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Application of Sedimentation Field-Flow Fractionation to Biological Particles: Molecular Weights and Separation

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Abstract

Sedimentation field-flow fractionation (SFFF) is an elution–centrifugation method which operates with an equilibrium solute layer thickness of the order of 10 μ. Retention is a function of molecular weight and density, thereby making it possible to obtain both fractionation and molecular weight values for particles and macromolecules. Equations for the characteristics of this method are given. Experimental data are obtained for bacteriophage T2 and compared with theoretical predictions. The molecular weight determined from these data are $(227 \pm 11) \times 10^6$ and $(236 \pm 7) \times 10^6$ for two different dilutions. Finally, it is noted that the potential speed and accuracy of the method are attractive, and are based largely on the small sedimentation layer thickness.

INTRODUCTION

Sedimentation field-flow fractionation (SFFF) is a major subclass of the general separation methodology, field-flow fractionation (FFF). A number of papers from this laboratory in the past few years have dealt with the theoretical and conceptual nature of FFF (*1–5*) and a number of specific experimental systems (*6–8*). Two recent papers have detailed the development of an experimental SFFF system and its application to the separation

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of polystyrene latex beads, and the development of two different programmed SFFF systems, one using a variable rotor speed and the other a variable solvent density (*9*, *10*). In earlier work, Berg, Purcell, and Stewart described preliminary experimental results on a system that falls in the SFFF category (*11–13*).

The purpose of this work is to investigate the applicability of SFFF to biological particles and macromolecules. To this end, theoretical results will be combined with the outcome of experimental work on bacteriophage T2. The experimental work, by itself, fails to demonstrate the full potential of SFFF because the existing system is limited to accelerations of a few hundred gravities and is thus limited to the larger particles of biological interest. Theory points the way to broader applications.

The potential applications of SFFF are twofold. First, the method has a considerable potential for the analytical fractionation of large macromolecules and particles. Second, the elution pattern in SFFF hinges on properties such as molecular weight, density, diffusion coefficients, and related quantities; one can thus obtain various physical parameters from measured elution properties. Here we are particularly interested in investigating the potential of SFFF for the rapid determination of molecular weights in the range corresponding to various phages and DNAs.

THEORY

The SFFF technique is based on the equilibrium sedimentation of particles and macromolecules into the slow flow region near the wall of a narrow flow tube. In this tube, or column, laminar flow is occurring in a direction perpendicular to the sedimentation force. This flow carries particles downstream, more so for particles near the center of a column than at its wall. The velocity with which a zone of particles is transported by flow hinges on the mean elevation of the particles above the wall. The mean elevation, or thickness, of a solute layer depends on the effective mass of the solute particles. Thus a differential velocity spectrum is created in which different types of particles are spread out along the column, and are eluted one after another, depending on their effective mass.

By the same token that fractionation can be achieved through the differential elution of particles of different effective mass, so too can effective mass and related parameters be measured as a function of the elution time of the particles. All that is required is a sound theoretical basis for the elution process whose conclusions are in agreement with experimentation. A theoretical framework exists for FFF in general and SFFF in particular; its conclusions have been found to agree closely with the experimental results on elution times obtained from a well-characterized SFFF system (*9*, *10*). The major conclusions of this theory are summarized briefly below.

Sedimentation drives solute particles toward one of the channel walls where, to a close approximation for high dilutions, an exponential equilibrium distribution is formed

$$c/c_0 = \exp(-x/l) \tag{1}$$

in which c is solute concentration, c_0 is the concentration at the wall, x is the distance from the wall, and l is the characteristic thickness—in channels of sufficient width, the mean thickness—of the exponential solute layer. Parameter l is related to the solute's molecular weight M by

$$l = \frac{\mathscr{R}T}{MG(1 - \bar{v}_s\rho)} \tag{2}$$

where $\mathscr{R}$ is the gas constant, T is temperature, G is the strength of the sedimentation field, $\bar{v}_s$ is the partial specific volume of solute, and ρ is the solvent density.

