Boundary Spreading in Sedimentation-Velocity Experiments

5. MEASUREMENT OF THE DIFFUSION COEFFICIENT OF BOVINE ALBUMIN BY FUJITA'S EQUATION

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In sedimentation-velocity experiments, a boundary is formed as the solute sediments away from an air-liquid meniscus. The rate of movement of this boundary gives the solute's sedimentation coefficient (S). In general, the boundary is not perfectly sharp; hence it is customary to speak of the boundary-gradient curve, the graph (after subtraction of a reference base line) of refractive index gradient against distance in the boundary region. Direct measurement of this curve may be made with the schlieren optical system (Philpot, 1938); cf. Fig. 1. The shape of the boundary-gradient curve is determined primarily by the solute's diffusion coefficient (D), its heterogeneity with respect to sedimentation behaviour, and its dependence of sedimentation coefficient upon concentration (c).

Recently, Fujita (1956) obtained an equation for the boundary-gradient curves observed when there is a single homogeneous solute with D constant but with S a linear function of c . Provided that an experimental system obeys the conditions assumed in deriving this equation, it is possible to measure D from the area and the maximum height of the boundary-gradient curve. In the past several attempts have been made to measure D from the boundary-gradient curves of sedimentation-velocity experiments by means of Faxén's equation (Faxén, 1929), which does not take account of the dependence of S on c . A procedure for doing this is given by Svedberg & Pedersen (1940, p. 286). It is now apparent that the omission of concentration dependence from Faxén's equation renders it inadequate for most experimental cases, except at concentrations far more dilute than those customarily used. One purpose of this article is to emphasize this fact.

Another purpose of this article is to illustrate a more general procedure for the measurement of diffusion coefficients from sedimentation-velocity experiments. Although values obtained in this way are less precise and more subject to error than those of conventional diffusion measurements, it is sometimes very useful to be able to measure the diffusion coefficient of a substance of which only 10 mg. can be obtained. At the same time, this measurement of D constitutes a sensitive test of the

homogeneity of the solute since there are significant differences in the boundary-gradient curves of a homogeneous and of a heterogeneous substance. Also, by comparing the values of D obtained from sedimentation experiments and from conventional diffusion experiments, it is possible to check the validity of boundary-spreading measurements in sedimentation.

Bovine albumin was chosen for this study because accurate diffusion studies (Gosting, 1955) had been made of this sample and because its values of S and D are in the range where the theory applies reasonably well.

EXPERIMENTAL

Materials. Armour crystalline bovine plasma albumin, lot no. R370295A, was dissolved in a buffer of pH 4.55 (glass electrode, 25°), containing 0.10M-KCl, 0.01M acetic acid and 0.01M-Na acetate. On the assumption that the viscosity and density increments of the buffer constituents are additive, this buffer was computed to have a density at 25° of 1.0024 and a viscosity relative to water of 1.005 (data from Svedberg & Pedersen, 1940, and Jones & Talley, 1933). Sedimentation coefficients were converted to 25° and the viscosity and density of water by the approximate relation $\eta/(1 - \bar{v}\rho) = \text{constant}$, where η is the solvent viscosity, ρ is the solution density and $\bar{v}$ is the solute's partial specific volume. The value of $\bar{v}$ used was 0.734 ml./g. (Dayhoff, Perlmann & MacInnes, 1952). According to the electrophoretic studies of Longworth & Jacobsen (1949), the albumin should be very nearly isoelectric in this buffer. Concentrations were measured by the optical density at 280 m μ , by using a value for $E_{1\%}^{1\text{cm}}$ of 6.60 (Tanford & Roberts, 1952). Since the samples were prepared by weight dilution, an additional check was made of their relative concentrations.

Some experiments of a few years back, which were made with another sample of Armour crystalline bovine albumin (lot no. 10 522) at pH 4.34 using the Oxford oil-turbine ultracentrifuge, were recalculated by methods discussed in this article. Details of these experiments are given in the preceding paper.

Temperature measurement. Sedimentation was observed by means of a Spinco Model E ultracentrifuge equipped with the company's new temperature-control system which enables one to maintain constant temperature during an experiment within $\pm 0.02^\circ$. The temperature control and measurement (or 'RTIC') unit maintains a set resistance in an electrical circuit which includes the rotor. A thermistor

with a high resistance (approx. 13000 Ω) and a temperature coefficient of -4% per degree is embedded in the base of the rotor. At the lower end of the rotor, electrical contact is made through a tungsten point dipping into mercury and, at the upper end, by a disk surrounded with mercury. The rotor chamber is refrigerated continuously and the bridge-circuit balance determines whether a wire radiation heater is on or off.

In order to correlate the temperature of the rotor with the dial reading of the RTIC unit, one takes the rotor outside its chamber and places it on a stand with electrical connexions to the RTIC circuit. Then the RTIC dial is read and simultaneously the temperature of the rotor is found from a thermometer held in thermal contact with the rotor by means of a metal container, filled with water, which fits snugly into a cell hole. Since these measurements are made with the rotor at rest, and since the resistance of the RTIC circuit might change upon rotation of the rotor, this calibration at rest was checked by the diphenyl ether method of Biancheria & Kegeles (1954) in which the rotor revolves at any desired speed.

