15 mg. did not exceed 5% of the dose given. Since this figure is, admittedly, an overestimate, the results indicate that, broadly speaking, none of the administered stilboestrol was present in the extracts. This does not, of course, imply that the urine did not contain oestrogenic substances. It is possible that metabolites of stilboestrol were present, still possessing oestrogenic activity, but whose structure precluded the formation of red-coloured products with antimony pentachloride. The formation of the anomalous brown pigments by such urine extracts may be significant in this connexion.

The negligible amounts of free stilboestrol found in the extracts of unhydrolyzed urine in the present experiments are possibly to be expected by analogy with other compounds and with the results of experiments with stilboestrol in other species. The main excretory product might well be a conjugated product. Some observations of Malpress are of interest in this connexion. Malpress (private communication) has so far found it impossible to estimate the ether-insoluble forms of stilboestrol in cow's urine, owing to the complete destruction of this oestrogen when hydrolysis by hydrochloric acid takes place *in the presence of other urinary constituents*. On the other hand, as he has shown (Malpress, 1946), hydrolysis of the glucuronide by hydrochloric acid *in simple solution* gives recoveries of the oestrogen which closely approximate those which can be calculated from postulates as to the action of hydrochloric acid on pure stilboestrol. The failure to

demonstrate conjugated stilboestrol in the present experiments may be due to similar causes.

SUMMARY

1. The behaviour of stilboestrol, dienioestrol, and isodienioestrol in Dingemane's antimony pentachloride reaction and Malpress's nitro reaction has been examined.

2. In solution in a variety of solvents, stilboestrol is stable, but dienioestrol and isodienioestrol are unstable. This instability of dienioestrol and of isodienioestrol in solution arises from their susceptibility to rapid atmospheric oxidation.

3. Attempts to demonstrate the presence of free or conjugated stilboestrol in the urine of subjects undergoing treatment with large therapeutic doses of this oestrogen failed to reveal the presence of significant amounts.

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The Osmotic Pressure of Insulin Solutions

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Sjögren & Svedberg (1931) examined solutions of crystalline insulin in the ultracentrifuge. From determinations of the sedimentation constant they concluded that it is a homogeneous protein, stable between pH 5 and 7. From sedimentation equilibrium measurements they calculated a molecular weight of 35,000. Polson (1937) determined the diffusion constant of insulin, and calculated from this ($D_{20} = 8.2 \times 10^{-7}$) and Sjögren & Svedberg's sedimentation constant ($S_{20} = 3.47 \times 10^{-13}$) the molecular weight 40,900. From Crowfoot's (1938) crystallographic measurements the weight of

the dry unit cell is about 36,000.* The unit cell has trigonal symmetry, and the molecular

* The value quoted by Crowfoot (1938) for the dry unit cell weight is 37,600. This has been calculated on the assumption that the moisture contents of air-dried insulin crystals are 5.35%. Miller & du Vigneaud (1937) pointed out the difficulties of obtaining accurate values for the moisture contents of insulin crystals and found 10.2% as the result of repeated analyses. The present author analyzed five samples of air-dried insulin crystals and found variations between 10 and 12% for the moisture contents. Completely dried crystals, if left at room temperature, absorbed water until they contained from 10 to 11%.

weight of insulin in the crystal is therefore 36,000 or 12,000.

The molecular weight of insulin was generally accepted to be about 36,000 up to the publication of the work of Miller & Andersson (1942), who carried out a thorough study of the sedimentation and diffusion constant and calculated the molecular weight 46,000 from their data ($S_{20} = 3.55 \times 10^{-13}$ and $D_{20} = 7.53 \times 10^{-7}$).

Gutfreund & Ogston (1946) re-examined the sedimentation constant with a view to observing any changes of insulin in alkaline solutions and on dilution. They found that $S_{20} = 3.35 \times 10^{-13}$ over a range of pH from 6.8 to 9.6 and at protein concentrations from 0.1 to 1.0 %. From this sedimentation and the diffusion constant ($D_{20} = 7.0 \times 10^{-7}$) obtained by Gutfreund (unpublished) the molecular weight 46,600 can be calculated.

On account of the discrepancies between the data for the molecular weight ranging from 35,000 to 46,000, a detailed study of the osmotic pressure of insulin solutions was undertaken. No previous osmotic pressure measurements have been recorded for this protein, probably because of the sparing solubility near the isoelectric point and the indication of dissociation outside the pH range 4.5–7.5 (Sjögren & Svedberg, 1931).