The effective molecular weight, M', is the mass of the particle in Dalton units minus the mass of the displaced fluid, which equals

$$M' = M(1 - \bar{v}_s\rho) \tag{3}$$

The substitution of this into Eq. (2) gives l in a very simple form:

$$l = \mathscr{R}T/M'G \tag{4}$$

So far the theory is the usual limiting form valid for equilibrium ultracentrifugation under conditions of low concentration and small l values. The connection to SFFF is made through the l expression of Eq. (4). The ratio of l to channel width w is the critical SFFF parameter, λ, which, as we shall see shortly, fixes the elution time of a solute peak

$$\lambda = l/w = \mathscr{R}T/wM'G \tag{5}$$

The degree of retention in a SFFF column is, as in chromatography, expressed in terms of the retention ratio R, the ratio of the passage time through the column of a nonretained substance to the passage time of the solute in question (*14*). With R given, retention time t_r or volume V_r can be stated in terms of the retention time t_0 or volume V_0 of the nonretained solute

$$t_r = t_0/R \tag{6}$$

$$V_r = V_0/R \tag{7}$$

Quantity V_0 is equal to the internal volume of the column and is thus obtainable from geometrical considerations or by independent measurement. Corrections for the volume of connecting tubing and the detector must, of course, be included.

The theoretical link between the experimental retention parameter R and the basic solute distribution parameter λ is provided by Eq. (3).

$$R = 6\lambda[\coth(1/2\lambda) - 2\lambda] \tag{8}$$

The migration of a solute zone is accompanied by its spreading. The factors which give rise to zone broadening in FFF are much like those responsible for peak spreading in chromatography, and have been discussed in some detail (*2*, *4*). The principle mechanism of zone spreading in an ideal column results from the slight perturbation by flow of the equilibrium distribution of Eq. (1). This process generates an effective plate height of (*2*)

$$H = \psi l^2 \mathscr{V}/D \tag{9}$$

where $\mathscr{V}$ is the migration velocity of the zone, D is the diffusion coefficient, and ψ is a dimensionless coefficient ranging from 0 to 4, the latter value being the limit approached as λ approaches zero. Zone velocity $\mathscr{V}$ can be expressed as $R\langle v\rangle$, where $\langle v\rangle$ is the mean flow velocity of the solvent.

EXPERIMENTAL

The centrifuge employed in this study was International Equipment Company model B-20. A column of length 45.7 cm and of rectangular cross section 0.0635 cm by 2.54 cm (7.38 ml volume) was coiled inside the centrifuge basket and connected to the outside by means of a special seal. The inner surface of the column was coated with a thin film of collodion. The temperature was held at about 2°C. The details of the remainder of the system have been described (*9*).

The T2 bacteriophage was obtained from Miles Research Laboratory. A Tris buffer solution (0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH = 7.6) containing 5 to 10 × 10^{12} particles/ml was used for the injected sample. The quantities injected varied from 10 to 70 μl. The carrier solvent was a phosphate buffer with pH 7.13 consisting of 0.05 *M* K_2HPO_4 and 0.02 *M* KH_2PO_4.

RESULTS

Retention

The variation of the retention ratio *R* of T2 bacteriophage with changes in sedimentation field strength G is shown in Fig. 1. This figure shows that a substantial retention of such species can be achieved, and moreover, this retention can be controlled in a systematic way through changes in *G*.

Equation (8) can be used to calculate the λ values corresponding to these retention experiments. According to Eq. (5), a plot of λ against $1/G$ should yield a straight line passing through the origin. A plot of this nature is shown in Fig. 2. The results are in accord with theory. The slope of the

