Optical Activity of Insulin. I. On the Nature of the Circular Dichroism Bands*

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ABSTRACT: Detailed studies of the optical rotatory dispersion and circular dichroism spectra of insulin were carried out to define the difficulties encountered at present in the analysis of protein optical activity in terms of specific conformations. Spectra were recorded at 27°, at elevated temperatures, and subsequent to reaction with *N*-acetylimidazole. Tyrosine acetylation resulted in a marked diminution and blue shifting of the near-ultraviolet circular dichroism spectrum; the 274-nm extremum was broadened into two distinct extrema, indicating a major contribution from tyrosine moieties to the near-ultraviolet circular dichroism spectrum. The contribution of unordered polypeptide segments to the far-ultraviolet circular dichroism spectrum of insulin was assessed from circular dichroism spectra of thermally denatured insulin. At 70° the far-ultraviolet circular dichroism spectrum of insulin is quite different from that usually obtained with unordered polypeptides in solution, suggesting the maintenance of appreciable amounts of residual order. Lack of a blue shift of the 209-nm extremum at 70° suggested that the negative band of the unordered conformation is located above 200 nm, as is found in polypeptide films. Heating to 70° resulted in a marked reduction of intensity in the near-ultraviolet region with no blue shift. The far-ultraviolet circular dichroism spectrum of native insulin contains two anomalous qualitative features: the position of the positive extremum at 196 nm and the high magnitude of the 209-nm extremum relative to that at 222 nm. These were analyzed in terms of shifts in position and intensity of α-helical and β-structure transitions. The principal limitations to interpreting these spectra exactly are the inadequacy of polypeptide models for protein polypeptide optical activity and the inability to assess quantitatively the contributions made by side chains to far-ultraviolet circular dichroism.

Although optical rotatory dispersion and circular dichroism are very useful methods for detecting conformational changes in proteins, their usefulness for obtaining specific information about peptide and side-chain conformations remains less certain. Furthermore, it is becoming increasingly apparent that there are a number of oversimplifications and limitations inherent in the current methods of analyzing these spectra, as evidenced, for example, from some of the discrepancies between optical rotatory dispersion–circular dichroism predictions and X-ray diffraction measurements (Jirgensons, 1969). The general problem of interpreting optical rotatory dispersion and circular dichroism spectra then resolves itself to one of distinguishing between what is approachable with improved theory and experiments and what are the actual limitations to these methods. Therefore, it appears important to examine critically the optical rotatory dispersion and circular dichroism spectra of those proteins whose spectra are most difficult to analyze in terms of specific conformations. The optical rotatory dispersion and circular dichroism spectra of insulin reported herein and in the accompanying paper (Ettinger and Timasheff, 1971) serve as excellent examples of these problems.

Circular dichroism spectra of insulin have been reported from several laboratories (Beychok, 1965; Mercola *et al.*, 1967; Timasheff *et al.*, 1967b; Morris *et al.*, 1968). The near-ultraviolet circular dichroism spectrum has been attributed to optical activity associated with tyrosine and phenylalanine residues (Morris *et al.*, 1968), as well as to that associated with disulfide bonds (Beychok, 1965). Although qualitative statements have been made focusing attention on the similarity of the far-ultraviolet circular dichroism spectrum of insulin to that of α-helical polypeptides (Mercola *et al.*, 1967; Timasheff *et al.*, 1967b), no detailed analysis has been reported. Measurements of various physical properties of insulin have yielded puzzling results when compared to those obtained with other globular proteins. The infrared absorption spectrum of insulin in D_2O contains an amide I band at 1654 cm^{-1} (Timasheff *et al.*, 1967a). Since insulin was judged to contain no more than 20 to 40% right-handed α helix by optical rotatory dispersion and circular dichroism measurements (Urnes and Doty, 1961; Markus, 1964; Mercola *et al.*, 1967), it was predicted that its infrared spectrum would contain an amide I band centered about 1640–1645 cm^{-1} (Susi *et al.*, 1967). The position of the observed band was unusual and virtually impossible to interpret definitively. Insulin was also found to be unusual in the nature of its response to helix-inducing solvents, such as 2-chloroethanol. The change in its optical rotatory dispersion b_0 parameter was much smaller than that observed with any other protein and has remained largely unaccounted for (Herskovits and Mescanti, 1965; Doty, 1960).

We have undertaken, therefore, a detailed circular dichroism study of insulin with the aim of resolving some of these problems. The optical rotatory dispersion and circular dichroism spectra of insulin at 27°, elevated temperatures, and subse-

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quent to reaction with *N*-acetylimidazole are reported here. The behavior of insulin in various solvent systems is the subject of an accompanying paper (Ettinger and Timasheff, 1971). To the extent which is, at present, possible, these results have been analyzed in terms of the recently reported X-ray diffraction analysis of porcine insulin at 2.8 Å resolution (Adams *et al.*, 1969). The use of the crystallographic data has increased the possibility of defining exactly what is and what may or may not be inferred from the optical rotatory dispersion and circular dichroism analyses of this protein.

