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Optical Activity of Insulin. II. Effect of Nonaqueous Solvents*

Murray J. Ettinger† and Serge N. Timasheff‡

With the Technical Assistance of Mrs. G. D. Strycharz

ABSTRACT: A detailed study has been made of the effects of acidic methanol and 2-chloroethanol on the optical activity of insulin. Changes in circular dichroism spectra have been correlated with changes in the optical rotatory dispersion a_0 and b_0 parameters by a mathematical analysis developed in these studies. The maximal effect on the a_0 parameter occurred at 70% 2-chloroethanol and 80% acidic methanol. On the other hand, the $-b_0$ parameter did not change in either solvent over the concentration range in which $-a_0$ attained its maximal value, but it did increase to a small extent at higher concentrations. Marked changes in the circular dichroism spectrum of insulin resulted from exposure to these solvents. The effect of either solvent in the near-ultraviolet was a large reduction in intensity without band shifting and an accentuation of fine structure. In the far-ultraviolet region, there was a large increase at 209 nm, a virtual disappearance of a distinct extremum at 222 nm, and an increase in intensity with a blue shift of the 196-nm extremum. Using a mathematical analysis aimed at improved resolution of overlaps of various transitions, the far-ultraviolet spectra of insulin in 0, 70, and 99% methanol were resolved into Gaussian zones, each of which contains one or a small number of actual transitions. The contributions of the various zones to the a_0 and b_0 parameters were evaluated. It was found that experimental a_0 and b_0 values represent the sum of larger contributions which partially cancel each other and that interpretations based on changes observed at individual wavelengths in the circular dichroism spectra would be hazardous. Far-ultraviolet circular dichroism spectral changes between 0 and 80% methanol were attributed primarily to solvent, nonconformational effects on α-helix and β-structure transitions, and to a small increase in unordered polypeptide conformation. Higher concentrations resulted in a small increase in α-helix content. Use of model compounds showed that the changes recorded in the near-ultraviolet circular dichroism spectra were related primarily to changes in side-chain conformation rather than to solvent nonconformational effects. The changes in self-association were correlated with changes recorded in both the near- and far-ultraviolet circular dichroism spectra of insulin in methanol.

Studies on the effects of nonaqueous solvents on the conformation of proteins have shown that a number of nonpolar, weakly hydrogen bonding organic solvents frequently lead to the denaturation of proteins, the end product being usually enriched with respect to α-helical content as evidenced by optical rotatory dispersion, circular dichroism, and infrared spectroscopy (Doty, 1960; Weber and Tanford, 1959; Tanford *et al.*, 1960; Urnes and Doty, 1961; Herskovits and Mescanti, 1965; Timasheff and Inoue, 1968; Timasheff, 1970). Concomitantly, it has been shown in several cases that this

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† This work was done during the tenure of a senior postdoctoral research associateship of the Agricultural Research Service, U. S. Department of Agriculture. Present address: Department of Biochemistry, State University of New York, Buffalo, N. Y. 14214.

‡ To whom correspondence should be addressed.

transformation of the protein structure is paralleled by a progressively increasing interaction with the organic solvent component (Timasheff and Inoue, 1968; Inoue and Timasheff, 1968; Timasheff, 1970). This has been attributed to increased solvent penetrability as the protein unfolds with the replacement of internal, interresidue hydrophobic interactions by external ones between amino acid residues and molecules of the organic solvent.

While the solvent-binding behavior of insulin in the water–2-chloroethanol system is very similar to that of other proteins (Timasheff and Inoue, 1968), the reported effect on the Moffitt–Yang optical rotatory dispersion parameters, a_0 and b_0, induced by dissolving insulin in pure 2-chloroethanol or acidic methanol (Doty, 1960; Urnes and Doty, 1961; Herskovits and Mescanti, 1965) has led to the conclusion that, in this protein, these solvents induce little or no change in conformation. Since the reported values were only for aqueous media or pure organic solvents, it seemed of interest to investigate the entire region of intermediate solvent concentrations. The method emphasized in this study was circular dichroism, since, because of the discrete narrow bands associated with various transitions, it affords a much better way than optical rotatory dispersion of separating the spectral components related to various peptide and side-chain chromophore contributions. In practice, the overlap between the circular dichroism bands in a protein spectrum is still large, with the result that changes in position or intensity of single transitions may be obscured by changes in other closely located transitions. In order to unravel this difficulty, at least in part, a treatment has been developed in which the circular dichroism spectrum is resolved mathematically into discrete Gaussian zones, each of which contains contributions from only one or a small number of transitions. This analysis has been applied to the insulin system, and the contributions of each zone to the Moffitt–Yang a_0 and b_0 parameters have been calculated. The results indicate that the individual contributions are considerably larger than their experimentally observed algebraic sums. The use of this zonal analysis, furthermore, greatly facilitates the assessment of changes in intensity and position of individual transitions and the separation between nonconformational solvent effects on the spectra and changes related to protein conformation of self-association. It is the purpose of this paper to describe the results of such circular dichroism and optical rotatory dispersion studies on insulin in the presence of organic solvents and their analysis by means of the zonal resolution.

Materials and Methods

The same insulin preparation as in our previous studies was utilized here (Ettinger and Timasheff, 1971). *N*-Acetyl-L-tyrosineamide, L-phenylalanine methyl ester, L-cystine, and poly-L-glutamic acid were all purchased from Mann Research Labs[1] and used without further purification. Methanol (Baker analyzed reagent grade) and 2-chloroethanol (Eastman Chemical Corp.) were redistilled prior to use through a fractionating column with a calcium chloride water trap.

Concentrations of insulin were determined from spectrophotometric measurements with a Cary Model 16 spectrophotometer using an absorptivity of 1.04 l./g at 277 nm (Herskovits, 1965). Insulin concentrations of 5 g/l. were used for optical rotatory dispersion experiments and 1 g/l. for circular spectroscopy. Absorbance measurements of aqueous insulin solutions were used to correct the weights of insulin in 2-chloroethanol or acidic methanol for water content. Water content of poly-L-glutamic acid was determined by Kjeldahl nitrogen determinations, of *N*-acetyl-L-tyrosineamide and L-cystine by vacuum drying at 70°, and of L-phenylalanine methyl ester by drying over phosphorus pentoxide. The circular dichroism spectra of these compounds were recorded under the following conditions: poly-L-glutamic acid, 0.8 mg/ml at pH 4.3; *N*-acetyl-L-tyrosineamide, 0.35 mg/ml at pH 2.05; L-cystine, 0.06 mg/ml at pH 1.7; L-phenylalanine methyl ester, 2 mg/ml at pH 2.0. Concentrations of methanol and 2-chloroethanol are reported as per cent volume of solvent in a total volume of the water–solvent mixture. An amount of HCl equal to that necessary to bring aqueous solutions to pH 3 was added to the solutions of 2-chloroethanol. Methanol–0.01 M HCl mixtures were diluted to appropriate concentrations at methanol concentrations exceeding 50%; with water–methanol mixtures containing less than 50% methanol, it was necessary to utilize a concentration of HCl equal to that required to solubilize insulin in aqueous solution at pH 3.

