Sedimentation and Diffusion Studies on Insulin: the Maximum Molecular Weight

By J. M. CREETH

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London W. 1

(Received 10 June 1952)

During a recent investigation of the sedimentation and diffusion constants of enzymic digests of insulin, comparative control experiments on the untreated, crystalline protein led to a lower value of the molecular weight than was expected. It was at first anticipated that the results would be explicable on the basis of the dissociation phenomena reported by Gutfreund (1948*a, b*) from osmotic studies, where the observed molecular weight was found to vary with the temperature, insulin concentration, and pH of the solution.

The osmotic method is well adapted for the study of dissociating systems, as it gives a (number average) value for the molecular weight directly, whereas the combination of sedimentation and diffusion measurements leads only indirectly to the molecular weight and may, in such systems, give a rather complex average. Nevertheless, in view of the present rather confused position concerning the molecular weights obtained from sedimentation and diffusion constants (previous values are summarized and criticized in the Discussion), it was felt that a limited study of the dissociation phenomena by these means, the accuracy and reproducibility of which have recently been established (Cecil & Ogston, 1948; Charlwood, 1952*a*; Creeth, 1952), would be rewarding, in that it should provide qualitative evidence of dissociation and, at least, should give a reliable value for the maximum molecular weight in the range in which the molecule is stable. This paper presents the results of such a study.

EXPERIMENTAL

Methods. Sedimentation constants were determined with the Spinco electrically driven ultracentrifuge, the details of experimental method and computation of results being exactly as described previously (Charlwood, 1952*a, b*). In one case, a determination was made at a low temperature ($\sim 7^\circ$); the rotor and cell were cooled to this temperature before being inserted in the rotor chamber, and the latter was kept cool during the run by periodic use of the refrigerator embodied in the machine. The temperature of the rotor fell by 1° during the run of approximately 2 hr. duration.

Diffusion measurements were carried out with the Gouy diffusimeter (Coulson, Cox, Ogston & Philpot, 1948; Ogston, 1949*a*), previously described (Creeth, 1952), the only modification of the apparatus being the construction of an additional thermostating bath to surround the capillary

exit tube from the cell and its ancillary boundary-forming device. By this means, all of the apparatus, with the exception of a short section of the capillary tube joining the two baths, is susceptible of accurate temperature control, and the need to work within about 1° of room temperature obviated. All determinations were made in duplicate, as has been the standard practice with this apparatus. In many cases it was desired to know if the diffusion coefficient varied with temperature in addition to the other variables studied; two pairs of runs were therefore performed on each solution, one at a temperature in the range $14\text{--}17^\circ$ (referred to in the tables as 'lower temperature') and the other at about $28\text{--}30^\circ$ ('higher temperature').

Although the operating temperature of the apparatus could be lowered to about 10° , by cooling the tap water passing through the copper coil in the main thermostating bath, this was not done as a routine as it involved the use of large quantities of ice. A temperature within the lower range referred to could be attained by the use of tap water without previous cooling.

All the observed diffusion coefficients have been corrected to the viscosity and absolute temperature of water at 20° , and are expressed in units of $10^{-7} \text{ cm}^2 \text{ sec}^{-1}$. Sedimentation constants were also corrected, and are stated in Svedbergs, i.e. $10^{-13} \text{ sec. units}$.

Materials. Work was largely performed on a particular sample of six-times recrystallized Zn-insulin obtained from Boots Pure Drug Co. Ltd.; other samples of crystalline material were obtained from Burroughs Wellcome and Co. Ltd., the British Drug Houses Ltd., and from Dr K. O. Pedersen of Uppsala. The latter sample was identical with the purified insulin used by Dr Pedersen in his work (1950), but had been stored at room temperature for 3 years. All solutions were dialysed in cellophane tubing, against about 100 vol. of buffer, for approximately 48 hr. before use; dialysis frequently had to be conducted at room temperature (about 20°) to prevent the insulin being precipitated. Where difficulty was encountered in dissolving insulin, the suspension was warmed to 35° for a few minutes. If precipitation subsequently occurred during dialysis, or the solution did not appear clear, the solution was centrifuged and the supernatant redialysed.

