The Statistical Estimation of Molecular Weights from Normal and Difference Ultracentrifuge Boundaries

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1. To determine molecular weights from boundary data taken from a sedimentation velocity experiment in an ultracentrifuge, the parameter s/D must be estimated. This can be obtained by using non-linear statistical methods to fit a mathematical model [the Fujita & MacCosham (1959) equation] to the results. 2. The statistical method chosen was the simplex method of Nelder & Mead (1965), which was found to be ideal for this problem. Internal errors were calculated at the end of the search for the minimum in the residuals, but in general these errors were found to not represent the overall true error of the experiment. 3. Calculations of molecular weights of myoglobin showed that instabilities at low concentrations of protein (less than 0.8 mg/ml) disturbed the calculation of s/D . If 1% (w/v) sucrose was included in the solvent, these instabilities were decreased, and extrapolating to infinite time the linear function of s versus $1/(\text{time})$ gave an acceptable value for s with an error of $\pm 4.8\%$. The estimates of the molecular weights were less well-defined and the mean value was low by 8%, with an estimated error of the mean of $\pm 3\%$. The conclusion was that vibration was responsible for the instabilities without sucrose. 4. The Fujita–MacCosham equation can be extended to make it possible to estimate ratios of sedimentation and molecular weights for difference boundaries. Tests using two solutions of orosomucoid in which a 2% decrease in velocity of one boundary was achieved by adding a calculated quantity of sucrose showed that the analysis gave realistic values for the two ratios, and the error for the ratio of sedimentation coefficients was $\pm 10\%$. The error was larger for the estimated ratio of the molecular weights, but the analysis gave the expected value for the ratio.

Recent years have seen marked improvements in both the variety and precision of methods for estimating molecular weights of biological macromolecules. Gel chromatography and electrophoresis are examples of simple procedures that provide estimates of the size of comparatively large molecules, but their accuracy depends on calibration with well-defined standards (Andrews, 1965; Weber & Osborne, 1969); hence, a reliable absolute procedure for estimating molecular weights is essential. Osmotic pressure, light scattering and ultracentrifugal measurements are recognized techniques for estimating molecular weights, and of these possibly the most popular method involves operating the analytical ultracentrifuge in the sedimentation-equilibrium mode. Here the speed of the rotor is adjusted to make sedimentation equal diffusion so that the concentration distribution is constant with respect to time. For molecular weights in excess of 1 000 000, the required speed is very low and because the resulting field is not sufficiently large, the earth's gravitational field cannot be neglected in the calculations. At higher speeds heavy solutes sediment, making it necessary to estimate s in the conventional manner, whereas D is estimated from the spreading of the boundary

(Trautman, 1963). There is the alternative pseudo-equilibrium procedure (Archibald, 1947), but this has the disadvantage that the results can be inaccurate. Probably future estimates of D for large molecules will be made by using fluctuation spectroscopy (Foord *et al.*, 1970), so combining this with estimates of s can provide an estimate of the molecular weight (M) (eqn. 1):

$$M = \frac{RT}{(1 - \bar{v}\rho)} \cdot \frac{s}{D} \left(1 + c \frac{d \ln \gamma}{dc} \right) \quad (1)$$

where s = sedimentation coefficient at concn. c ; $\bar{v}$ = partial specific volume of the solute; ρ = density of the solution; γ = activity coefficient of solute; R and T have the usual connotation. This procedure has the statistical advantage that it utilizes independent estimates of s and D : unfortunately, its use is limited to a few laboratories possessing the necessary combination of equipment. A further procedure is to estimate s/D from the shape of a sedimenting boundary by using a mathematical model, and this method is explored in the present paper.

The Lamm (1929) differential equation (eqn. 2) is the accepted mathematical model for a centrifuge

boundary, but before it can be used for analysing experimental results it must be integrated by employing realistic experimental conditions in the integration. With computers it is possible to obtain realistic integrations of the equation by using numerical procedures (Dishon *et al.*, 1966), but the numerical evaluation is lengthy and even with high-speed computers it is doubtful whether numerical integration could be included in a statistical package. Fujita & MacCosham (1959) have fortunately provided an integral solution which approximates closely to conditions found in velocity experiments. Spragg (1962) showed that this formulation is a suitable model for analysing centrifuge boundaries of dilute solutions of sucrose in water, but it has not been tested for larger molecules.

The principle of estimating s/D can be extended to analysing boundaries formed in difference experi-

Numerous solutions exist for the Lamm (1929) equation (eqn. 2) (for a summary of these solutions see Fujita, 1962), but possibly the solution by Fujita & MacCosham (1959) (eqn. 3) is the most useful for analysing transport experiments. The Fujita-MacCosham equation was derived for a two-component system (solvent and solute) in a semi-infinite centrifuge cell in which the solute is uniformly distributed through the solvent at the start of the experiment. The mathematical boundary conditions include an air/solution meniscus but do not allow for dependence of the diffusion and sedimentation coefficients on the concentration of the solute, nor for the presence of an acceleration period before the rotor reaches constant velocity. Also, allowance for ill-defined variables such as physical vibrations in the rotor (which produce atypical broadening of the boundary) cannot be included in the model.

$$c = \frac{c_0 \epsilon^{-\tau}}{2} \left\{ \left[1 - \Phi \left(\frac{\tau - Z}{2\sqrt{\epsilon\tau}} \right) \right] - \frac{2}{\sqrt{\pi}} \left(\frac{\tau}{\epsilon} \right)^{1/2} \text{Exp} - \frac{(\tau - Z)^2}{4\epsilon\tau} + \left(1 + \left[\frac{\tau + Z}{\epsilon} \right] \right) \left(1 - \Phi \left[\frac{\tau + Z}{2\sqrt{\epsilon\tau}} \right] \right) \text{Exp} \left(\frac{Z}{\epsilon} \right) \right\} \quad (3)$$

ments (Richards & Schachman, 1957, 1959; Schumaker & Adams, 1968*a,b*; Warner & Schumaker, 1970*a,b*). Kirschner & Schachman (1971) showed that as little as 0.2% difference in sedimentation coefficient could be measured with carefully aligned interference optics. These experiments are particularly valuable when studying conformation changes brought about by chemical reactions (Gerhart & Schachman, 1968; Shapiro & Ginsberg, 1968; Warner & Schumaker, 1970*a*; Goers & Schumaker, 1970), but this work implicitly assumes that the molecular weights of the molecules in the two regimes are equal. In principle, it is possible to estimate the ratio of the molecular weights from the Fujita-MacCosham equation, and this possibility was explored in the present work.

