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Boundary Spreading in Sedimentation-Velocity Experiments

4. MEASUREMENT OF THE STANDARD DEVIATION OF A SEDIMENTATION-COEFFICIENT DISTRIBUTION: APPLICATION TO BOVINE ALBUMIN AND β -LACTOGLOBULIN*

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Methods of studying the heterogeneity of proteins have been essential to the modern development of protein chemistry. One of the most useful methods has been analysis by means of the velocity ultracentrifuge, in which molecular species of different size, shape or density are resolved. This article provides a method for measuring the standard deviation of a sample's distribution of sedimentation coefficient. One would prefer to measure the distribution itself, but for several systems this is not possible while measurement of the standard deviation, p , of the distribution is possible.

One reason for studying the heterogeneity of a protein preparation is to find out whether homo-

geneous proteins exist in Nature. Because of the possibility of changes produced during fractionation or upon storage, measurements of heterogeneity must be interpreted with great caution. This subject has been reviewed by Pirie (1940) and recently by Colvin, Smith & Cook (1954). Another reason for studying the heterogeneity of a protein sample is to aid in interpreting other physical measurements. For example, heterogeneity enters into the measurements of sedimentation coefficient, diffusion coefficient and intrinsic viscosity in different ways, so that, in interpreting these measurements by a theory such as that of Scheraga & Mandelkern (1953) for the hydrodynamic behaviour, one must have detailed knowledge of the heterogeneity. A brief review of the measurement of heterogeneity by diffusion, electrophoresis

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and sedimentation experiments is given by Baldwin, Gosting, Williams & Alberty (1955b).

The general purpose of this series of papers has been to develop methods for the measurement of heterogeneity in the sedimentation behaviour of proteins and of other biological macromolecules. The first paper (Williams, Baldwin, Saunders & Squire, 1952) presented in outline a theory for taking into account the effects of diffusion on the measurement of heterogeneity when the distribution of sedimentation coefficient is continuous. The ordinate of the differential distribution curve was denoted by $g(S)$, the sedimentation coefficient being S . (In the preceding papers of this series, sedimentation coefficient has been denoted by s .) It was pointed out that, if concentration dependence effects could be ignored, then the curve of $g(S)$ against S could be obtained by extrapolation of an 'apparent distribution' to infinite time and also an independent determination could be made of the distribution's standard deviation, p , from the variation with time of σ^2 , the second moment about the mean of the boundary-gradient curve. Concurrently, Gosting (1952) gave a full theoretical account of the extrapolation to infinite time which is used in finding $g(S)$. Studies of dextran (Williams & Saunders, 1954) and gelatin (Williams, Saunders & Cicirelli, 1954) systems showed that, in general, concentration-dependence effects must be taken into account in finding $g(S)$ by means of a further extrapolation to infinite dilution. The second paper (Baldwin, 1954a) treated this problem by an approximate theory, and a method was developed for using the results of only one experiment to find $g(S)$, by means of a calculated correction for the effects of concentration dependence. This method was used by Ogston & Woods (1954) in their study of various dextrans.

There is a limit to the resolving power of present ultracentrifuges, and this restricts the class of systems for which $g(S)$ may be found by extrapolation to infinite time. This problem was investigated in the third paper (Baldwin, 1954b) by means of trial extrapolations to infinite time based on an analytical expression for the case that $g(S)$ is Gaussian. For a given field strength and duration of the experiment, the accuracy of the extrapolated distribution is related to the ratio of heterogeneity to diffusion coefficient for the system. It was found that, for many systems in which heterogeneity significantly influenced the spread of the boundary-gradient curves, the resolving power was too low for $g(S)$ to be found by extrapolation. Also, when the distribution of S is discontinuous, for example, when there is a discrete mixture of solutes whose boundary-gradient curves overlap, it is not yet possible to obtain the distribution by extrapolation to infinite time.

No such limitation affects the measurement of p from the variation of σ^2 with time; so long as heterogeneity measurably affects the spread of the boundary-gradient curves, one can calculate p . However, the dependence of S on concentration (c) is not included in the equation for finding p given by Williams *et al.* (1952) (see also Baldwin, 1954b). Experiments (Baldwin, 1953a) have shown that this is a serious limitation. An approximate, unpublished theory (Baldwin, 1953a) which takes into account the concentration-dependence effect has been quoted by Field & O'Brien (1955) and has been used (Baldwin, 1953c) in studying the neurotoxin of *Shigella shigae* prepared by Van Heyningen & Gladstone (1953). It is this theory, in a more rigorous form, which will be presented here. The end result is an equation for σ^2 which takes into account the dependence of S on c and which can be used to find p . The case considered is that of a heterogeneous system in which both the sedimentation and diffusion coefficients of the individual species are functions only of the total concentration. (Because of the demands of experiment, concentration is expressed on a refractive-increment basis.)

THEORY

Since p can be found from σ^2 , the second moment about the mean of $\partial n_c / \partial x$ against x , for the case in which the sedimentation and diffusion coefficients do not vary with concentration (Williams *et al.* 1952; Baldwin, 1954b), it was natural to hope that the concentration-dependent case could be solved by finding an expression for σ^2 . With this purpose in mind, a fairly general method has been developed for finding moments of the boundary-gradient curves observed in sedimentation, diffusion and electrophoretic experiments. It has been used (Baldwin, 1954b), when Faxén's (1929) equation for the sedimentation of a single solute applies, to find a simple expression in closed form for the positive even moments of the gradient curves. (Faxén's equation itself is expressed in an infinite series.) The method has also been used (Baldwin, Dunlop & Gosting, 1955a) in studying the diffusion of several solutes whose flows interact.

In outline, the procedure is as follows: First one writes an expression for any moment

$$(\mu_r, r = 0, 1, 2, \dots)$$

of the curve of $\partial n_c / \partial x$ against x in terms of the individual concentration gradient curves. This is then differentiated with respect to time. Next, by means of the expression for conservation of mass, one introduces the appropriate flow equation for each solute and thus its sedimentation and diffusion coefficients. The resulting expression is a differential equation for the rate of change of any specified

moment with time. This equation is put into suitable form for integrating with respect to time and the final step is integration.

Definition of p and $\bar{S}_0$, the standard deviation and the mean of the distribution of S_0 . The optical systems now used for ultracentrifugal analysis (cf. Philpot, 1938; Svensson, 1939; Philpot & Cook, 1948) record, as a function of position in the cell x , a quantity related linearly to n , the refractive index of the solution, or to its gradient $(\partial n/\partial x)_t$. In this paper it will be assumed that n can be adequately represented by the equation

$$n = n_0 + \sum_i R_i c_i, \quad (1)$$

where n_0 is the refractive index of the solvent at the same temperature and pressure (the solvent will be taken to include dialysable constituents such as buffer and salt ions), c_i is the volume concentration of the macromolecular solute i (in g./100 ml. of solution) and R_i is the differential refractive increment of i :

$$R_i \equiv \left(\frac{\partial n}{\partial c_i} \right)_{T, P, c_j \neq i}, \quad (2)$$

where T is the temperature, P is the pressure and j includes all macromolecular species other than i . In the sedimentation experiment, the temperature is assumed to be constant and R_i will be taken to be constant, independent of pressure and of the concentrations of the various solute species. Thus the gradient of n (i.e. its partial derivative with respect to x at constant t) is written

$$\left(\frac{\partial n}{\partial x} \right)_t = \left(\frac{\partial n_0}{\partial x} \right)_t + \sum_i R_i \left(\frac{\partial c_i}{\partial x} \right)_t, \quad (3)$$

where $(\partial n_0/\partial x)_t$ includes the gradient caused by buffer sedimentation. Hereafter the subscript t will be omitted. In experimental practice, reference base lines are used to subtract (approximately) $\partial n_0/\partial x$ from $\partial n/\partial x$; in any case it will be assumed here that $\sum_i R_i c_i$ (or its gradient) is readily measurable. For brevity, this quantity will be denoted by n_c :

$$n_c \equiv \sum_i R_i c_i, \quad (4a)$$

$$\frac{\partial n_c}{\partial x} = \sum_i R_i \frac{\partial c_i}{\partial x}. \quad (4b)$$