At first, the diphenyl ether method gave a temperature higher than the other by $1.0 \pm 0.1^\circ$, at 59 780 rev./min. Later, after replacing the mercury in the lower contact with a fresh, triple-distilled sample, the discrepancy was reduced to $0.2 \pm 0.1^\circ$ at 59 780 rev./min. The same sample of diphenyl ether was used throughout (Dow Chemical Co., Midland, Michigan; perfume grade). However, measurements made during this period of the sedimentation coefficient of bovine albumin show better agreement if the temperature is taken from the calibration at rest than if the results of the diphenyl ether method are used. The reasons for this are not understood at present. Temperatures quoted here are based on the calibration at rest. The sedimentation coefficients were measured in experiments when the discrepancy was 0.2° : the uncertainty in S corresponding to an uncertainty of 0.2° is 0.4% . The diffusion coefficients were measured from earlier experiments when the difference between the results of the two methods was 1.0° ; the diffusion coefficients reported here would be decreased by roughly 2% if the diphenyl ether temperatures were used instead. (The precision of measuring diffusion coefficients in the manner described here is, at best, only 5% .)

Speed measurement and instrument constants. In each experiment, the speed of rotation given by the odometer readings at the beginning and end of the period at full speed

agreed with the set speed (59 780 rev./min.) within the experimental error of taking the odometer readings ($<0.1\%$). The odometer records the number of revolutions of the rotor times $1/64000$; dividing 64 000 times the difference in odometer readings by the corresponding time interval gives the average speed.

The distance of the index hole from the centre of rotation increased by 0.035 cm. as the rotor was brought to full speed; this was measured in the manner suggested by Kegeles & Gutter (1951). At rest, the distance from the outer (flat) edge of the inner index hole to the centre of rotation is 5.715 cm.; this was found by measuring the outer diameter of the rotor with a micrometer and then the distance from the rotor edge to the index hole by means of a travelling microscope.

With the use of the ruled disk provided with the instrument, the magnification factor F (for distance in the direction of sedimentation) was found to be 0.4283 ± 0.0002 (cm. in cell/cm. on plate) with the old drive and 0.4289 ± 0.0003 with the new drive. This was measured with the rotor at rest and would not take into account any lens effects produced by distortion of the cell under running conditions.

Measurement of the boundary-gradient curve. A diagonal wire schlieren system was used: cf. Fig. 1. To read the photographic plates, a travelling microscope (Gaertner tool-maker's microscope M2001 RS) with a two-way movement and a projection attachment (M2001 PA) was used. The height of the gradient curve was measured, at a given position, at the centre of the pattern—no attempt was made to take readings at the upper or lower edges. This subject, as well as the use of reference base lines, is discussed in detail in the preceding paper.

CALCULATIONS

It is generally accepted, in studying free diffusion by means of refractive index-gradient curves, that the ratio of height to area of the boundary curve yields the most precise value for D . Consequently, it is desirable to use this ratio in measuring D from sedimentation-velocity experiments, and Fujita (1956) has derived the necessary equation for doing this. His equation (47) may be written as

$$\left(\frac{AF(\xi_m)}{H}\right)^2 = \frac{2D}{\omega^2 S_0} (e^\tau - 1) [1 + (1 - \lambda)^2]^\frac{1}{2}, \quad (1)$$

where $\frac{A}{H} = \int_{x_0}^{x_w} (\partial c / \partial x) dx / (\partial c / \partial x)_{\max.}$

$$= \int_{x_0}^{x_w} (\partial n_c / \partial x) dx / (\partial n_c / \partial x)_{\max.}, \quad (1a)$$

$$\xi_m = \left[\frac{\omega^2 S_0 x_2^0 k c_0}{2D} \right]^\frac{1}{2} \left[\frac{\lambda^\frac{1}{2}}{1 + (1 - \lambda)^\frac{1}{2}} \right], \quad (1b)$$

$$F(\xi_m) = \frac{\Phi'(\xi_m)}{1 + \Phi(\xi_m)} + 2\xi_m, \quad (1c)$$

$$\Phi(\xi_m) = \frac{2}{\sqrt{\pi}} \int_0^{\xi_m} e^{-y^2} dy, \quad (1d)$$

$$\lambda = kc_0(1 - e^{-\tau}), \quad (1e)$$

$$\tau = 2\omega^2 S_0 t, \quad (1f)$$

$$S = S_0(1 - kc). \quad (1g)$$

Fig. 1. Schlieren photographs at 8 min. intervals of the boundary-gradient curves produced by sedimentation of crystalline bovine plasma albumin in the Spinco Model E ultracentrifuge. Speed 59 780 rev./min., initial concentration 0.67 g./100 ml., diagonal wire angle 55° (on the basis of zero magnification at 90°). Sedimentation from right to left.

The symbol $\partial n_c/\partial x$ refers to that portion of the refractive-index gradient caused by the variation in protein concentration with distance (cf. preceding paper). (It is assumed that the specific refractive increment is independent of concentration and pressure.) Distance, x , is measured from the centre of rotation; x_0 is the position of the meniscus and x_w marks a position on the other side of the boundary where $\partial c/\partial x = 0$. Concentration is measured on a weight/volume basis and c_0 is the initial concentration of the sample, before sedimentation has begun. The solute's sedimentation coefficient at infinite dilution is S_0 , D is its diffusion coefficient (assumed constant), t represents time and ω is the angular speed of rotation.

