Studies of the Location of the Tyrosyl Residues in Insulin. II*

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ABSTRACT: Solvent perturbation studies have been carried out on the trypsin-modified desoctapeptide-insulin and the isolated B-chain fragments released by trypsin in order to gain further information concerning the environment and location of the three tyrosyl residues, A-14, A-19, and B-16 of the desoctapeptide-insulin core. The perturbation data obtained with a number of perturbants of varying dimensions and range effects have been analyzed in terms of various models.

The data obtained on desoctapeptide-insulin was most consistent with a model having one buried, one exposed, and one partly exposed tyrosyl residues. Since the parent hormone gave the best fit with one more buried tyrosyl, *i.e.*, with two buried, one exposed, and one partly exposed groups, it is concluded that tyrosyl residue B-26 removed by trypsin is one of the two buried groups. Analysis of the tyrosyl perturbation difference spectra of the B-chain peptides indicates that their release by trypsin results in exposure of the peptide tyrosyl B-26. Extension of the perturbation technique to analysis of the phenylalanyl difference spectra developed in this paper indicates that the adjacent peptide phenylalanyl residues B-24 and B-25 are also exposed during the course of this process. All three of these groups seem to be buried in the parent protein.

Limited digestion of insulin with trypsin results in the release of eight amino acids from the C-terminal end of the B chain (Nicol and Smith, 1956; Laskowski *et al.*, 1960; Young and Carpenter, 1961) with the core of the hormone, the so-called desoctapeptide-insulin remaining intact. Scheraga and coworkers (Laskowski *et al.*, 1956, 1960; Leach and Scheraga, 1960) have attributed the difference spectral changes accompanying trypsin digestion as being due to the disruption of a hydrogen bond involving tyrosyl residue B-26, which is part of the released B-chain peptide fragment, and an unspecified acceptor group of the remaining insulin core. Aoyama and coworkers (1965) have found that this tyrosyl residue is one of the two groups unreactive toward the reagent cyanuric fluoride (Kurihara *et al.*, 1963). Their observations suggested the possibility that this tyrosyl residue may be one of the two buried groups. The latter suggestion offered by the solvent perturbation studies of the accompanying paper (Menendez *et al.*, 1969) have been further tested in the present work, dealing with desoctapeptide-insulin core, together with the perturbation behavior of the short tryptic fragments of the B chain containing tyrosyl residue B-26 and phenylalanyl residues B-24 and B-25 (Figure 1).

Experimental Section

Materials. Bovine zinc-insulin was purchased from Mann Research Laboratories. Zinc-free insulin was prepared by the method of Sluyterman (1955). Two trypsin preparations were employed. One sample was a highly purified preparation, obtained through the courtesy of Dr. M. Laskowski, Sr. This sample was purified by acid dissociation (pH 2.0) and chromatographic separation of a trypsin–pancreatic trypsin-inhibitor complex. The second sample employed was a L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone modified commercial preparation purchased from Worthington Biochemical Corp. All other reagents, model compounds, and perturbants employed were described in the accompanying paper (Menendez *et al.*, 1969).

Preparation and Purification of Desoctapeptide-insulin. This insulin derivative was prepared essentially according to the limited digestion procedure, described by Young and Carpenter (1961). Zn-free solutions (0.5–1%) were digested for 4 hr, at pH 8.4 and room temperature, employing 1 or 2% purified trypsin or L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-trypsin (1:100 or 1:50, w/w, of trypsin to insulin). The digestion mixtures were prepared from acidic solutions of insulin and trypsin by adjusting the pH to 8.4 with appropriate KOH, KCl, and pH 8.4 Tris buffer solutions. The final solutions contained 0.1 M Cl^-, 0.01 M Tris, and 2–6 $\times$ 10^{-4} M $CaCl_2$. Trypsin stock solutions were initially made up in 0.001 M HCl and 0.05 M $CaCl_2$. The insulin and trypsin concentrations were based on optical density determinations using the per cent extinction coefficients of 10.4 at 278 mμ (Weil *et al.*, 1965) and 15.3 at 280–282 mμ (Trowbridge *et al.*, 1963), respectively.

After 4-hr digestion the crude desoctapeptide-insulin core was separated from the more soluble fragments of the B chain (Figure 1) by isoelectric precipitation at pH 5.4 (Young and Carpenter, 1961). This precipitate was collected by preparative centrifugation, washed with distilled water, and dissolved at pH 2.5 with 0.1 M KCl–HCl solutions. Further purification of the core preparations was made by gel filtration using a 1.5 $\times$ 42 cm Bio-Gel P-10 column, washed and equilibrated with pH 2.5, 0.1 M KCl–HCl. The column was usually charged with 10–15-ml portions of the insulin core containing 0.1–0.12 g of crude desoctapeptide-insulin; 3-ml fractions were collected, with the central fractions containing about 60% of the optically absorbing material being pooled. The purified