Since refractive increments are measured experimentally, it is convenient to define the heterogeneity of the sample on a refractive-increment basis. For the case that the sample contains a set of discrete components, the fractional amount of solute i , α_i , may be written

$$\alpha_i \equiv R_i c_i^0 / \sum_i R_i c_i^0, \quad (5)$$

where c_i^0 is the initial concentration of i before any sedimentation has taken place. Then, on this refractive increment basis, p is defined by

$$p^2 \equiv \sum_i \alpha_i (S_{i,0} - \bar{S}_0)^2, \quad (6a)$$

where $S_{i,0}$ is the sedimentation coefficient of species i at infinite dilution of all macromolecular solutes, and

$$\bar{S}_0 \equiv \sum_i \alpha_i S_{i,0}. \quad (6b)$$

Definition of μ_r , the r -th moment of the boundary-gradient curve. Remembering that n_c (equation 4a) is taken to be a measurable quantity, we define the r th moment of the gradient curve as

$$\mu_r \equiv \frac{\int_{x_0}^{x_w} x^r (\partial n_c / \partial x) dx}{\int_{x_0}^{x_w} (\partial n_c / \partial x) dx} \quad (r=0, 1, 2, \dots), \quad (7a)$$

and so, in terms of the individual concentration-gradient curves,

$$\mu_r = \frac{\sum_i R_i \int_{x_0}^{x_w} x^r (\partial c_i / \partial x) dx}{\sum_i R_i c_i^\beta}, \quad (7b)$$

where we require that all $\partial c_i / \partial x = 0$ at x_0 , the position of the meniscus, and at x_w , an arbitrary plane in the region of constant concentration ahead of the boundary (the region marked β in Fig. 1); c_i^β is the concentration of solute i in the β region. [It can be shown from equations (10) and (11) that, when S is a function of c only, there will be a region ahead of the boundary in which c does not vary with distance; cf. Trautman & Schumaker, 1954.]

The time derivative of μ_r . Equation (7b) may be differentiated by applying Leibnitz's rule [cf. Courant (1936) or Kaplan (1953, p. 217)] which requires that the indicated partial derivatives be continuous:

$$\frac{d\mu_r}{dt} \sum_i R_i c_i^\beta + \mu_r \sum_i R_i \frac{dc_i^\beta}{dt} = \sum_i R_i \int_{x_0}^{x_w} x^r \left[\frac{\partial}{\partial t} \left(\frac{\partial c_i}{\partial x} \right)_t \right] dx. \quad (8)$$

The order of differentiation can be inverted, again provided that the partial derivatives are continuous:

$$\left[\frac{\partial}{\partial t} \left(\frac{\partial c_i}{\partial x} \right)_t \right]_x = \left[\frac{\partial}{\partial x} \left(\frac{\partial c_i}{\partial t} \right)_x \right]_t. \quad (9)$$

The continuity equation for transport in a sector-shaped cell is

$$\left(\frac{\partial c_i}{\partial t} \right)_x = -\frac{1}{A} \left(\frac{\partial A J_i}{\partial x} \right)_t, \quad (10)$$

where A , the cell area, is given by

$$A = (\text{const.}) x, \quad (10a)$$

x being the distance from the centre of rotation. Equation (10) expresses simply the conservation of mass, stated in a differential form. The concentration, c , must be on a weight/volume scale and the flow, J (the amount transferred across unit area in unit time), must be expressed in corresponding units. Lamm's (1929) flow equation will be used, with the assumption that the diffusion and sedimentation coefficients are functions only of the various solute concentrations:

$$J_i = -D_i \frac{\partial c_i}{\partial x} + c_i S_i \omega^2 x. \quad (11)$$

In equation (11), D_i is the diffusion coefficient of i and ω is the angular speed of rotation.

After substitution of equations (9), (10) and (11) into equation (8), one may integrate by parts to obtain the following expression for the time derivative of μ_r :

$$\begin{aligned} \frac{d\mu_r}{dt} = & 2\omega^2 \mu_r \sum_i R_i c_i^\beta S_i^\beta / n_c^\beta \\ & + (r-2) \sum_i R_i \int_{x_0}^{x_w} x^{(r-2)} D_i (\partial c_i / \partial x) dx / n_c^\beta \\ & + (r-2) \omega^2 \sum_i R_i \int_{x_0}^{x_w} x^r \left[\frac{\partial}{\partial x} (c_i S_i) \right] dx / n_c^\beta, \quad (12) \end{aligned}$$

$$\text{where} \quad n_c^\beta \equiv \sum_i R_i c_i^\beta. \quad (12a)$$

In order to bring equation (12) into a form suitable for integrating with respect to time, it is necessary to state the exact concentration dependence of S_i and D_i .

The dependence of S_i upon the concentrations of the various solutes is conveniently expressed by means of a Taylor's series (cf. Kaplan, 1953, p. 369). Since p , $\bar{S}_0$ and μ_r have all been defined in terms of the refractive increments, $R_i c_i$, rather than the concentrations c_i , the equations are handled more readily if S_i is related to $R_i c_i$ instead of to c_i .

$$S_i = S_{i,0} - \sum_{j=1}^n a_{ij} R_j c_j - \sum_{j=1}^n \sum_{k=1}^n a_{ijk} R_j c_j R_k c_k - \dots, \quad (13)$$

where $S_{i,0}$ is the sedimentation coefficient of species i at infinite dilution of all n macromolecular species and the a_{ij} and a_{ijk} are partial derivatives evaluated at infinite dilution of all solutes:

$$a_{ij} \equiv - \left(\frac{\partial S_i}{\partial R_j c_j} \right)_{R_k c_k (k \neq j)}, \quad (13a)$$

$$a_{ijk} \equiv - \frac{1}{2} \left(\frac{\partial^2 S_i}{\partial R_j c_j \partial R_k c_k} \right)_{R_l c_l (l \neq j, k)}. \quad (13b)$$

In order to solve equation (12) we assume that all a_{ij} have the same value, a_1 , and also that all a_{ijk} are the same (a_2):

$$S_i = S_{i,0} - a_1 n_c - a_2 n_c^2 - \dots \quad (13c)$$

As the final equation (20) for p shows, the dependence of D_i on concentration has little effect on the shape of the boundary. A linear relation of D_i to n_c is assumed here:

$$D_i = D_{i,0} - K n_c. \quad (13d)$$

An expression for $d\sigma^2/dt$. The definition of σ^2 is simply

$$\sigma^2 \equiv \mu_2 - (\mu_1)^2, \quad (14)$$

where μ_2 and μ_1 are defined in turn by equation (7a). The first moment, μ_1 , has been denoted by $\bar{x}$ in this series of articles:

$$\mu_1 \equiv \bar{x}. \quad (14a)$$

Differentiation of equation (14) with respect to time gives

$$\frac{d\sigma^2}{dt} = \frac{d\mu_2}{dt} - 2\bar{x} \frac{d\bar{x}}{dt}. \quad (15)$$