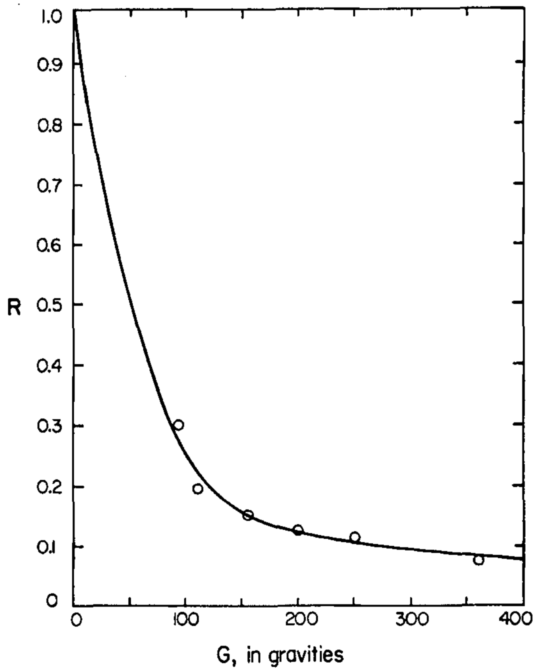

FIG. 1. Retention ratio *R* vs field strength *G* for T2 bacteriophage. The injected samples were 50 μl in volume and contained approximately 0.2 mg of T2. The flow rate was 6 ml/hr.

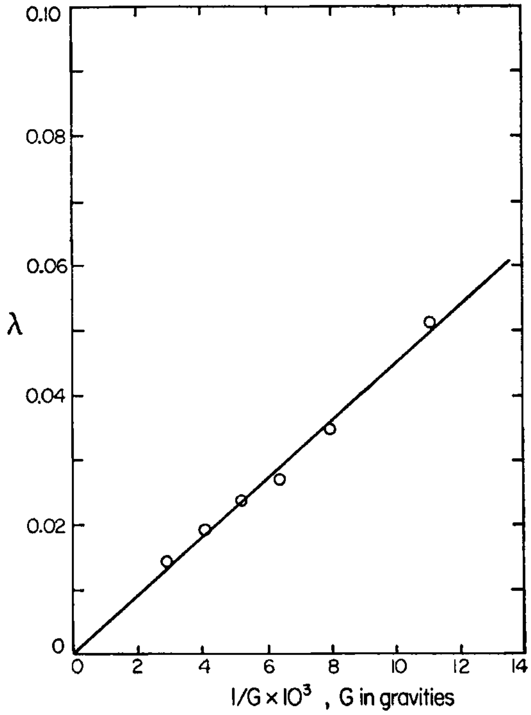

FIG. 2. The variation of retention parameter λ with reciprocal field strength $1/G$ for T2. The data from Fig. 1 were converted to λ values by means of Eq. (8) to obtain this plot.

line is determined by the effective molecular weight and will be used shortly to obtain a molecular weight value for this bacteriophage.

Plate Height

The widths of T2 elution peaks at their half-heights were measured at various flow velocities in order to determine plate height parameters. The results are shown in Fig. 3. Equation (9) predicts that the experimental points will fall on a straight line passing through the origin. There is clearly a qualitative departure from theory in this case. A quantitative comparison with theory must depend on an independent measurement of diffusion coefficient D, as shown by Eq. (9). For T2 this must be done with care because two discrete forms of this bacteriophage exist having different

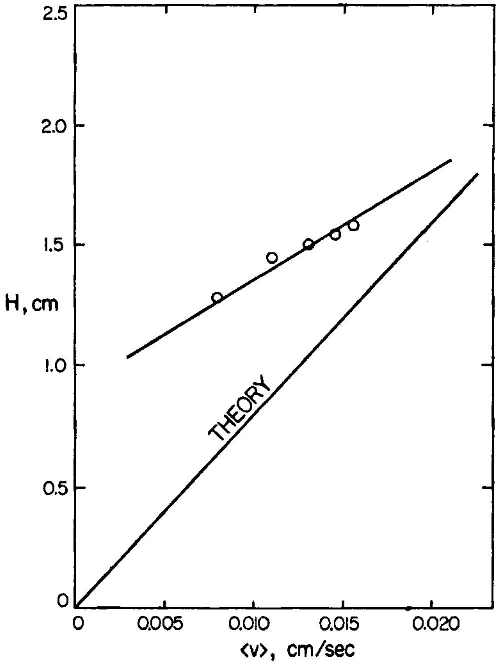

FIG. 3. Plate height H vs mean carrier flow velocity $\langle v \rangle$ for T2. Field strength, 201 g; sample size, 70 μl of solution containing 0.2 mg T2. The slope of the theoretical line is inversely proportional to D, where D is assumed here to be 1.32×10^{-8} cm^2/sec at 2 °C.

