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Sedimentation-Equilibrium Studies on the Heterogeneity of two β-Lactamases

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Solutions of crystalline β-lactamase I and β-lactamase II, prepared by Kuwabara (1970), were examined in the ultracentrifuge and their sedimentation coefficients, diffusion coefficients, molecular weights and heterogeneity determined. Each sample was shown to consist of a major component comprising at least 97% of the material and a minor component of much higher molecular weight. The molecular weights of the major components were 27800 for β-lactamase I and 35600 for β-lactamase II. Emphasis is placed on a straightforward practical way of analysing the sedimentation-equilibrium results on mixtures of two macromolecular components rather than on a strict theoretical solution. Appendices describe the theory of systems at both chemical and sedimentation equilibrium and the procedure for calculating the combined distribution of two components.

The power of the sedimentation-equilibrium technique for the evaluation of the thermodynamic parameters of macromolecular solutions and for the study of systems in chemical equilibrium is well understood. However, its potentialities for the analysis of simple mixtures have been somewhat neglected in comparison with the more straightforward velocity methods (sedimentation velocity and electrophoresis). It is shown here that the technique can be used to detect and to measure very small quantities of a second component. It is also shown that a few per cent of an impurity (or an aggregate of the main component) can have a significant effect on the solution properties. Thus, if reliable results are to be obtained on the major component, it is essential that the contributions of minor components be determined and subtracted from the whole.

With chemically reacting systems, each individual species is itself at sedimentation equilibrium as well as at chemical equilibrium at all points in the cell (Nichol & Ogston, 1965; see also Appendix I). Thus, when at sedimentation equilibrium, a system in chemical equilibrium behaves exactly like a simple mixture of the relevant components, since each of these is itself at sedimentation equilibrium, and can therefore be treated as such. Thus the treatment given here for mixtures is also applicable to reacting systems, especially self-aggregating ones. It should be noted, however, that the total quantity of each component in such a system will change when it is subjected to a gravitational field and comes to sedimentation equilibrium. Thus the application of the condition of conservation of mass to each component separately is not applicable to such a system. The treatment given here applies only to systems behaving ideally. If the assumption of ideality proves to be unjustified then only apparent molecular weights will be obtained, and with self-aggregating systems the full theory of Adams (1965*a*,*b*, 1967) would have to be used. However, many dilute protein solutions in buffered salt solutions at normal pH do behave very nearly ideally, so that the simpler treatment given here is justified.

MATERIALS AND METHODS

The samples of β-lactamase I and β-lactamase II were prepared by Kuwabara (1970) and were taken from his stage 7, first crystallization, of β-lactamase I, and stage 7, second crystallization, of β-lactamase II. Solutions of β-lactamase I were dialysed against 0.1 M-tris–acetate buffer, pH 7, and solutions of β-lactamase II were dialysed against 0.1 M-tris–acetate buffer, pH 7, containing 0.33 mM-$ZnSO_4$. These solutions were examined in a Beckman Spinco model E ultracentrifuge equipped with both schlieren and Rayleigh-interference optical systems. The optical systems had been accurately aligned by methods essentially the same as those described by Gropper (1964). Sedimentation-velocity studies were done at 20°C and at 59780 rev./min by using schlieren optics. Diffusion measurements were also made with the ultracentrifuge at 20°C by using a synthetic-boundary cell at low speed, and the concentration distributions within the boundaries were recorded by means of the Rayleigh interferometer.