Optical rotatory dispersion and circular dichroism spectra were recorded as previously described (Ettinger and Timasheff, 1971). Separate base lines were determined for each solvent mixture. Spectra were recorded immediately following the preparation of solutions of insulin in these solvents. Lorentz factors were calculated by the Sellmeir approximation (Urnes and Doty, 1961) from measurements of the refractive index at 589.3 and 435.8 nm with a Bausch and Lomb refractometer at 27° for each methanol or 2-chloroethanol concentration used.

A Wang Model 370 calculator, equipped with teletype and three card readers, was utilized with the programs for the zonal analysis of the circular dichroism curves and molecular weight calculations. To obtain the best resolution of a circular dichroism spectrum into Gaussian zones, the parameters which defined each Gaussian component of the spectrum were varied by trial and error until the sum of the components best fitted the observed spectrum. Each Gaussian component, k, was defined by the parameters of position, band width, and height from the following relationship: $[\theta']_\lambda = [\theta']_\lambda{}^0 \exp[-(\lambda - \lambda_k{}^0)^2/(\Delta_k{}^0)^2]$, where $[\theta']_\lambda$ is the ellipticity at wavelength λ; $[\theta']_k{}^0$ is the ellipticity at the maximum of the Gaussian curve at wavelength $\lambda_k{}^0$. $\Delta_k{}^0$ is one-half the width of the Gaussian component at the height where the intensity equals $(1/e) \times [\theta']_k{}^0$; primes indicate that Lorentz factors were included. Programs[2] were used with the calculator to obtain the sum of the ellipticities from all components at each wavelength from the above parameters. With each set of chosen parameters, the observed and calculated spectra were plotted on the teletype to aid in subsequent choice of Gaussian parameters which could be used to improve the fit. To correlate a Gaussian circular dichroism zone with a_0 and b_0 values, reduced mean rotations were calculated from the molar ellipticities of the zone by application of the Kronig–Kramers transform

$$[m']_\lambda = 2/\pi \int_0^\infty [\theta']_{\lambda'} \frac{\lambda'}{\lambda^2 - (\lambda')^2} d\lambda'$$

[1] Mention of this and other companies or products is for the convenience of the reader and does not constitute an endorsement by the U. S. Department of Agriculture.

[2] All programs used for this analysis were written by Dr. Kirk C. Aune.

where $[m']_\lambda$ is the reduced mean rotation at wavelength λ; $[\theta']_{\lambda'}$ is the molar ellipticity at wavelength λ' (Moffitt and Moscowitz, 1959). The integral was approximated as a sum using a 2-nm wavelength interval since there was no significant difference obtained with the use of a smaller interval.

Weight-average molecular weights were determined by the meniscus depletion method (Yphantis, 1964) using the Spinco Model E analytical ultracentrifuge at 25° with interference optics and a fine-slit mask. Since it was found that 2-chloroethanol or acidic methanol reacted with the commonly used double sector epon-filled centerpiece, a Kel-F double sector centerpiece (Spinco) was utilized. Speeds of 48,000 rpm in aqueous solutions and 40,000 rpm in methanol were determined to be sufficient for meniscus depletion. Insulin solutions were prepared at a concentration of 0.5 g/l. in 0.02 M sodium chloride and adjusted to pH 2.0 with HCl. The solutions were dialyzed against solvents using 23/32 No-Jax tubing (Union Carbide) to match solvent and solution. It was found that, although this dialysis tubing was not permeable to insulin in aqueous solutions, it did permit passage of insulin in acidic methanol. However, no error was observed in methanol at speeds equal to or less than 40,000 rpm from mismatched solvent and solution. Corrections for cell aberrations were determined with solvent in each sector in cells which had not been disassembled subsequent to the solvent–insulin run. Weight-average molecular weights were calculated from the average of three fringe displacements at each comparator distance, x, by an overlapping five-point least-squares analysis to determine the slope of the natural logarithm of the fringe displacement *vs.* r^2 relationship (Yphantis, 1964). A value of 0.72 ml/g was used for the partial specific volume of insulin (Oncley *et al.*, 1952).

Results

The complete solvent composition dependences of the optical rotatory dispersion a_0 and b_0 parameters of insulin in 2-chloroethanol and acidic methanol are shown in Figure 1. In both solvents, the $-a_0$ parameter passes through a maximum at a solvent composition intermediate between 0 and 100%. The value of a_0 increased from -80 to -222 between 0 and 70% 2-chloroethanol. In contrast, there was no significant concomitant change in $-b_0$. Between 70 and 100% chloroethanol, $-a_0$ decreased, *i.e.*, it approached more closely its value in water, while $-b_0$ possibly increased to a small degree. Similar changes in the values of a_0 and b_0 were obtained with insulin in acidic methanol. The maximal $-a_0$ value (reached at 80% methanol) was somewhat greater than that in 2-chloroethanol, the decrease in $-a_0$ at higher organic solvent contents was smaller, and the increase in $-b_0$ was somewhat greater. The effects of ethylene glycol monomethyl ether on the a_0 and b_0 parameters of insulin were similar to those of 2-chloroethanol. These changes in the a_0 and b_0 parameters are in good agreement with the literature values reported for 100% organic solvent (Doty, 1960; Urnes and Doty, 1961; Herskovits and Mescanti, 1965). The larger, but nevertheless relatively small, changes in the a_0 and b_0 values at intermediate solvent concentrations suggested the possibility that acutal changes in individual transitions, of which a_0 and b_0 are a resultant, may have been partially cancelled because of the opposite signs of the bands involved. In order to check this possibility, a detailed circular dichroism study was undertaken.

Striking changes in the circular dichroism spectra of insulin in 2-chloroethanol and acidic methanol were indeed observed.

