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An Equilibrium Ultracentrifuge Study of the Effect of Ionic Strength on the Self-Association of Bovine Insulin*

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ABSTRACT: Insulin self-associates in aqueous solution. Experiments are described in which the association constants for the association equilibria at pH 2 are determined at ionic strengths 0.05, 0.10, 0.15, and 0.20, by means of equilibrium sedimentation experiments. Calculation of the electrostatic free energy of association for appropriate models using both the Debye–Hückel and Verwey–Overbeek theories has been carried out and the results were compared with the experimental free energies of association. It is suggested that the Verwey–Overbeek theory works better for this system. The results support the thesis that the association equilibria represent a balance between a nonelectrostatic intermolecular attraction and an electrostatic repulsion.

In a recent paper (Jeffrey and Coates, 1966; which we will refer to as paper 1) we have described experiments in which the thermodynamic parameters characterizing the self-association of insulin, at pH 2 and ionic strength 0.1, have been determined by means of the equilibrium ultracentrifuge. This present paper describes experiments in which the methods described in paper 1 are applied to insulin solutions at pH 2 and ionic strengths of 0.05, 0.15, and 0.20. The number of polymeric species, their degree of polymerization, and the equilibrium constant determining their concentrations have been determined for these ionic strengths. In order to test the hypothesis that the ionic strength influences the equilibria largely by altering the electrostatic free energy of the species in solution; calculations of the latter have been attempted using simple models and the Debye–Hückel and Verwey and Overbeek (1948) theories.

Experimental Procedures

Preparation of Insulin Solutions. Bovine crystalline zinc insulin (batch no. A3) was supplied by the Australian Commonwealth Serum Laboratories. The zinc content was stated to be between 0.3 and 0.9%. Zinc is not bound by insulin at pH 2 (Cunningham *et al.*, 1955); thus after dialysis the concentration in the insulin solutions was reduced to about 1/2500 of the original value. The buffer used was an aqueous sodium chloride–glycine–hydrochloric acid solution; its ionic strength was varied by adjusting the concentration of sodium chloride in the mixture appropriately.

Solutions of insulin in the buffer were dialyzed at 2–6° against buffer. The pH values of diffusate and insulin solution were identical after dialysis and were always between 2.00 and 2.04, measured at 25°.

Measurement of Molecular Weights and Determination of Equilibrium Constants. Sedimentation equilibrium experiments and determinations of concentration were carried out in a manner identical with that described in paper 1. From the experimental data, graphs of apparent weight-average molecular weight *vs.* concentration were obtained for a range of solute concentrations. The plots were then analyzed by Steiner's (1952) method as applied in paper 1 with the assumption that the species involved in the equilibria carry no electrostatic charge and are thermodynamically ideal. From this analysis one obtains the set of equilibrium constants which most satisfactorily reproduce the data when expressed in a manner appropriate to the Steiner formulation (see paper 1). To enable a

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subjective assessment of the success of the analysis to be carried out, the derived equilibrium constants were used to calculate molecular weight *vs.* concentration curves for a series of arbitrary concentrations.

The total concentration of the protein component was always expressed in a manner consistent with the Casassa and Eisenberg (1960) formulation which allows three component charged systems to be formally treated as if they contain only two components. The protein component is designated 2*. The calculated curves can be readily compared with the original data (see, for example, Figure 1), in which measured concentration and molecular weight data are shown as points and the smooth curves are calculated from the derived equilibrium constants, using arbitrarily selected values of x_1c/m.

Results

Figure 1 shows the apparent weight-average molecular weight of insulin as a function of concentration, at pH 2, and ionic strengths of 0.05, 0.15, and 0.20. The equivalent graph for ionic strength 0.10 was published as Figure 1 of paper 1. It was found that at ionic strengths 0.15 and 0.20 the calculated curve did not fit the data very well if the results from the highest concentration experiment were included in the analysis; the dashed line on Figure 1a was calculated using all the data points shown. However, it was possible to obtain a reasonable fit when the points derived from the highest concentration experiment were omitted from the analysis; the full line in Figure 1b was obtained in this way. In a similar manner the full line in Figure 1c was derived omitting the points for the highest concentration experiment from the Steiner analysis.

