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The Molecular Weight of Insulin and its Dependence upon pH, Concentration and Temperature

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In a previous paper (Gutfreund, 1948) it was shown from the results of osmotic and diffusion-sedimentation measurements that the molecular weight of insulin is 47,000-48,000 in solutions of pH 7.0 containing 0.5-1.0% protein. Molecules of that size were found to dissociate into smaller units on dilution to protein concentrations of 0.2% or less. Sjögren & Svedberg (1931) have determined the sedimentation constant of insulin over a range of pH from 3.5 to 12.3 and found from their data that the molecular weight has a maximum at pH 6.8. They concluded that insulin dissociates both in acid and in alkaline solutions and also that this dissociation is reversible if the solution is not kept too long at a pH outside the stability region. Gutfreund & Ogston (1946) re-examined the sedimentation constant over a range of pH from 3.5 to 9.6 and found that the sedimentation velocity of the main boundary remained constant between pH 6.7 and 9.6. In some cases, however, some slower-sedimenting material was present.

Gutfreund & Ogston were interested in the sedimentation rate of the main boundary. A re-examination of the sedimentation diagrams showed the presence, in solutions more alkaline than pH 8, of some more slowly sedimenting material which did not form a clear boundary.

The recent work of Waugh (1944, 1945, 1946) showed that insulin undergoes drastic and reversible changes in acid solutions, and the studies of the influence of pH on the properties of insulin solutions

have therefore been continued and extended by means of both the osmotic pressure and ultra centrifugal technique.

EXPERIMENTAL

The sedimentation constants were determined at Oxford in the oil-turbine ultracentrifuge (Svedberg type). The sedimenting boundaries were observed by the Philpot (1938) method. The speed of the centrifuge during all the experiments was about 1000 rev./sec. A detailed description of the accuracy of the measurements is given by Gutfreund & Ogston (1946). All values of sedimentation constants (S_{20}) recorded are corrected to sedimentation in water at 20°.

For the osmotic-pressure measurements an apparatus of the type described by Adair (1925) was used. The pressure was measured directly as the height of solution in the capillary tube. The protein concentration of the equilibrated solution was calculated from Δn (refractive index difference between buffer and solution) assuming a specific refractive increment of 180×10^{-5} . Δn was measured in a Zeiss interferometer, the usual type of refractometers not being accurate enough for measurements on the very dilute solutions used throughout this work.

Since the solutions were very dilute all the values for molecular weights, recorded in this paper, were calculated by means of the simple formula

$$M = \frac{RTC}{P},$$

where M = molecular weight; P = osmotic pressure; R = gas constant; C = concentration of protein in g./l. of solution; and T = absolute temperature. Except where explicitly

stated, the osmotic pressure experiments were carried out in a cold room at $+2^{\circ}$.

The insulin used for all the investigations described in this paper was a sample of crystalline zinc insulin (Boots Pure Drug Co. Ltd.). Ultracentrifugal examination (Gutfreund & Ogston, 1946) showed that this material was homogeneous in solutions of pH 7.0–8.0. Electrophoretic analysis in the Tiselius apparatus, using the Philpot (1938) optical system for the observation of the moving boundaries, also confirmed the purity of the sample used.

the presence of a considerable amount of very slowly sedimenting material is evident from the boundary diagram. In a solution of pH 1.0 (Exp. 5) the sedimentation constant was somewhat higher than in a solution of pH 2.2. Furthermore, the boundary in the sedimentation diagram for Exp. 5 has a much more homogeneous appearance than that derived from all the other experiments on insulin in acid solutions.

Table 1. *Sedimentation constants of insulin*

Exp. no.	Insulin concentration (%)	pH	Molar composition of buffer solution				$S_{20} \text{ (corr.)} \times 10^{13}$
			Citric acid	HCl	NaCl	Na_2HPO_4	
1*	0.4	3.4	0.055	—	0.1	0.045	3.10
2	0.6	3.0	0.079	—	0.2	0.041	3.36
3	0.15	2.5	0.091	—	0.2	0.017	—
4	0.75	2.2	0.098	—	0.2	0.004	2.59
5	1.20	1.0	—	0.1	0.2	—	2.78
6†	0.84	8.0	—	—	0.1	0.0095 + 0.0005 KH_2PO_4	3.47

* Quoted from Gutfreund & Ogston (1946).

