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The Reversible Dissociation of Insulin and its Minimum Molecular Weight

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In two previous papers (Gutfreund, 1948*a, b*) it has been shown that the molecular weight of insulin is dependent upon pH, temperature and protein concentration of the solution. In neutral solutions of moderate protein concentration insulin appeared to be homogeneous with respect to molecular weight, $M = 48,000$ (Gutfreund & Ogston, 1946; Gutfreund, 1948*a*). Under optimum conditions for dissociation (pH 2.6) a steep extrapolation to zero concentration of osmotic pressure against insulin concentration gave a value of 12,000 for the molecular weight. From this evidence, as well as from crystallographic and chemical information, it was concluded that insulin in solution consists of aggregates of loosely bound sub-units of molecular weight 12,000. It is not claimed that aggregation into molecules containing four sub-units is necessarily an upper limit.

More recently two papers gave an account of ultracentrifugal and diffusion studies on insulin solutions, and the authors drew different conclusions from their results. Pedersen (1950) believes that the molecular weight of insulin is 42,000 and that molecules of that size dissociate into two sub-units of molecular weight 21,000. His data would,

however, be quite consistent with the hypothesis that under varying conditions of solution the mean molecular weight can have any value from 12,000 to 48,000 or more. Fredericq & Neurath (1950) found that in dilute phosphate buffer of pH 2.65, insulin dissociates into sub-units of molecular weight 6000, this value being calculated from diffusion and sedimentation constants extrapolated to zero concentration. This extrapolation is, however, very steep, and since measurements of the diffusion constant in dilute solutions are likely to give values which are too high, the extrapolated value of the molecular weight is likely to be too low.

The present paper is confined to the determination of the molecular weight of the sub-unit (minimum molecular weight) into which insulin can be dissociated reversibly. It has already been emphasized by Gutfreund (1948*b*) that the study of the effect of pH, temperature and protein concentration upon the aggregation of the sub-unit of insulin should be complemented with an investigation of the influence of salt concentration on this phenomenon. The present paper describes this further investigation, and shows how it confirms and clarifies the author's previous findings.

Table 1. *Composition of buffers*

Series	Molar composition of buffer	μ	pH
A	H ₃ PO ₄ :0.0033, NaH ₂ PO ₄ :0.01	0.013	2.69
B	H ₃ PO ₄ :0.017, NaH ₂ PO ₄ :0.05	0.067	2.65
C	H ₃ PO ₄ :0.033, NaH ₂ PO ₄ :0.10	0.133	2.60
D	H ₃ PO ₄ :0.110, NaH ₂ PO ₄ :0.33	0.44	2.56
E	H ₃ PO ₄ :0.017, NaH ₂ PO ₄ :0.05, NaCl:0.20	0.167	2.56
F	H ₃ PO ₄ :0.033, NaH ₂ PO ₄ :0.10, NaCl:0.40	0.533	2.55
G	Na ₂ HPO ₄ :0.0218, citric acid:0.089	0.213	2.60
H	Na ₂ HPO ₄ :0.0218, citric acid:0.089, NaCl:0.20	0.413	2.60

EXPERIMENTAL

The osmotic pressure of insulin solutions was measured by the simple method of Adair (1925). The osmometers were set up in boiling tubes holding 150 ml. of diffusate and left to equilibrate in a cold room at 3°. Collodion membranes holding about 4 ml. of protein solution were used, and the pressure was recorded directly from the height of the solution in the capillary tube mounted in the membrane above the level of the diffusate. Corrections for capillarity and density of the solution must be applied to these measurements. Since there is some danger that material of low molecular weight, such as the sub-unit of insulin, may diffuse through the membrane, only results from experiments which gave constant pressure readings over a period of 24 hr. were used. It was found that membranes prepared by the method of Adair (1925) are impermeable to material of molecular weight 12,000 if five coats of collodion solution (10 g. of Imperial Chemical Industries Ltd. Nitrocellulose HL 30/40 into a mixture of 100 ml. dried ether, 100 ml. dried ethanol and 4 ml. of ethylene glycol) are applied to the glass mould.

The insulin concentration in the solution, after osmotic equilibration, was determined by the micro-Kjeldahl procedure with all the precautions recommended by Chibnall, Rees & Williams (1943). A value of 15.6% was taken as the N content of dry ash-free insulin.

