having approximately 1 mg of insulin/ml. In the case of insulin derivatives, this extinction coefficient was corrected for differences in tyrosine content and/or molecular weight.

Ultracentrifuge Experiments. Sedimentation equilibrium experiments were conducted according to Van Holde and Baldwin (1958) in a Spinco Model E analytical ultracentrifuge equipped with absorption optics and a photoelectric scanner recording system. Avoidance of trace metal contamination was accomplished according to Thiers (1957). All ultracentrifuge experiments were carried out with solution column heights of 3 mm. Protein solutions were dialyzed against buffer for at least 16 hr at 4° and dilution of the samples was performed using the dialysate. Base-line scans were obtained in all cases as soon as the rotor attained the speed set for the experiment (24,000 rpm). The pen deflections calibrated into optical density units were correlated with the corresponding initial protein concentrations. Thus, protein concentrations could be calculated under the instrumental conditions of the experiments and checks on the linearity of pen deflections with regard to protein concentration could be made in each experiment. Monochromatic light of 232, 280, and 295 nm was used depending on whether low, intermediate, or high initial protein concentrations were used, respectively. This fact minimized the possible error in the calculation of protein concentrations which could arise due to the perturbation of the absorption spectra in the aromatic region occurring, especially at low protein concentrations, as a result of association-dissociation phenomena (Rupley *et al.*, 1967). In preliminary experiments, recordings taken after 18 and 24 hr of running the samples in the ultracentrifuge gave essentially identical molecular weight values and, therefore, 18 hr was the minimum time used in all experiments thereafter.

Treatment of the Ultracentrifuge Data. The solutions of insulin and its derivatives studied were assumed to be two-component, thermodynamically ideal systems and the effects of pressure and charge were disregarded. Molecular weights were estimated from the equation of Svedberg and Pederson (1940)

$$M_w^{(r)} = [2RT/(1 - \bar{v}p)\omega^2]\mathrm{d} \ln c^{(r)}/\mathrm{d}r^2 \quad (2)$$

where $M_w^{(r)}$ is the apparent weight-average molecular weight, R is the gas constant, T is the absolute temperature, $\bar{v}$ is the partial specific volume, p is the density of the solvent, ω is the angular velocity, and c is the protein concentration at a point in the cell located at r centimeters from the axis of rotation. In all calculations a value of $\bar{v}$ = 0.72 ml/g was used for insulin (Oncley *et al.*, 1952), and the same value was assumed for all the associating units

and the derivatives of insulin. In order to evaluate the term d ln $c^{(r)}/dr^2$ in eq 2, the linear regression of the log c *vs.* r^2 data was undertaken applying a least-squares procedure with the polynomials

$$\log c^{(r)} = \sum_{i=0}^{n} \beta_i X^i \qquad (3)$$

where $X = r^2$ and $n = 1, 2, 3$. The coefficients β_i were fitted by linear regression using computer programs available from the Berkeley Computer Library (Iscal, R. (1969), G2 Cal Regress Program, Computer Center Library, University of California, Berkeley; O'Connel, B. (1968), G2 Cal Cova Program, Computer Center Library, University of California, Berkeley). Three polynomials of first, second, and third degrees were thus obtained, and the best fit was decided from the estimated root mean square deviation (Hildebrand, 1956a). A third degree polynomial was chosen in most cases, reflecting the nonlinear point distributions with an upward curvature of log c *vs.* r^2 for the plots of insulin, desAsn-desAla-insulin and triMet-insulin systems which showed association–dissociation phenomena. Termwise differentiation of the fitted polynomial as a function of $X(= r^2)$ and substitution of the appropriate value of X provided the numerical values of the term d log $c^{(r)}/$d r^2. Weight-average molecular weights were then calculated as a function of protein concentration using eq 2.

Analysis of Self-Associating Systems. The aggregation scheme of ideal, associating systems of uncharged molecules in which one monomeric unit is added at a time has been described by Steiner (1952) by means of the expression

$$\bar{\alpha}_w/x_1 = 1 + 4K_{12}(x_1c/M_1) + 9K_{12}K_{23}(x_1c/M_1)^2 + 16K_{12}K_{23}K_{34}(x_1c/M_1)^3 + \ldots \qquad (4)$$

where $\bar{\alpha}_w$ is the ratio of the weight-average molecular weight over the molecular weight of the monomer M_1, x_1 is the mole fraction of the monomer at concentration c, and K_{12}, K_{23}, K_{34}, etc., are the association constants for the formation of dimers, trimers, tetramers, etc., respectively. A version of eq 4 derived by Jeffrey and Coates (1966) who considered an aggregation mechanism involving the formation of a dimer which then becomes the aggregating unit is expressed mathematically by the expression

$$\bar{\alpha}_w/x_1 = 1 + 4K_{12}(x_1c/M_1) + 16K_{12}^2K_{24}(x_1c/M_1)^3 + 36K_{12}^3K_{24}K_{46}(x_1c/M_1)^5 + \ldots \qquad (5)$$

where all the symbols are defined similarly as for eq 4. In eq 4 and 5 the variables amenable to experimental measurement are M_w and M_1, from which the average degree of polymerization $\bar{\alpha}_w$ is calculated, and the concentration c. The mole fraction of the monomer x_1, on the other hand, is not directly measurable but can be calculated from the expression (Steiner, 1952)