† Solution used in Exp. 5 adjusted to pH 8 and dialyzed against phosphate buffer (see text).

Two types of buffer systems were employed as solvent: for solutions of pH 2.2–5.0, McIlvaine's (1921) citric acid-phosphate buffer; and for those of pH 5.0–8.8, mixtures of Na_2HPO_4 and KH_2PO_4 as described by Clark (1928). Solutions of pH 1 were prepared in 0.1M-HCl.

After the protein solutions had been dialyzed against the appropriate buffer, the pH of the dialysate was determined with a glass electrode.

RESULTS

Gutfreund & Ogston (1946) found that insulin was polydisperse in solution of pH 3.4 and the first series of experiments described here was carried out to extend the study on the properties of more acidic insulin solutions. In Table 1 the sedimentation constants calculated from measurements on solutions of pH 3.4, 3.0, 2.2 and 1.0 respectively, are recorded together with details about composition of the buffer used as solvent and the protein concentration. Specimen photographs of the boundaries are shown in Fig. 1.

It can be seen from the width of the sedimenting boundaries shown in the sedimentation diagrams 1, 2, 3 and 4 that insulin in acid solutions is heterogeneous with respect to particle size. It can be presumed that these solutions contain insulin in various states of aggregation or dissociation. The spread of the heterogeneous boundaries decreases the accuracy of the values for S_{20} , particularly for the more dilute solutions. This, as well as dissociation due to dilution, may account for the difference in the value for S_{20} obtained from the ultracentrifuge Exps. 1 and 2. From Exp. 4 a lower sedimentation constant was calculated for the main boundary and

The combined effect of dilution and acidity on insulin was also investigated. A solution containing 0.15 % insulin in buffer of pH 2.5 was examined in

Fig. 1. Sedimentation diagrams for Exps. 1–6 numbered as in Table 1. The time (in min.) after reaching full speed was 80, 57, 80, 80, 60, 60, for diagrams 1–6 respectively.

the ultracentrifuge (Exp. 3) and the sedimentation diagram shows clearly a marked increase in the proportion of very slowly sedimenting material compared with the more concentrated solution of Exp. 4.

As no clear boundary was formed the sedimentation constant could not be computed from Exp. 3.

The solution of pH 1.0, which had been used for sedimentation Exp. 5, was afterwards adjusted to pH 8.0, and dialyzed against phosphate buffer of that pH and again examined in the ultracentrifuge (Exp. 6); the sedimentation constant of insulin in this solution, $S_{20} \times 10^{13}$, was 3.47, which agrees within the experimental error with the value recorded for the sedimentation constant of unchanged insulin.

In the previous paper on the osmotic pressure of insulin solutions (Gutfreund, 1948) experiments were described in which phosphate buffer of pH 7.0 was used as solvent. It was necessary to dilute the buffer to 0.01M to increase the solubility of insulin. In such dilute buffer a quite appreciable partial pressure due to excess ion activity was observed, and the necessary corrections were calculated from the results of membrane-potential measurements. In this paper the extrapolation procedure suggested by Adair & Robinson (1930) is used to interpret the results from measurements on solutions of insulin in 0.01M-phosphate buffer of pH 7.5. The graph in Fig. 2 shows P/C plotted against C and gives several

Fig. 2. Plot of P/C against C from osmotic-pressure measurements on solutions of insulin in 0.01M-phosphate buffer of pH 7.5. P =osmotic pressure; C =concentration of protein in g./100 ml. solution.

interesting results. At the centre of the curve (C equal to 0.4–0.5%) $P/C=4.9$, corresponding to a molecular weight of 47,400, which is in good agreement with the value previously obtained at pH 7.0 and similar protein concentration (0.4–0.8%). The value for P/C increases however both for $C > 0.5$ and $C < 0.3$. This is due to the membrane-potential effect in the more concentrated solutions and to the effect of dissociation, which has previously been described (Gutfreund, 1948) in the very dilute solutions.

Between pH 4.0–7.0 insulin is so insoluble as to make any investigations on solutions very difficult. Between pH 7.0 and 7.5 solutions up to nearly 1% could be obtained in very dilute buffer. At pH lower than 4.0 and higher than 7.5 it was possible to obtain insulin solutions, containing up to 1% protein, in the presence of sufficient salt to eliminate the partial pressure due to excess ion activity. I have therefore made all the buffer solutions of those outside pH regions, 0.2M with respect to NaCl. The following series of experiments was carried out at pH 1 (0.1M-

HCl, 0.2M-NaCl) to find out whether P/C remained constant over a range of concentrations 0.5–1.0% of protein, under those conditions.