Materials and Methods

A recrystallized bovine insulin preparation (Mann Research Labs) was used without further purification. The circular dichrosim spectrum of insulin which had been partially purified by equilibrium chromatography on a diethylaminoethanol-cellulose column (O'Donnell and Thompson, 1960) was not significantly different from the spectrum obtained with the unchromatographed insulin. *N*-Acetylimidazole (Eastman Chemical Corp.) was recrystallized from dried benzene and stored over calcium hydride. L-Threonine·HCl (Mann Research Labs) was used without further purification.

Concentrations of insulin were obtained 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 aimed at the determination of the a_0 and b_0 parameters; for optical rotatory dispersion spectra below 300 nm and all circular dichroism spectra, a concentration of 1 g/l. was used. Solutions were adjusted to pH 3.0 ± 0.03 with 0.1 N HCl in all cases, except in the circular dichroism experiments on the effects of acetylation; in these studies the circular dishroism spectra were obtained in pH 7.5 0.1 M borate buffer. Electrolyte was not added to the pH 3 solutions in order to minimize the tendency of insulin to self-associate (Steiner, 1952; Jeffrey and Coates, 1966a,b); under the conditions of the present experiments, insulin exists in the state of a monomer–dimer equilibrium (Jeffrey and Coates, 1966b). In the absence of added electrolyte, the circular dichroism spectra at pH 2.0 and 3.0, determined in our study, were found to be identical at the insulin concentrations used.

Optical rotatory dispersion and circular dichroism spectra were recorded on a Cary Model 60 spectropolarimeter with a Model 6001 circular dichroism attachment; the sample compartment was maintained at 27°. Slits were programmed to yield a 10 Å bandwidth at each wavelength. The instrument was calibrated monthly for circular dichroism accuracy with *d*-camphorsulfonic acid which had been recrystallized from ethyl acetate, and checked for optical rotatory dispersion precision at several wavelengths with L-threonine·HCl. Circular dichroism spectra were routinely recorded from 185 to 400 nm; optical rotatory dispersion spectra for the calculation of the a_0 and b_0 parameters were recorded from 350 to 550 nm. Overlapping spectra were obtained with the use of 0.01-, 0.1-, 1.0-, or 2.0-cm fused silica cells which were placed in specially constructed cell holders to ensure precise positioning. When deemed necessary to differentiate instrument noise from signal fine structure, a second spectrum was recovered over the spectrum obtained from the initial scan. Reduced mean residue rotations, $[m']$, and ellipticities, $[\theta]$, were calculated from established relationships (Urnes and Doty, 1961; Beychok, 1966). A value of 112 was used as the mean residue weight of insulin.

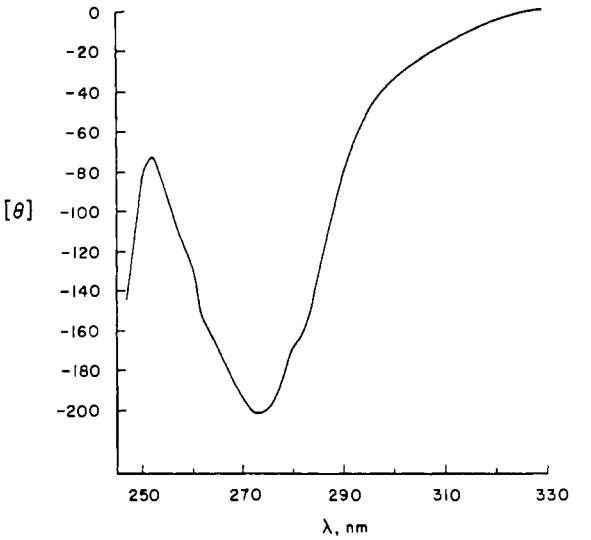

FIGURE 1: Near-ultraviolet circular dichroism spectrum of insulin at pH 3.0 (average of four determinations).

Lorentz factors, $3/(n^2 + 2)$, were calculated for water by the Sellmeir approximation (Urnes and Doty, 1961) using reported refractive index values at 434.1 and 589.3 nm which were corrected to the appropriate temperature (International Critical Tables, 1938).

Acetylation with *N*-acetylimidazole was performed by the method of Riordan *et al.* (1965), using 12.5 g/l. insulin solutions. The circular dichroism spectra of insulin subsequent to the acetylations were recorded immediately following the determination of the number of tyrosines reacted. Solution of insulin in the pH 7.5 borate reaction mixture was accomplished by addition of alkali prior to adjustment to the reaction pH. As a control, circular dichroism spectra were also recorded for unreacted insulin in the pH 7.5 borate buffer.

A circulating bath connected to jacketed cells of appropriate path length was used for temperature regulation during the thermal denaturation experiments. Agreement between bath and sample temperatures was ascertained with the aid of a Tele-thermometer thermister probe (Silver Springs Instr.).