RESULTS

The sedimentation constant values obtained for the various samples of insulin investigated are shown, with the relevant experimental conditions, in Table 1.

It is evident from these results that there is no marked difference between the insulin samples of different origin. Between the values obtained at

Table 1. *Sedimentation constants for insulin*(All solutions in phosphate buffer; $I=0.10$ except where stated.)

Material	Concentration (g./100 ml.)	pH	Temperature (T°)	$S_{20, w}$
Boots batch no. I 9633 B	0.4	7.4	17.0	3.10
Boots batch no. I 9633 B	0.4	7.4	25.9	3.11*
Boots batch no. I 9633 B	0.8	7.4	24.8	3.11
Boots batch no. I 9011 G	0.4	7.4	24.5	3.11
Boots batch no. I 11038 A	0.4	7.4	25.5	3.10
Burroughs Wellcome	0.4	7.4	22.0	3.15
B.D.H., batch no. 2083	0.4	7.4	21.8	3.13
Sample from Dr Pedersen	0.4	7.4	24.4	3.15
Boots batch no. I 9633 B	0.5	8.0	7.5	3.07
Boots batch no. I 9633 B	0.5	8.0	22.7	3.09
Boots batch no. I 9633 B	0.5	8.0	28.4	3.10
Boots batch no. I 11038 A	0.5	8.0	23.3	3.02

* Higher ionic strength; buffer composition: phosphate, $I=0.05$, sodium chloride, $I=0.25$.

pH 7.4 and those at pH 8, giving means of 3.12 and 3.07 respectively, there is a just significant difference ($0.05 > P > 0.02$). The run done at 0.8 % (w/v) concentration at pH 7.4 shows, in agreement with earlier observations (e.g. Gutfreund & Ogston, 1946; Pedersen, 1950), that there is no appreciable concentration dependence of the corrected sedimentation constant. Accordingly, the small difference between the two sets of figures can be ascribed to the effect of the pH change. It is also evident that, at pH 8.0, there is no temperature dependence of the sedimentation constant.

An attempt was made to simulate the conditions used by Oncley, Ellenbogen, Gitlin & Gurd (1952), who observed that the sedimentation constant of insulin increased as the ionic strength was raised from 0.10 to 0.68. The solubility of the insulin used here in phosphate-sodium chloride buffer, $I=0.60$, was too small, however, and the ionic strength had to be reduced to 0.30 before a suitable solution could be obtained. The sedimentation constant for this solution, however, was in good agreement with all others at this pH value.

In general, it has been found impossible to prepare solutions as concentrated as those used by Oncley *et al.* (1952); this may be due to the dissolution of the insulin directly, without prior solution in dilute acid, as has sometimes been used (Oncley, referred to by Waugh, 1948).

Following Jullander (1945) and Oncley *et al.* (1952), attempts were made in some cases to compute weight average sedimentation constants, S_w from the equation:

$$S_w = \frac{3S_m - S_t}{2}$$

Here S_m is the sedimentation constant calculated from the movement of the median of the boundary, and S_t that from the mode. The latter corresponds to the most probable sedimentation constant, and is the one usually calculated. At pH 7.4, no appreci-

able difference between S_m and S_t could be found, and at pH 8 the case showing most boundary spread gave a value of S_w only 0.8 % lower than S_t , which is well within the experimental error. It was therefore concluded, for the purpose of subsequent calculations, that S_w and S_t were identical. This is in agreement with Jullander's general conclusions on the characteristics of sedimenting protein boundaries.

Table 2. *The effect of temperature variation on the diffusion coefficient of insulin at pH 8.0*

Insulin concentration 0.5 g./100 ml.	Phosphate buffer, $I=0.10$
T°	$D_{20, w}$
10.7	8.81
14.5	8.67
20.0	8.81
27.8	8.80
28.3	8.81

Diffusion results. Except where stated otherwise, these refer exclusively to Boots insulin, batch I 9633 B. It was anticipated that the diffusion coefficients would be more sensitive to changes in particle size than the sedimentation constants (see, for example, Charlwood, 1952*b*). Accordingly, a wider range of variables was investigated, of which the first was the effect of temperature variation at a constant insulin concentration, ionic strength and pH; the results are shown in Table 2. These show immediately that there is no temperature dependence of the corrected diffusion coefficient under these conditions.

