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Physicochemical Properties of *p*-Carboxyphenylazoinsulins[1]

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The physicochemical properties of insulin coupled with *p*-carboxyphenyldiazonium sulfate have been studied by means of ultracentrifugation, solubility, paper electrophoresis and absorption spectrophotometry. The spectrophotometric and paper electrophoresis data indicate that all the diazonium salt is irreversibly bound and that the reaction occurs with tyrosine and histidine residues to form monoazo derivatives and with free amino groups to form bisazo derivatives. As more *p*-carboxyphenylazo groups are attached, there is a progressive shift and broadening of the region of minimum solubility to lower values of *p*H and a decreased solubility. In mildly alkaline solutions, where native insulin is in the form of dimers, trimers and tetramers, the increased electrostatic repulsive force between the insulin ions resulting from the introduction of 8.1 and 12.0 moles of ionized carboxyl groups per 12,000 g. of insulin, causes complete dissociation to the monomeric form (mol. wt. 12,000). The addition of 3.9 groups per molecule leads to an intermediate effect, *i.e.*, shifts the equilibrium toward a greater proportion of monomers and dimers. The sedimentation patterns of azoinsulins in acid solution show two peaks, a slow moving component which sediments like monomeric insulin, $S_{20,w} \sim 2.1$, and a fast moving component whose sedimentation coefficient increases as the ionic strength is raised or if more azo groups are attached but is relatively independent of protein concentration. It is suggested that the formation of soluble polymers results from the increased non-polarity of the insulin molecule and that the factors limiting the polymer size may resemble those responsible for determining the size of soap micelles.

Introduction

Recent evidence indicates that the true minimum molecular weight of insulin is 6,000.[3-7] However in aqueous solution, below *p*H 2 and at low ionic strength, insulin exists essentially as units having a molecular weight of 12,000.[8] If the *p*H is raised to 8 and/or the ionic strength increased, the monomer[9] reversibly associates forming dimers, trimers, tetramers and possibly some still larger units.[10-12] Above *p*H 8 dissociation to monomers and possibly to 6,000 molecular weight units occurs.[4]

The sensitivity of the equilibria to *p*H and ionic strength suggests that dissociation is due to electrostatic repulsion between similarly charged particles whereas association probably is due to short range attractive forces. The marked association observed with insulin containing non-polar azotolyl groups indicates that the interaction of lipophilic groups may provide the energy for association.[13]

In the present investigation the physicochemical properties of insulin treated with *p*-carboxyphenyldiazonium sulfate were examined by means of sedimentation in the ultracentrifuge, solubility, paper electrophoresis and absorption spectrophotometry. In neutral or mildly alkaline solutions the increased negative charge, resulting from the added ionized carboxyl groups, would be expected to aid dissociation while in acid solution increased association would occur due to enhanced non-polar interactions and perhaps intermolecular hydrogen bonds.

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(3) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3087 (1952).

(4) E. Fredericq, *Nature*, **171**, 570 (1953).

(5) D. W. Kupke and K. Linderstrøm-Lang, *Biochim. et Biophys. Acta*, **13**, 153 (1954).

(6) D. Yphantis and D. F. Waugh, cited by D. F. Waugh, *Ciba Foundation Coll. Endocrin.*, **9**, 122 (1956).

(7) E. D. Rees and S. J. Singer, *Arch. Biochem. and Biophys.*, **63**, 144 (1956); see also E. Fredericq, THIS JOURNAL, **79**, 599 (1957).

(8) J. T. Edsall, "The Proteins" (H. Neurath and K. Bailey, Eds.), Vol. I, part B, Academic Press, Inc., New York, N. Y., 1953, p. 549.

(9) The term monomer will be used to denote units of molecular weight 12,000 since these are the smallest units ordinarily observed in aqueous solution.

(10) J. L. Oncley, E. Ellenbogen, D. Gitlin and F. R. N. Gurd, *J. Phys. Chem.*, **56**, 85 (1952).

(11) P. Doty and G. E. Myers, *Disc. Faraday Soc.*, **13**, 51 (1953).

(12) R. F. Steiner, *Arch. Biochem. and Biophys.*, **44**, 120 (1953).

(13) W. L. Koltun and D. F. Waugh, cited by D. F. Waugh, *Adv. Prot. Chem.*, **9**, 325 (1954).

Materials and Methods

Insulin.—Zn-insulin crystals obtained from Eli Lilly and Co., Lot No. 535664, having an activity of 27 u./mg., were used. After recrystallization using a modification of the final crystallization technique of Romans, Scott and Fisher,[14] the insulin was dissolved in 0.05 *N* hydrochloric acid, precipitated and washed three times with anhydrous acid acetone, washed two times with anhydrous ethyl ether and dried over silica gel. The insulin was stored at 4°.