Substitution of equations (13c) and (13d) into equation (12) enables one to evaluate $d\mu_2/dt$ and $d\bar{x}/dt$ with the result that

$$\begin{aligned} \frac{d\sigma^2}{dt} = & 2\omega^2 \sigma^2 \sum_i R_i c_i^\beta S_i^\beta / n_c^\beta \\ & + 2\bar{x} \sum_i R_i \int_{x_0}^{x_w} x^{-1} D_i (\partial c_i / \partial x) dx / n_c^\beta \\ & + 2\omega^2 \bar{x} \sum_i R_i c_i^\beta S_{i,0} (\bar{x}_i - \bar{x}) / n_c^\beta - 2\omega^2 \bar{x} (2a_1 Q_1 + 3a_2 Q_2), \quad (16) \end{aligned}$$

where

$$\bar{x}_i \equiv \int_{x_0}^{x_w} x (\partial c_i / \partial x) dx / c_i^\beta, \quad (16a)$$

$$Q_1 \equiv \int_{x_0}^{x_w} (x - \bar{x}) n_c (\partial n_c / \partial x) dx / n_c^\beta, \quad (16b)$$

$$Q_2 \equiv \int_{x_0}^{x_w} (x - \bar{x}) n_c^2 (\partial n_c / \partial x) dx / n_c^\beta. \quad (16c)$$

Evaluation of the various summations in equation (16) (see Appendix) allows one to express $d\sigma^2/dt$ in such a way that it can be integrated with respect to time:

$$\begin{aligned} \frac{d\sigma^2}{dt} = & 2\omega^2 \sigma^2 (\bar{S}^\dagger + \dots) + 2\bar{D}_0 [1 + 4\omega^2 t (\bar{S}_0 - \bar{S}'_0) + \dots] \\ & - K n_c^0 + \dots + 2p^2 \omega^4 x_0^2 [1 + (\omega^2 t) [- (\frac{3}{2}) (q^3/p^2) \\ & + 2\bar{S}^\dagger] + \dots] + Q_{11} (-4\omega^2 \bar{x} a_1 + 2K/\bar{x}) - 6\omega^2 \bar{x} a_2 Q_2, \quad (17) \end{aligned}$$

$$\text{where} \quad \bar{S}^\dagger \equiv \bar{S}_0 - a_1 n_c^0 - a_2 (n_c^0)^2, \quad (17a)$$

$$n_c^0 \equiv \sum_i R_i c_i^0, \quad (17b)$$

$$\bar{D}_0 \equiv \sum_i \alpha_i D_{i,0}, \quad (17c)$$

$$\bar{S}'_0 \equiv \sum_i \alpha_i D_{i,0} S_{i,0} / \bar{D}_0, \quad (17d)$$

$$q^2 \equiv \sum_i \alpha_i (S_{i,0} - \bar{S}_0)^2. \quad (17e)$$

This equation can be compared with one previously derived for the case that S_i and D_i are independent of concentration (Baldwin, 1954*b*). When the expression for σ^2 given in that article (equation 23) is differentiated with respect to time and the result compared with the equation above, it may be seen that the two are identical once the terms of equation (17) in a_1 , a_2 and K are set equal to zero.

An equation for σ^2 . Since

$$\sigma^2 = \int_{t_0}^t \frac{d\sigma^2}{dt} dt + \sigma_0^2, \quad (18)$$

where σ_0^2 is the value of σ^2 at the time t_0 of the first photograph, integration of equation (17) gives

$$\begin{aligned} \sigma^2 = & \sigma_0^2 + 2\omega^2(\bar{S}^\dagger + \dots) \int_{t_0}^t \sigma^2 dt \\ & + 2\bar{D}_0[(t-t_0) + 2\omega^2(\bar{S}_0 - \bar{S}'_0)(t^2 - t_0^2) + \dots] \\ & - Kn_c^0(t-t_0) + \dots + p^2\omega^4x_0^2 \\ & \times \{(t^2 - t_0^2) + \omega^2(t^3 - t_0^3)[-(q^3/p^2) + (\frac{4}{3})(\bar{S}^\dagger)] + \dots\} \\ & - 2\omega^2 \int_{t_0}^t \bar{x}(2a_1Q_1 + 3a_2Q_2) dt + 2K \int_{t_0}^t (Q_1/\bar{x}) dt. \end{aligned} \quad (19)$$

The final equation for p , in terms of measurable quantities. By omitting from equation (19) the relatively minor terms (cf. Baldwin, 1954*b*) in $(\bar{S}_0 - \bar{S}'_0)$, q^3 and KQ_1 , and by defining the new variables B and $F(t)$, a simple equation is obtained for p , the standard deviation of the distribution of S :

$$p = [B/\omega^4x_0^2 F(t)]^{\frac{1}{2}}, \quad (20)$$

$$\begin{aligned} B \equiv & \sigma^2 - \sigma_0^2 - 2\omega^2\bar{S}^\dagger \int_{t_0}^t \sigma^2 dt - 2(\bar{D}_0 - \frac{1}{2}Kn_c^0)(t-t_0) \\ & + 2\omega^2 \int_{t_0}^t \bar{x}(2a_1Q_1 + 3a_2Q_2) dt, \end{aligned} \quad (20a)$$

$$F(t) \equiv (t^2 - t_0^2) + (\frac{4}{3})(\omega^2\bar{S}^\dagger)(t^3 - t_0^3). \quad (20b)$$

The plot of B against $F(t)$ is a straight line, with slope $p^2\omega^4x_0^2$, and is used to find p .

When it is not possible to make independent diffusion measurements, so that the value of $(\bar{D}_0 - \frac{1}{2}Kn_c^0)$ is unknown, equation (19) may be used in another way. We define σ_{corr}^2 by

$$\begin{aligned} \sigma_{\text{corr}}^2 \equiv & \sigma^2 - 2\omega^2\bar{S}^\dagger \int_{t_0}^t \sigma^2 dt \\ & + 2\omega^2 \int_{t_0}^t \bar{x}(2a_1Q_1 + 3a_2Q_2) dt, \end{aligned} \quad (21)$$

and plot σ_{corr}^2 against $(t-t_0)$. If the solute is homogeneous, the plot will be a straight line with slope $2(\bar{D}_0 - \frac{1}{2}Kn_c^0)$. If the solute is heterogeneous, the plot will have upward curvature.

Comparison with the case of a rectangular cell and constant field

In view of the numerous terms in equation (17), one might inquire if the approach to this problem is

unwieldy. The corresponding equation for the hypothetical case of a rectangular cell and constant field indicates that this is not the case. Equation (17) reduces, for this case, to

$$\begin{aligned} d\sigma^2/dt = & 2p^2\omega^4x_0^2 t \\ & + 2(\bar{D}_0 - \frac{1}{2}Kn_c^0) - 2(\omega^2x_0)(2a_1Q_1 + 3a_2Q_2), \end{aligned} \quad (22)$$

where the (constant) field strength has been denoted by ω^2x_0 . The equation could hardly be simpler, so that one must judge the number of terms in equation (17) to be a consequence of the experimental requirements of a sector-shaped cell and field strength, ω^2x , proportional to x .

METHODS OF CALCULATION

In order to calculate p from a given sedimentation experiment, the following groups of quantities are required. (i) Those which are computed from each schlieren photograph by numerical integration: $\bar{x}$, σ^2 , Q_1 , Q_2 and n_c^0 . (ii) Those which must be found from additional experiments: a_1 , a_2 and $(\bar{D}_0 - \frac{1}{2}Kn_c^0)$. (iii) Others. The angular speed of rotation, ω , and the time from the first photograph $(t-t_0)$ are found readily. Both t_0 and $\bar{S}^\dagger$ are found from equation (30), which relates the boundary position to time.

The schlieren curves produced by sedimentation of a protein and by the buffer in which it is dissolved are shown diagrammatically in Fig. 1*a* and *b* respectively. The latter schlieren curve (Fig. 1*b*) is termed the 'reference base line'; it is obtained under exactly the same conditions (the same cell, degree of filling, speed of rotation, time after reaching full speed and angle of the diagonal wire) as the former. The distance between two points in the cell, x_1 and x_2 , is magnified on the schlieren curve by a factor F :

$$X_2 - X_1 = F(x_2 - x_1), \quad (23)$$

where X is the co-ordinate axis on the photographic plate which corresponds to the direction of sedimentation in the cell.

