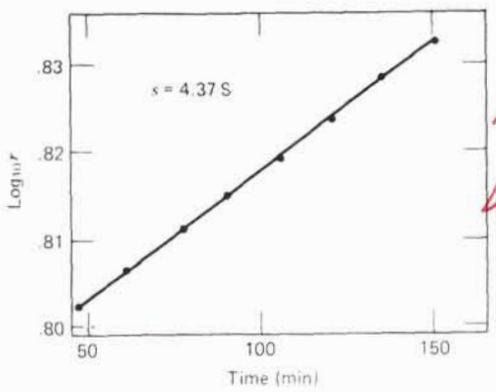

FIGURE 7-15. Determination of sedimentation coefficient from a plot of log (distance from center of rotation to middle of boundary) versus time in a sedimentation rate experiment, for a cellobiosylhydrolase enzyme from the wood-degrading fungus *Trichoderma viride*. Rotor speed was 48,400 revolutions/min. (P. R. Griffith and A. G. Marshall.)

formation) do *not* change with temperature or concentration, often unwarranted assumptions.]

$$s_{20°C,water} = s_{T°C,solution}\left(\frac{\eta_{T°C,solution}}{\eta_{20°C,water}}\right)\frac{(1-\bar{v}\rho)_{20°C,water}}{(1-\bar{v}\rho)_{T°C,solution}} \tag{7-34}$$

$$D_{20°C,water} = D_{T°C,solution}\left(\frac{293°K}{T°K}\right)\left(\frac{\eta_{T°C,solution}}{\eta_{20°C,water}}\right) \tag{7-35}$$

As seen in the next section, viscosity is simply a measure of the frictional force that slows the sedimenting macromolecule, so the viscosity corrections to s and D are intuitively reasonable. The remaining correction to s accounts for the difference in buoyancy between a measurement at 20°C in water and a measurement at $T°$ in the macromolecule-containing solution. Finally, the temperature correction for D accounts for the fact that D is proportional to absolute temperature (see Eq. 7-13).

The *shape*, *size*, and *extent of hydration* of a macromolecule can be derived from s values; the details require a brief discussion of viscosity of nonspherical molecules and are left until the next section.

7.B.2. Sedimentation Equilibrium

The other sedimentation situation that is simple enough to analyze results when there is zero *net flow* of macromolecules across a given section of the spinning solution. Flow of macromolecules across a region of cross-sectional area, A, consists of a flow outward due to sedimentation and a flow inward due to diffusion. At equilibrium, concentration at any one level of the solution is constant, and there is no net flow across that region. The algebraic form of these statements may now be elaborated (from Eq. 6-51):

$$\text{Flow inward} = -DA\frac{\partial[C]}{\partial r} \tag{6-51}$$

$$\text{Flow outward} = \frac{(1 - \bar{v}_{\text{solute}}\rho_{\text{solution}})M\omega^2 rA[C]}{Nf} \tag{7-36}$$

The form of Eq. 7-36 becomes apparent from recognition that

$$[(1 - \bar{v}_{\text{solute}}\rho_{\text{solution}})M][\omega^2 r] = \begin{pmatrix}\text{net}\\ \text{mass}\end{pmatrix}(\text{acceleration})$$
$$= \text{centrifugal force (outward) on one mole of solute particles}$$

while $(1/f)$ is just the (outward) velocity per unit (outward) force per molecule: the reader can quickly be convinced that Eq. 7-36 has the correct units of flow in mass per unit time across the stated area. Because at equilibrium there can be no net accumulation or depletion of solute with time in a given region of the solution

$$\text{Flow inward} + \text{flow outward} = 0 \tag{7-37}$$

or

$$D\frac{\partial[C]}{\partial r} = (1 - \bar{v}_{\text{solute}}\rho_{\text{solution}})M\omega^2 r[C]/Nf \tag{7-38}$$

Making (again) the substitutions, $f = (kT/D)$ and $R = Nk$, and rearranging

$$\int_{C_1}^{C_2}\frac{d[C]}{[C]} = d\,\log_e[C] = \int_{r_1}^{r_2}\frac{M\omega^2(1 - \bar{v}_{\text{solute}}\rho_{\text{solution}})}{RT}r\,dr \tag{7-39}$$