Extensive statistical analyses were used in the study, and to provide realistic estimates of the errors in the original record, an 'on-line' computer was used during collection of data. This was programmed to average data until the random errors were equal to a pre-set value (Spragg, 1967; Spragg & Goodman, 1969). The program used in the on-line computer embodies a time-averaging algorithm, so allowing the experimentalist the freedom to define the random error in the record. Thus, it can be inferred that an accurate fit occurs when the calculated random error equals that pre-set for the collection of the record.

Theoretical

$$\partial c / \partial r = \frac{1}{r} \cdot \frac{\partial}{\partial r} \cdot \left[r D \left(\frac{\partial c}{\partial r} \right) - s \omega^2 r^2 c \right] \quad (2)$$

where

$$\Phi(X) = (2/\sqrt{\pi}) \int_0^X \text{Exp}(-Z^2) dZ,$$

the error function; $Z = 2 \ln(r/r_m)$; $\tau = 2\omega^2 st$; $\epsilon = 2D/\omega^2 s r_m^2$; c = concentration at radius r ; ω = radial velocity (rev./s); t = time (s); r_m = radius of meniscus; c_0 = initial concn. of the solute. To shorten equations containing eqn. 3, it will be referred to as $f(r, \tau, \epsilon)$. If ϵ is known, then the molecular weight of the solute can be computed from a rearranged Svedberg equation (eqn. 4).

$$M = \frac{2RT}{(1 - \bar{v}\rho) \epsilon \omega^2 r_m^2} \left(1 + c \frac{\partial \ln \gamma}{\partial c} \right) \quad (4)$$

Normally γ is unknown and no attempt is made to calculate it in the present analysis, so the result is a measure of the apparent molecular weight at concentration c .

Non-linear statistics

The values of τ and ϵ must be estimated from experimental records consisting of concentration of the macromolecule versus radius. Several records of the values will be recorded during one experiment, each being collected at known times, in which zero is the time the rotor reached two-thirds of the final velocity (this calculation assumes linear acceleration of the rotor).

From eqn. (3) it follows that for any pair of ordinates (concentration and radius) eqn. (5) is true:

$$c_r - f(r, \tau, \epsilon) = 0 \quad (5)$$

provided that c_r is exact and measured with no error (c_r is the concentration at radius r). For any pair of

experimental ordinates the result of the subtraction will not be zero because of random error in c_i and insufficiency in the model. The amount the sum deviates from zero is a measure of the contribution of these two deviates to the total error. Thus, for n pairs of co-ordinates the total error (Q , eqn. 6) can be estimated by the sum of the squares of the residuals, that is:

$$\sum_{i=1}^{i=n} [c_i - f(r_i, \tau, \epsilon)]^2 = Q \quad (6)$$

It now remains to obtain unique estimates of τ and ϵ in which Q is minimal.

From differential calculus, Q will be minimal (or maximal) when $(\partial Q / \partial \tau)_\epsilon$ and $(\partial Q / \partial \epsilon)_\tau$ for eqn. (6) are simultaneously zero. Forming these partial derivatives and expanding the equation in a Taylor series produces two linear equations containing two coefficients (τ and ϵ), which can be estimated by an iterative procedure (Powell, 1966; McCalla, 1967; Swann, 1969). Experience of applying this technique to the present problem has shown that unless the initial guessed values of τ and ϵ (required to start the iterations) are close to the final values, successive cycles in the routine diverge from, rather than converge to, the minimum for Q .

Nelder & Mead (1965) produced an algorithm for solving non-linear minimizing problems which is less likely to diverge from the minimum. Their algorithm is based on the simplex method (Spendley *et al.*, 1962) and utilizes a topological procedure to follow contours contained within a three-dimensional map. In the present example this is constructed with Q (eqn. 6) as the contour parameter (Z axis) and τ and ϵ the two co-ordinates (x and y axes). Changing the values of τ and ϵ independently gives different values for Q , so the algorithm must logically move τ and ϵ until Q has reached a minimum. Fortunately, during the search it is necessary to store only three values for Q . The main advantage of this method over hill-climbing procedures (Powell, 1966; Swann, 1969) is that divergence is not possible; however, it is possible that the minimum located is not necessarily the required minimum. The search is terminated when further movements of τ and ϵ do not significantly change Q (eqn. 6), then the errors on the parameters can be calculated from Q (Nelder & Mead, 1965). In the present work it was decided to use a coefficient as the criterion for terminating the search. This is formed from the ratio of the standard error of the three values of Q (three are required for estimating two parameters) divided by the mean value of Q . For the present analysis, tests showed that if this ratio was 0.001, then a minimum for Q had been located.

Numerical precautions

Since the simplex method is essentially a controlled search of contours, and there exist more than one set of roots to eqn. (6), it is essential to ensure that the

correct pair is found by the search. The easiest test is to start the search from different positions in space, and if the same results are produced, then it is reasonable to assume that these represent a unique solution. This was used during the early stage of the present work, but for routine work the search area was restricted to a limited area in space. If the values for the parameters were moved outside this area, then Q was changed to an impossibly high value. Examples of indicators used to show that the search moved outside acceptable limits are large positive and any negative values for the parameters and unrealistic arguments for both the error function and exponential calculations. The effect of these constraints is to build an impenetrable cylinder around the search area which reflects the search back into the restricted region.

Difference experiments

Using eqn. (3) it is possible to define the difference in concentration $[\Delta c_i^{(r)}]$ between two solutions at any radius so eqn. (7) is equivalent to eqn. (6). This relationship assumes the solutions are contained in separate compartments in the rotor.

$$Q = \sum_{i=1}^{i=n} \{ \Delta c_i^{(r)} - [f(\tau_1, \epsilon_1, r_i) - f(\tau_2, \epsilon_2, r_i)] \}^2 \quad (7)$$

It is not an essential requirement for this procedure that the menisci be at the same radii, since Z (eqn. 3) is calculated from the ratio r_i/r_m . Thus, eqn. (7) should be re-written to include Z_1 and Z_2 in the function, but for the present work eqn. (7) expresses the model to be tested experimentally.