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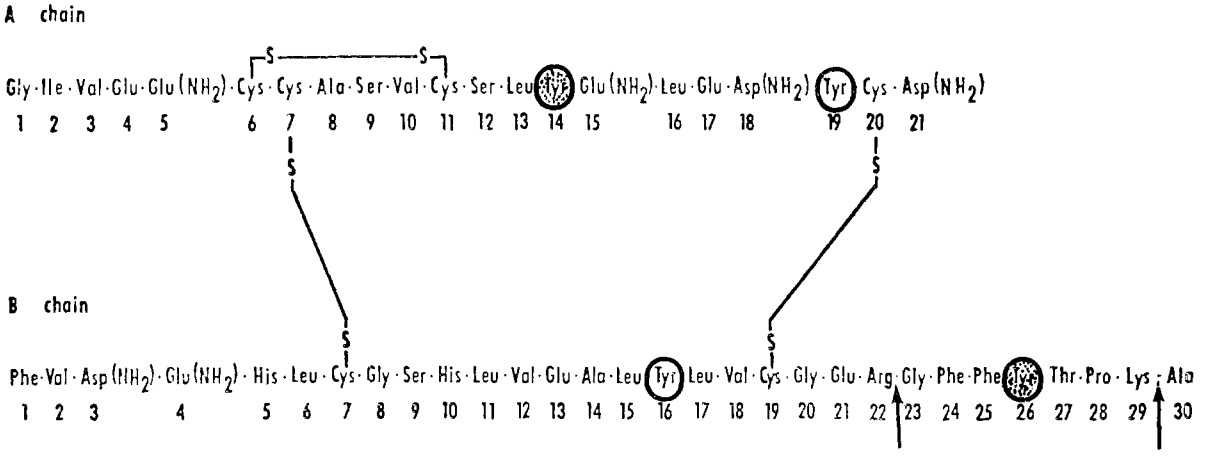

FIGURE 1: The primary sequence of amino acids in bovine insulin after Ryle *et al.* (1955). Arrows indicate the sites of cleavage by trypsin. The shaded tyrosyl residues A-14 and B-26 represent buried groups, unreactive toward cyanuric fluoride (Aoyama *et al.*, 1961).

TABLE I: The Amino Acid Composition of Purified Desoctapeptide-insulin and the Tryptic Peptides of Insulin.[a]

	Desoctapeptide-insulin (moles/monomer)			Tryptic Peptides (moles/monomer)		
	Prepn I	Prepn II	Theor	Prepn I	Prepn II	Theor
Aspartic acid	2.8	2.7	3.0	0.07	0.06	0
Threonine	0.02	0.13	0	1.1	1.0	1.0
Serine	2.9	3.0	3.0	0.05	0.02	0
Glutamic acid	7.1	6.7	7.0	0.06	0.08	0
Proline	0.02	0.01	0	0.74	0.83	1.0
Glycine	3.0	3.0	3.0	1.0	1.0	1.0
Alanine	1.8	2.0	2.0	1.2	0	1.0, 0
Half-cystine	6.5	6.0	6.0	0	0	0
Valine	5.0	5.0	5.0	0.04	0.04	0
Isoleucine	0.5	0.6	1.0	0.01	0.01	0
Leucine	6.1	5.9	6.0	0.02	0.06	0
Tyrosine	2.9	2.9	3.0	1.0[b]	1.1[b]	1.0
Phenylalanine	0.95	1.2	1.0	2.1	1.9	2.0
Lysine	0.07	0.1	0	1.0	1.0	1.0
Histidine	2.2	1.7	2.0	0.02	0.02	0
Purity estimated[c] (%)	97	92		95	95	

[a] Each sample was hydrolyzed in 6 N HCl for 20 hr at 110°; 50-μg samples were used. Analyses of the data were carried out according to the method described by Spackman *et al.* (1958). The data were normalized with respect to glycine rather than leucine used by Spackman *et al.* (1958) since the peptides contain no leucine other than leucine impurities. [b] Estimated spectrophotometrically from the absorption at 276–278 mμ using the molar absorbance value of 1340/mole of tyrosine. [c] Estimates of desoctapeptide-insulin impurities based on average lysine, proline, and threonine content and the tryptic peptides based on the aspartic acid, serine, glutamic acid, valine, leucine, and isoleucine impurities.

material was precipitated at pH 5.4, collected by centrifugation, and washed twice in turn with distilled water, 95% ethanol, and reagent grade ether (Sluyterman, 1955), and then dried under vacuum.

Purification of the Acid-Soluble Tryptic Peptides of Insulin. The pH 5.4 soluble B-chain fragments of insulin contained in the supernatant of the tryptic digests were purified by acidification (to pH 2.0), followed by centrifugation and gel filtration of the supernatant. The initial acidification of the supernatant resulted in the precipitation of some optically absorbing material which probably consisted of larger, pH 5.4 soluble fragments of insulin produced by trypsin (Laskowski *et al.*, 1956). Gel filtration was carried out by use of 1.5 × 42–43 cm Bio-Gel P-4 columns, washed, and equilibrated with pH 7.0, 0.1 M phosphate buffer. Purification of the peptides was achieved by applying 8–15-ml portions of the supernatant

