Polymerization Pattern of Insulin at pH 7.0†

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ABSTRACT: Sedimentation equilibrium results, obtained with bovine zinc-free insulin (with and without a component of proinsulin) at pH 7.0, I 0.2, 25 °C, and up to a total concentration of 0.8 g/l., are shown to be consistent with three different polymerization patterns, all involving an isodesmic indefinite self-association of specified oligomeric species. The analysis procedure, based on closed solutions formed by summing infinite series, yields for each pattern a set of equilibrium constants. It is shown that a distinction between the possible patterns can be made by analyzing sedimentation equilibrium results obtained in a higher total concentration range (up to 4 g/l.) with insulin freed of zinc and proinsulin, account being taken of the composition dependence of activity coefficients. The favored pattern, which differs from that previously reported in the literature, involves the dimerization of monomeric insulin (mol wt 5734), governed by a dimerization constant of 11×10^4 M^{-1} and the isodesmic indefinite self-association of the dimer, described by an association constant of 1.7×10^4 M^{-1}. This polymerization pattern is also shown to be consistent with the reaction boundary observed in sedimentation velocity experiments.

The polymerization behavior of insulin in solution at acid pH values has been well investigated (Steiner, 1952; Doty et al., 1952; Jeffrey and Coates, 1966) and, more recently, such studies have been extended to include examination of the system at neutral pH (Pekar and Frank, 1972). The latter work is of particular interest in relation to the definition of the biologically active species existing under physiological conditions (Jeffrey, 1974). Pekar and Frank (1972) used porcine insulin freed of zinc and proinsulin to obtain, from sedimentation equilibrium results at pH 7, I 0.2, values of apparent weight-average molecular weights, as a function of total protein concentration, in the range 0.09–4 g/l. These results were shown to be consistent with a model specifying that solutions comprised equilibrium mixtures of monomer, dimer, hexamer, and a series of higher polymers formed by the isodesmic indefinite self-association of the hexameric species. The three equilibrium constants derived on this basis were as follows: for dimer formation, $K_2 = 1.4 \times 10^5$ M^{-1}; for hexamer formation, $K_6 = 4 \times 10^8$ M^{-2}; and for the isodesmic self-association of hexamer, $K_{1,6} = 5 \times 10^3$ M^{-1}.

The analysis presented by Pekar and Frank (1972), in utilizing comparisons of calculated and experimentally obtained apparent weight-average molecular weights, is inherently less accurate than one based on direct simulation of sedimentation equilibrium distributions (plots of total concentration vs. radial distance), which includes consideration of composition-dependent activity coefficients. It is the purpose of this work to reexamine the system and to present an alternative polymerization pattern for insulin consistent with both sedimentation equilibrium and velocity results.

Theory

Formulation of Appropriate Models. It has been shown (Milthorpe et al., 1975) that the results of a sedimentation equilibrium experiment with a polymerizing solute in the form of total weight concentration of solute, $\bar{c}(r)$, vs. radial distance, r, may be analyzed directly to yield the thermodynamic activity of the monomer, $a_1(r)$. Thus,

$$\Omega_1(r) = \bar{c}(r)e^{\phi_1 M_1(r_F^2 - r^2)}/\bar{c}(r_F)$$
$$\lim_{\bar{c}(r)\to 0}\Omega_1(r) = a_1(r_F)/\bar{c}(r_F) \quad (1a)$$

$$a_1(r) = a_1(r_F)e^{\phi_1 M_1(r^2 - r_F^2)} \quad (1b)$$

where r_F is any selected reference radial distance in the experimentally observed distribution, $\bar{c}(r_F)$ being the corresponding total solute concentration. The quantity ϕ_1, equaling $(1 - \bar{v}_1\rho)\omega^2/2RT$ (with conventional notation), is assumed constant, independent of radial distance. Consider, first, the situation pertaining to a range of relatively low $\bar{c}(r)$ where nonideality terms are of insufficient magnitude to affect the distribution measurably. In this case, $a_1(r)$ may be taken to equal $c_1(r)$ and it is possible to obtain a closed solution for $\bar{c}(r)$ in terms of this variable even when isodesmic indefinite self-associations are encountered. This point will be illustrated with three models which prove to be pertinent in the reexamination of the insulin system. For all models,

$$\bar{c}(r) = \sum_i c_i(r) = M_1 \sum_i i m_i(r) \quad (2)$$

where $m_i(r)$ denotes the molar concentration of oligomeric species i ($i = 1$, monomer, and $m_1(r) = c_1(r)/M_1$).

