The Equilibrium Sedimentation of Hyaluronic Acid and of Two Synthetic Polymers

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1. The method of equilibrium sedimentation has been investigated as an alternative to osmotic-pressure measurement for determining thermodynamic properties of polymer solutions at relatively high concentrations. 2. The simplifications that must be made in the theoretical treatment are discussed. 3. Measurements have been made on samples of polyethylene glycol, neutralized polymethacrylic acid and hyaluronic acid. With the first and third, values of the 'non-ideality coefficients' have been obtained that agree with those obtained from osmotic measurements on the same materials. 4. Evidence has been obtained of the presence in hyaluronic acid preparations of a fraction that has either a lower degree of thermodynamic non-ideality or a higher density increment than the bulk of the sample. This fraction is not protein.

We were attracted to the use of the equilibrium sedimentation of hyaluronic acid, as a possible means of measuring its osmotic properties, because of the technical difficulties of making direct osmotic measurements on solutions of the order of concentration of its occurrence in the body (Preston, Davies & Ogston, 1965). In spite of the long period required for the attainment of sedimentation equilibrium, the amount of information obtainable from a single experiment should be equal to that obtained from many osmotic determinations; at the same time, the difficulties and errors that beset osmotic measurements on solutions of this sort (Davies, 1966) may be avoided. Our attention was drawn to the possibility of the method by the observations of Dr E. A. Balazs (personal communication) that, when solutions of hyaluronic acid are sedimented for long periods at high speed, an apparently compacted layer of solute is formed at the bottom of the cell. In his preliminary experiments this layer appeared dark, owing to the deflexion of light passing through it beyond the limits of the optical system; but calculations based on measurements of osmotic pressure (Preston *et al.* 1965) suggested that conditions could be found in which the optical schlieren pattern would be visible throughout the equilibrium distribution. When these conditions are used the equilibrium-gradient

curve of hyaluronic acid exhibits a maximum; although the equations for equilibrium sedimentation of a thermodynamically non-ideal solute predict the possible occurrence of such a maximum, we believe that attention has not been drawn to this before, and that it has not been observed experimentally. As shown below, the equilibrium-gradient curve can be used to obtain approximate values of virial coefficients of a thermodynamically non-ideal polymer.

We have performed equilibrium-sedimentation experiments with two preparations of hyaluronic acid (the UFR* from ox synovial fluid; and material deproteinized on DEAE-Sephadex; Preston *et al.* 1965) in the concentration range 0–17 mg./ml. The thermodynamic coefficients obtained from these experiments agreed satisfactorily with those obtained from direct osmotic measurements (in a lower range of concentration) by Preston *et al.* (1965). The experiments suggest, however, a type of polydispersity in the material that we have not yet been able to explain.

Partly as a check on the method with chemically better-defined material and partly in view of the possible use of this method with other high polymers, we have also made some measurements on a sample of polyethylene glycol, which is relatively homogeneous and well-characterized, and on a sample of polymethacrylic acid believed to be highly polydisperse.

Since the development of the ultracentrifuge,

* Abbreviation: UFR, ultrafilter residue.

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sedimentation equilibrium has been available as a means for determining relatively high molecular weights in solution (Svedberg & Pedersen, 1940; Schachman, 1959). Because of the partial separation of polydisperse material, and because of the marked effect of thermodynamic non-ideality on the equilibrium distribution, the method has been applied mainly to relatively homogeneous materials (such as proteins) whose solutions do not exhibit a high degree of thermodynamic non-ideality. Attention has been paid to the effects of heterogeneity and of non-ideality chiefly with the object of avoiding or eliminating them. Williams, Van Holde, Baldwin & Fujita (1958) described earlier attempts to make allowance for non-ideality in polydisperse systems, and drew attention to the difficulties of doing this, which are further discussed below. Wales (1951) described a method for determining the virial coefficients of polymers by obtaining several equilibrium distributions of the same solution at different speeds of rotation; he applied this method to several polymers with results that agreed with osmotic data. More recently, Fujita, Linklater & Williams (1960) have described a method of measuring the second virial coefficient of a polymer, and Fujita (1962), Osterhoudt & Williams (1965) and Casassa & Eisenberg (1964) have given detailed theoretical discussions of the effects of non-ideality on the equilibrium sedimentation of monodisperse and polydisperse polymeric systems.

THEORY

Inspection of the equation of Casassa & Eisenberg (1964) for the equilibrium sedimentation of a polydisperse solute makes it immediately clear that anything approaching the complete interpretation of such an experiment would be a hopeless task. One would need to know the density increment and refractive increment of each component of the mixture, together with their dependence on pressure and composition; one would need also to interpret the equilibrium distribution in terms of coefficients that would describe the thermodynamic properties of the solution as functions, not only of concentration, but of composition and pressure. Extensive approximations are therefore necessary.