To cast the result in an experimentally useful form, it is necessary to integrate Eq. 7-39 between the positions r_1 and r_2, where the concentration of macromolecule is $[C]_1$ and $[C]_2$:

$$\log_e[C]_2 - \log_e[C]_1 = \frac{M\omega^2(1 - \bar{v}_{\text{solute}}\rho_{\text{solution}})}{2RT}(r_2^2 - r_1^2) \tag{7-40}$$

Experimentally, one need only measure the macromolecular concentration as a function of distance away from the center of rotation, using a transparent ultracentrifuge cell and a suitable optical means for detection of concentration (such as light absorption or refraction), and construct a plot of $\log_e[C]$ versus r^2: the slope of the line will be proportional to the molecular weight of the macromolecule, where the constants of proportionality are readily measured in separate experiments. The important feature of this determination of molecular weight is that *it is not necessary to know the diffusion constant, D*.

The approach to (and the attainment of) sedimentation equilibrium are readily visualized graphically, as shown in Fig. 7-16.

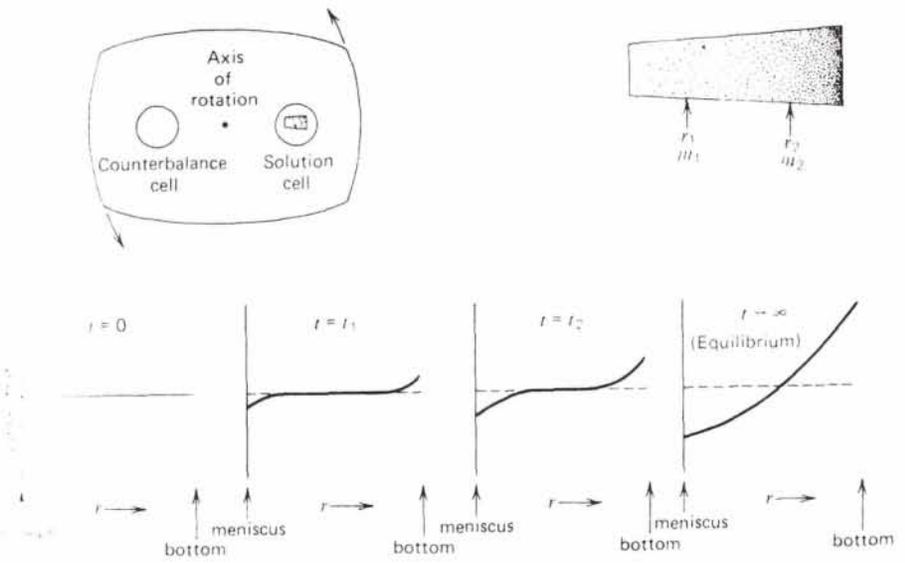

FIGURE 7-16. Schematic diagrams of the progress of a sedimentation equilibrium experiment. *Upper left:* the experimental arrangement. *Upper right:* the solution after equilibrium has been reached. *Bottom diagrams:* stages in the approach to equilibrium. r is the distance from the *center* of rotation, and m is molar concentration at r.

The remarkable accuracy of the sedimentation equilibrium technique in determination of molecular weights is evidenced by the data in Table 7-2, for molecules whose molecular weight is precisely known from the chemical formula for the molecule. The principal disadvantage of the method is that attainment of equilibrium generally requires at least 24 hours, so that measurements are time-consuming; on the other hand, sedimentation equilibrium probably provides the most accurate determination of molecular weight for large molecules in solution.

Table 7-2 Comparison of molecular weight, derived from application of Eq. 7-40 to sedimentation equilibrium data for concentration as a function of distance from the axis of rotation, with molecular weight calculated from the chemical formula for the same compound.*

Substance	Molecular Weight from Chemical Formula	Molecular Weight from Sedimentation Equilibrium
Sucrose	342.3	341.5
Ribonuclease	13,683	13,740
Lysozyme	14,305	14,500
Chymotrypsinogen *A*	25,767	25,670

*After K. E. van Holde, Physical Biochemistry, Prentice-Hall, Englewood Cliffs, N. J.: 1971.

sult is more general and precise, since it does not depend on any particular *mechanism* for the process of interest.

