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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, UNIVERSITY OF LIEGE]

The Molecular Weight of Insulin in Dioxane–Water Solutions

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The molecular weight of insulin in mixtures of water and dioxane has been determined by measurements of sedimentation constants, diffusion constants, viscosity and partial specific volume. In 20% dioxane the dissociation of insulin is strongly enhanced. In 40% dioxane, it is practically complete at all concentrations investigated. The minimum molecular weight is in both cases close to 6000.

Introduction

It has been established by numerous studies that insulin molecules dissociate when their charge increases. It has been pointed out by Oncley and Ellenbogen[1] that solvents with low dielectric constants should favor the dissociation by enhancement of the electrostatic repulsions between the molecular units. They reported, however, that precise measurements of sedimentation constants in such solvents are often difficult. The finding by Harfenist and Craig[2] of a molecular weight of 6,000 for insulin by countercurrent distribution confirmed the view that organic solvents favor the dissociation. We made a preliminary report[3] that the molecular weight of insulin is strongly reduced in mixtures of dioxane and water. More recently Rees and Singer[4] found by osmotic pressure measurements of insulin in dimethylformamide a molecular weight of 6,000.

In view of the recent controversy about the minimum molecular weight of insulin,[5] it seemed worthwhile to investigate more thoroughly the effect of mixed solvents with low dielectric constants. Mixtures of dioxane and water are very useful for this purpose; they allow the investigation of a great range of dielectric constants; insulin is very stable and very soluble in these solvents. In fact it can be recrystallized after several days of standing in the solutions. For sedimentation studies, mixtures of dioxane and water are particularly suitable because the densities of the components are very similar and there is very little separation of both, even during long centrifugations. Since the viscosity of the mixtures is fairly high, the sedimentation of insulin is slowed down, and it is necessary to use the synthetic boundary cell for measuring the sedimentation constants.

Experimental

Five times recrystallized beef insulin was kindly supplied by the Lilly Research Laboratories, Indianapolis (lots T-2344 and 535, 664). The dioxane was purified by distillation over sodium hydroxide. The presence of peroxides was checked frequently and the purification occasionally repeated.

The sedimentation constants were determined in a model E ultracentrifuge "Spinco," using the synthetic boundary cell.[6] The speed was 59,780 r.p.m. and the temperature around 22°. In 20% dioxane the photographs were taken every 8 minutes whereas in 40% dioxane, the time intervals were 16 minutes because of the very slow sedimentation rates. Enlarged tracings of ten photographs were made. In all instances the peaks were quite symmetrical and the motion of the boundary was determined by measuring the displacement of the maximum height. In 20% dioxane and in 40% dioxane, the displacements on the tracings were, respectively, about 8 and 4 mm. which were estimated within ±0.25 mm. The resulting errors on the sedimentation constant were, respectively, ±5% and ±10%. The results are expressed in Svedberg units.

Diffusion constants were determined at 25° in a Tiselius electrophoresis apparatus made by Pearson and equipped with the Longsworth scanning device. It was found necessary to dialyze the solutions against the solvent during 48 hr. at room temperature. The diffusion constants were computed by the height-area method with correction for zero time. They are expressed in cm.^{2} sec.$^{-1}$.

All constants are calculated for pure water at 20°, after proper corrections for viscosities or densities of the solvents.

Viscosities were measured in an Ostwald viscometer at 25°, the time of outflow being about 3 minutes for pure water. Special care was taken to avoid any evaporation of the mixed solvents, water–dioxane, in order to prevent modifications of viscosities by variations of the compositions. The results are expressed as viscosity increments, *i.e.*, $\lim_{\phi \to 0} (\eta_s/\phi)$, where ϕ is the volume fraction of the solute and η_s the specific viscosity of the solution.

The partial specific volume of 0.9% insulin in 20% dioxane was determined by pycnometry at 25°. It was found equal to 0.735 which is close to the value accepted in water, *i.e.*, 0.72.[1]

The *p*H of the water–dioxane mixtures was measured with a glass electrode after appropriate calibration.[7]

Results

Measurements in 20% Dioxane.—The sedimentation constants $s_{20,w}$ of insulin in 20% dioxane are given in Fig. 1, as a function of the protein concentration, in 0.1 *N* phosphate and in 0.1 *N* KCl, at *p*H 3 and 3.5. The sedimentation constant increases very slightly with the concentration at *p*H 3.5; at *p*H 3, it seems that the dissociation is complete. The values are a little higher in KCl than in phosphate. The same phenomenon occurs in aqueous solutions and was correlated to the binding of chloride ions[5]; it is probable that some binding occurs in dioxane–water solutions but to a lesser extent. In phosphate at *p*H 3, we obtain at low concentrations, the smallest value of the sedimentation constant, 1.05 *S*. It seems to correspond to the molecular unit of insulin.