Fig. 3. Plot of P/C against C from osmotic-pressure measurements on solutions of insulin in 0.1M-HCl, 0.2M-NaCl. P =osmotic pressure; C =concentration of protein in g./100 ml. solution.

Fig. 3 is a graphical representation of the measurements on solutions of pH 1. The molecular weight, calculated from the results of the six experiments recorded here, is $28,500 \pm 1100$ (standard deviation from the mean). The ratio P/C is constant over the range of C investigated, and it can therefore be concluded that the low molecular weight observed is due to dissociation in acid solutions and not to the effect of the electric charge of the protein molecules. These experiments also demonstrate the accuracy to be expected from osmotic-pressure measurements on solutions of similar concentration.

A series of measurements of the osmotic pressure of insulin solutions (protein concentration 0.6–0.9%) over a range of pH from 1.0 to 8.8 was then carried out, and the molecular weight values, recorded in Table 2, are in each case the means of the results calculated from several osmotic-pressure measurements.

Table 2. *The dependence of the molecular weight of insulin, in solutions of protein concentration 0.6–0.9%, upon the pH of the solution*

pH	Molecular weight calculated from osmotic-pressure measurements
1.0	28,500
2.2	22,000
2.5	22,000
3.0	22,000
7.0	47,700
7.5	47,400
8.0	40,000
8.8	28,000

It is clearly indicated by these observations that insulin dissociates into subunits, both in acid and alkaline solutions. The reversibility of this dissociation was examined by adjusting both acid and alkaline solutions of insulin to pH 7.5 and it was found that re-association to a molecular weight of 47,000–48,000 occurred.

The observed re-aggregation of the insulin subunits between pH 2.2 and 1.0 suggested that it might be possible to study the fibrous polymerization in acid solutions described by Waugh (1946). However, on changing the concentration of HCl

from 0.1 to 1.0M most of the insulin in solution was precipitated and no measurements were made on more acidic solutions.

An observation, indicating the presence simultaneously of insulin in different states of aggregation, was then made. A buffer solution of pH 8.0 was saturated with insulin at a temperature of 30°. This solution, containing after centrifugation 1.1% insulin, was stored in a cold room over night and a crop of crystals was formed. Though the mean molecular weight of insulin in this solution was 40,000, the crystals which deposited were found to be isomorphous with those described by Crowfoot (1938).

From the results of the experiments so far described it is clear that in acid solutions the pH of maximum dissociation is between 2 and 3. The results of the ultracentrifuge Exps. 3 and 4 show that the degree of dissociation is dependent upon insulin concentration. The combined effect of pH and dilution was studied by a series of osmotic-pressure measurements on solutions of pH 2.5 containing 0.04–2.4% of insulin. The results of these experiments are recorded in Fig. 4 where the molecular

Fig. 4. Plot of molecular weight of insulin against concentration calculated from osmotic-pressure measurements in citric acid-phosphate buffer (pH 2.5). Empty circles represent measurements at 2°, full circles represent measurements at 23°.

weight is plotted against insulin concentration. Two curves are shown: one represents the results of measurements at 2°, the other those of measurements at 23°. Both curves can be extrapolated to a value of $12,000 \pm 500$ (standard deviation of the individual observations) for the molecular weight at zero concentration. A consideration of the accuracy of the measurements is particularly important for very dilute solutions (0.04–0.1% of protein). The accuracy of the molecular weight calculated from an osmotic-pressure measurement depends upon the accuracy of the estimation of the protein concentration and pressure. For the smallest values of concentration and pressure, measured for the calculation of the data in Fig. 4, the accuracy was $\pm 5\%$ in each case.

DISCUSSION

From the results reported in this paper and from the data given by Gutfreund (1948) it can be concluded that the maximum molecular weight of insulin is 48,000. This value is found for neutral solutions (pH 7.0–7.5) of protein concentration 0.4–0.9%. Under these special conditions insulin is homogeneous with respect to particle size. In the previous paper it has been shown that insulin of molecular weight 48,000 is not very stable, as dissociation into smaller units occurs on dilution to protein concentrations of less than 0.3%.