Archibald Method Another common sedimentation analysis is based on the fact that at *any* time during a sedimentation experiment, there can be no net flow of substance across either the *meniscus* or the *bottom* of the centrifuge cell. Thus, Eq. 7-40 will always be valid at those positions in the cell, and may be used to obtain the molecular weight from Eq. 7-43, using the concentrations at just those two points. Practically, however, the concentrations at these points are not measured directly, but are obtained by extrapolation of the concentration-distance profile (see Fig. 7-16), either to the meniscus or to the bottom of the cell. The resultant molecular weight obtained is not nearly as accurate as the measurements based on true equilibrium throughout the cell (since the latter are based on a much *larger* number of *directly* determined data points), but may be obtained *rapidly* since complete equilibrium is not required.

7.B.3. Density Gradient Sedimentation

Although the previous sedimentation techniques can give the molecular weight for a *purified* macromolecule, they are not especially suitable for measuring the molecular weights of each of several different types of macromolecules in a *mixture*. For mixtures, it is first necessary to *separate* macromolecules of different molecular weight, which may be achieved using "density gradient" techniques based on construction of a solution in which the solution density varies smoothly from a high density at the bottom of the sedimentation cell to a low density at the top.

When sedimentation *rate* experiments are conducted in a sucrose density gradient (Fig. 7-17), macromolecules sediment at a rate (approximately) proportional to their sedimentation coefficients. Thus, by introducing macromolecules of known sedimentation coefficient into an unknown sample, allowing sedimentation to proceed, and then withdrawing aliquots of solution and assaying for the presence of each macromolecular species, it is possible to determine the sedimentation coefficients of each of several unknown macromolecules in a solution (Fig. 7-17). When the sucrose gradient is arranged so that macromolecules of different molecular weight sediment at (different) *constant* rates, the method is called "isokinetic" density gradient sedimentation.

For sedimentation *equilibrium*, the density gradient may be generated during the experiment, using a solution of, for example, CsCl salt (see Fig. 7-19 for an example). A given macromolecule in such a solution will either sink or rise until it reaches the ("isopycnic") point in the cell where the density of the solution is the same as that of the macromolecule [i.e., $\rho_{solution} = (1/\bar{v}_{solute})$]. A short calculation (see Problems) then shows that if the solution density varies linearly over a short distance, the macromolecules of a given molecular weight will distribute themselves according to the now-familiar Gaussian curve, centered at the isopycnic point. The great ad-

vantage of both density gradient sedimentation techniques is that once the components of the mixture have been separated by sedimentation, aliquots of solution may be withdrawn from different levels of the centrifuge tube and assayed *chemically* (and thus very *specifically*) for their constituents.

EXAMPLE *Sedimentation of an Enzyme Mixture in an Isokinetic Sucrose Gradient*

In this example, the density gradient is prepared *before* the sedimentation experiment, by, for example, carefully layering a sucrose solution of decreasing density into the sedimentation tube. A thin layer of (less dense) macromolecule-containing solution is then layered on top of the sucrose gradient