For each boundary (identified by the subscripts 1 and 2) there are unique values for τ and ϵ ; thus, providing one pair is known (τ_1 and ϵ_1), then it is possible to estimate τ_2 and ϵ_2 by the procedure of Nelder & Mead (1965, described previously). Numerically, eqn. (7) is subject to an increasing proportion of rounding-error as the difference between the numerical values of the parameters approaches zero. To explore the limits of stability for eqn. (7) and at the same time to test the statistical analyses, it was necessary to generate analogue data containing random errors and analyse these. To do this, a series of values for Δc was calculated from eqn. 3 by subtraction (as in eqn. 7); this data was accurate to one part in ten thousand over most of the range (this accuracy is determined by the algorithm employed to calculate the error function). The use of analogue data to test mathematical models has been successfully applied by Myhill (1967, 1968), Gilbert (1967) and Roark & Yphantis (1969) to a range of biochemical techniques. The data $[y_i^{(r)}]$ are calculated by adding the random error to the error-free ordinates (y_i). The errors are calculated by using pseudo-random numbers (based on a normal distribution of

errors, N_i), to scale the assumed standard error (σ , eqn. 8)

$$y_i^{(a)} = y_i \pm \sigma(N_i) \quad (8)$$

This set of data can then be fitted by the statistical routine to give values of τ and ϵ together with their associated standard errors. This method of computing analogue data assumes that the variables are independent and not statistically correlated. No attempt has been made to compute data for the more realistic but more difficult case where the variables are correlated.

Calculation of sucrose concentrations for the difference experiments

The α_1 -glycoprotein, orosomucoid, was used to test the numerical procedure for analysing a difference boundary. This protein was dissolved in a solvent containing 1M-NaCl and sucrose: a fractionally greater amount of sucrose was added to one of the solutions and this decreased the rate of sedimentation by an amount defined by eqn. (9):

$$s_1/s_2 = \frac{(1 - \bar{v}\rho_1)\eta_2}{(1 - \bar{v}\rho_2)\eta_1} \quad (9)$$

where the subscripts 1 and 2 refer to the two solutions and η is the viscosity. It is assumed that $\bar{v}$ for the protein is the same in the two solutions. This assumption is reasonable in these experiments, because the differences in the sucrose concentrations are small. The viscosity and density for sucrose can be calculated from its concentration by using the empirical relationships of Barber (1966). In the present work one of the concentrations of sucrose was fixed and the second value was computed by an iterative method based on the Newton-Raphson procedure and eqn. 9. The reference solution was assumed to contain 0.5g of sucrose per 99.5g of 1M-NaCl.

Experimental

Velocity experiments with one solution

Solution conditions. A commercial freeze-dried sample of sperm-whale metmyoglobin (Miles-Seravac Ltd., Maidenhead, Berks., U.K.) was dissolved in 0.1M-sodium phosphate buffer (pH 7.0). Residual polymeric material was removed by chromatography on Sephadex G-50 (30cm $\times$ 1cm column). Fractions (5ml) were collected and the fractions from the centre of the peak were used in the centrifuge experiments. The extinction of the solution was adjusted to the required value immediately before the ultracentrifuge experiment.

Ultracentrifuge conditions. Two 12mm Kel-F centrepieces for the Beckman model E ultracentri-

fuge were employed: one of these was filled to within 2mm of the top of the cell with solvent and the other to approximately the same level with solution. A two-hole centrifuge rotor was used for the experiments and was run at 56100 rev./min and the temperature was maintained by a previously calibrated rotor-temperature control unit.

Difference experiments

Solution conditions. Orosomucoid was prepared from urine of patients suffering from nephrotic syndrome by chromatography on DEAE-cellulose followed by Sephadex G-50 (Spragg *et al.*, 1969) to give a preparation that was shown to be homogeneous by both ultracentrifugation and immuno-diffusion. The purified orosomucoid was stored as a freeze-dried powder and samples were dissolved in the appropriate sucrose solution (see the Theoretical section) containing 1M-NaCl to give a concentration of approx. 0.8 mg/ml (pH 4.1). The E_{280} of the two solutions was adjusted to make them exactly equal by adding solvent to the more concentrated solution while monitoring the difference in extinction with a Unicam SP. 500 spectrophotometer. Solutions having an E_{280} of approx. 0.6 were used for the differential experiments. The ultracentrifuge was operated at 52260 rev./min and Kel-F centrepieces were used in the cells. The temperature of the rotor was kept constant at approx. 20°C.

Method of aligning the menisci

The system (see below for a description of the computer system) requires two individual cells in separate holes in the rotor for calculation of extinction values. This makes the difference experiments more difficult than the simpler procedure of using double-sector centrepieces to hold the two solutions (Kirschner & Schachman, 1971) if the menisci are to be superimposed. It is useful, but not essential for the analysis by eqn. (7), that the menisci be at the same radial position during the experiment.

Weighing the liquids into the cells proved unreliable because small differences in the volumes of the two centrepieces meant that the menisci were displaced. Eventually a procedure was developed in which one cell was filled and the distance of the meniscus from the top of the cell was measured with a cathetometer. This cell was then replaced by the second cell and its meniscus position was measured. Sufficient solution was added to the cell having the larger distance to make the two measurements equal. The cells were then sealed, placed in the rotor and spun at approx. 6000 rev./min, and the schlieren image was examined. If the two menisci were not superimposed, the procedure was repeated, but since the schlieren image does not provide information to differentiate between

the cells, this must be obtained from the absorption of the light. The light-pulses change in a reproducible manner when scanning across the meniscus making it possible to relate the menisci to holes in the rotor: the pulses from the rotor reference hole are an absolute reference. All these pulses were examined with a double-beam oscilloscope. One beam of the oscilloscope carried the pulses from the reference hole (one pulse per revolution) and the other carried the pulses from the cells. Thus, relating the cells to the reference position in the rotor is comparatively simple. We were surprised to find that this method proved reliable and with experience the alignment was achieved with the minimum adjustment.

Estimating the extinction coefficient of orosomucoid

Approx. 10mg of the freeze-dried protein was weighed into 1M-NaCl solution and the E_{280} was measured with a Unicam SP.500 spectrophotometer. The moisture content of a second sample was determined at the same time and due allowance made in the weighings. Replicate estimations gave an average of 0.81 ml/mg for the extinction coefficient.