Coupling of *p*-Carboxyphenyldiazonium Sulfate to Insulin.—Solutions of *p*-carboxyphenyldiazonium sulfate were prepared from the sodium salt of *p*-aminobenzoic acid immediately before use.[15]

The coupling reaction was carried out under the following conditions: borate buffer, *p*H 8.4; ionic strength, μ, 0.15; insulin concentration 6 mg. per ml.; initial diazonium salt concentration 0.0095 *M*; temperature 22.5°. The buffering capacity was sufficient to maintain the *p*H during the reaction.

The extent of coupling was determined by measuring the disappearance of free diazonium salt. At selected times the reaction was stopped by precipitating the insulin in 19 times the volume of acidified acetone at 0°. Residual diazonium salt was determined by treating 1.0 ml. of the supernatant with 9.0 ml. of 0.01 *M* resorcinol in acetate buffer, *p*H 4.5, μ 0.20, at room temperature for 30 minutes to assure maximum color development (formation of *p*-carboxyphenylazoresorcinol). Maximum color is developed in 15 minutes and is stable for at least 2 hr. The concentration of *p*-carboxyphenylazoresorcinol was determined spectrophotometrically from the relation $O.D._{385} = \epsilon_{385}c$, where $O.D._{385}$ is the optical density at 385 mμ, the wave length of maximum absorption; ϵ_{385}, the molecular extinction coefficient, is 21,500, and c is the molar concentration. The concentration of diazonium salt is equal to the concentration of *p*-carboxyphenylazoresorcinol since coupling was carried out using a large excess of resorcinol.

Preparation of Stock Azoinsulins.—Insulins having on the average 3.9, 8.1 and 12.0 moles of *p*-carboxyphenylazo groups per 12,000 g. of insulin were prepared by stopping the reaction at 6, 16 and 33 minutes, respectively. The modified protein was dissolved in phosphate buffer, *p*H 7.4, μ 0.15, dialyzed for 24 hr. against distilled water, lyophilized and stored at 4°.

Azoinsulins are designated as I_n, the subscript denoting the number of added groups. The moles diazonium salt per 12,000 g. of insulin was calculated assuming that all the

(14) R. G. Romans, D. A. Scott and A. M. Fisher, *Ind. Eng. Chem.*, **32**, 908 (1940).

(15) K. H. Saunders, "The Aromatic Diazo Compounds," Longmans, Green and Co., New York, N. Y., 1949.

salt which disappeared, exclusive of slight decomposition which occurred during the reaction, had reacted with the protein. No diazonium salt was found in supernatants of azoinsulins after repeated precipitation nor was any detected leaving the proteins during dialysis, indicating that all the salt is irreversibly bound to insulin.

Absorption Spectra.—Absorption measurements were made with a Beckman spectrophotometer, model DU, using 1-cm. cells. Molecular extinction coefficients, ϵ, were calculated from the equation

$$\log_{10}(I_0/I) = \epsilon cd$$

where I_0/I is the ratio of the intensity of the light emerging from the pure solvent to that from the solution, c is the molar concentration of absorbing species and d is the thickness of the cell in centimeters.

Sedimentation.—Ultracentrifuge runs were made in the model E Spinco ultracentrifuge, using a synthetic boundary cell and a standard 12-mm. 4° cell with a plastic (Kel-F) centerpiece. All runs were made at 59,780 r.p.m. (260,000 × g), and at 20.0 ± 0.2°. The rotor temperature was measured and controlled by means of the Spinco rotor temperature indicator and control unit. Sedimentation coefficients were corrected to the value expected for sedimentation in a medium having the viscosity and density of water at 20°.

Paper Electrophoresis.—Paper electrophoresis studies were carried out with the Spinco model "R" paper electrophoresis apparatus at *p*H 2.1 and *p*H 5.5–11.6. The following buffers, each of ionic strength 0.05, were used: *p*H 2.1, glycine–HCl; *p*H 5.5, sodium acetate–acetic acid; *p*H 6.4, 7.4, KH_2PO_4–K_2HPO_4; *p*H 8.6, sodium veronal–veronal; *p*H 10.2, borate–KOH; *p*H 11.6, glycine–KOH.

All runs were made using 0.3 mg. of protein per paper strip. A constant current of 4.0 milliamperes was applied for 12.5 hr. At the conclusion of the run the paper strips were stained with Spinco Dye B-1 for 4 hr., fixed with Spinco Fixative B-1 and dried at 110°.

Solubility.—Precise solubility measurements were not attempted. Aliquots of stock protein solutions, 0.5–2.0% in 0.05 *N* hydrochloric acid and in phosphate buffer *p*H 7.4, μ = 0.10, were added to 10–20 × the volume of buffer. After 4 hr. at 24° with frequent stirring, the solutions were centrifuged and the concentration of protein in the supernatant determined spectrophotometrically. Solubility was examined over the *p*H range 2.1–6.2 using the following buffers: *p*H 2.1 to 2.9 glycine–HCl; *p*H 3.5 to 5.4, sodium acetate–acetic acid; *p*H 6.2, KH_2PO_4–K_2HPO_4.

