REFERENCES

- Archibald, R. M. (1944). *J. biol. Chem.* **154**, 643.
 Archibald, R. M. (1945). *Chem. Rev.* **37**, 161.
 Archibald, R. M. (1947). *Rev. Euclides, Madrid*, **7**, 251.
 Ferdman, D. L., Frenkel, S. R. & Silakova, A. I. (1942). *Biochimica*, **7**, 43.
 Gale, E. F. (1945). *Biochem. J.* **39**, 46.
 Hamilton, P. B. (1945). *J. biol. Chem.* **158**, 397.
 Harris, M. M. (1943). *J. clin. Invest.* **22**, 569, 577.
 Krebs, H. A. (1948). *Biochem. J.* **43**, 51.
 Parnas, J. K. (1928). *Acta Biol. exp., Varsovie*, **1**, no. 3, 1.
 Prescott, B. A. & Waelsch, H. (1947). *J. biol. Chem.* **167**, 855.
 Van Slyke, D. D. (1913). *J. biol. Chem.* **16**, 187.
 Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.

A Method for Determining the Sedimentation Constant of Material of Low Molecular Weight: Studies on Oxidation Products of Insulin

By H. GUTFREUND, *Department of Colloid Science, University of Cambridge*
 AND A. G. OGSTON, *Department of Biochemistry, University of Oxford*

(Received 28 May 1948)

In a recent paper one of us (Gutfreund, 1948) has shown that insulin molecules probably consist of sub-units of molecular weight 12,000; these units polymerize to molecules of weight 48,000 in neutral solutions of moderate insulin concentration (0.5–1%). The mean molecular weight is, however, dependent upon pH, temperature and concentration. From the determination of terminal amino groups of insulin, Sanger (1945) suggested that these sub-molecules, of molecular weight 12,000, are made up of four peptide chains bound together by disulphide linkages. Two of these chains have terminal glycyl residues and two have terminal phenylalanyl residues. Sanger (1947) has shown that the disulphide linkages can be split by oxidation with performic acid, without affecting any other part of the insulin molecule. Some preliminary studies on the peptides resulting from this oxidation of insulin have been reported by Sanger (1947).

It was the purpose of the work described in this paper to develop methods for the determination of sedimentation constants (S_{20}) less than 1×10^{-13} , and to study fractions from oxidized insulin both by ultracentrifugal sedimentation and by diffusion. Sanger (1947) suggested that the peptides obtained on oxidation of insulin should have a molecular weight of about 3000. This value is between the ranges of molecular weights which have been studied by methods suitable for macromolecules (osmotic pressure, sedimentation and diffusion, etc.) on the

one hand, and those used for simpler compounds (freezing-point depression and similar procedures) on the other hand. It was necessary to modify the procedure of computing sedimentation constants to make it useful for the purpose of studying these polypeptides.

EXPERIMENTAL

Ultracentrifugal examinations were carried out in a Svedberg oil turbine ultracentrifuge, and the Philpot (1938) optical system was used for the observation of the boundaries. The speed of the centrifuge was about 1010 r.p.s. Diffusion constants were determined by the method of Coulson, Cox, Ogston & Philpot (1948).

Dr F. Sanger kindly prepared for us all the oxidized insulin and fractions thereof used in this work. The material was prepared and fractionated as described by Sanger (1949). Crystalline zinc insulin (obtained from Boots Pure Drug Co. Ltd.) was used as starting material. Two fractions (A and B) were examined. Fraction A contains the peptides with terminal glycyl residues while fraction B contains those with terminal phenylalanyl residues. The purity of each fraction, as shown by end-group assay, was about 95%.

It was found that oxidized insulin diffused slowly through collodion or cellophan membranes on dialysis; as it was essential to get the solutions of oxidized insulin into equilibrium with a salt solution of known composition, these were dialyzed against a large volume of $m\text{-Na}_2\text{HPO}_4$. Up to half the nitrogenous material was lost from the solutions.

Method of calculation of the sedimentation constant in the absence of a clear boundary of sedimentation

Two methods are in general use for the observation of sedimentation constants of proteins; first the light absorption method, giving a direct plot of concentration c against distance x in the cell, and secondly the refractive index method, giving a plot of concentration gradient dc/dx against x . Whichever method is used, the sedimentation constant is normally calculated from the movement of the mid-point of the protein boundary with time. This procedure is accurate only if the calculations are made from measurements on boundaries which have completely left the meniscus of the solution and have not reached the bottom of the cell. The peptides studied during this investigation have such small sedimentation and high diffusion constants that one end of the boundary is at the bottom of the cell by the time the mid-point (or peak) has left the meniscus, and it is, therefore, impossible to get successive pictures of the whole boundary (see Fig. 1).