It is felt that the omission of the highest concentration data from the analysis is justified by virtue of the theory developed in paper 1 where it was shown that little error was introduced into the values of the successive equilibrium constants by treating insulin at pH 2 and ionic strength 0.10 as if it were uncharged. The theory also showed that the error would become larger under conditions of lower ionic strength and if polymeric species higher than the hexamer were present in appreciable concentration. The latter situation arises at high ionic strength and high protein concentration, when high-order terms in x_1c/m become more significant in the Steiner polynomials.

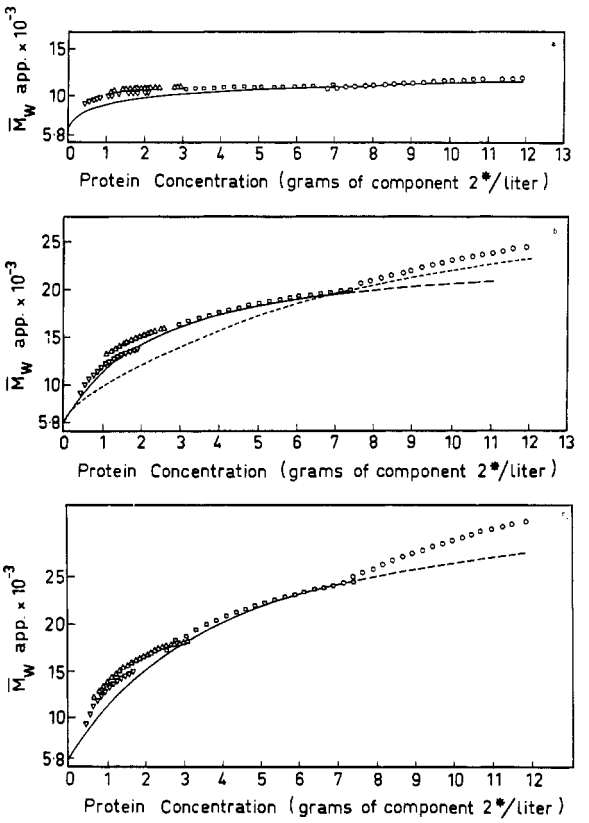

FIGURE 1: The apparent weight-average molecular weight of insulin as a function of concentration, at pH 2 and 25°. Ionic strengths: (a) 0.05, (b) 0.15, and (c) 0.20. Each set of symbols refers to a particular experiment. For explanation of dashed lines see text.

TABLE I: Equilibrium Constants for Insulin Equilibria at pH 2 and 25°.[a]

Ionic Strength	0.05	0.10	0.15	0.20
$k_2 \times 10^{-2}$	79.5	102.0	38.3	66.4
$SE \times 10^{-2}$	±0.04	±2.1	±3.1	±7.0
$k_4 \times 10^{-2}$	3.1	7.8	111.0	32.2
$SE \times 10^{-2}$	±0.03	±0.5	±18.8	±12.5
$k_6 \times 10^{-2}$	—	6.7	8.9	61.8
$SE \times 10^{-2}$	—	±0.5	±1.7	±26.0
$k_8 \times 10^{-2}$	—	—	—	22.1
$SE \times 10^{-2}$	—	—	—	±9.5

[a] Concentrations are expressed in moles per liter.

Examination of the results obtained at ionic strength 0.05 showed that the low ionic strength made necessary the correction of the tetramer formation constant k_4 for the effect of charge on the form of the polynomial coefficients. The derived equilibrium constants and their standard errors as calculated by the computer are shown in Table I.

Discussion

Examination of the table shows that the molecular weights of the largest units present at pH 2 and ionic strengths of 0.05, 0.10, 0.15, and 0.20, are, respectively, 23,200, 34,800, 34,800, and 46,400. This result is in general agreement with those of other workers, despite the fact that their results were all interpreted in terms of a monomer unit of 12,000 molecular weight.