transport properties (*15–17*). For calculational purposes a D value measured at pH 7.85 has been selected (*18*). This is in close proximity to the pH of 7.13 used in this work, and should therefore engender the same structural form. Corrected to 2°C, this D value is 1.32×10^{-8} cm^2/sec. The plate height line calculated on this basis is shown in Fig. 3. The gap between the experimental points and the theoretical prediction is significant, involving a departure from theory from 25 to 100% on the high side. A similar departure was noted in earlier SFFF work with polystyrene beads, and various reasons for the departure were explored (*9*). The most likely reason for the divergence of values is the interaction of particles, leading to concentration-dependent results. In Fig. 4 we plot the maximum theoretical concentration, c^L_{00}, of solute in a zone at the end of the column versus flow velocity. Shown also is the fractional departure of plate height from its theoretical value. The two curves show the same trend, which at

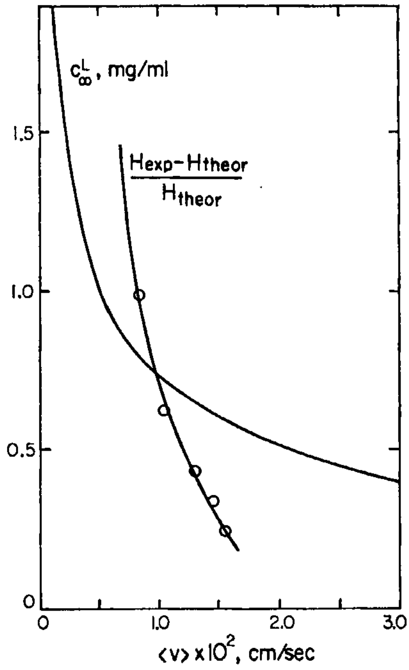

FIG. 4. Curves showing the maximum concentration c_{∞}^{L} of T2 in the zone just prior to elution, and the fractional departure of plate height from its theoretical value, as a function of mean flow velocity $\langle v \rangle$.

least suggests a relationship between them. However, the crucial question is whether independent evidence suggests nonlinear interactions at concentrations of this order of magnitude. The plate height could not be measured accurately on several peaks generated from smaller samples because plate height accuracy is sensitive to the increased noise level encountered. Other evidence suggests very little change in the transport properties of T2 up to 1 mg/ml (*19*), except for a possible anomaly at about 0.1 mg/ml (*15*, *16*). These data do not in general support a plate height divergence of the observed magnitude.

The factors that finally assume responsibility for the plate height divergence are likely to have little effect on retention and the physical constants derived from retention methods. Plate height is enormously more sensitive to noise, dead volume, solute interactions, and a host of other factors than is retention. This is borne out by the entire chromatographic literature and by our specific observations on polystyrene beads in the earlier studies.

Fractionation

Dilute samples of T4, T7, and tobacco mosaic virus (TMV) were available, and an effort was made to fractionate each, in turn, from T2. The *G* values were, respectively, 219*g*, 238*g*, and 279*g*. Unfortunately, the detector response to these dilute samples was so slight that the emerging peaks could not be distinguished unequivocally from noise in the system. Nonetheless, small peaks were observed in all three cases at a retention volume appropriate to the molecular weight and density of the particle. These peaks were well separated from the larger T2 peak, as expected from the calculations.

Molecular Weight of T2

The data of Figs. 1 and 2 were employed in Eq. (5) to calculate *M'*. From this, the molecular weight *M* of T2 was calculated assuming its specific volume to be 0.66 (*16*). These *M* values are shown as the circles in Fig. 5. The values are reasonably consistent over a fourfold range in λ values, with no major trends apparent. The mean and standard deviation calculated from these points are $M = (227 \pm 11) \times 10^6$.