Results

The near-ultraviolet circular dichroism spectrum of insulin in pH 3.0, salt-free HCl, shown in Figure 1, exhibits a comparatively large negative extremum with a molar ellipticity of −202 deg cm² per dmole at 274 nm. Repeated recordings of the spectrum consistently revealed the existence of slight fine structure between 280 and 285 nm and between 255 and 270 nm, confirming previous reports on multiple contributions to this region of the spectrum (Morris *et al.*, 1968). The circular dichroism spectrum of insulin from 185 to 250 nm, shown in Figure 2, contains extrema at 222, 208, and 196 nm with molar ellipticities equal to −9960, −12,550 and 22,500, respectively. This spectrum resembles closely the circular dichroism spectrum of helical polyglutamic acid with two notable exceptions. In the spectrum of helical polyglutamic acid, the 209-nm extremum has a smaller magnitude than the 222-nm extremum (Ettinger and Timasheff, 1971), whereas in the insulin spectrum, the 209-nm extremum is of considerably greater magnitude than the 222-nm band. Furthermore, rather than containing an extremum at 191–192 nm, as is found in helical polypeptide spectra, the insulin spectrum possesses an extre-

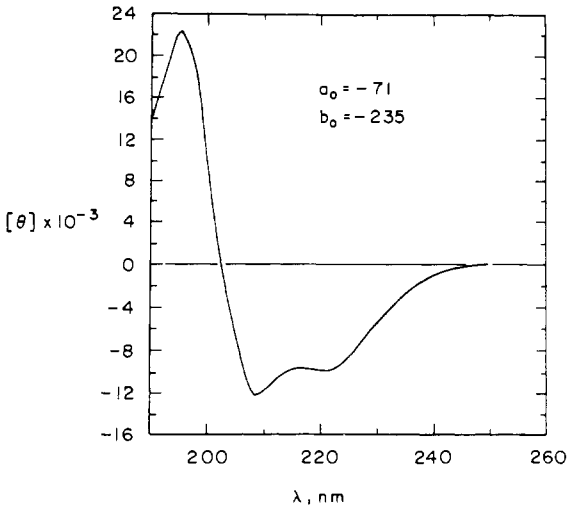

FIGURE 2: Far-ultraviolet circular dichroism spectrum of insulin at pH 3.0 (average of four determinations). The Moffitt–Yang parameters, a_0 and b_0, represent the average values calculated from twelve recordings of the optical rotatory dispersion spectrum between 350 and 550 nm.

mum at 196 nm, *i.e.*, at the position of β-structured polypeptides (Townend *et al.*, 1966). In addition to arising from a strong β-conformation contribution, the 196-nm extremum might originate from optical activity associated with other conformational types, as will be discussed in more detail below. As is apparent from the Kronig–Kramers transform relationship (Moffitt and Moscowitz, 1959), one consequence of the position of the positive extremum at 196 nm is the relatively low a_0 value of −71, while b_0 is −235. Typically, a protein with a b_0 value of −235 would be expected to yield a much more negative value of a_0 (usually −300 to −400). The contribution to $-b_0$ from near-ultraviolet side-chain transitions, calculated with the Kronig–Kramers transform from the circular dichroism spectrum of Figure 1, was only −18 (Ettinger and Timasheff, 1971) and was, thus, insignificant.

The optical rotatory dispersion spectrum of insulin below 350 nm, shown in Figure 3a, showed anomalous dispersion between 270 and 290 nm, corresponding to the large circular dichroism band in this region. In the far-ultraviolet region, shown in Figure 3b, a trough was noted at 233 nm characteristic of α-helical polypeptides (Simmons *et al.*, 1961). The observed optical rotatory dispersion peak at 201 nm is characteristic neither of helical polypeptides the circular dichroism spectra of which display a peak at 198 nm nor of β-structured polypeptides which commonly contain a peak at 205 nm (Davidson *et al.*, 1966; Sarkar and Doty, 1966).

Insulin is a favorable molecule for specific O-acetylation of its tyrosine residues with *N*-acetylimidazole, since it has only one lysine and two N-terminal groups as other possible reactants (Ryle *et al.*, 1955), although reaction with aliphatic hydroxyls cannot be totally excluded (Jencks and Carriulo, 1958; Gorbunoff, 1970). Therefore, insulin was treated with *N*-acetylimidazole in order to assess the relative contribution of tyrosine residues to the observed circular dichroism spectrum in the 274-nm region. Concentrations of the reagent utilized were such that they resulted in the average acetylation of 1.3 tyrosines in one experiment and 4.0 tyrosines in another. As shown in Figure 4, increasing tyrosine acetylation is reflected in a marked diminution and blue shift in the near-ultraviolet circular dichroism spectrum. Accompanying these changes, there was broadening of the 274-nm extremum and resolution of this band into two distinct extrema. These changes suggest that tyrosines make an appreciable contribution to this region of the insulin circular dichroism spectrum; the interpretation of these changes, however, was complicated by the observation that changes were also noted in the far-ultraviolet region of the insulin spectrum, as shown in Figure 5. After reaction of 1.3 tyrosines, the ellipticity at 209 nm increased together with a blue shifting and diminution of the ellipticity at 196 nm. A further increase at 209 nm, along with blue shifting and diminution of the 196-nm extremum, was noted when 4.0 tyrosines had reacted; furthermore, the 222-nm extremum had virtually disappeared. That these far-ultraviolet spectral changes represent alterations in peptide conformation was suggested by their resemblance to changes observed on thermal denaturation, described below. While the changes observed in the 274-nm region on acetylation probably contain some contributions from changes in phenylalanine and/or disulfide bond conformation, the acetylation results strongly indicate that transitions arising from tyrosine phenolic moieties make a major contribution to the near-ultraviolet circular dichroism spectrum of insulin. It should be pointed out that the circular dichroism spectra of unmodified insulin in pH 7.5 borate were somewhat different from those recorded at pH 3.0; in particular, the amplitudes at 274 and 196 nm were greater at the higher pH (see Figures 1, 2, 4, and 5). Such a pH dependence of the insulin circular dichroism spectra is similar to that reported by Morris *et al.* (1968).