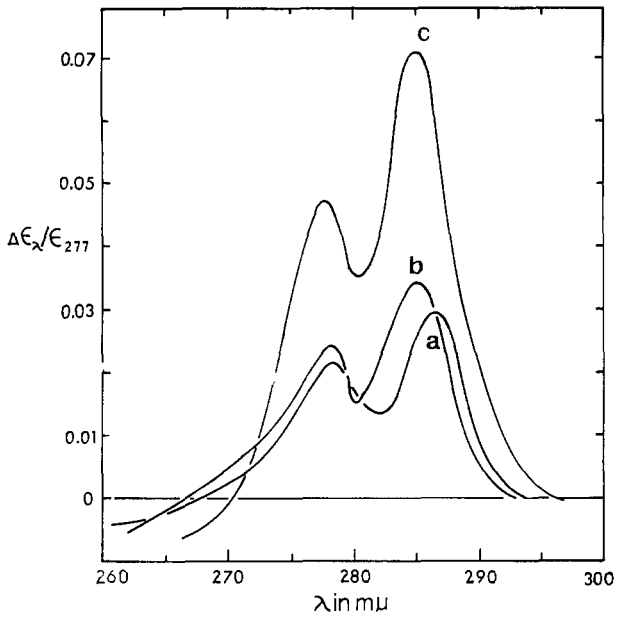

FIGURE 2: Representative perturbation difference spectra of insulin and desoctapeptide-insulin obtained with 20% ethylene glycol as perturbant. Curve a, silver–insulin, 0.05 M KNO_3, pH 7.2; curve b, desoctapeptide-insulin plus 2.4×10^{-4} M $AgNO_3$–0.05 M KNO_3, pH 7.5; curve c, 2-mercaptoethanol-reduced insulin in 8 M urea, 0.1 M Cl^-, and 1.5×10^{-3} M mercaptoethanol, pH 7.4. Protein concentration 0.08–0.1%.

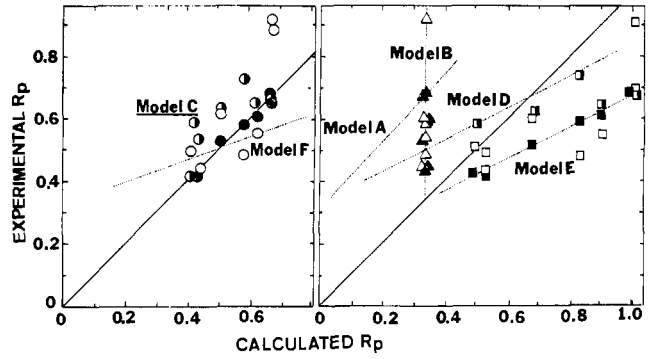

FIGURE 3: Correlation diagram showing the relation between the experimental and calculated, R_p, parameters for desoctapeptide-insulin in neutral, pH 7.0, 0.1 M Cl^- solutions (○, △, □) in pH 7.4, 0.05 M KNO_3 solutions (◑, ◧) and in pH 7.0–7.5, 0.05 M KNO_3 solutions containing stoichiometric quantities of silver ion (●, ▲, ■). The calculated R_p values (given in Tables IV) were based on two buried and one partly buried tyrosyl residue for model A; one exposed and two buried tyrosyl residues for model B (△, ▲); one buried, one exposed, and one partly buried for model C (○, ◑, ●); one buried and two partly buried for model D; one exposed and two partly buried for model E (□, ◧, ■); and three partly buried groups for model F. The solid lines drawn with a slope of one represent the maximally attainable correlation values. For reasons of clarity the data points of models A, D, and F, represented by dotted lines, have not been included.

adjusted to pH 7.0 ($A_{278\,m\mu}$ = 1.2–2.4), and collection and pooling of the central fractions of the effluent. As in the case of the desoctapeptide-insulin, 3-ml fractions were collected. The purified pooled peptide preparations were stored in the frozen state.

The purity of two desoctapeptide-insulin preparations, used for most of our difference spectral studies, is estimated at 92–97%, while the corresponding B-chain peptides resulting from the same preparations are found to be 95% pure (Table I). Amino acid analyses on which these estimates are based were kindly performed for us by Dr. Spyros Vratsanos of the Department of Microbiology, College of Physicians and Surgeons, Columbia University.

An interesting finding, related to the composition of the B-chain peptides (Figure 1), is that at the lower trypsin concentrations used, the main tryptic fragment produced seems to be the B-chain octapeptide (preparation I),[1] rather than the heptapeptide usually obtained with higher trypsin concentrations (preparation II). The latter peptide results from cleavage of both arginine–glycine bond 22–23 and lysine–alanine bond 29–30 at the C-terminal end of the B chain (Sanger and Tuppy, 1951; Young and Carpenter, 1961).

Experimental Methods. The difference spectral techniques employed and the preparation of solutions have been fully described in previous publications (Herskovits and Laskowski, 1962; Herskovits, 1967; Herskovits and Sorensen, 1968) and in the accompanying paper (Menendez *et al.*, 1969). Measurements were made on a Cary 14 recording spectrophotometer equipped with a 10× scale multiplier.

Optical rotatory dispersion and circular dichroism measurements were made in a Cary 60 recording spectropolarimeter equipped with a Cary 6002 circular dichroism attachment.

Insulin, desoctapeptide-insulin, and the tryptic peptide concentrations were based on absorption measurements using the per cent or molar extinction coefficients of 10.4 and 6080 (Weil *et al.*, 1965), 10.4 and 5000, and 1340, at 276–278 mμ, respectively.

Results

Solvent Perturbation of Desoctapeptide-insulin Core. The solvent perturbation data obtained on the two chromatographically purified desoctapeptide-insulin preparation, I and II are summarized in Table II. As in the case of the parent insulin studies, the perturbation data were analyzed in terms of exposed, buried, and partly buried tyrosyl residues, using the insulin *F* parameters of the accompanying paper (Menendez *et al.*, 1969). Typical solvent perturbation difference spectra, on which the data of this table are based, are shown in Figure 2. For reasons of comparison, the insulin perturbation difference spectra and the difference spectra of fully unfolded disulfide-cleaved insulin in 8 M urea obtained with the same perturbant, 20% ethylene glycol, have also been included in this figure. As described in the accompanying paper and in footnote *c* of Table III, the fraction of exposed tyrosyl residues was based on the disulfide-cleaved insulin data in 8 M urea, corrected for solvent effects.