The effect of sample size was investigated by substituting a sample con-

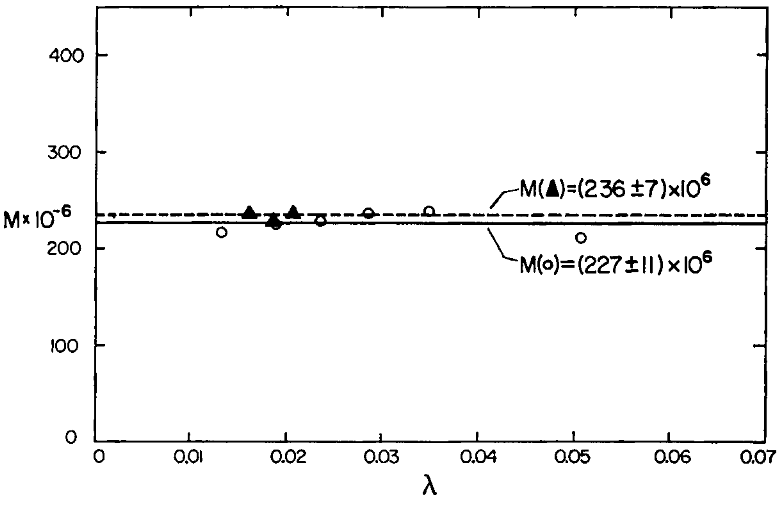

FIG. 5. Experimental molecular weight values for T2 derived from data acquired at various λ values and with sample sizes of 0.2 mg (○) and 0.1 mg (▲).

taining approximately 0.1 mg of T2 for the 0.2 mg injected in the runs discussed above. The three values of molecular weight calculated from these retention data are shown in Fig. 5 as triangles. Averaged, the three yield $M = (236 \pm 7) \times 10^6$. This value is slightly higher than that reported above, but still reasonably consistent. The decreasing retention volume (and thus decreasing apparent molecular weight) with increasing sample size was noted before for polystyrene beads and was attributed to column overloading.

The reasonable consistency of the experimental M values over a range of λ values (and thus l values) and concentration lends confidence to the efficacy of this method in determining molecular weight. Unfortunately, the literature values for M are scattered in the range $(180 \text{ to } 300) \times 10^6$ (*18–20*), and thus offer little aid in judging accuracy.

We note that the SFFF results for polystyrene beads of known size and weight yielded good agreement between theory and experiment, verifying good agreement between theory and experiment, verifying in a general way the accuracy of the method (*9*). An analysis of the data reported in Ref. 9 for the three polystyrene particle sizes (1087 Å, 1756 Å, and 2339 Å) yielding the most reproducible results shows that the measured retention (molecular weight) is higher than the predicted values by amounts ranging from 0 to 5%, with an average of 2.5%. This result must be treated cautiously, however, because there is some uncertainty in the density of the polystyrene beads used in that work.

EXTENSIONS AND LIMITATIONS OF THE SFFF METHOD

Magnitude of *l*

A compact solute layer, characterized by a small characteristic layer thickness l of the order of 10 μ, is the basic requirement for effective fractionation or for efforts to determine physical constants like molecular weight. First of all, the system must exhibit considerable retention, with the retention ratio R preferably < 0.5, or else the retention volume will cease to depend strongly on molecular weight, and both fractionation and the accuracy in determining the parameters underlying retention will suffer. Furthermore, there is a crucial time element tied up in parameter l, because the generation of solute peaks narrow enough to achieve good fractionation and physical-constant accuracy requires a number of diffusional excursions over distance l (*4*). The time required for each such excursion increases with the square of l, in accord with the Einstein equation. Thus it is important to make l as small as possible in order to reduce the time required for an experiment.

Magnitude and Implication of *l*

While SFFF and other forms of FFF should be operated in such a manner that low *l* values will result, there are definite practical limits to the reduction of *l*. For some systems and some solutes, one simply cannot generate a field strong enough to reduce *l* significantly. The experimental system used in this work, for instance, is limited to a few hundreds of gravities; a suitable *l* cannot, therefore, be reached for solutes having a molecular weight less than about 50×10^6. More basic limitations have been discussed (*5*): they are encountered when *l* approaches the dimensions of surface irregularities or of the solute species themselves. They may also be encountered when *l* is small enough to multiply solute concentration to the point where major overloading effects result.