As pointed out above, the far-ultraviolet circular dichroism spectrum of insulin contains two features which appear to be strikingly unusual. These are the relatively high magnitude of the 209-nm extremum and the position of the positive band at 196 nm. The b_0 value, the qualitative features of the circular dichroism spectrum from 200 to 240 nm, and the position of the infrared amide I band in D_2O all suggest that the insulin molecule contains an appreciable amount of α helix (perhaps 20–40%). This has been confirmed by the preliminary report on the X-ray diffraction pattern of insulin at 2.8 Å resolution (Adams *et al.*, 1969), which has also revealed the presence of antiparallel β structure in the insulin dimer. Given this content of α helix, one would predict from current models of peptide conformation that the circular dichroism spectrum of insulin should contain extrema of nearly equal magnitudes at 209 and 222 nm and a peak at 191 and 192 nm, since the ellipticities observed for α-helical polypeptides are far greater than those reported for β-structured or random coiled polypeptides.[1] It is apparent then that the anomalies at 209 and 196 nm are a primary barrier to any definite analysis of the far-ultraviolet circular dichroism spectrum of insulin in terms of specific conformational types.

One probable source of error or oversimplification in this sort of approach to the analysis of protein circular dichroism

[1] Spectra were calculated for insulin, assuming various values of α helix, β structure, and random conformation consistent with the a_0 and b_0 values determined from optical rotatory dispersion. These calculations predicted an extremum at 191 nm rather than 196 nm, as observed. Furthermore, there were marked disparities between the calculated and observed spectra. Helical polyglutamic acid, β-structured poly-L-lysine, and randomly coiled poly-L-lysine were used to determine the standard spectra. Furthermore, when similar calculations were attempted to account for changes observed in the circular dichroism spectra at various concentrations of methanol (Ettinger and Timasheff, 1971), it was impossible even to predict the direction of change at any wavelength.

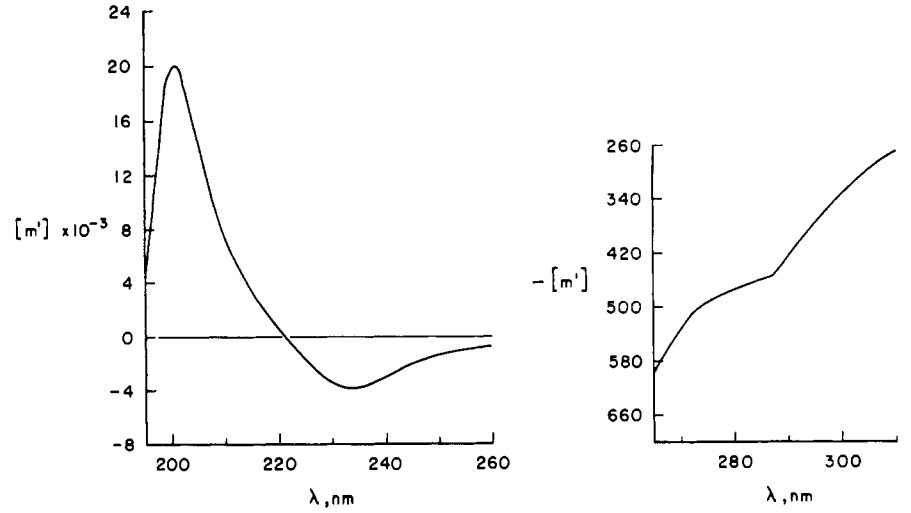

FIGURE 3: Optical rotatory dispersion spectra of insulin at pH 3.0 (average of duplicate determinations): right, near-ultraviolet spectrum; left, far-ultraviolet spectrum.

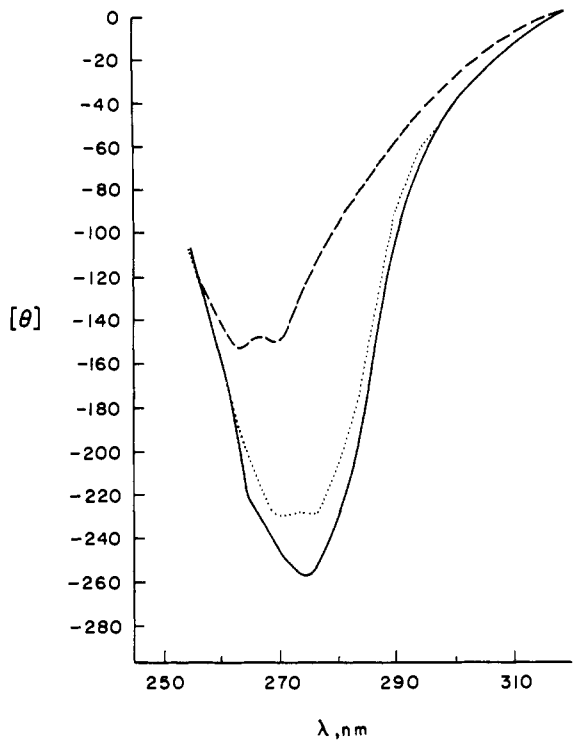

FIGURE 4: Near-ultraviolet circular dichroism spectra of insulin in pH 7.5 borate buffer, subsequent to reaction with *N*-acetylimidazole: unreacted insulin (——); insulin treated with a five-times excess of reagent (calculated with respect to four tyrosine equivalents per mole of insulin); the product contained 1.3 acetylated tyrosines (· · ·); insulin treated with a 120-times excess of reagent, the product containing 4.0 acetylated tyrosines (---).