To determine whether temperature dependence occurred at other concentrations under these conditions (Gutfreund's (1948*a*) temperature-dependent molecular weight values referred to a much lower concentration as well as a lower pH), diffusion coefficients were determined over the concentration range 0.2–0.8 g./100 ml., at two temperatures (see

Experimental section). The results are shown in Table 3. The relation between the concentration and refraction increment was obtained by the use of the figure 1.94×10^{-4} for the specific refraction increment of insulin. This was calculated from the data given by Doty, Gellert & Rabinovitch (1952) using the equation given by Perlmann & Longworth (1948). It is clear that over the whole range down to 0.3 g./100 ml. the diffusion coefficient is independent of concentration and temperature. The value at the lowest concentration is significantly higher, and doubtless reflects the dissociation which, from the osmotic measurements reviewed later, would be expected to occur in this region.

Table 3. *Variation of the diffusion coefficient of insulin with concentration, at pH 8*

(All solutions in phosphate buffer, $I=0.10$; concentrations expressed in terms of the refraction increment, v =number of wavelengths retardation; $v=29.36$ corresponds to 0.80 g./100 ml. solution.)

v	$D_{20, w}$	
	Lower temp.	Higher temp.
7.95	9.22	9.30
9.82	8.70	8.87
14.04	—	8.80
15.79	8.81	—
16.67	8.74	—
18.37	—	8.93
29.36	—	8.85

The values for the diffusion coefficient are much higher than those obtained by previous workers, and in view of Gutfreund's (1948*b*) evidence, that dissociation may occur outside the pH range 7.0–7.5, the diffusion coefficients of solutions of concentration approximately 0.4 g./100 ml. were determined over a limited range of pH; the results obtained are shown in Table 4.

Table 4. *Variation of the diffusion coefficient of insulin with pH*

(Insulin concentration = 0.4–0.5 g./100 ml. Phosphate buffers, $I=0.10$.)

pH	$D_{20, w}$	
	Lower temp.	* Higher temp.
7.0	—	8.30
7.2	—	8.25
7.4	8.22*	8.29*
7.6	8.60	8.62
8.0	8.80	8.82

* Average values, see Table 5.

These results are in good qualitative agreement with the findings of Gutfreund (1948*b*), there being evidently no appreciable change of diffusion coefficient over the range pH 7.0–7.4, while above this a marked increase occurs, indicative of dissocia-

tion into sub-units. No significant difference is evident between the results obtained at the two temperatures.

At this stage, it appeared that the stability conditions for insulin had been established with sufficient precision for a more detailed study of the relationship between dissociation and concentration to be undertaken. The pH value of 7.4 was chosen, as at this pH the solubility is just sufficient for most of the solutions to be studied at the two temperatures.

The results obtained are shown in Table 5, from which it can be seen that, as at pH 8.0, the diffusion coefficient does not vary over the range 0.8–0.3 g./100 ml., but that below the latter concentration there is a rise indicative of the onset of dissociation. Again the influence of temperature, in the limited range in which study is possible, appears to be negligible.

Table 5. *Variation of the diffusion coefficient of insulin with concentration, at pH 7.4*

(All solutions in phosphate buffer, $I=0.10$. v =number of wavelengths retardation.)

v	$D_{20, w}$	
	Lower temp.	Higher temp.
5.93	9.45	—
6.65	9.59	9.55
7.20	8.97	9.10
8.76	8.74	8.74
9.45	8.86	8.96
9.80	—	8.77
11.01	8.40	8.60
12.07	8.23	—
13.98	8.21	8.25
17.65	—	8.34
25.18	8.23*	

* Run at 20.0°.