Recording data from the ultracentrifuge

A Beckman model E analytical ultracentrifuge was modified by adding a photo-electric scanner (Spragg *et al.*, 1965), which included a 250 W xenon arc lamp filtered by an interference filter (Spectral Services Inc., Waltham, Mass., U.S.A.) to supply light at 287nm. Two cells were used in the present system and the extinction was computed by measuring the light transmitted through individual cells and computing the ratio with an on-line digital computer (Spragg, 1967). To minimize 'noise' in this ratio, a program was composed which at any radial position averaged the ratio until the signal-to-noise reached a pre-set value (Spragg & Goodman, 1969). With this system it was possible as a routine to achieve a s.e.m. extinction of ± 0.005 for any radial position. Unfortunately the absorption characteristics of the windows of the cells were not sufficiently reproducible to maintain this low value of random error, and when measurements were taken along the complete radial length of the cells containing water, the s.e.m. increased to ± 0.02 unit. This value for the error was used for all the experiments. Allowance was made for errors introduced by stationary dirt on the windows by recording a base-line and subtracting this from the experimental record.

In addition to correcting error from windows and assessing random 'noise' on the records, an attempt was made to minimize errors introduced by 'white light' penetrating the interference filter. The amount of 'white light' reaching the photo-multiplier tube was measured at the beginning of the experiment by

removing the u.v. light with either a saturated solution of tyrosine in 0.1M-HCl, or more conveniently by placing a sheet of Perspex in the beam of light (the latter method removed light at wavelengths less than 320nm). The transmitted light was measured with the u.v. filter in place and this value was subtracted from the measurements before the transmission ratio was calculated. This method is only reliable if the percentage transmittance of the solution is high, which means that extinctions in excess of 0.6 unit cannot be examined. The computer system does not contain sufficient core-store to make it possible to collect and store records of the 'white light' at multiple radial positions in the cell so the present correction can only be approximate.

Results

Determination of molecular weight

The use of spectrophotometric principles to record the distribution of a solute provides a record of concentration versus radius at measured intervals of time. Although this procedure has distinct advantages in centrifuge equilibrium experiments, the record is less useful in velocity experiments where the more familiar schlieren record has many advantages (here the derivative of the concentration with respect to the radius is recorded). For analysing sedimentation velocity experiments Trautman *et al.* (1969) proposed an approximate but linear analysis, which contains terms to detect heterogeneity as well as providing initial estimates of the sedimentation and diffusion coefficients. These estimates are approximate because of the assumptions made to give linear equations (Trautman *et al.*, 1969), but they can be used as starting values for the present analysis.

Before considering the more difficult case where it is suspected that more than one component is present, it is necessary to show that the analysis is reliable with a homogeneous sample. Myoglobin was chosen for this work because it is a well-defined protein that cannot dissociate, and in Table 1 are listed the estimated molecular and statistical parameters from an analysis of a typical velocity experiment made with myoglobin. The mean estimate for the sedimentation coefficient from seven records collected at different times was $1.482 \times 10^{-13} \pm 0.037 \times 10^{-13}$ s at 13°C, which when converted to give the $s_{20,w}$ gave $1.778 \times 10^{-13} \pm 0.049 \times 10^{-13}$ s (four significant figures are quoted for the sedimentation coefficient and molecular weights in this and other tables so that the estimated standard errors can be compared). The internal standard errors (these are the errors returned from the fitting for each set of results) were generally similar in value to that of the standard error of the mean. The estimated mean molecular weight was 4980 ± 840 (assuming a partial specific volume of

Table 1. *Estimate of molecular weight and sedimentation coefficient of myoglobin dissolved in 0.1 M-phosphate buffer (pH 7.0)*

The rotor velocity was 56100 rev./min and the temperature was 13°C. The E_{280} of the solution was 0.40 unit. S.E., standard error on the ordinate estimated from Q (eqn. 6).

Time (s)	$10^{13} \times s$ (s)	$10^{-3} \times \text{Mol. wt.}$	No. of pairs per frame	S.E.
5040	1.504 ± 0.048	5.25 ± 0.16	28	0.058
5520	1.430 ± 0.040	7.56 ± 0.04	28	0.059
5880	1.526 ± 0.040	2.78 ± 0.74	28	0.065
6300	1.486 ± 0.048	3.63 ± 0.34	28	0.047
6720	1.500 ± 0.001	6.633 ± 0.019	28	0.031
7200	1.534 ± 0.022	1.73 ± 0.18	28	0.054
7620	1.422 ± 0.001	6.64 ± 0.015	28	0.025
Mean	1.482	4.89		0.048
S.E.M.	± 0.0373	± 0.84		± 0.0097

0.743 ml/g; Theorell, 1934), but the estimates of the internal standard errors were consistently smaller than the standard error of the mean. These statistical inconsistencies are relatively trivial when the estimates for the molecular weights are compared with the true value for myoglobin (17830; Edmundson & Hirs, 1962).

Because the calculation of the sedimentation coefficient from τ (eqn. 3) requires that the sedimentation time be known accurately, deviations of this coefficient from the expected value could be caused by inaccurate measurements of the true time. Error in time measurement should not affect the calculation of the molecular weight from ϵ (eqn. 3). Hence, it seems perturbations were introduced during the experiment that are not allowed for by the mathematical model. Examination of the calculated standard error of the ordinate (Table 1) showed that the estimates were consistently higher than the expected value of 0.02 (see the Experimental section for defining the expected standard error), showing that the experimental data was 'forced to fit' the mathematical model and the errors on the ordinate are really estimates of the inadequacy of the model as well as estimates of the random error on the measurements.

Numerous experiments covering a range of concentrations between 0.1 and 0.8 mg/ml and temperatures from 13°C and 21°C confirmed the results given in Table 1, and the conclusion was reached that at these concentrations density instabilities caused boundary spreading at a rate in excess of that pro-

duced by diffusion. To decrease this effect, a density gradient was formed in the cell during the experiment by dissolving myoglobin in a sodium phosphate buffer containing 1% (w/v) sucrose. Since the sucrose was distributed uniformly through the solution at the beginning of the experiment but redistributed during the run, records collected early in the experiment will be less reliable than the later records. Trial experiments showed that with 1% (w/v) sucrose, the major part of the redistribution of the sucrose occurred within the first 1.5 h. The results are shown in Table 2, when the estimates of the mean sedimentation coefficient and molecular weight were $1.464 \times 10^{-13} \pm 0.024 \times 10^{-13}$ s and 15710 ± 480 respectively. The $s_{20,w}$ was calculated to be $1.520 \times 10^{-13} \pm 0.030 \times 10^{-13}$ s, which was less than the result without sucrose (Table 1) and less than the accepted value for myoglobin (between 1.7×10^{-13} and 1.9×10^{-13} s; Rossi Fanelli *et al.*, 1964). Plotting the estimated coefficients versus the reciprocal of the time (Fig. 1) showed that the value for the coefficient increased linearly with time. Fitting the results to a curvilinear polynomial showed that the best representation of the relationship was a straight line, having an extrapolated value for the sedimentation coefficient at infinite time of $1.66 \times 10^{-13} \pm 0.08 \times 10^{-13}$ s and a slope of $-3.2 \times 10^{-10} \pm 1.4 \times 10^{-10}$ s² (the quadratic and higher terms were not significant at the 95% probability level when the fitting was repeated by using higher powers). Correcting the extrapolated estimate gave an $s_{20,w}$ of $1.70 \times 10^{-13} \pm 0.08 \times 10^{-13}$ s, which was not significantly different from the mean estimate of $s_{20,w}$ calcu-