Model 1. The model proposed by Pekar and Frank (1972), may now be specified by $i = 1,2,6,12, \ldots, \infty$, although the previous workers truncated their series at $i = 54$. Equation 2 becomes with the introduction of the isodesmic self-association constant, $K_{1,6}$,

$$\bar{c}(r) = M_1\{m_1(r) + 2m_2(r) + 6m_6(r)S\}$$
$$S = \sum_{k=0}^{\infty}(k+1)K_{1,6}{}^k m_6{}^k(r) \quad (3a)$$

It is a simple matter to show that $S\{1 - K_{1,6}m_6(r)\}$ equals the sum of an infinite number of terms constituting a geometric progression with first term unity and common ratio $K_{1,6}m_6(r)$. It follows that $S = 1/\{1 - K_{1,6}m_6(r)\}^2$; $(K_{1,6}m_6(r) < 1)$ and that eq 3a becomes

$$\bar{c}(r) = M_1 \times \left\{m_1(r) + 2K_2 m_1{}^2(r) + \frac{6K_6K_2{}^3 m_1{}^6(r)}{\{1 - K_{1,6}K_6K_2{}^3 m_1{}^6(r)\}^2}\right\} \quad (3b)$$

In a similar way, a closed solution for

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$$(M_{w,r}/M_1) = \left\{\sum_i ic_i(r)\right\} \Big/ \bar{c}(r)$$

may be obtained for this model,

$$\frac{M_{w,r}}{M_1} = \frac{M_1 m_1(r) + 4M_1K_2m_1^2(r)}{\bar{c}(r)} + \frac{36M_1K_6K_2^3m_1^6(r)\{1 + K_{1,6}K_6K_2^3m_1^6(r)\}}{\bar{c}(r)\{1 - K_{1,6}K_6K_2^3m_1^6(r)\}^3} \quad (3c)$$

The usefulness of being able to determine $c_1(r)$ and hence $m_1(r)$ by application of eq 1 is now apparent, since use of eq 3b, for example, permits ready construction of the corresponding plot of $\bar{c}(r)$ vs. r for particular values of the equilibrium constants, such as those reported previously (Pekar and Frank, 1972).

An important feature of model 1 is that the hexamer is the basic unit undergoing indefinite self-association. Certainly, it will become apparent that the results in this work also indicate the operation of an indefinite self-association. However, there is no a priori reason why the hexamer should function as the basic unit for this process. Accordingly, models 2 and 3 explore the simplest possible alternatives that either monomer or dimer, respectively, is the basic unit.

Model 2. This is specified by $i = 1,2,3,\ldots,\infty$. In this case, the experimental plot of $\bar{c}(r)$ vs. r may be used not only to determine $c_1(r)$, but also to evaluate first estimates of the successive equilibrium constants involved in the indefinite self-association by a method based on Laplace transformation (Nichol et al., 1976). Accordingly, it is again pertinent to formulate a closed solution for $\bar{c}(r)$ to permit a test of the applicability of the model and to refine the first estimates of the equilibrium constants, if appropriate. Howlett et al. (1973) showed for this model that

$$\bar{c}(r) = \frac{M_1 m_1(r)\{1 + 2m_1(r)(K_2 - K_{1,1}) + K_{1,1}m_1^2(r)(K_{1,1} - K_2)\}}{\{1 - K_{1,1}m_1(r)\}^2} \quad (4a)$$

where dimer formation is governed by K_2 and, thereafter, the successive addition of monomer by the isodesmic self-association constant, $K_{1,1}$. By placing $K_2 = K_{1,1}$, eq 4a applies to an isodesmic self-association of monomer. It is also noted that similar solutions in closed form may readily be formulated if a limited number of more than two equilibrium constants are indicated by the initial Laplace analysis. In the terms specified by eq 4a, the analogue to eq 3c is

$$\frac{M_{w,r}}{M_1} = \frac{M_1\{m_1(r) + 4K_2m_1^2(r)\}}{\bar{c}(r)} + \frac{M_1K_2m_1(r)\{1 + K_{1,1}m_1(r)\}}{K_{1,1}\bar{c}(r)\{1 - K_{1,1}m_1(r)\}^3} - \frac{M_1K_2m_1(r)\{1 + 4K_{1,1}m_1(r)\}}{K_{1,1}\bar{c}(r)} \quad (4b)$$

Model 3. The specification $i = 1,2,4,6,\ldots,\infty$ implies that monomer coexists in equilibrium with dimer which acts as the basic unit for indefinite self-association (considered here to be isodesmic). This formulation differs from model 1 in that only bimolecular collisions are envisaged and from model 2 in that odd-numbered polymers are considered not to be formed. It acknowledges (as do models 1 and 2) that dimer and hexamer are known to be stable species (Jeffrey and Coates, 1966; Blundell et al., 1972). The appropriate equations are again formulated by summing infinite series to give,

$$\bar{c}(r) = \frac{M_1m_1(r)\{(1 - K_2K_{1,2}m_1^2(r))^2 + 2K_2m_1(r)\}}{\{1 - K_2K_{1,2}m_1^2(r)\}^2} \quad (5a)$$

$$\frac{M_{w,r}}{M_1} = \frac{M_1m_1(r)}{\bar{c}(r)} + \frac{4M_1K_2m_1^2(r)\{1 + K_2K_{1,2}m_1^2(r)\}}{\bar{c}(r)\{1 - K_2K_{1,2}m_1^2(r)\}^3} \quad (5b)$$

where $K_{1,2}$ is the isodesmic self-association constant of dimer.