From these results it appears that the diffusion coefficient of insulin tends to the value of about 8.2 in the stability region. In order to determine whether this value really does represent the minimum attainable, diffusion experiments were performed under conditions which might be expected to favour further association. By lowering the ionic strength the solubility at low temperatures is increased, and the first of these auxiliary experiments was performed with an insulin concentration of 0.55 g./100 ml. and ionic strength 0.04 (pH 7.4). At 14°, the lowest attainable, this gave $D_{20, w} = 8.51$.

The solution in buffer of $I=0.30$, referred to under the sedimentation experiments, gave $D_{20, w} \sim 8.6$ (measured at 30.1°). This solution had to be dialysed at 35°, to avoid precipitation; to minimize bacterial action the period of dialysis was cut to 12 hr. It is possible, therefore, that this result is slightly high because of incomplete dialysis.

From these experiments, it appears that neither of these sets of conditions, namely, higher protein

concentration at a low temperature and low ionic strength, and high ionic strength and (perforce) high temperature, causes any reduction in the diffusion coefficient below the value obtained in the established stability range.

Table 6. *Diffusion coefficients for insulin of various origins*

(Insulin concentration 0.4 g./100 ml. phosphate buffer, $I=0.10$, pH 7.4, $T=20-23^\circ$.)

Description of insulin	$D_{20, w}$
Boots batch no. I 9633 B	8.25
Boots batch no. I 9011 G	8.20
Boots batch no. I 11038 A*	8.90
Burroughs Wellcome	8.82
B.D.H., batch no. 2083	8.69
Sample from Dr Pedersen	8.16

* This sample was more than 4 years old.

Finally, Table 6 shows the results obtained on insulin samples of different origin, together with the average value for Boots batch I 9633 B inserted for comparison. The conditions chosen for these experiments were such as to minimize dissociation, that is, an insulin concentration of about 0.4 g./100 ml. at pH 7.4. The results show clearly that considerable differences in diffusion coefficient may occur in samples of different origin, but that there can be little doubt that two of the three Boots samples and the purified sample from Dr Pedersen, are directly comparable. The rather wide variations in diffusion coefficient are in contrast to the sensibly constant values of the corresponding sedimentation constants, and form another example of the relative insensitivity of the latter characteristic.

All the above diffusion coefficients have been calculated from the movement of the outermost minimum in the record of the interference pattern, the position of which is very largely determined by the magnitude of the refractive index gradient in the centre of the boundary (Ogston, 1949*a*). The values obtained accordingly correspond to the D_A values obtained by the Lamm (1937) scale, or diagonal schlieren, methods, and, as stated by Ogston (1949*b*) for a mixture of components each diffusing ideally, the mean diffusion coefficient, D , obtained by this procedure, is that average given by Quensel (1942):

$$\frac{1}{\sqrt{D}} = \sum \frac{\alpha_i}{\sqrt{D_i}},$$

where α_i is the proportion, in terms of refractive increment, of component of diffusion coefficient D_i .

It has long been recognized that the ratio of D_m (the diffusion coefficient obtained from the second moment of the gradient curve) to D_A is some measure of the homogeneity of the diffusing substance (Gralén, 1944), and where the specific refractive

increments of all components are equal (as is almost certainly the case with the insulin monomer-polymer system), D_m is the weight average diffusion coefficient of the mixture:

$$D_m = \sum \alpha_i D_i.$$

A method of determining D_m from the Gouy interference pattern has recently been developed by Ogston (1949*b*). It is doubtful whether it may be applied reliably to solutions of less than about 20 wavelengths retardation, but for stronger solutions, it is only slightly less accurate than the procedure used to give D_A . Accordingly, D_m values were computed for the most concentrated insulin solutions referred to in Tables 3 and 5. The values obtained were 8.90 (compared with $D_A=8.78$) at pH 8.0 and 8.16 ($D_A=8.23$) at pH 7.4.

The difference between the two values at pH 7.4 is negligible. At the higher pH, the difference is only about 2.5% and is still well within the experimental error. Accordingly, these figures cannot be used to give a reliable value of the degree of dissociation under these conditions, and an attempt to calculate the maximum possible degree of dissociation, by ascribing a probable value to the error attendant upon D_m and assuming the value of 8.2 for the diffusion coefficient of the polymer, was abandoned, as it led to a value of <10 for the diffusion coefficient of the monomer. Such a low value must be considered unlikely, both on general grounds and in view of the results in acid solutions obtained by Fredericq & Neurath (1950).