Table 2. Estimates of the molecular weight and sedimentation coefficients of myoglobin dissolved in 0.1 M-phosphate buffer (pH 7.0) containing 1% sucrose

The rotor speed was 59780 rev./min and the temperature was 20°C. The initial E_{280} of the solution was 0.56. S.E. is the standard error on the ordinate estimated from Q (eqn. 6).

Time (s)	$10^{13} \times s$ (s)	$10^{-3} \times \text{Mol.wt.}$	No. of pairs per frame	S.E.
4322	1.408 ± 0.003	13.64 ± 0.92	17	0.014
4935	1.465 ± 0.015	15.09 ± 0.74	20	0.019
5547	1.427 ± 0.015	14.24 ± 0.90	17	0.018
6159	1.392 ± 0.001	15.78 ± 1.34	21	0.017
6747	1.488 ± 0.001	16.81 ± 1.42	22	0.018
7347	1.549 ± 0.017	16.64 ± 1.08	21	0.024
8338	1.536 ± 0.031	16.40 ± 1.80	23	0.020
Mean	1.467	15.61		0.019
S.E.M.	± 0.024	± 0.48		± 0.002

ated from Table 1, but is lower than the value quoted in the literature for other myoglobins (Rossi Fanelli *et al.*, 1964).

An ill-defined trend of increasing molecular weight occurred with time (Table 2), but it was not possible to relate either these results or results from similar experiments to a simple time-dependent relationship. The molecular weights obtained after 6747s, however, were equal within the internal errors to the expected value of 17830 (Edmundson & Hirs, 1962). Further, when sucrose was present in the solution it was found that the estimated standard errors on the experimental ordinate were approximately equal to the pre-set value (see the Experimental section) showing that the errors were probably true estimates of the random error in the data and were not caused by an inadequate mathematical model (the Fujita-MacCosham equation, eqn. 3). Comparing the calculated shape of the boundary with that recorded experimentally (Fig. 2) showed that, over the major part of the curve, the experimental points were distributed evenly on both sides of the calculated curve. In the region near the plateau, however, the deviation between the calculated and observed profiles was not random, but showed a marked bias in which the calculated values were consistently larger than the experimental values.

The Fujita-MacCosham equation (eqn. 3) is derived for a two-component system, but it is of interest to follow the effect that heterogeneity will have on the analysis. For these tests a relatively complex system was examined in which myoglobin

was mixed with 2% (w/v) sodium dodecyl sulphate by adding weighed amounts of sodium dodecyl sulphate to the myoglobin solution. Centrifuge equilibrium experiments with this mixture have shown that the estimate of the molecular weight of myoglobin under these conditions is low (Barnett & Spragg, 1971). It is thought that the low value reflects the formation of polymicelles with the protein, which were absent from the sodium dodecyl sulphate solution alone. This provides an extreme of heterogeneity for testing the analysis, and of course, with these experiments it was unnecessary to include sucrose in the solution because the unbound sodium dodecyl sulphate formed a density gradient for stabilizing the boundary.

In Table 3 are listed the estimated sedimentation coefficients and molecular weights for data collected over a period of 10050s. Because both the estimated molecular weights and sedimentation coefficients decreased markedly with time, it served no purpose to estimate a mean for the parameters. As in the experiment in which pure myoglobin was dissolved in sucrose solution, the changes in the sedimentation coefficient could be described by a linear model relating the coefficient with reciprocal time (Fig. 1). Fitting the data to a linear regression equation gave an estimate for the coefficient at infinite time of $1.44 \times 10^{-13} \pm 0.09 \times 10^{-13}$ s and a slope of $0.133 \times 10^{-13} \pm 0.015 \times 10^{-13}$ s². These values were calculated assuming that the partial specific volume of myoglobin in sodium dodecyl sulphate was 0.743 ml/g; however, the detergent binds tightly to the protein,

Fig. 1. Relationship between the sedimentation coefficient and reciprocal time for sperm whale myoglobin at a concentration of approx. 0.3 mg/ml

The standard error of the mean for each point is indicated by the bar. ▲, Results for the solution in 0.1M-sodium phosphate buffer containing 1% (w/v) sucrose. The extrapolated value of s at infinite time is $1.66 \times 10^{-13} \pm 0.08 \times 10^{-13}$ s. ●, Results for myoglobin dissolved in 0.1M-sodium phosphate buffer containing 2% (w/v) sodium dodecyl sulphate. The extrapolated value for the coefficient at infinite time is $1.44 \times 10^{-13} \pm 0.09 \times 10^{-13}$ s.

and it seems from the work of Reynolds & Tanford (1970), and confirmed for myoglobin by Barnett & Spragg (1971), that a more realistic value for the partial specific volume would be 0.82 ml/g. If this partial specific volume is used to correct the extrapolated value from Fig. 1, it gives $s_{20,w}$ of $2.01 \times 10^{-13} \pm 0.13 \times 10^{-13}$ s, which is not significantly different from the previous estimates (Tables 1 and 2, discussed above) when allowance is made for the errors contained in the estimate of the partial specific volume. The correction term is relatively large (a multiplication factor of 1.44) and a small error in the estimate of the partial specific volume will have a magnified effect on the coefficient (for example, decreasing the partial specific volume to 0.80 ml/g gives a $s_{20,w}$ of $1.88 \times 10^{-13} \pm 0.12 \times 10^{-13}$ s). These difficulties make it impossible to calculate a more accurate value for the coefficient from this experiment, but the results showed that even in complex systems combining the statistical analysis with the empirical relationship between the sedimentation coefficient and reciprocal-time gave an acceptable estimate for the coefficient.