Measurements of the sedimentation velocity were carried out in the Svedberg oil-turbine ultracentrifuge at Oxford. The diagonal schlieren optical system of Philpot (1938) was used for the observation of sedimenting boundaries. The procedures recommended by Cecil & Ogston (1948) were followed for the observations, measurements and computations of sedimentation constants. The solvent buffers were made up to the composition given in Table 1 and the pH values were checked with a glass electrode. For all but a few experiments specially marked, five times recrystallized Zn insulin from Boots Pure Drug Co. Ltd. was used. Dr H. Neurath pointed out to the present author that some of the differences in results obtained may be due to variation in the different samples used. Unfortunately, samples of the particular Eli Lilly insulin used by Dr Neurath were not available. Some experiments were therefore carried out with a small portion of a sample of Eli Lilly T2536 Zn insulin, which was made available by Mrs D. Hodgkin.

RESULTS AND ANALYSIS OF OSMOTIC PRESSURE MEASUREMENTS

The results of measurements of the osmotic pressure of ideal or very dilute solutions can be used directly

for the calculation of the mean molecular weight M from the equation

$$M = \frac{RT}{P/C}, \quad (i)$$

where R is the gas constant, T the absolute temperature, P the osmotic pressure in cm. of water and C the protein concentration in g./l. Deviations from the ideal laws at finite protein concentrations have been discussed in a variety of reviews (e.g. Gutfreund, 1950). The procedure of measuring the osmotic pressure of a series of solutions over a range of protein concentration and of calculating the molecular weight from P/C extrapolated to $C=0$ gives reliable results if (1) the mean molecular weight is independent of protein concentration, and (2) the partial pressure due to Donnan effects is not very great.

In the present study the maximum protein concentration of solutions used for osmotic pressure measurements was about 10 g./l. and most of the deviation normally encountered in thermodynamic studies of solutions of large molecules are negligible under such conditions. The anomalies which have to be dealt with in the study of insulin in acid solutions are due to the dependence of the molecular weight of insulin on protein and salt concentration. If the latter effect is studied in buffer solutions of low ionic strength, the Donnan effect becomes very marked. The buffer solutions used ranged in pH from 2.55 to 2.69. Assuming that all the basic groups and none of the acidic groups are ionized in this range of pH, insulin would have one positive charge per molecular weight of 1000 (see review of chemical analysis, Sanger, 1949). The partial osmotic pressure P_i due to the unequal distribution of the $\text{-H}_2\text{PO}_4$, H^+ and Na^+ ions of the principal buffer system used, can be calculated from the Gibbs-Donnan membrane equilibrium equation

$$[\text{H}^+ + \text{Na}^+]_s \times [\text{-H}_2\text{PO}_4]_s = [\text{H}^+ + \text{Na}^+]_d \times [\text{-H}_2\text{PO}_4]_d, \quad (ii)$$

where the subscripts S and D stand for solution and diffusate respectively. Electric neutrality in the protein solution requires that

$$m\text{Z}^+ + [\text{H}^+ + \text{Na}^+]_s = [\text{-H}_2\text{PO}_4]_s, \quad (iii)$$

where mZ^+ is the product of the molarity of the protein and the charge valence. The volume of the diffusate is taken as sufficiently large for the equality $[H^+]_D + [Na^+]_D = [-H_2PO_4]_D$ to hold. From the above relations it is clearly possible to derive an expression for P_i as a function of mZ^+ , $[H^+ + Na^+]_D$ and $[-H_2PO_4]_D$. Details of such derivations are given in two recent reviews, Adair (1947) and Gutfreund (1950). In Fig. 1 a plot of P_i against

Fig. 1. Plot of P_i in cm. of water against mZ^+ for different ionic strength of 1:1 valence type buffer mixtures.

mZ^+ is shown for three different buffer compositions. The graphs were computed from the equation

$$P_i = RT \{ \sqrt{(mZ^+)^2 + 4[-H_2PO_4]^2} - 2[-H_2PO_4] \}. \quad (iv)$$

Strictly speaking, the osmotic activity of the ions and not their concentrations should be taken for the above calculations. Corrections by means of osmotic coefficients cannot easily be applied to mixtures of low salt concentration, since insufficient data are available. No osmotic activity correction of P_i has been attempted in the present case, since it would not be likely to reduce the value of P_i by more than 10%.

Table 1 shows the composition of the various buffer solutions used for the osmotic pressure measurements, together with their pH and ionic strength μ calculated from the equation $\mu = \frac{1}{2} \sum mv^2$, where m is the molarity and v the valence of each kind of ion in the solution. The letters A to H will be used throughout to indicate the type of buffer used for the experiment.