We begin by considering the equilibrium sedimentation of a non-ideal homogeneous solute, assuming that its specific refractive increment and density increment $\partial\rho/\partial c$ (Casassa & Eisenberg, 1964) are independent of concentration and pressure, and that its activity coefficient γ is independent of pressure. We can then use the specific refractive increment to measure concentration (with use of either schlieren or interference optics) and write for sedimentation equilibrium:

$$\frac{d \ln c}{dr^2} = \frac{\omega^2 M \partial \rho / \partial c}{2RT \left(1 + \frac{d \ln \gamma}{d \ln c} \right)} \quad (1)$$

where c is the concentration in g./ml., r is the radius of rotation, ω the angular velocity and M the molecular weight.

We choose to express the activity coefficient as a virial expansion of the form:

$$\ln \gamma = M(\alpha_1 c + \alpha_2 c^2 + \alpha_3 c^3 + \dots) \quad (2)$$

where $\alpha_1, \alpha_2, \dots$ are constant coefficients. From application of the Gibbs-Duhem equation it follows (for comparison) that the osmotic pressure is expressed by:

$$\frac{\pi}{RTc} = \frac{1}{M} + \frac{\alpha_1}{2} c + \frac{2\alpha_2}{3} c^2 + \frac{3\alpha_3}{4} c^3 + \dots \quad (3)$$

Although this is not one of the several usual forms of the virial expansion of the osmotic equation (see Tompa, 1956, equations 6.4 to 6.6), it is closely related to them, the coefficients $\alpha_1, \alpha_2, \dots$ (which, to avoid confusion, we shall call 'non-ideality coefficients') being proportional to the second, third etc. virial coefficients.

At this point we must notice that, although virial expansions of the type of eqn. (3) are usually taken to be convergent, although most theoretical treatments predict convergence, and although many experimental measurements of osmotic pressures are satisfactorily represented by cutting off the series at the c^2 term, we cannot be sure *a priori* that this is a justifiable assumption. More complex behaviour has been observed with certain systems (Schick, Doty & Zimm, 1950; Flory & Daoust, 1957; Leonard & Daoust, 1962). However, since no progress can be made without this assumption, we shall proceed tentatively, retaining the cubic term at this stage.

Differentiating eqn. (2) and putting it into eqn. (1), we obtain:

$$\frac{dc}{dr^2} = \frac{\omega^2 \partial \rho / \partial c}{2RT \left(\frac{1}{Mc} + \alpha_1 + 2\alpha_2 c + 3\alpha_3 c^2 + \dots \right)} \quad (4)$$

Eqn. (4) shows the effects of $\alpha_1, \alpha_2, \dots$ on the equilibrium gradient (schlieren) pattern, whose ordinate measures dc/dr : if $\alpha_1 = \alpha_2 = \alpha_3 = \dots = 0$, dc/dr^2 and dc/dr increase indefinitely with c and therefore with r^2 , the curvature of dc/dr^2 and of c against r^2 being everywhere positive (Fig. 1,

Fig. 1. Diagram to show the effects of non-ideality coefficients on the variation of dc/dr^2 with r^2 (and therefore with c) in sedimentation equilibrium. Curve I: $\alpha_1 = \alpha_2 = \alpha_3 = \dots = 0$; curve II: $\alpha_1 > 0$, $\alpha_2 = \alpha_3 = \dots = 0$; curve III: $\alpha_1 > 0$, α_2 etc. > 0 .

curve I); if $\alpha_1 > 0$ and $\alpha_2 = \alpha_3 = \dots = 0$, dc/dr^2 approaches a limiting value as c (and r) $\rightarrow \infty$ (Fig. 1, curve II) and c approaches linearity against r^2 ; if $\alpha_1 > 0$ and all α_2, α_3 etc. > 0 , dc/dr^2 passes through a maximum (Fig. 1, curve III) and the plot of c against r^2 has an inflexion beyond which its curvature is negative.

Differentiating eqn. (4) with respect to r^2 :

$$\frac{d}{dr^2} \left(\frac{dc}{dr^2} \right) = \frac{\omega^2 \partial \rho / \partial c}{2RT} \cdot \frac{\frac{1}{Mc^2} - 2\alpha_2 - 6\alpha_3 c - \dots}{\left(\frac{1}{Mc} + \alpha_1 + 2\alpha_2 c + 3\alpha_3 c^2 + \dots \right)^2} \cdot \frac{dc}{dr^2} \quad (5)$$

If it is to be possible to observe equilibrium sedimentation over a wide range of concentration by the conventional optical methods, dc/dr^2 or dc/dr must not be too large at any point in the cell; eqns. (4) and (5) show that this condition is favoured by a large value of α_1 in relation to $1/M$.