The changes with time for the estimates of the molecular weights are complex when sodium dodecyl sulphate is present, and the estimates decreased in an approximately hyperbolic way with respect to increasing time finally to give a relatively constant estimate of 11600 ± 200 after 6872 s (assuming a partial specific volume of 0.743 ml/g). Correcting this estimate for the binding of the detergent gave the value of 16700 ± 300 , which is less than the accepted value (17830; Edmundson & Hirs, 1962) but not unreasonably low.

The standard error for the experimental ordinate

Fig. 2. Comparison between the calculated ordinates (—) and experimental values (●) for one scan at 287 nm of an experiment with myoglobin dissolved in 0.1M-sodium phosphate buffer (pH 7.0) containing 1% (w/v) sucrose

This scan was obtained 112 min after two-thirds the final rotor velocity had been reached.

Table 3. *Estimates of molecular weights and sedimentation coefficients of myoglobin dissolved in a 2% solution of sodium dodecyl sulphate in 0.1 M-phosphate buffer (pH 7.0)*

Results are calculated for $\bar{v} = 0.743$. The rotor velocity was 59780 rev./min and the temperature was 20°C. The E_{280} of the initial solution was 0.54 unit. s.e. = standard error on the ordinate calculated from Q (eqn. 6).

Time (s)	$10^{13} \times s$ (s)	$10^{-3} \times \text{Mol.wt.}$	No. of pairs per frame	s.e.
3727	2.758 ± 0.145	21.44 ± 0.25	7	0.163
4352	2.523 ± 0.001	21.05 ± 0.01	6	0.074
4959	2.604 ± 0.090	20.62 ± 0.18	9	0.071
5615	2.420 ± 0.002	17.19 ± 0.01	12	0.040
6247	2.176 ± 0.054	16.21 ± 0.11	11	0.044
6872	2.176 ± 0.001	11.80 ± 0.008	10	0.032
7504	2.049 ± 0.001	11.47 ± 0.01	11	0.027
8135	2.003 ± 0.001	12.51 ± 0.004	12	0.033
8752	1.914 ± 0.002	11.45 ± 0.01	9	0.028
9376	1.878 ± 0.030	11.23 ± 0.06	9	0.023
10050	1.922 ± 0.001	11.33 ± 0.01	13	0.036

(Table 3) for records collected at the beginning of the experiment were higher than expected. When the molecular weight reached an approximately constant value, the error approached the expected value showing that initially the mathematical model was inadequate to describe the experimental results. Later, however, it gave a reasonable fit but was never as satisfactory as with myoglobin in sucrose (Table 2).

Experiments with difference boundaries

The tests showed that the Fujita-MacCosham equation (eqn. 3) is a suitable model for analysing boundaries containing low concentrations of pure protein provided that precautions are taken to stabilize the boundaries against density disturbances. This made it possible to use the equation to analyse difference boundaries from the centrifuge by using the model given in eqn. (7).

By following normal practice for testing a new numerical procedure, analogue data was generated (see the Theoretical section for the methods) for three values of random error, i.e. zero, 0.005 and 0.02 extinction unit; the last represented the expected experimental error for the on-line computer system.

The data for zero standard error (the so-called error-free data) is subject to rounding error from the calculation of the error function (see eqn. 3). In Table 4 are given the results from this analogue data for the case where the ratio between the sedimentation coefficients was 0.98 and the molecular weight was constant. Because previous work (see Tables 1, 2 and 3) showed that the internal standard errors were not always reliable estimates of the true experimental error, these values are omitted from Table 4.

Fitting the error-free analogue data to eqn. (7) showed that the procedure gave approximately correct answers for the ratios of both the sedimentation coefficients and molecular weight; however, the standard errors of the means were higher than expected (about 0.0023 when they should have been ± 0.0001). This difference between the expected error and that calculated is a measure of the total rounding error for eqn. (7) (see discussion on numerical analysis in the Theoretical section). Readjusting the accuracy with which the simplex procedure located the minimum did not materially affect the error. The s.e.m. values calculated for both ratios in all three cases were similar (Table 4), although the calculated standard error on the ordinates (for

Table 4. *Difference boundary calculations with analogue data in which the ratio of sedimentation coefficients (s_1/s_2) 0.98, with the ratio of molecular weights (M_1/M_2) assumed to remain constant*

The absolute value for $s_1 = 2.60 \times 10^{-13}$ s and for molecular weight = 37000. The absolute value for the extinction (c_0 , eqn. 3) was 0.5 for these calculations. S.D. = assumed standard deviation for the experimental measurements.

S.D. ...	0.000		0.005		0.020	
Time (min)	s_1/s_2	M_1/M_2	s_1/s_2	M_1/M_2	s_1/s_2	M_1/M_2
10	0.9831	1.001	—	—	—	—
25	0.9802	0.999	0.9804	1.000	0.9810	0.999
40	0.9767	0.998	0.9790	1.001	0.9736	1.000
55	0.9796	0.999	0.9774	0.998	0.9774	1.001
70	0.9804	1.002	0.9808	0.999	0.9802	1.000
85	0.9792	1.000	0.9840	1.001	0.9884	0.998
Mean	0.9799	0.999	0.9803	0.999	0.9801	0.999
S.D.	± 0.0021	± 0.0020	± 0.0024	± 0.0010	± 0.0017	± 0.0010

simplicity omitted from Table 4) were always approximately equal to the values used to calculate the analogue data. The relatively high contribution of rounding error to the total uncertainty of the estimates meant that the analysis is restricted to estimating ratios that are much less than 0.998.

Kirschner & Schachman (1971) found that by using Rayleigh interference optics to detect and measure the boundaries as little as 0.2% difference between the sedimentation coefficients could be measured. Analogue data was computed for this small difference and perturbed by random errors having a standard error of 0.005 extinction unit. Visual examination of the data for the boundary suggested that it consisted of a random scatter of points; however, fitting the data for three separate times gave a mean difference of 0.3% between the coefficients. This is an unacceptably high deviation from the difference used to calculate the data, showing that with absorption optics the extinction must be measured with errors less than 0.005 before this method can equal the results from interference measurements.