The sedimentation-equilibrium experiments were done at 3°C at speeds of 10589 rev./min for β-lactamase II and 12590 rev./min for β-lactamase I. The initial concentrations of the most concentrated solutions were determined by comparing the refractive index of the solution with that of its diffusate in a Brice–Phoenix differential refractometer. The more dilute solutions were obtained by careful dilutions of the initial solution with diffusate. All concentrations are quoted in terms of fringes, where one fringe is equal to $2.20\times10^{4}\Delta n$ where Δn is the difference in refractive index between the solution and its diffusate. The solutions were centrifuged in 3 mm columns contained in either 12 mm or 30 mm aluminium-filled-Epon double-sector centrepieces for at least 16 h. The resulting concentration distributions were determined by using the Rayleigh interferometer and the concentration-gradient distributions were determined with schlieren optics. Base-line interference photographs were taken at the beginning of the run and also after redistributing the contents of the cell after the run and bringing the rotor back to its original operating speed. The latter photographs were generally used for the measurements. The position of the hinge point (that point in the solution column at equilibrium where the concentration was the same as before centrifugation) was determined by the white-light fringe method of Richards & Schachman (1959), the refractive index of the solvent having been raised to that of the solution by addition of butane-1,3-diol. The addition of butane-1,3-diol to the solvent not only enables the white-light fringe to be observed but also improves the quality of the monochromatic fringes, since they are more nearly zero-order fringes (Richards, Teller & Schachman, 1968).

The partial specific volumes of the two enzymes were calculated from their amino acid composition as described by Schachman (1957; see also McMeekin & Marshall, 1952), since insufficient material was available for a reliable experimental determination. For this purpose a value of 0.666 was used for the partial specific volume of the amino sugars present in β-lactamase II as recommended by Gibbons (1966). The presence of amino sugars in β-lactamase II introduces some uncertainty into the calculation of the partial specific volume, since it is not known whether they are present as free amino sugars or as their *N*-acetyl derivatives; also, some destruction of the amino sugars is possible as a result of the hydrolysis procedures used before the amino acid analysis. However, these uncertainties amount only to about 1.5% in the calculated value of the partial specific volume, resulting in an uncertainty of about 3% in the calculated molecular weight of β-lactamase II.

All photographs were measured by means of a microcomparator (Projectorscope; Precision Grinding Instrument Co.). The plates were aligned with respect to the comparator axes by means of the fringe patterns produced by the reference holes, and the radial co-ordinates were measured with respect to the shadow of the wire built into the counterbalance, the position of which had previously been calibrated with respect to the reference hole in the side of the rotor. Measurements of the fringe displacement with respect to the hinge point were made at intervals of 0.2 mm on the plate, resulting in about 30 readings across the solution column. Weight-average molecular weights were determined from the slopes of plots of $\ln c$ versus r^2, where c is the total concentration, determined from the interferograms, and r is the distance from the centre of rotation. Since these plots were not straight lines, the gradients at about 26 points along the curve were determined either by drawing tangents to the experimental curve or by fitting a straight line through each successive set of five adjacent points by the method of least squares. The latter method was found to be satisfactory for experiments at higher concentrations, where the scatter of points was very small, but the former method was preferred for more dilute solutions, where the scatter was rather greater. The *z*-average molecular weights were determined from the schlieren patterns by the method of Lamm (1929) by using either the statistical-fit method or tangents as described above. The $(z+1)$-average molecular weight for β-lactamase II was determined by the method of Wales (1948).

RESULTS AND DISCUSSION

Sedimentation-velocity studies showed that the sedimentation coefficients at 20°C in 0.1 M-tris–acetate buffer were 2.8 S for β-lactamase I and 2.7 S for β-lactamase II. Neither value was appreciably dependent on concentration. The diffusion coefficients were 8.2×10^{-7} cm²/s for β-lactamase I and 7.2×10^{-7} cm²/s for β-lactamase II.

The results of the sedimentation-equilibrium experiments are shown in Fig. 1 for β-lactamase I and in Fig. 2 for β-lactamase II, which show the weight-average molecular weight, M_w, and the *z*-average molecular weight, M_z, plotted as a function of r^2 [Fig. 2 also shows the $(z+1)$-average molecular weight, M_{z+1}, as a function of r^2]. In each case the results for M_w are taken from two experiments at different concentrations to show that over most of the cell M_w was independent of concentration.

