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

Celia J. Menendez,† Theodore T. Herskovits, and Michael Laskowski, Jr.

ABSTRACT: The location of tyrosyl residues in insulin were investigated by the solvent perturbation technique of difference spectroscopy.

Together with the results of the urea-denatured protein obtained with a number of perturbants of varying dimensions and range effects, the solvent perturbation data were examined in terms of a number of alternative models. The data obtained on zinc and zinc-free insulin were found to be most consistent with a model having one exposed, two buried, and one partly buried tyrosyl residues. Zn-free insulin in the acidic pH region, where it is largely a dimer, gave results consistent with a model having two to three exposed, three to four buried, and two to three partly exposed tyrosyl residues.

The environment and location of the four tyrosyl residues in insulin have been the subject of a number of investigations (Laskowski *et al.*, 1956, 1960; Leach and Scheraga, 1960; Inada, 1961; De Zoeten and De Bruin, 1961; De Zoeten and Havinga, 1961; Kurihara *et al.*, 1963; Aoyama *et al.*, 1965; Riordan *et al.*, 1965; Weil *et al.*, 1965; Herskovits, 1965; Sokolovski *et al.*, 1966; Cory and Frieden, 1967; Covelli and Wolff, 1967). The difference spectral studies of Scheraga and coworkers (Laskowski *et al.*, 1956, 1960; Leach and Scheraga, 1960) have suggested that two of the four tyrosyls in insulin are "bound" and hydrogen bonded, while the remaining two are "free" and presumably exposed. Shibata and coworkers (Kurihara *et al.*, 1963; Aoyama *et al.*, 1965) have identified the latter tyrosyl residue as A-19 together with the second "free" B-16 residue by use of tyrosyl-specific reagent, cyanuric fluoride. These two tyrosyls were found to react with this reagent, whereas residues A-14 and B-26 were found to be unreactive. The studies of Sokolovski *et al.* (1966) with another tyrosyl-modifying reagent, tetranitromethane, have also indicated that two of the tyrosyls are again in the unreactive class. Together with the earlier solvent perturbation studies obtained with 20% sucrose and dimethyl sulfoxide (Weil *et al.*, 1965; Herskovits, 1965) these studies suggest that the two unreactive tyrosyl residues are buried in the interior folds of native insulin. On the other hand, the reactivity of all four tyrosyls with *N*-acetylimidazole (Riordan *et al.*, 1965) as well as the more extensive iodination (Gruen *et al.*, 1959; De Zoeten and De Bruin, 1961; De Zoeten and Havinga, 1961; Covelli and Wolff, 1967) and susceptibility of these residues to oxidation by tyrosinase (Cory and Frieden, 1967) suggest that this simple description of the location of the tyrosyls may be an oversimplification.

The presence of partly exposed groups accessible and reactive with certain chemical reagents or perturbants but not to others because of steric or other conformational factors (Witkop, 1961; Gorbunoff, 1967; Cohen, 1968) peculiar to insulin may offer an explanation for this apparently anomalous behavior of this protein.

In the hope of presenting a clearer picture concerning the location of the tyrosyl residues in insulin, we have examined the solvent perturbation difference spectra of Zn-and Zn-free insulin. The accompanying paper (Menendez and Herskovits, 1969) presents a similar study of desoctapeptide-insulin and the isolated peptide fragments of the B chain obtained by tryptic digestion of insulin (Nicol and Smith, 1956; Young and Carpenter, 1961).

Experimental Section

Materials. Two preparations of bovine Zn-insulin of essentially identical difference spectral and optical rotatory properties (Herskovits, 1965) were employed in this study. One sample was a Mann Research Laboratories product, while a second was obtained from the Eli Lilly Laboratories through the courtesy of Dr. Otto K. Behrens. Zn-free insulin was prepared by the method of Sluyterman (1955). *N*-Acetyl ethyl esters of tyrosine and phenylalanine were purchased from Mann Research Laboratories. Polyethylene glycol (Carbowax 300) was a Union Carbide product, whereas deuterium oxide (99.8%) was a Bio-Rad Laboratories product. All other perturbants and reagents were spectroscopic grade or of the purest commercially available quality.

Methods. The preparation of most solutions and the difference spectral techniques employed have been fully described in previous publications (Herskovits and Laskowski, 1962; Herskovits, 1967). Measurements were made in a Cary 14 recording spectrophotometer (at 25 ± 1°), equipped with a scale multiplier which permits the expansion of the optical density scale by a factor of 10. Most of the measurements were made with use of the scale expander in matched 1.00-cm path-length cylindrical double cells. Before each set of experiments the base line was adjusted with appropriate solvent and perturbant blanks as previously described (Herskovits and Laskowski, 1962; Herskovits, 1967). Base-line adjustments were especially critical in the case of insulin in KNO_3 solutions, because of the fairly strong ultraviolet

* From the Department of Chemistry, Fordham University, New York, New York 10458, and the Department of Chemistry, Purdue University, Lafayette, Indiana 47907. *Received June 2, 1969.* This work was supported by Research Grants GM 10831 and GM 14468 from the National Institute of Health, U. S. Public Health Service.