Having established the lowest difference which gave a satisfactory answer, an ultracentrifuge experiment was made in which the ratio between the sedimentation coefficients was known and also considerably larger than the limits set by the numerical procedure. An s_1/s_2 ratio of 0.98 was used, and the known molecular weight ratio was kept at 1.0. In this test two solutions were centrifuged; both contained sucrose and the same concentration of protein. In one the solvent was 0.5 g of sucrose in 99.5 g of 1 M-NaCl and the second contained 0.4 g of sucrose in 99.6 g of 1 M-NaCl. This difference in concentration of sucrose was calculated to produce an s_1/s_2 ratio of 0.98. The second solution was used as the solvent and the

extinctions of the two solutions were equalized at 280 nm. In 1 M-NaCl this sample of orosomucoid at this concentration (0.8 mg/ml) had a sedimentation coefficient of $2.60 \times 10^{-13} \pm 0.01 \times 10^{-13}$ s as determined in a separate experiment with schlieren optics, and sedimentation-equilibrium experiments showed the molecular weight to be 37000 ± 800 .

After the experiment, the records were fitted to eqn. (7) for each of the five times to give a mean estimate for s_1/s_2 of 0.974 ± 0.002 and M_2/M_1 of 1.008 ± 0.012 (Table 5). The error on s_1/s_2 was surprisingly low and appeared to be caused by rounding error in the arithmetic (see above). Estimates for both ratios were approximately equal to the expected values, but the standard error on M_2/M_1 was disappointingly high. The small difference between the calculated s_1/s_2 and the expected value (0.98) can be accounted for by small uncertainties in viscosity and density from Barber's relationships (Barber, 1966). In all the frames the estimated standard error for the experimental ordinate was approx. 0.02, and equal to the expected value, showing that eqn. (7) was a realistic model for this type of experiment.

When analysing difference boundaries, it is important to determine whether significant differences could be measured with the same solutions in both centrifuge cells. Should a significant result be found, then the cell windows are suspect. Tests for this effect were made by placing the same solution of orosomucoid in sucrose and NaCl in both cells and a mean s_1/s_2 of 1.009 ± 0.008 and M_2/M_1 of 1.005 ± 0.02 was calculated from the record. These results were consistent with the expected lowest detectable differences showing that the estimated 2% difference in sedimentation coefficient (Table 5) was real. It was noteworthy from the aspect of the rounding error to find that when random numbers having a mean of zero and

Table 5. *Experimental values for a difference experiment with orosomucoid in which one solvent contained 0.5 g of sucrose per 99.5 g of 1 M-NaCl, and the second contained 0.51 g of sucrose per 99.49 g of 1 M-NaCl*

The ratio s_1/s_2 should be 0.980 and the molecular weights should remain constant. The assumed absolute values for the sedimentation coefficient (s_2) and molecular weight (M_2) were 2.60×10^{-13} s and 37 000 respectively. Both contained protein at equal extinction (0.54 unit at 280 nm). The rotor velocity was 56 100 rev./min and zero time was taken at two-thirds the final velocity of the rotor. S.E. is the estimated standard deviation in the experimental ordinate calculated from Q (eqn. 7).

Time (min)	s_1/s_2	M_2/M_1	No. of pairs per frame	S.E.
47.7	0.968 ± 0.008	1.044 ± 0.089	18	0.028
58.2	0.974 ± 0.013	0.970 ± 0.027	23	0.014
68.8	0.970 ± 0.009	1.015 ± 0.043	17	0.020
79.1	0.976 ± 0.018	0.989 ± 0.050	21	0.019
89.3	0.980 ± 0.005	1.020 ± 0.030	20	0.030
Mean	0.974	1.008		0.022
S.E.M.	± 0.002	± 0.012		± 0.007

standard error of 0.02 were supplied as data, the estimated value for s_2/s_1 was 1.001 ± 0.001 and for M_2/M_1 , 1.015 ± 0.015 .

Locating the difference boundary when the random error in the experimental ordinate is 0.02 extinction unit for a total absorbance of 0.42 can be a problem, as shown in Fig. 3. Here both analogue (Fig. 3a) and experimental data (Fig. 3b) are given together with error-free profiles superimposed on the perturbed data. The spread of the experimental values (which for Fig. 3a were computed by assuming a standard error of 0.02 unit) often make it difficult to find the boundary. The analogue data is particularly useful in this situation and forms an integral part of the experiment, since from this it is possible to define the regions in the experimental record where the boundary is most likely to occur and these ordinates can be removed for analysis.

Finally, mention should be made of the effect that optically unbalanced windows have on the calculations. If the base-line is not uniform along the cell, then false, but stationary, boundaries will be recorded. An allowance must be made for these perturbations by subtracting estimates of the base-line from the data before analysing the data. Estimates of the base-line can be obtained from the experimental records by using data from the early scans as estimates of the base-line for later times while that from later scans are used to correct the early scans. Thus the records taken from regions of the cell where the boundary is known to be absent can be used to correct the data for that region when the boundary moves to the region.

Discussion

Early attempts to estimate molecular weights by determining sedimentation and diffusion coefficients from separate experiments and from boundary data (for a summary of the evolution of centrifuge techniques see Williams, 1969) were superseded by the centrifuge equilibrium procedure. This is unfortunate, because the velocity experiments have many features that make them superior to the equilibrium experiments, especially for examination of mixtures. Hence, the present study was made in the hope that a general analytical technique would emerge that could be used for estimating molecular weights from the records of a velocity experiment. Whether the analysis can be applied to a wide range of molecules is unknown, but the present results show that useful estimates of sedimentation coefficients and molecular weights for molecules in the medium range of molecular size can be obtained.

Provided that suitable precautions are taken during the experiment, then the Fujita-MacCosh equation (eqn. 3) is an approximate description of a sedimenting boundary formed from a relatively low concentration of protein. It was disturbing to find that estimates of molecular weights were inaccurate if a stabilizing density gradient was omitted; however, in its absence it was possible to calculate sedimentation coefficients from the equation. The amount of stirring that occurred without stabilization was small because not only does the boundary continue to pivot about the centroid (as shown by the correct estimate of the sedimentation coefficient in the absence of sucrose)

Fig. 3. Comparison between experimental and theoretical data for a difference boundary of orosomucoid (the absolute extinction was 0.56 unit) having a 2% difference in sedimentation coefficient but the same molecular weight

(a) Analogue data calculated assuming a variance of 2×10^{-4} extinction unit; continuous line is the error-free profile; (b) set of data from an experiment in which sucrose is added to one cell to retard the boundary by 2%; the continuous line is the profile fitted by the statistics.

but also it can be decreased to a negligible value by the presence of a comparatively shallow density gradient within the cell. One possible source of disturbance was vibration transmitted along the driving spindle. These vibrations must increase at higher speeds because by using the same centrifuge and operating at considerably lower speeds it was possible to obtain accurate estimates of the molecular weight of low concentrations of myoglobin through centrifuge-equilibrium experiments, without adding sucrose to the solution. Further, workers have estimated diffusion coefficients from artificial boundaries formed in a centrifuge cell at low speeds (for example Fisher *et al.*, 1971). The alternative explanation, that a temperature gradient across the cell produced

the instabilities, is less likely. In the model E ultracentrifuge, heat is supplied to the centre of the rotor and cold at the edge, these being ideal conditions for stability (provided that the temperature is greater than 4°C).