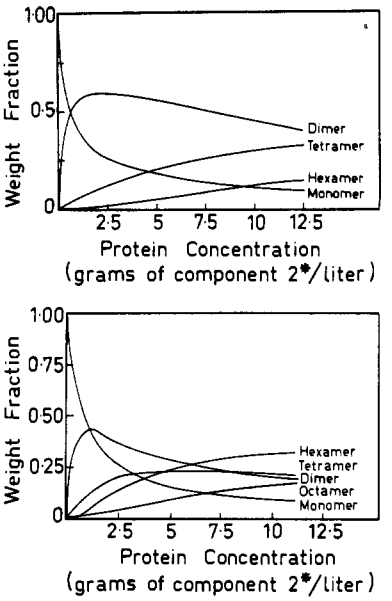

FIGURE 2: The weight fraction of the various insulin species present in solution at pH 2 and 25° as a function of total insulin concentration. Calculated from the equilibrium constants shown in Table I. Monomer molecular weight 5800. Ionic strengths: (a) 0.10 and (b) 0.20.

It will be noticed that the precision of the results decreases at higher ionic strength, this is an unfortunate but inescapable artifact of the method of analysis which involves curve fitting to polynomials of increasingly high orders. It must be understood that the standard errors quoted are derived directly from the standard errors of the coefficients of the polynomials and thus do not take account of any systematic errors included in the original molecular weight *vs.* concentration data. It is noteworthy that the criterion for selecting one reaction scheme compared with another, and for not including results from the highest concentration experiments, was that the acceptable scheme should give the lowest variance for the fit of measured x_1c/m values compared with values calculated from the regression equation; with the additional restriction that all the regression coefficients should be positive.

The effect of ionic strength on the relative concentrations of the various polymeric species is shown in Figure 2, in which the weight fraction of each species present at ionic strengths of 0.10 and 0.20 is plotted against the total concentration of insulin. The curves were calculated using the equilibrium constants shown in Table I; they show clearly how the higher ionic strength encourages the formation of higher proportions of the higher polymers, however the dimer is prominent at both ionic strengths because of its high association constant.

Calculation of the Electrostatic Contribution to the Standard Free Energy of Association. On the grounds that insulin tends to self-associate more extensively if the ionic strength is increased (Gutfreund, 1948) or if the electrostatic charge on the monomers is decreased, Oncley *et al.* (1952) suggested that there is a more or less constant attractive force between insulin monomers, opposed by a mutual electrostatic repulsion. If this is the case, the effect of ionic strength on the free energy of association should be entirely accounted for by changes in the electrostatic contribution to the free energy.

Calculation on the Basis of the Debye–Hückel Theory. We have calculated the electrostatic free energy of association of a model system intended to simulate the insulin system. In this model the associating and associated species are represented as uniformly charged spheres of appropriate diameters. The electrostatic part of the chemical potential of an insulin ion of radius b_i and charge z_i (Oncley *et al.*, 1952) can be calculated from the following equation which is based on the Debye–Hückel theory (Scatchard, 1943)

$$\mu_i^e = \frac{N\epsilon^2 z_i^2}{2D}\left[\frac{1}{b_i} - \frac{K}{1+Ka_i}\right]$$

where e is the electronic charge, D is the dielectric constant (taken as that for water at 25°), and a_i is the sum of the radii of the insulin particle and the chloride ion. K is the Debye–Hückel parameter equal to $0.329 \times 10^8 \times I^{1/2}$ in water at 25°. The principal approximations in applying this equation to insulin over and above those of the Debye–Hückel theory itself are the assumptions of spherical shape and uniform surface distribution of charge.

The magnitude of z_i was arrived at as follows. Tanford and Epstein (1954) found that at pH 1.965 in 0.075 M potassium chloride and 25° (similar conditions to ours) 11.0 moles of hydrogen ion are bound/12,000 molecular weight units; their ion-binding studies showed one chloride ion bound/12,000 molecular weight unit at pH 2. On the basis of these results we have taken a charge of +10 for the 12,000 molecular weight unit. The radius of the sphere of equal volume to the insulin molecule was calculated from our molecular weight values and a partial specific volume of 0.72 ml/g. The radius of the chloride ion was taken as 2.5 A.