The other co-ordinate of the schlieren curve, Z , is linearly related to $\partial n/\partial x$:

$$Z = Gab \cot \psi [\partial n/\partial x + f(x)]. \quad (24)$$

In equation (24) $f(x)$ expresses the prismatic deflexion of the schlieren curve caused by deformation of the cell windows: cf. Svedberg & Pedersen (1940, p. 268). (It is a function not only of x but of the particular cell, the degree of tightening of the guard rings, and the density and refractive index of the solution.) Denoting Z in the reference baseline experiment by Z_0 , we have

$$Z_0 = Gab \cot \psi [\partial n_0/\partial x + f_0(x)]. \quad (24a)$$

If $f(x)$ can be set equal to $f_0(x)$ (cf. Ogston & Woods, 1954), then

$$Z - Z_0 \equiv Z_c = Gab \cot \psi (\partial n_c/\partial x). \quad (24b)$$

Measurement of the cell thickness, a , the optical lever arm, b , and the magnification factors F and G , is discussed by Longworth (1946) and Cecil & Ogston (1948). In equation (24) the angle of the diagonal wire, ψ , is measured from a line parallel to the slit image at the diagonal wire.

By means of a comparator, values of (Z, X) are read from the photographic plate at a fixed interval ΔX so chosen that there will be twenty or so readings taken in the boundary region. The stage of the comparator is free to travel in two directions, at right angles to each other. In order to align the photographic plate on the stage, the meniscus trace is set parallel to one axis of the comparator because this trace represents an infinite gradient of refractive index and is thus parallel to the Z axis. Since $\partial n_c/\partial x$ is expected to be zero in the region of constant concentration ahead of the boundary (the β region of Fig. 1), the origin for Z_c is found by superimposing the reference baseline (Fig. 1b) and the schlieren curve of Fig. 1a in the β region.

In view of the theoretical (Kegeles & Gosting, 1947) and practical uncertainties of measuring $\partial n_c/\partial x$ (approx. 1%), the method of numerical integration known as trapezoidal integration is

sufficiently accurate to find $\bar{x}$, σ^2 , Q_1 and Q_2 and n_c^0 . Given a set of n values of y ($y_1, y_2, \dots, y_n$), where y is a continuous function of z and the values of y are spaced at the constant interval Δz , the integral $\int y dz$, evaluated between the limits of z at y_1 and y_n , is approximated by

$$\int y dz = \Delta z \left\{ \sum_{i=2}^{n-1} y_i + \frac{1}{2}(y_1 + y_n) \right\}. \quad (25a)$$

If $y_1 = y_n = 0$, this reduces to

$$\int y dz = \Delta z \sum y_i. \quad (25b)$$

The calculations are simplified by choosing an arbitrary origin X' from one of the set of readings (X, Z) and by writing $X - X'$ as $h\Delta X$. Thus h takes the values 1, 2, 3, ..., to the right of X' and -1, -2, -3, ..., to the left of X' . The position in the cell, x' , corresponding to X' on the photographic plate is

$$x' = x_i + (X' - X_i)/F, \quad (26)$$

where x_i is the distance from the centre of rotation to an edge of the index hole in the balance cell, and X_i marks this edge of the index trace on the photographic plate. Then the following equations are obtained from equations (25a) and (25b) for $\bar{x}$, σ^2 , etc.:

$$\bar{x} = x' + \left(\frac{\Delta X}{F} \right) \frac{\sum h Z_c}{\sum Z_c}, \quad (27a)$$

$$\sigma^2 = \left(\frac{\Delta X}{F} \right)^2 \frac{\sum h^2 Z_c}{\sum Z_c} - (x' - \bar{x})^2, \quad (27b)$$

$$Q_1 = \left(\frac{\Delta X}{F} \right) \frac{\sum h n_c Z_c}{\sum Z_c} + \frac{(x' - \bar{x})}{2} n_c^0, \quad (27c)$$

$$Q_2 = \left(\frac{\Delta X}{F} \right) \frac{\sum h n_c^2 Z_c}{\sum Z_c} + \frac{(x' - \bar{x})}{3} (n_c^0)^2, \quad (27d)$$

$$n_c^0 = \frac{\Delta X}{F G a b \cot \psi} \sum Z_c, \quad (27e)$$

where the value of n_c in equations (27c) and (27d) for the j th set of readings of (X, Z) is given by

$$(n_c)_j = \frac{\Delta X}{F G a b \cot \psi} \left\{ \sum_{i=2}^{j-1} (Z_c)_i + \frac{1}{2}(Z_c)_1 + \frac{1}{2}(Z_c)_j \right\}. \quad (27f)$$

In order to see how accurately σ^2 , n_c^0 and Q_1 are given by this kind of numerical integration with a limited set of $\partial n_c/\partial x$ values, they were calculated from tabulated values of the Gaussian function, or probability integral. It was assumed that values of $\partial n_c/\partial x$ less than 0.5% of the maximum gradient would not be detected experimentally, and so these were omitted from the calculations. Taking seventeen values of $\partial n_c/\partial x$ spaced throughout the

Fig. 1. Diagrams of the schlieren curves observed during sedimentation (a) of a protein solution and (b) of the buffer in which the protein is dissolved. Sedimentation is from left to right. The position of the meniscus trace is labelled X_0 and the edge of an index trace is marked X_I .

gradient curve (including the maximum gradient and eight values on each side of the maximum) gave values for σ^2 , n_c^0 and Q_1 which were 0.8, 0.06 and 0.4% too low respectively. (For a Gaussian curve, Q_1 is given by $\sigma n_c^0/2\sqrt{\pi}$.) Increasing the number of $\partial n_c/\partial x$ values to 35 reduced the error very little; the calculated values were then 0.7, 0.05 and 0.3% too low. At present, the uncertainty introduced by this use of numerical integration is not a limiting source of error.

In order to check the experimental accuracy of measurements of $\partial n_c/\partial x$, n_c^0 also is calculated from each photograph and then values from successive photographs compared. According to Trautman & Schumaker (1954)

$$n_c^0 = \int_{x_0}^{x_w} x^2 (\partial n_c/\partial x) dx/x_0^2, \quad (28a)$$

and thus (cf. equation 7a)

$$n_c^0 = n_c^0 (\mu_2/x_0^2) = n_c^0 (\bar{x}^2 + \sigma^2)/x_0^2, \quad (28b)$$

where σ^2 is only of the order of 0.05% of $\bar{x}^2$. Another check on the accuracy of $\partial n_c/\partial x$ values is the comparison of values for n_c^0 found from the sedimentation experiment with $(n_c^0)_{D.R.}$, the value found by a differential refractometer.

To obtain $\bar{D}_0 - \frac{1}{2}Kn_c^0$, a free diffusion experiment is carried out in which the mean concentration is equal to half the initial concentration of the sedimentation experiment. Then this average diffusion coefficient is calculated from $\sigma^2/2t$, where the definition of σ^2 is still that given in equations (14) and (7) (cf. Gralén, 1944). To convert the diffusion coefficient to the temperature and buffer of the sedimentation experiment one may use the approximate relation (cf. Longworth, 1954)

$$\eta D/T = \text{constant}, \quad (29)$$

where η is the solvent viscosity and T is the absolute temperature.

