DIFFUSION COEFFICIENTS FROM THE ZONAL ULTRACENTRIFUGE USING MICROGRAM AND MILLIGRAM QUANTITIES OF MATERIAL

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Received March 24, 1970

SUMMARY

A method is presented for the determination of the diffusion coefficients of very small quantities of material in the zonal ultracentrifuge, yielding an error of 3.5% or less (the data here, from a series of experiments, gives less than 2% error overall). Both theory and supporting experimental data are presented.

INTRODUCTION

Translational diffusion coefficients have not been previously measured in the zonal centrifuge. One of the most serious problems affecting resolution in zonal systems is the occurrence of the phenomenon known variously as droplet or streamer sedimentation.[1,2] This may occur when a less dense macromolecular solution is layered above a more dense solution of highly diffusible, density gradient substance. Density inversion may take place at the interface due to unequal diffusion, and convection ensues. The majority of our previous experiments have been performed statically. That is, the studies were not undertaken in a spinning centrifuge rotor, but in cuvettes or centrifuge tubes on the bench.[3] Various theories have appeared in the literature purporting to define the experimental conditions required to prevent droplet sedimentation.[4,5] As yet, experimental evidence to support these is lacking. A sensitive hydrodynamic tool is needed to detect droplet sedimentation, and for this we have chosen the diffusion coefficient of the macromolecular component in the zone. If the diffusion coefficient may be measured precisely and accurate values obtained, then anomalous spreading should be readily detectable by a sudden large increase in the diffusion

coefficient.

THEORY

The problem here is to extract the diffusion coefficient from the experimental data, in this case, a profile of some macromolecular property distribution. This resolves itself into solving the equations for radial diffusion centrifugally and centripetally from a thin annulus. Vinograd and Bruner give this solution.[6]

$$c(r,t) = \left\{ \frac{c(r_o,o)}{2(\pi D\alpha t)^{1/2}} \exp\left[- \frac{(r_o-r)^2}{4D\alpha t} \right] \right\} \frac{r_o}{r_c} \left(\frac{r_c}{r}\right)^{1/2} \delta r_o \qquad (1)$$

where c is the concentration at radius r and time t,

r_o is the initial center of mass of the zone,

r_c is the center of sedimentation,

δr_o is the initial zone width,

D is the diffusion coefficient,

$\alpha = \frac{\exp(2\omega^2 st)-1}{2\omega^2 st}$,

where s = the sedimentation coefficient, and ω the angular velocity.

A plot, therefore, of $\ln[(\frac{r_o}{r})^{1/2} \cdot \frac{1}{c(r,t)}]$ against $(r_o-r)^2$ yields a line of slope $(4D\alpha t)^{-1}$. We have assumed that $r_o = r_c$ and for our purposes the difference between them is not measurable.

MATERIALS

Sodium bromide was Mallinckrodt Analytical Reagent Lot #VKH B and required filtering to remove suspended matter before use. Equine cytochrome-c was Calbiochem A grade Lot 71348.

PROCEDURE

The zonal rotor (B-XV, titanium, Oak Ridge National Labs) was loaded with a sodium bromide solution of uniform density, the rotor speed being maintained at 3000 rpm. When full, 10 ml of an 0.05% solution of cytochrome-c in sodium bromide at a concentration decrement of 2w/w% was pumped slowly into the center of the rotor and the time noted (t_1). This was followed slowly by 460 ml of sodium bromide at a further concentration

decrement of 2w/w%. These decrements were chosen to give a stable interface as per reference 5. When the 460 ml overlay was in the rotor, a device for measuring $\int\omega^2 t$ continuously (Beckman Instruments, Inc.) was started. Inlet and outlet lines were disconnected, the rotor capped, and allowed to run at 3000 rpm for about 20 hours. At the end of this time, the rotor was emptied by pumping heavy sodium bromide to the edge of the rotor, and the temperature of the effluent monitored with a Tele-thermometer (Model 42SC, Yellow Springs Instrument Co., Inc., Ohio). The time (t_2) was noted when 400 ml effluent had been collected in 15 ml fractions, and the summation of $\omega^2 t$ stopped. The optical density profile of the effluent was constructed, using radius as the abscissa rather than volume.

CALCULATIONS

A smooth curve is drawn through the experimental points and the values of c at the appropriate r on the curve measured. The peak, r_o, may be readily found from the plot. Knowing these quantities it is a simple matter to plot $\ln[(\frac{r_o}{r})^{1/2} \cdot \frac{1}{c(r,t_2)}]$ against $(r_o-r)^2$ for both sides of the curve. A least squares routine is used to obtain the slope. Corrections for the viscosity of the sodium bromide and the temperature of the experiment to those of water at 20° are made by the usual formula

$$D_{20,w} = D \cdot \frac{\eta}{\eta_{20,w}} \cdot \frac{293}{T},$$

where η and T denote viscosity and absolute temperature respectively.

RESULTS

A typical profile is shown in Fig. 1, with the corresponding plot in Fig. 2. As can be seen, the fit is extremely good, so that we only accept correlation coefficient values of 0.9990 or better, with standard errors of the diffusion coefficient of 3.5% or less. Results are seen in Table 1.