† Part of this work was taken from the thesis for the Ph.D. degree, Fordham University, 1969. A preliminary report of parts of this work has appeared (Menendez and Herskovits, 1968). Present address: Department of Chemistry and Biological Sciences, Columbia University, New York City, N. Y.

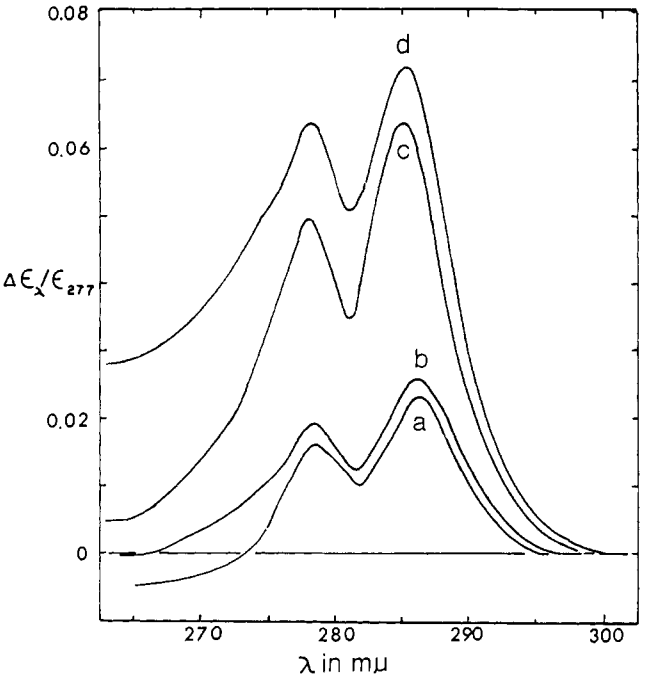

FIGURE 1: Solvent perturbant difference spectra of native and urea-denatured insulin. Perturbant, 20% (v/v) glycerol curve a, silver–insulin, 0.05 M KNO_3 (pH 6.7), and 1.8×10^{-4} M Ag^+; curve b, Zn-insulin, 0.1 M Cl^- (pH 2.5); curve c, Zn-free insulin in 8 M urea–0.1 M Cl^- (pH 7.0); curve d, mercaptoethanol-reduced insulin in 8 M urea, 0.1 M Cl^-, and 1.5×10^{-2} M 2-mercaptoethanol (pH 3.7). Insulin concentration, 0.066–0.095%.

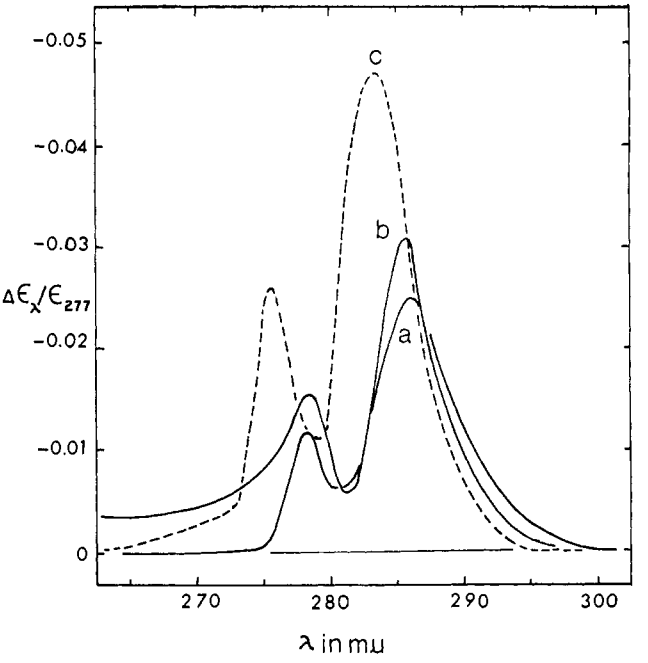

FIGURE 2: Solvent perturbation difference spectra of insulin and insulin model compound mixture produced by 90% deuterium oxide. Curve a, silver–insulin, 0.05 M KNO_3 (pH 7.1), and 2.4×10^{-4} M Ag; as curve b, Zn-free insulin, 0.1 M Cl^- (pH 1.8); curve c, insulin model compound mixture consisting of 1×10^{-3} M *N*-acetyl-L-tyrosine ethyl ester and 0.75×10^{-3} M *N*-acetyl-L-phenylalanine ethyl ester–0.01 M Cl^- (pH 7.0). Protein concentration, 0.082–0.12%.