$$\ln (x_{1,c}/x_{1,0}) = \int_{c_0}^{c} [(\bar{\alpha}_w^{-1} - 1)/c]dc \qquad (6)$$

in the case of both eq 4 and 5. The integral in eq 6 was calculated by fitting a plot of $(\alpha_w^{-1} - 1)/c$ *vs.* c with appropriate arbitrary functions (Goldman, 1971) using a Fortran IV nonlinear regression program available from the Berkeley Computer Library (Baer, R. M. (1967), G2 Cal NLin Program, Computer Center Library, University of California, Berkeley) and integrating numerically these functions by means of another Fortran IV program (Hutchinson, D. (1967), D1 Cal Quad Program, Computer Center Library, University of California, Berkeley) which uses the Newton–Coates method (Hildebrand, 1956b). The numerical values of x_1 as a function of c were thus obtained and the terms $\bar{\alpha}_w/x_1$ and x_1c/M_1 of eq 4 and 5 were calculated. These data were fitted to the polynomials (4) and (5) describing the self-association models using the nonlinear regression Fortran IV program of Baer (see above). All possible mechanisms of aggregation up to the hexamer were tested. The minimum least-squares sum of the deviations plus an agreement better than 600 between the predicted and observed molecular weights over the entire concentration range were used as criteria for best fit.

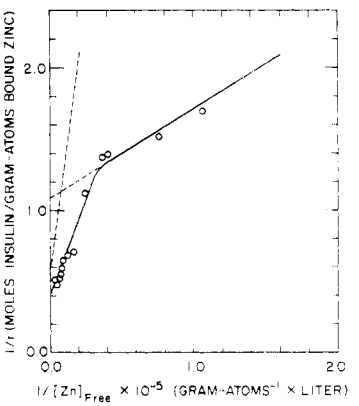

FIGURE 1: Double reciprocal plot of the binding of zinc by insulin at pH 8.0. The dashed lines represent the linear least-squares fits of the independent contributions of the two classes of zinc binding sites discernible under the conditions of the experiment. The studies were performed at an insulin concentration of 0.5 mM in 0.169 M Tris-chloride buffer at pH 8.0, $\mu = 0.1$.

Results

Binding of Zinc by Insulin at pH 8.0. A double reciprocal plot of the binding of zinc by 5×10^{-4} M insulin at pH 8.0 is given in Figure 1. The existence of multiple binding sites with different affinities for the metal is apparent from the data distribution into two nearly straight line segments with a breaking point in between. As seen in Table I, two classes of zinc binding sites are apparent in insulin at this pH. The site of highest affinity (site 1) shows an apparent stoichiometry (n_1) of nearly 1 g-atom of zinc/mol of insulin and an apparent association constant (K_1) of 1.86×10^5 M^{-1}. For the sites of lowest affinity (site 2), n_2 amounts to 1.76 g-atoms of zinc/mol of insulin and K_2 is equal to 7.3×10^3 M^{-1}. In the concentration range of zinc where site 2 began to be occupied by the metal, precipitation of insulin was observed. However, precipitation did not coincide with the concentration of zinc at which the breaking point in the double reciprocal plot occurred which indicated that the latter was not an artifact.

Binding of Zinc by DesAsn-desAla-Insulin at pH 8.0. The pattern of zinc binding by desAsn-desAla-insulin is very similar to that of insulin (Figure 2). At pH 8.0 heterogeneous binding characterized by values of $n_1 = 0.97$ and $K_1 = 3.78 \times 10^5$ M^{-1} for site 1, and $n_2 = 1.46$ and $K_2 = 6.49 \times 10^3$ M^{-1} for site 2, were observed. Analogously to insulin, protein precipitation occurred when site 2 started to interact with zinc.

Binding of Zinc by TriMet-Insulin at pH 8.0. TriMet-insulin also resembled insulin very closely in its pattern of zinc binding at pH 8.0 (Figure 2). The values of n_1 and K_1

TABLE I: Zinc Binding by Insulin and Its Derivatives at pH 8.0.

Derivative	Site 1 n_1	Site 1 K_1 (M^{-1})	Site 2 n_2	Site 2 K_2 (M^{-1})
Insulin	0.92	1.86×10^5	1.76	7.3×10^3
DesAsn-desAla-insulin	0.99	3.78×10^5	1.46	6.5×10^3
TriMet-insulin	1.03	1.89×10^5	2.33	7.0×10^3
Desoctapeptide-insulin			1.71	3.8×10^4

were found to be 1.03 and 1.85×10^5 M^{-1}, and of n_2 and K_2 were equal to 2.33 and 6.98×10^3 M^{-1}, respectively (Table I). As was found in the case of insulin and desAsn-desAla-insulin, triMet-insulin also underwent precipitation at average saturations higher than 1 g-atom of zinc/mol of protein.

Binding of Zinc by Desoctapeptide-Insulin at pH 8.0. Unlike native insulin, desoctapeptide-insulin showed homogeneous binding of zinc (Figure 3). A stoichiometry of 1.71 g-atoms of zinc/mol of protein and an apparent association constant of 3.81×10^4 M^{-1} were estimated. Protein solubility was observed over a wider range of zinc concentration than in the case of insulin.