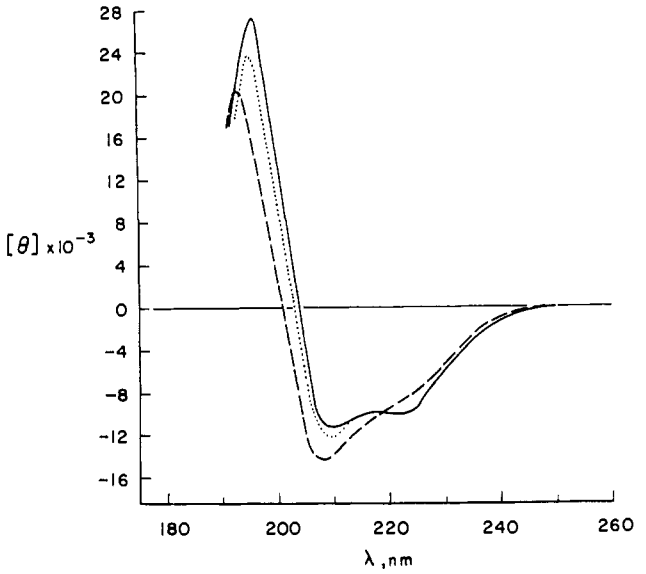

FIGURE 5: Far-ultraviolet circular dichroism spectra of insulin in pH 7.5 borate buffer, subsequent to reaction with *N*-acetylimidazole: unreacted insulin (——); insulin with 1.3 acetylated tyrosines (· · ·); insulin with 4.0 acetylated tyrosines (---). The concentrations of reagent were the same as indicated in the legend to Figure 4.

spectra is that the common polypeptide models used as prototypes of peptide conformation in proteins are not necessarily adequate or appropriate. In particular, the validity of employing the circular dichroism spectrum of poly-L-lysine at pH 7.7 as a model for random conformation in proteins[2] has been questioned seriously by the results obtained with polypeptide films (Stevens *et al.*, 1968; Fasman *et al.*, 1970). While these spectra differed considerably from those of randomly coiled polypeptides in solution, they did in fact resemble more closely circular dichroism spectra obtained with proteins in a denatured, but constrained state (Stevens *et al.*, 1968; Fasman *et al.*, 1970). Therefore, in an attempt to arrive at a clearer understanding of the circular dichroism spectrum of insulin, circular dichroism spectra were obtained under conditions in which appreciable unfolding is known to occur. Thermal denaturation was chosen, since it results in products which can be observed in the far-ultraviolet region of the circular dichroism spectra while common chemical denaturants, such as guanidine·HCl, have too high an absorbance in this region. At 45°, the circular dichroism spectrum showed an increase in magnitude of the 209-nm extremum and a decrease at the 196-nm maximum (Figure 6). At 70°, where thermal unfolding had progressed to a greater degree (Figure 7), the circular dichroism spectrum shows: a marked diminution of intensity throughout, virtual disappearance of the 222-nm extremum,

[2] The term "random" used here refers to that portion of the polypeptide conformation which lacks repeatable measureable order rather than to true random coil behavior.

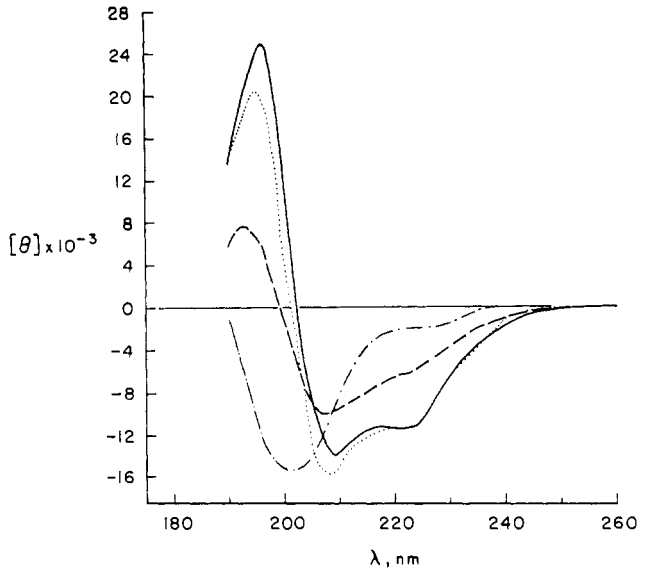

FIGURE 6: Far-ultraviolet circular dichroism spectra of insulin at pH 3.0 recorded at 27.0° (——); 45.0° (···); 69.8° (---) (average of duplicate determinations); (—·—): circular dichroism spectrum of a poly-L-lysine film at pH 7 (in arbitrary intensity units).