The measurement of sedimentation coefficients is discussed in the next paper and will not be considered in detail here except to point out differences between the calculations for a homogeneous and for a heterogeneous system. Equation (A 10) of the appendix may be written (cf. Goldberg, 1953)

$$\ln (\mu_2/x_0^2)^{\frac{1}{2}} - (\omega^2 t)^2 (\bar{S}^{\dagger}) [a_1 n_c^0 + 2a_2 (n_c^0)^2 - (p^2/\bar{S}^{\dagger})] + \dots = \bar{S}^{\dagger} \omega^2 t. \quad (30)$$

Thus to calculate $\bar{S}^{\dagger}$ from a given experiment, one needs preliminary estimates of a_1 , a_2 , p and t_0 . Since the term in $(\omega^2 t)^2$ is usually not very large, these preliminary estimates need not be accurate. Then the left-hand side of equation (30) is plotted against $t - t_0$, the time from the first photograph; the slope gives $\bar{S}^{\dagger} \omega^2$ and the intercept, when the left-hand side = 0, gives $\int_0^{t_0} \omega^2 dt$. The value of t_0 required for equation (20b) is given by the ratio of

this integral to the value of ω^2 at full speed. (Another way of plotting this kind of equation is described in the next paper.) The value of $\bar{S}^{\dagger}$ is converted to standard conditions by the approximate relation

$$S\eta/(1 - \bar{v}\rho) = \text{constant}, \quad (31)$$

where $\bar{v}$ is the solute's partial specific volume and ρ is the density of the solution. Finally the plot of $\bar{S}^{\dagger}$ against n_c^0 yields values for a_1 and a_2 and these are compared with the preliminary estimates used in calculating $\bar{S}^{\dagger}$, to determine whether recalculation is necessary.

When the optical constants a , b and G are unknown, the following procedure can be used to evaluate $a_1 Q_1$ and $a_2 Q_2$. From equations (27c-f) and (28b), $Gab Q_1$, $(Gab)^2 Q_2$ and $Gab n_c^0$ can be calculated. The plot of $\bar{S}_2$ against C^0 , where C^0 is the initial total concentration,

$$C^0 = \Sigma c_i^0 \quad (32)$$

yields $a_1 \bar{R}$ and $a_2 \bar{R}^2$, the definition of $\bar{R}$ being

$$\bar{R} \equiv n_c^0/C^0 \quad (33)$$

(cf. equation 17b). Then

$$a_1 Q_1 = (a_1 \bar{R}) (Gab Q_1) (C^0)/(Gab n_c^0), \quad (34a)$$

$$a_2 Q_2 = (a_2 \bar{R}^2) [(Gab)^2 Q_2] [C^0/Gab n_c^0]^2, \quad (34b)$$

Next $\int \sigma^2 dt$ and $\int \bar{x}(2a_1 Q_1 + 3a_2 Q_2) dt$ are found by equation (25a) with Δt for Δz and, for y , σ^2 and $\bar{x}(2a_1 Q_1 + 3a_2 Q_2)$ respectively. At this point all the quantities required to calculate B and $F(t)$ (equations 20a and b) are known. Note that equation (20a) calls for values of a_1 , a_2 , $\bar{S}^{\dagger}$ and $\bar{D}_0 - \frac{1}{2}Kn_c^0$ for the solvent and temperature of the sedimentation experiment. Finally p^2 is found from the plot of B against $F(t)$ and then p is computed and converted to standard conditions by equation 31.

EXPERIMENTAL

Materials. Two samples of Armour crystalline bovine plasma albumin were investigated. Lot no. 10 522 was examined in a buffer containing 0.15 M-NaCl, 0.03 M total acetate (acetic acid and Na acetate), pH 4.34 (glass electrode, 20°). The buffer used in studying Lot no. R 370 295 A contained 0.10 M-KCl, 0.01 M acetic acid and 0.01 M-Na acetate; its pH (glass electrode, 25°) was 4.55. The sample of bovine β -lactoglobulin, prepared by the modified (Smithies, 1953) method of Cecil & Ogston (1949), was the gift of Dr J. M. A. Tilley. This sample was studied in the same buffer (0.1 M-NaCl, 0.1 M-Na acetate, 0.04 M acetic acid) used by Cecil & Ogston (1949) in their study of the sedimentation and diffusion of β -lactoglobulin.

Methods. Calibration of the Spinco ultracentrifuge, used in studying albumin sample no. R 370 295 A, is described in the following article. The Oxford oil-turbine ultracentrifuge, used in studying the lactoglobulin and the other albumin sample, also has been described elsewhere: Cecil & Ogston (1948) and Baldwin (1953b). The diagonal bar-schlieren system was focused under running conditions. A mono-

chromator isolates the green line of a 250 w, box-type air-cooled mercury lamp. The rotor speed is measured by an apparatus designed and built by Dr R. Cecil which records the revolutions of a phonic motor. Since one shaft of the rotor is magnetized, there is a current generated as the rotor turns and this current is amplified and used to drive the phonic motor. The rotor thermocouple was calibrated with diphenyl ether in the manner described by Cecil & Ogston (1948).

Refractive increments were measured, after dialysis, with the differential refractometer of Cecil & Ogston (1951); a diffraction pattern produced by a single slit was used to measure the deflections.

RESULTS AND DISCUSSION

β-Lactoglobulin. No measurable heterogeneity was found. The values of B , and hence of p (equation 20), did not differ significantly from zero; the largest value was only 1% of $(\sigma^2 - \sigma_0^2)$. Consequently the plot of σ_{corr}^2 (equation 21) against $(t - t_0)$ should be a straight line with slope $2(\bar{D}_0 - \frac{1}{2}Kn_c^0)$. This plot is shown in Fig. 2. The value of D computed from its slope may be compared with the diffusion measurements of Cecil & Ogston (1949); interpolation of their data gives a value for $D_{20, w}$ of $7.7_2 \times 10^{-7}$ cm.² sec.⁻¹ at a mean concentration of 0.4 g./100 ml. From the slope of Fig. 2 a value for $D_{20, w}$ of $7.7_6 \times 10^{-7}$ cm.² sec.⁻¹ is obtained when equation (29) is used to convert the value of D from the temperature of the sedimentation experiment (28.2°) to that quoted by Cecil & Ogston. This agreement is somewhat fortuitous, since the experimental uncertainty of measuring D from a sedi-

mentation experiment is about 5% (cf. the next paper).

It would be interesting to know whether this sample contained one or both of the components observed by Aschaffenburg & Drewry (1955) on filter-paper electrophoresis. Unfortunately, none of this sample remains. In the light of the work of Ogston & Tilley (1955) and of Aschaffenburg & Drewry (1955), it is to be expected that different samples of β -lactoglobulin will show different behaviour. Cecil & Ogston (1949) found evidence for a rapidly diffusing impurity in certain β -lactoglobulin samples, and Creeth (personal communication) has also found this, in an amount which should be detectable in sedimentation experiments. If one takes a value of B equal to 5% of

$$2(\bar{D}_0 - \frac{1}{2}Kn_c^0)(t - t_0)$$

as the least significant evidence of heterogeneity in sedimentation behaviour, then the least measurable value of p for this sedimentation experiment is computed to be 5% of $\bar{S}_0$. If there were two components present in equal amount, they would have to differ in S_0 by 10% of $\bar{S}_0$ or less (cf. equation 35b) with this value of p .

In deriving the theory given here, chemically reacting systems were excluded. Consequently it is important to note that Ogston & Tilley (1955) found that β -lactoglobulin associates at low temperatures. However, they concluded that association is negligible above 20°.