Osmotic pressure and membrane potentials

The results of osmotic pressure measurements were used for the calculation of the molecular weight of insulin. As shown by Adair & Robinson (1930), this can be done by the use of the following equation:

$$P - P_i = P_p = RTg_p M_p,$$

where P = observed osmotic pressure; P_i = ion pressure difference; P_p = pressure due to protein, i.e. colloid osmotic pressure; g_p = osmotic coefficient (correcting for repulsive and attractive forces); and M_p = mol. protein/l. of solvent. The correction factors can usually be eliminated simultaneously by use of the equation

$$M = 10RT/\pi_0,$$

where M = molecular weight; $\pi_0 = P/C$ extrapolated to zero concentration; C = concentration of protein in g./100 ml. of solution. (The factor 10 in the above equation brings this up to conc./l. of solution.)

If the charge of the protein particles in solution is high and the salt concentration of the solvent buffer low, P_i may still be significant at low-protein concentrations. This has to be checked by membrane potential measurements or chemical analysis of diffusible ion concentration on both sides of the membrane (Adair & Robinson, 1930; Adair & Adair, 1934). For the work described in this paper the membrane potential was used for the calculation of P_i after the equation developed by Adair (1928):

$$P_i = 15.4 E^2 \Sigma C_i Z_i^2,$$

where P_i is the ion pressure difference in mm. of mercury, E is the membrane potential, and $\Sigma C_i Z_i^2$ is the sum of the buffer ion concentration in the dialysate, multiplied by the square of their valence. All the experiments described in this paper were carried out on solutions of protein concentration below 1 %, and under those conditions the factor g_p can be neglected for nearly spherical molecules, such as insulin. Throughout this paper we have therefore used the simple formula $P_p = P - P_i = RT (C/M)$ for molecular weight calculations.

EXPERIMENTAL

Two types of osmometer were used for the experiments described in this paper. The simple apparatus developed by Adair (1925) was employed for the first two series of measurements. The osmotic pressure is read directly from the height of the solution in the capillary. When equilibrium is reached the capillary tube is removed and two samples of protein are taken from the membrane. The first is used for capillarity measurements and the second for estimation of protein concentration. Wherever required, membrane potential measurements were then carried out on the remaining solution. The technique described by Adair & Adair (1934) was followed in every detail for the measurement of membrane potentials.

The corrections for capillarity in the experiments recorded in Tables 1 and 2 are between 13 and 20 mm., depending on the inner diameter of the capillary. A study of the duplicate measurements of capillarity suggested that it would not be possible to get accurate results from osmotic pressure experiments on solutions containing less than 0.5 % protein, as a small error in the correction would have a large effect on the molecular weight calculation. If very wide capillary tubes are used the correction can be reduced, but equilibration is prolonged considerably. A new osmometer, employing toluene as pressure head in the capillary, has been developed by Mr G. S. Adair (to be published). With this apparatus much more reliable capillarity corrections can be made. The osmometers have been used according to the technique developed by Adair (personal communication), and the results of measurements on dilute solutions are recorded in Tables 3 and 4.

The following insulin preparations were used: (1) crystalline insulin from Boots Pure Drug Co. Ltd., (2) Squibb crystalline insulin. Nitrogen, moisture and ash contents of all samples of insulin received were examined. For the nitrogen estimation the micro-Kjeldahl procedure, with all precautions suggested by Chibnall, Rees & Williams (1943), was used. This analysis was important as the concentration of insulin in solutions after osmotic pressure measurements were calculated either from nitrogen contents or from refractive index difference (Δn) measurements. Δn was estimated in a Zeiss interferometer which was standardized with insulin solutions, their concentration previously being determined from micro-Kjeldahl nitrogen. The values obtained for the nitrogen contents of three samples of Boots crystalline insulin and one sample of Squibb crystalline insulin were all within 1 % of the value quoted by Chibnall *et al.* (1943), i.e. 15.4 % nitrogen in moisture- and ash-free protein.

The samples of protein were the same as those used by Gutfreund & Ogston (1946) for their ultracentrifuge experi-

Table 1. *Molecular weight of Boots crystalline insulin in buffer pH 7.0*

(In 0.1M-phosphate; temp. 18°.)