What is readily apparent from this figure (see also Table IV) is the fact that the tyrosyl residues in the desoctapeptide core are on an average more exposed than in the parent

[1] Amino acid analysis of the unhydrolyzed peptide preparations revealed no significant quantities of free alanine. This argues against the possibility that the presence of alanine in the hydrolysate of our peptide preparation I (Table I) was due to our inability to separate the heptapeptide from free alanine by means of the Bio-Gel P-4 column used.

TABLE II: Summary of Difference Spectral Parameters, $\Delta\epsilon_{286-288}/\epsilon_{276-278}$, of Desoctapeptide-insulin (DOI).

Perturbant[a]	DOI Preparation I		DOI Preparation II	
	0.05 M NO_3^-, pH 7.4[b]	0.05 M NO_3^- + Ag^+, pH 7.0–7.5[c]	0.1 M Cl^-, pH 7.0[d]	0.05 M NO_3^- + Ag^+, pH 7.7–7.5[c]
90% deuterium oxide		−0.032		
20% sucrose	0.023	0.023	0.031 (0.030)	0.023
20% ethylene glycol	0.040	0.038	0.035	0.036
20% methanol	0.035	0.028	0.023	0.028
20% glycerol	0.039	0.033	0.038	0.035
20% hexaethylene glycol	0.070	0.060	0.063	0.058
10% dimethyl sulfoxide		0.030		0.028
20% dimethyl sulfoxide	0.084		0.073	

[a] 20 volumes of liquid perturbant was used per 100 volumes of final solution, with the exception of sucrose solutions that contained 21.6 g/100 ml of solution (w/w), dimethyl sulfoxide solutions that were 10 and 20% (v/v) and deuterium oxide solutions that were 90% (v/v). [b] pH 7.4, 0.05 M KNO_3–0.01 M Tris buffer. [c] pH 7.0–7.5, 0.05 M KNO_3–0.01 M Tris buffer plus stoichiometric quantities of added $AgNO_3$. [d] Preparation II, pH 7.0, 0.1 M KCl–0.01 M phosphate buffer. The data in parentheses refer to preparation I.

TABLE III: Summary of Differences Spectral Parameters of the Purified Tryptic Peptide of Insulin.[a]

Perturbant[b]	Preparation I			Preparation II		
		Av Tyr Exposure[c]			Av Tyr Exposure[c]	
	$\Delta\epsilon_{286-288}/\epsilon_{276-278}$	R_p	(R_M)	$\Delta\epsilon_{286-288}/\epsilon_{276-278}$	R_p	(R_M)
20% sucrose	0.036	1.06	(1.06)	0.033	0.97	(0.97)
20% ethylene glycol	0.067	1.07	(0.94)	0.062	1.00	(0.87)
20% glycerol	0.058	0.95	(0.91)	0.05	0.92	(0.88)
20% hexaethylene glycol	0.121	0.85	(0.90)	1.104	0.73	(0.77)
20% dimethyl sulfoxide	0.154	1.08	(0.91)	0.136	0.96	(0.80)

[a] pH 6.8, 0.1 M phosphate buffer. [b] See footnote *a* of Table II. [c] Calculated by use of the relations $R_p = \Delta\epsilon_{286-288}/\epsilon_{276-278}$ (protein in water)$/f \times \Delta\epsilon_{286-288}/\epsilon_{276-278}$ (disulfide-cleaved protein in 8 M urea), where $f = \Delta\epsilon_{286-288}/\epsilon_{276-278}$ (model mixture in water)$/\Delta\epsilon_{286-288}/\epsilon_{276-278}$ (model mixture in 8 M urea) and $R_M = \Delta\epsilon_{286-288}/\epsilon_{276-278}$ (protein in water)$/\Delta\epsilon_{286-288}/\epsilon_{276-278}$ (model mixture in water). The f values and model data are taken from the accompanying paper (Menendez *et al.*, 1969).

protein. The removal of the apparently buried tyrosyl residue B-26 gives rise to the modified protein core having one buried, one exposed, and one partly buried tyrosyl residues per monomer unit (model C of Table IV), as compared with two buried, one exposed, and one partly buried groups in the parent protein (Menendez *et al.*, 1969). As shown in Table IV and Figure 3 none of the other models considered by using other possible combinations of exposed, buried, and partly buried groups, gave sufficiently satisfactory fit of the experimental data, comparable with model C.

Mercola *et al.* (1967b) have presented ultracentrifugal evidence suggesting that above pH 7 desoctapeptide-insulin exists in the monomeric state. The studies of Gubensek and Rupley (1968) also indicate less extensive association in the acidic pH region. Unfortunately as in the case of Zn-free insulin in the absence of nitrate and silver ion (Menendez *et al.*, 1969), the scatter in the difference spectra obtained makes analysis and interpretation of the data more difficult.

The Tyrosyl and Phenylalanyl Perturbation Difference Spectra of the B-Chain Tryptic Peptide of Insulin. The difference spectra of the chromatographically purified tryptic fragments of the B chain, containing tyrosyl residue B-26, have also been studied and analyzed (Table III and Figure 4). The data of Table III indicate that once this fragment is released the unreactive and presumably buried tyrosyl residue B-26 (Aoyama *et al.*, 1965) becomes essentially fully exposed, giving perturbation parameters ($\Delta\epsilon_{286-288}/\epsilon_{276-278}$) or R_p values comparable with those of free tyrosine. This is suggested by the fact that the ($\Delta\epsilon_{286-288}/\epsilon_{276-278}$) values relative to those of the fully unfolded protein, summarized in the last two columns of this table, are very nearly equal to unity.