DISCUSSION

Earlier values of the molecular weight of insulin show considerable discrepancies. In historical order, they may be summarized as follows: Sjögren & Svedberg (1931) using the equilibrium ultracentrifuge obtained S_{20} (uncorrected)=3.47, and $M=35000$; Polson (1939) determined the diffusion coefficient finding $D_{20, w}=8.2$: from this figure and Sjögren & Svedberg's value for S , an independent value of $M=40900$ was calculated. The next evidence was crystallographic: Crowfoot (1938) showed that the weight of the dry unit cell was 36000 (this figure has been corrected from the original, using modern values for the proportion of water in 'dry' insulin, following Gutfreund (1948*a*)) and that the cell had trigonal symmetry, thus indicating that the molecular weight in the solid state was 36000, and that this referred to an aggregate of 3, or $3n$, identical units. Miller & Andersson (1942), using the sedimentation velocity method, found $S_{20, w}=3.55$ for the pH range 7.0-7.5. Diffusion measurements in two different forms of apparatus gave a mean value $D_{20, w}=7.53$. Using the value of 0.749 for the partial specific volume

(Sjögren & Svedberg, 1931), these workers calculated $M = 46\,000$. Gutfreund & Ogston (1946), however, obtained a lower sedimentation constant than Miller & Andersson (1942), finding $S_{20,w} = 3.34$, a value which was constant over the pH range 6.7–9.0.

Gutfreund's osmotic pressure measurements (1948*a*, *b*) showed that, at pH 7.0–7.5, the molecule was stable in the concentration range 0.4–1.0 g./100 ml., but that dissociation, leading to a lower average molecular weight, occurred at concentrations lower than about 0.3 g./100 ml. Dissociation, moreover, was a function of the temperature in the concentration region 0.10–0.16 g./100 ml. Measurements in acid and alkaline solutions showed that the range of stability was rather narrow, dissociation occurring at pH values above 7.5 and below 4. In the first of these papers, Gutfreund gave a value for the diffusion coefficient of insulin of 7.0, which combined with Gutfreund & Ogston's (1946) value for the sedimentation constant, gave $M = 47\,000$. Gutfreund's final conclusion was that the molecular weight of the sub-unit was 12 000, that three such units formed the molecule in the dry state, but that four units formed a stable aggregate, of molecular weight 48 000, in neutral solution.

This conclusion was criticized by Pedersen (1950), who presented further evidence from dynamic measurements: values of $S_{20,w} = 3.46$ and $D_{20,w} = 7.59$, with a new value for the partial specific volume $\bar{v} = 0.735$ were used to calculate $M = 42\,000$. Pedersen also pointed out that Gutfreund & Ogston's (1946) $S_{20,w}$ and Gutfreund's (1948*a*), $D_{20,w}$ values, if used with the new value for $\bar{v}$, gave $M = 44\,000$.

Fredericq & Neurath (1950) found $S_{20,w} = 3.45$ at pH 7.0 and 0.25 g./100 ml. concentration; other values, referring to acid solutions, were taken to indicate that the molecular weight of the sub-unit was 6000, a view which was subsequently modified (Tietze & Neurath, 1952) to agree with Gutfreund's (1952) conclusion that the relevant figure was 12 000.

Oncley *et al.* (1952) came to the same conclusion regarding the size of the sub-unit, but concluded on the basis of sedimentation velocity measurements that probably only three units associated in neutral solution, pointing out that the value of 48 000, resting largely on the low figure used for the diffusion coefficient, leads to a frictional ratio higher than would be expected from viscosity measurements. At pH 7.3, the value of $S_{20,w}$ reported by these workers was about 3.4, but in solutions of very high ionic strength (0.68), this was increased to over 4; the latter figure, together with a value referring to acid solutions, was used to describe the equilibrium between monomer and polymer in acid solutions.