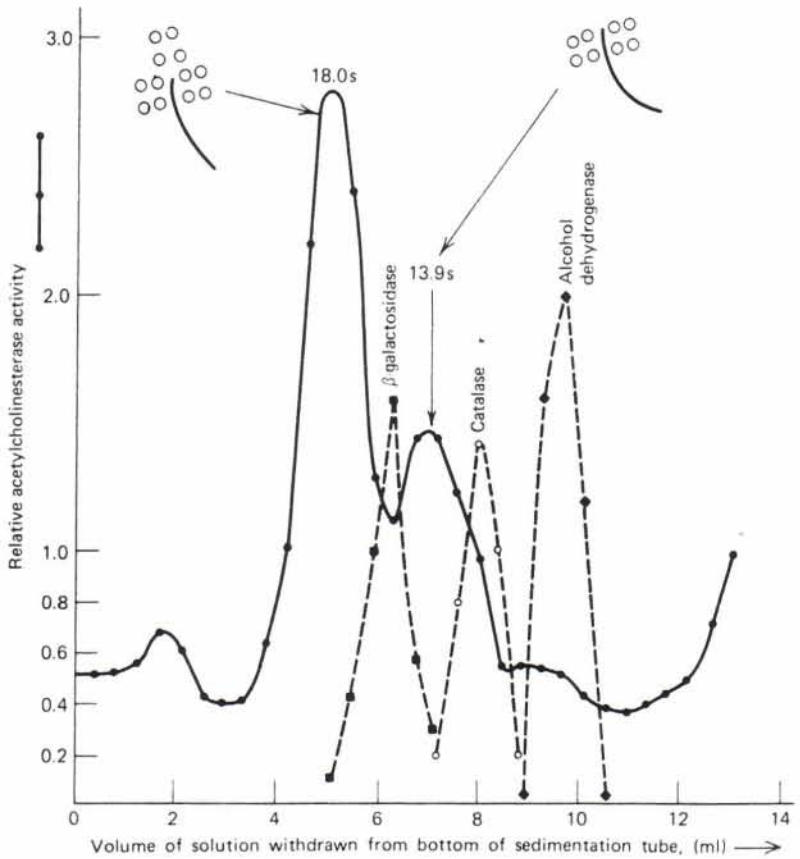

FIGURE 7-17. Sucrose-gradient sedimentation pattern for a mixture of three known enzymes (β-galactosidase, catalase, and alcohol dehydrogenase) and a sample of purified native acetylcholinesterase. Dotted lines show the activities of various aliquots toward substrates of the three known enzymes. Acetylcholinesterase activity provides an indicator of the relative amounts of the two major forms of the enzyme, whose proposed structures (based on electron micrograph evidence) are shown in the figure. [From P. J. Morrod, A. G. Marshall, and D. G. Clark, *Biochem. Biophys. Res. Commun. 63*, 335 (1975).]

solution, and sedimentation is allowed to proceed, with the various enzymes in the mixture sedimenting at different (constant) rates. Assays specific for the various enzymes are then performed on aliquots withdrawn from various depths in the centrifuge tube, with the results shown in Fig. 7-17. In this study, it was possible to determine the relative amounts of two forms of acetylcholinesterase enzyme, using three enzymes of known sedimentation coefficient.

EXAMPLE *Replication of DNA*

The most famous example of density gradient sedimentation is in study of the pairing of DNA molecules, providing direct evidence for the two-stranded

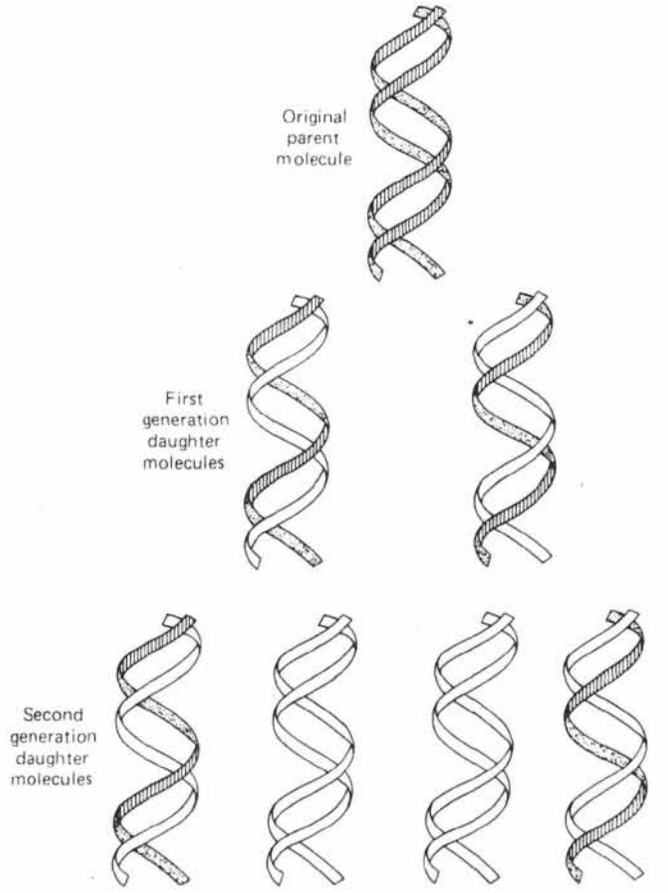

FIGURE 7-18. Mechanism of DNA replication proposed by Crick and Watson. After one replication, each daughter molecule consists of one parental chain (black) paired with one newly synthesized chain (white). Since the parental (single) chain remains intact throughout, the second and subsequent generations will each exhibit two molecules containing one parental chain. [See F. H. C. Crick and J. D. Watson, *Proc. Roy. Soc. London*, Ser. A 223, 80 (1954).]