Two of three phenylalanyl residues of insulin are also part of the tryptic fragment released. The difference spectra

TABLE IV: Comparison of Theoretical and Experimental Values of the Apparent Fraction of Exposed Tyrosyl Residues, R_P, in Desoctapeptide-insulin (DOI).[a]

Perturbant[b]	Model A[c]	Model B[d]	Model C[e]	Model D[f]	Model E[g]	Model F[h]	DOI-Prep I: 0.05 M NO_3^-, pH 7.4	DOI-Prep I: 0.05 M NO_3^- plus Ag^+[i]	DOI-Prep II: 0.1 M KCl[j]	DOI-Prep II: 0.05 M NO_3^- plus Ag^+[i]
90% deuterium oxide	0.33	0.33	0.68	0.67	1.00	1.00		0.68		
20% sucrose	0.33	0.33	0.67	0.67	1.00	1.00	0.68	0.68	0.91	0.69
20% ethylene glycol	0.28	0.33	0.62	0.57	0.90	0.85	0.65	0.61	0.55	0.58
20% methanol	0.25	0.33	0.58	0.49	0.83	0.74	0.74	0.59	0.48	0.59
20% glycerol	0.17	0.33	0.50	0.33	0.67	0.50	0.64	0.53	0.61	0.57
20% hexaethylene glycol	0.10	0.33	0.42	0.19	0.52	0.29	0.49	0.42	0.44	0.41
10% dimethyl sulfoxide	0.08	0.33	0.41	0.16	0.49	0.24		0.42		0.45
20% dimethyl sulfoxide	0.08	0.33	0.41	0.16	0.49	0.24	0.59		0.52	

[a] Calculated by use of the relation $\Sigma n_i R_i / \Sigma n_i$, where n_i represents the number of tyrosyls of each class i. The R_i values used were 1 for exposed groups, 0 for buried groups, and $R_i = F$ for partly buried groups (Herskovits and Laskowski, 1968). The F values used were taken from the accompanying paper (Menendez *et al.*, 1969). [b] See footnote *a* of Table II. [c] Two buried and one partly buried. [d] One exposed and two buried. [e] One exposed, one buried, one partly buried. [f] One buried, two partly buried. [g] One exposed, two partly buried. [h] Three partly buried. [i] pH 7.0–7.5, 0.05 M KNO_3–0.01 M Tris buffer plus stoichiometric quantities of added $AgNO_3$. [j] pH 7.0, 0.1 M Cl^-–0.01 M phosphate buffer.

TABLE V: Analysis of the Phenylalanyl Perturbation Difference Spectra of the B-Chain Tryptic Peptide of Insulin and an Equimolar Desoctapeptide-insulin–Tryptic Peptide Mixture Obtained with 20% Dimethyl Sulfoxide.

Location of the Phe Maxima and Minima (mμ)		$(\Delta\epsilon_{\lambda_{max}} - \Delta\epsilon_{\lambda_{min}})$				
λ_{max}	λ_{min}	Uncor Exptl Values	Contributions Due to Tyr[a]	Cor Exptl Values[b]	Model Values/mole of Phe[c]	No. of Phe Groups Exposed Rel to Model[c]
			Tryptic Peptide[d]			
265–266	267–268	23	0	23	12	1.92
258–260	263–265	39	6.0	33	17	1.94
253–254	256–258	39	3.5	35.5	15.5	2.28
247–249	251–252	23	5.0	18	9.0	2.00
					Average value	2.0 ± 0.15
			Equimolar Tryptic Peptide-desoctapeptide-insulin Mixture[e]			
265–266	267–268	19	0	19	12	1.60
258–260	263–265	40	16	24	17	1.35
253–254	256–258	39	9.3	29.7	15.5	1.90
247–249	251–252	34	13	21	9.0	2.20
					Average value	1.76 ± 0.30

[a] Values based on the molar absorbance difference value of *N*-acetyl-L-tyrosine ethyl ester taken from the data of Herskovits and Sorensen (1968). For the peptide this constitutes 1 mole of tyrosine while for the desoctapeptide-tryptic peptide mixture 2.65 moles of tyrosyls are involved (see text). [b] The uncorrected experimental value minus the value of 1 mole of tyrosine for the tryptic peptide. For the peptide-desoctapeptide-insulin mixture the uncorrected value minus the value for 2.65 moles of tyrosine. [c] Values per 1 mole of *N*-acetyl-L-phenylalanine ethyl ester. [d] Tryptic peptide (preparation I), pH 6.8, 0.1 M phosphate buffer. [e] Equimolar mixture of purified peptide and desoctapeptide-insulin (preparation III), pH 7.6, 0.05 M phosphate buffer.