Circular Dichroism Studies. The circular dichroic spectrum of insulin under various experimental conditions is given in Figure 4. At pH 8.0, 5×10^{-4} M zinc-free insulin showed negative minima at 208, 222, and 273 nm and spectral fine structure was represented by shoulders at 256, 262, and 283 nm. Addition of 2.5×10^{-4} M $ZnSO_4$ induced a decrease in the intensity of the dichroic minimum at 208 nm and an increase at 273 nm, with a small bathochromic shift to 275 nm in the latter and conservation of the pattern of fine structure in the 250–300-nm region of the spectrum. In zinc-free insulin, a 200-fold dilution brought about a decrease in the troughs at 222 and 273 nm. The addition of $ZnSO_4$ to the dilute insulin solution (2.5×10^{-6} M) at a final concentration of 1.12×10^{-6} M also decreased the minimum at 208 nm with no effect at 222 nm.

The effect of pH on the CD of zinc-free insulin is given in Figure 5. The spectrum at pH 2.0 was virtually superimposable to the one at pH 8.0. At pH 11.0, the CD of insulin underwent substantial changes characterized by the loss of structure in the 250–300-nm range where the minimum at 273 nm was reduced to a small shoulder superimposed on a steep and almost featureless curve.

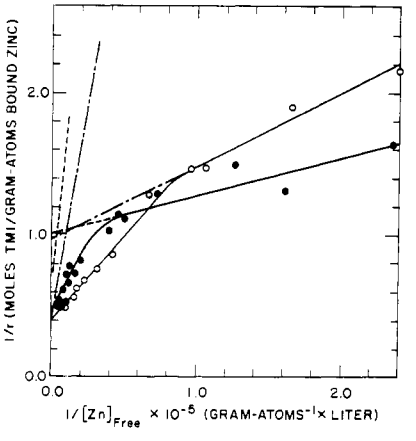

FIGURE 2: Double reciprocal plots of the binding of zinc by desAsn-desAla-insulin (●) and triMet-insulin (○) at a concentration of insulin derivatives of 0.5 mM in 0.169 M Tris buffer at pH 8.0, $\mu = 0.1$. The dashed lines represent the linear least-squares fits of the independent contributions of the two classes of binding sites: (- - -) desAsn-desAla-insulin and (– – –) triMet-insulin.

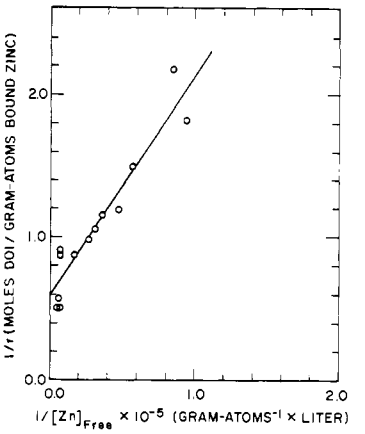

FIGURE 3: Double reciprocal plot of the binding of zinc by desoctapeptide-insulin at 0.5 mM concentration in 0.169 M Tris at pH 8.0.

A miscellany of metal ions other than zinc were also studied with regard to their effect on the CD of insulin as shown in Figure 6. Thus, Fe^{2+}, Cu^{2+}, Pb^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, and Ni^{2+} increased to various extents the magnitude of the minimum at 275 nm. Addition of Fe^{3+} and Mg^{2+} ions did not alter significantly the CD spectra of insulin. The CD of 5×10^{-4} M desoctapeptide-insulin at pH 8.0 is shown in Figure 7. In the absence of metal ions, the minima at 222 and 273 nm and the fine structure in the 250–300-nm range were significantly decreased as compared with insulin. Zinc ions did not perturb the CD spectra of desoctapeptide-insulin.

DesAsn-desAla-insulin exhibited a dichroic spectrum (Figure 8) similar to that of desoctapeptide-insulin. Under the same experimental conditions of pH and protein concentration, it showed in the absence of zinc a decrease in the

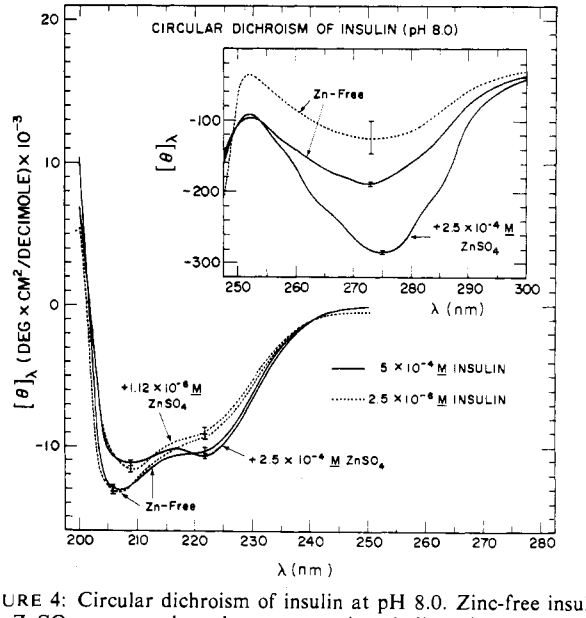

FIGURE 4: Circular dichroism of insulin at pH 8.0. Zinc-free insulin and $ZnSO_4$ were used at the concentrations indicated in the graph. Vertical bars represent the noise level.