Equation (1) may be used to find D simply by plotting, for a given sedimentation experiment, $[AF(\xi_m)/H]^2$ against $(e^\tau - 1)[1 + (1 - \lambda)^2]^2$, measuring the slope of this plot and then multiplying the slope by $\omega^2 S_0/2$. For brevity, this plot will be referred to as the height-area plot. To obtain the values required for this plot, one must determine A and H from each schlieren photograph and one must know the value of τ at the time of each photograph; λ and ξ_m both are computed from τ and from quantities which are constant during a given sedimentation experiment. In order to evaluate S_0 , which enters into τ , and k , which enters into λ and ξ_m , one must perform a series of sedimentation experiments at different concentrations and find the rate of movement of the boundary at each concentration.

Measurement of the area under the gradient curve. Assuming that the photographic plates have been measured and reference base lines subtracted, one begins by computing the areas under the boundary-gradient curves.

$$A = \Delta x \Sigma (\partial n_c/\partial x), \quad (2)$$

where $(\partial n_c/\partial x)$ values may be left in the arbitrary units in which they are measured from the photographic plates since the units cancel in the ratio A/H . The distance between successive $(\partial n_c/\partial x)$ readings is Δx , which is measured in units of cm. in the cell. The error introduced by using trapezoidal integration (equation 2) to find A is readily made less than 0.1% (cf. the preceding paper).

Values of A decrease with time because of the dilution which occurs upon sedimentation in a sector-shaped cell and changing field. In order to compare values of A from successive photographs, it is necessary to correct for this dilution effect. Multiplying A by $(\bar{x}/x_0)^2$, where $\bar{x}$ is the position of the first moment, gives (to a good approximation) the constant A_0 (cf. the preceding paper):

$$A_0 = A(\bar{x}/x_0)^2. \quad (3)$$

If the boundary-gradient curve is symmetrical, $\bar{x}$ coincides with x_H , the position of the maximum gradient H .

Since Fujita (1956) has shown that for a linear dependence of S on c , the gradient curve for a single component is quite symmetrical, marked asymmetry in itself indicates heterogeneity. With the bovine albumin studied here, it was necessary to make allowance for the presence of a leading component. When values of $(\partial n_c/\partial x)$ on the trailing side of x_H were subtracted from those on the leading side, spaced at corresponding distances from x_H , it was found that the trailing edge of the leading component's gradient curve was zero at x_H . Consequently, H could be taken to include only the gradient of the main component and A_0 for the main component was estimated by computing the area on the trailing side of x_H (again by trapezoidal integration), doubling this area and then multiplying it by $(x_H/x_0)^2$. In Table 2 this quantity is denoted by $(A_0)_1$, and A_0 is the corrected area under the entire gradient curve, obtained by equation (3). This method of finding $(A_0)_1$ is valid when Faxén's (1929) equation applies. A study of its validity for concentration-dependent systems must wait for solution of the continuity equation for the case of two concentration-dependent solutes. In the meantime, it should not be used for markedly concentration-dependent systems.

In order to prevent the fluctuation of values found for $(A_0)_1$ from successive photographs from appearing in the height-area plot, and perhaps obscuring any upward curvature, the values for A used in the computations are calculated from the average of the values for $(A_0)_1$ by

$$A = (x_0/x_H)^2 (\bar{A}_0). \quad (4)$$

Evaluation of the diffusion coefficient. Once values have been obtained for A , H , ω , S_0 , τ , x_0 , k and c_0 , the calculations are simple and straightforward, except that one must assume a value for D in order to calculate ξ_m . If the measured value of D is significantly different from the assumed value, then one must continue with successive approximations until the two values of D agree. In computing $F(\xi_m)$, *Tables of Probability Functions* (Vol. 1, Federal Works Agency, Works Projects Administration, City of New York, 1941) are very useful (available from the Superintendent of Documents, Government Printing Office, Washington, D.C., U.S.A.).

Finally, D is found from the slope of the height-area plot. The point (0, 0) does not necessarily fall on this plot because ω , and thus the solute's sedimenting velocity, is changing as the rotor comes up to speed and because during this period the process of diffusion is restricted rather than free diffusion. These effects compensate to some extent and in both

Fig. 2. The height-area plot from which D is evaluated: its slope is $2D/\omega^2 S_0$. Points represented by filled circles have been corrected for concentration dependence; the open circles show the same data without this correction. Fig. 2A (above): $c_0 = 0.67$ g./100 ml.; Fig. 2B: $c_0 = 1.35$ g./100 ml.

Fig. 3. The data of Fig. 2 plotted as apparent diffusion coefficients. Filled circles again show data corrected for concentration dependence, open circles show the same data without this correction. Fig. 3A (above): $c_0 = 0.67$ g./100 ml.; Fig. 3B: $c_0 = 1.35$ g./100 ml.