Consideration of Nonideality. The analysis of sedimentation equilibrium results obtained in a range of low $\bar{c}(r)$ may lead to a situation where different models (and associated equilibrium constants) equally well describe the results. It is thus required to test each model against results obtained in a higher concentration range where nonideality effects can no longer be neglected. Ogston and Winzor (1975) pointed out that the activity coefficient of each species i, $y_i(r) = a_i(r)/c_i(r)$, is a function of solution composition, which in sedimentation equilibrium varies with radial distance. Thus, for all i

$$\ln y_i(r) = \frac{\alpha_{ii}c_i(r)}{M_i} + \sum_{j \neq i} \frac{\alpha_{ij}c_j(r)}{M_j} \quad (6)$$

where α_{ii} and α_{ij} are, respectively, constant coefficients expressing interactions of species i with itself and with other species j. It is implicit in eq 6 that concentrations on the molar and molal scales are assumed identical and that higher order terms are negligible. Values of α_{ii} and α_{ij} may be estimated on the following basis,

$$\alpha_{ii} = U_{ii} + (z_i^2/2I); \quad \alpha_{ij} = U_{ij} + (z_iz_j/2I) \quad (7)$$

where U_{ii} and U_{ij} are covolumes (M^{-1}); z_i and z_j are the net charges on i and j and I is the ionic strength of the uniunivalent supporting electrolyte. If all species are regarded as hydrated spheres, it may be shown (Tanford, 1961) that,

$$U_{ij} = (\bar{v} + w\bar{v}_{solvent})(M_i^{1/3} + M_j^{1/3})^3/1000 \quad (8)$$

where $\bar{v}$ (ml/g) is the anhydrous partial specific volume of each oligomeric species; w is the number of g of bound solvent/g of anhydrous solute; $\bar{v}_{solvent}$ is the partial specific volume of solvent, and M_i and M_j refer to anhydrous molecular weights. It has been assumed in eq 8 that $(\bar{v} + w\bar{v}_{solvent})$ is identical for each oligomeric species. Equation 8 encompasses the particular case $i = j$, thereby permitting calculation of U_{ii}. Further discussion of the approximations involved in estimating α_{ii} and α_{ij} follows later.

It could now be recalled that application of eq 1 to a sedimentation equilibrium distribution spanning a relatively high range of $\bar{c}(r)$ yields $a_1(r)$ and thus the activities of all other polymeric species at each r may be calculated without assumption using the equilibrium constants appropriate to each model. These values of activities may, in turn, be used as first estimates of $c_i(r)$ and $c_j(r)$ in eq 6, the summation term being taken to include those species contributing significantly to the total concentration. Thus, with the use of the calculated α_{ii} and α_{ij} values, first estimates of all relevant $y_i(r)$ are obtained, which may be divided into the available $a_i(r)$ values to obtain improved estimates of $c_i(r)$. Accordingly, the process may be repeated until these values converge, whereupon eq 2 gives $\bar{c}(r)$ for the model under examination. Direct comparison of the experimental results with those obtained for the various models may now be made, as account has been taken of the composition dependence of all activity coefficients.

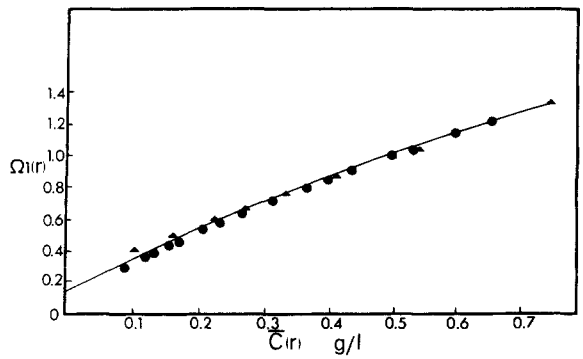

FIGURE 1: A plot of $\Omega_1(r)$, defined by eq 1a, vs. total solute concentration found in two experiments conducted with unfractionated zinc-free insulin at pH 7.0, I 0.2, and 25 °C. (▲) experiment 1, where $\omega = 4191$ rad/s, $r_b - r_m = 0.2307$ cm, $\bar{c}^0 = 0.427$ g/l., and $\bar{c}(r_m) = 0.035$ g/l.; (●) experiment 2, where $\omega = 4608$ rad/s, $r_b - r_m = 0.6701$ cm, $\bar{c}^0 = 0.111$ g/l., and $\bar{c}(r_m) \sim 0$ g/l., Chervenka (1970) design. A value of $\bar{c}(r_F)$ of 0.498 g/l., common to both experiments, was used to construct the plot, the solid line which attempts to average the data extrapolating to $a_1(r_F)/\bar{c}(r_F) = 0.14_5$.