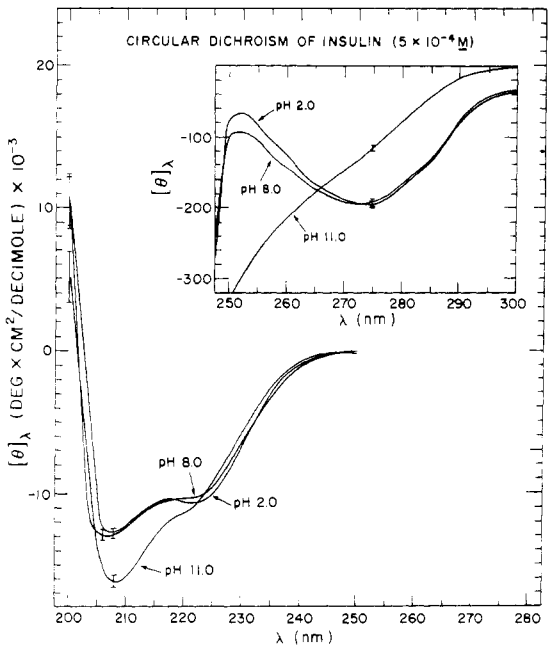

FIGURE 5: Effect of pH on the circular dichroism of insulin. Concentration of zinc-free insulin was 5×10^{-4} M. Vertical bars represent the noise level.

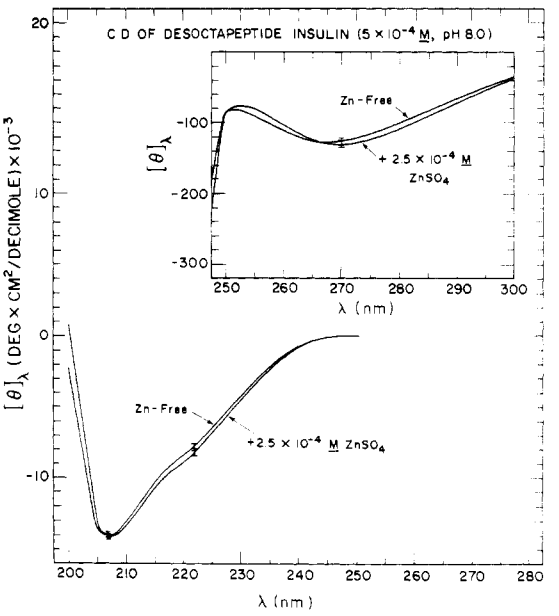

FIGURE 7: Circular dichroism of desoctapeptide-insulin. Protein and $ZnSO_4$ concentrations are indicated in the graph. Vertical bars represent the noise level.

minima at 222 and 273 nm, with regard to insulin, and the fine structure in the 250–300 nm range was markedly diminished. Zinc ions produced a small but significant attenuation of the 208-nm band. CD spectral measurements on 5×10^{-4} M triMet-insulin at pH 8.0 resembled that of native insulin. Although a small decrease in the magnitude of the minima at 222 and 273 nm was observed, the fine structure in the 250–300-nm region was still present (Figure 9). Interaction with 2.5×10^{-4} M $ZnSO_4$ resulted in a decrease of the 208-nm minimum and an increase in the negative band at 273 nm (Figure 9).

Equilibrium Sedimentation Studies. The dependence of the weight-average molecular weight of zinc-free insulin on protein concentration is shown in Figure 10. In the same figure, the continuous line represents the best fit obtained with a monomer–dimer–tetramer–hexamer model of aggregation using eq 5 and the assumption of a monomer molecular weight of 5800. The corresponding association constants are given in Table II. All the other models tested, namely aggregation schemes involving all the intermediate steps of a monomer associating up to a hexamer species (eq 4) and a monomer–dimer–tetramer mechanism gave either negative values for some of the constants or fits less satisfactory than that obtained with the monomer–dimer–tetramer–hexamer scheme.

Data from sedimentation equilibria experiments per-

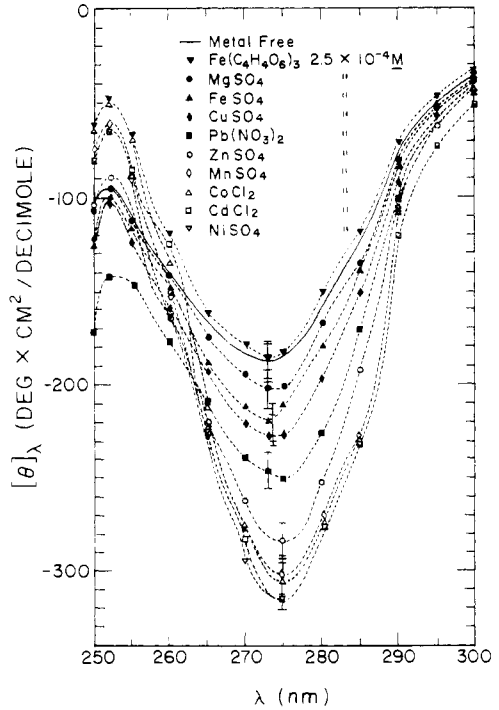

FIGURE 6: Metal ion effects on the circular dichroism of insulin. Experiments were performed at 5×10^{-4} M zinc-free insulin at pH 8.0. The noise level is represented by vertical bars.

