The Effect of Rotor Velocity on Sedimentation Coefficients

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Sedimentation-coefficient measurements on human IgG, fibrinogen and α_2 -macroglobulin and pig thyroglobulin were made at rotor velocities of 15 220–59 780 rev./min and 25.0°C in capillary-type synthetic-boundary cells or ordinary cells. At the lowest velocity, IgG and fibrinogen gave results several per cent higher than at other velocities, whereas α_2 -macroglobulin and thyroglobulin gave values only about 1.5% higher. This behaviour of IgG and fibrinogen is attributed primarily to imperfect initial boundaries.

Polson (1967) reported that the sedimentation coefficients of ovalbumin, human γ -globulin and *Jasus Calandii* haemocyanin are about 10% higher when measured at lower velocities than when measured at the high velocity normally used for studying such materials. If this phenomenon were general, as Polson (1967) pointed out, a consistent discrepancy would be expected between molecular weights calculated from combination of sedimentation coefficients measured at high velocity and diffusion coefficients and those obtained directly from sedimentation equilibrium at relatively low velocity. At one time the errors involved in these techniques might have obscured such a difference, but with the increased accuracy now possible a consistent bias should be detectable, but none has been claimed.

For technical reasons referred to by Polson (1967), measurements of sedimentation coefficients at low velocities must be done in a synthetic-boundary cell, unless molecules of high sedimentation coefficient are involved. The same author described the difficulties he experienced with the valve-type cell (Pickels, Harrington & Schachman, 1952; Schachman & Harrington, 1954) in getting initial boundaries that were free from defects even judged merely by the rather insensitive criterion of visual inspection. Since the quality of artificial boundaries had been improved when Kegeles (1952) and Klainer & Kegeles (1955) introduced capillary-type cells, it seemed highly desirable that measurements should be made with this kind of cell to see whether they substantiated the findings of Polson (1967).

EXPERIMENTAL

Proteins. Human IgG* was prepared by diluting 320 ml of buffered human serum and then adding an equal volume of 4M-(NH₄)₂SO₄. The precipitate was filtered

off, dissolved, dialysed and fractionated on columns of Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden) as described by Charlwood (1969). Material from the second zone eluted from each column was pooled, buffered and concentrated by a second precipitation with an equal volume of 4M-(NH₄)₂SO₄ and dissolved in and dialysed against 17.5mM-sodium phosphate buffer, pH 6.3. This solution was applied to a column (2.2 cm $\times$ 60 cm) of DEAE-Sephadex A-50 (Pharmacia) prepared in and thoroughly washed with the same buffer, which was then pumped through the column at 30 ml/h. The most concentrated fractions of eluate when mixed gave a stock solution of IgG containing about 17.5 g/l which was dialysed against 0.15M-NaCl–0.02 *I*-sodium phosphate buffer, pH 7.0.

Human fibrinogen (AB Kabi, Stockholm, Sweden; lot 16879) was dissolved in and dialysed against 0.15M-NaCl–0.02 *I*-sodium phosphate–10mM-trisodium citrate buffer, pH 6.9. Before use it was centrifuged for a short time in the ordinary laboratory centrifuge to remove undissolved material.

Pig thyroglobulin (19S), prepared by the method of Brownstone (1969) and given by Dr R. Pitt-Rivers, was dialysed against the same solvent as the IgG.

Human α_2 -macroglobulin was a gift from Professor R. A. Kekwick, who prepared it from the G2 ether-separated fraction of plasma (Kekwick & McKay, 1954) by steps that included preparative ultracentrifugation, gel filtration and chromatography on DEAE-cellulose. It was dialysed against 0.2 *I*-sodium phosphate buffer, pH 8.0.

Concentration determinations. Measurements in a differential refractometer (Cecil & Ogston, 1951) relative to the respective solvents gave the concentrations of stock solutions of the proteins, which were diluted appropriately for ultracentrifuge experiments. Slightly more dilute solutions were made for synthetic-boundary-cell use so that, as far as possible, conditions should be equivalent to those in the corresponding experiments in ordinary cells, in which some sector dilution has occurred by the time the boundary reaches the radial position at which a synthetic boundary is formed. A specific refractive increment of 0.00185 was used when concentrations were converted to a weight basis.

* Abbreviation: IgG, immunoglobulin G.