TABLE I: Comparison of Experimental Sedimentation Equilibrium Distributions Obtained with Insulin at pH 7.0, I 0.2, and 25 °C with Distributions Calculated on the Basis of Stipulated Models.[a]

	Standard Deviations[b] (microns)		
Expt	Model 1	Model 2	Model 3
1	10	11	10
2	40	36	34
3	7	12	14
4	132	232	15
5	150	280	36

[a] Model 1, $K_2 = 1.4 \times 10^5$ M^{-1}, $K_6 = 4 \times 10^8$ M^{-2}, $K_{1,6} = 5 \times 10^3$ M^{-1}; Model 2, $K_2 = 8 \times 10^4$ M^{-1}, $K_{1,1} = 3.9 \times 10^4$ M^{-1}; Model 3, $K_2 = 11 \times 10^4$ M^{-1}, $K_{1,2} = 1.7 \times 10^4$ M^{-1}. [b] Based on ten comparisons of $\bar{c}(r)$ over the measurable range and expressed in terms of fringe displacement.

Materials and Methods

Bovine insulin containing 0.78 g of zinc/100 g of anhydrous protein, 3–4% proinsulin, and approximately 5% monodesamido insulin was obtained from the Australian Commonwealth Serum Laboratories. The insulin was freed of zinc by dialyzing, in Visking $^{18}/_{32}$ tubing, 20-ml volumes (10 mg/ml of insulin in 0.01 M HCl) against 1 l. of the same solvent at 4 °C for 48 h with three changes of the dialyzate. Atomic absorption spectroscopy revealed that the samples so treated contained less than 0.01 ppm of zinc, while disc gel electrophoresis on polyacrylamide at pH 7.8 showed that the samples retained their proinsulin and monodesamido insulin content: such samples are termed unfractionated insulin in reference to their use in certain experiments cited in the text. For other experiments, the zinc-free insulin was fractionated as follows: a solution (20 mg of protein in 5 ml of 0.05 M Tris-HCl, pH 7.8, I 0.05) was loaded onto a 2.5 × 5 cm column of 7% polyacrylamide gel equilibrated with the same buffer and subjected to electrophoresis at 40 mA for 15 h at 20 °C, employing a LKB Uniphor 7900 apparatus. Fractions from the main peak were concentrated by ultrafiltration using a UM-2 membrane. This fractionated insulin was shown to be free of proinsulin but still retained a small amount of the monodesamido component.

Stock solutions of fractionated and unfractionated insulin at pH 7.0, I 0.2 were prepared by dialyzing against buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.001 M EDTA) at 25 °C and dilutions were made by weight with the buffer. Diluted solutions were dialyzed over night preceding each sedimentation experiment and final concentrations were determined spectrophotometrically at 276 nm employing an extinction coefficient of 1.05 ODU/cm (mg/ml) (Frank and Veros, 1968). Zinc-free insulin was found to have a solubility of only 4.5–5 g/l. in the buffer employed and, accordingly, sedimentation equilibrium experiments were designed so that a concentration of about 4 g/l. was not exceeded at any time during an experiment.

Sedimentation equilibrium experiments were conducted at 25 °C using a Spinco Model E analytical ultracentrifuge and Rayleigh interference optics. Equal amounts by weight of the inert fluorocarbon FC43 were placed in each sector of the cells before introducing solution and dialysate into the respective channels. The fluorocarbon FC43 was obtained from Beckman and used without purification: in a control experiment performed with a saturated insulin solution at pH 7, no precipitation was observed at the solution–oil interface. In certain designated experiments, a synthetic boundary centerpiece was employed and the experiment was conducted as described by Chervenka (1970): in others, equal amounts of solution and solvent were introduced into a cell with standard centerpiece. The photographic records of sedimentation equilibrium distributions were measured with a Nikon microcomparator and, for experiments other than those of the meniscus depletion design (Chervenka, 1970), the method of Teller (1973) was used to calculate the total concentration at the meniscus. In general, experiments were run for 20 h and the attainment of equilibrium was checked by measuring the penultimate and final exposures. The monomer molecular weight of beef insulin was taken as 5734 (Ryle et al., 1955), a value of 0.73 ml/g was used for the partial specific volume (Frank and Veros, 1968), and densities were measured with an Anton Paar DMA-02C precision density meter.

Sedimentation velocity patterns were obtained, at 25 °C in the same buffer, employing schlieren optics: those obtained using a synthetic boundary cell were analyzed in terms of the square root of the second moment (Goldberg, 1953); while those showing incomplete resolution at the meniscus in a standard double-sector cell were analyzed by the method of Baldwin (1953).

Results

Sedimentation Equilibrium over a Range of Low $\bar{c}(r)$. Figure 1 presents a plot of $\Omega_1(r)$, defined by eq 1a, vs. $\bar{c}(r)$ referring to two equilibrium experiments conducted with unfractionated zinc-free insulin, details being reported in the caption. At first sight, it may appear that the sets of points from the two experiments deviate slightly at the lower concentrations. However, these deviations are within the estimated errors in $\Omega_1(r)$, which increase with decreasing concentration (ca. 1% at 1 g/l. and 10% at 0.1 g/l.). Accordingly, the solid line represents an attempt to average all data and yields by use of eq 1a a mean value of $a_1(r_F)$ of 0.072 g/l. at $r_F = 7.1450$ cm (experiment 1) and $r_F = 7.1035$ cm (experiment 2). This mean value of $a_1(r_F)$ was now used in eq 1b to calculate $a_1(r)$ for selected r values in each experiment and these values were used in eq 3b to calculate $\bar{c}(r)$ as a function of r employing the values of K_2, K_6, and $K_{1,6}$ reported in the introduction (Pekar and Frank, 1972). Comparison of these $(\bar{c}(r), r)$ points with the

[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; ODU, optical density units.

corresponding experimental points for both experiments led to the standard deviations (ten comparisons over the entire measurable range) reported in Table I (model 1).