An unfortunate side-effect of adding sucrose is that the velocity of the boundary does not reflect the velocities of the macromolecules throughout the solution (at constant field strength, i.e. $\omega^2 r$; Trautman & Schumaker, 1954), because of higher densities and viscosities near the bottom of the cell. In effect, this will mean that there will not be a true plateau in the cell where $dc/dr = 0$. The linear relationship between sedimentation and $1/(\text{time})$, shown in Fig. 1, is caused by this lack of correspondence between the mathematical model and the experimental results, so the effect is an artificial acceleration as time increased and the boundary reached larger radii. The inverse relationship with time can be compared with the procedure of Gosting (1952) and Baldwin (1954) for evaluating distributions of sedimentation coefficients in mixtures. In the present analysis the slope is negative, whereas in heterogeneity it is positive (Fig. 1, ●). Again, the model does not allow for a non-linear dependence of sedimentation rate on radius, but since the effect is second-order a usable combination can be formed in which the mathematical model provides a set of estimates, which are then corrected for the effect of the sucrose. The result is an acceptable value for the sedimentation coefficient.

It seems probable that in sucrose the trend in the molecular-weight estimates for pure myoglobin reflected the time required for sucrose to redistribute. Low values for both parameters were observed during the early stages of the experiment (Table 2) and could reflect the instability present during the reorientation of the gradient. It is a little worrying, however, to find that later in the experiment the estimates were approximately correct, showing that the boundary must have returned to the shape that it would have reached despite the stirring in the early stages of the experiment. This reversion seems most unlikely, and since the errors remain relatively constant throughout the experiment, it seems that the trends observed for the early part of the experiment are caused in part by approximations incorporated into eqn. (3). The fact that apparent molecular weights are calculated from the analysis with no allowance for effects of concentration on the diffusion and sedimentation coefficients cannot account for the trends found experimentally. The decrease in concentration caused by sectorial dilution would be approx. 3% for the distance the boundary moved. When this small dilution is combined with the low concentrations of the protein then the virial coefficients would have negligible values.

Introducing heterogeneity into the experimental system in the form of myoglobin with sodium dodecyl

sulphate had a considerable effect on the results. The trend in the estimates of the molecular parameters (Table 3) reflected the complex equilibria between myoglobin, free detergent and its micelles. The sedimentation-equilibrium experiments (Barnett & Spragg, 1971) support the present estimates of molecular weights for myoglobin-detergent complex, which in turn show that the partial specific volume of myoglobin in sodium dodecyl sulphate is between 0.80 and 0.82 ml/g. The relationship between the sedimentation coefficient and $1/(\text{time})$ follows the pattern predicted by Baldwin (1954) for heterogeneous mixtures and differs both in sign and magnitude of the slope from that observed for pure myoglobin in sucrose (see Fig. 1). Despite the uncertainties produced by extrapolating to infinite time, it seems probable that the present analysis could aid the study of paucidisperse systems by providing estimates of the molecular weights which can be correlated with the concentration changes.

Applying the present analysis to difference boundaries gave realistic estimates of differences in sedimentation coefficients. For absorption optics it is necessary to limit these experiments to cases where the differences are greater than 2%, and at this level it appears possible to obtain realistic estimates of the molecular-weight ratios. The relatively large errors for the molecular-weight ratios leave much to be desired; however, it may be possible to decrease these errors by combining the present analysis with the method of Kirschner & Schachman (1971). Their procedure provides accurate estimates of the ratio of sedimentation coefficients (equivalent to τ in eqn. 3) so leaving one parameter (ϵ) to be estimated from eqn. (6). Employing interference records for this type of analysis presents extra experimental problems, because the absolute concentration of the protein at zero time (c_0 , eqn. 3) is required, and measuring this from interference records requires special experimental procedures. Fortunately, the measurement of c_0 can be approximate since test calculations in which c_0 was changed and the analysis repeated showed that provided that gross errors were not present in c_0 (greater than $\pm 10\%$) the estimations of the ratios were not significantly different. In fact, although the present analysis assumes that s_1 and M_1 (eqn. 7) are known, calculations show that, as with c_0 , some error in these values also can be tolerated by the analysis without significantly changing the ratios. Decreasing the number of parameters to one will decrease the uncertainty with which this parameter is estimated, as well as simplifying the minimization of Q (eqn. 6). Instead of using the simplex method (Nelder & Mead, 1965) the minimum would be found by the Newton-Raphson procedure and the errors estimated directly from Q at the end of the search.

Throughout this study experimental results have been related to mathematical models by collecting

records at defined levels of error. This data was then used to test the model by comparing the expected errors with those evaluated numerically. Unless these two were approximately equal it was assumed that the model was insufficient to account for the data. When the calculated error was equal to the error set for the collection of data then it was assumed that an acceptable correlation occurred between the experimental record and the mathematical model. This approach would not have been possible without the on-line computer.

There remains the question of whether this analytical method can be used for estimating molecular parameters of large molecules. From the present work it would seem probable that the method could provide useful estimates of molecular weights for large molecules provided that the centrifuge experiment is designed to allow time for the boundary to diffuse. Calculations suggest that if a speed is chosen to give a time-interval of approx. 3 h for the molecule to sediment from 6 cm to 7 cm, then molecular weights even of viruses can be estimated. For example, a particle having a sedimentation coefficient of 186×10^{-13} s would require a speed of between 6000 and 8000 rev./min to fulfil this criterion. These predictions have yet to be tested experimentally. A further factor would be the large dependence of the parameters on concentration, since the Fujita-MacCosham equation (eqn. 3) makes no allowance for this dependence. It would seem, however, that with particles that are more dense than proteins, the difference in density across the boundary would be larger and instability would not occur until lower concentrations than those given for the present analysis had been reached.

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