For a given salt concentration, the effect of *p*H is weaker in dioxane–water than in pure water (Table I).

Because of the high compressibility coefficients of organic solvents, we thought that the partial

(1) J. L. Oncley and E. Ellenbogen, *J. Phys. Chem.*, **56**, 87 (1952).
(2) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3087 (1952).
(3) E. Fredericq, *Nature*, **171**, 570 (1953).
(4) E. D. Rees and S. J. Singer, *ibid.*, **176**, 1072 (1956).
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(6) E. G. Pickels, W. F. Harrington and H. K. Schachman, *Proc. Natl. Acad. Sci.*, **38**, 943 (1952).
(7) E. Fredericq, *J. Polymer Sci.*, **XII**, 287 (1954).

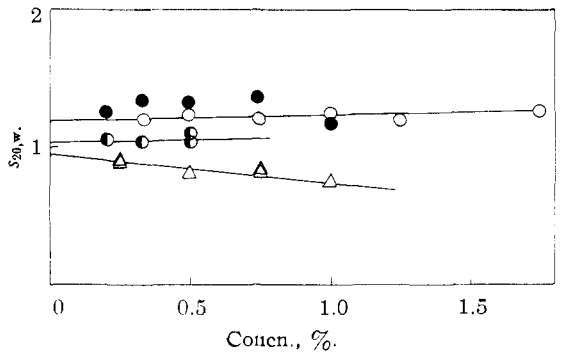

Fig. 1.—Sedimentation constant of insulin as a function of protein concentration, at ionic strength 0.1: ●, 20% dioxane, 0.1 *N* KCl, *p*H 3; ○, 20% dioxane, 0.1 *N* phosphate, *p*H 3.5; ◐, 20% dioxane, 0.1 *N* phosphate, *p*H 3; △, 40% dioxane, 0.1 *N* KCl, *p*H 3.5.

specific volume could be influenced by the speed of rotation and that the sedimentation constant could vary with the speed. We made two experiments for checking this possibility. We determined the values of $s_{20,w}$ for two given solutions at two different speeds. (1) In 0.1 *N* phosphates, *p*H 2.9, the values of $s_{20,w}$ at 60,000 and 50,000 r.p.m. were, respectively, 1.1 and 1.2. (2) At *p*H 4, at 60,000 and 44,800 r.p.m., they were, respectively, 1.53 and 1.48. The second set of values, which is the more accurate, shows that almost doubling the gravity field does not change the value of $s_{20,w}$ within experimental error. It can be concluded that the partial specific volume is not affected by the speed of the run.

TABLE I

EFFECT OF *p*H ON THE SEDIMENTATION CONSTANTS OF 0.25% INSULIN, AT IONIC STRENGTH 0.1

Buffer, 0.1 *N* phosphates

*p*H	$s_{20,w}$ Water[a]	$s_{20,w}$ 20% dioxane
2.9	1.4	1.05
3.5	1.9	1.2
4.0	2.8	1.5

[a] Data from ref. 8 and new measurements.

The dissociating effect of dioxane is quite reversible. If the dioxane is completely removed by dialysis, a value of $s_{20,w}$ identical to that in aqueous solution is found again.

The diffusion constant of insulin in 0.1 *N* phosphate at *p*H 3 in 20% dioxane was determined at three protein concentrations: results were, respectively, $D_{20,w} = 13.5 \times 10^{-7}$ (0.5%), 14.9×10^{-7} (0.33%), 14.2×10^{-7} (0.2%).

The viscosity increment was determined under similar conditions, at concentrations ranging between 0.4 and 0.8%. It was found constantly equal to 4 ± 0.2 (intrinsic viscosity 2.9 ml./g.)