absorption of the nitrate ion in the wavelength region of interest. Potassium nitrate solutions were found to have a broad absorption maximun centering at 300–302 mμ with a molar extinction coefficient, ϵ_M, of about 7.0. For these reasons, the careful adjustment of the water and perturbant blanks with respect to both nitrate and silver ion concentration were found to be necessary. In all cases dilutions of both solvent and perturbant blanks were made from common freshly prepared KNO_3 and $AgNO_3$ solutions. The same KNO_3 stock solutions were used to prepare solutions in KNO_3 and solutions containing stoichiometric quantities of silver ion (Marcker and Graae, 1961). These solutions were usually prepared by dissolving insulin in cold 0.1 M KNO_3 adjusted to pH 8.5 with concentrated KOH. Experiments were usually carried out after overnight equilibrations in the cold. Difference spectral measurements on solutions containing Ag^+ were made about 1 hr after the addition of $AgNO_3$ to the insulin solutions. Insulin concentrations were based on absorption measurements using the per cent or molar extinction coefficient of 10.4 and 6080 at 278 mμ (Weil *et al.*, 1965).

Optical rotatory dispersion measurements were performed on a Cary 60 recording spectropolarimeter. Measurements on Zn-free insulin in aqueous solutions and in the presence of 20% ethylene glycol, glycerol, hexaethylene glycol, methanol, and 90% deuterium oxide indicated no significant changes in mean residue rotation at 233 mμ, suggesting that these perturbants had no adverse effect on the conformation of insulin. The mean residue rotation, $[m']_{233}$, obtained on these solutions in the presence of 0.1 M Cl^- and 0.01 M phosphate buffer (pH 6.9), was equal to $-3600 \pm 100°$. In comparison the $[m']_{233}$ value of denatured insulin in 8 M urea (pH 3.6) was $-2470°$.

Results

Solvent Perturbation Studies. Typical solvent perturbation difference spectra, characteristic of tyrosine-rich proteins, are shown in Figures 1 and 2. The 20% glycerol difference spectra have positive absorbance difference values at the major 286–288- and 278–281-mμ tyrosine peaks, characteristic of most of the perturbants employed (Table I). These perturbations are due to red shifts or solvent shifts to longer wavelength, in contrast to the blue shifts produced by 90% deuterium oxide (Laskowski, 1966), shown in Figure 2. As in our previous studies (Herskovits and Laskowski, 1962, 1968; Williams *et al.*, 1965) both unfolded, disulfide-cleaved protein data obtained in 8 M urea and the model mixture data were used for the quantitative evaluation of the fraction and apparent number of tyrosyls exposed to various perturbants. Some of the latter protein and model mixture data are shown in the same two figures.

Tables I and II present a summary of the difference spectral data, obtained on Zn- and Zn-free insulin in various solvents, insulin in 8 M urea and mercaptoethanol-reduced insulin in 8 M urea, together with insulin model mixture data (consisting of 4 to 3 molar mixtures of tyrosine and phenylalanine) in both aqueous and 8 M urea solutions. The difference spectral data are expressed in terms of the reduced difference spectral parameter, $\Delta\epsilon_{286-288}/\epsilon_{276-278}$, where $\Delta\epsilon_{286-288}$ is the molar absorptivity difference at the first tyrosine maximum (or minimum in case of 90% D_2O) at 286–288 mμ and $\epsilon_{276-278}$ is

TABLE I: Summary of Difference Spectral Parameters, $\Delta\epsilon_{286-288}/\epsilon_{276-278}$, of Zn-Insulin and Zn-Free Insulin.

	Zn-Insulin			Zn-Free Insulin			
Perturbant[a]	0.1 M Cl^-, pH 1.8–2.2	0.1 M Cl^-, pH 7.0–8.0	0.05 M KNO_3 + Ag^+, pH 7.2–7.7[b]	0.1 M Cl^-, pH 1.8–2.2	0.1 M Cl^-, pH 7.0–7.5	0.05 M KNO_3, pH 7.2–7.7[c]	0.05 M KNO_3 + Ag^+, pH 7.2–7.7[b]
90% deuterium oxide	−0.027			−0.031	−0.025		−0.024
20% sucrose	0.020	0.026	0.018	0.023	0.027	0.019	0.018
20% glycerol	0.026	0.033	0.023	0.032	0.031	0.023	0.023
20% ethylene glycol	0.027	0.038	0.029	0.031	0.032	0.028	0.028
20% methanol	0.021	0.040		0.020	0.042	0.029	0.023
20% hexaethylene glycol	0.048	0.049	0.046	0.054	0.047	0.049	0.045
10% dimethyl sulfoxide	0.027	0.032	0.023	0.027	0.025	0.021	0.023

[a] 20 volumes of liquid perturbants 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% (v/v), and deuterium oxide solutions that were 90% (v/v). [b] Insulin in the presence of stoichiometric quantities of silver nitrate. [c] In presence of 0.01 M Tris buffer.

TABLE II: Difference Spectral Results of Urea and Disulfide-Cleaved Insulin and Their Model Mixture Analog.