Results

Absorption Spectra.—The absorption spectra of unreacted insulin, I_0, and *p*-carboxyphenylazoinsulins are shown in Fig. 1. In contrast to the single absorption maximum at 277 mμ of I_0 the modified insulins show a broad maximum between 275 and 285 mμ, with indications of peaking at 277 and 282 mμ. As the protein becomes extensively coupled, a single peak at 280 mμ is observed. The optical density at 277 mμ, O.D.$_{277}$, may be expressed by

$$\text{O.D.}_{277} = (\epsilon_0 + a\epsilon_1)c \qquad (1)$$

where ϵ_0, the molecular extinction coefficient of unreacted insulin, is 11,340, ϵ_1, the molecular extinction coefficient per mole diazonium salt per 12,000 g. of insulin, is 4,140, a is the number of moles diazonium salt per 12,000 g. of insulin and c is the molar concentration of insulin using a molecular weight of 12,000. It may be seen that for any given insulin concentration the O.D.$_{277}$ rises linearly as the number of groups coupled increases. This is further evidence that all the diazonium salt is irreversibly bound to the insulin.

The absorption maximum at approximately 340 mμ (338, 340 and 344 mμ for $I_{3.9}$, $I_{8.1}$ and $I_{12.0}$, respectively) corresponds closely to the absorption region of tyrosinemonoazo derivatives whereas the shoulder at 380 mμ is in the absorption region of histidinemonoazo derivatives.[16,17] However, in contrast to the absorption at 277 mμ, the color intensity at 340 mμ is not linearly proportional to the number of groups attached. The progressive decrease of the molecular extinction coefficient at 340 mμ, ϵ_{340}, per azo group as the moles diazonium salt per mole insulin increases, Fig. 2, indicates that in addition to coupling with tyrosine and histidine the diazonium salt reacts with other amino acid residues to give less colored products. The extrapolated value of ϵ_{340} per azo group is approximately 21,000 which is in the range of those reported for azobenzene derivatives and for diazonium salts

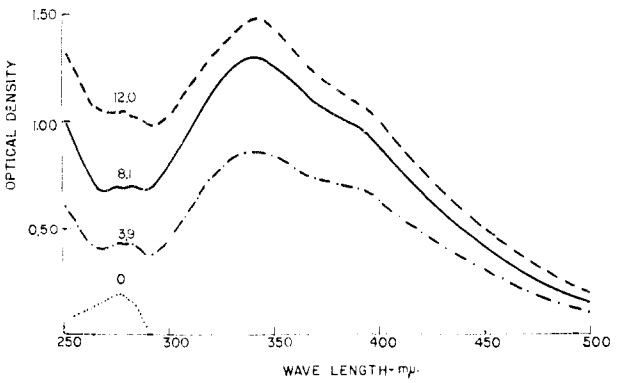


Fig. 1.—Absorption spectra of native and *p*-carboxyphenylazoinsulins in phosphate buffer, *p*H 7.4, μ = 0.10, protein concentration 0.20 mg./ml. The number above each curve indicates the moles of diazonium salt per 12,000 g. of insulin.

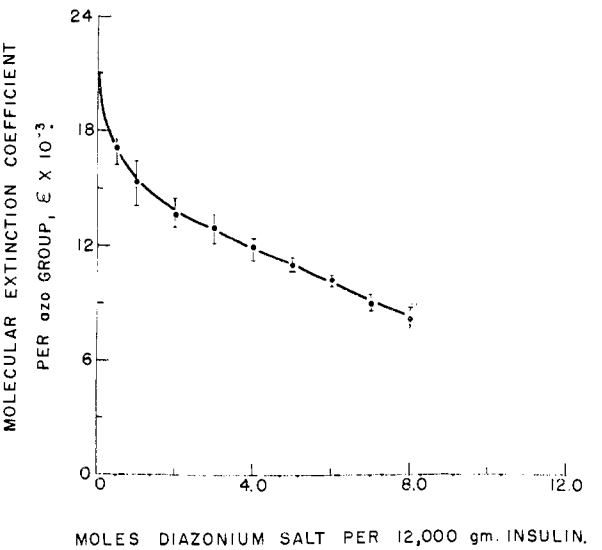


Fig. 2.—The dependence of the molecular extinction coefficient per azo group at 340 mμ on the moles diazonium salt added per 12,000 g. of insulin. Open circles are values obtained for the stock azoinsulins.

(16) H. G. Higgins and D. Fraser, *Australian J. Sci. Research, Ser. A*, **5**, 736 (1952).