Fig. 1. Philpot schlieren diagrams of fraction A 20, 60, 100 and 140 min. after full speed had been attained. Oxidized insulin concentration 0.74%.

It has been suggested by Tiselius, Pedersen & Svedberg (1937) that, in such cases, the movement of the quantity of protein across an arbitrary plane in the cell could be used for the calculation of the sedimentation constant. This method was elaborated by Svedberg & Pedersen (1940), but no practical use appears to have been made of it so far, and no working procedure has been described; we have, therefore, developed the following method for the

calculation of the sedimentation constant of very slowly sedimenting substances from photographs obtained by the Philpot (1938) optical method.

Fig. 2 is a diagram of the ultracentrifuge cell, with a plot of the concentration and of the gradient of concentration. A fixed plane, distant X cm. from the centre of rotation, is chosen so that the concentration in its neighbourhood varies only with time t

Fig. 2. A, diagram of the ultracentrifuge cell showing the dimensions used in the calculation; B, plots of c and dc/dx against x .

and not with the distance x from the centre of rotation. The sedimentation constant can be obtained from the rate at which material passes the plane X . The concentration at X (and throughout the cell) is initially c_0 , but after time t (assuming that the speed of rotation ω and the sedimentation constant s do not vary) it has fallen to

$$c_{x,t} = c_0 e^{-2s\omega^2 t}. \quad (1)$$

The rate at which material is passing X at time t is, therefore,

$$\begin{aligned} \frac{dQ}{dt} &= c_{x,t} s \omega^2 X \psi X d. dt \\ &= \psi d c_0 s \omega^2 X^2 e^{-2s\omega^2 t} dt. \end{aligned}$$

Integrating between 0 and t , the amount of material which has passed across X from the upper region of the cell is

$$\Delta Q_t = \psi d \cdot \frac{c_0 X^2}{2} (1 - e^{-2s\omega^2 t}). \quad (2)$$

This quantity ΔQ_t can be obtained from the schlieren diagrams. The concentration at x , t is given by

$$c_{x,t} = c_{x,t} - \int_x^X \frac{dc}{dx} dx. \quad (3)$$

The quantity of material above X at time t is then given by

$$Q_t = \int_{x_0}^X c_{x,t} \psi x dx$$

which, from (1) and (3),

$$\begin{aligned} &= \psi d \left\{ \int_{x_0}^X c_0 e^{-2s\omega^2 t} x dx - \int_{x_0}^X x \int_x^X \frac{dc}{dx} dx \cdot dx \right\} \\ &= \psi d \left\{ c_0 (X^2 - x_0^2) e^{-2s\omega^2 t} / 2 - \int_{x_0}^X x \int_x^X \frac{dc}{dx} dx \cdot dx \right\}, \quad (4) \end{aligned}$$

The quantity initially above X was

$$Q_0 = \int_{x_0}^X c_0 \psi x dx = \psi d \cdot c_0 (X^2 - x_0^2) / 2. \quad (5)$$

Since $\Delta Q_t = Q_0 - Q_t$ and writing Q'_t for the second term in the bracket of (4),

$$c_0 X^2 (1 - e^{-2s\omega^2 t}) / 2 = c_0 (X^2 - x_0^2) / 2 - c_0 (X^2 - x_0^2) e^{-2s\omega^2 t} / 2 + Q'_t,$$

whence
$$Q'_t = c_0 x_0^2 (1 - e^{-2s\omega^2 t}) / 2.$$

This expression is exact. It can be solved, without further approximation, by converting it into the form

$$\log \left\{ 1 - \frac{2Q'_t}{c_0 x_0^2} \right\} = -\frac{2s\omega^2}{2 \cdot 303} t. \quad (6a)$$

By plotting the log term against t , a straight line of slope $-2s\omega^2/2 \cdot 303$ should be obtained. Where $s\omega^2 t$ is small, equation (6a) approximates to

$$Q'_t = c_0 x_0^2 s \omega^2 t. \quad (6b)$$

This method of solution amounts to the assessment of the amount of material which would have passed the plane at x_0 had the solution not been terminated there by the meniscus; this is measured by the amount of material which is absent below x_0 compared with expectation, making allowance for the 'dilution factor'.