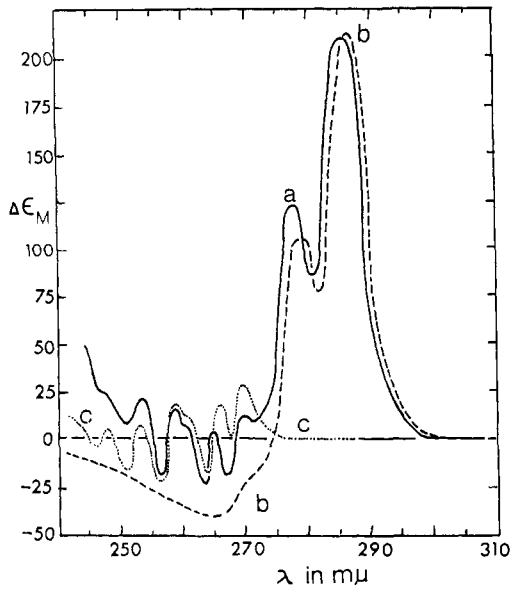

FIGURE 4: Comparison of the difference spectra of the purified tryptic octapeptide (preparation I) with the free tyrosyl and phenylalanyl difference spectra, obtained with 20% dimethyl sulfoxide. The data are represented as molar absorbance difference values $\Delta\epsilon_M$. Curve a, tryptic peptide, pH 7.0, 0.1 M phosphate buffer; curve b, 1 mole of *N*-acetyl-L-tyrosine ethyl ester (data taken from Herskovits and Sorensen, 1968); curve c, 2 moles of *N*-acetyl-L-phenylalanine ethyl ester, pH 6.8, 0.1 M phosphate buffer. Peptide concentration 0.020%.

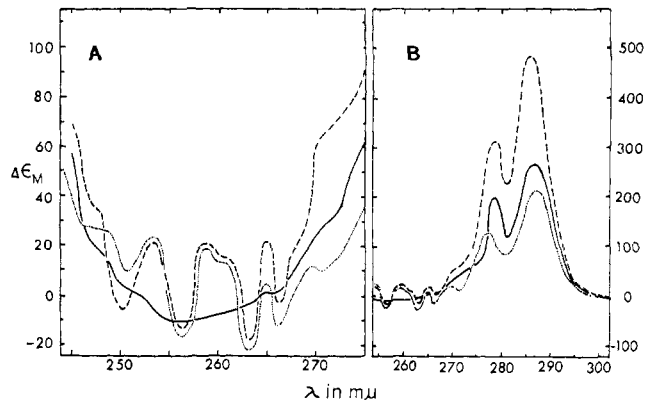

FIGURE 5: The phenylalanyl and tyrosyl perturbation difference spectra of insulin tryptic peptide and equivalent mixture of purified tryptic peptide and desoctapeptide-insulin. The perturbant used was 20% dimethyl sulfoxide. (——) Insulin pH 2.1, 0.1 M Cl^-; (· · · · ·) tryptic peptide, pH 7.0, 0.1 M phosphate buffer; (– – – –) tryptic peptide-desoctapeptide-insulin mixture, pH 7.6, 0.035 M phosphate buffer. Protein concentration, 0.1–0.33%.

of the tryptic B-chain peptide clearly show the five maxima and minima in the 240–270-mμ spectral region characteristic of free phenylalanine (Laskowski *et al.*, 1956; Cross and Fisher, 1966; Herskovits, 1967). A quantitative comparison of the phenylalanyl difference spectra of the tryptic fragment with 2 moles of phenylalanine acetyl ethyl ester is shown in the same figure. The difference spectra were obtained with 20% dimethyl sulfoxide, since this perturbant is known to produce the largest spectral shifts.

The data of this figure afford a useful illustration related to the analysis of phenylalanyl perturbation data. Because the difference spectra of this chromophore are usually superimposed on a curved base line resulting in part from the tail-end contributions of tyrosyl residues in the 240–270-mμ region, it was felt that measuring the molar absorbance differences from minima to maximn of the four phenylalanyl difference spectral peaks in this wavelength region would offer the best solution of this problem. The perturbation parameters calculated for the tryptic peptide and for 1 mole of *N*-acetyl-L-phenylalanine ethyl ester are given in the third and sixth columns of Table V. With a single mole of exposed tyrosine present in the peptide (Table III), it was necessary to correct for its contribution to the observed ($\Delta\epsilon_{\lambda_{max}} - \Delta\epsilon_{\lambda_{min}}$) values. This requires the subtraction of the tyrosyl perturbation at each phenylalanyl wavelength maximum and minimum. The required ($\Delta\epsilon_{\lambda_{max}} - \Delta\epsilon_{\lambda_{min}}$) values per mole of tyrosine (based on the *N*-acetyl-L-tyrosine ethyl ester data) are given in column four. The fraction of exposed phenylalanyl residues in the tryptic peptide were calculated by dividing the corrected peptide perturbation parameter ($\Delta\epsilon_{\lambda_{max}} - \Delta\epsilon_{\lambda_{min}}$) by the ($\Delta\epsilon_{\lambda_{max}} - \Delta\epsilon_{\lambda_{min}}$) values for 1 mole of *N*-acetyl-L-phenylalanine ethyl ester. The corrected peptide data and estimates of the fraction of exposed phenylalanyl residues at the four phenylalanyl difference spectral maxima considered are given in the last columns of this table. The exposure of 2.0 (±0.15) moles of phenylalanine indicates that the phenylalanyl residues in the tryptic peptide are fully exposed. This is to be expected since the tryptic peptides of insulin are relatively short, consisting of only seven or eight amino acids.