Figs. 2A and B it may be seen that the best straight line through the various points passes close to the point $(0, 0)$. The plots of the apparent diffusion coefficient, D^* , against t in Fig. 3 are illustrative only and were not used in finding D . [The values of D^* in Fig. 3 are obtained by dividing the ordinates of Fig. 2 by the corresponding abscissae times $(2/\omega^2 S_0)$, after subtracting from the abscissa the intercept of the plot.]

Computations when the solute is heterogeneous. When the Oxford experiments were computed according to equation (1), upward curvature was observed in each of the height-area plots, indicating that in this sample of albumin the main component itself is heterogeneous. Since in this case the intercept of the height-area plot cannot be found by placing the best straight line through the data, it was assumed that the curves pass through the point $(0, 0)$ and the D^* values of Fig. 4 were computed on this basis.

To examine the question of whether the curvature is significant, and whether the appearance of heterogeneity (Fig. 4) could be caused instead by the requirement that the height-area plot pass through the point $(0, 0)$, one may proceed in the following way. First equation (1) is re-

Fig. 4. Some older data for another sample of albumin, recalculated for this paper as apparent diffusion coefficients (corrected for the dependence of S on c) against time. In each case, the intercept is the value of D predicted from the diffusion measurements of Gosting (1955). All three experiments were carried out with the same solution ($c_0 = 0.61$ g./100 ml.) in order to check their reproducibility.

written, after expanding $(e^x - 1)$ into a Maclaurin series, as

$$W = 2Dt + 2DS_0\omega^2t^2 + \frac{4}{3}DS_0^2\omega^4t^3 + \dots, \quad (5)$$

where
$$W \equiv \frac{1}{2} \left\{ \frac{AF(\xi_m)}{H[1 + (1-\lambda)^{\frac{1}{2}}]} \right\}^2. \quad (5a)$$

Let W at the time of the first photograph (t_0) be denoted by W_0 :

$$W_0 = 2Dt_0 + 2DS_0\omega^2t_0^2 + \frac{4}{3}DS_0^2\omega^4t_0^3 + \dots \quad (5b)$$

Next subtract equation (5b) from (5) and sum over all values of W (one for each schlieren photograph, or $n+1$):

$$\sum_{i=0}^n (W_i - W_0) = 2D \sum_{i=0}^n (t_i - t_0) + 2DS_0\omega^2 \sum_{i=0}^n (t_i^2 - t_0^2) + \frac{4}{3}DS_0^2\omega^4 \sum_{i=0}^n (t_i^3 - t_0^3) + \dots \quad (6)$$

If there is heterogeneity, the right-hand side of equation (6) should be significantly less than the left-hand side. For the non-concentration-dependent case when the distribution of S is Gaussian (cf. Baldwin, 1954), the difference would be approximately

$$p^2\omega^4x_0^2 \left\{ \sum_{i=0}^n (t_i^2 - t_0^2) + S_0\omega^2 \sum_{i=0}^n (t_i^3 - t_0^3) \right\},$$

where p is the standard deviation of S_0 . To use equation (6), one must know D from independent diffusion measurements and one must also know t_0 to find the terms in $(t^2 - t_0^2)$ and $(t^3 - t_0^3)$. However, these are minor terms and a small error in t_0 is unimportant; for the major term, involving $(t - t_0)$, the value of t_0 itself is not required.

Measurement of sedimentation coefficients. It has been customary to measure sedimentation coefficients from the slope of the plot of $\ln(x_H/x_0)$ against ω^2t and to refer to the sedimentation coefficient measured in this way as being that at the initial concentration of the experiment. In recent years, two features of this method have been examined critically. Goldberg (1953) showed that the true boundary position, x_b , is given by a moment

equation and that x_b differs slightly from x_H for a symmetrical gradient curve. Kegeles & Gutter (1951) pointed out that, since the concentration changes during the experiment, it would be more logical to refer to the sedimentation coefficient measured in this way as being that at the concentration found midway through the experiment. Alberty (1954) noted that one effect of the dependence of S upon c is to introduce curvature into the plot of $\ln(x_b/x_0)$ against ω^2t ; he suggested that this should be taken into account in computing S and gave equations for doing this. From a solution of the continuity equation, Fujita (1956) obtained an equation relating $\ln(x_H/x_0)$ to ω^2t for a linear dependence of S on c and Trautman, Schumaker, Harrington & Schachman (1954) have given a method for computing S which takes into account both concentration dependence and variation in temperature during an experiment.

In view of the accurate speed and temperature control now available, it seemed worth while to study methods of computing S to determine whether full use is being made of the experimental accuracy. When S is a quadratic function of c

$$S = S_0(1 - k_1c - k_2c^2), \quad (7)$$

the equation relating $\ln(x_b/x_0)$ to ω^2t for a homogeneous solute is (cf. Alberty, 1954, and preceding paper)

$$\ln(x_b/x_0) - (S^+) (S_0) (k_1c_0 + 2k_2c_0^2) + \dots = S^+\omega^2t, \quad (8)$$

where S^+ is the sedimentation coefficient at the beginning of the experiment

$$S^+ \equiv S_0(1 - k_1c_0 - k_2c_0^2). \quad (8a)$$

If one defines an apparent sedimentation coefficient, S^* , by the equation

$$S^* \equiv \{\ln(x_b/x_0)_t - \ln(x_b/x_0)_{t_0}\} / \omega^2(t - t_0), \quad (9)$$

where $(t - t_0)$ is the time from the first photograph, then S^* is easily calculated (cf. Table 1) as well as being based on a time origin which can be measured precisely.