The values of the standard deviations for models 2 and 3, also shown in Table I, were found in an entirely analogous way employing, instead of eq 3b, eq 4a and 5a, respectively. Comment is required, however, on the values of the equilibrium constants used in this connection. As has been noted, first estimates of the successive equilibrium constants for model 2 may be found by analysis of an experimental result (in which the total concentration at the meniscus does not approximate zero) by a method based on Laplace transformation (Nichol et al., 1976). This analysis applied to experiment 1 gave first estimates of the dimerization constant $K_2 = 7 \times 10^4$ M^{-1} and $K_{I,1} = 4.1 \pm 0.8 \times 10^4$ M^{-1}, the latter being the average value of eight apparent constants describing the successive addition of monomer to dimer. These proved valuable in searching for improved estimates in the same ranges: the search was conducted by employing eq 4a to determine the set of $\{K_2, K_{I,1}\}$ which gave the minimum standard deviation when experimental and calculated $(\bar{c}(r), r)$ points, for experiment 1, were compared. The result is reported in Table I where the entries for model 2 refer to $K_2 = 8 \times 10^4$ M^{-1} and $K_{I,1} = 3.9 \times 10^4$ M^{-1}, values evidently close to the original estimates, and, moreover, ones which gave a reasonable fit to experiment 2. For model 3, eq 5a is appropriate and requires the specification of K_2 and $K_{I,2}$: values of K_2 starting with 8×10^4 M^{-1} (from model 2) and increasing in increments of 1×10^4 to 14×10^4 M^{-1} (from model 1) were examined, the corresponding values of $K_{I,2}$ being calculated on the basis of eq 5a and one $(\bar{c}(r), r)$ point. Each set of apparent $\{K_2, K_{I,2}\}$ values was used to generate the entire $\bar{c}(r)$ vs. r distribution for experiment 1, the set leading to the minimum standard deviation, shown in Table I, being $K_2 = 11 \times 10^4$ M^{-1}, $K_{I,2} = 1.7 \times 10^4$ M^{-1}. Again it is evident that this set reasonably describes experiment 2. Indeed, on the basis of these initial experiments 1 and 2, it is not possible to favor any of the three suggested models, nor to attempt further improvement of the numerical values of the equilibrium constants assigned to each. Thus, all relevant standard deviations are equal to or less than 40 μm, a value which may well reflect total errors including those involved in measuring interferograms and in determining the total concentration at the meniscus.

Two further points merit comment in relation to experiments conducted over a relatively low $\bar{c}(r)$ range. First, comparison is warranted between experiments 1 and 2, conducted with unfractionated zinc-free insulin, and the result of a sedimentation equilibrium experiment performed with a fractionated sample. This experiment 3 (Table I) was of the Chervenka (1970) design with angular velocity, $\omega = 4608$ rad/s, column height, $r_b - r_m = 0.6874$ cm, and initial loading concentration, $\bar{c}^0 = 0.112$ g/l. As before, an $\Omega_1(r)$ plot was constructed and the derived $a_1(r)$ values used in eq 3b, 4a, and 5a to obtain the standard deviations reported in Table I. In addition to confirming that all three models adequately describe the results extending to $\bar{c}(r) = 0.8$ g/l., the standard deviations for experiment 3, when compared with those of the previous experiments, show that the 4% proinsulin content of unfractionated samples does not contribute significantly to the distributions up to this total concentration. Indeed, this was confirmed by simulating for experiment 1 the distribution of proinsulin on the basis that it exhibited an identical polymerization pattern to insulin (Pekar and Frank, 1972) and was of monomer molecular weight 8684 (Nolan et al., 1971): in terms of fringe displacement proinsulin contributes a maximum of only 24 μm

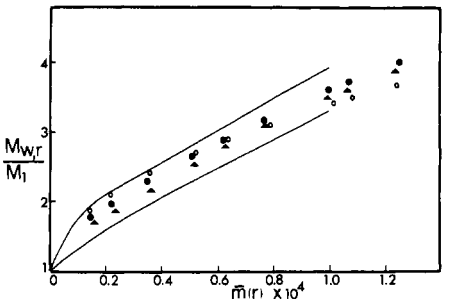

FIGURE 2: A plot of the apparent weight-average molecular weight, $M_{w,r}$, divided by the molecular weight of monomer ($M_1 = 5734$) vs. total protein concentration on the molar scale. The solid lines define a domain within which the experimental points of Figure 2 of Pekar and Frank (1972) reside. The points were calculated from the experimentally determined $(c_1(r), r)$ points of this work (experiment 3): (▲) Model 1 using eq 3c; (○) Model 2 using eq 4b; (●) Model 3 using eq 5b.