The most significant feature of Figs. 1 and 2 is that in the top half of the centrifuge cell the M_w and M_z values are the same, indicating that the material in this part of the cell was homogeneous,

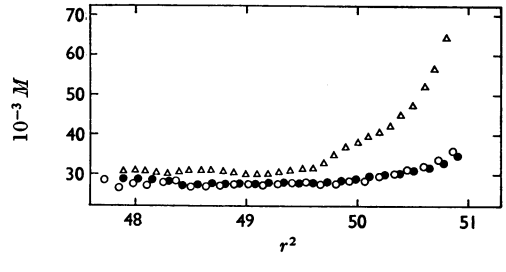

Fig. 1. Weight-average and *z*-average molecular weights of β-lactamase I as a function of the distance, *r*, from the centre of rotation at sedimentation equilibrium. ○, M_w when the initial concentration was 18.5 fringes; ●, M_w when the initial concentration was 9.25 fringes; △, M_z when the initial concentration was 18.5 fringes.

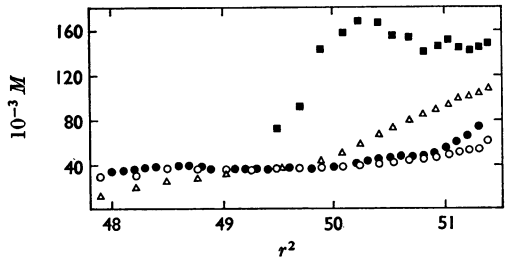

Fig. 2. Weight-average, z-average and $(z+1)$-average molecular weights of β-lactamase II as a function of the distance, r, from the centre of rotation at sedimentation equilibrium. ○, M_w when the initial concentration was 18.4 fringes; ●, M_w when the initial concentration was 6.13 fringes; △, M_z when the initial concentration was 18.4 fringes; ■, M_{z+1} when the initial concentration was 18.4 fringes.

but that lower down the cell they diverge markedly. The lack of dependence of M_w on the concentration, in the range 6–18 fringes, over most of the cell also indicates ideal behaviour. In Fig. 2 the curve for M_z appears to fall below that for M_w near the top of the cell. Apparent M_z values can only fall below M_w values when the non-ideality terms in the sedimentation-equilibrium equations are significant (Haydon & Peacocke, 1968), but the more accurate M_w results at different concentrations indicate that these terms are not significant. However, the difference between M_w and M_z in this region can be accounted for if the relevant measurements on the schlieren photographs were in error by only 40 μm. Schlieren patterns are never very clear near the meniscus (especially when the off-set double-slit mask is in use for interference optics) and in any case the lines are about 300 μm wide, so this error is considered to be within reasonable limits. The error has a very serious effect on the $(z+1)$-average, which is why these results are not plotted below $r^2 = 49.4$. (In fact the values of M_{z+1} actually obtained from the results plotted in Fig. 2 rose again at low values of r^2, so that near the meniscus the average molecular weights appeared to increase in the order M_z, M_w, M_{z+1}; this is an impossible situation that can only be resolved if it is assumed that M_z is in error and the three values become equal near the meniscus.) The deduction of M_{z+1} involves not only M_w and M_z but also dM_z/dc (Wales, 1948). This latter quantity is very subject to errors in M_z (which is itself sensitive to small errors in the reading of schlieren photographs), so that the values of M_{z+1} obtained must inevitably be rather inaccurate. Even so, the scatter of points at values of r^2 greater than 50 is only ±10%, so that the sudden threefold rise of M_{z+1} between $r^2 = 49.4$ and $r^2 = 50$ must be real. In a mixture of two species differing five- or six-fold in molecular weight M_{z+1} is not very dependent on the weight fraction of the heavier material present when this exceeds about 20%. These observations therefore suggest very strongly that this sample of β-lactamase II consisted of a mixture of two components, one with a molecular weight of about 35600 and the other with a much higher molecular weight. At the rotor speed used in this experiment (12590 rev./min) the heavier material would be effectively removed from the top part of the cell, so that only the smaller component was observed there. Since M_w, M_z and probably M_{z+1} all become equal there, this component must be homogeneous. It remains to calculate the molecular weights of the two components and the weight fraction of each in the original sample.

In the discussion that follows, subscripts 1 and 2 are used to designate quantities referring to the lighter and heavier components respectively. Thus M_1 is the molecular weight of the lighter material and M_2 is the molecular weight of the heavier material. Similarly c_1 and c_2 represent the concentrations of the two components at some particular position in the cell at distance r from the centre of rotation, and c represents the total concentration of protein. Superscripts ^{0} are used to designate the original concentrations before centrifugation. The weight fraction of the heavier component is designated x, and for convenience M_2/M_1 is called n. (Where the heavier component is an aggregate of the lighter component the significance of n is obvious, but this is not necessarily its meaning.) It is convenient for the analysis to proceed in terms of n, since this term is necessarily a constant and is derivable from observations pertaining to all levels of the cell.