We find that making the correction for α is unnecessary, since this is 0.1% of the value of $D_{20,w}$ under the conditions used, and is negligible compared with errors in temperature measurement, for example. Therefore, it

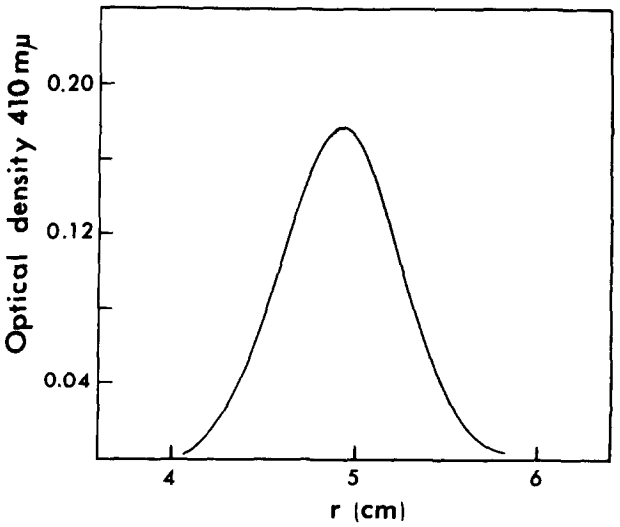

Figure 1. Concentration profile of cytochrome-c after approximately 20 hrs. diffusion in the BXV zonal rotor.

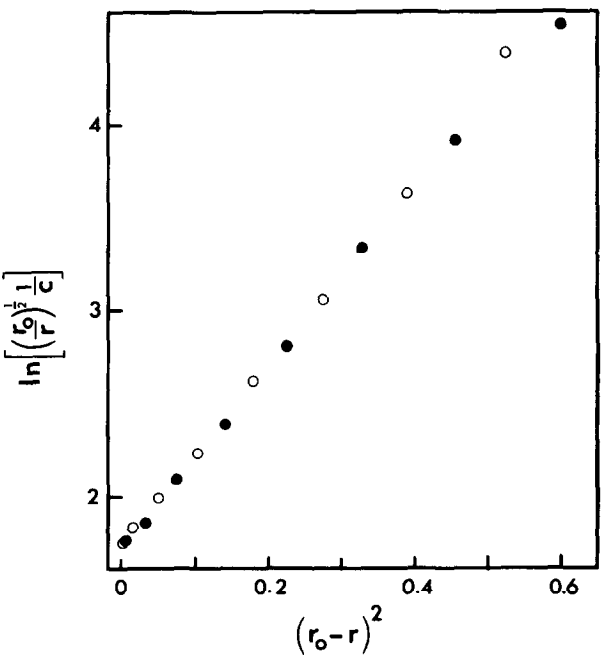

Figure 2. Plot of $\ln[(\frac{r_o}{r})^{1/2} \cdot \frac{1}{c(r,t_2)}]$ against $(r_o-r)^2$ for the data of Figure 1. Data points are ● leading edge, ○ trailing edge.

TABLE 1

Run	$D_{20,w}$ (in Ficks) Trailing	Leading	All points
31	11.86 ± 0.28 (.9996)	* (.9981)	* (.9926)
32	11.32 ± 0.27 (.9996)	11.88 ± 0.25 (.9997)	11.66 ± 0.23 (0.9991)
33	11.56 ± 0.40 (.9990)	11.59 ± 0.06 (1.0000)	11.59 ± 0.23 (0.9991)

Above values give a weighted mean $\bar{D}^{c}_{20,w}$ = 11.60 ± 0.15 F

*Standard error greater than 3.5% and correlation coefficient less than 0.9990. Numbers in brackets are correlation coefficients of least squares fit.

is not necessary to measure $\int \omega^2 t$, and we only do this now to correlate with work at higher or lower speeds.

DISCUSSION

At the commencement of this work, we did not expect to obtain diffusion coefficient values with such accuracy and precision, anticipating perhaps 10% error. The precision actually obtained will aid us greatly in detecting small hydrodynamic disturbances, which was the main object of this work. It also prompted us to author this communication because of the potential applications of the technique. Present methods of measuring the diffusion coefficients of macromolecules rely on the optical properties of their solutions, mainly refractive index, and can consume large quantities of material. This method relies only on that the macromolecule has some property which is accurately measurable, radioactivity, enzyme activity, optical density, chemical determination of protein, for example. The method would seem particularly useful for enzymes since the concentration of enzyme required in the initial band would be between 50 and 250 micrograms total, depending on the activity, and would tend to be in the lower part of this range. Satisfaction of the assumptions of equation (1) are more truly approached as the bandwidth approaches zero. This does not seem to have

affected our results, however, using a bandwidth of 0.5 mm. Therefore, with enzyme solutions, the effective concentration used may be from 5 to 25 micrograms per milliliter. The concentration of sodium bromide required to support this would (using reference 5 as a guide) be from 0.0008 M to 0.02 M depending on the combination of D and enzyme concentration per milliliter. D has been assumed to vary from 4F to 11F, corresponding to molecular weights from about 13,000 to 300,000. The most likely concentration of sodium bromide used would be 0.005 M, and it is conceivable that this may reduce the enzyme activity somewhat. Most systems, however, could tolerate a 50% reduction in activity due to this cause, and this much reduction is unlikely.

An objection which might be raised here is the error which would be introduced from uncertainty in the biological assays. It is important to remember that a smooth curve is drawn through the points. At these concentrations, provided dissociation and association are not taking place, the profile is Gaussian, and this assists in the curve drawing. We have used biological assays many times to follow a sedimenting boundary in the preparative centrifuge, and have used the data for a mathematical analysis of the shape of that boundary to extract the sedimentation coefficient.[7] Results carry about 5% error, and we therefore believe that the biological assay errors will not be a negating factor.

ACKNOWLEDGMENTS

We wish to thank Dr. N. G. Anderson of Oak Ridge National Laboratories for the generous loan of the zonal rotor. This work was supported by a research grant from the National Institutes of Health, Public Health Service, Grant No. 13914.

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