Table 1. *Sample calculation of S^**

$10^3 \log(x_H/x_0)$	$10^3 Dt / 2 \cdot 303 x_0^2$	$10^3 \log [(x_b)_t / (x_b)_{t_0}]^a$	$10^{-3}(t - t_0)^{b,d}$	$S_{25,w}^*(s)^c$
0.557	0.001	0	0	—
2.473	0.003	1.918	2.40	4.79 ₅
2.853	0.003	2.298	2.88	4.78 ₈
3.242	0.003	2.687	3.36	4.79 ₉
3.627	0.004	3.073	3.84	4.80 ₁
4.005	0.004	3.451	4.32	4.79 ₃

^a $\log [(x_b)_t / (x_b)_{t_0}] = \log(x_b/x_0)_t - \log(x_b/x_0)_{t_0}$, where x_b is the boundary position, x_0 is the position of the meniscus and t_0 is the time of the first photograph. According to Faxén's (1929) equation, $\ln(x_b/x_0) = \ln(x_H/x_0) + Dt/x_0^2 + \dots$

^b The time from the first photograph.

^c $S^* \equiv 2 \cdot 303 \log [(x_b)_t / (x_b)_{t_0}] / \omega^2(t - t_0)$. $S_{25,w}^* = S^* \left(\frac{\eta}{\eta_{25,w}} \right) \frac{(1 - \bar{\nu}\rho)_{25,w}}{(1 - \bar{\nu}\rho)}$.

^d In order to plot $S_{25,w}^*$ against $t + t_0$, it is necessary to find t_0 and to add $2t_0$ to $t - t_0$. For this purpose, t_0 may be estimated with satisfactory accuracy as $\log(x_b/x_0)_{t_0}$ multiplied by the initial value of $(t - t_0) / \log [(x_b)_t / (x_b)_{t_0}]$. Thus for this experiment $t_0 = (0.558 \times 10^{-3}) (2.40 \times 10^3) / (1.918 \times 10^{-3})$, or $t_0 = 0.70 \times 10^3$ sec.

Comparison of equations (8) and (9) shows that S^* is well-defined theoretically

$$S^* = S_0^* \{1 + (S_0 \omega^2)(t + t_0)(k_1 c_0 + 2k_2 c_0^2) + \dots\}, \quad (10)$$

but that it is a function not only of c_0 but also of $(t + t_0)$. One might avoid this difficulty by plotting S^* against $t + t_0$ and extrapolating to $t + t_0 = 0$ in order to find S_0^* , a function only of c_0 (cf. Alberty, 1954); however, there are experimental difficulties in doing this. Fig. 5 shows the effect on S^* of a fixed uncertainty in reading the positions x_0 and x_H : since the percentage uncertainty in $(x_H - x_0)$ decreases as the distance between x_H and x_0 increases, the values of S^* become increasingly accurate as time increases. Thus it is not desirable to try to extrapolate S^* back to $t + t_0 = 0$.

By taking values of S^* from a set of experiments at fixed $(S_0 \omega^2)(t + t_0)$, these values of S^* are a function only of c_0 :

$$S^* = S_0 \{1 - k_1 c_0 [1 - (S_0 \omega^2)(t + t_0)] - c_0^2 [k_2 - 2k_2 (S_0 \omega^2)(t + t_0) + k_1^2 (S_0 \omega^2)(t + t_0)] - \dots\}. \quad (11)$$

As equation (11) shows, once $(S_0 \omega^2)(t + t_0)$ is fixed the plot of S^* against c_0 may be extrapolated to $c_0 = 0$ in order to find S_0 , and then the plot of c_0 against $(S_0 - S^*)/S_0 c_0$ yields, from its slope and intercept, quantities from which k_1 and k_2 may be found. When k_1 and k_2 are known, the slope of the plot of S^* against $t + t_0$ is known from equation (10), and the graphs from which the values of S^* were taken can be re-examined, to see whether the lines correspond with theory.

Fig. 5. The plot used to measure S . An 'apparent' sedimentation coefficient, S^* , defined by equation (9), is plotted against $t + t_0$, where t_0 is the time of the first photograph. The line drawn through the points has the theoretical slope given by equation (10). The line through each point gives the uncertainty in S^* caused by an uncertainty of 0.001 cm. in reading the positions of x_H and x_0 on the photographic plate. For this experiment $c_0 = 0.87$ g./100 ml.

RESULTS AND DISCUSSION

Values found for the diffusion coefficient

Fig. 2 shows the height-area data from experiments at $c_0 = 0.67$ and 1.35 g./100 ml. The slope of Fig. 2A gives a value for D of $7.0_2 \times 10^{-7}$, as compared with 6.85×10^{-7} predicted from the diffusion measurements of Gosting (1955). In Fig. 2B the observed value is $6.7_6 \times 10^{-7}$ and the predicted value is 6.94×10^{-7} . It should be remembered that the precision of the values shown in Figs. 2 and 3 is considerably greater than their accuracy. The average value of $(A_0)_1$, from which the values of A were computed, is uncertain by $\pm 2\%$ and this uncertainty is doubled in the data of Figs. 2 and 3 since A is squared in computing D . Thus the observed value of D cannot be expected to be more accurate than $\pm 5\%$, and the agreement between observed and predicted values is satisfactory.