(17) The rise in the absorption maximum from 338 to 344 mμ, as more groups are attached, may indicate that coupling is first to tyrosine and then to histidine residues. On the other hand, these slight changes may be due to differences in the local environment at the bonding sites.

coupled to serum albumin, 19,000–26,000.[18] Using this value for the azo linkage to tyrosine and histidine and assuming that the azo linkage to other amino acids does not absorb at 340 mμ, the moles of diazonium salt per 12,000 g. of insulin linked to residues other than tyrosine and histidine may be estimated. The results are shown in Table I. In the extensively reacted insulin, $I_{12.0}$, only about one-third of the salt is bound to tyrosine and histidine *via* the azo linkage.

TABLE I

AMINO ACID RESIDUES INVOLVED IN COUPLING INSULIN WITH *p*-CARBOXYPHENYLDIAZONIUM SULFATE

Azo groups per 12,000 g. insulin	ε_{340} per azo group	Fraction coupled to tyrosine and histidine	Fraction coupled to other amino acids	Azo groups coupled to other amino acids
3.9	12,800	0.61	0.39	1.5
8.1	9,000	.43	.57	4.6
12.0	7,400	.35	.65	7.8

Tyrosine and histidine residues containing two *p*-carboxyphenylazo groups absorb at 480–490 mμ.[16] Since no absorption peaks are observed in this region, it is concluded that such bis compounds are not present in the modified insulins.

Paper Electrophoresis.—The effect of coupling *p*-carboxyphenylazo groups on the electrophoretic mobility of insulin was examined in acid and alkaline solutions using paper electrophoresis. At *p*H 2.1 the relative mobilities, calculated as the fraction of the mobility of normal insulin, are 0.88, 0.54 and 0.33 for $I_{3.9}$, $I_{8.1}$ and $I_{12.0}$, respectively. Assuming that the observed mobility is directly related to the charge, then the azo coupled insulins migrate as if they had 1.3, 5.0, and 7.4 less positive charges than normal insulin. These are suggestively close to the number of groups bound to residues other than tyrosine and histidine estimated from the optical data, 1.5, 4.6 and 7.8, respectively (Table I), and might indicate that for each group not coupled to tyrosine and histidine a positive charge is removed. The agreement probably is fortuitous in view of the simplifying assumptions relating the mobility directly to charge. Moreover, the 6 free amino groups, which are the most likely positive groups to react, would not account for all of the 7.8 groups not reacted to tyrosine and histidine in $I_{12.0}$. The slow movement of the azoinsulins at *p*H 2.1 probably reflects their decreased solubility and/or increased binding to the paper, making it appear as if the positive charge is lower than is actually the case.

The results obtained with normal insulin at *p*H 5.5, 6.4, 7.4, 8.6, 10.2 and 11.6 and with the azo coupled insulins at *p*H 6.4, 7.4 and 8.6 are presented in Fig. 3. In curve A the charge on $I_{3.9}$, $I_{8.1}$ and $I_{12.0}$ was determined by adding 3.9, 8.1 and 12.0, respectively, to the charge of unreacted insulin under these conditions.[19,20] The latter values obtained directly from the titration data of Tanford and Epstein[21] were used to plot the data for unreacted insulin, curve B. No corrections were made at *p*H 10.2 and 11.6 for the effect of potassium ion binding. In curve C the charge on the azo coupled insulins was determined assuming that each azo group coupled to residues other than tyrosine and histidine not only contributes a negative charge but simultaneously removes a positive charge. If the mobility *vs.* charge relationship observed for normal insulin is considered as a standard curve, then the azoinsulins appear to move faster than expected on the basis of simply adding negative charges and slower if for each azo group not coupled to tyrosine and histidine one positive charge is removed. The best fit is obtained if two of these azo groups are coupled to one amino group removing one positive charge. These points are indicated by crosses. The agreement with the standard curve indicates that, in addition to reacting with tyrosine and histidine, the diazonium salt reacts with amino groups to form bisazo derivatives.

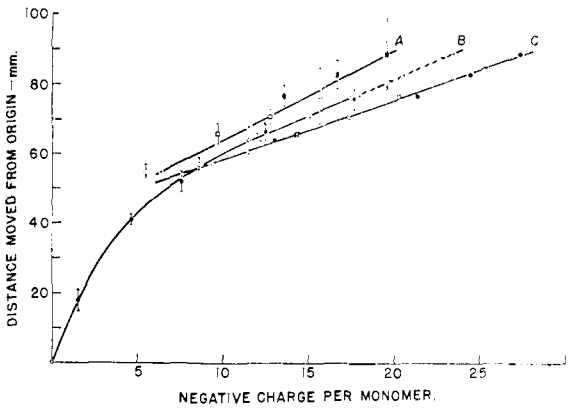


Fig. 3.—The distance moved *vs.* negative charge per 12,000 g. of insulin: ●, native insulin; ○, $I_{3.9}$; □, $I_{8.1}$; ■, $I_{12.0}$. For details see the text.