From the definitions of M_w and M_z in a two-component mixture it is easily shown that:

$$M_w = M_1 + x(M_2 - M_1) \quad (1)$$

and

$$M_w M_z = M_1^2 + x(M_2^2 - M_1^2) \quad (2)$$

Eliminating x from eqns. (1) and (2), rearranging and introducing $n(= M_2/M_1)$ gives:

$$n = \frac{M_w(M_z - M_1)}{M_1(M_w - M_1)} \quad (3)$$

Thus, given M_1, and M_w and M_z as functions of r^2, n may also be calculated as a function of r^2.

The results for β-lactamase I and β-lactamase II are given in Table 1. The values of n for each solution are fairly constant in spite of quite large changes in M_w and M_z, which confirms that only two species are present in detectable quantities. If other species were present, n would be expected to show a steady change with r^2. Since n appears to be constant and does not change

Table 1. *Weight-average and z-average molecular weights and the values of n deduced from them by eqn. (3) at various levels in the centrifuge cell below the point where these two average molecular weights began to differ*

The values refer to experiments on solutions whose original concentrations were 18.5 fringes for β-lactamase I and 18.4 fringes for β-lactamase II. The values of M_1, deduced from the top part of the cell where M_w was independent of r, were 27800 for β-lactamase I and 35600 for β-lactamase II.

β-Lactamase I				β-Lactamase II			
r^2	M_w	M_z	n	r^2	M_w	M_z	n
50.00	29100	38200	8.2	50.40	39100	51600	5.7
50.10	29500	39300	6.9	50.54	39900	57300	6.0
50.20	29900	40200	6.3	50.67	40100	63900	7.6
50.30	30400	42100	5.9	50.80	41300	70100	7.5
50.40	31300	44700	5.4	50.92	42900	76300	7.0
50.50	32000	47300	5.3	51.03	44400	82200	6.9
50.60	33000	51800	5.5	51.12	45900	87600	6.7
50.70	34000	56400	5.6	51.21	48200	93300	6.3
50.80	35200	64000	6.2	51.30	51700	99000	5.8

systematically with r^2, it is permissible to take an average value of n to allow M_2 to be calculated as follows.

Substituting n into eqn. (1) and rearranging gives:

$$x = \frac{(M_w/M_1) - 1}{n - 1} \qquad (4)$$

This enables the values of M_1 and n to be combined with M_w to give x as a function of r^2. The original results give c (the total concentration) as a function of r^2, hence c_2 ($= xc$) can be deduced as a function of r^2, and a graph plotted of $\ln c_2$ versus r^2 (Fig. 3), from the slope of which M_2 can be calculated. Although n is involved in the calculation of c_2, it does not affect the slope of a plot of $\ln c_2$ versus r^2 because $(n-1)$ is a constant and appears as a multiplying factor in the expression for c_2 and so disappears when $\ln c_2$ is differentiated with respect to r^2. The plot of $\ln c_2$ versus r^2 may be extrapolated to the bottom of the cell and, if necessary, to the top of the solution column, to yield values of c_2 at these two points. (The correct value of n must be used to deduce c_2 before this extrapolation can be done.)

The condition of conservation of mass in a sector-shaped cell may be written:

$$\frac{c_b - c_m}{c^0(r_b^2 - r_m^2)} = \frac{M(1-\bar{v}\rho)\omega^2}{2RT} \qquad (5)$$

where c_b and c_m are the concentrations of one component at the bottom meniscus and the top meniscus respectively, r_b and r_m are the radial co-ordinates of these two menisci, and the other symbols have their usual significance. The values of c_2 at the ends of the solution column deduced above may be substituted into eqn. (5) and c_2^0 deduced. This is the concentration of the second component in the solution before centrifugation. Since the total concentration of the original solution is known its composition can now be specified. As a final check, the concentration distribution of such a mixture may be calculated theoretically and compared with the experimental curve (see Appendix II). These curves are shown in Figs. 4 and 5, which are based on the results given in Table 2.