	$\Delta\epsilon_{286-288}/\epsilon_{276-278}$			
Perturbant[a]	Insulin[b] in 8 M Urea	2-Mercaptoethanol[c]-Reduced Insulin in 8 M Urea	Model Mixture[d] Water	Model Mixture[d] 8 M Urea
90% deuterium oxide			−0.047	
20% sucrose	0.047	0.047	0.034	0.047
20% glycerol	0.063	0.072	0.064	0.076
20% ethylene glycol	0.069	0.071	0.071	0.081
20% methanol	0.058	0.062	0.055	0.072
20% hexaethylene glycol	0.101	0.123	0.135	0.117
10% dimethyl sulfoxide	0.060	0.074	0.085	0.089

[a] See footnote *a* of Table I. [b] $\Gamma/2 = 0.1$, pH 3.5–7.8. [c] $\Gamma/2 = 0.1$, pH 7.0. [d] Mixtures of *N*-acetyl ethyl esters of tyrosine and phenylalanine, molar ratio = 4:3.

the molar absorptivity of the protein or model mixture at 276–278 mμ (Herskovits and Laskowski, 1962; Herskovits, 1967).

Tables III and V present detailed analyses of the difference spectral data of both Zn- and Zn-free insulin obtained in the neutral and acidic pH regions in terms of exposed, partly buried, and buried tyrosyl residues. The fraction of exposed tyrosyls (R_P and R_M) has been calculated on the basis of both the model mixture data and the unfolded disulfide-cleaved protein data, corrected for solvent effects (Williams *et al.*, 1965; Herskovits, 1967). The fraction of exposed groups can be simply expressed as

$$R_P = \frac{\Delta\epsilon_{286-288}/\epsilon_{276-278}\ (\text{protein})}{f \times \Delta\epsilon_{286-288}/\epsilon_{276-278}\ (\text{disulfide-cleaved protein in 8 M urea})} \quad (1)$$

$$R_M = \frac{\Delta\epsilon_{286-288}/\epsilon_{276-278}\ (\text{protein})}{\Delta\epsilon_{286-288}/\epsilon_{276-278}\ (\text{model mixture})} \quad (2)$$

The factor f in eq 1 corrects for the effects of 8 M urea on the reduced parameter $\Delta\epsilon_{286-288}/\epsilon_{276-278}$, which is required to keep the disulfide-cleaved protein dissolved and in fully unfolded random conformation (Herskovits and Laskowski, 1962, 1968). It is equal to the ratio of this parameter for the model mixture in water and in 8 M urea, $\Delta\epsilon_{286-288}/\epsilon_{276-278}$ (model mixture in H_2O)/$\Delta\epsilon_{286-288}/\epsilon_{276-278}$ (model mixture in 8 M urea). The R_p entries of the last columns of these tables are based on the silver–insulin data of Table I used in conjunction with the disulfide-cleaved protein and model data of Table II.

As in our recently published studies on ribonuclease (Herskovits and Laskowski, 1968), the various models considered for the tyrosyls in insulin in these tables were based on esti-

TABLE III: Comparison of Theoretical and Experimental Values of the Apparent Fraction of Exposed Tyrosyl Residues, R_P, in Insulin.[a]

Perturbant[b]	Model A[c]	Model B[d]	Model C[e]	Model D[f]	Model E[g]	Zn-Free Insulin: 0.1 M Cl^-, pH 7.0–7.5	Zn-Free Insulin: 0.05 M NO_3^-, pH 7.2–7.7[h]	Zn-Free Insulin: 0.05 M NO_3^- + Ag^+ + 7.2–7.7[i]	Zn-Insulin: 0.1 M Cl^-, pH 7–8.0	Zn-Insulin: 0.05 M NO_3^- + Ag^+, pH 7.2–7.7[i]
90% deuterium oxide	0.74	0.73	0.49	0.48	0.72	0.53		0.51		
20% sucrose	0.75	0.75	0.50	0.50	0.75	0.79	0.56	0.53	0.76	0.53
20% ethylene glycol	0.71	0.67	0.46	0.48	0.64	0.52	0.45	0.45	0.61	0.47
20% glycerol	0.62	0.50	0.38	0.25	0.38	0.51	0.38	0.38	0.54	0.38
20% methanol	0.68	0.62	0.44	0.37	0.55	0.88	0.61	0.48	0.84	
20% hexaethylene glycol	0.57	0.39	0.32	0.15	0.20	0.33	0.35	0.32	0.34	0.32
10% dimethyl sulfoxide	0.56	0.37	0.31	0.12	0.18	0.35	0.30	0.32	0.45	0.32

[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. For exposed groups $R_i = 1$, for buried groups $R_i = 0$, and for partly buried groups $R_i = F$. The experimental F values used are taken from column 4 of Table IV. [b] See footnote *a* of Table I. [c] Two exposed, one buried, and one partially buried. [d] One exposed, two partially buried, and one buried. [e] One exposed, two buried, and one partially buried. [f] Two buried and two partially buried. [g] One buried and three partly buried. [h] In 0.05 M KNO_3 and 0.01 M Tris buffer. [i] In 0.05 M KNO_3 plus stoichiometric quantities of silver nitrate.

TABLE IV: Difference Spectral Parameters of Insulin in 8 M Urea.