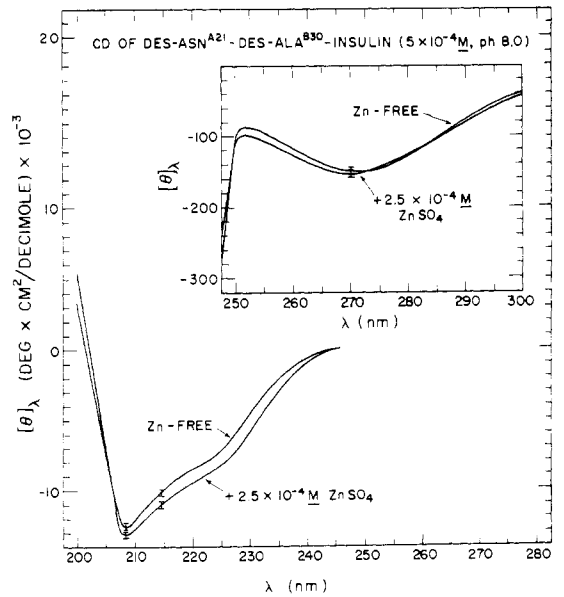

FIGURE 8: Circular dichroism of desAsn-desAla-insulin. Experimental conditions were similar to those in Figure 4.

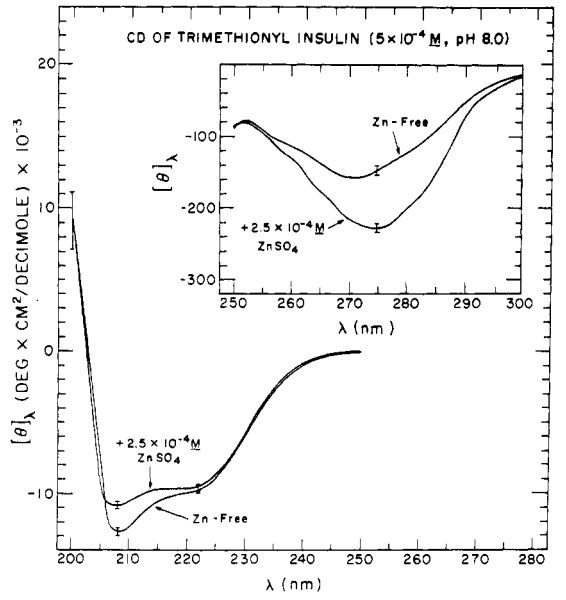

FIGURE 9: Circular dichroism of triMet-insulin. The experimental conditions were similar to those in Figure 4.

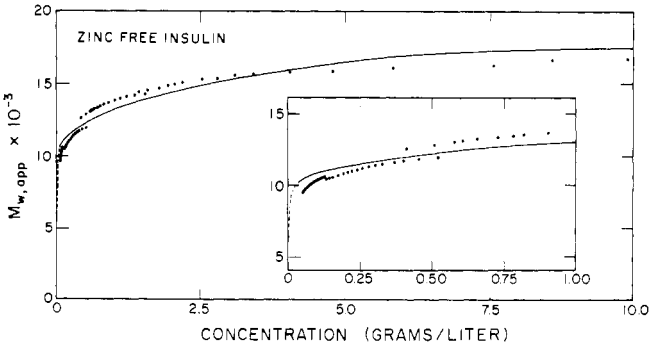

FIGURE 10: Apparent weight-average molecular weight of zinc-free insulin as a function of protein concentration. Tris-HCl buffer (0.169 M) (pH 8.0), ionic strength 0.1. was used. Temperature, 23°. The dots are the experimental points and the continuous line is the curve calculated for a monomer–dimer–tetramer–hexamer model of aggregation using the association constants listed in Table II.

formed at several initial concentrations of zinc-free desoctapeptide-insulin gave straight lines when plotted as log c *vs.* r^2. The molecular weight of this derivative of insulin was calculated from the slope of these plots and found to be 5300, in reasonable agreement with the values obtained from the amino acid composition (4822).

The results of similar experiments with zinc-free desAsn-desAla-insulin and triMet-insulin are shown in Figure 11. Assuming molecular weights of 5500 and 6100 for desAsn-desAla-insulin and triMet-insulin, respectively, the model fitting best the data on these two derivatives was found to be a monomer–dimer–trimer–tetramer mechanism in both cases. The values for the corresponding association constants are given in Table II.