The first two series of osmotic pressure measurements were carried out on insulin solutions in buffers B and C. The results of these experiments are recorded in Figs. 2-4. It can be seen that the difference in the results obtained from these two series is only due to the greater values for P_i in the more dilute phosphate solutions. The values for P_i were estimated from the graphs in Fig. 1 on the basis of insulin having one positive charge per unit of mole-

cular weight 1000 at pH 2.6. The slopes of the lines representing the corrected plots of $(P - P_i)/C$ against C give a measure of the increase of the mean molecular weight with insulin concentration. The calculation of the molecular weight extrapolated to zero concentration is done by numerical substitution in Eqn. (i), which gives at 3° : $M = \frac{23400}{P/C}$. (P in cm. of water and C in g./l.) The four graphs shown in Figs. 2-4 extrapolate to $P/C = 2$ and in spite of the

Fig. 2. Plot of P/C against C , where P is the total osmotic pressure in cm. of water and C insulin concentration in g./l. of buffer B.

Fig. 3. Plot of $(P - P_i)/C$ against C , where $(P - P_i)$ is in cm. of water and C the insulin concentration in g./l. of buffer B.

Fig. 4. Plot of $(P - P_i)/C$ (⊗) and P/C (⊙) against C , where P and P_i are in cm. of water and C the insulin concentration in g./l. of buffer C.

uncertainties of the extrapolation this value is accurate to ± 0.1 . Comparison of these experiments with those previously reported (Gutfreund, 1948b) showed that a decrease in salt concentration affects the aggregation of insulin at finite protein concentrations. As can be seen from Fig. 1, when $\mu = 0.0133$ the corrections for P_i become very large and the results become less accurate. Values obtained for $(P - P_i)/C$ from measurements on insulin solutions in buffer A fell within the same limits as those recorded in Fig. 3 for the measurements in buffer B. Osmotic pressure measurements in buffer H were carried out for comparison with a previous study (Gutfreund, 1948b) and the results (see Fig. 5) are in very good agreement with those previously reported. Experiments carried out in buffer G are also plotted

in Fig. 5. Both these graphs again extrapolate to $P/C = 2$ at $C = 0$.

In Fig. 6 isolated experiments are recorded to give further information about the effect of an increase in salt concentration upon the mean molecular weight of insulin. The peculiar solubility properties of insulin limit the range of protein and salt concentration. These experiments were designed primarily to find the optimum conditions for reversible dissociation into sub-units and only to a lesser extent to obtain any information about the mechanism of aggregation.

Fig. 5. Plot of P/C against C , where P is the osmotic pressure in cm. of water and C the insulin concentration in g./l. of buffer G (○) and H (⊗) respectively.

Fig. 6. Plot of P/C against C , where P is the osmotic pressure in cm. of water and C the insulin concentration in g./l. of buffer D (⊗), E (○) and F (●) respectively.

The osmotic pressure of 0.23 and 0.125 % solutions of Eli Lilly insulin in buffer B was measured and the values for $(P - P_i)/C$ were 1.88 and 1.85 respectively. This is in fair agreement with the results of measurements on Boots insulin used for the rest of the experiments.

RESULTS OF ULTRACENTRIFUGE MEASUREMENTS

Three solutions of insulin in buffer B were examined in the ultracentrifuge. They contained (1) 0.5 % Boots insulin, (2) 0.25 % Boots insulin, and (3) 0.22 % Eli Lilly insulin, respectively. Cecil & Ogston (1948) suggested the graphical computation of S_{20} from a plot of $\Sigma(\Delta x)$ against $\Sigma(\Delta t')$, where Δx is the movement of the centre of the boundary during time Δt and $\Delta t' = \Delta t(\eta_{20}/\eta_0) X \omega^2$; η_{20} and η_0 are the viscosities of water at 20° and at the rotor temperature respectively; X is the mean distance of the boundary from the centre of rotation and ω the mean angular velocity. S_{20} the sedimentation constant in cm./sec./dyne at 20° can then be calculated from

$$S_{20} = \Sigma(\Delta x) / \Sigma(\Delta t'). \quad (v)$$

For the best use of all the information the sedimentation constant should be computed from the regression of x on t' by the following equation

$$S_{20} = \frac{\Sigma \{x(t' - \bar{t}')\}}{\Sigma (t' - \bar{t}')}.$$

Fig. 7 shows the plot of $\Sigma(\Delta x)$ against $\Sigma(\Delta t')$ for the second experiment, a 0.25 % solution of Boots insulin. The sedimentation constant calculated from this run $S_{20} \times 10^{13} = 1.65 \pm 0.07$ (s.d. from the regression coefficient). The other results were for the first run $S_{20} = 1.62 \times 10^{-13}$ and for the third run $S_{20} = 1.67 \times 10^{-13}$. All these values are corrected to sedimentation in water at 20°, and Cecil & Ogston's (1948) correction factor for the thermocouple has also been introduced.