As Gutfreund (1948*a*) stated, some of the discrepancies in the dynamic quantities noted above

may represent real variations in molecular weight, originating in dissociation of the substance with concentration, temperature, or pH. However, as most of the investigations recorded were performed in the known stability range, other grounds of criticism should be considered. Recent work (Cecil & Ogston, 1948, 1949; Kegeles & Gutter, 1951; Charlwood, 1952*a*; Creeth, 1952) has shown that the sedimentation constants of several proteins published by the Swedish workers are somewhat high, and this has been confirmed for insulin by Pedersen (1952). Moreover, the work of Gutfreund & Ogston (1946) was performed before the introduction of the standard conditions for running the oil turbine, and the results are hence likely to be some 2 or 3 % too high (Ogston, 1952). Similar criticism can be applied to the American workers' values for insulin.

For the diffusion coefficients, Miller & Andersson's (1942) values show a total variation of nearly 20 %, whereas for the much lower value quoted by Gutfreund (1948*a*), no details of method, concentration or other experimental conditions were given. Pedersen's (1950) values show D_m about 4 % lower than D_A , but on theoretical grounds any inequality must be in the opposite sense.

Molecular weights determined from these dynamic data must accordingly be subject to comparatively large errors, but, on the whole, tend to give a lower value than indicated from the osmotic data.

The mean value obtained in this investigation for the sedimentation constant of insulin within its stability range, 3.12, is considerably lower than the figures cited above, the nearest being that obtained by Gutfreund & Ogston (1946), after correction by the factor 0.978 given by Cecil & Ogston (1948), which is 3.27, and the revised value of 3.30 given by Pedersen (1952). As the Spinco ultracentrifuge used here has been shown to give the same results as the oil turbine at Oxford (Charlwood, 1952*a*) when the latter is run under standard conditions, there is no reason for doubting the validity of the value 3.12. As Oncley *et al.* (1952) pointed out, in the sedimenting boundary of any insulin solution there must be some levels at which the concentration is low enough to permit dissociation. In view of the boundary characteristics described in the experimental section, it is unlikely that any error from this cause is associated with the above value.

From the diffusion data, it appears that the diffusion behaviour in the stability range approximates to the ideal ($D_A \sim D_m$) and that the constant has a value of about 8.2. This agrees with Polson's (1939) determinations, but differs quite widely from the other results. A possible reason for the discrepancies has been advanced by Pedersen (1952); if the solutions have been prepared by slight warming, it is very easy to obtain supersaturation, and such solutions, as Gutfreund (1948*a*) noticed,

tend to precipitate on standing. If, during the rather protracted duration of a diffusion experiment by the classical procedures, material tends to come slowly out of solution but remains in suspension, low results may be anticipated. Such behaviour would not be expected to influence the sedimentation similarly, as any suspended material would be very rapidly removed. During the comparatively short duration of the diffusion experiments described here, it is unlikely that phenomena attendant upon supersaturation would have an appreciable effect, especially in view of the precautions outlined in the experimental section.

If these values for the sedimentation and diffusion constants are inserted into the Svedberg equation

$$M = \frac{RTS}{D(1 - \bar{v}\rho)}$$

(where $\bar{v}$ is the partial specific volume of the protein and ρ the density of the medium to which the sedimentation and diffusion constants are referred, namely water at 20°) one obtains the molecular weight values of 34800, if $\bar{v} = 0.735$ (Pedersen, 1950), or 36700 on the older figure, $\bar{v} = 0.749$ (Svedberg & Pedersen, 1940). Even lower values for $\bar{v}$ have recently been claimed (Oncley *et al.* 1952), the use of which would, of course, give a lower figure for the molecular weight, but in the absence of detailed evidence the use of the more conventional value is probably to be preferred. The result of this investigation, therefore, is to give the maximum molecular weight of insulin as approximately 36000. The frictional ratio is 1.20. The values for the dynamic quantities used here do not, of course, refer to zero concentration, as is theoretically required. However, in view of the virtual independence of concentration shown by the sedimentation constant, and the apparently similar behaviour in diffusion demonstrated by the results (down to 0.3 g./100 ml.) of Table 5, it is unlikely that if the extrapolation were possible it would yield a greatly different value of the molecular weight.