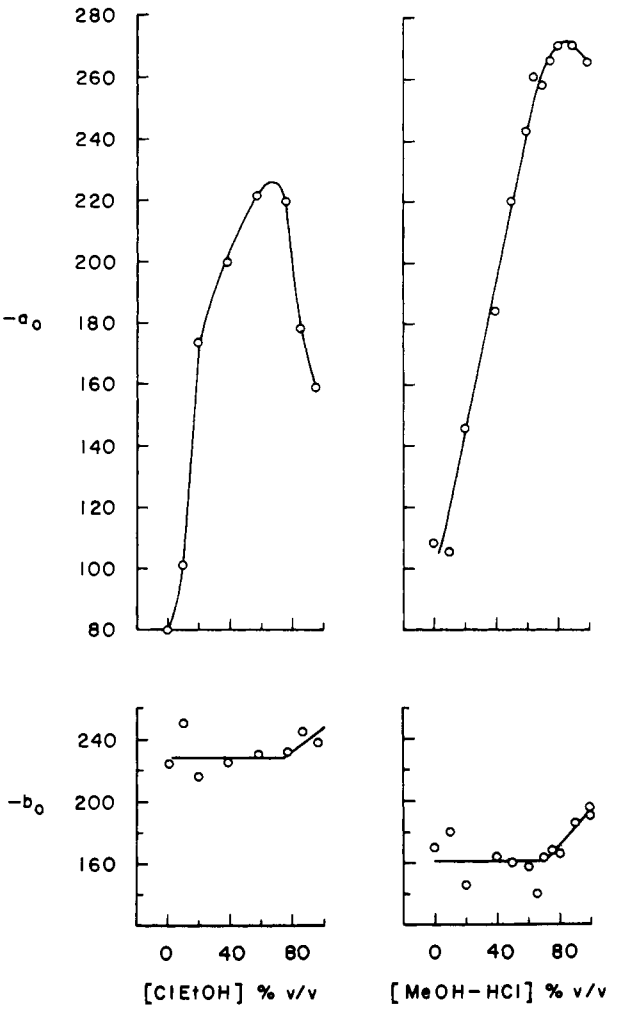

FIGURE 1: Variance of the a_0 and b_0 parameters of insulin with 2-chloroethanol (ClEtOH) and acidic methanol (MeOH–HCl) concentrations. (Averages of duplicate determinations with 2-chloroethanol and five determinations with acidic methanol.)

The spectra obtained in 0, 57, and 95% chloroethanol, between 250 and 300 nm, are shown in Figure 2. These are characterized by a progressive decrease in ellipticity above 257 nm, and a marked accentuation of the fine structure, which is barely apparent in the spectrum obtained in aqueous solutions (Figure 2). The effects of methanol on the near-ultraviolet spectrum were similar to those obtained with chloroethanol, as shown in Figure 3. There appeared to be little or no shifting of band positions in this spectral region with change in solvent composition, as demonstrated by the fact that the difference spectrum calculated from the spectra obtained in water and in the various solvents followed the spectrum of insulin in water. Had the spectrum of insulin been shifted to the red or blue in these solvents, the difference circular dichroism would have contained extrema at positions other than those observed in the original spectrum.

In 57% chloroethanol, the far-ultraviolet circular dichroism spectrum of insulin showed an increase in ellipticity in the vicinity of the 209-nm extremum and a virtual disappearance of a distinct extremum at 222 nm, as shown in Figure 4. A further increase in magnitude at 209 nm was recorded in 95% chloroethanol along with a partial restoration of an extermum at 222 nm. With chloroethanol it was impossible to obtain circular dichroism spectra below 200 nm, because of its ab-

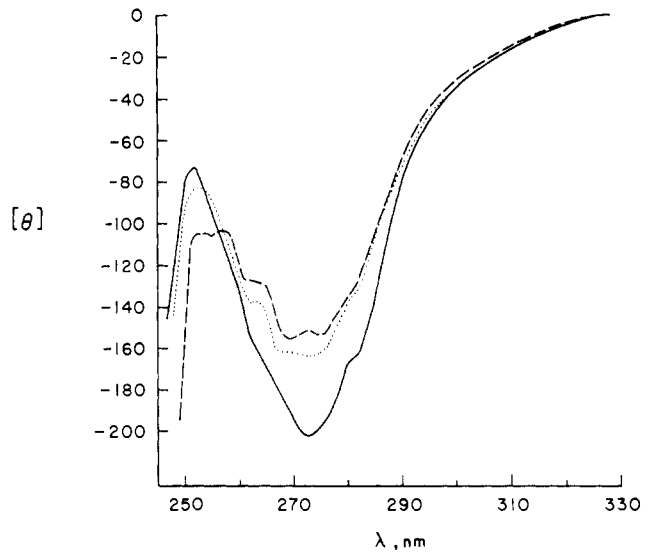

FIGURE 2: Near-ultraviolet circular dichroism spectra of insulin in 2-chloroethanol: 0% (—); 57% (···); 95% (---) (averages of duplicate determinations).

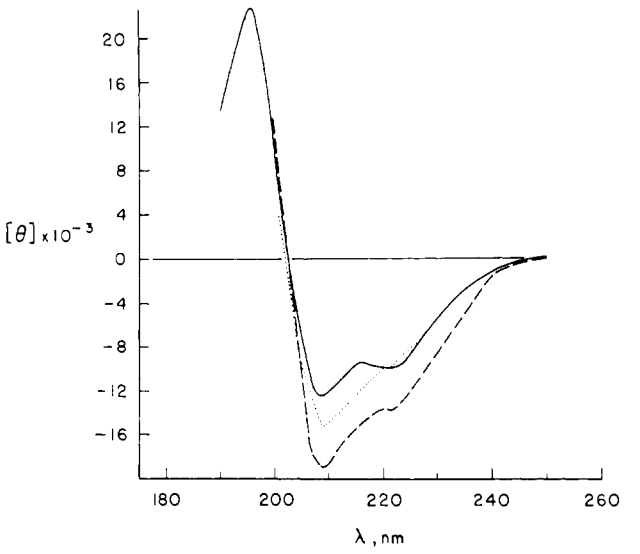

FIGURE 4: Far-ultraviolet circular dichroism spectra of insulin in 2-chloroethanol: 0% (—); 57% (···); 95% (---) (averages of duplicate determinations).

sorption. In acidic methanol, however, it was possible to demonstrate the effect of these solvents on the 196-nm extremum present in aqueous systems. As shown in Figure 5, in 70% methanol the magnitude of the maximum at 196 nm was unaltered, but the peak was blue shifted. In 99% methanol, an increase in the magnitude of the extremum in the 196-nm region was observed.

From the above results, it is evident that significant changes occur in the circular dichroism spectrum of insulin when the medium is gradually altered. It is, however, hazardous to make any specific interpretation of changes observed at single wavelengths in the circular dichroism spectra because of band overlaps. Such overlaps are an important obstacle to interpreting *changes* in circular dichroism spectra, and it must be overcome prior to the analysis of the spectra themselves. With this purpose in mind, the circular dichroism spectra presented above were resolved into distinct zones, and the changes in each of these zones were followed separately as a function of change in solvent composition. The contributions from each zone to changes in the a_0 and b_0 parameters were then calculated to establish the contributions made by the individual zones to the overall changes in optical activity far from the wavelengths of the transitions.

The far-ultraviolet circular dichroism spectra obtained with insulin in 0, 70, and 99% acidic methanol were each resolved into three Gaussian zones centered close to 196, 204, and 222 nm. This was found to be the minimum number of zones required to fit the recorded spectra. It must be emphasized that these zones are mathematically derived, and do not represent

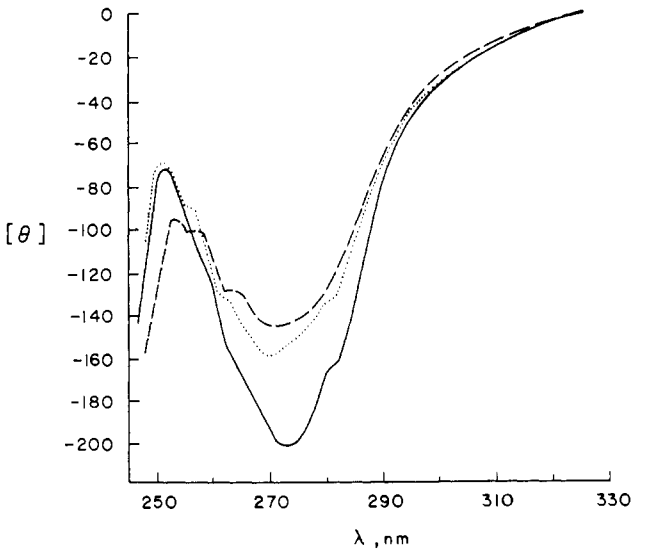

FIGURE 3: Near-ultraviolet circular dichroism spectra of insulin in acidic methanol: 0% (—); 70% (···); 99% (---) (averages of three determinations).