Perturbant[a]	Insulin in 8 M Urea: $(\Delta\epsilon_{286-288}/\epsilon_{276-278})$[b]	Insulin in 8 M Urea: Fraction of Accessible Tyrosyls,[c] R_p	Tyrosyl Adjoining Disulfide Bridge: $(\Delta\epsilon_{286-288}/\epsilon_{276-278})$[d]	Tyrosyl Adjoining Disulfide Bridge: Fractional Perturbation[e] (F): Insulin	Tyrosyl Adjoining Disulfide Bridge: Fractional Perturbation[e] (F): RNase
90% deuterium oxide		(0.96)[f]		(0.96)[f]	(0.77)[f]
20% sucrose	0.047	1.00	0.047	1.00	0.63
20% glycerol	0.063	0.88	0.036	0.50	0.59
20% ethylene glycol	0.069	0.97	0.060	0.85	0.53
20% methanol	0.058	0.94	0.046	0.74	0.69
20% hexaethylene glycol	0.101	0.82	0.036	0.29	0.42
10% dimethyl sulfoxide	0.060	0.81	0.018	0.24	0.21

[a] See footnote *a* in Table I. [b] $\Gamma/2 = 0.1$, pH 3.5–7.8. [c] $(\Delta\epsilon_{286-288}/\epsilon_{276-278})$ values relative to $(\Delta\epsilon_{286-288}/\epsilon_{276-278})$ values of disulfide-cleaved insulin in 8 M urea. [d] Calculated by the relation $4\Delta\epsilon_{286-288}/\epsilon_{278-278}$ (insulin in 8 M urea) $- 3\Delta\epsilon_{286-288}/\epsilon_{276-278}$ (disulfide-cleaved insulin in 8 M urea). [e] From Herskovits and Laskowski (1968). [f] Calculated or evaluated graphically (Figure 3).

mates for R_i, for partly buried groups using the 8 M urea data (Table IV). These estimates are based on the following considerations. In general, the R_P value of a protein can be expressed as the sum of perturbations due to individual chromophoric groups, that is, $R_p = \Sigma n_i R_i / \Sigma n_i$, where n_i represents the number of chromophores of class i. In addition to the exposed and buried chromophores, with R_i values of 1 and 0, R_i values for the partly buried groups need to be known, which in principle should have values ranging from 1.0 to 0, depending upon the degree of exposure of these groups to solvent access and the extent of interaction of these groups with the perturbant used. The latter effects designated as "range effects" (Herskovits and Laskowski, 1962; Laskowski, 1966; Herskovits, 1967) are found to vary from one perturbant to another. It is really these effects of the perturbants on the experimentally determined R_i values for partly buried groups which permit us to select one mode or one combination of exposed, buried, and partly buried groups over another

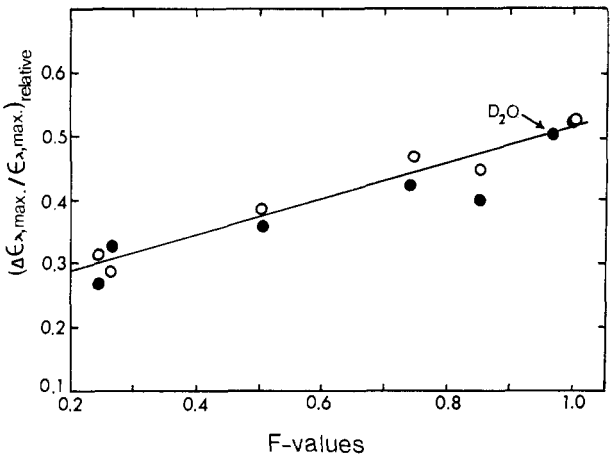

FIGURE 3: The dependence of the apparent fraction of exposed tyrosyls (R_p or R_M) upon the F parameter of insulin (see text and footnotes *c* and *d* of Table IV for definition). The apparent fraction of exposed groups, R_p, relative to the fully unfolded disulfide-cleaved protein are represented by the open circles while the fraction of exposed groups, R_M, relative to the model compounds are given by the shaded circles.