In general, the boundary-gradient curves produced by sedimentation of a homogeneous protein will not be Gaussian in shape because of the dependence of S on c (Fujita, 1956). However, the concentration dependence shown by β -lactoglobulin is so slight (Cecil & Ogston, 1949) that at the concentration studied here ($c^0 = 0.78$ g./100 ml.) the boundary-gradient curves were found to be Gaussian. The plot of $\log \partial n_c / \partial x$ against $(x - x_R)^2$ was linear on both sides of x_R (the position of the maximum gradient) and values of σ calculated by equation (27b) agreed with those from the equation for a Gaussian curve within experimental error. (For a Gaussian curve, $\sigma = A/H\sqrt{2\pi}$, where A is the area and H the maximum height of the boundary-gradient curve.) The greatest difference between the two values of σ , in six photographs, was 2.1%; the mean difference was 1.2%. Consequently values of σ were taken from A and H , since these are more precise than the values of σ calculated by (27b), and also Q_1 was calculated from the equation for a Gaussian curve ($Q_1 = \sigma n_c^0 / 2\sqrt{\pi}$). The value for $a_{1(25, w)}$ was calculated to be $0.055 \times 10^{-13} / \bar{R}$, from the data of Cecil & Ogston (1949). From the results of Perlmann & Longworth (1948), 1.86×10^{-3} was computed for $\bar{R}$ at 25° and the mercury green line. The areas under six successive boundary-gradient

Fig. 2. Plot of σ_{corr}^2 (equation 21) against the time from the first photograph, for a sample of lactoglobulin. When a solute displays no heterogeneity in sedimentation behaviour, this plot is a straight line with slope equal to twice the diffusion coefficient.

curves were used to calculate n_c^0 (equation 28b) for comparison with $(n_c^0)_{D.R.}$, the value found with a differential refractometer (D.R.). The average value of the ratio $(n_c^0)_{D.R.}/(n_c^0)_{U.C.}$ (U.C., ultracentrifuge) was found to be 0.999, with a standard error of 0.009.

Bovine plasma albumin. Different values of p (Table 1) were found for the two different samples of albumin, although both samples are crystalline and were obtained from Armour and Co. For sample 10522,

$$p_{25,w} = 1.08 \pm 0.09s,$$

while for sample R 370 295A

$$p_{25,w} = 0.69 \pm 0.02s$$

(s = Svedberg units). However, sample 10522 was studied at a somewhat lower pH (4.34) than that used with R 370 295A (pH 4.55) and dependence on pH of various physical properties of albumin has been reported by several workers: cf. Tanford, Buzzell, Rands & Swanson (1955) and Harrington, Johnson & Ottewill (1956).

In view of these different values for p , it is of interest to compare other properties of the two samples which are related to their heterogeneity. The ratio of the mean sedimentation coefficient to that of the main component (Table 2) is not significantly different for the two samples. In the next paper, measurements are discussed which allow one to estimate the amount of a faster-sedimenting component in both samples; the fractional amount, α_2 , of this component was not found to be significantly different in the two samples. By use of Fujita's (1956) solution to the continuity equation, the homogeneity of the main component was investigated for both samples and is reported in the next paper. Here again the two samples differed: the main component of R 370 295A showed no measurable heterogeneity, whereas that of 10522 did show heterogeneity.

In order to see how well the values of p and $\bar{S}_0/S_{1,0}$ can be interpreted in terms of only two

solutes (1 and 2), equations were developed which relate each of these quantities to

$$\frac{(S_{2,0} - S_{1,0})/S_{1,0}}{S_{1,0}} = \frac{(\bar{S}_0/S_{1,0}) - 1}{\alpha_2}, \quad (35a)$$

$$\frac{S_{2,0} - S_{1,0}}{S_{1,0}} = \frac{p}{S_{1,0}(\alpha_1 \alpha_2)^{\frac{1}{2}}}. \quad (35b)$$

With sample R 370 295A, both of these equations give the same value for $(S_{2,0} - S_{1,0})/S_{1,0}$ within experimental error. Taking $\bar{S}_0/S_{1,0}$ as 1.040 ± 0.005 and α_2 as 0.12 ± 0.02 gives

$$(S_{2,0} - S_{1,0})/S_{1,0} = 0.33 \pm 0.10.$$

Taking p as $0.69 \pm 0.02s$ and $S_{1,0}$ as $5.01s$ gives

$$(S_{2,0} - S_{1,0})/S_{1,0} = 0.42 \pm 0.05.$$

Taken together, these lead to an estimate for $(S_{2,0})_{25,w}$ of $7.0 \pm 0.3s$. For sample 10522, the estimate of $\bar{S}_0/S_{1,0}$ is much more uncertain so that it is not possible to say whether the higher value of p can be explained by a higher value of $S_{2,0}$ or by heterogeneity of the leading component. It can be shown from equation (6a) that the heterogeneity of the main component, which is discussed in the next paper, contributes only 5% to the overall value of p . The heterogeneity in diffusion behaviour found by Gosting (reported by Baldwin *et al.* 1955b) with different samples of Armour crystalline bovine albumin could be interpreted in terms of only two solutes.

In Expts. A1, A2, A3 and B1, S was treated as a linear function of n_c with a_1 (equation 13c) equal to $0.057 \bar{S}_0/\bar{R}$; for the Expt. B2 at the higher concentration, $a_1 = 0.054 \bar{S}_0/\bar{R}$ and $a_2 = 0.004 \bar{S}_0/\bar{R}^2$. These values are taken from the dependence on concentration of the sedimentation coefficient of the main component (see next paper). From the results of Perlmann & Longworth (1948) one can compute a value of 1.92×10^{-3} for $\bar{R}$ at 25° , $0.1M$ -NaCl and the mercury green line. The value taken for $\bar{D}_{25,w}$ ($6.73 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$) is that found by Gosting (Baldwin *et al.* 1955b) for sample R 370 295A at a mean concentration of 0.515 g./100 ml. According to Creeth (1952), the dependence of D on c is very small (at pH 6.8). The temperatures of the sedimentation experiments were as follows: A1, 26.8° ; A2, 27.0° ; A3, 27.3° ; B1, 24.5° ; B2, 25.1° .

Methods of checking the calculation of p

In computing p the first problem is to check on the measurements of $\partial n_c / \partial x$. The results in Table 2 show that these values of $\partial n_c / \partial x$ are reasonably precise, as judged by the agreement of values for n_c^0 from successive photographs. The solutions used for the sedimentation experiments had been dialysed and a differential refractometer (Cecil & Ogston, 1951) was used to measure the difference in

Table 1. *Heterogeneity of two samples of crystalline bovine albumin*

Expt. no. ^a	C^{0b}	$p_{25,w} (\pm \text{S.D.}) (s)^c$
A1	0.61	1.13 ± 0.03
A2	0.61	1.09 ± 0.01
A3	0.61	0.96 ± 0.07
B1	0.67	0.68 ± 0.02
B2	1.35	0.71 ± 0.02

^a Expts. A1, A2 and A3 were carried out with sample no. 10522 and with an oil-turbine ultracentrifuge. In Expts. B1 and B2, sample no. R 370 295A was studied in a Spinco ultracentrifuge.

^b Initial concentration of the experiment in g./100 ml.

^c The standard deviation of the sample's distribution of sedimentation coefficient, followed by the standard error of measurement, in Svedberg units.

refractive index between solvent and solution. Table 2 shows that the values obtained with the differential refractometer agree with the values computed from the areas under the schlieren diagrams of the sedimentation experiments. This agreement means that the method of placing base lines is satisfactory provided that one can assume that (i) there are no large aggregates in the sample which sediment immediately to the bottom of the cell and do not contribute to the boundary curve, and (ii) that any differences in concentration of buffer constituents on the two sides of the dialysis membrane also occur on the two sides of the sedimenting boundary.

A small error in positioning the base line on the Z axis has a relatively large effect on the value for σ^2 . This is shown in Table 3, where the values of σ^2 have been recalculated (and then denoted by σ'^2) after shifting the position of the base line on the Z axis enough to make the value of n_0^0 from each photograph agree with the value found by the differential refractometer, $(n_0^0)_{D.R.}$. This means that the variability of values for n_0^0 from successive schlieren photographs should be a fairly good guide to the reliability of the experiment. If these values do not show reasonable agreement, it will prove more profitable to find out why than to proceed further in computing p .