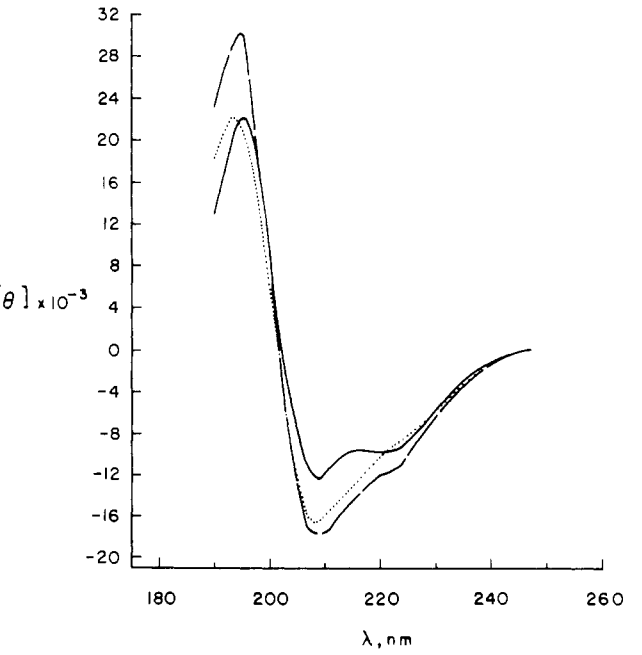

FIGURE 5: Far-ultraviolet circular dichroism spectra of insulin in acidic methanol: 0% (——); 70% (···); 99% (---) (averages of three determinations).

TABLE I: Parameters Defining the Resolved Gaussian Zones of the Spectra of Insulin in Acidic Methanol.[a]

Concn of MeOH (%)	$[\theta']_k^0$	Δ_k^0	λ_k^0
0	23,800	8.7	197.5
	−15,800	8.3	204.0
	−7,000	12.0	222.5
70	25,800	8.7	196.6
	−19,200	9.2	204.0
	−7,100	12.2	222.4
99	31,400	8.7	196.8
	−21,200	8.9	204.0
	−8,800	12.0	222.3

[a] $[\theta']_k^0$ = the molar ellipticity multiplied by the Lorentz factor at the maximum of the Gaussian zone centered at λ_k^0. Δ_k^0 is one-half the width of the zone at height equal to $1/e \times [\theta']_k^0$. The resolutions were obtained by trial and error fits to recorded spectra (Figure 5).

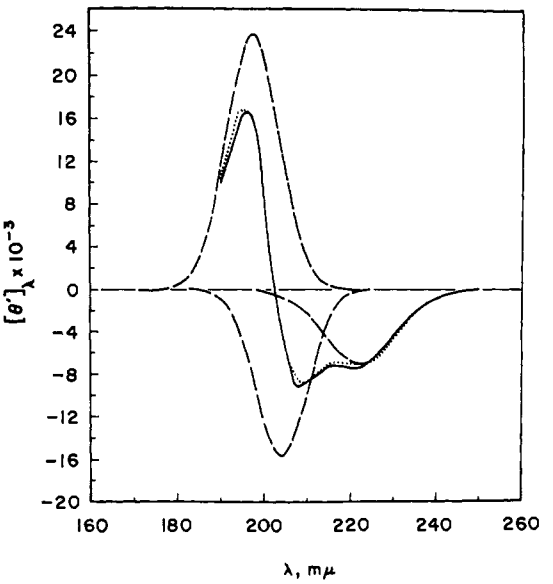

FIGURE 6: Resolution of the far-ultraviolet circular dichroism spectrum of insulin (——) into three Gaussian zones (---) by trial and error fit. Resultant spectrum from the sum of the Gaussian zones (· · ·).

actual transitions. A minimum of eight optical activity transitions could be predicted for the far-ultraviolet circular dichroism spectrum of insulin. A unique fit to eight contributions is impossible, however, as a result of the error involved in recording the initial spectra. The fits obtained in such a manner were excellent, as exemplified by the resolution of the far-ultraviolet circular dichroism spectrum of insulin in aqueous medium, shown in Figure 6. Parameters defining the Gaussian zones (labeled as the 196, 204, and 222 zones) which yielded the best fits to the recorded spectra are listed in Table I. It is evident that addition of methanol brings about large intensity changes within the zones; these partly cancel each other and result in the experimentally observed much smaller changes, shown on Figure 5. The largest changes take place in the 196 and 204 zones which have opposite signs. While, as stated before, these zones as such do not correspond to actual electronic transitions, changes within a zone must reflect changes in transitions contained in that zone. These changes can, therefore, be used in a structural analysis more definitively than the entire spectrum because of the smaller number of transitions to be considered.

In order to establish the contribution made by each zone to the higher wavelength optical rotations, the Kronig–Kramers transform was applied to each one of these zones and to the entire near-ultraviolet circular dichroism spectrum, as a fourth zone (labeled as the 270 zone). The contributions of each zone to the a_0 and b_0 parameters were then calculated for each of the resolved spectra from the reduced mean residue rotations calculated in this manner. The sums of the a_0 and b_0 contributions from the zones resulted in excellent agreement with the experimental values, as shown in Figure 7, where the triangles are the calculated values and the circles are the experimental ones. This demonstrates that the four-zone analysis of the circular dichroism curves did, in fact, account for the optical activity indicated by the a_0 and b_0 parameters.

A detailed analysis of the contribution of each circular dichroism zone to optical rotation is summarized in Figure 7. The 270 zone contributed a very small progressive decrease in $-a_0$ with increasing methanol concentration. The positive a_0 contribution from the 196 zone greatly increased with an increase in methanol concentration, the largest increase occurring between 70 and 99% methanol. A change, similar in magnitude but opposite in sign, occurred concomitantly in the 204 zone. Several inferences can be drawn immediately from these results. First, it can be stated that the changes in a_0 values determined from rotations measured between 350 and 550 nm arise from much larger changes in the a_0 contributions of each circular dichroism zone; these, however, have opposite effects on the resultant $-a_0$ value because of the opposite signs of the transitions. Second, this analysis has identified the predominant changes in the zones leading to the changes observed in a_0 and b_0. One may note that the increase in $-a_0$ from 0 to 70% methanol is associated predominantly with the changes in optical activity within the 204 zone, partly compensated by the contribution from the 196 zone. The decrease in $-a_0$ from 70 to 99% methanol is related to a predominance of the positive contribution from transitions associated with the 196 zone over the sum of the negative contributions of those occurring within the 204 and 222 zones. A similar analysis with 2-chloroethanol demonstrated that the greater decrease in $-a_0$ that was obtained at high concentrations of 2-chloroethanol over that obtained in methanol can be attributed to a smaller increase in the $-a_0$ contribution from the 204 zone in chloroethanol.