It should be emphasized that the compositions of the two preparations, presented in Table 2, are based on the assumption that the heavier material is not in chemical equilibrium with the lighter component. With an equilibrium between a monomer and an n-mer, the effect of the gravitational field is to increase the quantity of the n-mer present in the cell. This arises because the hinge point for a heavy molecule is nearer the bottom of the cell than for a lighter molecule. However, when the two constituents are in chemical equilibrium the concentration of n-mer must be the same as in the original solution at the overall hinge point (as determined by the white-light fringe). This must be lower than the value of c_2^0 deduced from eqn. (5). Thus the question whether or not the heavier material is in chemical equilibrium with the lighter material may be resolved by calculating the quantity of heavier material present in experiments run either at different speeds or at different initial concentrations. The apparent proportion of the heavier material should increase as either the initial concentration or the speed is increased. The calculations described above, and illustrated in Figs. 1–5, have been repeated on a solution of β-lactamase I of half the original concentration and on a solution of β-lactamase II of one-third the

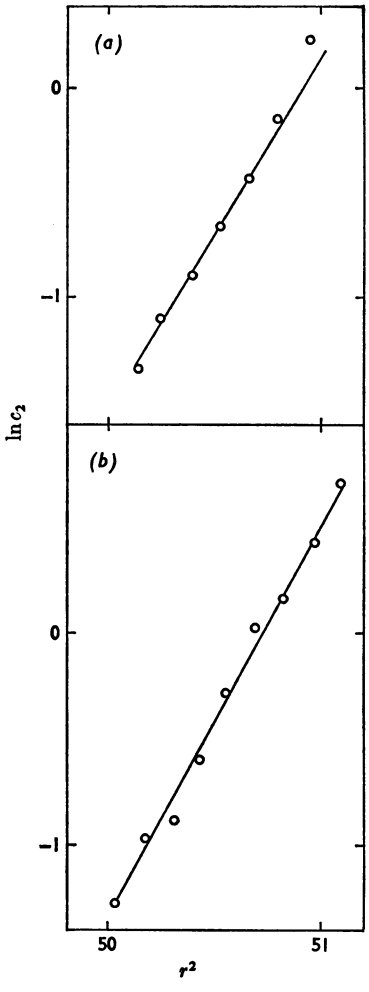

Fig. 3. Plot of $\ln c_2$ as a function of r^2 for (*a*) β-lactamase I and (*b*) β-lactamase II at sedimentation equilibrium, where c_2 is the concentration of the heavier component deduced as described in the text and r is the distance from the centre of rotation.

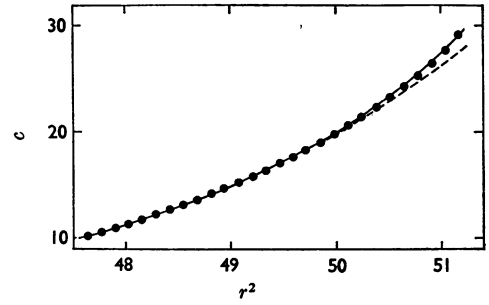

Fig. 4. Concentration distribution of β-lactamase I as a function of the distance from the centre of rotation at sedimentation equilibrium compared with the theoretical distribution calculated from the data in Table 2. ——, Theoretical distribution; ●, experimental points (original concentration 18.5 fringes); – – – –, theoretical distribution for the lighter material only; c is the concentration in fringes, and r is the distance from the centre of rotation.