From all those values, it can be concluded that at *p*H 3, in phosphate, the molecular constants vary very little with the concentration of insulin. If we take the experimental data in the range 0.3 to 0.5%, we calculate a molecular weight of 7,000, a frictional ratio of 1.19 and an axial ratio close to 1.

(8) E. Fredericq and H. Neurath, THIS JOURNAL, **72**, 2684 (1950).

Measurements in 40% Dioxane.—Estimations of the molecular weight of insulin in 40% dioxane were made by sedimentation and viscosity measurements. The determination of sedimentation constants is rendered difficult by the high relative viscosity of the solvent and its fairly high density: those two factors slow down the sedimentation rate; uncorrected values of *s* are close to 0.4 *S*. Despite the use of longer times of centrifugation, the error on $s_{20,w}$ is about ± 10%. However, the values of $s_{20,w}$ reported on Fig. 1 give a straight line and indicate unequivocally that the variation of $s_{20,w}$ with the concentration of insulin conforms to undissociating systems. There is only a small negative slope in agreement with the normal concentration dependence of the sedimentation constant. We may conclude that insulin is completely dissociated in 40% dioxane at all the concentrations used. The extrapolated value of $s_{20,w}$ at zero concentration is 0.95 *S*.

Viscosity measurements were made under similar conditions (in 0.1 *N* KCl at *p*H 3). For insulin concentrations ranging from 0.3 to 0.6%, a viscosity increment of 4.5 ± 0.1 was found. This indicates an increase either in solvation or in asymmetry and explains the low value found for $s_{20,w}$. It is very likely that it is due to some swelling of insulin in dioxane rather than to an unfolding. It appears that the mixtures of water and dioxane are better solvents for insulin than are the pure components, the solubility of the protein being maximum for about 40 to 50% dioxane. This may be understood when considering that insulin has about equal numbers of polar and non-polar residues. The former have a stronger attraction for water, the latter for dioxane. In the mixed solvents, the interactions with the solute will be particularly strong and the solvation will be higher than in water. Some swelling may result. Assuming a spherical shape for the molecule, one can deduce from the viscosity increment 4.5, a frictional ratio of 1.22. Combining this value with $s_{20,w} = 0.95$, a molecular weight of 6,000 is obtained.

Discussion

In confirmation of other data, our results show that in solvents with low dielectric constants the dissociation of insulin is strongly enhanced. In 40% dioxane, a complete dissociation is reached. The hydrodynamic constants indicate that the molecular weight of the molecular unit is close to 6,000 or, in other words, that it is identical with the chemical unit of Sanger, the molecular weight of which is 5,750. The frictional ratios obtained in dioxane solutions (1.19 and 1.22) are markedly higher than in pure water ($1.10^{4.5}$). The consideration of the hydrodynamic constants indicates, however, that the shape of the molecules is also spherical in dioxane solutions but that there is a higher solvation or a swelling.

There is now an increasing evidence that the minimum molecular weight of insulin is 5,750, in aqueous solutions as well as in organic solvents.[5]

The dissociating effect of organic solvents was predicted by Oncley and Ellenbogen.[1] These authors also have calculated the value of the repulsive forces between insulin molecules in water by the

Debye–Hückel equation. We tried to use a similar computation for our solvents water–dioxane, using dielectric constants of 65 and 45, respectively, for 20% and 40% dioxane. The charge was considered as maximum since the titration curves[7] indicate that almost the maximum positive charge is reached at *p*H 3 in 20% dioxane and at *p*H 3.5 in 40% dioxane. The number of bound anions was taken similar to the value in aqueous solutions, *i.e.*, one anion per two chemical units.[9] Slight differences in the number of bound anions would not introduce significant differences in the energies of repulsion.