P_p corrected to 0° (mm. H ₂ O)	Protein conc. (%)	Mol. wt.
9	0.14	36,000
9	0.145	35,300
11	0.18	37,800
14	0.21	34,700
14	0.22	36,400
Mean		$36,060 \pm 1080$ (S.D.)

ments and shown to be homogeneous. Examination in the Tiselius electrophoresis apparatus also confirmed their purity.

RESULTS

The major difficulty encountered during all the experiments on insulin solutions was the low solubility and with that the measurement of very small osmotic pressures. The first series of measurements was carried out on solutions of insulin in 0.1M-phosphate buffer (pH 7.0) at 18°. Though it was possible to obtain supersaturated solutions, containing 1 %

Table 2. *Molecular weight of crystalline insulin preparations in buffer pH 7.1*

(In 0.01M-phosphate; temp. 0°.)

Preparation	P (mm. H ₂ O)	P_i (mm. H ₂ O)	P_p (mm. H ₂ O)	Protein conc. (%)	Mol. wt.
Boots	23	2	21	0.46	51,000
	42	9	33	0.64	45,000
	35	7	28	0.58	48,000
	43	10	33	0.70	49,000
	36	7	29	0.58	46,000
	47	12	35	0.77	51,000
	40	8	32	0.60	43,000
	34	7	27	0.58	50,000
Squibb	51	13	38	0.77	47,000
	35	3*	32	0.65	47,000
			Mean		$47,700 \pm 3000$ (S.D.)

* This experiment was carried out in 0.04M-phosphate buffer pH 7.1.

Table 3. *Molecular weight of Squibb crystalline insulin (temperature 0.05°)*

P_p (mm. H ₂ O)	Protein conc. (%)	Mol. wt.
A. In phosphate buffer (0.02M, pH 7.0).		
6.4	0.112	40,800
9.2	0.154	38,800
8.8	0.164	42,800
7.7	0.126	38,200
6.5	0.116	41,200
8.4	0.146	40,300
7.7	0.132	38,800
Mean		$40,300 \pm 1400$ (S.D.)
B. In phosphate buffer (0.02M, pH 7.5).		
7.6	0.137	41,700
7.1	0.115	37,800
10.0	0.180	41,500
Mean		$40,300 \pm 1800$ (S.D.)

Table 4. *Molecular weight of Squibb crystalline insulin*

(In 0.02M-phosphate buffer pH 7.0; temp. 20.5°.)

P_p (mm. H ₂ O), corrected to 0°)	Protein conc. (%)	Mol. wt.
8.1	0.122	34,900
9.6	0.136	30,500
9.4	0.143	35,400
9.8	0.115	27,100
10.2	0.132	39,800
10.2	0.143	32,200
10.8	0.159	34,100
Mean		$32,000 \pm 2800$ (S.D.)

protein, under these conditions precipitation occurred during osmotic equilibrium, and the final protein concentration was always below 0.25%. Table 1 shows the results of this first series of measurements, giving a mean molecular weight for insulin of 36,000. The membrane potential under those conditions of protein and buffer concentration was measured and found to be negligible

$$(P_i < 0.05 \text{ mm. H}_2\text{O}).$$

It was observed that, on reducing the phosphate concentration of the buffer from 0.1 to 0.01M, solutions containing up to 0.8 % protein could be obtained at pH 7.0 and a temperature of 0°. Table 2 shows the results of a series of experiments carried out under those conditions. As can be seen the membrane potential effect, and with that the excess pressure of diffusible ions, becomes significant, both on account of higher protein concentration and of the reduced salt concentration in the buffer. The standard deviation from the mean molecular weight, $47,700 \pm 3000$, is much larger than that obtained from measurements on ovalbumin and lactoglobulin (Gutfreund, 1944, 1945), but this is due to the relatively small pressures measured and also due to the correction for P_i which, changing with the square of the membrane potential, may account for much of the deviations.

It was thought possible that the difference between the molecular weight calculated from measure-

ments on solutions between 0.5 and 0.8% protein and on solutions containing 0.15–0.25%, may be due to dissociation of insulin on dilution. The accuracy of the results of Table 1 can be disputed on account of the difficulties of capillarity measurements in the type of osmometer used. The toluene osmometer, mentioned above, was therefore employed for a series of experiments which is recorded in Table 3. These results indicate, first, a dissociation of insulin on dilution, and secondly, that no further dissociation takes place on changing the pH from 7.0 to 7.5. As a further test for dissociation of insulin in dilute solutions, a series of measurements was carried out under identical conditions of pH and buffer and protein concentration, at a higher temperature. Table 4 shows the record of a number of experiments carried out at 20.5°. A marked increase of dissociation on raising the temperature can be observed.