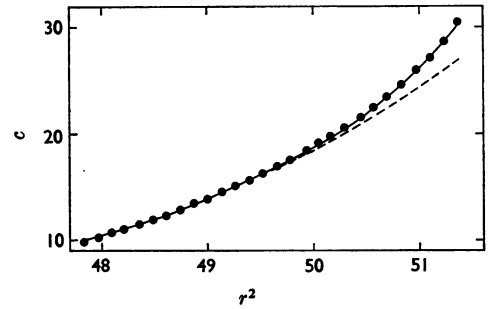

Fig. 5. Concentration distribution of β-lactamase II as a function of the distance from the centre of rotation at sedimentation equilibrium compared with the theoretical distribution deduced from the data in Table 2. ——, Theoretical distribution; ●, experimental points (initial concentration 18.4 fringes); – – – –, theoretical distribution for the lighter component only. c is the concentration in fringes, and r is the distance from the centre of rotation.

original concentration. If the heavier material were in chemical equilibrium with the lighter material (it would be a hexamer) then twofold dilution would decrease the concentration of the aggregate 64-fold ($64 = 2^6$), and threefold dilution would decrease the concentration of the aggregate 729-fold ($729 = 3^6$). In fact no such decrease was observed. The molecular weights were found to be unchanged, and the actual quantity of the high-molecular-weight material was 1.0% for β-lactamase I and 4.7% for β-lactamase II at the lower concentrations. The small decrease for β-lactamase I is probably not significant, but the increase for β-

Table 2. *Composition of the solutions of β-lactamase I and β-lactamase II that were used to construct the theoretical concentration distributions at sedimentation equilibrium plotted in Figs. 4 and 5*

	β-Lactamase I	β-Lactamase II
M_1	27800	35600
M_2	173000	244000
c_1^0 (fringes)	18.22	17.74
c_2^0 (fringes)	0.27	0.65
$100\,c_2^0/(c_1^0+c_2^0)$	1.5	3.5
n	6.3	6.6
Speed (rev./min)	12590	10589
Temperature	276°K	276°K
$\bar{v}$	0.73	0.71

lactamase II from 3.5 to 4.7% is more significant. The reason for this apparent increase is not obvious. However, a theoretical reconstruction of the concentration distribution for this solution assuming 3.5% of the heavier material was a rather poor fit to the experimental curve (being in error by as much as half a fringe near the bottom of the cell, where the concentration was about 10 fringes). Therefore the result would seem not to be spurious. Whatever the explanation, the results on diluted solutions of both enzymes indicate clearly that the heavier material is not in rapid chemical equilibrium with the lighter one.

Since the amount of contaminating material is so small, the sedimentation-velocity patterns must effectively be due to the major component only, and hence the sedimentation and diffusion coefficients must refer to the lighter component. The sedimentation coefficients were combined with the molecular weights derived above to yield values for the frictional ratios (f/f_0). These are 1.16 for β-lactamase I and 1.70 for β-lactamase II. The former value is fairly typical of a globular protein, but β-lactamase II is evidently either more asymmetric or more hydrated than is β-lactamase I. Frictional ratios cannot alone distinguish between these two possibilities, but if each is assumed to be hydrated to an extent of 0.3 g of water/g of protein (a fairly typical value) then the axial ratios of the equivalent prolate ellipsoids, calculated from the equation of Perrin (1936), are 2.1 for β-lactamase I and 4.2 for β-lactamase II. Alternatively, if β-lactamase II is assumed to have the same axial ratio as β-lactamase I, then it must be hydrated to an extent of 2.3 g of water/g of protein. The truth probably lies between these two extremes.

The results obtained in this work on β-lactamase I are in close agreement with those published by Hall & Ogston (1956). The difference in molecular weight (Hall & Ogston quote 30600) results from the fact that these authors assumed a partial specific volume of 0.75, compared with the value of 0.73 used in this work. If the values of the sedimentation coefficients and diffusion coefficients quoted here are substituted in the Svedberg equation, one obtains a molecular weight of 29000 for β-lactamase I and 33000 for β-lactamase II. Considering the possible errors in determining the two coefficients separately, compared with the precision obtainable in sedimentation-equilibrium experiments, these values must be considered as being in very good agreement with those obtained by the latter method. Sedimentation-velocity and diffusion experiments could not be expected to reveal the very small amounts of second components that were so readily detected by sedimentation equilibrium.

We thank Dr S. Kuwabara and Professor E. P. Abraham, F.R.S., for supplying us with samples of β-lactamase I and β-lactamase II, and Dr A. J. Haydon, whose preliminary experiments on these two enzymes stimulated our interest in them. We acknowledge financial support from the Medical Research Council.