In an analogous manner, the resultant lack of variance in $-b_0$ between 0 and 70% methanol was related to the increase in $-b_0$ associated with the 196 zone and compensated for by a decrease in $-b_0$ from the 270 zone and an increase in $+b_0$ from the 204 zone (Figure 7). The increase in $-b_0$ from 70 to 99% methanol was related to increases in the contributions of the 196 and 222 zones which were predominant over the increase in the $+b_0$ contribution from the 204 zone. This zonal analysis, in addition to pointing out the contributions of various spectral regions to a_0 and b_0, has been found to be useful also in the interpretation of the relations between changes in the circular dichroism spectrum of insulin and

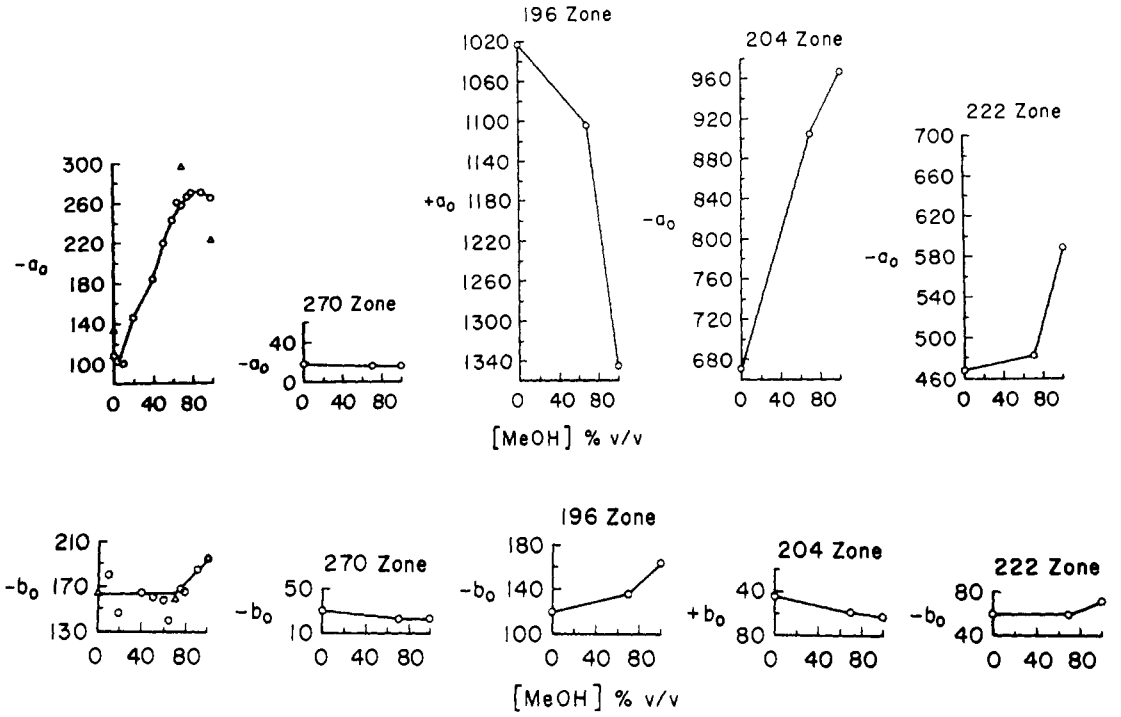

FIGURE 7: Contributions to the a_0 and b_0 values of insulin of the resolved Gaussian zones as a function of acidic methanol concentration, determined from the Kronig–Kramers transform. In the first pair of graphs, the circles are experimental points; the triangles are calculated values, *i.e.*, the sums of the contributions of the individual zones.

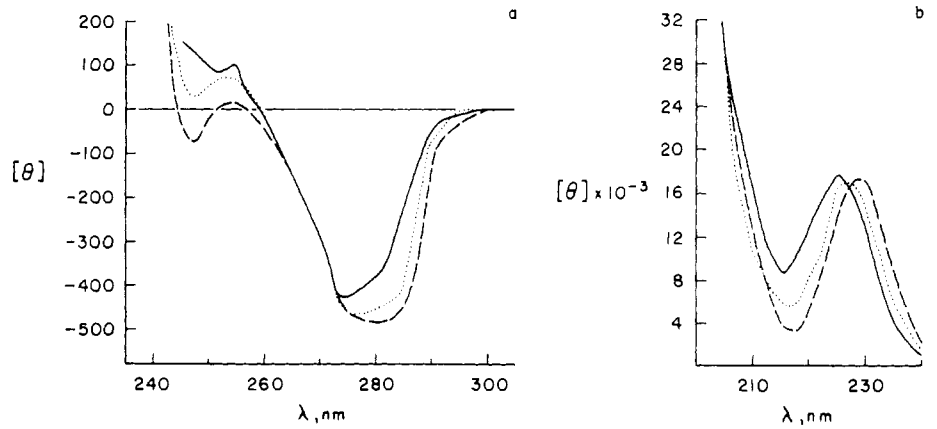

FIGURE 8: Effect of acidic methanol on the circular dichroism spectrum of *N*-acetyltyrosinamide: % acidic methanol; 0 (pH 2.05) (——); 70 (· · ·); 99 (---); averages of duplicate determinations: (a) near-ultraviolet spectra, (b) far-ultraviolet spectra.

conformational changes which occur in this protein when solvent composition is altered.

The reported changes in the optical rotatory dispersion and circular dichroism of insulin induced by addition of the organic solvents can either reflect conformational changes within the protein or they may be the result of nonconformational solvent effects due mostly to changes in polarity or polarizability of the environment of the groups responsible for the various transitions. In order to differentiate between these two possibilities, circular dichroism experiments were carried out in the same solvents on pertinent model compounds both in the near- and far-ultraviolet.