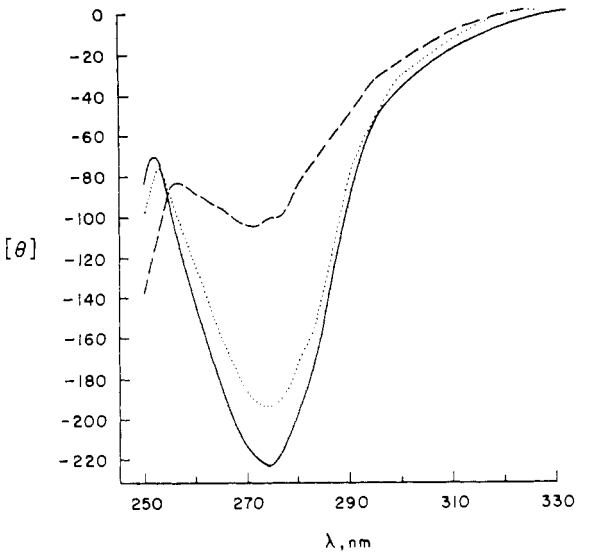

FIGURE 8: Near-ultraviolet circular dichroism spectra of insulin at pH 3.0 recorded at 27.0° (——); 45.0° (···); 69.8° (---) (average of duplicate determinations).

and a blue shifting of the 196-nm extremum to 193 nm (Figure 6). The 70° circular dichroism spectrum of insulin is very different from that usually obtained with unordered polypeptides in film or solution. These spectra are characterized by a large negative extremum at 200–205 nm in the film and at 197 nm in solution; for comparison, a typical film spectrum is shown by the dot–dash curve of Figure 6. The positive band at 193 nm observed with insulin suggests that a considerable amount of order still remains in this protein at 70°, in a manner analogous to the case of thermally denatured ribonuclease, which can be denatured further by guanidine·HCl (Aune *et al.*, 1967).

In the near-ultraviolet region, the principal change upon thermal denaturation was a progressive reduction in magnitude of the 274-nm extremum with increasing temperature, shown in Figure 8. It is striking, however, that, in contrast to the case of tyrosine acetylation, there was no shifting of the 274-nm extremum with increasing temperature.

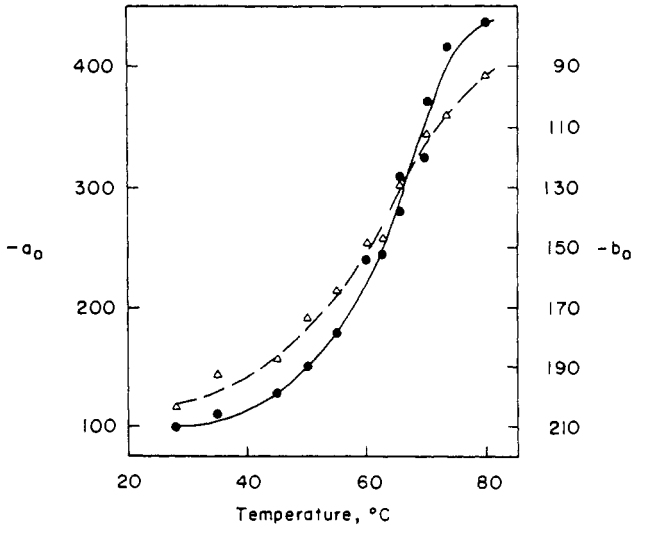

FIGURE 7: Thermal transition curves for insulin at pH 3.0 in terms of a_0 (——) and b_0 (△—△--△) (average of duplicate determinations).

Discussion

The circular dichroism spectra reported in this paper are in general agreement with those previously published (Beychok, 1965; Mercola *et al.*, 1967; Morris *et al.*, 1968; Timasheff *et al.*, 1967b). Insulin contains the following amino acids whose side-chain chromophores might contribute to its near-ultraviolet circular dichroism spectrum: four tyrosines, four disulfide bonds, and three phenylalanines; there is no tryptophan (Ryle *et al.*, 1955). Two different assignments have been proposed to account for the circular dichroism bands present in insulin in the near-ultraviolet region. One postulate was that the principal origin of the 274-nm band(s) was from transitions associated with cystine residues. This suggestion was based on the observation that the band(s) blue shifted rather than red shifted at pH 11.5, as would be predicted from tyrosine absorption, and that they disappeared on treatment with mercaptoethanol (Beychok, 1965). Correspondence between the positions of the absorption and circular dichroism bands led to the alternate assignment of this circular dichroism region predominantly to tyrosine and phenylalanine transitions (Morris *et al.*, 1968). As reported above, acetylation of tyrosine residues resulted in large changes in this circular dichroism spectral region. Changes occurred in the far-ultraviolet region as well, suggesting some possible peptide conformational alterations. The far-ultraviolet changes, however, were much smaller than those observed when insulin was subjected to thermal denaturation (Figure 6) or when it was placed in acidic methanol or 2-chloroethanol (Ettinger and Timasheff, 1971), even though the changes in the near-ultraviolet region were of similar magnitude in the three cases. Furthermore, acetylation of insulin failed to produce any changes in its infrared spectrum in the amide I band region, demonstrating that this treatment caused no major alterations in peptide conformation. It appears, therefore, that the changes in the 274-nm region induced by acetylation are

directly related to the modification of the tyrosine residues. Indeed, X-ray diffraction results have indicated that tyrosines A19 and A14 are hydrogen bonded to residues A1 and A5 and that tyrosine B26 is intimately involved in the interaction of insulin monomers to yield the dimer (Adams *et al.*, 1969). Thus, O-acetylation should certainly disrupt the hydrogen bonding interactions, affecting the environment of residues A14 and A19 and possibly that of B26 as well.