(a value within experimental error) to the highest $\bar{c}(r)$ recorded of 840 μm. On the other hand, this finding does suggest that experiments conducted in a higher $\bar{c}(r)$ range should be performed with insulin free of proinsulin.

Secondly, Figure 2 presents a plot of $M_{w,r}/M_1$ vs. the total concentration expressed on a molar scale. The solid lines define the domain within which the experimental points of Figure 2 of Pekar and Frank (1972) fall. The points were calculated using eq 3c, 4b, and 5b with the values of $c_1(r)$ found from the $\Omega_1(r)$ plot of experiment 3: analogous points for experiments 1 and 2 also fell within the envelope. It is clear then that experimental results obtained in this work with bovine-fractionated insulin are in agreement with those obtained previously with porcine samples. Moreover, it is now evident that evaluation of apparent $M_{w,r}$ values from $d \ln \bar{c}(r)/d(r^2)$ estimates introduces sufficient scatter to render impossible the distinction between the relevance of models 1, 2, or 3.

Behavior in the High $\bar{c}(r)$ Range. Two sedimentation equilibrium experiments were conducted with fractionated zinc-free insulin to explore the possibility that a distinction between the models might be possible in a higher $\bar{c}(r)$ range. Details of these experiments are as follows: experiment 4, $\omega = 1466$ rad/s, $r_b - r_m = 0.3552$ cm, $\bar{c}^0 = 1.158$ g/l., and $\bar{c}(r_m) = 0.530$ g/l.; experiment 5, $\omega = 1466$ rad/s, $r_b - r_m = 0.3600$ cm, $\bar{c}^0 = 1.276$ g/l., and $\bar{c}(r_m) = 0.570$ g/l. Plots of $\Omega_1(r)$ vs. $\bar{c}(r)$, evaluated with a common $\bar{c}(r_F) = 0.574$ g/l., formed a smooth curve which overlapped with that constructed for experiment 3 (fractionated insulin) employing the same $\bar{c}(r_F)$ value. The values of $a_1(r)$ for experiments 4 and 5 derived on this basis (eq 1) were first used in eq 3b, 4a, and 5a to calculate hypothetical $\bar{c}(r)$ values (termed $\bar{a}(r)$) for each model: only if nonideality effects are negligible would $\bar{a}(r) = \bar{c}(r)$. The results are shown in Figure 3 where they are compared with the experimental $(\bar{c}(r), r)$ points for experiment 4. A similar plot for experiment 5 confirmed the observation apparent from Figure 3 that no model adequately describes the experimental results when nonideality is neglected and that, only for model 3, does the $\bar{a}(r)$ curve lie above the $\bar{c}(r)$ plot.

Consideration of nonideality involved use of eq 7 and 8 to calculate the sets of α_{ii} and α_{ij} appropriate to each model: z_1 was taken as -2 (Tanford and Epstein, 1954), conservation of charge on polymerization being assumed, and $(\bar{v} + w\bar{v}_{solvent})$ was set equal to 0.8 (a value calculated from the sedimentation coefficient of the monomer on the basis of spherical geometry). Equation 6 was then applied iteratively, as previously described, using $a_i(r)$ values calculated for each model from reported equilibrium constants (Table I) and $a_1(r)$ values from

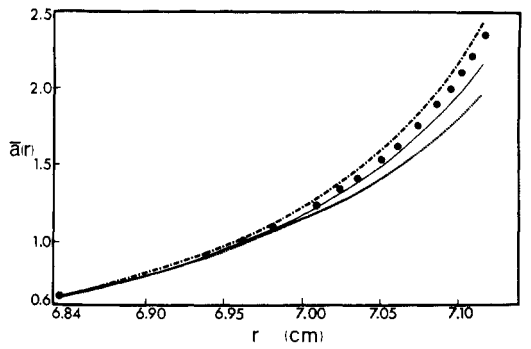

FIGURE 3: The relationship between radial distance, r, and the quantity $\bar{a}(r)$ calculated using $a_1(r)$ values found from experiment 4 and eq 3b for model 1 (—); eq 4a for Model 2 (· · ·), and eq 5a for Model 3 (– · –). In so doing, the assumption was made that nonideality effects were negligible. Included for comparison are the experimental points (●) of total concentration vs. radial distance for experiment 4.