Exposure of the buried or partly buried phenylalanyl residues accompanying the release of this peptide on digestion of insulin with trypsin would explain the prominent phenylalanyl difference spectra observed by Scheraga and coworkers (Laskowski *et al.*, 1956, 1960). The perturbation difference spectra of insulin and desoctapeptide-insulin in the 240–270-mμ region reveal no clearly discernible phenylalanyl difference spectra, suggesting that the three phenylalanine residues in native insulin must be largely buried (see Figures 2 and 5). A quantitative analysis of the phenylalanyl data similar to that of the tryptic peptide was also carried out on equimolar mixtures of purified desoctapeptide-insulin and tryptic peptide (Table V). In this case the necessary tyrosyl correction constitutes 2.65 moles of tyrosine. This value is based on the tyrosyl perturbation data of the mixture at the 286–288 mμ (Figure 5B). The estimated average value of phenylalanyl exposure of 1.76 ± 0.3 for the mixture represent to some extent the increased experimental uncertainties involved due to larger tyrosyl contributions to the perturbation difference spectra in the 240–270-mμ region. The phenylalanyl exposure of less than two groups may also suggest the possibility that some association between the desoctapeptide-insulin core and the tryptic peptide does occur.

Optical Rotatory Dispersion and Circular Dichroism Studies. In order to gain further information concerning the possible effects of trypsin on the secondary and tertiary structure of insulin the optical rotatory dispersion and circular dichroism spectra of desoctapeptide-insulin, the purified tryptic peptides of the B chain, and the intact parent insulin were investigated and compared. The results of Figure 6

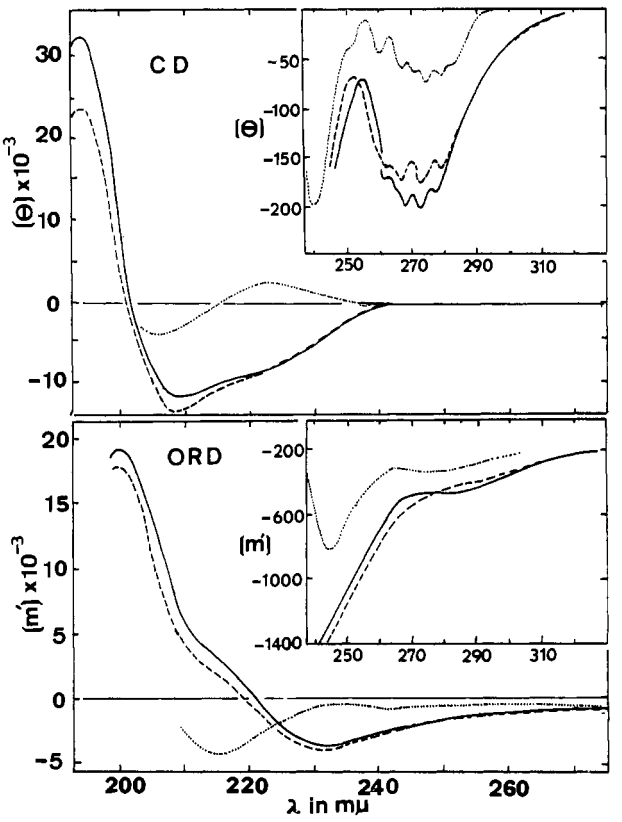

FIGURE 6: The optical rotatory dispersion and circular dichroism of Zn-free insulin, desoctapeptide-insulin, and the purified heptapeptide of insulin. (———) Zn-free insulin, 0.1 M Cl^-, and 0.01 M phosphate buffer, pH 6.8. (——) Desoctapeptide-insulin, 0.1 M Cl^-, and 0.01 M phosphate buffer, pH 7.4. (·····) Insulin-heptapeptide, pH 7.0, 0.1 M phosphate buffer. Protein concentrations, 0.0047–0.053%.

indicate that the optical rotatory dispersion and circular dichroism spectra of insulin and desoctapeptide-insulin are closely similar, suggesting that the core conformation of insulin has been largely preserved in the process of limited tryptic hydrolysis. The intensities of the 230–233-mμ rotatory dispersion and 222- and 209-mμ dichroic bands, associated with the polypeptide conformation, are essentially identical. The somewhat lower value of the optical rotation at the 198–200-mμ band may reflect the loss of some α-helical organization.

Analysis of the 313–578-mμ rotatory dispersion data of Zn- and Zn-free insulin obtained in the pH 6.8–7.8 region, in 0.1 M phosphate buffer gave Moffitt–Yang b_0 parameters (Moffitt and Yang, 1956) ranging from 150 to 160°. The corresponding value in acidic solutions, pH 2.1–2.2, 0.1 M Cl^-, were 190–220°. The b_0 of the two desoctapeptide-insulin preparations I and II in the neutral pH region were essentially the same as those of the parent hormone, namely, 150–160°, suggesting again that trypsin digestion produced no drastic changes in the insulin conformation.

The circular dichroism and optical rotatory dispersion spectra of insulin and desoctapeptide-insulin in the aromatic region (Figure 6) exhibit moderate difference, reflecting the fact that one of the B-chain tyrosyl residues has been liberated by trypsin. The spectra and rotatory parameters of the tryptic peptide (Figure 6) suggest that the liberated peptide has suffered some conformational alterations, probably as a result of relaxation of some of the steric constrains imposed by the three-dimensional structure of the insulin core. The b_0 values of +30 and +70° obtained on the tryptic peptide preparations I and II (Menendez, 1969) and the relatively low intensities of the optical rotatory and circular dichroic bands in the ultraviolet region (represented by the dotted lines in Figure 6) indicate that the isolated tryptic peptides are largely devoid of α-helical organization (Urnes and Doty, 1961; Holzworth *et al.*, 1962; Beychok, 1967).