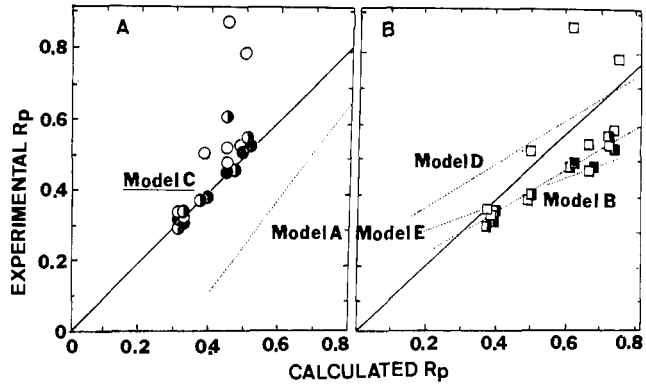

FIGURE 4: Correlation of the experimental and calculated R_p parameters of Zn-free insulin in neutral, pH 7.0–7.5, 0.1 M Cl^- (○,□), in 0.05 M KNO_3, pH 7.2–7.7 (◐,◨) and in 0.05 M KNO_3, pH 7.2–7.7 containing stoichiometric quantities of Ag^+ ion (●,■). The calculated R_p values (described in the text and footnote *a* of Table III) were based on two exposed, one buried, and one partly buried tyrosyl residues for model A; one exposed, one buried, and two partly buried groups for model B (□,◨,■); one exposed, two buried, and one partly buried group for model C (○,◑,●); two buried, and two partly buried for model D; and one buried and three partly buried groups for model E. The solid lines, drawn with a slope of one, represent maximal attainable correlation values. For reasons of clarity the data points of model A, D, and E, represented by dotted lines, have not been included.

model, with a different set of combination of chromophoric groups. In the case of ribonuclease satisfactory agreement has been obtained between the experimental R_p values and the calculated values based on two exposed, two buried, and two partly buried tyrosyls, with only a single class *i* of partly exposed groups. The R_i values for this class of two tyrosyls were based on the perturbation data of the protein in 8 M urea, assuming that in the native protein the partly buried groups are blocked or shielded from contact with perturbant to about the same extent as the tyrosyls adjoining the two disulfide bridges in the urea-denatured protein.[1] The most gratifying feature of the above analysis, with the limitations inherent in the assumption of only three classes of tyrosyls, was the satisfactory agreement between this description of the location of the six tyrosyls in solution and the location and environment of these groups revealed by the detailed three-dimensional X-ray structure of ribonuclease in the crystalline state (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967).

The same analysis has been extended to insulin in the present paper, with the single tyrosyl A-19 adjoining disulfide bridge A-20–B-19 in 8 M urea serving to give estimates of the R_i values for the partly buried group or groups in insulin. The required R_i values, designated as F in the last two columns of Table IV, were calculated from the differences of the observed $\Delta\epsilon_{286-288}/\epsilon_{276-278}$ values for insulin and disulfide-cleaved insulin in 8 M urea (see footnote *d* of Table IV). Since it is difficult to carry out experiments in 8 M urea with sufficient volume remaining to accommodate 90 vol % of deuterium oxide (*e.g.*, the volume occupied by 8 moles of urea is about 35% of the total), the F value required for this perturbant was obtained by linear extrapolation, using the experimental R_p and R_M *vs.* F values obtained with the various perturbants of this study. As shown in Figure 3 the R_M value of 0.51 for 90% deuterium oxide corresponds to an F value of 0.96.

[1] In insulin there is only a single such tyrosyl, residue A-19 located next to two cystinyl groups forming the disulfide bridge A-20–A-19.

Figure 4 presents correlation plots of the experimentally obtained R_p parameters of insulin and the calculated R_p, parameters based on various combinations of exposed, buried, and partly buried tyrosyl residues considered. The solid lines, drawn with a slope of one, represent the maximum correlation attainable. Model C, with one exposed, two buried, and one partly buried tyrosyl residues, presents the best correlation obtained between the experimental and calculated R_p parameters.

An analysis of our insulin data obtained in acidic solutions is presented and compared with the neutral pH data in Table V. At the protein concentration employed in our present study, insulin in acidic solutions is predominantly in the dimeric form (Gutfreund, 1948, 1952; Doty and Myers, 1953; Marcker, 1960; Jeffrey and Coates, 1966; Rupley *et al.*, 1967). As a result, the model data of this table were calculated assuming eight tyrosyls per insulin dimer. Unlike in the case of insulin in the presence of nitrate and silver ion, the experimental R_p values appear to be in less satisfactory agreement with any single set of calculated R_p values. This suggests that perhaps more than one set of partly buried groups is present, with substantially different degrees of exposures, or possibly the effect of other complications arising from the effects of the perturbants employed on the dimer–tetramer equilibrium of insulin in acidic solutions (Gutfreund, 1948, 1952; Doty and Myers, 1953; Marcker, 1960). Such effects have been noted in the low pH association region of α-lactalbumin, with sucrose and deuterium oxide serving as perturbants (Kronman and Holmes, 1965).

In relation to the acid data it may be significant that even though exposure of insulin to low pH gives rise to acid difference spectra (Laskowski *et al.*, 1956, 1960), in fact no net exposure of tyrosyls seems to be involved in the acid region (Menendez, 1969). This is analogous to the acid difference

TABLE V: Comparison of Theoretical and Experimental R_p Values in Acidic and Neutral Insulin Solutions.[a]