To show more clearly the magnitude of the concentration dependence correction, the data have been replotted in Fig. 3 as apparent diffusion coefficients, D^* (cf. Calculations). By the end of the experiment at $c_0 = 1.35$, the uncorrected value of D^* falls almost to half the corrected value. Since 1.35 g./100 ml. is in the range where sedimentation studies of globular proteins are commonly made, and since bovine albumin shows less marked concentration dependence than many, this correction of the apparent diffusion coefficients for the dependence of S on c is clearly an important correction. Values of D^* which have not been corrected for the dependence of S on c cannot be expected to agree with measurements of D from conventional diffusion experiments. Where such agreement has been found (cf. Svedberg & Pedersen, 1940, p. 286, Lauffer, 1942; Davisson, Gibson, Ray & Vestling, 1953) it must now be presumed that the proteins studied were heterogeneous and that this compensated for neglect of the concentration-dependence correction. Dieu & Oth (1954) have reported values of uncorrected apparent diffusion coefficients for bovine albumin which are less than the predicted value of D , but values for ovalbumin which they say are in agreement with the predicted value of D .

It should be emphasized that the presence of a faster sedimenting component in this sample of albumin introduces some uncertainty into the calculation of D by Fujita's equation. The procedure for finding $(A_0)_1$ is based on Faxén's equation, not on an equation which takes into account the dependence of S on c . In other words, the effect of the leading component on the shape of the gradient curve of the main component is not known. Also the procedure for finding k in $S = S_0(1 - kc)$ is based on the assumption that only one component is present. The uncertainty in the calculated value of D increases with increasing concentration, for both

these reasons. Thus the agreement of predicted and observed values for D is more significant in the experiment at $c_0 = 0.67$ than at $c_0 = 1.35$ g./100 ml. Also, the plot of S against c shows slight downward curvature above a concentration of 1 g./100 ml. However, the precision of measuring the gradient curves is better at the higher concentration.

The predicted value of D was computed in the following way. According to the measurements of Gosting (1955), $\bar{D}$ (the weight-refractive average referred to 25° and the viscosity of water) is $6.73_2 \times 10^{-7}$ for this sample at a mean concentration of 0.515 g./100 ml. (Values of D refer to the mean concentration of the solutions on the two sides of the boundary; in the experiments reported here the mean initial concentrations are 0.33 and 0.68 g./100 ml. Creeth (1952) reports that the concentration dependence of D for bovine albumin (at pH 6.8) is negligible in this range of concentration.) Taking α_2 (the fraction, on a refractive increment basis, of the leading component) as 0.12, one may compute from the fringe deviation graph of the diffusion experiment that D_1/D_2 (the ratio of diffusion coefficients of the main and leading components) is 1.40 (L. J. Gosting, private communication). Since $\bar{D} = \alpha_1 D_1 + \alpha_2 D_2$, it follows that $D_1 = 1.035 \bar{D}$ or 6.97×10^{-7} . Values of D for the particular temperature and buffer of the sedimentation experiment were then calculated on the assumption that $D\eta/T$ was constant, where η is the solvent viscosity and T the absolute temperature. The values used for k were 0.057 at $c_0 = 0.67$ g./100 ml. and 0.059 at $c_0 = 1.35$ g./100 ml.

If the expression for D is taken to be

$$RT(1 + \partial \ln y / \partial \ln c) / f$$

(cf. Onsager & Fuoss, 1932), where f is the frictional coefficient and y is the activity coefficient on the c scale, then a slight pressure dependence of D is to be expected. The viscosity of the solvent, which affects f , depends on pressure and the thermodynamic term also is, in general, a function of pressure (cf. Guggenheim, 1950).

Evidence of heterogeneity in one sample

Fig. 4 shows three experiments carried out with a single solution of bovine albumin. A different batch of albumin (Armour, lot no. 10 522) was used from the one considered above, the pH was lower (4.34), and the measurements were made with the Oxford oil-turbine ultracentrifuge. The fractional amount of the leading component, α_2 , was found to be 0.11 by the procedure outlined in Calculations. In Fig. 4 it is apparent that the values of D^* increase with time: this indicates heterogeneity (cf. Williams, Baldwin, Saunders & Squire, 1952; Baldwin, 1954). However, the solution to the differential equation has not been carried out for a

heterogeneous concentration-dependent system; so there is no theoretical expression for the slope of Fig. 4. For a solute without dependence of S on c and a Gaussian distribution of S , the slope would be approximately $(\frac{1}{2})p^2\omega^4x_0^2(1 + \bar{S}_0\omega^2t + \dots)$ (Baldwin, 1954; equation (5) which, combined with equation (1), gives $\partial(C/C_0)/\partial x$ when $g(S)$ is Gaussian, contains a misprint: the term $p^2\omega^4x_0^2$ should be $p^2\omega^4x_0^2/2\pi$). Taking this as the expression for the slope gives a value for $p_{25, w}$ of $0.35 \text{ s} \pm 0.04 \text{ s (s.d.)}$, or 7% of S_0 ($s = \text{Svedberg units}$). The alternative calculation of p from the difference between the left-hand and right-hand sides of equation (6) gives

$$p_{25, w} = 0.36 \text{ s} \pm 0.04 \text{ s (s.d.)}.$$

This agreement between the two values of p rules out the possibility that the upward slope of D^* against t in Fig. 4 could have been caused by requiring the height-area plots to pass through the point (0, 0). Since a difference of 5% between D^* and the predicted value of D would not be considered significant, the value for p given above is near the limit of detectability.