Discussion

The equilibrium dialysis experiments on zinc binding by insulin reported in this paper are in agreement with previous workers (Cunningham *et al.*, 1955; Summerell *et al.*, 1965) in that at pH values around 8.0, insulin exhibited heterogeneous binding which indicates the presence of several binding sites. When the data were analyzed by the reciprocal plot of Klotz (1953), two types of sites with different affinities and stoichiometries were indicated. At pH 8.0 the site with the strongest affinity for zinc (site 1) exhibited a stoichiometry of binding approaching 1 g-atom of zinc/monomer while the weakest binding site (site 2) had a stoichiometry approaching 2 g-atoms/monomer. Thus at pH 8.0, insulin is capable of binding a total of 3 g-atoms of zinc/monomer. These results show that the specific binding of zinc as found in the X-ray studies of 2 zinc insulin (Adams *et al.*, 1969; Blundell *et al.*, 1971) is a feature of this particular crystalline form and does not represent the total capacity of the molecule to bind zinc ions nor the only manner in which zinc ions can be bound in other experimental conditions. These conclusions are supported by recent studies of Grant *et al.* (1972) which appeared after the present work was completed. Grant *et al.* (1972) compared the zinc ion binding of porcine insulin with porcine proinsulin at pH 7.0 and also determined the effect of zinc ion on the aggregation of the molecules. Low concentrations of zinc ions, where approximately 0.3 g-atom/monomer is bound, promoted the specific formation of hexameric species for both molecules. Some of the binding studies of Grant *et al.* (1972) were conducted at concentrations of zinc ions somewhat lower than those reported here. They measured zinc ion binding at concentrations which yielded ratios of free to bound ions of 0.01–0.1 free:1 bound whereas most of our measurements as well as some of those of Grant *et al.* (1972) were conducted in concentrations which yielded ratios of 0.1–10 free:1 bound. From the data for insulin involving ratios of free to bound zinc ions of less than 0.1, Grant *et al.* (1972) calculated an apparent association constant of 1.9×10^6 M^{-1} with a capacity for zinc ion of 0.284 g-atom/monomer. Our data did not reveal this fractional site. When Grant *et al.* (1972) used concentrations which yielded ratios of free to bound zinc ions greater than 0.1, their results for pH 7.0 indicated that the insulin bound a total of 1.0 g-atom/monomer.

At pH 8.0, desAsn-desAla-insulin and triMet-insulin exhibit a pattern of zinc binding that is very similar to that of insulin. Indeed from the binding data alone it is difficult to distinguish between the three compounds. This has some implications with regard to the functional role of zinc in the expression of the hormonal activity of insulin. DesAsn-desAla-insulin exhibits about 5% (Slobin and Carpenter, 1963a) and triMet-insulin has less than 50% (Levy and Carpenter, 1966,1967) of the hormonal activity of insulin. Therefore, the hormonal active site cannot be made up exclusively of the zinc binding sites. The X-ray crystallographic studies have shown the participation of histidine-B10 in the binding of zinc in the crystal (Adams *et al.*, 1969; Blundell *et al.*, 1971). Probably this same histidine residue is implicated in the binding of zinc by the titration experiments of Tanford and Epstein (1954a,b). The absence of histidine-B10 in the insulin of some species (Dayhoff, 1969) eliminates the binding of Zn^{2+} to this residue as a necessary prerequisite for hormonal activity. Further, the variety of metals that can yield crystalline insulins (Scott, 1934; Schlichtkrull, 1956) or can associate with insulin in solution, as determined by circular dichroic effects, indicates a lack of specificity for Zn^{2+} at the metal binding sites. If a zinc–insulin complex is needed for hormonal activity, it is unlikely that the hormone would show a lack of specificity in metal binding. Finally, although the actual free zinc ion concentration in serum is unknown, it is probably considerably less than 5×10^{-5} M, calculated from the

TABLE II: Aggregation of Zinc-Free Insulin and Derivatives.

Derivative	Mode of Aggregation	Aggregation Constants $\times 10^{-3}$ K_{12} (M^{-1})	K_{24} or K_{23} (M^{-1})	K_{46} or K_{34} (M^{-1})
Insulin	$0 \rightarrow 00 \rightarrow \begin{matrix} 00 \rightarrow 000 \\ 00 \rightarrow 000 \end{matrix}$	2.2×10^2	0.036	0.22
DesAla-desAsn-insulin	$0 \rightarrow 00 \rightarrow 000 \rightarrow 0000$	1.74	1.11	0.03
TriMet-insulin	$0 \rightarrow 00 \rightarrow 000 \rightarrow 0000$	4.8	1.23	2.0
Desoctapeptide-insulin	$0 \not\rightarrow 00$			

total zinc concentration of serum, owing to binding of zinc ions by serum proteins. If this is so, then one can calculate from the association constant for the zinc-insulin complex that only a small fraction of the total insulin in serum could be complexed with zinc. Consideration of all of these points makes it highly unlikely that a zinc-insulin complex is necessary for hormonal activity.

Although the above discussion casts doubt on a role for zinc in the hormonal action of insulin, it does not eliminate a biological role for the zinc-insulin complex. It has been known for a number of years that the β cells of islet tissue contain a relatively large proportion of zinc (Okomato, 1949,1955). Electron micrographs have shown that in some instances the granules of β cells contain crystalline inclusions similar in properties to the 2 zinc insulin crystals (Howell *et al.*, 1969). Thus, zinc is implicated in the storage of insulin in the pancreas. The theory of Maske (1957) for the control of insulin storage and release through mediation of a zinc-insulin complex is certainly viable in terms of the association constants reported in this paper and has received further detailed analysis by Goldman (1971).