In the near-ultraviolet region, tyrosine residues appear to make the predominant contribution to the circular dichroism spectrum of insulin; phenylalanine and cystine chromophores probably contribute as well (Ettinger and Timasheff, 1971). The spectra of *N*-acetyl-L-tyrosineamide, L-phenylalanine methyl ester, and L-cystine in increasing amounts of methanol are shown in Figures 8 and 9. In general, the changes observed in the near-ultraviolet circular dichroism spectrum of insulin in chloroethanol or methanol differ both qualitatively and quantitatively from changes in the circular dichroism spectra of these amino acid residues in methanol. As the methanol concentration is increased, the near-ultraviolet circular dichroism spectrum of *N*-acetyltyrosineamide was red shifted and increased in intensity as shown in Figure 8a. In addition, the presence of a negative band below 260 nm became more apparent in methanol than in water. Except for a possible relation of this last effect to the effects of methanol on the fine structure of the insulin circular dichroism spectrum, the changes in the tyrosine spectra in methanol appear to be unrelated to those found in insulin, where the intensity was decreased by methanol or chloroethanol addition (Figures 2 and 3). Horwitz *et al.* (1969) have recently resolved the circular dichroism spectrum of phenylalanine in the near-ultraviolet region into

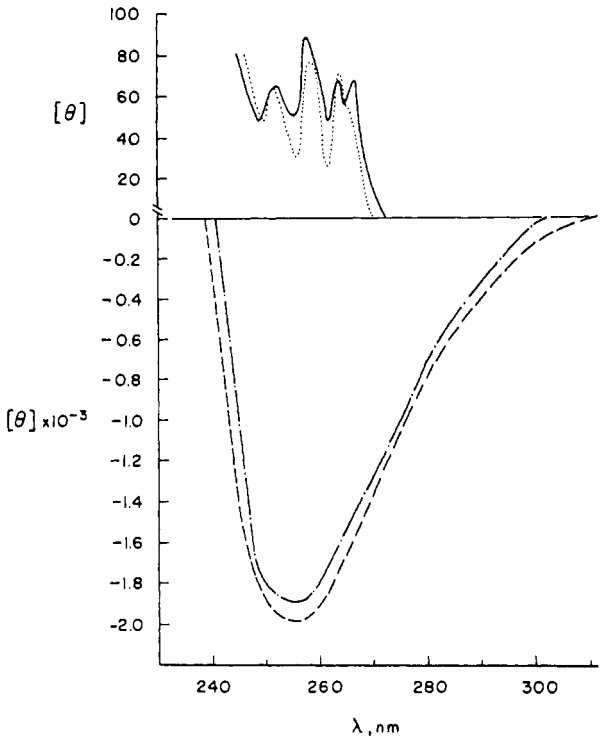

FIGURE 9: Effect of acidic methanol on the circular dichroism spectra of amino acid derivatives (averages of duplicate determinations). Upper spectra: L-phenylalanine methyl ester; in water at pH 2.0 (——) and in 70% acidic methanol (· · ·). Lower spectra: L-cystine in water at pH 1.7 (---); in 70% acidic methanol (– · – · –).

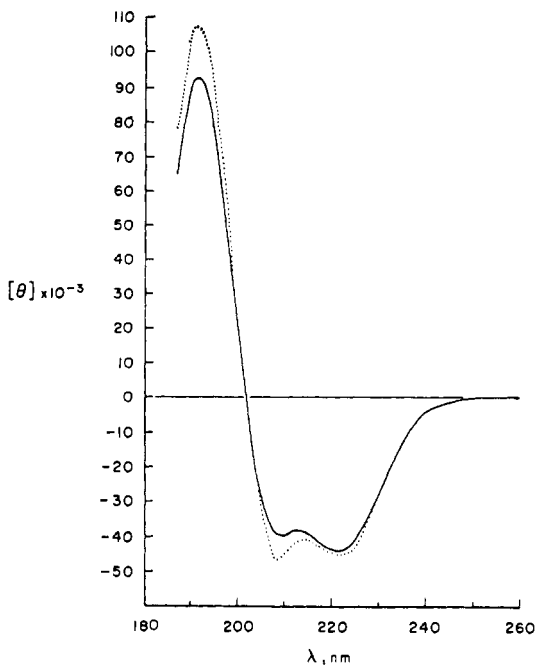

FIGURE 10: Circular dichroism spectra of poly-L-glutamic acid in water at pH 4.3 (——) and in a 70% methanol solution containing an equal amount of HCl as the aqueous solution (· · ·) (averages of duplicate determinations).

at least six overlapping positive and negative contributions and demonstrated that N-substitution of L-phenylalanine methyl ester does not alter its spectrum. The effect on the circular dichroism spectrum of L-phenylalanine methyl ester induced by changing the medium from aqueous to 70% methanol is shown in Figure 9. The principal change in the spectrum between 252 and 270 nm was a general shift to less positive values. Similar effects on optical activity of the phenylalanine residues in insulin could make a contribution to the enhancement of fine structure observed between 260 and 274 nm in chloroethanol or methanol (Figures 2 and 3). The effect of 70% methanol on the optical activity of disulfide bonds is shown in Figure 9; it is seen that the circular dichroism spectrum of L-cystine was slightly reduced in magnitude by transfer to 70% methanol. Derivatives of L-cystine with amino and carboxyl groups blocked are known to give rise to similar circular dichroism spectra as L-cystine, but only with reduced intensities (Beychok, 1966). Thus, although the reduction in the ellipticity of L-cystine in methanol was much smaller than the reduction observed in insulin under identical conditions, it is possible that a nonconformational solvent effect on disulfide bond optical activity might be responsible for at least part of the observed effect of methanol. Taken together, however, the results of the effects of methanol on the circular dichroism spectra of each of these amino acid derivatives suggest that the changes recorded in the near-ultraviolet spectra of insulin in methanol or 2-chloroethanol cannot be accounted for in terms of nonconformational solvent effects. They must, therefore, stem in greatest part from actual conformational changes of the side-chain residues responsible for the spectra.

The question of the contribution of changes in the far-ultraviolet optical activity of side-chain residues due to solvent nonconformational effects to the observed changes in the far-ultraviolet circular dichroism spectrum of insulin is difficult to assess, because the contribution from these residues to this spectral region are not known for the native protein (Ettinger and Timasheff, 1971). The 225-nm extremum exhibited by *N*-acetyltyrosineamide has been attributed to the aromatic moiety of this compound (Beychok and Fasman, 1964; Pflumm and Beychok, 1969). The change in medium from aqueous to increasingly methanolic resulted in a red shift of this band, without any changes in intensity, as shown in Figure 8b. A similar effect on the tyrosine optical activity in insulin could make a small contribution to the ellipticity increases at 208-nm observed with insulin in methanol (Figure 3). Effects on the extrema at lower wavelengths observed with this derivative, or with derivatives of cystine or phenylalanine, could not be interpreted due to the difficulty in differentiating carboxyl, ester, or amide group transitions from aromatic or disulfide contributions (Quadrifoglio and Urry, 1968a; Goodman and Toniolo, 1968).

Since the far-ultraviolet region is dominated by transitions of the peptide chromophores, the effect of methanol on the circular dichroism spectrum of helical poly-L-glutamic acid was determined in order to assess the magnitude of solvent nonconformational effects on the optical activity of the α helix. The results, shown in Figure 10, indicate some qualitative, if not quantitative, similarities to the effects of methanol on the far-ultraviolet circular dichroism spectrum of insulin. In polyglutamic acid, the ellipticity was found to increase at 191 and 208 nm, but no band shifts were observed. This is similar to the reported large increase in the 196-nm circular dichroism extremum of the silk fibroin β-structured protein on addition of methanol (Iizuka and Yang, 1968). The recently reported X-ray diffraction results on the insulin dimer, which is the predominant form in aqueous medium at low pH, have revealed the presence of intermolecular β structure in the area of subunit contact (Adams *et al.*, 1969). A similar

effect has been noted for the 197-nm band in β-structured poly-L-serine (Quadrifoglio and Urry, 1968b); this increase was interpreted, however, as reflecting the conversion of random structure to β conformation. While the increase in intensity near 196 nm in the circular dichroism spectrum of insulin in methanol resembles the effects reported both for β structure and α helix models, in insulin this was accompanied by blue shifting of this extremum which was not observed with either model.