Solubility.—The marked effect of adding *p*-carboxyphenylazo groups on the solubility of insulin is shown in Fig. 4. As more groups are attached there is a progressive shift and broadening of the region of minimum solubility to lower values of *p*H and a decreased solubility. Minimum solubility occurs approximately at *p*H 4.3, 3.9 and 3.6 for $I_{3.9}$, $I_{8.1}$ and $I_{12.0}$, respectively, compared to *p*H 5.4 for I_0.[22] At constant *p*H, increasing the ionic strength decreases the solubility.

When the carboxyl groups are un-ionized, the effect of the carboxyphenylazo radical is to increase the non-polar attractive forces and possibly intermolecular hydrogen bonds, which results in a decreased solubility. Even when maximally charged, *i.e.*, at *p*H ~ 2, $I_{12.0}$ is virtually insoluble. However, above *p*H 5 the additional ionized carboxyl groups increase the electrostatic repulsion between molecules and hence increase the solubility. The greater the number of added groups the more pronounced is the effect.

(18) E. W. Gelewitz, W. L. Riedeman and I. M. Klotz, *Arch. Biochem. and Biophys.*, **53**, 411 (1954).

(19) Since the *pK* for carboxyl groups is approximately 4, above *p*H 5.5 only the carboxylate ion is present.

(20) The diffuse band obtained with the azo coupled insulins, approximately twice that of native insulin, indicates some electrophoretic inhomogeneity.

(21) C. Tanford and J. Epstein, THIS JOURNAL, **76**, 2163 (1954).

(22) If it is assumed that for every two azo groups coupled to residues other than tyrosine and histidine one positive charge is removed, the isoelectric points for $I_{3.9}$, $I_{8.1}$ and $I_{12.0}$ are computed to be 4.3, 3.9 and 3.6, respectively.

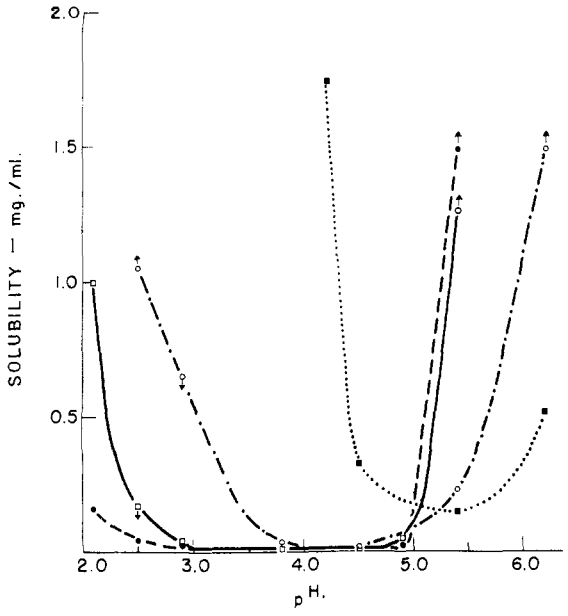


Fig. 4.—The effect of *p*H on the solubility of native and *p*-carboxyphenylazoinsulins, ionic strength 0.1: ■, native insulin; O, $I_{3.9}$; □, $I_{8.1}$; ●, $I_{12.0}$; ↑, solubility greater than indicated; ↓, less soluble than indicated.

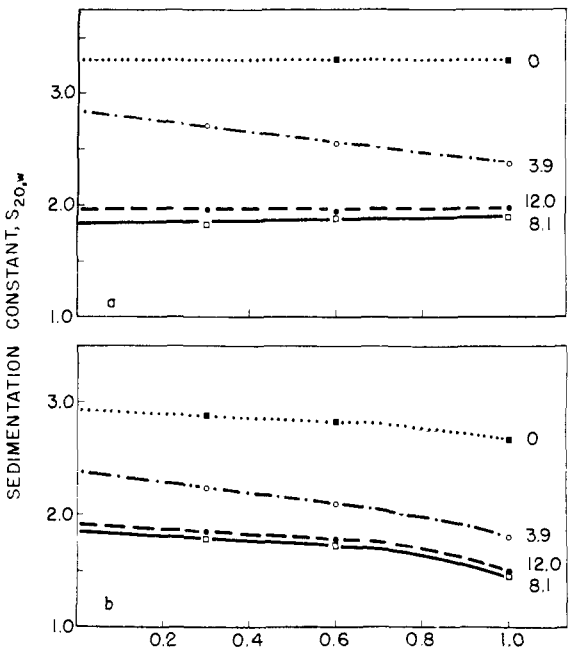


Fig. 5.—The dependence of $S_{20,w}$ on concentration, ionic strength 0.10: a, *p*H 7.5; b, *p*H 8.5. The number beside each curve indicates the moles of diazonium salt per 12,000 g. of insulin.