There are too many imponderables at this stage to formulate a practical optimum for *l* values. The experimental work displayed in Fig. 2 follows the expected straight-line trend over the range of *l* values from about 10 to 30 μm. [Quantity *l* is calculated as λw, Eq. (5).] Similarly, polystyrene beads followed theoretical trends with *l* values ranging from 6 to 120 μm (*9*). Experiments in thermal FFF have followed expected behavior with *l*'s ranging from 8 to 100 μm (*8*). It is probable that the optimum *l* lies at values well below the lowest *l*'s reported (*5*). The practical range of *l* values

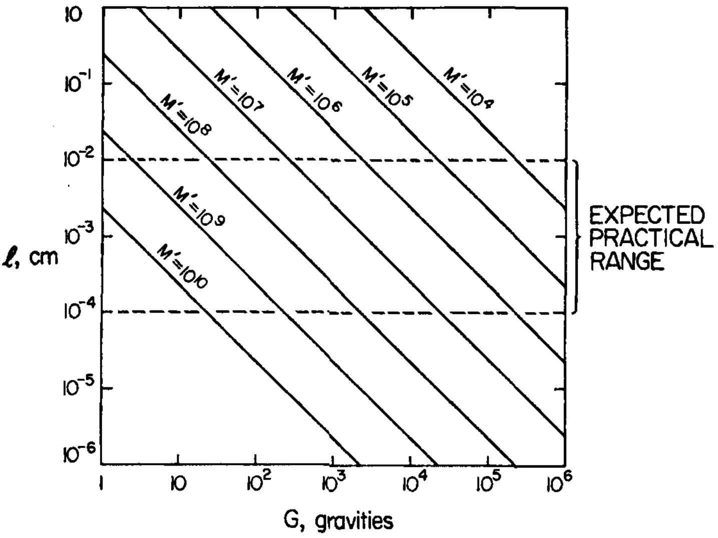

FIG. 6. Plots of the characteristic layer thickness *l* vs field strength *G* for molecules with various effective molecular weight *M'*.

would appear from present knowledge of the subject to fall in the range from 1 to 100 μm.

Equation (4) shows that l is inversely related to field strength G, which points out the necessity for higher G's to diminish l. This matter is given quantitative expression in Fig. 6, which shows the l value expected from solutes of different effective molecular weight M' as a function of G. For a G value of 10^5 g's, M' values of about 20,000 lie at the top of the practical l range. This corresponds to a molecular weight of about 50,000, and represents, to an initial approximation, the smallest species for which SFFF can presently be imagined as applicable. At the other extreme, DNAs and bacteriophages in the approximate M' range from 10^7 to 10^8 require only about 10^3 g's.

Time Requirements

The time required for either fractionation or molecular weight determination is critical. Experiments with macromolecules and particles are inherently slow because of sluggish transport. The time scale of SFFF experiments hinge on these properties also. However, the diffusion distance, l, is small enough that reasonable time characteristics can be expected. Below we will formulate the time requirements of the SFFF method.

The minimum time t_p required to generate one theoretical plate, in chromatography (*14*) or in FFF (*5*), is the plate height H arising from nonequilibrium effects divided by peak velocity $\mathscr{V}$. This ratio is termed the nonequilibrium coefficient, and is designated by C. From Eq. (9)

$$t_p = C = \frac{H}{\mathscr{V}} = \psi \frac{l^2}{D} \tag{10}$$

a time that corresponds roughly to the period needed to diffuse distance l.