The electrostatic part of the free energy of association is then calculated, for example, by subtracting the electrostatic free energy of two monomers from that of a single dimer. Any other association reaction can be treated similarly.

Calculation on the Basis of the Verwey–Overbeek Theory. Verwey and Overbeek (1948) have provided a method for calculating the potential energy of interaction, due to electrostatic repulsion, of two identically charged spherical particles surrounded by charged double layers, when Ka_i is of the order of unity. K is the Debye–Hückel parameter; a_i is the radius of the spherical particle. For insulin molecules under the conditions of the experiments described here this condition is fulfilled. The tabulated values of Verwey

TABLE II: Electrostatic Free Energy of Association (kcal/mole).

Ionic Strength	0.05	0.10	0.15	0.20
		Debye–Hückel Approach		
Monomer–dimer	4.29	3.64	3.19	2.93
Dimer–tetramer	11.72	9.58	8.45	7.76
Dimer, tetramer–hexamer	—	15.46	13.58	12.43
		Residual Free Energy		
Monomer–dimer	−9.61	−9.11	−8.08	−8.14
Dimer–tetramer	−15.15	−13.53	−13.97	−12.54
Dimer, tetramer–hexamer	—	−19.31	−17.60	−17.60
		Verwey and Overbeek Approach		
Monomer–dimer	1.41	1.08	0.92	0.82
Dimer–tetramer	3.47	2.59	2.16	1.90
		Residual Free Energy		
Monomer–dimer	−6.73	−6.55	−5.81	−6.03
Dimer–tetramer	−6.90	−6.54	−7.68	−6.68
		Observed Total Free Energy of Association (kcal/mole)		
Monomer–dimer	−5.32	−5.47	−4.89	−5.21
Dimer–tetramer	−3.43	−3.95	−5.52	−4.78
Dimer, tetramer–hexamer	—	−3.85	−4.02	−5.17

and Overbeek have been used to find the energy of interaction of touching identical spheres of equivalent volume to insulin monomers, dimers, etc., with the appropriate value of the Debye–Hückel parameter K, as a function of the surface potential of the spheres. The surface potential of the uniformly charged spherical particles was evaluated on the basis of the Debye–Hückel theory. This approach follows that of Steiner (1952). As before the dielectric constant of water at 25° was used.

The Residual Free Energy of Association. If it is considered that the observed free-energy changes for the different association reactions are composed of two terms, one due to electrostatic repulsion, the other term accounting for the attractive forces between the insulin particles; the residual (attractive) free energy may be calculated by subtracting the electrostatic contribution from the experimentally observed free energy. The results of such a calculation for the four different ionic strengths are shown in Table II.

Examination of the table shows that the two different methods of calculation lead to two different sets of results. Probably this is largely due to the approximation of the associated species to a sphere when the Debye–Hückel theory is used or to the inadequacies of the theory itself when applied to this system. However, it can be seen that increasing ionic strength has only a small effect on the electrostatic repulsion between the insulin *monomers;* this probably explains why no systematic dependence of k_2 on ionic strength was detected in the experiments. For the *higher polymers* it appears that there is a tendency for the total free energy of association to become more negative with increasing ionic strength, although the trend is not well characterized because of the experimental uncertainties.

On calculation of the residual free energy it is found that the Debye–Hückel approach leads to fairly large negative values decreasing in magnitude with increasing ionic strength. On the other hand, the Verwey–Overbeek approach leads to negative values of the residual association free energy quite similar to those for the monomer–dimer association and showing little if any systematic dependence on ionic strength.

A possible interpretation of these results is that the Verwey and Overbeek approach gives a more reasonable description of the electrostatics of the system; particularly since the residual free energy is apparently independent of the ionic strength implying that if the effect of ionic strength is confined to the electrostatic interaction it has been completely accounted for in the calculation.

One consequence of this study is the implication that for insulin at this pH value a large proportion of the free energy of association is due to the electrostatic interactions involved. Accordingly experiments are now being undertaken with α-chymotrypsin which can be studied near its isoelectric point, and should thus be representative of systems in which the electrostatic contribution to the free energy of association is small.