Rather than producing simply a progressive decrease in magnitude of the circular dichroism band(s) in the near-ultraviolet region, as observed with thermal denaturation, acetylation resulted in blue shifting along with the generation of new fine structure (the second extremum). This difference between the effects of the two treatments might be explained best by assuming that cystines and phenylalanines also make contributions to the native insulin spectrum with the tyrosines most probably predominating. As a consequence of its cooperative nature, thermal denaturation would be expected to affect the optical activity of each of these residues, while acetylation would be expected to affect tyrosine optical activity more selectively. The observed blue shift of the near-ultraviolet spectrum on acetylation is consistent with this hypothesis, since phenylalanine and its derivatives have been shown to have circular dichroism transitions with extrema between 250 and 270 nm (Horwitz *et al.*, 1969), while the circular dichroism spectrum of *N*-acetyltyrosineamide has an extremum at 274 nm (Beychok, 1967; Ettinger and Timasheff, 1971). Acetylated tyrosine residues might also contribute to the observed blue shift since their absorption spectrum is also blue shifted relative to that of tyrosine (Riordan *et al.*, 1965). The contribution of disulfides and phenylalanines to the near-ultraviolet region is further indicated by the observation that the near-ultraviolet fine structure exhibited solvent sensitivity analogous to that observed with phenylalanine and disulfide bond model compounds (Ettinger and Timasheff, 1971).

There are three distinct possible origins of the apparent anomalies in the circular dichroism spectrum of insulin at 209 and 196 nm. These are: contributions to the far-ultraviolet circular dichroism spectrum from side-chain transitions, the inadequacy of polypeptide models for the three conformational types (helix, β, and random structures), and the effects of self-association on conformation. The last is considered in the accompanying paper (Ettinger and Timasheff, 1971).

The quantitative analysis of tyrosine, phenylalanine, and cystine contributions to the far-ultraviolet circular dichroism spectra of proteins is an essentially impossible task in the present state of knowledge. A central problem, thus far, in attempting assignments is that it has been difficult to resolve the far-ultraviolet circular dichroism spectra of derivatives of these amino acids into circular dichroism contributions from the chromophore in question and those from carboxyl or amide optical activity (Quadrifoglio and Urry, 1968a; Goodman and Toniolo, 1968). Therefore, at present, only qualitative suggestions can be made for possible aromatic and disulfide contributions to the insulin spectrum. Coleman and Blout (1968) have shown that the disulfide bond should make a negative circular dichroism contribution with an extremum at 200 nm to the spectrum of *N,N'*-diacetyl-L-cystinebismethylamide. Since insulin contains four disulfide bonds in only 51 amino acids, it is quite possible that these may contribute to the high value of the 209-nm maximum in its spectrum. Their exact contribution, however, would be a strong function of the dihedral angles of the disulfide bonds.

Assessment of tyrosine and phenylalanine contributions to the far-ultraviolet circular dichroism spectrum is equally difficult. Beychok and Fasman (1964) have attributed a positive band at 225 nm found in random poly-L-tyrosine to this side-chain chromophore. This extremum is also present in the spectrum of *N*-acetyl-L-tyrosineamide (Beychok, 1967; Pflumm and Beychok, 1969; Ettinger and Timasheff, 1971). Although the magnitude and even the sign of a tyrosine contribution to the insulin spectrum in this region is impossible to predict *a priori*, a positive contribution near 225 nm could partially account for the lowering of the ellipticity at 222 nm relative to that at 209 nm. A decrease on acetylation of the 225-nm tyrosine band could contribute to the increase in the 209-nm negative extremum; this, however, could not affect the ratio of the 209- to 222-nm band strengths. The circular dichroism spectrum of L-phenylalanine ethyl ester contains a band at 218 nm, most probably attributable to the carbonyl of the ester bond, and apparently, a second positive band below 209 nm (Ettinger and Timasheff, 1971). The presence of a similar transition in insulin could contribute to the positive ellipticity values below 200 nm.

The current status of the application of polypeptides as models of protein conformation has been recently defined by attempts at calculations of protein circular dichroism spectra from those of polypeptides in three standard conformations (Greenfield and Fasman, 1969). Such calculations have shown the inadequacy of the use of a small number of polypeptide models for this purpose. First, the circular dichroism spectrum of randomly coiled poly-L-lysine at pH 7.7 may not be a realistic model for random or unordered conformation within a protein. Indeed, there appears to be no compelling reason for assuming any one circular dichroism spectrum for the unordered conformation, since the term when applied to protein conformation merely refers to an amorphous group of conformational types possessing the common characteristic of of being devoid of measurable repeating order. Although the circular dichroism spectra of thermally denatured insulin indicated that there was still appreciable ordered peptide conformation in insulin at 70°, it may be significant that the 209-nm extremum was not blue shifted. This would be consistent with an increase in negative contribution to the insulin far-ultraviolet circular dichroism spectrum at a wavelength higher than 197 nm, which is the position of the band characteristic of randomly coiled polypeptides in solution. Unordered polypeptide films have negative circular dichroism extrema between 200 and 205 nm, as do constrained denatured proteins (Fasman *et al.*, 1970). A negative contribution from such a band to the insulin spectrum would lead to higher ellipticites at 209 and 196 nm than those predicted, using randomly coiled polypeptides in solution as models. Calculations based on this hypothesis have shown that use of the film spectrum, while contributing to the unusual 196- and 209-nm characteristics, still cannot entirely account for these features.