If we replace coefficient ψ by its maximum value of 4, use Eq. (4) to eliminate l, and substitute $\mathscr{R}T/Nf$ for D, where N is Avogadro's number and f is the friction coefficient, we obtain

$$C = \frac{4Nf\mathscr{R}T}{M'^2G^2} \tag{11}$$

Friction coefficient f can be written as

$$f = 6\pi\eta\left(\frac{f}{f_0}\right)\left(\frac{3M'}{4\pi N\Delta\rho}\right)^{1/3} \tag{12}$$

where η is viscosity, $\Delta\rho$ is $(1/\bar{v}_s) - \rho$, and (f/f_0) is the friction coefficient for the particle divided by the value it would assume if reshaped into a sphere of the same density.

The substitution of Eq. (12) into Eq. (11) leads to

$$C = \underbrace{12\pi\left(\frac{6}{\pi}\right)^{1/3}}_{46.773} \frac{f/f_0}{M'^{5/3}G^2} \frac{N^{2/3}\mathscr{R}T\eta}{\Delta\rho^{1/3}} \tag{13}$$

We are most interested in the strong variation of C with M' and G', and to a secondary extent in the effect of f/f_0. We focus on these terms in the logarithmic form

$$\log C = 1.6700 + \log\frac{f}{f_0} - \frac{5}{3}\log M' - 2\log G + \log\left(\frac{N^{2/3}\mathscr{R}T\eta}{\Delta\rho^{1/3}}\right) \tag{14}$$

Parameters in the last term are assigned typical values: $T = 293\,^\circ\mathrm{K}$, $\eta = 0.01$ poise, and $\Delta\rho = 0.5$ g/ml. This gives

$$\log C = 20.0276 + \log\frac{f}{f_0} - \frac{5}{3}\log M' - 2\log G \tag{15}$$

in which G is expressed in gravities. Values of $\log C$ are plotted as a function of M' and G in Fig. 7. The effect of f/f_0 for species of various length–width ratios is illustrated on the $M' = 10^6$ plot using prolate ellipsoids of revolution and associated friction coefficients (*21*). The region of practical l values is also shown in this figure.

We conclude from Fig. 7 that operation midway in the practical range of SFFF would require roughly 10 sec per plate for the high-speed rotation of species with $M' \sim 10^5$ or 10^6, and roughly 100 sec per plate for species with $M' \sim 10^7$ or 10^8. These times would be 100-fold less if operation were to prove possible at $l = 10^{-4}$ cm.

The number of plates required for moderate fractionating power and reasonable accuracy in determining physical constants is of the order of magnitude of 10^2. Thus the method would typically require run times from 10^3 to 10^4 sec—roughly 1 hr. These estimates would require revision upward if much more than 100 plates were required or if peak dispersion could not be reduced to its theoretical value. They could be revised downward, to roughly 1 min, if operation at $l = 10^{-4}$ cm proved possible.

Additional time is required for the initial "relaxation time," the time required to essentially form the exponential solute layer (*3*, *9*). This is equivalent to the run time in equilibrium sedimentation, except it is shortened enormously by the small w and l values. In theory this time could

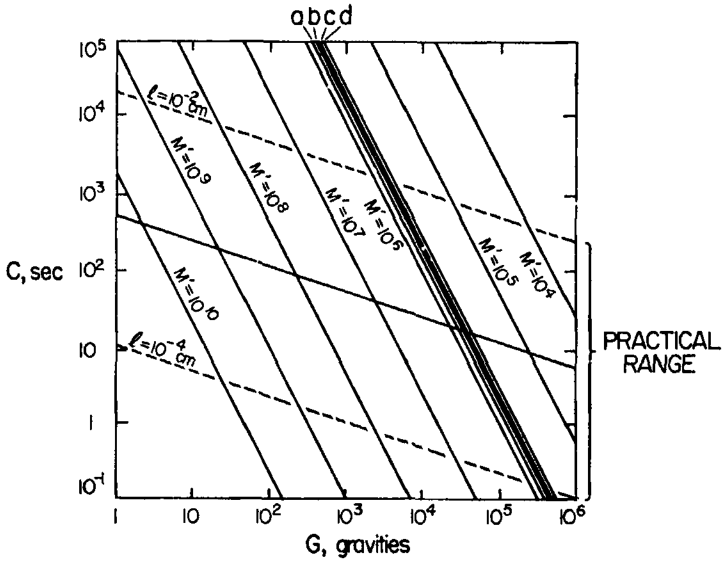

FIG. 7. The time required to generate one theoretical plate, equivalent to C, plotted as a function of field strength G and the effective molecular weight M' of spherical particles. Lines a, b, c, and d illustrate the magnitude of shape factors; these correspond to prolate ellipsoids of revolution having axial ratios of 10, 20, 30, and 40, respectively.