Perturbant[b]	Model C[c]	Model F[d]	Model G[e]	Zn-Free Insulin			Zn-Insulin		
				0.1 M Cl^-, pH 1.8–2.2	0.1 M Cl^-, pH 7.0–7.5	0.05 M NO_3^-, pH 7.2–7.7[f]	0.1 M Cl^-, pH 1.8–2.2	0.1 M Cl^-, pH 7–8.0	0.05 M NO_3^- + Ag^+, pH 7.2–7.7[g]
90% deuterium oxide	0.49	0.61	0.62	0.66	0.53		0.57		
20% sucrose	0.50	0.63	0.63	0.68	0.79	0.56	0.58	0.76	0.53
20% glycerol	0.38	0.44	0.50	0.52	0.51	0.38	0.43	0.54	0.38
20% ethylene glycol	0.46	0.56	0.58	0.50	0.52	0.45	0.44	0.61	0.47
20% methanol	0.44	0.52	0.56	0.42	0.88	0.61	0.44	0.84	
20% hexaethylene glycol	0.32	0.35	0.44	0.38	0.33	0.35	0.34	0.34	0.32
10% dimethyl sulfoxide	0.31	0.34	0.44	0.38	0.35	0.30	0.38	0.45	0.32

[a] Calculations of R_p values described in text and footnote *a* of Table III. [b] See footnote *a* of Table I. [c] Two exposed, four buried, and two partially buried. [d] Two exposed, three partially buried, and three buried. [e] Three exposed, three buried, and two partially buried. [f] In 0.05 M KNO_3 and 0.01 M Tris buffer. [g] In 0.05 M KNO_3 plus stoichiometric quantities of $AgNO_3$.

spectra observed in the case of α-lactalbumin (Kronman and Holmes, 1965) and recently in ovomucoid (Donovan, 1967).

Discussion

Our earlier perturbation studies on insulin obtained with sucrose and dimethyl sulfoxide as perturbants (Weil *et al.*, 1965; Herskovits, 1965) have suggested that about two or more of the four tyrosyl residues are buried in the interior parts of the native hormone. Analysis of present data in terms of exposed and both buried and partially buried tyrosyl residues suggests, that in addition to the two buried groups, another tyrosyl is partly buried. With the exception of the sucrose and methanol data, which gave consistently high R_p values in neutral 0.1 M KCl (Table III and Figure 4), the data obtained with the other five perturbants of this study appear to be in fairly satisfactory agreement with model C. The R_M parameters for this model were based on two buried, one partly, and one exposed tyrosyl. Considerable improvement in the correlation of the experimental and calculated R_p parameters was obtained by the use of 0.05 M KNO_3 solvents and also by the addition of stoichiometric quantities of silver ion to the pH 8.5 nitrate solutions (Marcker and Graae, 1961). The appropriate data are represented by the filled and half-filled circles in Figure 4A. The open circles represent the data obtained on neutral insulin solutions. The fact that the high sucrose and alcohol R_p values are reduced in acid solutions (Table V) suggests that strong binding of sucrose and alcohol to the native hormone at pH values and ionic conditions close to its physiologically active state has occurred, or that these perturbants alter the state of dimer to hexamer association in neutral solutions (Cunningham *et al.*, 1955; Andersen, 1956; Marcker, 1960). Thus, the addition of silver ion to neutral insulin solutions need not be interpreted as causing insulin to dissociate into monomers,[2] especially since the data obtained in nitrate solutions alone produces a satisfactory correlation with the calculated R_p values for model C.

Aoyama and coworkers (1965) have found tyrosyl residue A-19 and residue B-16 was reactive toward cyanuric fluoride. Of the four tyrosyls in insulin, two groups are also known to be reactive toward tetranitromethane (Sokolovski *et al.*, 1966). While in these latter studies the reactive and unreactive groups have not been identified, we may assume that the same two tyrosyls are probably also reactive, while the buried groups A-14 and B-26 are of the unreactive class. Since the best fit of our perturbation data is obtained with one exposed, two buried, and one partly buried groups, it is reasonable to assume that the partly buried group should be the reactive residue A-19. Actual identification of this group is offered by the observation that in 8 M urea this tyrosyl remains partly blocked to solvent access.[3] Reductive cleavage of the neighboring disulfide bridge A-20–B-19 leads to the obliteration of the partial blocking of this residue to solvent (Table II). The unusual iodination behavior of this group would also tend to support this contention. De Zoeten and coworkers

[2] There are grounds for questioning the report (Marcker, 1960) that addition of silver ion to dilute solutions of insulin causes its dissociation to the monomeric form. Sedimentation velocity and Sephadex experiments carried out in Dr. Rupley's laboratory (J. A. Rupley, private communication) suggest that the effects of silver ion may not be those stated by Marcker (1960).

[3] Similar effects have been noted in case of other proteins such as serum albumin (Herskovits and Laskowski, 1962), ribonuclease (Herskovits and Laskowski, 1968), lysozyme (Williams *et al.*, 1965), catalase (Herskovits, 1969), etc. This has not been the case with α-chymotrypsinogen (Williams *et al.*, 1965), aldolase (Herskovits and Sorensen, 1968), and myoglobin and hemoglobin (Herskovits, 1969) where the amino acid sequence shows no tyrosyl or tryptophyl residues adjoining disulfide bridges.

TABLE VI: A Comparison of the Experimental and Theoretical R_p Values of Silver–Insulin Based on Insulin and RNase Model Parameters.