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Phenazines and Phenoxazinones from Some Novel *Nocardiaceae**

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ABSTRACT: Microorganisms forming a novel group of *Nocardiaceae* were seen to produce 1,6-phenazinediol 5,10-dioxide (iodinin) crystals. Careful study of one strain in submerged culture revealed the formation of 1,6-phenazinediol, 1,6-phenazinediol 5-oxide, 2-amino-3H-phenoxazin-3-one, and 2-acetamidophenoxazin-3-one, all substances which are produced along with iodinin by *Microbispora aerata*, *Brevibacterium iodinum*, and *Streptomyces thioluteus*. In addition, four other compounds in yields of 0.1–0.5 mg/l. were detected and identified by comparison with authentic samples. Two, previously known from other microorganisms, were phenazine-1-carboxylic acid and 3,6-dibenzylidene-2,5-dioxopiperazine. A new compound was 1-phenazinol 10-oxide (orange crystals, mp 165–167°; λ_{max}^{EtOH} 468 (ϵ 2120), 387 (3600), and 279 mμ (67,800)) which was also formed from 1-phenazinol by disrupted cells of *B. iodinum* and was synthesized chemically in low yield by the peracetic acid oxidation of 1-phenazinol.

Also new was 2-amino-1-carboxy-3H-phenoxazin-3-one (dark crystals, decomposing at 310–320°; λ_{max}^{EtOH} 425 (ϵ 10,620), 442 (10,380), and 233 mμ (28,220); methyl ester, orange crystals, mp 210–215°) which was also synthesized chemically in low yield by the ferricyanide oxidation of a mixture of *o*-aminophenol and 3-hydroxyanthranilic acid. The position of the carboxy group in the new phenoxazinone (1 not 9) was proved by its conversion in aqueous base to 2-hydroxy-3H-phenoxazin-3-one.

The morphology of a novel group of *Nocardiaceae* (Lechevalier and Lechevalier, 1965) is being investigated in these laboratories. The first organism of this group, called the "Malloch strain," and received from Dr. R. E. Gordon, formed slants with lustrous coppery needles on the mycelium and in the agar. This is the characteristic appearance of iodinin, 1,6-phenazinediol 5,10-dioxide, as we have seen it before on cultures of *Microbispora aerata* and *Brevibacterium iodinum*.[1] When the Malloch strain was grown in submerged culture on a variety of media used for actinomycetes (Becker *et al.*, 1964), it produced the mixture of phenazines and phenoxazinones that usually accompany iodinin (Gerber and Lechevalier, 1964). These mixtures were extracted from whole broth by chloroform and separated by column chromatography on silica gel, thin layer chromatography on silica gel G, and paper chromatography on fully acetylated paper. The separated pure substances were identified by ultraviolet and visible absorption spectra and shown to be identical with authentic samples by their color, fluorescence, and R_F values in at least four different solvent systems, usually three on paper and one on thin layer plates. Identified in this way were iodinin, 1,6-phenazinediol, 1,6-phenazinediol 5-oxide (Gerber and Lechevalier, 1965), 2-amino-3H-phenoxazin-3-one, and 2-acetamidophenoxazin-3-one. In addition to these known materials, when the Malloch strain was grown on a soybean meal–peptone–glucose–salt medium, we isolated four unknown compounds, all in amounts too small for ordinary analyses. Therefore we deduced their structures from spectra, color tests, and chromatographic behavior, then confirmed them by comparison with authentic synthetic products.

The first of these was observed as a separate orange band from column chromatography. Its absorption spectrum was characteristic of a phenazine, maxima at 468, 387, and 279 mμ. The yields of pure material, orange crystals, mp 165–167°, were about 0.5 mg/l. of whole broth. With sodium hydrosulfite this orange phenazine was reduced to 1-phenazinol, identical

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[1] Previously referred to as *Pseudomonas iodina*. As Sneath (1956) observed, it is a Gram-positive diptheroid bacterium and is probably best included with the brevibacteria.