Computation

Enlarged projections ($\times 4 \cdot 5$) of the boundary photographs were traced on to graph paper; the base lines were fitted from the corresponding photographs of a control run done on buffer alone. Values of $\int_x^X \frac{dc}{dx} dx$ were obtained for a series of values of x in each tracing by counting squares, and were converted into units of refractive increment by use

of the appropriate optical constants. Each value was then multiplied by the corresponding value of x (the actual distance from the centre of rotation) and each product $x \int_x^X \frac{dc}{dx} dx$ was plotted against x . Graphical integration of each resulting curve between x_0 and X gave the value of Q'_t .

Fig. 3. Plot of $-\log \left\{ 1 - \frac{2Q'_t}{c_0 x_0^2} \right\}$ (in terms of refractive index) against time of sedimentation t from reaching full speed, for fractions A and B.

Fig. 3 shows the values of $-\log \left\{ 1 - \frac{2Q'_t}{c_0 x_0^2} \right\}$ plotted against t for the two fractions of oxidized insulin. The variations of ω^2 and of the temperature together affect the rate of sedimentation by less than 1% and their mean values have therefore been used; the regression coefficient b and the standard deviation of b have been calculated in each case. The sedimentation constant s is then given by

$$s = 2 \cdot 303 b / 2\omega^2.$$

The sedimentation constant in water at 20°, S_{20} (corr.), is obtained by applying the usual corrections to s , including that for error of reading of the cell temperature (Cecil & Ogston, 1949).

RESULTS

The values of S_{20} (corr.) and of D_{20} (corr.) (measured at the same concentrations) are given in Table 1; the molecular weights have been obtained from these values, assuming a value of 0.75 for the partial specific volume.

Table 1. *Sedimentation and diffusion constants of oxidized insulin fractions*

	S_{20} (corr.) $\times 10^{13}$	S.D. of S_{20} (corr.) $\times 10^{13}$	D_{20} (corr.) $\times 10^7$	Molecular wt.
Fraction A	0.53	0.034	17.6	2900
Fraction B	0.91	0.054	12.6	7000

DISCUSSION

The diffusion results indicated that fraction A was nearly homogeneous, whereas fraction B was heterogeneous. The method of preparation would also suggest that fraction A is the more homogeneous, fraction B probably containing a certain amount of incompletely oxidized insulin.

The simplest assumption from Sanger's (1945) data is that the unit of insulin is split by oxidation into four peptide chains, two of these having glycyl end-groups (fraction A) and the other two having phenylalanyl end groups (fraction B). The molecular weight estimated for the former agrees moderately with Sanger's (1947) estimate of 2500 from analytical data. By difference from the total weight of 12,000, the molecular weight of fraction B should then be about 3100; the discrepancy between this and the value estimated suggests that fraction B contains a considerable proportion of incompletely

oxidized material, which would seriously affect the values of both sedimentation and diffusion constants.

The main purpose of this paper was to describe a general method for studying these polypeptides in the ultracentrifuge; the results of the examination of the oxidation products of insulin are necessarily only preliminary. It is possible that some fraction of much smaller molecular weight was lost during dialysis and the assumption of two chains with glycyl end groups and two with phenylalanyl end groups, making up the unit of molecular weight 12,000, may well be an oversimplification. The increase in molecular weight on oxidation (600 per 12,000) has not been taken into consideration in the above calculation.

SUMMARY

1. Details are given of a method of obtaining the sedimentation constant of material of too small a molecular weight to form a clear sedimenting boundary in the ultracentrifuge.

2. This method has been applied to the study of the peptides obtained by partial degradation of insulin with performic acid. The diffusion constants of two fractions have also been measured and estimates made of their molecular weights.

One of us (H. G.) is indebted to Prof. R. A. Peters, F.R.S., for hospitality in his laboratory.

REFERENCES

- Cecil, R. & Ogston, A. G. (1949). *Biochem. J.* **44**, 33.
 Coulson, C. A., Cox, J. T., Ogston, A. G. & Philpot, J. St L. (1948). *Proc. Roy. Soc. A*, **192**, 382.
 Gutfreund, H. (1948). *Biochem. J.* **42**, 544.
 Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
 Sanger, F. (1945). *Biochem. J.* **39**, 507.
 Sanger, F. (1947). *Nature, Lond.*, **160**, 295.
 Sanger, F. (1949). *Biochem. J.* **44**, 126.
 Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*. Oxford: University Press.
 Tiselius, A., Pedersen, K. O. & Svedberg, T. (1937). *Nature, Lond.*, **140**, 848.