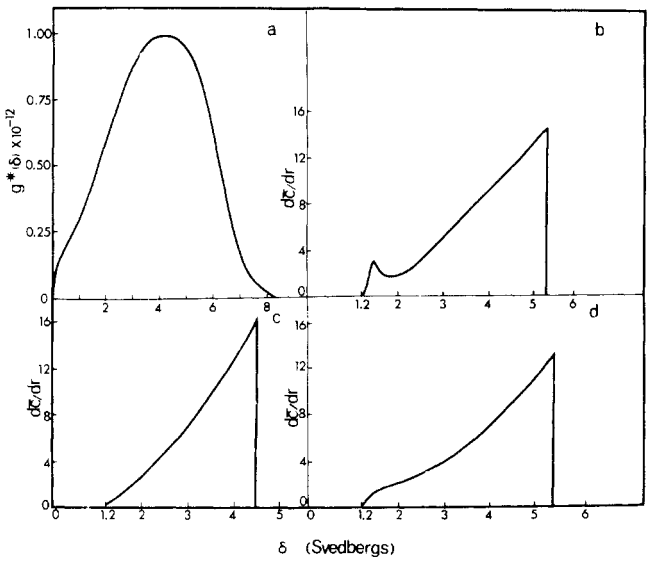

FIGURE 4: A comparison of the forms of calculated and experimental reaction boundaries pertaining to the sedimentation of insulin at pH 7.0, I 0.2, and 25 °C. (a) A plot of $g^*(\delta)$ vs. δ derived from an exposure taken (at 8720 s) in a sedimentation velocity experiment conducted at an angular velocity of 5.027×10^3 rad/s; (b,c,d) calculated idealized plots (neglecting diffusional spreading and composition dependence of sedimentation coefficients) of the weight-concentration gradient vs. the sedimentation coefficient parameter, δ, referring, respectively, to models 1, 2, and 3, and the same conditions as for a.

the $\Omega_1(r)$ plot. A typical result referring to experiment 4 and model 3 is shown in Table II for the particular r value of 7.1172 cm corresponding to the highest $\bar{c}(r)$ measured. The first two columns of Table II show the contributions of species up to the 24-mer: in practice, the calculations were extended to $i = 50$. The next three columns illustrate the rapid convergence of the iterative procedure used to determine the activity coefficient of each relevant species i. The last column tabulates $c_i(r)$ obtained by dividing $a_i(r)$ by the final estimate of $y_i(r)$. The sum of these $c_i(r)$ values (2.329 g/l. for $i = 1,2, \ldots, 50$) was compared with the experimental $\bar{c}(r)$ at $r = 7.1172$ cm. The procedure was repeated for nine other values of r, for both experiments 4 and 5, and for models 1 and 2 to give the standard deviations reported in Table I. The definite result emerges that only model 3 describes within experimental error the entire set of sedimentation equilibrium results when realistic account is taken of composition-dependent activity coefficients. These calculations also revealed that below $\bar{c}(r)$ values of 0.9 g/l. the

TABLE II: Illustration of the Determination of Activity Coefficients and Species Concentrations Appropriate to the Point ($\bar{c}(r)$ = 2.359 g/l., r = 7.1172 cm) Observed in Experiment 4 Utilizing Model 3 (Table I).

Species Act.[a], $a_i(r)$		Est. Act. Coeff.,[b] $y_i(r)$			$c_i(r)$
i	(g/l.)	1	2	3	(g/l.)
1	0.102	1.0122	1.0117	1.0117	0.101
2	0.392	1.0191	1.0183	1.0183	0.385
4	0.464	1.0319	1.0304	1.0305	0.450
6	0.411	1.0442	1.0422	1.0422	0.394
8	0.324	1.0562	1.0536	1.0537	0.307
10	0.239	1.0682	1.0650	1.0651	0.224
12	0.169	1.0801	1.0763	1.0765	0.157
14	0.117	1.0920	1.0877	1.0879	0.108
16	0.079	1.1040	1.0990	1.0993	0.072
18	0.052	1.1160	1.1104	1.1107	0.047
20	0.034	1.1281	1.1219	1.1222	0.030
22	0.022	1.1402	1.1334	1.1337	0.019
24	0.014	1.1525	1.1450	1.1453	0.012

[a] Determined with $a_1(r)$ from the experimental $\Omega_1(r)$ plot and $K_2 = 11 \times 10^4$ M^{-1}, $K_{1,2} = 1.7 \times 10^4$ M^{-1}. [b] Determined from eq 6 utilizing α_{ii} and α_{ij} calculated from eq 7 and 8.

correction for nonideality effects for all models was less than 15 μm, a finding justifying their neglect in the analysis of experiments 1, 2, and 3.

Sedimentation Velocity Results. Figure 4a presents a plot of $g^*(\delta)$ vs. δ, as defined by Signer and Gross (1934) to account for radial dilution and the inhomogeneous centrifugal field, found from an exposure taken 8720 s from the start of an experiment conducted with unfractionated zinc-free insulin (3.5 g/l., other details being reported in the caption). The weight-average sedimentation coefficient, $\bar{s}_{25,b}$, for this experiment was 4.1 S, a value confirmed in a separate experiment using a synthetic boundary cell and the analysis procedure of Goldberg (1953). It is noted from Figure 4a that, while a slight asymmetry is detectable on the trailing side of the boundary, the peak is essentially unimodal. It is of interest to compare this result with that predicted theoretically on the basis of models 1, 2, and 3. In this connection, application of numerical procedures (Cox 1967; 1969; Gilbert and Gilbert, 1973) designed to account for effects due to mass migration, chemical reaction, frictional interaction, and diffusional spreading would require the specification of the sedimentation and diffusion coefficients (and their composition dependence) for each relevant oligomeric species, a difficult task for the present system. It is, however, possible to calculate idealized schlieren patterns, which neglect diffusion and composition-dependence effects and assume a homogeneous field, but which show the essential features of the reaction boundary dictated by mass migration and chemical reaction (Gilbert, 1959). Figure 4b,c,d presents such plots for models 1, 2, and 3, respectively, calculated for the same time as Figure 4a. The calculations involved the assignment only of s_i values which were estimated using $s_i = (f_0/f_i)i^{2/3}s_1$ with s_1 = 1.2 S (Jeffrey and Coates, 1965) and the frictional coefficient (f_1/f_0) set equal to 1.2. At first sight, model 3 (Figure 4d) appears to be favored in that it predicts the slight shoulder on the trailing side of the boundary evident in Figure 4a: moreover, $\bar{s}_{25,b}$ corresponding to 3.5 g/l., calculated by standard procedures, was 4.1 S for this model, in agreement with the experimental value. However, it must be stressed that the effect of diffusional spreading has been neglected in the theoretical calculations and accordingly, it is not possible to exclude models 1 or 2 on the basis of the forms of