The dependence of the weight-average molecular weight of zinc-free insulin at pH 8.0 on protein concentration (Figure 10) indicates the ability of this hormone to aggregate. The use of absorption optics on the centrifuge made it possible to follow this aggregation in dilute solution where there was a substantial portion of monomer present in the mixture. In agreement with the previous data of Jeffrey and Coates (1966) obtained on zinc-free insulin at pH 2.0, a

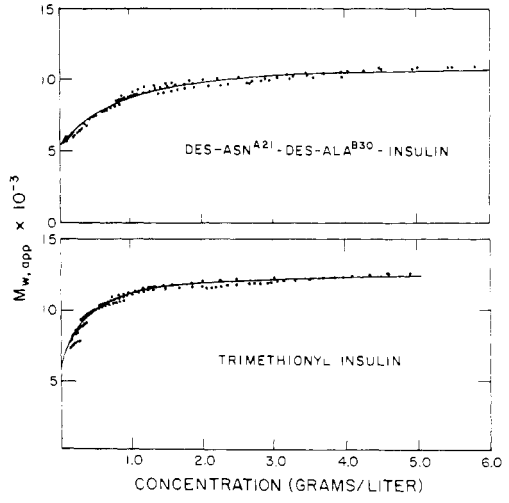

FIGURE 11: Apparent weight-average molecular weight of desAsn-desAla-insulin (upper) and triMet-insulin (lower) as a function of concentration. Experimental conditions were similar to those listed in Figure 10.

mode of aggregation at pH 8.0 which involved monomer-dimer-tetramer-hexamer was found to describe the polymerization better than a mechanism involving the addition of one unit at a time. The equilibrium constants corresponding to each step are shown in Table II and were used to calculate the theoretical curve shown in Figure 10. The agreement between the curve and the experimental points is quite good even though assumptions of ideality and no charge effects make the computed association constants "apparent" rather than true thermodynamic parameters. Perhaps the most significant point to be derived from these calculations is the fact that at pH 8.0 dimer formation (in the absence of zinc ions) is much stronger than tetramer or hexamer formation. The same observation was made by Jeffrey and Coates (1966) at pH 2.0. However, our calculated value for $K_{12} = 2.22 \times 10^5$ M^{-1} at pH 8.0 is somewhat higher than the value calculated by Jeffrey and Coates of $K_{12} = 1.02 \times 10^4$ M^{-1} at pH 2.0. Since the present work was completed two other studies on the aggregation of insulin have appeared. Lord *et al.* (1973) studied the aggregation at pH 2.0 by following changes in absorption spectra in the aromatic region and found a value of 4×10^4 M^{-1} for the formation of dimer—a value somewhat higher than that of Jeffrey and Coates but still lower than that observed here at pH 8.0. Pekar and Frank (1972) compared the self-association of insulin and proinsulin at pH 7.0. Using as their model of aggregation a system of monomer-dimer-hexamer-*n*-mer, they calculated an association constant of $K_{12} = 1.4 \times 10^5$ M^{-1} at pH 7.0 for dimer formation of insulin—a value in good agreement with our calculations even though we used a slightly different model and pH.

Crystallographic studies of 2 Zn-insulin (Adams *et al.*, 1969; Blundell *et al.*, 1971) show that the crystalline form is made up of a hexamer composed of a trimer of dimers. Zinc ions are not involved in dimer formation but are involved in the association of dimers to give the hexamer. Association of monomers to dimers involves the C-terminal portions of the B chain and results in the formation of an antiparallel β-pleated sheet between residues B23-28 of each of the two monomers. That dimer formation is a necessary prerequisite for higher aggregation is indicated by the relative magnitude of the association constants reported here for insulin and also by the fact that desoctapeptide-insulin, which is missing the residues involved in dimer formation, does not aggregate. Although both desAsn-desAla-insulin and triMet-insulin show a tendency to aggregate in zinc-free solution at pH 8.0, the aggregation constants for dimer formation are smaller than that for insulin by a factor of 100 (Table II). Consequently, in these derivatives there must be some perturbation of the C-terminal portion of the B chain which prevents the formation of the β struc-

ture associated with dimerization in insulin. Changes in CD spectrum of these derivatives support this interpretation. It should be recalled that desoctapeptide-insulin (Young and Carpenter, 1961) and desAsn-desAla-insulin (Slobin and Carpenter, 1963a) exhibit very low hormonal activity while desAla^{B30}-insulin possesses full activity (Slobin and Carpenter, 1963b) and crystallizes identically to the native hormone (Steiner and Clark, 1968). These latter two observations indicate a hormonal role for the C-terminal asparagine on the A chain which can be correlated with a structural role in maintaining the conformation of the C-terminal residues of the B chain. It is tempting to speculate that dimer formation may be a prerequisite to the expression of hormonal activity. However, there are several factors that argue against this idea: (1) triMet-insulin possesses about 50% of the hormonal activity of insulin (Levy and Carpenter, 1966) but as shown here has a much decreased ability to form dimers; (2) tetra(nitrotyrosine)-insulin (Boesel and Carpenter, 1972) shows no tendency to aggregate at pH 7.4 but possesses 60–70% of the hormonal activity; and (3) from the aggregation constants presented in this paper as well as those of Pekar and Frank (1972) it can be calculated that, at the concentration in which insulin occurs in the blood, a very small fraction is in the form of the dimer (Goldman, 1971; Pekar and Frank, 1972). These arguments are not to be construed as indicating that the conformation of the C-terminal end of the B chain is not important for hormonal activity but rather that the expression of this conformation through the ability to form the specific dimer interactions that are exhibited by native insulin is not an essential (although frequently prevalent) property of biologically active molecules.