Sedimentation.—The effect of adding *p*-carboxyphenylazo groups on the reversible association exhibited by insulin was examined by means of ultracentrifugation. The concentrationde pendence of the sedimentation coefficient, $S_{20,w}$, at *p*H 7.5 and 8.5, ionic strength 0.10, is shown in Fig. 5. At both *p*H values the extrapolated $S_{20,w}$ of $I_{8.1}$ and $I_{12.0}$ is 1.84 ± 0.05 and 1.94 ± 0.05, respectively, which corresponds to monomeric units of mol. wt. 13,200 and 13,800. The monomeric unit of unreacted insulin, mol. wt. 12,000, has an extrapolated $S_{20,w} = 1.75$.[23] Thus under conditions where native insulin is in the form of dimers, trimers and tetramers of varying proportions, the increased electrostatic repulsive force between the insulin ions resulting from the introduction of 8.1 and 12.0 groups appears sufficient to cause complete dissociation to the monomeric form. The addition of 3.9 groups leads to an intermediate effect, *i.e.*, shifts the equilibrium toward a greater proportion of monomers and dimers.

Sedimentation experiments in acid solution, *p*H ~ 1.8, were limited mainly to $I_{3.9}$ because of the decreased solubility of the more extensively reacted insulins. $I_{12.0}$ was completely insoluble while $I_{8.1}$ was sufficiently soluble only at low ionic strength (0.05 *N* hydrochloric acid). Ultracentrifuge runs with $I_{3.9}$ were carried out in 0.05 *N* hydrochloric acid and the ionic strength varied up to 0.15 by the addition of sodium chloride.

The sedimentation patterns show two well defined peaks, a slow moving component which sediments like monomeric insulin, $S_{20,w} \sim 2.1$, and a fast moving component whose sedimentation coefficient, $S^{P}_{20,w}$ increases as the ionic strength is raised but is independent of protein concentration, Table II, column 6. The asymmetry and spreading of the fast moving peak indicates a distribution of soluble polymers, having the average sedimentation coefficient indicated. In certain cases, $I_{3.9}$, $\mu = 0.15$ and $I_{8.1}$, $\mu = 0.05$, still higher molecular weight components are observed. These are completely sedimented within 6 minutes after full speed is attained and recovered as a gel at the conclusion of the run. Estimates of the concentration of these heavier components, column 5, were obtained from area measurements of the sedimentation pattern, The fraction of protein represented by monomer and polymer, column 4, was measured directly and the fraction forming the gel obtained by difference.

The distribution of material between monomer and polymer as a function of ionic strength and total protein concentration could not be determined accurately. Measurements of areas were not entirely satisfactory because the monomer was present in small amounts and the peaks were not fully resolved. Nevertheless, from area measurements, the concentration of monomer present in $I_{3.9}$ appears to remain constant, between 0.1–0.2% and to be independent of total protein concentration and ionic strength. With $I_{8.1}$ the concentration of monomer was estimated as 0.06–0.12%.

Discussion

The spectrophotometric and paper electrophoresis studies provide some evidence as to the nature of the amino acid residues in insulin to which the *p*-carboxyphenylazo groups are coupled. The low molecular extinction coefficient per azo group of extensively coupled insulins indicates that the reaction is not primarily with tyrosine and histidine, as has been generally accepted for proteins,[24] but

(23) H. K. Schachman and W. F. Harrington, *J. Polymer Sci.*, **12**, 379 (1954).

(24) R. M. Herriott, *Adv. Prot. Chem.*, **3**, 169 (1947).

TABLE II

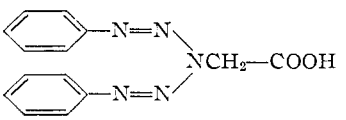
SEDIMENTATION OF *p*-CARBOXYPHENYLAZOINSULINS IN ACID SOLUTIONS

Azo groups 12,000 g. insulin	Solvent	Prot. concn., g./100 ml.	Concn. ($m + p$), g./100 ml.	Concn. gel., g./100 ml.	$S^{p}_{20,w}$	Mol. wt.[b]	N_0[c]	C_0[d] × 10^5	W_m,[e] kcal./mole
3.9	0.05 *N* HCl	0.30	0.30	0.00	4.8	60,000	5	3.34	14.8
		0.60	0.60	.00	4.8				
		1.00	1.00	.00	4.7				
					4.8[a]				
3.9	.05 *N* HCl	0.30	0.30	.00	7.7	125,000	10	1.60	17.9
	.05 *M* NaCl	0.60	0.60	.00	7.7				
		1.00	1.00	.00	8.2				
					7.8[a]				
3.9	.05 *N* HCl	0.30	0.26	.04	11.8	240,000	20	0.835	20.0
	.10 *M* NaCl	0.60	.47	.13	11.4				
		1.00	.70	.30	11.7				
					11.8[a]				
8.1	.05 *N* HCl	1.00	.9	.1	17	400,000	33	0.225	22.7