Ultracentrifuge experiments. These were done at 15220, 31410, 44770 and 59780 rev./min at 25.0°C in a Spinco model E ultracentrifuge fitted with temperature control. Schlieren records obtained by the optical system, which included a Wratten 77A filter to isolate 546nm light, and a phase-plate, were measured with a modified comparator (Charlwood, 1968). Measurements were confined to those exposures in which the boundary was clear of the meniscus but the plateau region had not been lost. In each experiment 10–20 suitable exposures were made.

The regression of $\ln r$ on t (where r is the radial position of peak maximum and t is the time) was calculated. From the measured speed of rotation (ω) sedimentation coefficients (s) were then obtained, all corrected to water at 20°C by means of density and viscosity data (Svedberg & Pedersen, 1940) for the salts. In these calculations, although the result is insensitive to it, the partial specific volume ($\bar{v}$) was taken as 0.720 for human IgG (Schultze, Schmidtberger & Haupt, 1958), 0.725 for human fibrinogen (Armstrong, Budka, Morrison & Hasson, 1947), 0.723 for pig thyroglobulin (Derrien, Michel, Pedersen & Roche, 1949), and 0.735 for human α_2 -macroglobulin (Schultze *et al.* 1958). From the radial positions of the peaks and the initial concentrations of solutions, a mean concentration was calculated corresponding to each observed sedimentation coefficient.

All experiments were done in capillary-type synthetic-boundary cells or ordinary 12mm cells in a D rotor, except most of those at 15220 rev./min for which a J rotor was used. In a few cases the boundary was separated from the meniscus by using a 12mm cell in the D rotor at a higher velocity, the velocity was decreased to 15220 rev./min and sedimentation was followed. To ensure accuracy of comparisons, the thermistor calibrations were checked and, in some instances, the thermistor was transferred from one rotor to the other. Cells were filled with standard volumes of solutions by means of a syringe microburette (Scientific Products Inc., Flushing, N.Y., U.S.A.).

RESULTS

For each set of experiments, i.e. for a particular protein or, in the case of IgG, for those experiments at a particular concentration, the value of the sedimentation coefficient at 59780 rev./min (or the mean value if two experiments had been done) was taken as standard. All other values in the set were divided by it to give relative sedimentation coefficients, which were plotted against the square of the velocity (Figs. 1 and 2).

The results were analysed in various ways. First, the regressions of relative sedimentation coefficients on ω^2 were calculated for (a) all experiments, (b) all except those at 15220 rev./min, (c) experiments on IgG and fibrinogen only, (d) those on IgG and fibrinogen, excluding ones at 15220 rev./min and (e) experiments on thyroglobulin and α_2 -macroglobulin only. Regression coefficients were highly significant for (a)–(d) ($P < 0.001$), but the regression in (e) did not approach significance. In (b) and (d) the effect corresponded to a decrease of only about

Fig. 1. Relative sedimentation coefficients of IgG and fibrinogen as a function of square of rotor velocity. ∇ , $\blacksquare$, $\blacktriangle$ and $\bullet$, Experiments done in the synthetic-boundary cell. ∇ and $\blacktriangledown$, IgG (0.25%); $\square$ and $\blacksquare$, IgG (0.50%); $\triangle$ and $\blacktriangle$, IgG (0.74%); $\circ$ and $\bullet$, fibrinogen (0.26%).

Fig. 2. Relative sedimentation coefficients of thyroglobulin and α_2 -macroglobulin as a function of square of rotor velocity. $\bullet$ and $\blacksquare$, Experiments done in the synthetic-boundary cell. $\circ$ and $\bullet$, Thyroglobulin (0.15%); $\square$ and $\blacksquare$, α_2 -macroglobulin (0.17%).

1.5% between the lowest and highest velocities, whereas for (a) and (c) it was 2.7 and 3.8% respectively. A comparison of results obtained in the 12mm and synthetic-boundary cells at 44770 and 59780 rev./min with IgG and fibrinogen showed no statistically significant effect of the cell type.

Since it was the results at 15220 rev./min for IgG and fibrinogen that were responsible for the larger part of the dependence of relative s on ω^2 , the measurements on these records were examined in further detail. In any particular experiment the total time between the first and last exposures used in the ordinary calculations was subdivided and exposures falling in the earlier and later halves were used to arrive at sedimentation coefficients for the earlier and later stages. The mean value (1.04₃) of the ratios of earlier s to later s was very significantly above unity ($P < 0.001$). Corresponding calculations for 59780 rev./min, whether involving both types of cell or confined to the synthetic-boundary type,

gave $P > 0.05$. In addition, s values for the later halves of the experiments at 15220 rev./min were significantly higher than those for the corresponding solutions at 59780 rev./min ($P < 0.001$). Despite all these findings, it was difficult to detect curvature in any of the plots of $\ln r$ on t , except by mathematical analysis. This is illustrated by Fig. 3, which is based on experiments in which departure from linearity was greatest. Even in these examples the standard error of the slope of the regression was only about 0.5% of the slope.