Eqn. (5) shows further that at the maximum of the schlieren curve:

$$\alpha_2 = \frac{1}{2Mc_{\max}^2} - 3\alpha_3 c_{\max} - \dots \quad (6)$$

Putting this result into eqn. (4) gives:

$$\left(\frac{dc}{dr^2} \right)_{\max} = \frac{\omega^2 \partial \rho / \partial c}{2RT \left(\frac{2}{Mc_{\max}} + \alpha_1 - 3\alpha_3 c_{\max}^2 - \dots \right)} \quad (7)$$

Eqns. (6) and (7) provide a means for estimating α_1 and α_2 only if the term in α_3 and all higher terms are negligible. In recognition of the uncertainty whether this is the case, discussed above, we shall denote by α_1^* and α_2^* quantities defined operationally by:

$$\alpha_2^* \equiv \frac{1}{2Mc_{\max}^2} \quad (6a)$$

$$\alpha_1^* \equiv \frac{\omega^2 \partial \rho / \partial c}{2RT (dc/dr^2)_{\max}} - \frac{2}{Mc_{\max}} \quad (7a)$$

In what follows, the insertion of α_1^* and α_2^* into eqns. (2)–(7) implies the neglect of terms in α_3, α_4 etc. If the series in eqn. (2) is properly convergent, it is to be expected that α_1^* will be a good approximation to α_1 , but even so α_2^* may deviate considerably from α_2 if terms in α_3 and higher terms are not negligible.

Knowledge of the value of M is required to estimate α_1^* and α_2^* . With a homogeneous solute, this can be estimated by extrapolation of equilibrium-sedimentation data to zero concentration, as described by Yphantis (1964) (provided that the conditions of sedimentation are such that c approaches zero at the meniscus) or by other means. However, provided that α_1^* is large compared with $4\alpha_2^*c$ (not necessarily larger in absolute magnitude than α_2^*) it follows from eqn. (6a) that α_1^* is large in comparison with the term $2/Mc_{\max}$ appearing in eqn. (7a). With this proviso, the estimate of α_1^* from eqn. (7a) will be insensitive to that of M .

This method for estimating α_1^* and α_2^* uses, in effect, only a single point on the schlieren curve (the maximum of dc/dr^2) and therefore does not test the consistency of the values of α_1^* and α_2^* over a range of concentration. This

test could, of course, be performed by putting the starred values back into eqn. (4) and comparing the predicted with the experimental curve. Alternatively, eqn. (4) in terms of starred quantities can be converted into one of several forms; for example by integration and rearrangement:

$$\frac{\ln c}{M} + \alpha_1^* c + \alpha_2^* c^2 = \frac{\omega^2 \partial \rho / \partial c}{2RT} r^2 + I \quad (8)$$

where I is the integration constant. It appears at first sight possible to use this equation as a multivariate regression to estimate M, α_1^* and α_2^* from the whole curve. In practice, this least-squares fitting (tested on computed curves of accuracy made comparable with those obtainable by experiment) is not successful, the high degree of correlation between the variables leading to unacceptable errors of estimation. However, least-squares fitting does become practicable if the number of variables is reduced by using an independent estimate of M and writing eqn. (8) as:

$$Z = \frac{\omega^2 \partial \rho / \partial c}{2RT} r^2 - \frac{\ln c}{M} = \alpha_1^* c + \alpha_2^* c^2 - I \quad (9)$$

Polydisperse solutions. It has been pointed out that in practice extensive approximations are necessary for polydisperse solutes. The method described demands (because of optical limitations) solutes of a high degree of non-ideality, if measurements are to be carried to higher concentrations; this means chain-polymers and these are always in some degree polydisperse.

In comparison with the measurement of osmotic pressure, equilibrium sedimentation is at a disadvantage in some respects. In the former, the composition is constant, independently of total concentration, and one can therefore operate with mean values of molecular weight, virial coefficients etc.; in the latter, variations within the material of the molecular weight, the density increment and the thermodynamic properties of the individual species will result in a variation of composition through the equilibrium distribution, so that average values referring to the whole material (however defined) are strictly inapplicable. Moreover, variation of specific refractive increments with composition will prevent the refractive record from representing exactly the distribution of mass concentration.