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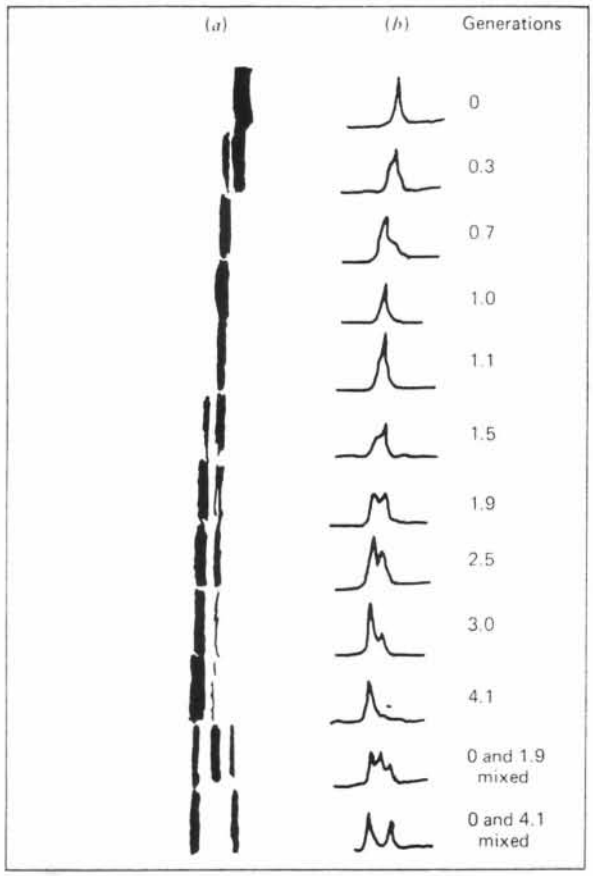


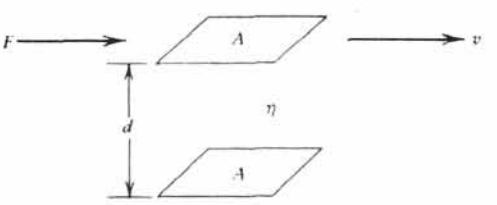
FIGURE 7-19. An application of density-gradient centrifugation. (*a*) Ultraviolet absorption photographs showing DNA bands resulting from density-gradient centrifugation of lysates of bacteria sampled at various times after the addition of an excess of ^{14}N substrates to a growing ^{15}N-labeled culture. Each photograph was taken after 20 hours of centrifugation at 44770 rpm under the conditions described in the text. The density of the CsCl solution increases to the right. Regions of equal density occupy the same horizontal position on each photograph. The time of sampling is measured from the time of the addition of ^{14}N in units of the generation time. The generation times were estimated from measurements of bacterial growth. (*b*) Microdensitometer tracings of the DNA bands shown in the adjacent photographs. The microdensitometer pen displacement above the base line is directly proportional to the concentration of DNA. The degree of labeling of a species of DNA corresponds to the relative position of its band between the bands of fully labeled and unlabeled DNA shown in the lowermost frame, which serves as a density reference. A test of the conclusion that the DNA in the band of intermediate density is just half-labeled is provided by the frame showing the mixture of generations 0 and 1.9. When allowance is made for the relative amounts of DNA in the three peaks, the peak of intermediate density is found to be centered at 50 ± 2 per cent of the distance between the N^{14} and N^{15} peaks. [From M. S. Meselson, F. W. Stahl, and J. Vinograd, *Proc. Natl. Acad. Sci. U.S.A. 44*, 671 (1958).]