The β conformation, as well, is known to exist in more than one type. It has been shown that the optical rotatory dispersion and circular dichroism spectra of β-structured polypeptides belong to at least two classes, I-β and II-β (Ikeda and Fasman, 1967; Fasman and Potter, 1967; Stevens *et al.*, 1968). The circular dichroism spectrum of β-structured poly-L-lysine (I-β) has extrema at 217 and 195 nm, while that of poly-*S*-carboxymethylcysteine (II-β) has extrema at 227 and 198 nm. In addition, the spectrum of β-structured poly-L-serine contains extrema at 222 and 197 nm (Quadrifoglio and Urry, 1968b). The β conformation has also been shown to be sensitive to the immediate environment, in that the circular dichroism spectrum of β-structured silk fibroin is extremely sensitive to solvent polarity (Iizuka and Yang, 1968). In terms

of these considerations, a more positive and/or slightly red shifted β-structure contribution in insulin below 200 nm relative to the spectrum of poly-L-lysine could account for the position of the positive extremum at 196 nm.

If an unusually high 195–197-nm contribution from β structure does exist in insulin, a disproportionately weak 217-nm band would be expected, as has been deduced for β-lactoglobulin (Townend *et al.*, 1967; Timasheff *et al.*, 1967b). This would account for the observed helical-like spectrum of insulin from 200 to 240 nm. Intermolecular β structure has been found in insulin at 2.8 Å resolution, and it would be of interest to learn whether any intramolecular β structure is also present.

The circular dichroism spectra of the α-helical conformation may also vary as has been shown by the theoretical calculations of Woody (1968). In addition to including Gaussian contributions ensuing from amide $n-\pi^*$, $\pi-\pi^*$ parallel, and $\pi-\pi^*$ perpendicular transitions, a fourth non-Gaussian $\pi-\pi^*$ perpendicular component was included in his resolved spectrum for the α helix. A small red shift of the negative, $\pi-\pi^*$ parallel component would then give rise to an α-helical circular dichroism spectrum with a higher ellipticity at 209 than 222 nm (Woody, 1968), much like that recorded with insulin. Although helical polyglutamic acid does not display this characteristic, poly-L-alanine does (Quadrifoglio and Urry, 1968a). The 209-nm band in insulin demonstrates marked solvent sensitivity (Ettinger and Timasheff, 1971). This is fully consistent with the explanation that the intensity of the 209-nm band in insulin is related to a small red shift of the $\pi-\pi^*$ parallel component compared to its position in helical polyglutamic acid.

Considerations of these components in the circular dichroism spectra of helical polypeptides indicates another possible origin of the position of the positive band at 196 nm in insulin. A reduction in the band width of the $\pi-\pi^*$ perpendicular component, a slight red shifting of the $\pi-\pi^*$ perpendicular component, or an increase in magnitude of this last component would all have the effect of red shifting the positive far-ultraviolet band of an α helix. Any one of these effects could lead to an observed circular dichroism spectrum with a band at 196 nm rather than at 191–192 nm. Therefore, it would appear that an unusual α-helical contribution in insulin with characteristics somewhat different than the α-helical conformation in homopolymer polypeptides could be responsible for both the 209- and 196-nm features of the circular dichroism spectrum. In this regard, the X-ray diffraction data revealed that "the A chain has an involved much folded structure with short stretches of near helical conformation" (Adams *et al.*, 1969). In addition, two stretches of α helix, slightly opened at each end, were also found in the B chain.

The presence of an unusual α-helical conformation in insulin could be used, as well, to rationalize its unexpected infrared spectrum with an amide I band at 1654 cm^{-1} (Timasheff *et al.*, 1967a). The shift to higher frequency is also quite consistent with the presence of a somewhat strained and open structure.

The analysis of the circular dichroism spectrum of insulin reported in this paper can serve as a good example of the serious limitations which exist in the interpretation of the circular dichroism spectra of proteins in terms of specific types of conformations. In particular, at present, it is still impossible to assess quantitatively the contributions made by side-chain chromophores to the far-ultraviolet circular dichroism spectra. A serious limitation also arises from the inadequacy of polypeptide models to predict quantitatively the circular dichroism contributions from specific conformations. It has been possible, however, to demonstrate a strong tyrosine contribution to the near-ultraviolet circular dichroism spectrum of insulin. It has also been possible to analyze some of the unusual features of the far-ultraviolet circular dichroism spectrum of insulin in terms of theoretical considerations about the α-helix spectrum and the known variability in the contribution of β structures.

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

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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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