Discussion

Analysis of the perturbation data of desoctapeptide-insulin in neutral solutions (Table IV and Figure 3) suggests that the most satisfactory fit of the data result from the combination of one exposed, one partly buried, and one buried tyrosyl residue. Since the perturbation parameters of the parent hormone were best fitted with one additional buried tyrosyl, *i.e.*, with two buried, one exposed, and one partly buried group (Menendez *et al.*, 1969), it would appear that tyrosyl B-26, removed by trypsin as part of the released B-chain peptide (Figure 1), is one of the two unreactive (Aoyama *et al.*, 1965) and presumably buried groups. The other unreactive group toward the reagent cyanuric fluoride, identified by Aoyama *et al.* (1965), is residue A-14.

The possibility that the released tyrosyl B-26 could be fully or partly exposed has also been considered. Models A and B were calculated on the basis of such assumptions. In addition, the possibility that removal of the peptide could result in exposure of previously buried or partly buried groups have been entertained. Models D and F were calculated on the basis of such considerations. The tyrosyls described by model C could result from release of the single exposed group with subsequent uncovering and exposure of one of the two buried groups. Similarly, the disposition of tyrosyl in model E could be due to the release and removal of a buried tyrosyl, accompanied by partial exposure of the second buried group. The location of the tyrosyls in model E could also be the result of the loss of a partly buried group and the subsequent exposure of the two buried groups. Such combination of groups could also arise as a result of conformational alteration or other sequence of events not considered by us.

Despite the somewhat greater scatter of the 0.1 M chloride data than the corresponding data obtained in 0.05 M nitrate solutions, none of the models, with the exception of model C, gives a sufficiently close correlation or fit of the experimental and calculated perturbation parameters to require serious consideration. An especially good fit for model C is obtained with the nitrate data containing silver ion (represented by close circles in Figure 3A). It is significant that the removal of the buried tyrosyl B-26 from insulin, required by this model, is also in agreement with the difference spectral data of Scheraga and coworkers (Laskowski *et al.*, 1956, 1960; Leach and Scheraga, 1960) provided we assume that the bound and presumably buried tyrosyl B-26 and phenylalanyls B-24 and B-25 become exposed in the course digestion with trypsin.

The fact that solvent perturbations of the three phenyl-

alanyl groups in insulin are largely absent (Figure 5A) and that two groups are nearly fully perturbed following trypsin digestion and exposure of phenylalanyl residues B-24 and B-25 in the released B-chain tryptic peptide, supports this contention. This is suggested by the perturbation data of the purified and recombined tryptic peptide and desoctapeptide-insulin, analyzed in Table V. Interestingly, the buried and chemically unreactive tyrosyl residue B-26 is in the same sequence of buried hydrophobic groups released by trypsin. This tyrosyl has been identified as one of the two groups unreactive toward cyanuric fluoride, the other unreactive group being tyrosyl A-14 in the A chain (Aoyama *et al.*, 1965).

To some extent, the strength of these contentions depend upon the assumptions that the secondary and tertiary structure of insulin core, other than the removal of the B-chain terminal peptides, remains unaltered during both the process of tryptic digestion and the ensuing preparative steps employed in the isolation of desoctapeptide-insulin. Moderate changes in the structure of the insulin core may indeed have occurred, as has been suggested by the circular dichroism studies of Mercola *et al.* (1967a) on bovine insulin and desoctapeptide-insulin. Our own circular dichroism and optical rotatory dispersion measurements, while not strictly comparable with the studies of Mercola *et al.* (1967a,b), because of the milder, neutral pH conditions employed in most of our work and the fact our desoctapeptide-insulin preparations were not subjected to 7 M urea treatment,[2] would indicate only slight changes in the structure of insulin following trypsin digestion and purification. Changes in the 222- and 209-mμ circular dichroism bands and the 230–233- and 195–198-mμ optical rotatory dispersion minima and maxima associated with the conformation of the polypeptide chain in proteins (Urnes and Doty, 1961; Holzwarth *et al.*, 1962; Beychok, 1967) suggest only marginal changes in the core conformation of insulin. In contrast, in the case of RNase and its subtilisin-modified s-protein derivative (which has 20 amino acids missing from the N-terminal end of RNase) appreciably greater changes in the optical rotatory dispersion and circular dichroism parameters have been noted[3] (Herskovits and Menendez, 1969). The latter changes reflect the loss of one of the three helical segments shown by the X-ray structure of RNase (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967).

[2] Mercola *et al.* (1967a) used 2% trypsin (2:100, w/w) for the digestion of Zn-free insulin at pH 9.5 and 37° (4 hr). They employed DEAE-Sephadex (A-25) with 7 M urea and a salt gradient for the chromatographic separation and purification of desoctapeptide-insulin. In our procedure the milder digestion method of Young and Carpenter (1961) was employed, using 1 or 2% purified trypsin or L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone-trypsin, with the additional difference that the digestion was carried out at pH 8.4 and room temperature (see Methods).

[3] The Zn-free insulin and the three desoctapeptide-insulin preparations have mean residue rotations, $[m']_\lambda$, of -3500 ± 200 and -3700 to $-3800°$ at 233 mμ, and mean residue ellipticities, $[\theta]_\lambda$, at the 209-mμ extremum of $-11,600 \pm 1,000$ and $-12,600$–$14,400$ deg cm^2 per dmole, respectively. These values are not significantly different when compared with the values obtained on RNase and the s-protein with $[m']_\lambda$ values of -4000 ± 200 and $-3200°$ at the 226–229-mμ trough and $[\theta]_\lambda$ values of -9200 ± 500 and -6250 deg cm^2 per dmole at 209 mμ (Herskovits and Menendez, 1969).

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