The value of 36000 is not in agreement with the recent work of Doty *et al.* (1952), in which evidence for the existence of the tetrameric form of insulin was adduced from light-scattering studies at so low a pH as 3.2. The work of Gutfreund & Ogston (1946) at this pH indicated that the sedimentation constant was slightly lower than at pH 7, and Gutfreund's osmotic data (1948*b*) confirmed the occurrence of dissociation in this region, at the relevant concentration. There is clearly scope for the extension of diffusion work to this pH region.

The reason for the discrepancy between the values obtained by the osmotic and dynamic methods is not clear. The number average molecular weight given by the former method should, in the case of a mixture, be a lower value than that given by the

latter, for, as Jullander (1945) has shown, the combination of weight average sedimentation and diffusion constants, while not giving a weight average molecular weight, will generally give a value between the weight and number averages.

Apart from this, the evidence concerning the dissociation of insulin with concentration and pH is in good qualitative agreement with the results obtained by Gutfreund. While the limitations of conclusions formed from the rather restricted range of the results must be borne in mind, it does appear that temperature has little effect on the degree of dissociation under the conditions of the experiments reported here. This behaviour bears a superficial similarity to that discussed by Klotz & Curme (1948) (see also Fiess & Klotz, 1952) for the binding of copper ions to bovine serum albumin, where the assumption of a non-temperature-dependent electrostatic energy of interaction led to an expression for the binding in agreement with experimental observations. Oncley *et al.* (1952), however, found for insulin that the sedimentation behaviour could be explained on the basis of a repulsive electrostatic force acting together with a nearly constant attractive force of unspecified origin. Gutfreund (1952) also concluded that at low pH values, where the sub-units are strongly positively charged, dissociation would be favoured unless the ionic strength was high. In view of the inability to duplicate the higher sedimentation constants reported by Oncley *et al.* (1952) for solutions of higher ionic strength, it is probably unwise to speculate on the nature of the interaction in neutral solutions, beyond the observation that the lack of temperature-dependence indicates that the free energy of the aggregation-disaggregation reaction is associated largely with the entropy change.

SUMMARY

1. The sedimentation and diffusion characteristics of solutions of crystalline insulin have been investigated in the pH range 7–8; the variation of diffusion coefficient with concentration and pH confirms the evidence of dissociation advanced from other studies.

2. Temperature change, within the range 14–30°, has no apparent effect on the dissociation equilibrium.

3. The substance is apparently stable in the range pH 7.0–7.4 and at concentrations greater than 0.3 g./100 ml. Under these conditions, the sedimentation constant is 3.12 and the diffusion coefficient about 8.2. These values differ from previously accepted values.

4. The diffusion and sedimentation results have been combined to give a maximum molecular weight of about 36000 for this protein, in agreement with X-ray data on the solid substance.

The author wishes to acknowledge his indebtedness to Dr K. O. Pedersen, of the Institute of Physical Chemistry, Uppsala, for the gift of the crystalline insulin referred to, and for communicating some of his unpublished results; to Messrs Burroughs Wellcome Ltd., for the gift of another

sample of insulin; to Dr H. Gutfreund for communicating an abstract of some of the American work prior to publication; to Dr P. A. Charlwood of this Institute for his criticism of the manuscript; and to Prof. E. C. Dodds, F.R.S., for his interest and support.