The drift with time of D^* would be evident in Expts. A1 and A2 even if D were not known from independent evidence. However, in Expt. A3 the scatter is such that this drift might be overlooked; if one were to calculate D from this experiment as if there were no heterogeneity, the value would be 10% too high. This emphasizes the need for accurate data in using sedimentation measurements to find D , and it also illustrates the difficulty of finding D from sedimentation experiments when the solute is heterogeneous. Even in Expts. A1 and A2, where the heterogeneity is evident, the uncertainty in the intercept is considerably greater than it would be with comparably precise data for a homogeneous solute.

Area measurements

Table 2 shows the results of measuring areas under the boundary-gradient curves. The agreement of the values found from successive photographs, after correction for the dilution effect, is quite good in view of the theoretical and practical uncertainties of the schlieren optical system (cf. Kegeles & Gosting, 1947; Trautman & Burns, 1954) and the difficulties of establishing the correct base lines. As Table 2 shows, there is no significant dependence on time of the areas once a correction for the dilution effect has been made. The ratio, α_1 , of $(A_0)_1$ to A_0 (cf. Table 2) was found to be 0.89₄ at $c_0 = 0.67$ and 0.88₅ at $c_0 = 1.35$ g./100 ml. If a correction for the Johnston-Ogston effect (Johnston & Ogston, 1946) is made by their (approximate) equations (2) and (3), these values for α_1 become 0.88₈ at $c_0 = 0.67$ and 0.87₄ at $c_0 = 1.35$ g./100 ml.

Table 2. *Areas from schlieren diagrams*

Measurements from two experiments of the area under the boundary-gradient curve times the correction for the dilution effect. The areas were found by trapezoidal integration; values given here are in arbitrary units. The left-hand set of columns gives the area under the entire curve, A_0 , and the right-hand set shows that attributed to the main component, $(A_0)_1$. The diagonal-wire angle gave a higher magnification (by a factor of 1.50₂) at the lower concentration; the areas from the experiment at $c_0 = 0.67$ agree, within the precision of the measurements, with those from the experiment at $c_0 = 1.35$ g./100 ml.

Photograph no.	(a) A_0	(b) A_0	(a) $(A_0)_1$	(b) $(A_0)_1$
1	95.7	127.3	85.5	115.0
2	94.6	125.9	85.1	111.0
3	94.9	127.2	84.1	111.6
4	92.7	127.7	83.3	112.4
5	93.4	126.4	82.5	111.2
6	93.5	126.3	84.4	111.7
Average	94.1 ± 1.2 %	126.8 ± 0.6 %	84.1 ± 1.3 %	112.2 ± 1.4 %
	(a) $c_0 = 0.67$ g./100 ml.		(b) $c_0 = 1.35$ g./100 ml.	

Table 3. *Sedimentation coefficients of bovine albumin*

c_0^a	$S_{25, w}^b$	S_{calc}^c
0.29	4.92 ₇	4.930
0.59	4.83 ₉	4.843
0.87	4.77 ₂	4.759
1.17	4.66 ₃	4.667
1.17	4.66 ₃	4.667
1.76	4.47 ₅	4.472

^a The initial concentration of the experiment in g./100 ml.

^b The measured sedimentation coefficient at the initial concentration of the experiment, in Svedberg units (s).

^c Calculated from the equation $S_{25, w} = 5.01 (1 - 0.054c - 0.004c^2)$ s.

Precision of the sedimentation coefficients

Several studies have recently been made of the concentration dependence of S for bovine albumin (Koenig & Pedersen, 1950; Kegeles & Gutter, 1951; Creeth, 1952; Miller & Golder, 1952; Taylor, 1952; Shulman, 1953). Yet, on examination of the data in these papers, it became apparent that in none of them, not even in the very careful study of Kegeles & Gutter, was the slope of S against c known with the accuracy required for these boundary-spreading calculations. Consequently, a study of the concentration dependence of S was made; the results are given in Table 3. In Fig. 6, the need for such a study may be seen; values given by various authors have been plotted as S/S_0 against c . This plot was chosen, instead of S against c , in order to focus attention on the accuracy with which the concentration dependence is known rather than on the absolute values of S .