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APPENDIX I

It is normally assumed that any chemical equilibrium constant is independent of the gravitational potential (ϕ), i.e. it is unaffected by the presence of a gravitational field of $-\mathrm{d}\phi/\mathrm{d}x$ gravitational force per unit mass. The formal thermodynamic proof of this (following Guggenheim, 1967) may be briefly recapitulated as follows. Let any chemical equation be represented by:

$$0 = \sum_J \nu_J J \qquad \text{(i)}$$

where J represents the various chemical species and ν_J the number of moles of each involved in the usual chemical equation with the added convention that ν_J is negative for reactants and positive for products. The most general form for the condition of equilibrium in the absence of a gravitational field is (when ϕ is zero):

$$\sum_J \nu_J \mu_J = 0 \qquad \text{(ii)}$$

where the μ_J are molar chemical potentials. In the presence of a gravitational field, within a region where the gravitational potential may be regarded as virtually constant (in practice this means a lamina perpendicular to the field and of thickness small in relation to the variation of ϕ), then this condition for chemical equilibrium becomes:

$$\sum_J \nu_J(\mu_J + \phi M_J) = 0 \qquad \text{(iii)}$$

where the M_J are the molecular weights of the species J, and because of the conservation of mass in chemical processes are related by:

$$\sum_J \nu_J M_J = 0 \qquad \text{(iv)}$$

Multiplication of eqn. (iv) by a constant ϕ and subtraction of the product from eqn. (iii) restores eqn. (ii) as still the condition for chemical equilibrium in the presence of a gravitational potential ϕ; consequently, the thermodynamic equilibrium constants are independent of the value of the gravitational potential ϕ, whatever the relation of ϕ to the spatial co-ordinates may be, and hence are also independent of the presence of a gravitational field.

On the normal scale of laboratory experiments the variation of ϕ due to the earth's field within, say, a few millilitres of a solution of a macromolecule is negligible, and the situation is that envisaged in eqn. (iii) with ϕ non-zero but virtually the same in all parts of the solution. However, in the gravitational field of the ultracentrifuge ϕ only remains constant within infinitesimally small laminae perpendicular to the direction of the field. There are a series of laminar phases (..., α, β, ...) each with its characteristic gravitational potential ..., ϕ^α, ϕ^β, ..., etc. (Table 1).

With this formalism in mind, it is now possible to consider the question: 'If there is chemical equilibrium at one level and sedimentation equilibrium throughout, will there be chemical equilibrium at all levels?' The answer is 'there is' and the proof is as follows.

Let (1) chemical equilibrium be established in phase α at the gravitational potential ϕ^α, as denoted by the asterisk in Table 1; (2) sedimentation equilibrium be established for each and every component throughout the system, so that for J, for example, the quantity $(\mu_J + \phi M_J)$ is the same in all the phases ..., α, β, ..., which is the thermodynamic condition of sedimentation equilibrium (from which all the relations between concentration and r follow); (3) the components 1, 2, ..., J, ... be all involved in the chemical reaction of eqn. (i). Let superscripts denote phases. Then for the phase α because of eqn. (ii):

$$\sum_J \nu_J \mu_J^\alpha = 0 \qquad \text{(v)}$$

and also:

$$\sum_J \nu_J(\mu_J^\alpha + \phi^\alpha M_J) = 0 \qquad \text{(vi)}$$

since eqn. (iv) applies within the phase α, which is specified by having the same gravitational potential, ϕ^α, throughout. Because sedimentation equilibrium is established (2) we can also write for each and every component that comparing phase α, where there is chemical equilibrium, with any other phase β:

$$\mu_J^\alpha + \phi^\alpha M_J = \mu_J^\beta + \phi^\beta M_J \qquad \text{(vii)}$$

and so:

$$\mu_J^\beta - \mu_J^\alpha = (\phi^\alpha - \phi^\beta) M_J \qquad \text{(viii)}$$