On this basis we find in 20% dioxane and in 40% dioxane, respectively, 2,250 and 3,200 cal. for the change in electrostatic free energy resulting from the association of two molecular units, that we call ΔF^e. If we let ΔF^a be the term for the attractive forces involved in the process, we can write for the total free energy change of association ΔF

$$\Delta F = \Delta F^a + \Delta F^e \qquad (1)$$

In water, we found an equilibrium constant of 500 for the association of two units. Then $\Delta F = -3{,}600$ cal. ΔF^e is calculated as 1,800 cal. and from equation 1, $\Delta F^a = -5{,}400$ cal. If the attractive forces in the association process were the same in water and in the mixed solvents, then

in 20% dioxane, $\Delta F = -5400 + 2250 = -3150$ cal.

in 40% dioxane, $\Delta F = -5400 + 3200 = -2200$ cal.

and the corresponding equilibrium constants would be 220 and 44, respectively. However, an inspection of the sedimentation curves on Fig. 1 indicates that the association of the molecular units is extremely weak in 20% dioxane and practically nonexistent in 40% dioxane. Consequently, the equilibrium constants calculated above are much too high, their true values must be inferior to unity. For obtaining such figures, the absolute value of ΔF^a in equation 1 must be much lower than 5,400.

The lowering of the absolute value of the attractive term in dioxane solutions may be explained by the following considerations. This term results from two kinds of forces: (1) the attractions between insulin units; (2) the attractions between insulin and solvent molecules. During the association process, there is a negative free energy change resulting from the first forces and a positive free energy change resulting from the decreased solvation of the associated particles. From the considerations already developed, it is evident that the attractions between insulin and solvent molecules are much higher in dioxane–water than in pure water. Consequently, the total attractive term ΔF^a will be higher in dioxane–water than in water, and its absolute value will be smaller.

From this discussion, we see that the weak association of insulin units in dioxane solutions results not only from the lower dielectric constant of the medium but also for an important part, from the interactions between insulin particles and solvent molecules. A similar situation was encountered in the study of the binding of organic ions by proteins in water–dioxane mixtures.[10]

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(10) E. Fredericq, *ibid.*, **64**, 639 (1955).

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Molecular Stability and Sub-structure of Bovine Fibrinogen

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As indicated by determination of molecular weight (light scattering) and sedimentation constant, fibrinogen retains its characteristic size and shape up to *p*H 10.8 and independently of protein concentration and ionic strength. Above *p*H 12.8 the molecule decomposes and yields one A fragment of $M = 230{,}000$ and $S = 5.5$ (at 0.1% protein) and approximately eight B fragments of $S = 1.77$, $D = 12.4 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ (at 1% protein) and thus $M = 14{,}500$. The dimensions calculated for the anhydrous A fragment are 724 × 27 Å. (length and diameter of prolate ellipsoid assuming 0.3 g. of water per gram protein) and 600 × 30 Å. (assuming 0.75 g. of water per gram protein). If the anhydrous B fragment carries 0.3 g. of water per gram protein, it has dimensions of 67 × 22 Å. Combinations of fragments would readily yield the nodose structures seen in electron micrographs. At values intermediate between *p*H 10.8 and 12.8 the fibrinogen molecule may undergo a number of rapid changes in frictional resistance without a change in molecular weight, the maximum change in S recorded here being from $S^0_{20} = 8.1$ to $S^0_{20} = 5.5$ (*p*H 12.2). The clear increase in frictional resistance is due to molecular expansion and is attributed to a partial liberation of the B fragments rather than to an isotropic swelling. At *p*H 12.2 there is a slow denaturation such that after 60 min. at 4° a rapid return to *p*H 7.7 finds 90% of the protein insoluble. Below *p*H 12.2 denaturation proceeds too slowly and above irreversible changes appear. After denaturation a maximum recovery of solubility and clottability at *p*H 7.7 may be obtained by incubation for 4–6 hours at intermediate *p*H values, the most effective being *p*H 10.82 at room temperature. After such treatment, 90% of the original protein is soluble and 70% of the soluble protein is clottable. The sizable fraction of protein which is soluble but non-clottable has a molecular weight and sedimentation coefficient typical of fibrinogen and thus resembles fibrinogen in size and shape. A rapid return from *p*H 12.2 to 7.7 thus stabilizes structural changes leading to insolubility while at *p*H ~10.8 energy barriers are such as to allow a gradual rearrangement to a more native family of structures. From the foregoing it is clear that fibrinogen does not dissociate under conditions where it is altered by thrombin and thus the complexity of the single critical contact between thrombin and fibrinogen, predicted from kinetic data, may be determined when the necessary associated chemical events are known.

(1) Fellow American Cancer Society. A part of this work was reported in J. E. Fitzgerald, Ph.D. Thesis, Department of Biology, Massachusetts Institute of Technology, May, 1955.

(2) Public Health Service Fellow of the National Heart Institute.

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