The sedimentation coefficients shown in Table 3 appear to be considerably more precise than any yet reported for albumin. As the third column of the table shows, they fit the equation

$$S_{25, w} = 5.01(1 - 0.054c - 0.004c^2) \text{ s,}$$

Fig. 6. Comparison of the results for the concentration dependence of S with some of the data in the literature. Open circles, data of Kegeles & Gutter (1951); half-filled circles, data of Koenig & Pedersen (1950); filled circles, this paper. To compare measurements of the concentration dependence of S rather than of absolute values, all sedimentation coefficients were divided through by S_0 , the sedimentation coefficient for infinite dilution. The values taken from the literature are plotted against the average concentration of the experiment. (For the data of Koenig & Pedersen, the average concentration was taken to be 0.89 time the initial concentration.)

with a maximum deviation of 0.2% and a standard deviation of 0.14% of S_0 . In analysing reasons for the precision of these values, three factors should be mentioned. First, the control of temperature by the RTIC unit ($\pm 0.02^\circ$) is better than in the earlier studies on albumin. Secondly, the precision gained by the use of a travelling microscope, as compared to the use of enlarged tracings, is considerable, particularly when the microscope has a two-way movement so that the plates may be aligned accurately. Thirdly, the method of calculation makes good use of the experimental accuracy.

There appears to be downward curvature in the plot of S against c , so that plotting $1/S$ against c would make the curvature greater, not less. Cecil & Ogston (1949), in their very accurate study of the sedimentation behaviour of β -lactoglobulin, observed this same kind of curvature so that one may wonder if it is not a general property of the globular proteins. If curvature is overlooked and the extrapolation for finding S_0 based on data from too high a concentration range, the value of S_0 will be too high.

Values of S^* were read from the graphs of S^* against $t+t_0$ at $t+t_0=5.76 \times 10^3$ sec. After S_0 had been found from the plot of S^* against c_0 , values of S^* (the sedimentation coefficient at the initial concentration c_0) were calculated from equation (10). The values of S were converted to the viscosity and density of water at 25° , rather than at 20° , because Longworth (1954) has shown a slight dependence of f/η (where f is the frictional coefficient) on temperature.

Molecular weight of bovine albumin

Use of the Svedberg equation (Svedberg, 1925) gives a molecular weight of 66 500 with the values at pH 4.6 of

$$S_{25,w} = 5.01 \times 10^{-13} \text{ sec.},$$

$$D_{25,w} = 6.97 \times 10^{-7} \text{ cm}^2 \text{ sec.}^{-1}$$

[the value of Gosting (1955), corrected for the presence of a slower-diffusing impurity by means of the fringe deviation graph of the diffusion experiment and by sedimentation analysis for the amount of the impurity], $\bar{v}=0.734$ ml. g.⁻¹ (Dayhoff *et al.* 1952) and ρ for H₂O = 0.9971 at 25° . It should be pointed out that no attempt has been made to correct the values of S for pressure dependence and that this correction may well be significant. For a comparison of these values for M , S , and D with other values in the literature, see Harrington, Johnson & Ottewill (1956).

SUMMARY

1. The use of Fujita's theory for the concentration-dependent sedimentation of a single solute is discussed and illustrated with experiments on bovine plasma albumin.

2. With this theory, the diffusion coefficient of bovine albumin has been measured from experiments with the velocity ultracentrifuge. The values obtained for D were in agreement within experimental error ($\pm 5\%$) with the value known from Gosting's diffusion studies.

3. As the theory shows, the correction of apparent diffusion coefficients for the dependence of S on c is large. Previous work, in which this correction was ignored, should be re-evaluated.

4. The main component of one sample of bovine

albumin showed measurable heterogeneity in sedimentation behaviour.

5. A method for calculating sedimentation coefficients is discussed. The method takes into account the dependence of S on c and the effect on S of the uncertainty in measuring the photographic plates.

6. Sedimentation coefficients of bovine albumin measured from six experiments fit the equation $S_{25,w} = 5.01(1 - 0.054c - 0.004c^2)$ s with a standard deviation of 0.14% .

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A Chromatographic Method of Analysis for Thiols

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Although there are a number of methods for the detection and estimation of thiol compounds (Patterson & Lazarow, 1954), no fully adequate chromatographic procedure has been hitherto available. The reduced forms of coenzyme A (Stadtman, 1952), cysteine (Toennies & Kolb, 1951), and glutathione (Miller & Rockland, 1952) have been detected on paper by means of thiol reactions, but the chief obstacle has been the ease of autoxidation of the thiol groups, leading to secondary spots and the excessive formation of 'comets' or tailing.

The specific problem of α -amino acid-thiol separations was first resolved by Hanes, Hird & Isherwood (1950) and expanded by Gutcho & Laufer (1954). In their methods the thiol group is masked with

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N-ethylmaleimide (Friedman, Marrian & Simon-Reuss, 1949), a reaction that proceeds rapidly to completion in dilute aqueous solution over a wide pH range (Leopold & Price, in preparation). After chromatographic development of the *N*-ethylmaleimide-thiol conjugates, the compounds are detected with ninhydrin. A disadvantage of this method was that these conjugates were distinguished from other amino acids and peptides only by their R_f values. More recently (Benesch, Benesch, Gutcho & Laufer, 1956) a method has been found for detecting the conjugates of *N*-ethylmaleimide and any thiol. An alternative approach, suggested by Gutcho & Laufer (1954), is that the thiol-masking reagent contains a potential chromophore. Such a procedure is in fact the basis of a histochemical technique which has been developed successfully (Barnett & Seligman, 1954). The most promising substance of this sort, described recently