The standard error of a value for p is a fairly good guide to the reproducibility of the experiment, as the results of A 1, A 2 and A 3 show (Table 1). These experiments were carried out with the same initial solution of bovine albumin in order to test this. The values for p are reproducible within twice the standard error or less. [Mather (1946) discusses computation of the standard error when the slope of a straight line is being found.]

The contribution to B of the term involving the dependence of S on c can be varied by varying the initial concentration of the experiment. Thus in Expt. B 1, where $C^0 = 0.67$ g./100 ml., the final value of the integral involving a_1 and a_2 is 26 % of the

corresponding value of B (cf. Table 4). In B 2, where $C^0 = 1.35$ g./100 ml., this integral is 44 % of the final value of B . The values of p from the two experiments agree within 4 %, or roughly the sum of their standard errors. In the previous theory for computing p (Williams *et al.* 1952; Baldwin, 1954*b*),

Table 2. *Other data from the experiments with albumin*

Expt. no.	$(n_0^0)_{D.R.}/(n_0^0)_{U.C.}$ ($\pm$ S.D.) ^a	$\bar{S}_0/S_{1,0}$ ($\pm$ S.D.) ^b
A 1	0.985 $\pm$ 0.010	1.047 $\pm$ 0.012
A 2	1.003 $\pm$ 0.010	1.047 $\pm$ 0.012
A 3	0.986 $\pm$ 0.020	1.017 $\pm$ 0.016
B 1	1 $\pm$ 0.012 ^c	1.038 $\pm$ 0.005
B 2	1 $\pm$ 0.006 ^c	1.041 $\pm$ 0.005

^a Refractive increment of the albumin in the initial solution, as measured by a differential refractometer, divided by the mean value calculated from the areas under the boundary-gradient curves of the ultracentrifuge experiment. The standard error gives the variation of $(n_0^0)_{U.C.}$ values about the mean.

^b Ratio of the mean sedimentation coefficient to that of the main component, at infinite dilution.

^c The value of $(n_0^0)_{D.R.}$ was not determined; the standard error of $(n_0^0)_{U.C.}$ is given.

Table 3. *Effect of an error in positioning the reference base line on values for σ^2*

Photo no. ^a	$(n_0^0)_{D.R.}/(n_0^0)_{U.C.}$ ^b	σ'^2/σ^2 ^c
1	0.989	0.999
2	0.960	0.850
3	0.977	0.900
4	0.992	0.966
5	1.014	1.053

^a Expt. A 3.

^b Ratio of values for the refractive increment of the protein in the initial solution as found with a differential refractometer and from the area of the boundary-gradient curve of the ultracentrifugal experiment.

^c Ratio of the second moment about the mean of the boundary-gradient curve after (σ'^2) and before (σ^2) adding a constant amount to each value of $\partial n_c/\partial x$ such that $(n_0^0)_{U.C.}$ then agrees exactly with $(n_0^0)_{D.R.}$.

Table 4. *An example of the relative importance of the various quantities from which p is computed*

Photo no. ^a	$\sigma^2 - \sigma_0^2$	$2\omega^2 \bar{S}^\dagger \int_{t_0}^t \sigma^2 dt$	$2\bar{D}(t - t_0)$	$4\omega^2 a_1 \int_{t_0}^t \bar{x} Q_1 dt^b$	B^c
1	0	0	0	0	0
2	0.95	0.05	0.66	0.12	0.36
3	1.94	0.11	1.31	0.25	0.77
4	2.88	0.20	1.96	0.41	1.13
5	4.54	0.30	2.62	0.57	2.18
6	5.83	0.44	3.27	0.76	2.88

^a Expt. B 1: BPA, lot no. R 370 295 A, $C^0 = 0.67$ g./100 ml. All values have been multiplied by 10^3 and have units of cm^2 .

^b In general, this integral contains a term in a_2 , but $a_2 = 0$ in this case.

^c B is defined by equation 20*a* as $(\sigma^2 - \sigma_0^2) - 2\omega^2 \bar{S}^\dagger \int_{t_0}^t \sigma^2 dt - 2\bar{D}(t - t_0) + 2\omega^2 \int_{t_0}^t \bar{x}(2a_1 Q_1 + 3a_2 Q_2) dt$.

^d The plot of B against $F(t)$ (defined by equation (20*b*)), is a straight line with slope $p^2 \omega^2 x_0^2$, p being the standard deviation of the distribution of sedimentation coefficient.

the dependence of S on c was not considered. The effect of omitting this factor may be seen in Fig. 3, where values of B have been computed by the present theory and also by the older theory, for Expt. B2. It may be seen that the dependence of S on c is a significant factor in the calculation of p .

Applicability of the theory

In using this method to find p , the following considerations should be kept in mind. (i) No account has been taken of any dependence on pressure of S , D and R . This is not likely to be a serious source of error for aqueous solutions but might be when organic solvents are used. (ii) For systems which are both strongly concentration dependent and markedly heterogeneous, the representation of the dependence of S_i on n_c in equation (13c) will be inadequate when the initial concentration is more than a certain value. For the purpose of these calculations, equation (13c) will be considered satisfactory so long as any error introduced into B by the use of this equation is less than the experimental uncertainty of σ^2 . Provided that $\bar{S}^+/\bar{S}_0 > 0.9$,

Fig. 3. Plot of B against $F(t)$ for sample R370295A of crystalline bovine plasma albumin at an initial concentration of 1.35 g./100 ml. The points shown by filled circles have been calculated by equation (20); the open circles show the same data calculated by the older theory, without correction for the dependence of sedimentation coefficients on concentration. The slope of this plot (through the filled circles) is $p^2\omega^4x_0^2$, p being the standard deviation of the sample's distribution of sedimentation coefficient.

this should not be a serious source of error. And, of course, it is not a source of error for a homogeneous system. (iii) If chemically reacting systems were to be considered, additional terms would have to be added to the continuity equation (10). (iv) The flow equation (11) does not include transport by convection, and it has been shown by Harrington & Schachman (1953) (see also Trautman, Schumaker, Harrington & Schachman, 1954) that this occurs in systems of two solutes when the boundaries separate completely from one another. (In the experiments reported here with bovine albumin, one continuous boundary-gradient curve was observed.) Also a more complicated flow equation would be required if there were significant transport caused by an electrical potential gradient (cf. Svedberg & Pedersen, 1940, p. 23). (v) The requirement that all $\partial c_i/\partial x = 0$ at x_0 (e.g. equations 7b and 12) excludes systems where S is so small, or D so large, that the boundary-gradient curve does not resolve from the meniscus.

On the other hand, the following factors have been taken into account: (i) heterogeneity in S , D and R ; (ii) dependence on total concentration of S and D ; (iii) the fact that the rotor does not achieve full speed instantaneously and also the initial period of restricted diffusion. Equations (17) and (19) apply once the boundary region has resolved from the meniscus, regardless of previous history. In computing B (equation 20a) it is not necessary to know the time from the beginning of diffusion but only the time $(t-t_0)$ from the first photograph. In computing $F(t)$ (equation 20b) it is necessary to know the integral $\int_0^{t_0} \omega^2 dt$ as well, but this is readily obtained by use of equation (30).

Use of moments contrasted with solution of the continuity equation

In general, solution of the continuity equation (10) to give the shape of the boundary-gradient curves gives more information than does an equation for the moments of these curves. However, there are formidable mathematical difficulties in the way of solving the continuity equation for the shape of the boundary-gradient curve for the case presented here, in which the distribution of S is left unspecified and the flow equation for an individual solute includes a dependence of S on total concentration. The case of only two such concentration-dependent solutes has not yet been solved and the solution for a single solute (Fujita, 1956) is a major achievement.