nature of DNA in solution. Shortly after the DNA replication mechanism shown in Fig. 7-18 was proposed by Watson and Crick, the following experiment was carried out (see Fig. 7-19). Bacteria that had been grown in a medium containing heavy nitrogen (^{15}N) were suddenly switched to a medium containing only ^{14}N. According to the Crick-Watson replication model, by the end of the first generation, one should observe (double-stranded) DNA molecules with a molecular weight exactly half-way between the values for purely ^{15}N and purely ^{14}N DNA. After two generations, there should be an equal mixture of $^{15}N^{14}N$ and $^{14}N^{14}N$ DNA molecules. These predictions are borne out by experiment (Fig. 7-19). The spectacular resolution furnished by the density gradient experiment is apparent when it is realized that ^{15}N and ^{14}N differ by a factor of only (1/15) in mass, and since a nucleotide is only 8% nitrogen, the separation shown in Fig. 7-19 amounts to resolving between two molecules whose molecular weights differ by about 1/2 of 1 percent!

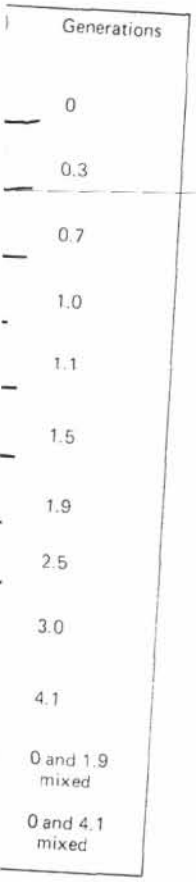
7.C. VISCOSITY

Viscosity is the parameter that characterizes the motion of molecules due to a mechanical "*shear*" force, just as ionic mobility and sedimentation coefficients characterize the response to an *electrical* or *centrifugal* driving force. Definition of viscosity leads directly to a description of the flow of a fluid through a narrow capillary, which affords a simple means for measurement of viscosity. Finally, it is possible to relate viscosity to frictional coefficients for molecules of various shapes, resulting in a number of routes toward determination of molecular shape from sedimentation, diffusion, or viscosity data.

In a description of the attractive forces between 10^{23} molecules by one parameter (viscosity), it is clear that any proposed mechanism must depend much more heavily on plausibility than rigor. The plausibility argument begins from consideration of two parallel plates, each of area A, separated by a distance, d, where the plates themselves are taken to be very thin layers of fluid. The question is, how much force must be supplied to keep the upper plate moving at velocity, v, relative to the lower plate? It is plausible



that more force will be required for plates of bigger area, for plates that are closer together, and for plates moving at larger relative velocities. Finally, it is recognized that the force required to move one plate over the other will depend on the particular fluid involved; all these intuitive suppositions may be collected in an equation:



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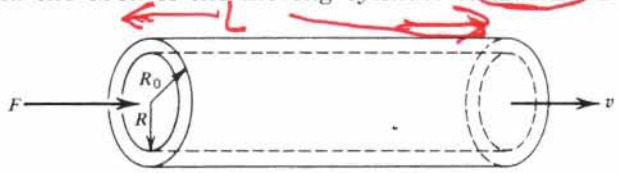
$$F = \frac{Av}{d}\,\eta \tag{7-44}$$

where η is defined as the viscosity of the medium, and may be thought of as the force required to maintain unit velocity difference between two plates of unit area that are one distance unit apart. Equation 7-44 typifies one of the most important aspects in analysis of any experiment, namely the separation of the *geometry* of the experiment from the *intrinsic* ("physical") property under study.

7.C.1. Flow of Fluid in a Capillary

Treatment of flow through a capillary requires no new intuition beyond Eq. 7-44, but does involve a change in the geometry of the experiment. Consider now a cylinder of radius, R_0, and length, L. We are asked to find the force required to push a smaller cylinder of fluid past the outer cylinder at velocity, v. Recognizing that the distance between the cylinders is $(R_0 - R)$ and the area of the moving cylinder is $(2\pi RL)$, Eq. 7-44 gives



$$v = \frac{F(R_0 - R)}{2\pi RL\,\eta} \tag{7-45}$$

An economy in notation is realized by using the relation

Pressure = Force/area

$$P = \frac{F}{\pi R^2} \tag{7-46}$$

to substitute for F in Eq. 7-45

$$v = \frac{\pi R^2 P(R_0 - R)}{2\pi RL\eta} = \frac{PR(R_0 - R)}{2L\eta} \tag{7-47}$$