[a] Extrapolated value. [b] Assuming spherical polymer. [c] N_0 = monomers per polymer. [d] C_0 = critical micelle concentration, mole fraction. [e] W_m = attractive energy per monomer.

other residues which give less colored products also react. Similar results were obtained with a variety of diazonium salts coupled to serum albumin[18] and with *p*-tolylazofibrinogen[25] where it is estimated that only about a third of the irreversibly[26] bound dye groups are attached to tyrosine and histidine. Although the interpretation of the electrophoretic migration on paper in terms of the charge on the migrating ion is uncertain, it appears probable that in addition to coupling with tyrosine and histidine residues forming monoazo derivatives, two azo groups may react with one amino group to form bisazo derivatives.

Busch, *et al.*,[27] isolated a colorless addition compound between benzenediazonium chloride and glycine which is thought to have the structure



A similar reaction with the 2 ϵ-amino groups of lysine and 4 terminal amino groups per 12,000 g. of insulin would account for the low values of ϵ per azo group and the electrophoretic migration.

The solubility and ultracentrifuge data are in accord with the view that in acid solution the increased association results from enhanced lipophilic interaction and/or hydrogen bonding, whereas above *p*H 5 the additional negative charges furnished by the *p*-carboxyphenylazo group aid dissociation by increasing the electrostatic repulsive forces between molecules.

Estimates of the effect of increased electrostatic repulsion on the association–dissociation equilibria in basic solution were made using the treatment of Oncley and Ellenbogen.[10] The total free energy change, Δu_{1-n}, in associating n monomers to a polymer, *i.e.*, for the reaction $n\ \mathrm{I} \rightleftarrows \mathrm{I}_n$ may be expressed by

$$\Delta u^{s}_{1-n} = \Delta u^{s}_{1-n} + \Delta u^{a}_{1-n} \qquad (2)$$

where Δu^{e}_{1-n} is the electrostatic free energy change and depends on the charge of the insulin ion and on the ionic strength and Δu^{a}_{1-n} is the attractive free energy change.

From sedimentation studies of native insulin in acid solutions it is estimated that the attractive energy for trimerization is between −18,000 and −21,000 cal. or −6,000 to −7,000 cal. per monomer assuming trimerization results in the formation of three common faces.[10] The additional *p*-carboxyphenylazo groups should not contribute appreciably to the attractive energy per monomer since the close proximity of the ionized carboxyl groups to the phenyl ring would prevent the interaction of these lipophilic groups.

In Table III are shown the free energy changes calculated for dimerization ($n = 2$), trimerization ($n = 3$) and tetramerization ($n = 4$), using a value of −6,500 cal. for the attractive free energy per monomer and calculating the charge assuming that for each 2 azo groups not coupled to tyrosine and histidine a positive charge is removed. At *p*H 7.5, $\mu = 0.1$, the most prevalent forms of normal insulin are expected to be trimers and tetramers; for $I_{3.9}$ the equilibrium would be shifted to a greater proportion of dimers and trimers, whereas for $I_{8.1}$ and $I_{12.0}$ only monomers are likely to be present. The experimental findings agree with these conclusions.

TABLE III

ESTIMATED FREE ENERGY CHANGES OF POLYMERIZATION OF INSULIN AT *p*H 7.5, $\mu = 0.10$

Azo groups/ 12,000 g. insulin	Negative charge/ 12,000 g. insulin	Kcal./mole Δu_{1-2}	Δu_{1-3}	Δu_{1-4}
0.0	4.8	−11.6	−17.5	−20.5
3.9	9.5	− 7.7	−11.6	− 4.5
8.1	15.2	+ 0.6	+ 1.1	+28.0
12.0	20.7	+12.3	+17.7	+72.6

The dissociation of native insulin at *p*H 10, ($z \sim -12$), to 6,000 molecular weight units is considered to be due mainly to electrostatic repulsion.[4] However, the failure to observe 6,000 units with I_0 in acid solution ($z \sim +12$) except at very low concentrations,[6] and with $I_{8.1}$ and $I_{12.0}$ at *p*H 7.5–8.5,

(25) J. Fitzgerald and W. L. Koltun, THIS JOURNAL, in press.

(26) In contrast to insulin, serum albumin and fibrinogen also reversibly bind diazonium salts.

(27) M. Busch, N. Patrascanu and W. Weber, *J. prakt. Chem.*, **140**, 117 (1934).

where these have even a greater net negative charge than I_0 at *p*H 10, indicates that factors other than simple electrostatic repulsion are involved. Charged groups located in the bonding region or regions of two 6,000 units probably are required to dissociate the monomer. The negative charges furnished by the *p*-carboxyphenylazo groups most likely are distributed over the surface of the monomer and not in the bonding regions. Since dissociation occurs near the pK for the ionization of the hydroxyl groups of tyrosine, the stabilization of the 12,000 unit may involve tyrosine residues either by hydrogen bonding or non-polar interactions.