However, the situation may not be as bad as it appears. If the solute is a homologous polymer there is good reason for thinking that the specific refractive and density increments should vary little with molecular weight and therefore with composition, so that single values will serve for the whole distribution with a reasonable degree of accuracy. The coefficient α_1 (equivalent to the conventional second virial coefficient) also appears not to vary rapidly with molecular weight both experimentally (Bawn, Freeman & Kamaliddin, 1950) and theoretically (see Flory, 1953; Tompa, 1956; Fixman, 1960); an average value of α_1^* , as defined by eqn. (7a), can therefore be expected to represent the thermodynamic behaviour of a mixture fairly well. Less is known about α_2 (equivalent to the third virial coefficient), but this (as shown below) may make a relatively small contribution to the distribution. Finally, eqn. (4) shows that, at higher concentrations, provided that α_1 is high in relation to $1/M$, the distribution is dominated by the non-ideality terms, and, because of the small variation of α_1 with molecular weight, little fractionation with respect to composition is to be expected in this region. It remains true that in the

upper part of the distribution (in the region approaching zero concentration and thermodynamically ideal behaviour) there will be a relative excess of material of the lowest molecular weight, and a corresponding relative deficiency further down the cell. However, the change of composition in the more concentrated solution need not be large and such a change is not expected to have an important effect either on the mean value of α_1 or on the estimate of its value.

Limitations of the method. Taking the foregoing argument into consideration, it is desirable that the material should be such that a sufficient concentration is attainable within the optical limits of the system to justify the use of an average molecular weight in the greater part of the distribution. The optics of the Spinco model E ultracentrifuge limit the maximum angular deflexion of light in the cell to 4×10^{-2} radian, so that the maximum permissible gradient of refractive index is $4 \times 10^{-2}/b \text{ cm.}^{-1}$, when b is the cell thickness. If the specific refractive increment is, say, 0.15 ml./g. , then the limiting value of dc/dr is $0.26b$. This, with eqn. (4), gives a guide to the choice of b and ω^2 .

EXPERIMENTAL

Polyethylene glycol. This was a sample of Carbowax 6000 (Carbide & Carbon Chemicals Co., New York, N.Y. U.S.A.). Solutions were made by weight in water and the concentrations were expressed as g./ml. by use of the partial specific volume. The value of the latter was determined to be 0.837 by pycnometric measurement of the density of a solution of concentration $2.170 \times 10^{-2} \text{ g./ml.}$ at 25° ; the corresponding value of the density increment $\partial\rho/\partial c$ was 0.165 . The specific refractive increment was determined at $546 \text{ m}\mu$ in a differential refractometer (Cecil & Ogston, 1951) to be 0.137 ml./g. Alexandrowicz (1959b, and personal communication) has estimated the molecular weight of this material to be 8000 ± 500 by osmotic pressure and light-scattering, and this value has been used in the present work.

Polymethacrylic acid. This unfractionated sample was a gift from Professor D. O. Jordan; it was the free acid, freeze-dried. Its moisture content was determined by drying to constant weight in air at 110° . Solutions were made up by weight in 0.2 M-NaCl to approximately the required concentration; they were neutralized by addition of the calculated weight of solid NaHCO_3 and then dialysed against $0.2 \text{ M-NaCl-}0.12 \text{ M-NaHCO}_3$ (Preston *et al.* 1965). The change of concentration during dialysis was measured by weighing the mopped Visking sac before and after dialysis (Preston *et al.* 1965). The final pH was $6.9-7.0$. A pH titration in 0.2 M-NaCl by means of a Leeds and Northrup Coulometric Analyzer (platinum cathode and silver anode) showed that the acid is close to 100% neutralized at pH 7.

The specific refractive increment of the material neutralized to pH 7.0 and dissolved in 0.2 M-NaCl , measured against 0.2 M-NaCl , was found to be 0.296 at $546 \text{ m}\mu$ expressed as ml./g. of free acid. After dialysis against 0.2 M-NaCl , the value was 0.229 ml./g. of free acid measured against diffusate. The difference between these two values arises from the unequal distribution of diffusible ions, due to the Gibbs-Donnan equilibrium; it corresponds to a difference of 0.560 mole of $\text{NaCl}/\text{base-mole}$ of polymethacrylic acid, between solution and diffusate. The value obtained after dialysis was used in interpreting the light-scattering and sedimentation measurements (Casassa & Eisenberg, 1964).

Fig. 2. Zimm plot of light-scattering results obtained with polymethacrylate, pH 7, in 0.2 M-NaCl .

The partial specific volume of sodium polymethacrylate dissolved in 0.2 M-NaCl was taken to be 0.390 ml./g. of sodium salt, from the data of Andersson (1963); the corresponding value of $\partial\rho/\partial c$ is 0.6075 . However, for application to sedimentation equilibrium, this value has to be corrected to the value that it would have if measured against equilibrium diffusate (Casassa & Eisenberg, 1964), by allowing for the unequal distribution of NaCl , as estimated from the refractometric measurements (see above). The resulting value of $\partial\rho/\partial c$ is 0.493 , c being expressed as before as g. of free acid/ml.