As has been reported by various investigators (Grosjean and Tari, 1964; Beychok, 1965, 1966; Carpenter and Hayes, 1966; Goldman and Carpenter, 1967,1969b; Mercola *et al.*, 1967; Menendez and Herskovits, 1970; Ettinger and Timasheff, 1971; Brugman and Arquilla, 1973), the optical rotatory dispersion and CD spectra of insulin show main cotton effects centered at 192, 208, and 222 nm, and a minor band at 273 nm (Figures 4 and 5).

273-nm Band. When Beychok (1965,1966) reported the presence of a negative ellipticity band at 273 nm, he attributed the band to a strained disulfide bridge rather than to aromatic residues primarily because it did not undergo a red shift at alkaline pH. However, the facts that the band was missing or attenuated in desoctapeptide-insulin and desAsn-desAla-insulin (Carpenter and Hayes, 1966; Brugman and Arquilla, 1973) (Figures 7 and 8) and that it was concentration dependent (Goldman and Carpenter, 1967; Morris *et al.*, 1968) (Figure 4) support the assignment of the band to aromatic residues (tyrosine and phenylalanine) which exhibit optical activity as a function of aggregation of the molecule. Attenuation of the band at 273 nm is correlated with conditions (dilution, Figure 4; high pH, Figure 5) which bring about disaggregation (Fredericq, 1953); while a strengthening of the band is associated with conditions (addition of metal ions, Figures 4 and 6) which enhance aggregation (Goldman, 1971). A number of the metal ions which strengthen the band at 273 nm (Figure 6) have been shown by Schlichtkrull (1956) to be able to replace zinc ions in the formation of crystalline insulin.

The ellipticity band centered at 273 nm is complex in that there are shoulders at 256, 262, and 283 nm which are indicative of contributions from phenylalanine as well as tyrosine (Figure 4). The crystallographic structure of insulin (Adams *et al.*, 1969; Blundell *et al.*, 1971) allows tentative assignment of particular aromatic chromaphores to the observed CD bands. In the insulin dimer, tyrosines-B26 and phenylalanine-B24 of each of the participating monomers take part in the formation of an "aromatic" cage whose twofold symmetry is violated by the adjoining phenylalanine-B25. All of these residues are part of the B23–28 stretch involved in the intermonomer antiparallel β structure. In agreement with the assignment of the band at 273 nm to the formation of a dimer between the B chain of two molecules is the fact that desoctapeptide-insulin, which lacks the residues involved in dimer formation as well as those contributing to the optical activity, has a greatly diminished 273-nm band. Although the residues involved in dimer formation are present in desAsn-desAla-insulin, this molecule as well as triMet-insulin exhibits a much lower tendency to aggregate which is reflected in an attenuation of the band at 273 nm. The CD bands at 273 and 283 nm can be assigned to tyrosine-B26 while those at 256 and 262 nm may arise from phenylalanine-B24 and/or -B25. In this regard, multiple vibronic bands have been observed in both tyrosine (Horwitz *et al.*, 1970; Strickland *et al.*, 1970) and phenylalanine (Horwitz *et al.*, 1969; Simmons *et al.*, 1969) chromophores. Tyrosine-B16 has also been reported to be involved in the dimerization of insulin (Blundell *et al.*, 1971) and may also be contributing to the aromatic CD bands.

222-nm Band. This band can be assigned in large part to β structure (Quadrifoglio and Urry, 1968). The β structure is a predominant feature of the dimer. In agreement with this assignment the band is attenuated by dilution and in those derivatives which either do not aggregate (desoctapeptide-insulin, Figure 7) or aggregate in a manner different from insulin (desAla-desAsn-insulin, Figure 8).

208-nm Band. This band may be attributed largely to α helix (Ettinger and Timasheff, 1971) which is a characteristic feature of the monomer involving residues B10–19 and additional contributions from A2–6 and A13–19 which are arranged in distorted helices (Blundell *et al.*, 1971). In agreement with this assignment the band is not affected by dilution nor by conversion of insulin to desoctapeptide-insulin which does not aggregate or to desAsn-desAla-insulin which aggregates in a different manner from insulin. The band is attenuated by the addition of zinc ions (or other divalent metal ions) (Figure 4). The crystalline structure shows that a zinc ion is coordinated with histidine-B10. This residue is a component of the principal α helix of the molecule (B10–19) and perturbation due to zinc binding is not unexpected.

In conclusion, the fact that the main features of the CD spectra and the results from the equilibrium sedimentation data on these derivatives of insulin with variable hormonal activities can be rationalized in terms of the reported crystallographic structure of insulin strongly suggests that the dissolved and crystalline forms of the hormone are largely similar.

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