Fig. 7. Plot of $\Sigma(\Delta x)$ in cm. measured on the photographic record against $\Sigma(\Delta t')$ in cm. measured on the photographic record $\times 10^{-11}$ /sec. for sedimentation velocity run on 0.25 % insulin in buffer B . (See Eqn. v for explanation of symbols.)

The sedimentation diagrams obtained showed traces of faster sedimenting material, but there was no indication of any more slowly sedimenting material on the buffer side of the boundary. The latter effect would be observed if the main boundary peak represents material which undergoes further dissociation on dilution, as was the case for insulin solutions of the same protein concentration and pH, but higher salt concentration (Gutfreund, 1948b).

DISCUSSION

The results of the osmotic pressure measurements recorded in this paper, give further evidence for the hypothesis that insulin molecules in solution consist of aggregates of sub-units of molecular weight 12,000. The mean molecular weight is a function of pH, temperature, protein concentration and ionic strength of the buffer. The reliability of a value obtained for the molecular weight of the sub-unit from the extrapolation, to zero insulin concentra-

tion, of measurements carried out in one buffer system may be subject to suspicion. It has been shown, however, that the graphs representing the dependence of molecular weight upon concentration in a variety of buffers all extrapolate—with different slopes—to the same value of $P/C = 2.0 \pm 0.1$ at $C = 0$. This agreement is taken as conclusive evidence that the minimum molecular weight of insulin is 11,200–12,300. Mean molecular weight values in heterogeneous solutions obtained from osmotic pressure measurements are always lower than those obtained from kinetic methods, since the former gives a number average which is much more sensitive to the low molecular weight material present than the weight average obtained by the other methods. This sensitivity of osmotic pressure to the presence of small fractions of low molecular weight magnifies the disagreement between the above results and those of Fredericq & Neurath (1950), who state that the molecular weight calculated from the sedimentation and diffusion constants extrapolated to $C = 0$ is 6000. Fredericq & Neurath's experiments were carried out in phosphate buffer pH 2.6, $\mu = 0.1$ over a range of protein concentration 1.0–0.2%. Under those conditions the highly charged insulin molecules will give anomalous values for the sedimentation and diffusion constants, and it is very unlikely that the uncorrected extrapolation is justified. Fredericq & Neurath observed a fall of the sedimentation constant and increase of the diffusion constant on dilution. In our own experiments we found in fact a slight rise in the sedimentation constant with decreasing insulin concentration. This is to be expected from the charge effect. Furthermore, the extrapolation of the diffusion constant is subject to large errors because the method used by Fredericq & Neurath is likely to give progressively low values in very dilute solutions.

Ultracentrifuge runs were carried out as a further test of the hypothesis that the reversible dissociation of insulin is, under the conditions used, limited to division into sub-units of molecular weight 12,000. Though, of course, the molecular weight calculated from the sedimentation constant alone depends upon the hydration and asymmetry of the particles, with the aid of Wyman & Ingalls (1943) nomograms one can easily fix the likely limits for the molecular weight, from the sedimentation constants observed for solutions of insulin in buffer *B*, as 10,000 and 14,000, with $12,000 \pm 500$ as the most plausible value. Though there were traces of material of higher molecular weight present, there was no indication of any which sedimented more slowly than the main boundary. These results indicate that the homogeneous sub-unit of insulin has a sedimentation constant $S_{20} = 1.65\text{--}1.67 \times 10^{-13}$. Previous experiments at the same pH, but higher salt con-

centration, showed that the sedimentation constant decreased with decreasing insulin concentration, but it was quite clear from the sedimentation diagrams that the minimum had not been reached (Gutfreund, 1948b).