The molecular weight of the polymethacrylic acid was estimated by measurement of light-scattering. Successive additions of a neutralized stock solution in $0.2 \text{ M-NaCl-}0.12 \text{ M-NaHCO}_3$, whose concentration was estimated refractometrically against diffusate, were made to diffusate and the scattering was measured in a Sofica (Le Mesnil Saint Denis, Seine-et-Oise, France) light-scattering photometer at $436 \text{ m}\mu$ at angles between 30° and 150° . The results, extrapolated by the method of Zimm (1948), are shown in Fig. 2. From the intercept at zero angle and zero concentration M_w is 3.2×10^5 .

Hyaluronic acid. Two samples were used. One was an 'ultrafilter residue' (UFR) that contained approx. 20% of protein, apparently bound to the hyaluronic acid; the other (S18) was an unfractionated sample from which the protein had been removed by DEAE-Sephadex. Both samples were prepared as described by Preston *et al.* (1965). The specific refractive increments of these materials, measured against equilibrium diffusate (Casassa & Eisenberg, 1964), were calculated from the data of Preston *et al.* (1965). Concentrations were measured refractometrically against equilibrium diffusate. The solvent was $0.2 \text{ M-NaCl-}0.12 \text{ M-NaHCO}_3$, pH $6.8-7.0$ (Preston *et al.* 1965). The density increment was taken to be 0.31 .

Sedimentation. This was performed in a Spinco model E ultracentrifuge with An-D rotor and schlieren optics. The temperature was controlled near 23° . The final (equilibrium) speed was chosen so as to keep the final refractive gradient within the required limits and, with one exception (polyethylene glycol, run C), the concentration at the meniscus near zero. During the initial parts of the run higher speeds were used so as to accelerate the attainment of equilibrium.

Table 1. *Details of equilibrium runs on polyethylene glycol and values of coefficients α_1^* and α_2^**

Times (hr.) are given in parentheses after the speeds.

	Run A	Run B	Run C
$10^3 \times$ Initial concn. (g./ml.)	3.59	29.54	100.0
Column height (cm.)	1.03	1.01	0.30
Initial speed (rev./min.)	52640 (22)	52640 (46)	52640
Final speed (rev./min.)	39460 (25)	50740 (13)	52640 (29)
$10^3 \times$ Final concn. at meniscus (g./ml.)	0	1.42	81.6
$10^3 \times$ Final concn. at bottom (g./ml.)	16.42	83.2	115.6
α_1^* (eqn. 7a)	—	5.4×10^{-3}	—
α_2^* (eqn. 6a)	—	2.1×10^{-2}	—
α_1^* (eqn. 9)	$4.3 \times 10^{-3*}$	$5.3 \times 10^{-3\dagger}$	$4.6 \times 10^{-3\dagger}$
α_2^* (eqn. 9)	—	$1.8 \times 10^{-2\dagger}$	$3.3 \times 10^{-2\dagger}$
Figure no.	3, 6	4	5

* By graphical plot.

† By least-squares fitting.

For the polymers a 3mm. single-sector 4° cell with Epon centre-piece was used, the base lines being determined in separate experiments with solvent alone. For most runs with hyaluronic acid a 12mm. double-sector ($2\frac{1}{2}^\circ$) cell was used, one sector being filled with solvent; for one run a 1.5mm. single-sector 4° cell, with aluminium centre-piece, was used. In all cases a thin layer of Kel-F polymer oil was included at the bottom of the cell. The runs were continued for a period after equilibrium appeared to have been reached, and this was checked by the identity of the terminal distribution with that measured 6–8hr. earlier.

Measurement and calculations. The plates were measured with two-co-ordinate comparator (Gaertner Toolmakers Microscope, type M2001, AS-P) after alignment of the radial direction along the x co-ordinate of the comparator. The differences of the y -co-ordinate readings of the schlieren patterns between experiment and base line, taken at a number (20–50) of equally spaced points between the meniscus or the position at which schlieren trace and base line were indistinguishable and the bottom of the column, gave (with use of the appropriate optical constants and specific refractive increment) values of dc/dr at each point. Successive trapezoidal integration of dc/dr then gave a value of c representing the excess of concentration at each point over that at the meniscus. A second trapezoidal integration of cr through the whole column (equivalent to $\int cr/dr$) measured the apparent total quantity of solute in the cell; comparison of this with the original filling gave, where this was required, the concentration at the meniscus and so the actual concentration at each point in the cell.

Where a maximum was present, this was located by plotting $(1/r)(dc/dr)$ in its vicinity, to give the maximum value of dc/dr^2 and the corresponding value of c .

Other calculations are described below.