Of considerable interest is the increased association in acid solution, leading to the formation of soluble polymers in equilibrium with monomer. The facts that (1) there appears to be a critical micelle concentration below which no polymer is detected, (2) this concentration decreases as more *p*-carboxyphenylazo groups are added and (3) the size of the polymer is relatively independent of total protein concentration but increases if the ionic strength is raised or more non-polar groups are attached, suggest that the formation of polymers may be a micellar phenomenon. The factors operating to limit the polymer size may resemble those responsible for determining the size of soap micelles as discussed by Debye[28] and Reich[29] and by Waugh[13] in connection with protein associations.

If the Debye theory is applied to account for the formation of soluble polymers of azoinsulins, the results shown in Table II, columns 7–10, are obtained.[30] Extrapolation of W_m, the attractive energy per monomer, leads to a value of approximately −7,000 cal. for unreacted insulin which is in good agreement with that reported by Oncley and Ellenbogen.[10] The increase of W_m per added azo group is about −2,000 cal.

Recently Reich[29] has shown that the Debye theory leads to incorrect calculations of the micelle size distribution. Reich's theory, which minimizes the free energy of the system rather than the free energy per micelle, leads to an unsymmetrical micelle size distribution. A sharp rise occurs as the number of molecules, N per micelle approaches the most energetically favored number, N_0, whereas a more gradual decline is expected for $N > N_0$. A similar asymmetric distribution of polymers formed by the azoinsulins is observed upon ultracentrifugation. The size of the most stable micelle is shown to depend not only on the ionic strength but on the ratio, S/A, where A is the total surface of the molecule and S is the fraction covered by polar groups. At zero ionic strength, $A - S$, the fraction of hydrocarbon surface of $I_{3.9}$, is estimated to be 0.40, which is suggestively close to the fraction of non-polar amino acids in insulin, 0.41.[13] Further possible application of Reich's theory to the azoinsulin system would require knowledge of entropy and enthalpy changes per monomer occurring upon aggregation.

(28) P. Debye, *J. Phys. Chem.*, **53**, 1 (1949).
(29) I. Reich, *ibid.*, **60**, 257 (1956).
(30) Debye's equations which apply to disk shaped micelles were used, although the micelle size was determined assuming a spherical structure.

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[CONTRIBUTION FROM THE DIVISION OF ORGANIC CHEMISTRY OF THE ORTHO RESEARCH FOUNDATION]

Synthetic Oxytocics. I. Synthesis and Reactions of 3-Indolyl-2′-pyridylcarbinols and of 2,3-(2′,3′-Indolo)-hexahydroquinolizines

BY HENRY BADER AND WILLIAM OROSHNIK

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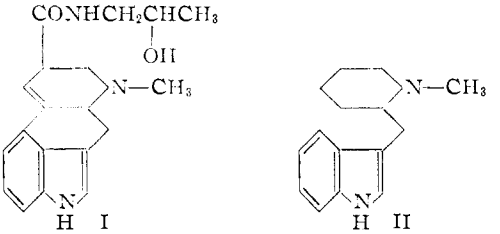
3-Indolyl-2′-pyridylcarbinol was prepared from 3-indolealdehyde and 2-pyridyllithium. It was catalytically reduced to 3-indolyl-2′-piperidylcarbinol (V) and to 2-skatylpiperidine. The latter compound could be N-methylated by lithium aluminum hydride reduction of its N-formyl derivative. It also was transformed by a modified Pictet–Spengler condensation into the 2,3-(2′,3′-indolo)-hexahydroquinolizine (VII, R = H) or its 1′-hydroxymethyl derivative, according to reaction conditions. A similar condensation with the carbinol V gave VII, R = OH.

The highly complex structure of ergonovine, the isopropanolamide of lysergic acid (I), has motivated many searches for a potent oxytocic drug among simpler compounds, particularly those which represent fragments of the lysergic acid molecule. In some recent work along these lines, Akkerman and Veldstra[1] accomplished the synthesis of compound II and several of its derivatives, thereby attaining the ring structure of dihydrolysergic acid opened at the 10, 11-junction. The method of synthesis consisted essentially of the condensation of isatin with the appropriate α-picoline and reduction of the resulting dioxindole (after N′-methylation) with sodium in butanol. The over-all yields obtained were only about 5%. Unfortunately, none of the compounds showed very significant oxytocic activity.



In a similar program in this Laboratory, the synthesis of compound II also was achieved, and its pharmacological properties were found to closely

(1) A. M. Akkerman and H. Veldstra, *Rec. trav. chim.*, **73**, 629 (1954).