In order to assess the possible effects on the circular dichroism spectra which might result from altered self-association behavior, equilibrium sedimentation experiments were carried out in methanol as well as in aqueous media. In water, at pH 2, 0.02 ionic strength, the weight average molecular weight of insulin was found to increase with increasing radial distance and protein concentration in the centrifuge cell. The distribution of weight-average molecular weights suggested that, under these conditions, insulin was present primarily in the state of a monomer–dimer equilibrium, in agreement with the results of Jeffrey and Coates (1966a,b). The circular dichroism spectra under these conditions were identical with those obtained at pH 3 in the absence of supporting electrolyte. In methanol, with 0.02 M salt and the same amount of HCl as used in water, only monomer was present; *i.e.*, the weight-average molecular weight remained at 5850 throughout the cell.[3] This is similar to the observations when dioxane or pyridine were used as solvent (Fredericq, 1957; Yphantis and Waugh, 1957). Therefore, changes in the self-association behavior must be considered in the interpretation of the above optical rotatory dispersion–circular dichroism results. These changes in the degree of association of insulin must be reflected in specific conformational changes, since, on dissociation, the intermolecular β structure must be disrupted. They may also lead to effects similar to those reported for associating α helical polypeptides (Cassim and Yang, 1967; Hammes and Schullery, 1968).

Discussion

The near-ultraviolet circular dichroism spectrum of insulin has been related predominantly to tyrosine transitions, with some contributions from phenylalanine residues and disulfide groups (Ettinger and Timasheff, 1971). The changes in the near-ultraviolet circular dichroism spectra observed in 2-chloroethanol and acidic methanol resemble those accompanying thermal denaturation in their marked reduction in intensity without any shifts in band position. The broadening of the 274-nm extremum into two apparent extrema is similar to the effect observed on O-acetylation of the tyrosine residues; in that case, however, there was a marked blue shifting of the entire zone (Ettinger and Timasheff, 1971). Thus, these alterations of the near-ultraviolet circular dichroism spectrum in the organic solvents seem to reflect most probably a conformational perturbation involving the pertinent side-chain residues. Furthermore, the effects of methanol on the spectra of the model amino acid derivatives were of a nature which could hardly be related to the effects of the same solvent on the circular dichroism spectrum of insulin. The changes in the spectra of *N*-acetyltyrosineamide and L-phenylalanine methyl ester were of opposite sign to those observed in insulin and the reduction in the ellipticity of L-cystine was much smaller than that of insulin.

In water, insulin exists predominantly as a dimer. The results of the X-ray diffraction studies have shown that tyrosine B26, phenylalanine B24, and phenylalanine B25 are intimately involved in the formation of the dimer (Adams *et al.*, 1969). The dissociation of the insulin dimer in methanol must, therefore, result in an altered conformation around at least three residues. Changes in tyrosine exposure associated with changes in self-association of insulin have been reported (Gubensek and Rupley, 1969), while a change in tyrosine environment in the presence of methanol has been inferred from solvent perturbation experiments (Herskovits, 1965). The loosening of the structure with increased freedom of motion of the chromophoric groups when the dimer is disrupted should result in a reduction in intensity (Strickland *et al.*, 1969). It would appear, therefore, that the reduction of the circular dichroism intensity in the near-ultraviolet and the accentuation of the fine structure in the spectrum are best attributed to an increase in the freedom of motion of the tyrosine residues with possibly some minor nonconformational solvent effects.

The effects of solvent on the secondary structure of insulin can be summarized as follows: the full concentration dependence of the a_0 and b_0 parameters on the concentrations of 2-chloroethanol or acidic methanol have demonstrated that the largest changes in the a_0 parameter occur at a solvent composition intermediate between water and 100% organic solvent. Furthermore, contrary to what was apparent from measurements of a_0 and b_0 values alone, the circular dichroism spectra of insulin in these solvents revealed that large effects on optically active transitions occurred throughout the spectra.

Resolution of the circular dichroism spectra into Gaussian zones helped to clarify some of the consequences of the overlap in transitions in the far-ultraviolet spectrum. Specifically, the virtual disappearance of the 222-nm extremum in 57% chloroethanol or 70% methanol was related to an increase in contributions from transitions associated with the 204 zone rather than to any change in the 222 region. Similarly, the apparent constancy of the 196-nm extremum in 70% methanol actually represents an increase in intensity in this region of the spectrum, since there is a compensating negative increase in the 204 zone. Finally, the increase at 209 nm which was observed between 70 and 99% methanol was primarily a result of the increase in the 222 zone, rather than any change in the 204 zone.

To interpret further the correlations which were established between changes in the circular dichroism zones and changes in the a_0 and b_0 values, it is convenient to consider separately the changes between 0 and 70% methanol and those between 70 and 99% methanol.

The increase in $-a_0$ on passing from 0 to 70% methanol has been associated with contributions from transitions in the 204 zone (this zone includes the 209-nm extremum). The unusually large magnitude of the 209-nm extremum has been previously interpreted (Ettinger and Timasheff, 1971) by applying a theoretical analysis of the circular dichroism spectra of α-helical polypeptides with resolution into

[3] Since in these experiments, the solvent was 99% methanol, any multicomponent effects on the partial specific volume of insulin, $\bar{v}$, would be negligible. The assumption that $\bar{v}$ of insulin in methanol is not significantly different from that in water seems reasonable, in particular, in view of the finding that in 20% dioxane the two values are very close (Fredericq, 1957). Calculations show that a $\bar{v}$ of 1.0 would have to be assumed if insulin were to be present as dimer rather than monomer throughout the centrifuge cell. Furthermore, it has been shown by osmotic pressure measurements that the molecular weight shifts toward 6000 when dimethylformamide is used as solvent (Rees and Singer, 1956).

four components (Woody, 1968). In particular, it has been pointed out that a red shift in the predicted parallel π–π^* component could account for the intensity at 209 nm. A further red shift in this negative α helix component of the circular dichroism spectrum is a likely explanation of the increases in the 204 zone when insulin is exposed to methanol. An additional probable facet of the increases in this zone is a blue shift in the positive perpendicular π–π^* components of the α-helix contribution, as evidenced by the blue shifting of the 196 zone in methanol. Since the red shift of the parallel π–π^* component would be essentially cancelled by a blue shift in the perpendicular π–π^* components, there is no shift observed in the 204 zone. These effects on transitions associated with an α helix would appear to be related largely to nonconformational solvent effects, since very little change occurred in the 222 zone which corresponds almost exactly to the n–π^* transition of an α helix (Woody, 1968), indicating no significant change in the amount of that conformation. Furthermore, the circular dichroism spectrum of helical polyglutamic acid in methanol also exhibited an increase in magnitude of the 209-nm extremum and no change at 222 nm, although the increase at 209 nm was of a smaller magnitude than that observed with insulin. The increase in the ellipticity of the 209-nm extremum or of the 204 zone in methanol is probably not a reflection of the effect of the dissociation of insulin into monomers on the optical activity of α helices, since it is opposite in direction to changes that would be predicted for such an effect. Cassim and Yang (1967) have reported that self-association of α-helical polyglutamic acid was accompanied by an increase in intensity and a red shift at 209 and 222 nm. In our experiments, the intensity increased under conditions of decreasing self-association of insulin with no shifts in the positions of these extrema.