REFERENCES

- Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
 Cecil, R. & Ogston, A. G. (1949). *Biochem. J.* **44**, 33.
 Charlwood, P. A. (1952*a*). *Biochem. J.* **51**, 113.
 Charlwood, P. A. (1952*b*). *Biochem. J.* **52**, 279.
 Coulson, C. A., Cox, J. T., Ogston, A. G. & Philpot, J. St L. (1948). *Proc. Roy. Soc. A* **192**, 382.
 Creeth, J. M. (1952). *Biochem. J.* **51**, 10.
 Crowfoot, D. (1938). *Proc. Roy. Soc. A* **164**, 580.
 Doty, P., Gellert, M. & Rabinovitch, B. (1952). *J. Amer. chem. Soc.* **74**, 2065.
 Fiess, H. A. & Klotz, I. M. (1952). *J. Amer. chem. Soc.* **74**, 887.
 Fredericq, E. & Neurath, H. (1950). *J. Amer. chem. Soc.* **72**, 2684.
 Gralén, N. (1944). Inaugural Dissertation, Uppsala.
 Gutfreund, H. (1948*a*). *Biochem. J.* **42**, 156.
 Gutfreund, H. (1948*b*). *Biochem. J.* **42**, 544.
 Gutfreund, H. (1952). *Biochem. J.* **50**, 564.
 Gutfreund, H. & Ogston, A. G. (1946). *Biochem. J.* **40**, 432.
 Jullander, I. (1945). *Ark. Kemi Min. Geol.* **21 A**, no. 8.
 Kegeles, G. & Gutter, F. J. (1951). *J. Amer. chem. Soc.* **73**, 3770.
 Klotz, I. M. & Curme, H. G. (1948). *J. Amer. chem. Soc.* **70**, 939.
 Lamm, O. (1937). *Nova Acta Soc. Sci. upsal.* **10**, no. 6.
 Miller, G. L. & Andersson, K. J. I. (1942). *J. biol. Chem.* **144**, 459.
 Ogston, A. G. (1949*a*). *Proc. Roy. Soc. A*, **196**, 272.
 Ogston, A. G. (1949*b*). *Biochem. J.* **45**, 189.
 Ogston, A. G. (1952). Private communication.
 Oncley, J. L., Ellenbogen, E., Gitlin, D. & Gurd, F. R. N. (1952). *J. phys. Chem.* **56**, 85.
 Pedersen, K. O. (1950). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 140.
 Pedersen, K. O. (1952). Private communication.
 Perlmann, G. E. & Longworth, L. G. (1948). *J. Amer. chem. Soc.* **70**, 2719.
 Polson, A. (1939). *Kolloidzshr.* **87**, 149.
 Quensel, O. (1942). Dissertation, Uppsala.
 Sjögren, B. & Svedberg, T. (1931). *J. Amer. chem. Soc.* **53**, 2657.
 Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*. Oxford University Press.
 Tietze, F. & Neurath, H. (1952). *J. biol. Chem.* **194**, 1.
 Waugh, D. F. (1948). *J. Amer. chem. Soc.* **70**, 1850.

The Metabolism of Collagen from Liver, Bone, Skin and Tendon in the Normal Rat

BY A. NEUBERGER AND H. G. B. SLACK

*The National Institute for Medical Research, Mill Hill, London, N.W. 7 and
 The Rheumatism Research Centre, The University of Manchester*

(Received 18 June 1952)

In a previous communication (Neuberger, Perrone & Slack, 1951) the metabolism of collagen from normal rat tendon was studied. These experiments have now been extended to include collagen obtained from tendon, skin, bone and liver. Some of the results have been the subject of a preliminary communication (Perrone & Slack, 1951).

EXPERIMENTAL

Treatment of animals and administration of labelled glycine

Male albino rats of the Institute stock were used throughout. The animals consisted of three groups of rats: (1) old rats with body weight ranging from 350 to 390 g., all being more than 14 months old, in two series of four rats and three rats

respectively; (2) young adults with body weight ranging from 200 to 250 g. in two series of four and five rats, and a further series of young adults of known age in two groups of three rats each; (3) young rats with body weight ranging from 50 to 70 g. in two series of five and three rats respectively.

All animals were apparently in normal health and had not been used for other experiments. Throughout these experiments they were given the Institute stock diet. Each rat received 10 μ c./100 g. initial body weight of glycine labelled with 14 C in the methylene position. The specific activity of the glycine was 1 mc./m-mole. The required dose was weighed on a microbalance, dissolved in 1 ml. of distilled water and administered by intraperitoneal injection. The animals were killed by stunning at varying times after the injection.

Growth of animals. The old rats showed no marked or consistent changes in body weight (Table 1). The young