RESULTS AND DISCUSSION

Polyethylene glycol. Because of the low molecular weight of this material, not all the features predicted by eqn. (4) were demonstrable in a single run, and three experiments were therefore performed at

Fig. 3. Schlieren diagram (60° angle) of equilibrium sedimentation of polyethylene glycol, run A; for details see Table 1. m, Meniscus; b, bottom-of-solution column.

widely differing initial concentrations (Table 1 and Figs. 3–5). In run B the maximum was present and values of α_1^* and α_2^* were calculated from this by use of eqns. (6a) and (7a). The value of α_1^* was also obtained from run A (where the concentration is relatively low, and the α_2^* term is unimportant) by the plot of Z (eqn. 9) against c , which did not depart significantly from linearity. The values of α_1^* and α_2^* were also obtained from runs B and C by least-squares fitting of eqn. (9). As shown in Table 1, the results obtained are in reasonable agreement, in spite of the differences of concentration range, and this justifies (at least for this case) the approximations made. Another test was the plot from run A of $(\ln c + \alpha_1^*Mc)$ against

r^2 ; the α_2^* term being small, eqn. (9) shows that this should give a straight line if there is no significant variation of composition (with respect to molecular weight) with r^2 . In fact the plot is linear through more than 99% of the range of concentration (Fig. 6).

Alexandrowicz (1959a,b, and personal communication) studied the same material by osmotic pressure and light-scattering. He gave his results in terms of the osmotic coefficient ϕ :

$$\frac{\pi}{RTc} = \frac{\phi}{M} \quad (10)$$

Expressing his values for ϕ as a function of concentration gives:

$$\phi = 1 + 16c + 53c^2 \quad (11)$$

Fig. 4. Schlieren diagram (a) and base line (b) (60° angle) of equilibrium sedimentation of polyethylene glycol, run B; for details see Table 1. m, Meniscus; b, bottom-of-solution column.

Fig. 5. Schlieren diagram (a) and base line (b) (60° angle) of equilibrium sedimentation of polyethylene glycol, run C; for details see Table 1. m, Meniscus; b, bottom-of-solution column.

Comparison of eqns. (10) and (11) with eqn. (3) gives equivalent values $\alpha_1^* = 4 \times 10^{-3}$ and $\alpha_2^* = 10^{-2}$. Our estimate of α_1^* is in fair agreement with this, though that of α_2^* is substantially higher. It should be pointed out that our measurements covered a wider range of concentration than did those of Alexandrowicz (1959a,b).

Polymethacrylic acid. This material was chosen because, unlike the polyethylene glycol, it is

Fig. 6. Plot of $(\ln c + \alpha_1^* M c)$ against r^2 for equilibrium sedimentation of polyethylene glycol, run A; for convenience the ordinate scale is $[6 + \log c + (\alpha_1^* M c / 2.303)]$. The range of $10^3 c$ is from 0.106 to 16.415.

Fig. 7. Schlieren diagram (60° angle) of equilibrium sedimentation of polymethacrylate, run D₁; for details see Table 2. m, Meniscus; b, bottom-of-solution column.

Fig. 8. Schlieren diagram (60° angle) of equilibrium sedimentation of polymethacrylate, run *D*₂; for details see Table 2. m, Meniscus; b, bottom-of-solution column.

Fig. 9. Schlieren diagram (60° angle) of equilibrium sedimentation of polymethacrylate, run *E*; for details see Table 2. m, Meniscus; b, bottom-of-solution column.

polyanionic and was expected to be highly polydisperse. Two runs were done, with the same initial concentrations, but at three different final speeds of rotation. The results (Figs. 7–9) show that in each case a maximum of dc/dr occurred, as predicted by eqn. (4). Values of α_1^* and α_2^* were estimated from the maximum by use of eqns. (6a) and (7a) (Table 2). In contrast with polyethylene glycol (Fig. 6), the plot of $(\ln c + \alpha_1^*Mc)$ against r^2 exhibits two ranges of approximate linearity (Fig. 10), which shows that the assumptions on which eqn. (9) are based were not fulfilled in this case; either α_1^* varies, or there is significant separation in the distribution of material of widely differing molecular weight. In view of the shape of the light-scattering plot (Fig. 2), which indicates a high degree of polydispersity, the latter seems likely to be the major effect. The quantity of material represented by the linear portion of the plot at higher r^2 (the region from which α_1^* was estimated) represents two-thirds of the whole. The remaining one-third would then represent material of lower molecular

Fig. 10. Plot of $(\ln c + \alpha_1^*Mc)$ against r^2 for equilibrium sedimentation of polymethacrylate, run *E*; for convenience, the ordinate scale is $[5 + \log c + (\alpha_1^*Mc/2.303)]$. The range of 10^3c is from 0.02 to 22.09.