be reduced to only a few C units at low λ values by relaxing initially at very high rotational speeds, and then allowing a second relaxation from the overcompressed layer first formed. The second relaxation would entail diffusion over distance l, and would therefore require a time interval of the order of magnitude of C. This latter approach was not used here and the experimental relaxation times were therefore of the order of 1 hr.

For comparing SFFF time requirements to those existing for other direct techniques, we refer to Yphantis (*22*) who has developed the theory for the time requirements of equilibrium ultracentrifugation. In an application of this to bacteriophage T7 using a 2.4-mm liquid column, Bancroft and Freifelder (*23*) estimated the required time as 80 hr, although they allowed the run to continue for at least 120 hr. The larger phage studied here, T2, would require about 300 hr under corresponding conditions as opposed to 80 hr for T7 because of the fourfold larger diffusion coefficient.

Various indirect methods for determining molecular weights are, of course, more rapid than equilibrium ultracentrifugation. The limitations of these methods with respect to DNA have been discussed by Freifelder *(24)*.

The runs for T2 in this study varied roughly between 10 and 20 hr. Theory suggests that these runs could be shortened by using smaller *l* values and by seeking an optimum flow velocity suitable to the experimental requirement. For instance, the relative flatness of the experimental curve in Fig. 3 suggests that high flow velocities could be used to advantage in SFFF. Several experiments which were tried after the completion of the main body of the work verified this conclusion. Figure 8 shows the results of one such experiment. Using a flow velocity 10 times higher than normal (0.103 cm/sec), we eluted a T2 peak in 85 min after relaxation was completed. The calculated molecular weight was 211 × 10^6, in reasonable agreement with the earlier results. The total time from injection to the elution of the critical part of the peak—its central region—was about 2.5 hr. This time could undoubtedly be reduced by further increases in flow rate and reductions in the overly-conservative hour-long relaxation time. Further gains will accrue if means are found to reduce plate height to its theoretical value.

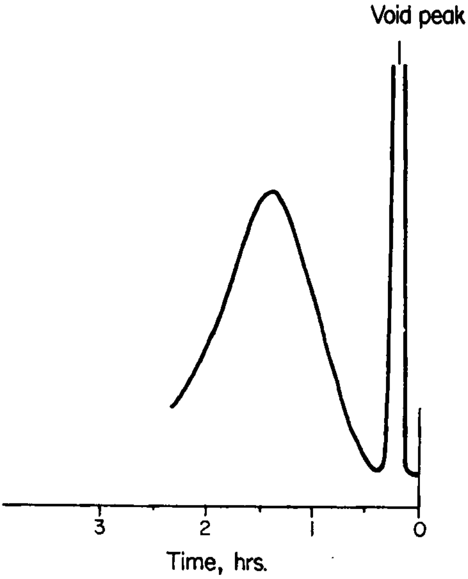

FIG. 8. The high-speed elution of a T2 peak at 259*g*'s and a flow velocity of 0.0103 cm/sec (60 ml/hr).

In summary, the preliminary experiments reported here for bacteriophage T2, combined with earlier results on polystyrene latex beads, show that SFFF can generate differential migration in a column with migration velocities being a predictable function of molecular weight. The potential for both fractionation and molecular-weight determination are thus established. The possible advantages of this method in determining molecular weight include: fast operation; the versatile characteristics of an elution method which decreases the complexity but increases the choice of detection methods; and the natural existence of fractionation which can be used to purify samples as their molecular weights are being determined. A great deal more work will be necessary to examine the areas of applicability and the levels of accuracy, and to determine to what degree the potential advantages listed above can be experimentally realized.

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