To test the effects of boundary anomalies or optical artifacts that might be present to a greater extent in the early stages, s values were also calculated with the omission of the first two exposures in each case from the experiments on IgG and fibrinogen at 15220 rev./min. The result was always lower than when these two exposures were included, although the decrease in no instance exceeded 1%. Statistically the decrease was significant ($P < 0.001$), whereas no significant effect was found when similar calculations were done from the equivalent results at 59780 rev./min, whether confined to those obtained with the synthetic-boundary cell or not.

Calculations of the peak positions in terms of the second moment about the axis of rotation were carried out on records from experiments at 15220 rev./min with IgG (0.75%) and fibrinogen. Sedimentation coefficients thus derived did not differ significantly from those based on the usual peak maximum location (Table 1).

Fig. 3. Plots of $(100 \ln r + \text{constant})$ against t at 15220 rev./min for 0.26% fibrinogen (●) and 0.74% IgG (○). Lines shown are calculated regression lines. Time units are 40 min for fibrinogen and 26.7 min for IgG.

Those experiments in which the boundary was detached from the meniscus and the velocity then decreased so that sedimentation could be observed in a weaker field gave relative values of s even higher than those in the corresponding synthetic boundary cell experiments. These values, 1.118 and 1.088 for the highest concentration of IgG and 1.065 for fibrinogen, were not included in the general analysis. One other experiment attempted with IgG (1.0%) in the synthetic boundary cell in the D rotor gave a boundary visibly anomalous in shape.

DISCUSSION

A true dependence of s on rotor velocity might be attributable to molecular orientation or to hydrostatic effects either on the solution or on $\bar{v}$. As Polson (1967) pointed out, the substances he studied do not sediment fast enough to overcome the random orientation distribution brought about by rotational diffusion. Although hydrostatic compression of the solutions would operate to give a lower s at higher speeds (Polson, 1967) the magnitude of the effect would be quite small.

Thus in the present experiments the increase in density of water in the part of the plateau region furthest from the axis, calculated from the compressibility constants of Gibson & Loeffler (1941) is about 0.038, 0.34 and 1.2% at 15220, 31410 and 59780 rev./min respectively. The corresponding changes in $(1 - \bar{v}\rho)$ are 0.1, 0.93 and 3.4%, so that, on this basis, s would apparently decrease by about 2.5% between 31410 and 59780 rev./min. The observed change was about $1.5 \pm 0.4\%$ (S.D.), but it is difficult to assess the comparison because, when compressibility effects operate, there is no longer a true plateau region and it is not possible to define simply the level in the cell to which the measured s value corresponds. The compression at levels nearer the meniscus will, of course, be less than that calculated above. A strict comparison would also have to take into account the demonstration by Schachman (1959) that rotor stretching, unless allowed for, causes s to be slightly overestimated. This effect increases with speed to about 0.5% at top speed.

Thus apart from small effects detected only by statistical analysis, and compatible in magnitude with expectations based on compressibility data,

Table 1. Comparison of sedimentation coefficients based on peak positions and second moments of schlieren patterns

Substance	r_1 (peak)	r_1 (moment)	r_2 (peak)	r_2 (moment)
IgG	6.3729	6.3734	6.5419	6.5413
Fibrinogen	6.3579	6.3627	6.6389	6.6477
	s (peak) (S)		s (moment) (S)	
IgG	6.55		6.52	
Fibrinogen	7.82		7.92	

the only abnormal results have been obtained in experiments at the lowest rotor velocity, either in the synthetic-boundary cell or when an initial period in a stronger field was used, with materials having $s_{20,w}$ values that are not particularly high, i.e. under conditions such that absolute rates of movement were low, but boundary shape changes due to diffusion most marked, and peak maxima least sharp.