the reaction boundaries. For example, the pronounced bimodality apparent in Figure 4b may, with diffusional spreading, be evident in practice only as a shoulder on the trailing side (compare Figure 8 of Gilbert and Gilbert, 1973). The calculated values of $\bar{s}_{25,b}$ for models 1 and 2 were 3.9 and 3.5 S, respectively, the former being in reasonable agreement with the experimental value (4.1 S).

Discussion

Inspection of Table I shows that, of the models examined, only model 3 describes, with acceptable precision, sedimentation equilibrium distributions obtained over the total concentration range dictated by the solubility of zinc-free insulin in the environment studied. Although it is evident that equilibrium sedimentation offered advantage over velocity sedimentation in distinguishing between models for the present system, it is reassuring to note that the polymerization pattern specified by model 3 (formation of dimer, $K_2 = 11 \times 10^4$ M^{-1} and its isodesmic indefinite self-association, $K_{1,2} = 1.7 \times 10^4$ M^{-1}) is consistent with the sedimentation velocity behavior of the protein, both with regard to the $\bar{s}_{25,b}$ value and the form of the reaction boundary.

Three points merit further comment in relation to the sedimentation equilibrium results. First, this work provides a further illustration of the observation that it is insufficient to consider a restricted concentration range in analyzing results in terms of a particular model: thus, models 1, 2, and 3 are all consistent with results obtained up to $\bar{c}(r)$ values of 0.8 g/l. Nevertheless, such experiments at relatively low concentrations are invaluable in defining appropriate sets of values of possible equilibrium constants, particularly as the use of the $\Omega_1(r)$ function together with the ability to sum infinite series to form closed solutions for $\bar{c}(r)$ facilitates such analysis in a range where nonideality effects may reasonably be neglected. Secondly, the necessity of considering results pertaining to higher values of $\bar{c}(r)$ automatically implies that nonideality effects must now be considered. This was the major point neglected by Pekar and Frank (1972) in their ingenious analysis of $M_{w,r}/M_1$ points corresponding to a wide range of $\bar{c}(r)$ values. The agreement between their calculated and experimental points at high $\bar{c}(r)$ would not be expected, if their model inclusive of the specified equilibrium constants were correct, in view of the operation of nonideality effects. The situation is clarified by Figure 3, where either model 1 or 3 might, at first sight, be judged satisfactory. However, as consideration of nonideality effects can only lower the ordinate values, it is clear even from this Figure that model 3 is favored. This conclusion was verified (Table I) in quantitative terms when a realistic account was taken of the composition dependence of the array of activity coefficients: certainly, estimates of α_{ii} and α_{ij} used for this purpose are based on assumptions concerning charge and covolumes, but these are apparently not serious in the treatment of the second order, but important, nonideality effect. Thirdly, the question arises whether model 1 with altered values of K_2, K_6, and $K_{1,6}$ (or indeed other models involving three or more equilibrium constants) might also satisfactorily describe the available results. Evidently, such a possibility cannot be excluded but it does not seem profitable to explore it in relation to sedimentation equilibrium results, since the precision of fit obtained with the simple model 3 (involving only two equilibrium constants) could not be improved.

In the latter connection, it could be noted that the revised polymerization pattern specified in model 3 shares certain qualitative features with that suggested by Pekar and Frank (1972): both involve an isodesmic indefinite self-association (but with different operative basic units) and both predict at physiological concentrations (0.1–3 ng of insulin/ml) a marked predominance of the monomeric form. This, however, does not minimize the importance of having a simpler and improved definition of the polymerization pattern of insulin. In the first place, the detailed pattern may well determine the form of binding curves obtained with insulin as acceptor and glucose (for example) as ligand (Anzenbacher and Kalous, 1975). Secondly, it is relevant to the consideration of storage of insulin in the β cell (Pekar and Frank, 1972). Finally, it is basic to the interpretation of future experiments designed to explore the effects of imposing selected constraints on the equilibria operative in the zinc-free system. In these respects, it is hoped that the present findings will be of use.

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