Table 2. Details of equilibrium runs on polymethacrylic acid at pH 7 and values of coefficients α_1^* and α_2^*

	Times (hr.) are given in parentheses after the speeds		
	Run <i>D</i> ₁	Run <i>D</i> ₂	Run <i>E</i>
10 ³ × Initial concn. (g./ml.)	3.427	3.427	3.427
Column height (cm.)	0.946	0.946	0.912
Initial speed (rev./min.)	52 640	52 640 (21)	50 740 (5)
Final speed (rev./min.)	52 640 (21)	35 600 (13)	24 630 (16)
10 ³ × Final concn. at meniscus (g./ml.)	0	0	0
10 ³ × Final concn. at bottom (g./ml.)	43.14	28.59	22.09
α_1^* (eqn. 7a)	1.32×10^{-3}	1.20×10^{-3}	0.98×10^{-3}
α_2^* (eqn. 6a)	3.1×10^{-3}	4.1×10^{-3}	5.3×10^{-3}
Figure no.	7	8	9, 10

Bioch. 1967, 102

weight. In view of the low value of the α_2^* as compared with the α_1^* term, the estimate of α_1^* is only slightly dependent on the value assumed for the molecular weight; and in view of the likelihood that α_1^* is not strongly dependent on molecular weight this provides at least an approximate value of α_1^* for the whole material. The estimate of α_2^* (whose value depends directly on that assumed for M ; eqn. 6) indicates only its order of magnitude.

Hyaluronic acid. The equilibrium-sedimentation distributions of the hyaluronic acids (Figs. 11 and 12) resemble those of polyethylene glycol and

polymethacrylic acid in some respects, but differ from them in others. The much steeper rise of dc/dr from the base line is a consequence of its much greater molecular weight. There is again a maximum in dc/dr , but this subsequently passes through a minimum and then rises steeply again to the bottom of the cell.

The optical appearance of the steep initial rise and the maximum made us suspect that this might represent a sharp phase transition; however, the maximum occurs at a concentration of only 2mg./ml., and there is no other evidence of discontinuity of properties at this concentration (Preston *et al.* 1965). It seems more likely to be a diffraction effect arising from the high rate of change of gradient of refractive index in this region.

Values of α_1^* and α_2^* (Table 3) were estimated from the maximum, by use of eqns. (6a) and (7a). Eqn. (9) was not used because of the occurrence of the secondary rise of dc/dr . The value of the α_2^* term in eqn. (7a) is small compared with α_1^* , so that the latter is little affected by the value assumed for the molecular weight. The estimates of α_1^* agree satisfactorily with the osmotic data of Preston *et al.* (1965); their preferred value of A_4 , namely 1.94, corresponds to an α_1^* value of 3.9×10^{-3} .

There are several possible explanations of the increase of dc/dr at the bottom of the cell. The first is that, during the approach to equilibrium at higher speed, a concentrated layer was formed at the bottom of the cell that did not subsequently have time to equilibrate. This explanation is made unlikely by the observation that no detectable change of the pattern occurred during the last 6–8 hr. at the final speed. It is eliminated by the result, in run *J*, of lowering the speed for a period and then increasing it again to the speed of equilibration (Fig. 13). On lowering the speed, the gradients at the bottom of the cell decreased, but returned to their previous values on again increasing the speed; since a gradient that is too high because of failure to equilibrate cannot do anything but decrease with time, this shows that equilibrium had been attained.

The second possible explanation is that the non-ideality of the material requires for its description the retention of a term $3\alpha_3c^2$ in eqn. (4), with a negative value of α_3 . This explanation is eliminated by the result of run *H* (Fig. 14), in which the volume of solution, of identical concentration, was about one-quarter of that in run *G*, and the concentration at the bottom was only about one-half. Since the effect, if this explanation were correct, would be uniquely dependent on concentration, the rise of gradient at the bottom of the cell should have been absent from run *H*.

The third explanation is that the material is heterogeneous; comparison of runs *G* and *H* (Figs. 12 and 14) supports this explanation. It is unlikely,

Fig. 11. Schlieren diagram (70° angle) of equilibrium sedimentation of protein-containing hyaluronic acid (UFR), run *F*; for details see Table 3. m, Meniscus; b, bottom-of-solution column.

Fig. 12. Schlieren diagram (70° angle) of equilibrium sedimentation of protein-free hyaluronic acid (S18), run *G*; for details see Table 3. m, Meniscus; b, bottom-of-solution column.