Since Fujita's equation is limited, strictly, to the case of only one solute, it can be used to detect heterogeneity but not to measure it. Consequently it is most useful, from the standpoint of studying

heterogeneity, with systems which are on the borderline of detectable heterogeneity. With his equation, one can make use of the area and maximum height of the boundary-gradient curves and these can be measured more accurately than can σ . Also, the calculations required for use of Fujita's theory are less laborious than the ones required here. In the next paper, it was found possible to obtain some information about the homogeneity of a main component, when a small amount of another component is present. The theory given here cannot cope with a task such as this.

The chief use envisaged for this theory is in characterizing the heterogeneity of systems which are not sufficiently heterogeneous, or which diffuse too rapidly, for the distribution of S to be obtained by extrapolation to infinite time (cf. Baldwin, 1954b). Also, for systems in which $g(S)$ can be found by extrapolation to infinite time, it will be a valuable check on the accuracy of the extrapolated distribution to compute p both by this method and from $g(S)$ itself.

SUMMARY

1. A theory is developed for computing p , the standard deviation of the distribution of sedimentation coefficient in a sample containing macro-

molecular solutes. No assumption as to the form of the distribution is required.

2. The theory takes into account dependence of the sedimentation and diffusion coefficients upon the total concentration.

3. A sample of bovine β -lactoglobulin, prepared by the modified method of Cecil & Ogston (1948), showed no measurable heterogeneity in sedimentation behaviour. The resolving power of the method is sufficient to detect a value of p equal to 5% of the mean sedimentation coefficient, despite present limitations on the accuracy of measuring boundary-gradient curves.

4. Two different samples of Armour crystalline bovine plasma albumin were found to have different values of p .

5. An equation is derived also for computing the mean sedimentation coefficient of a mixture, when the individual sedimentation coefficients depend on the total concentration.

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APPENDIX

Evaluation by expansion into series of the terms in equation (16). In order to find expressions for c_i^p and S_i^p , the concentration and sedimentation coefficient of i in the uniform region ahead of the boundary, the following procedure may be used (cf. Alberty, 1954). Let

$$S_i^p = S_i^* + bt + \dots, \quad (\text{A } 1)$$

$$\text{where } S_i^* \equiv S_{i,0} - a_1 n_c^0 - a_2 (n_c^0)^2 \quad (\text{A } 1a)$$

and b is to be determined. Since all $\partial c_i / \partial x = 0$ in this region, substitution of equation (11) into (10) gives

$$\frac{dc_i^p}{dt} = -2c_i^p S_i^p \omega^2. \quad (\text{A } 2)$$

Integration of equation (A 2), after substituting equation (A 1), yields

$$c_i^p = c_i^0 \exp(-2\omega^2 S_i^* t + \dots), \quad (\text{A } 3)$$

$$\text{or } c_i^p = c_i^0 (1 - 2\omega^2 S_i^* t + \dots). \quad (\text{A } 3a)$$

$$\text{Thus } n_c^p = \sum_i R_i c_i^0 (1 - 2\omega^2 S_i^* t + \dots) \\ = n_c^0 (1 - 2\omega^2 \bar{S}^* t + \dots). \quad (\text{A } 4)$$

[For the definition of $\bar{S}^*$ and other symbols in this section, see equations (16) and (17).]

Substitution of equation (A 4) into equation (13c) and comparison of the resulting equation with (A 1) shows that

$$b = 2\omega^2 \bar{S}^* [a_1 n_c^0 + 2a_2 (n_c^0)^2]. \quad (\text{A } 5)$$

Thus, using equation (A 1) for S_i^p and (A 3a) for c_i^p , one finds that the first term on the right-hand side of equation (16) is

$$\sum_i R_i c_i^p S_i^p / n_c^p = \bar{S}^* \{1 + 2(\omega^2 t) [a_1 n_c^0 + 2a_2 (n_c^0)^2 - (p^2 / \bar{S}^*)] + \dots\}. \quad (\text{A } 6)$$

Turning to the next term in equation (16), we first expand $(1/x)$ as

$$\frac{1}{x} = \frac{1}{\bar{x}} \left[1 - \left(\frac{x - \bar{x}}{\bar{x}} \right) + \dots \right], \quad (\text{A } 7)$$

and so

$$2\bar{x} \sum_i R_i \int_{x_0}^{x_w} x^{-1} D_i (\partial c_i / \partial x) dx / n_c^p = 2 \sum_i R_i D_{i,0} c_i^p / n_c^p \\ - K n_c^p - 2 \sum_i R_i D_{i,0} c_i^p (\bar{x}_i - \bar{x}) / \bar{x} n_c^p + 2K Q_1 / \bar{x} + \dots \quad (\text{A } 8)$$

To obtain an expression for $(\bar{x}_i - \bar{x})$, one may write (cf. equation 14) that

$$\bar{x} = (\mu_2)^{1/2} [1 - (\sigma^2 / 2\mu_2) + \dots]. \quad (\text{A } 9)$$

Substitution of equation (A 6) into equation (12) for $r = 2$ yields, after integration,

$$\mu_2 = x_0^2 \exp. \bar{S}^\dagger \{ 2\omega^2 t + 2(\omega^2 t)^2 [a_1 n_0^0 + 2a_2(n_0^0)^2 - (p^2/\bar{S}^\dagger)] + \dots \}. \quad (\text{A } 10)$$

Provided that the concentration-dependence terms are small enough for σ^2 to be approximated in equation (A 9) by the first terms of the non-concentration-dependent case (Williams *et al.* 1952; Baldwin, 1954*b*), one obtains for equation (A 9)

$$\bar{x} = [x_0 \exp S^\dagger \{ \omega^2 t + (\omega^2 t)^2 [a_1 n_0^0 + 2a_2(n_0^0)^2 - (p^2/\bar{S}^\dagger)] + \dots \} - x_0(p\omega^2 t)^2 (1 + \dots)/2 - \bar{D}_0 t (1 + \dots)/x_0]. \quad (\text{A } 11)$$

Similarly,

$$\bar{x}_i = \{x_0 \exp [\bar{S}^\dagger \omega^2 t + \frac{1}{2} b \omega^2 t^2 + \dots] - D_{i,0} t (1 + \dots)/x_0\}. \quad (\text{A } 12)$$

Thus

$$(\bar{x}_i - \bar{x}) = \{x_0 \exp (\bar{S}^\dagger \omega^2 t) \{ (S_{i,0} - \bar{S}_0) (\omega^2 t) + (\omega^2 t)^2 [\frac{3}{2} p^2 + \frac{1}{2} (S_{i,0} - \bar{S}_0)^2] + \dots \} + (\bar{D}_0 - D_{i,0}) (t/x_0) + \dots \}. \quad (\text{A } 13)$$

With this result, one can now evaluate equation (A 8):

$$2\bar{x} \sum_i R_i \int_{x_0}^{x_w} x^{-1} D_i (\partial c_i / \partial x) dx / n_0^\beta = 2\bar{D}_0 [1 + 3(\omega^2 t) (\bar{S}_0 - \bar{S}_0') + \dots] - K n_0^0 + 2K Q_1 / \bar{x} + \dots \quad (\text{A } 14)$$

The information required to evaluate the third term on the right-hand side of equation (16) is also now available:

$$2\omega^2 \bar{x} \sum_i R_i S_{i,0} c_i^\beta (\bar{x}_i - \bar{x}) / n_0^\beta = 2p^2 \omega^4 x_0^2 t \times \{ 1 + (\omega^2 t) [2\bar{S}^\dagger - (3/2) (q^2/p^2)] + \dots \} + 2\bar{D}_0 (\omega^2 t) (\bar{S}_0 - \bar{S}_0') (1 + \dots). \quad (\text{A } 15)$$

Substitution of equations (A 6), (A 14) and (A 15) into equation (16) yields equation (17).

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