Multiplication by ν_J and summation over all J yields:

$$\sum_J \nu_J \mu_J^\beta - \sum_J \nu_J \mu_J^\alpha = \sum_J (\phi^\alpha - \phi^\beta)\nu_J M_J \qquad \text{(ix)}$$

$$= (\phi^\alpha - \phi^\beta) \sum_J \nu_J M_J \qquad \text{(x)}$$

The quantity $(\phi^\alpha - \phi^\beta)$ is a common factor for all the components and for this important reason can be taken outside the summation on the right-hand

Table 1. *Chemical and gravitational potentials at different levels in a field*

Laminar phases	Component ... Gravitational potential	1	2	. .	J	. .
.	.	.	.	. .	.	. .
.	.	.	.	. .	.	. .
*α	ϕ^α	μ_1^α; $\phi^\alpha M_1$	μ_2^α; $\phi^\alpha M_2$	. .	μ_J^α; $\phi^\alpha M_J$	. .
.	.	.	.	. .	.	. .
.	.	.	.	. .	.	. .
β	ϕ^β	μ_1^β; $\phi^\beta M_1$	μ_2^β; $\phi^\beta M_2$	. .	μ_J^β; $\phi^\beta M_J$	. .
.	.	.	.	. .	.	. .
.	.	.	.	. .	.	. .

side of eqn. (ix), as in eqn. (x). Since mass is conserved in the chemical process (eqn. iv), the right-hand side of eqn. (ix) is zero and, since chemical equilibrium is established in phase α (eqn. v), it follows that:

$$\sum_J \nu_J \mu_J^\beta = 0 \qquad \text{(xi)}$$

Hence chemical equilibrium is also established in phase β and so in all other phases, if it is established at one level and if sedimentation equilibrium of the individual components has also been established [conditions (1) and (2) above].

This conclusion is, in some ways, intuitively obvious since, if it were not so, there would be net formation of one of the molecular species in a level at which chemical equilibrium is not attained and so net transport in one direction down or up in the cell, contrary to the establishment of sedimentation equilibrium. However, the proof is necessary to establish whether or not the usual equations representing the distribution with respect to r of macromolecular solutes can be applied when these solutes are in chemical equilibrium.

The condition that sedimentation equilibrium of each component is separately established, so that $(\mu_J + \phi M_J)$ is constant throughout all phases and ϕ values of the system, has been shown to be thermodynamically consistent with chemical equilibrium also being established so that at each level eqn. (ii) applies. For a system in which both kinds of equilibrium are set up the equations:

$$(\mu_J + \phi M_J) = \text{constant} \qquad \text{(xii)}$$

and

$$\mathrm{d}\mu_J + M_J \mathrm{d}\phi = 0 \qquad \text{(xiii)}$$

apply to each molecular species, where the differentiation in eqn. (xiii) refers to passage from a level r and potential ϕ to $(r+\mathrm{d}r)$ and $(\phi+\mathrm{d}\phi)$. Hence the various equations that represent the distribution at sedimentation equilibrium of each individual component can be applied when these components participate in a chemical equilibrium at each level.

The preceding proof refers to any system of the kind depicted whether or not it is thermodynamically ideal and so widens the scope of the derivation previously made by Nichol & Ogston (1965).

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APPENDIX II

At sedimentation equilibrium the concentration, c, of a single macromolecular component at any distance, r, from the centre of rotation is given by:

$$c = C \exp(ML r^2) \qquad \text{(xiv)}$$

where L is $(1-\bar{v}\rho)\omega^2/2RT$ and C is an integration constant. If a solution contains two non-interacting macromolecular components the total concentration at any level in the cell is the sum of the two individual concentrations. Hence:

$$c = c_1 + c_2 = C_1 \exp(M_1 L_1 r^2) + C_2 \exp(M_2 L_2 r^2) \qquad \text{(xv)}$$

where the subscripts refer to the two macromolecular components.

In the experiments described in the main paper c_2 became negligible near the top meniscus. Measurements of the concentration distribution in this region enabled M_1 and hence C_1 to be calculated. M_2 was deduced as described in the Results and Discussion section of the main paper, and measurements of c near the bottom meniscus enabled C_2 to be calculated. The total concentrations, c, of the solution at many different values of r^2 (intervals of 0.2) were calculated from eqn. (xv) and used to construct the curves shown in Figs. 4 and 5 of the main paper.