The investigation of the effect of pH on the molecular weight of insulin showed that dissociation of insulin occurred both in acid and alkaline solutions. In acid solutions the optimum pH for dissociation is pH 2.0–3.0. pH 1 is the critical pH for the beginning of the re-aggregation resulting in more acidic solutions, in the formation of a fibrous precipitate. The results of ultracentrifugal and osmotic-pressure measurements are in agreement about the dissociation in acid solutions and the beginning of re-aggregation in solutions of pH 1. When establishment of equilibrium between dissociated and associated insulin molecules is not slower than the resolution produced by sedimentation, one can expect to get a spreading boundary giving a mean sedimentation constant, rather than two or more separate component boundaries. It is at present difficult to give a definite explanation for the results obtained by Gutfreund & Ogston (1946) on ultracentrifugal examination of insulin in alkaline solutions. They observed no change in the sedimentation constant nor any spreading of the boundary. However, as stated on p. 544 of this paper, some slowly-sedimenting material could be seen in the sedimentation diagrams from experiments on solutions more alkaline than pH 8.0. It is possible that the difference in the appearance of sedimenting boundaries in acid and alkaline solutions is due to a difference in the speed of the dissociation equilibrium under the two respective conditions. A very slow equilibrium may account for the type of boundaries obtained in alkaline solutions.

The last series of osmotic-pressure measurements described in this paper was undertaken to investigate the size of the units into which insulin dissociates. Though the results indicate that the molecular weight of insulin in infinitely dilute solution is 12,000, there is no direct proof from these data as to the homogeneity of the dissociation products. In spite of variations of temperature, pH and concentration, the molecular weight was never found to be below 12,000 and the two curves from measurements at 2° and 23° converge at the point of molecular weight 12,000 (Fig. 4); this can be assumed to be the molecular weight of the dissociation product.

The results of the crystallographic investigations (Crowfoot, 1938) and of the chemical analysis (Chibnall, 1946) do not by themselves prove the existence of a subunit of molecular weight 12,000, but they are consistent with that hypothesis. The unit cell in crystalline insulin is composed of three identical parts and the unit-cell weight is 36,000 (re-calculated for a moisture content of 10–11% for insulin crystals). The minimum molecular weight calculated from amino-acid analysis is 12,100 with a standard deviation of 530, from the values quoted by Chibnall (1946).

The reversibility of the dissociation reaction of insulin was demonstrated by the ultracentrifugal Exps. 5 and 6, by osmotic-pressure measurements and by the work of Waugh (1946). Gutfreund (1948) calculated the total heat change occurring on dissociation of insulin molecules of molecular weight 48,000 into four identical subunits. At pH 7.0, ΔH was found to be 29,000 g. calories (for 1 mol. insulin). Similar calculations were not done for the data recorded in Fig. 4, as a series of measurements at each temperature for one point of the concentration scale has to be made to get results of sufficient accuracy for subsequent calculation of the relative concentrations of associated and dissociated insulin. The data so far obtained are, however, quite accurate enough to show the relation between concentration and molecular weight.

Since the degree of dissociation of insulin molecules is dependent upon pH, temperature and concentration, it can be concluded that the forces holding the subunits together involve the ionizing groups of the protein. It is very difficult to differentiate between electrostatic forces and hydrogen bonds as the latter are often affected by the ioniza-

tion of certain groups. A study of the influence of the ionic strength of the solvent buffer upon dissociation, which is now being pursued, may give further information on that point.

SUMMARY

1. Insulin has a maximum molecular weight (47,000–48,000) in solutions of pH 7.0–7.5 at protein concentrations of 0.4–0.9%. Under those conditions it is homogeneous with respect to particle size.

2. Molecules of this weight dissociate reversibly on change of pH to above 7.5 or below 4, as well as on dilution. The optimum pH for dissociation was found to be 2.0–3.0.

3. On studying the combined effect of pH, dilution and temperature upon dissociation it was found that the minimum molecular weight was $12,000 \pm 500$. Other evidence suggesting that insulin molecules in solution were composed of subunits of molecular weight 12,000 is summarized.

4. It is suggested that units of molecular weight 12,000 are bound by weak electrostatic forces.

5. The dissociation phenomena have been studied by osmotic pressure and by ultracentrifugal measurements. The two methods gave results in good agreement.

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