The comparison of s values for the earlier and later stages of low speed experiments with IgG and fibrinogen showed a downward trend with time, which is in the opposite direction to that which must be there due to sector dilution and to the concentration dependence of s . This latter trend would be quite small for the substances studied here since, in the most pronounced cases, changes in concentration in the plateau region corresponded to alterations in s of only 0.78% for IgG and 0.50% for fibrinogen. There is clearly a predominant contrary effect. This was much too large to be attributed to errors in the positions assigned to the references and seems most likely to have arisen from imperfect formation of the initial boundary resulting in abnormal shape of the peak and anomalous location of its maximum. Although both diffusion and sedimentation may be expected to cause a reversion towards a more normal situation (and there is evidence that this does occur because s is significantly lower later in each experiment), it is under precisely these conditions of low absolute rates of movement that anomalies of this sort would have the biggest effect on s . Decrease of velocity during an experiment must be assumed also to give rise to boundary distortions, possibly through hydrostatic changes or because of thermal effects.

The calculations of second moments of schlieren patterns (Table 1) raise a question requiring discussion. When a boundary location is defined in terms of the second moment, theory shows that the s value obtained corresponds to that of material in the plateau region, and should be independent of boundary shape. Even if the s values obtained at low speed from peak measurements (Table 1) are greater than they should be because of distortion of the boundaries, it might be expected that moment calculations would still give the true s values. The agreement in Table 1 between calculations carried out by the two methods could arise as follows. Either there are processes (such as convective transport) that occur but are not taken account of in the second-moment theory, or the information in Table 1 is too limited and therefore subject to statistical errors that mask any difference.

Even where there were trends of s with time, it

was not usually possible to detect by eye any undoubted curvature of the usual $\ln r$ against t plot. It is well known that in diffusion an imperfectly formed boundary may appear satisfactory to visual inspection and give an excellent linear plot of h^{-2} versus t (h being the maximum height of the schlieren curve), but that the regression of h^{-2} on t may have a spuriously high value. It seems that something similar is possible in sedimentation.

It is concluded that the effects observed here at 15220 rev./min and by Polson (1967) are anomalies brought about by imperfect boundary formation, although the capillary-type cell appears superior to the valve-type in producing smaller effects. Apart from this, the magnitude of the variation of s with rotor velocity is explicable in terms of hydrostatic-pressure changes. Of course, a particular protein system might be particularly susceptible to pressure changes (either with respect to their effect on $\bar{v}$, or with respect to a tendency to associate or dissociate, e.g. myosin, studied by Josephs & Harrington, 1968) and exhibit bigger effects, but there is no necessity to bring in any such special concepts here.

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REFERENCES

- Armstrong, S. H., jun., Budka, M. J. E., Morrison, K. C. & Hasson, M. (1947). *J. Am. chem. Soc.* **69**, 1747.
- Brownstone, A. D. (1969). *Analyt. Biochem.* **27**, 25.
- Cecil, R. & Ogston, A. G. (1951). *J. scient. Instrum.* **28**, 253.
- Charlwood, P. A. (1968). *Analyt. Biochem.* **26**, 490.
- Charlwood, P. A. (1969). *Biochem. J.* **115**, 897.
- Derrien, Y., Michel, R., Pedersen, K. O. & Roche, J. (1949). *Biochim. biophys. Acta*, **3**, 436.
- Gibson, R. E. & Loeffler, O. H. (1941). *J. Am. chem. Soc.* **63**, 898.
- Josephs, R. & Harrington, W. F. (1968). *Biochemistry, Easton*, **7**, 2834.
- Kegeles, G. (1952). *J. Am. chem. Soc.* **74**, 5532.
- Kekwick, R. A. & McKay, M. E. (1954). *The Separation of Protein Fractions from Human Plasma with Ether*, p. 18. London: H.M. Stationery Office.
- Klainer, S. M. & Kegeles, G. (1955). *J. phys. Chem.* **59**, 952.
- Pickles, E. G., Harrington, W. F. & Schachman, H. K. (1952). *Proc. natn. Acad. Sci. U.S.A.* **38**, 943.
- Polson, A. (1967). *Biochem. J.* **104**, 410.
- Schachman, H. K. (1959). *Ultracentrifugation in Biochemistry*, p. 17. New York: Academic Press Inc.
- Schachman, H. K. & Harrington, W. F. (1954). *J. Polym. Sci.* **12**, 379.
- Schultze, H. E., Schmidtberger, R. & Haupt, H. (1958). *Biochem. Z.* **329**, 490.
- Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*, p. 446. Oxford University Press.