Further evidence about the division of insulin into sub-units of molecular weight 12,000 can be obtained from crystallographic studies. It depends upon the particular crystal class and symmetry whether the unit cell dimensions give values equal to or multiples of the molecular weight. Crowfoot (1938) showed that the unit cell size of the rhombohedral form of insulin, if corrected for 10% hydration of the air-dried crystal, corresponds to a molecular weight of 36,000. These crystals have the space group *R*3, which implies trigonal symmetry. There are, therefore, three identical units within the unit cell, but it is not possible to decide, in this particular case, whether the molecular weight is 36,000 or 12,000. The reasoning developed by Weissenberg (1928) enables one under certain conditions of symmetry to fix the size of the 'crystal molecule' as a fraction of the unit cell. This was possible in the case of orthorhombic insulin crystals grown by Crick & Gutfreund (1950) from acid phosphate buffers. Different crystal forms have been obtained, and one of them is being studied by Mrs D. Hodgkin, F.R.S., and Mr H. Mendel at Oxford. Their preliminary investigations showed that there are four sub-units of molecular weight about 12,000 in the unit cell and the space group is *P*₂₁₂₁₂₁. Under these conditions the molecular weight is essentially that of the asymmetric unit (12,000), since symmetry conditions show that any bonding which occurs between two sub-units of that size would have to occur between every unit of a chain extending through the macroscopic crystals.

Crystals of insulin sulphate examined by Low (1950) were also orthorhombic and contained four equal units of molecular weight 12,000 in the unit cell. These crystals, however, have the space group *P*₂₁₂₁₂ which does permit the presence of units of molecular weight 24,000 with a twofold symmetry for each of them.

Chemical evidence does not at present show quite conclusively whether the minimum molecular weight calculated from analytical data is 12,000 or 6000, but it certainly is compatible with the unit of $M = 12,000$ (Sanger, 1949) and there is, of course, no reason why this unit should not consist of two identical halves. It is to be hoped that further chemical evidence or physical investigations will show whether the six S—S bridges in the sub-unit are holding the four peptide chains together or whether some of these bridges are used to link a chain with itself, while the pairs of chains are coupled by another kind of bond.

The effect of salt concentration and of the type of ions in the solution upon the aggregation of the sub-unit of insulin can be summarized by the statement that increase in salt concentration causes aggregation, and that there is some indication that sodium chloride has a more marked effect than phosphates. Fredericq & Neurath have shown that potassium thiocyanate favours aggregation even more strongly than sodium chloride. During the last few years it has been shown that a large number of the crystalline proteins have a variable mean molecular weight in solutions. The differences in the conditions of solution, such as salt concentration, pH, protein concentration and temperature, probably cause far more discrepancies in the data obtained than the impurities of the protein preparations to which they are often attributed. Several examples of reversible dissociation into sub-units have been described by Pedersen (1950), Gutfreund (1949) and Tsao, Bailey & Adair (1951). Urea, strong electrolytes and increase in hydrogen-ion concentration cause dissociation of protein into sub-units. High concentrations of strong electrolytes probably act by sheltering the charges on the protein molecules. They cause dissociation in isoelectric haemoglobin or tropomyosin solutions, where the sub-units are held together by opposite charges, while they cause aggregation under conditions when the strong positive charges would otherwise hold the sub-units apart, as described in the above experiments on insulin. Insulin is very stable and such reagents as urea and 0.1M acid do not cause any irreversible changes since the overall structure is likely to be held so rigidly by the S—S linkages (Sanger, 1949) that a temporary rupture of bonds of electrostatic nature would not cause any change in configuration within the sub-unit. In other proteins the denaturation effect of these reagents is probably due to the

slow breaking of hydrogen bonds and a resulting disorder in the protein structure. The large entropy changes of unfolding or stretching chains cause irreversible denaturation and in some cases such a process accompanies the otherwise reversible dissociation into sub-units.

SUMMARY

1. It has been shown that the degree of aggregation of insulin in solutions depends not only upon pH, temperature and insulin concentration but also upon the electrolyte concentration and the type of electrolyte present in the solution.

2. At the optimum pH for dissociation (pH 2.5–2.7) the molecular weight calculated from osmotic pressure measurements extrapolated to zero protein concentration is about 12,000 irrespective of salt concentration. The molecular weight at finite protein concentration is, however, larger the greater the salt concentration. There is some indication that sodium chloride has a more marked effect on the aggregation than phosphates.

3. The sedimentation constant of insulin under conditions of maximum dissociation was found to be 1.65×10^{-13} , which is likely to be the sedimentation constant for the homogeneous unit of $M = 12,000$.

4. Some general features of reversible changes occurring in solutions of crystalline proteins are discussed together with structural information which can be adduced from them.

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