The increase in magnitude of the 196 zone in the presence of 70% methanol is consistent with a nonconformational solvent effect on a β structure (Iizuka and Yang, 1968). Such effects would have to be on intramolecular β structure, since the intermolecular β structure of the insulin dimer must be absent in methanol. There was no mention, however, of any intramolecular β structure in the preliminary report on the diffraction studies of insulin dimer (Adams *et al.*, 1969). A solvent effect on α-helix transitions could also account for the increases in the 196 zone, since an increase was recorded at 191 nm in the spectrum of α-helical polyglutamic acid in methanol (Figure 10). The observed blue shifting of the position of the 196 zone with increasing methanol concentration can be easily rationalized either in terms of solvent effects on the transitions associated with the β- or α-helical conformations or of subtle changes in α-helical regions, altering these from strained, somewhat opened structures to ones resembling more closely those observed in synthetic polypeptides.

The similarities in the changes of the far-ultraviolet circular dichroism spectrum of insulin upon addition of organic solvents to changes observed on acetylation or thermal denaturation (Ettinger and Timasheff, 1971) also suggest the possibility of an increase in unordered conformation. Thus, part of the increase in the 204 zone between 0 and 70% methanol may be related to an increase in disordered conformation, consistent with the increase in the $+b_0$ contribution from the 204 zone.

Turning now to the changes which occur on going from 70 to 99% methanol, the decrease in $-a_0$ (more pronounced with 2-chloroethanol) and increase in $-b_0$ have been associated with predominating contributions from the 222 and 196 zones. Since the 222 zone can be considered to be essentially the resolved n–π^* helix band, the increase in magnitude of this band most likely reflects an actual increase in α-helical content. Nonconformational solvent effects are probably of secondary importance, since in the case of α-helical polyglutamic acid, transfer to a methanolic medium resulted in no significant changes in the intensity of this band. The further large increase in the 196 zone and its $+a_0$ and $-b_0$ contributions on going from 70 to 99% methanol are also consistent with an increase in α-helical content, although an additional contribution from solvent nonconformational effects is also likely.

The zonal analysis of circular dichroism spectra described in this paper, and the calculation of the contributions of each zone to the a_0 and b_0 parameters, should prove to be a useful aid in the analysis of changes in protein circular dichroism spectra when the protein and its environment have been modified. While each zone undoubtedly contains contributions from several transitions, it represents, nevertheless, a great simplification from the total spectrum. In the case of insulin, this analysis has permitted detection of changes which were not evident from an examination of entire circular dichroism spectra, and it has been possible to show the gradual occurrence of subtle conformational changes on methanolic dissociation of the native insulin dimer and disruption of the intermolecular β structure as well as to clearly demonstrate nonconformational solvent effects on the circular dichroism contributions of ordered sections of the molecule. The conformational changes which do occur seem to proceed by the pathway of an initial small increase in unordered structure, followed by refolding into α-helical conformation. The extent of these conformational changes is of smaller magnitude than that observed with other proteins (Weber and Tanford, 1959; Tanford *et al.*, 1960).

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Magnetic Susceptibility Evidence for a Binuclear Iron Complex in Hemerythrin*

T. H. Moss, C. Moleski, and J. Lyndal York

ABSTRACT: Magnetic susceptibilities have been determined for oxy, deoxy, and several met derivatives of hemerythrin. Below 205°K all the methemerythrin derivatives were very clearly diamagnetic.

These findings directly support long-standing ideas that the two iron atoms per hemerythrin monomer are sufficiently closely linked to form an antiferromagnetically coupled pair.

The iron ions in most of the common iron porphyrin proteins appear to be perfect paramagnets, in the sense that their magnetic properties can be explained in terms of spins isolated completely from one another in a diamagnetic protein matrix. Recent electron spin resonance (Tsibris *et al.*, 1968; Beinert and Orme-Johnson, 1969), magnetic susceptibility (Moss *et al.*, 1969; Moleski *et al.*, 1970), and Mössbauer measurements (Moss *et al.*, 1968) have demonstrated, however, that another large class of iron proteins, that of the non-heme iron sulfur proteins (ferredoxins etc.), exhibits exactly the opposite property. In these, magnetic ions are grouped in clusters with spins closely coupled. Hemerythrin, the oxygen-transport protein of sipunculid worms and certain other invertabrae, is neither a heme protein nor a member of the ferredoxin class (Klotz and Keresztes-Nagy, 1963). However, on the basis of absorption spectra, and the fact that the protein binds O_2 and anionic ligands in the ratio of 1:2 Fe atoms (Keresztes-Nagy and Klotz, 1965), it has been proposed that the irons are linked in a bridged structure, in which any unpaired ionic spins could be coupled antiferromagnetically. Supporting this viewpoint, recent approximate room temperature magnetic susceptibility measurements indicated (Okamura *et al.*, 1969) a decrease in susceptibility on converting deoxyiron(II) hemerythrin into an iron(III) form. The magnitude of the decrease compared to experimental error was such as to allow either low-spin ($S = {}^1/_2$) or diamagnetically coupled ion pairs in the iron(III) form, but, in addition, Mössbauer measurements failed to detect any magnetic hyperfine broadening at 4.2°K. Such broadening is normally observed in applied fields if there are unpaired electrons associated with the iron ions. The combination of Mössbauer and single temperature susceptibility measurements led these authors as well to conclude that the iron atoms formed a coupled antiferromagnetic structure. By measuring susceptibilities at low temperatures, as a function of temperature, we are able to measure with high accuracy the absolute, rather than relative, paramagnetism of a series of hemerythrin compounds. We present here data which put a very low limit on the maximum paramagnetism of the iron(III) derivatives and their individual subunits, excluding completely the $S = {}^1/_2$ case, and confirming directly the antiferromagnetic coupling of the iron atoms into diamagnetic pairs in the subunits of oxidized hemerythrin.

Experimental Section

The method of purification of hemerythrin was essentially that previously described (Florkin, 1933). The worms were cut with scissors and the contents squeezed out and filtered

* From the IBM Watson Laboratory, Columbia University, New York, New York 10025 (T. H. M. and C. M.), and the Department of Biochemistry, University of Arkansas School of Medicine, Little Rock, Arkansas 72201 (J. L. Y.). This research was partially supported by Grant GM 16514 from the National Institutes of Health to J. L. Y.