Perturbant[a]	Calculated Model Values[b]						Experimental Values[d]	
	Based on Insulin *F* Values[c]			Based on RNase *F* Values[c]				
	C	D	E	C	D	E	Zn-Free Insulin	Zn-Insulin
90% deuterium oxide	0.49	0.48	0.72	0.44	0.39	0.58	0.51	
20% sucrose	0.50	0.50	0.75	0.41	0.32	0.47	0.53	0.53
20% glycerol	0.38	0.25	0.38	0.40	0.30	0.44	0.38	0.38
20% ethylene glycol	0.46	0.48	0.64	0.38	0.27	0.40	0.45	0.47
20% methanol	0.44	0.37	0.55	0.40	0.35	0.52	0.48	
20% hexaethylene glycol	0.32	0.15	0.20	0.35	0.21	0.31	0.32	0.32
10% dimethyl sulfoxide	0.31	0.12	0.18	0.30	0.11	0.16	0.32	0.33

[a] See footnote *a* of Table I. [b] Model C: one exposed, two buried, and one partially buried tyrosyls; model D: two buried and two partly buried tyrosyls; model E: one buried and three partly buried tyrosyls. [c] *F* values taken from Table IV. Calculation described in text and footnote *a* of Table III. [d] pH 7.2–7.8, 0.05 M KNO_3 plus stoichiometric quantities of $AgNO_3$.

(1961) have found that this tyrosyl reacts only with a single iodine atom per mole and that its rate of iodination is greater than the remaining three groups. The later groups react with up to 2 moles of iodine/mole of tyrosine.

In relation to model C and the above discussion of our insulin data, the perturbation studies of desoctapeptide-insulin of the accompanying paper (Menendez and Herskovits, 1969) should be mentioned. This trypsin-digested derivative of insulin contains only three tyrosyl residues per insulin monomer unit, residues B-26 having been removed with seven other residues from the C-terminal end of the B chain (Young and Carpenter, 1961). The best fit of the data due to perturbations of the remaining A-14, A-19, and B-16 tyrosyls suggests the presence of a single buried group and as in the case of the unmodified, parent protein, one exposed, and one partly buried group. This finding is in accord with the idea that the unreactive residue B-26 removed by trypsin is buried.

Despite the fairly satisfactory fit of our data with the disposition of the tyrosyls considered in model C, and the fact that none of the other models consisting of various combinations of exposed, partially buried, and buried groups (Tables III and V) gave comparably good fits, it is important to note that the most uncertain feature of such fit or calculation remains the question concerning the correct choice of experimental R_t or *F* parameters for partly buried groups. For this reason we have also made calculations with the *F* values derived from our previous studies on urea-denatured RNase (Table IV). Table VI presents a comparison of the experimental data obtained on neutral nitrate solutions in the presence of silver ion with those two sets of calculated R_p parameters.

What is apparent from this comparison is that despite the somewhat altered R_p parameters based on the RNase *F* values, model C still remains the best choice giving the best correlation of the data. A somewhat less satisfactory fit is obtained with model E, comprising three partly buried and one buried group, based on the RNase *F* parameters. This model does not give a very satisfactory fit with the insulin *F* parameters. However, it does suggest the possibility that other models, with perhaps a gradation of partly exposed to buried or nearly buried groups, not considered by us because of the lack of appropriate mode parameters, may equally well describe the experimental data.

The data obtained on Zn- and Zn-free insulin in acidic solutions indicate a somewhat greater degree of tyrosyl exposure (Table I), suggesting perhaps a slight conformational alteration in the structure of insulin. This structural change is to some extent also reflected by the increase in levorotation at 233 mμ and the more negative Moffitt and Yang (1956) b_0 parameters relative to the b_0 of the neutral insulin solutions (Menendez, 1969). Analysis of the acid data, similar to those carried out on the neutral insulin solutions suggest the presence of perhaps two to three exposed, two to three partly buried, and three to four buried tyrosyls per insulin dimer (Table V, model C–G), which appears to be the predominant species of aggregates at the concentration and pH conditions employed on our work (Gutfreund, 1948, 1952; Doty and Myers, 1953; Jeffrey and Coates, 1966; Rupley *et al.*, 1967). The somewhat less satisfactory fit obtained with the latter data when compared with the neutral insulin data in the KNO_3 solvents (Table V) would suggest that a single set of R_t values for partly buried groups may not be sufficient to describe the perturbation behavior of the tyrosyls in the acidic form of the hormone.[4]

Added in Proof

After this and the accompanying paper (Menendez and Herskovits, 1969) were submitted for publication the detailed three-dimensional structure of zinc insulin in the crystalline

[4] The poorer fit could presumably be also due to the effects of the various perturbants used on the state of association or dimerization of acid insulin (Herskovits, 1967; Kronman and Holmes, 1965) and the ensuing changes on perturbation parameters.

state, at 2.8-Å resolution, was published (Adams *et al.*, 1969).

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