DISCUSSION

In solutions containing 0.5–1.0% protein the molecular weight of insulin is $\sim 48,000$. This is shown both by the results of osmotic pressure measurements and combined diffusion and sedimentation constants. In dilute solutions, containing less than 0.25% protein, the molecular weight is lower, indicating dissociation. A further proof of the dissociation phenomena is given by the decrease of mean molecular weight with temperature. It has not been possible to upset the equilibrium completely to the side of the dissociation product and so to determine the size of the smaller unit, but both crystallographic evidence (Crowfoot, 1938) and chemical evidence (Chibnall, 1946) would be consistent with the assumption that subunits of molecular weight 12,000 are loosely bound to form insulin molecules. Further investigations on the molecular weight of the dissociation product will be described in a subsequent paper.

On the assumption that the dissociation of insulin on dilution is a reversible reaction $A_4 \rightleftharpoons 4A_1$ (A_4 is a molecule of weight 48,000 and A_1 a molecule of weight 12,000), the results recorded in Tables 3 and 4 are used for the calculation of the concentration of the A_4 and A_1 units, from the mean molecular weight at 0.5 and 20.5° respectively. Though particles of the type A_2 (molecular weight 24,000) and A_3 (molecular weight 36,000) may also be present, a correct measure of the number of bonds broken between these subunits is obtained by considering the equilibrium $A_4 \rightleftharpoons 4A_1$ only.

If x is the concentration of A_4 units and $(1-x)$ the concentration of A_1 units, the equilibrium constant $K = \frac{x}{(1-x)^4}$, x is calculated from the equation

$$\frac{1}{M} = \frac{x}{M_1} + \frac{(1-x)}{M_2},$$

where M = mean molecular weight, M_1 = molecular weight of A_4 unit, M_2 = molecular weight of A_1 unit. The free-energy change is calculated from

$$\Delta G = -RT \ln K:$$

$$\text{at } 0.5^\circ: \quad x = 0.93, \quad K = 38,730, \quad G = -57,292 \text{ cal.}$$

$$\text{at } 20.5^\circ: \quad x = 0.83, \quad K = 994, \quad G = -40,166 \text{ cal.}$$

Van't Hoff's reaction isochore can be used for the calculation of the total heat change ΔH , from the two equilibrium constants:

$$\ln \frac{K_1}{K_2} = -\frac{\Delta H}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right),$$

$$\Delta H = -29,106 \text{ cal.}$$

From the relation $\Delta H = \Delta G + T\Delta S$ we can work out the entropy change ΔS . Taking a round mean value for $\Delta G = -48,000$ cal. (at 283° absolute),

$$\Delta S = +67 \text{ cal./degree.}$$

This is a first attempt to study reversible dissociation of proteins in relation to the energies involved. Though it is not justifiable to lay too much stress on the values obtained for ΔG and ΔH , they do give some indication of the type of reaction involved. This method of approach to the study of dissociation reactions is being continued and expanded for insulin and other proteins showing similar effects.

It is very likely that the dissociation effect, demonstrated here, accounts for the variation in the figures for the molecular weight of insulin, previously reported. Any measurements carried out in solutions would vary with concentration. The most interesting effect is the apparent formation of molecules consisting of three units (of molecular weight 12,000) in the crystal, while in solution the molecule can consist of up to four units. It is, of course, possible that still larger aggregates may form at higher concentrations. The calculation of molecular weight from crystallographic data is, under favourable conditions, the most accurate available, and it is a genuine difference in size of the molecule and not a disagreement of results, which is suggested.

Gutfreund & Ogston (1946) could not find any signs of dissociation in their ultracentrifuge experiments at concentrations below 0.2%, but it is obvious that such small amounts as 10 or even 20% of dissociation product would not be visible in the sedimenting boundary of such dilute solutions.

SUMMARY

1. It is shown that the molecular weight of insulin, calculated from osmotic pressure measurements on solutions containing 0.5–0.8% protein at pH 7.0, is $48,000 \pm 3000$.

2. Insulin tends to dissociate on dilution to below 0.25% protein concentration. The dissociation effect increases with temperature.

3. Some preliminary calculations, on the energies involved in the dissociation reaction, have been carried out.

4. A possible cause for the discrepancies between previously reported molecular weight values is discussed.

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