Table 3. *Details of sedimentation equilibrium runs on hyaluronic acid preparations and values of coefficients α_1^* and α_2^**

Times (hr.) are given in parentheses after the speeds

Material	Run <i>F</i>	Run <i>G</i>	Run <i>H</i>	Run <i>J</i>
	UFR	S18	S18	S18
$10^3 \times$ Initial concn. (g./ml.)	1.61	2.23	2.23	2.23
$10^3 \times$ Final concn. at bottom (g./ml.)	14.7	17.6	approx. 8	16.9
Column length (cm.)	1.13	0.97	0.23	0.76
Cell thickness (cm.)	1.2	1.2	1.2	0.15
Initial speed (rev./min.)	50740 (6)	50740 (5½)	50740 (2½)	50740 (5)
Final speed (rev./min.)	21740 (42)	23150 (36)	23150 (49)	20410 (8)
				10000 (3)
				20410 (5)
α_1^* (eqn. 7a)	3.6×10^{-3}	4.4×10^{-3}	4.9×10^{-3}	4.3×10^{-3}
α_2^* (eqn. 6a)	1.1×10^{-2}	0.9×10^{-2}	—	0.7×10^{-2}
Figure no.	11	12	14	13

Fig. 13. Schlieren diagrams (60° angle) of sedimentation of protein-free hyaluronic acid (S18), run *J*; for details see Table 3. (a) Equilibrium distribution at 20410 rev./min.; (b) effect of reducing speed to 10000 rev./min. for 3 hr.; (c) return to approximately equilibrium distribution after again increasing the speed to 20410 rev./min. for 5½ hr. m, Meniscus; b, bottom-of-solution column.

however, for reasons discussed above, that mere polydispersity of homologous material could cause this effect, particularly in view of the dominance of α_1^* over $1/Mc$ in this region. It is more likely to arise from the presence of a chemically or physicochemically distinct fraction. If such a fraction had either

Fig. 14. Schlieren diagram (70° angle) of equilibrium sedimentation of protein-free hyaluronic acid (S18), run *H*; for details see Table 3. In this run the cell contained about one-quarter the volume of solution, and the concentration at the bottom of the cell was about one-half that in run *G* (Fig. 12). m, Meniscus; b, bottom-of-solution column.

a substantially lower degree of non-ideality in the mixture or a substantially greater value of $\partial\rho/\partial c$, it would tend to sediment more markedly than the bulk of the material. The possibility that the effect is due to the separate sedimentation of the protein, which is present in UFR, is excluded at once by the fact that the S18 material, which is almost protein-free, gives the same sedimentation pattern (Figs. 11 and 12). This must mean that the material that shows excess accumulation at the bottom of the cell is a fraction of 'hyaluronic acid' itself.

General discussion. In spite of the reservations, discussed above, about the application of highly simplified equations to the equilibrium sedimentation of polydisperse material, it appears that the analysis works fairly well. The agreement between predicted and experimental curves for the polyethylene glycol validates the assumption that the series is convergent in the range of concentration

used; at the worst, α_2^*c/α_1^* reaches about 0.7 at the bottom of the cell and is considerably smaller with the other materials. Further, the values of α_1^* are in satisfactory agreement with those found for α_1 (equivalent to the second virial coefficient) from osmotic measurements for two polymers as different in chemical nature and molecular weight as polyethylene glycol and hyaluronic acid. A plausible, though unchecked, value of α_1^* was obtained for a highly polydisperse sample of polymethacrylic acid.

The hyaluronic acid and polymethacrylic acid were nearly completely ionized under the experimental conditions. This fact has been allowed for by using values for both density and refractive increments measured against equilibrium diffusate (Casassa & Eisenberg, 1964). The osmotic value of α_1 for hyaluronic acid was obtained under similar experimental conditions; both this and α_1^* are 'operational' values that refer to the conditions used (state of ionization, temperature and ionic strength).

These estimates are not seriously affected by any error in the estimation of the average molecular weight. In agreement with expectation one can therefore conclude that arbitrary curtailment of the virial expansion at the c^2 term does not lead to serious error in the second virial coefficient. It is noteworthy that the values of α_1^* lead to very similar values for the parameter χ in the Flory-Huggins equation (Flory, 1953; eqn. XII-31''), namely: polyethylene glycol, 0.44; polymethacrylate, 0.43; hyaluronic acid, 0.42.

The relation between α_2^* and α_2 is uncertain, not only because of neglecting the terms in c^3 and above in the virial expansion, but also because of the relatively small magnitude of α_2^* and its dependence on M (eqn. 6a). Thus the values quoted for α_2^* can probably indicate no more than the order of magnitude of α_2 , though the presence of a maximum in the gradient curve indicates with certainty a positive contribution by terms in α_2 and above.

In most of these experiments we have deliberately used long columns of solutions, in order to display the character of the sedimentation equilibria over wide ranges of concentration. The use of shorter

columns would both shorten the time required for equilibration and diminish such errors in the estimates of average non-ideality coefficients as may arise from the fractionation of polydisperse material.

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