Finally, by choosing R as infinitesimally close to R_0, so that $(R_0 - R) \to -dR$ and $v \to dv$, and integrating from the surface of the capillary to R_1 (recall that $v \to 0$ as $d \to 0$ in Eq. 7-44), the fluid velocity at any point in the interior of the capillary is obtained:

$$\int_{v \text{ at } R_0}^{v \text{ at } R_1} dv = \frac{-P}{2L\eta}\int_{R_0}^{R_1} R\,dR$$

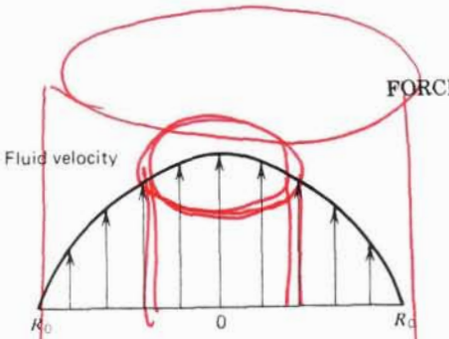


FIGURE 7-20. Fluid velocity in a capillary of radius, R_0, as predicted for laminar flow by Eq. 7-48.

$$v_{\text{at } R_1} = \frac{P}{4L\eta}(R_0^2 - R_1^2) \tag{7-48}$$

A plot of fluid velocity as a function of position within the capillary is thus a simple parabola, as shown in Fig. 7-20. Capillary flow is an example of Newtonian or laminar ("layered") flow for which the lines of flow are straight, as opposed to turbulent or nonNewtonian flow in which vortices or whorls show up in the flow lines. Turbulent flow is thought to be instrumental in initiating the blood-clotting process, by introducing strong shear forces near the edges of a (jagged) cut or projection that facilitate the agglutination of platelets to the rough surface. Clotting in the normal vascular system is prevented by the smooth inner surface of blood vessels that prevents adherence of platelets and thus prevents release of pro-coagulants from the platelets. Similarly, blood flow from a sharp cut is more laminar than from a rough cut, which is why razor cuts clot so slowly. Finally, it is difficult for a clot to form in a rapidly flowing vessel (e.g., an artery), because the flowing blood carries away the pro-coagulants so quickly that their concentrations never rise high enough to sustain clot formation.

A simple measure of viscosity is provided by the Ostwald viscosimeter shown in Fig. 7-21. In the Ostwald device, one measures the length of time required for the bulb of liquid shown at upper right in the diagram to discharge between the two "fiducial" marks at the top and bottom of the bulb. In order to accommodate the result for fluid *velocity* (Eq. 7-48) to the experiment of fluid *flow* (volume/time), one need only notice that the volume of fluid passing through the (infinitesimal) cross-section bounded by R and $R - dR$ per unit time is given by the velocity of the fluid times the area of the infinitesimally thin ring, $v(2\pi R\, dR)$. The total flow of fluid through the total cross-sectional area of the capillary is thus obtained by integration:

$$\text{Flow (volume/time)} = \frac{\pi P}{2L\eta}\int_{R=0}^{R=R_0}[R_0^2 - R^2]\, R dR$$

$$\frac{v}{t} = \frac{\pi P R_0^4}{8L\eta} \tag{7-49}$$

(It is interesting to note that Eq. 7-49 was first derived by Poseuille, an anatomist studying the flow of blood through blood vessels.) Since the pres-

$$\eta_{\text{unknown}} = \eta_{\text{known}} \frac{\rho_{\text{sample}}\, t_{\text{sample}}}{\rho_{\text{standard}}\, t_{\text{standard}}} \tag{7-52}$$

The glass capillary viscosimeter (viscometer) is the simplest to build and easiest to use, but operates at a fixed (large) shearing force that is set by the height of the column of fluid. Elongated molecules, such as DNA, tend to orient with their long axes along the flow lines, when the shear rate is high, and elaborate viscometers based on mechanical or magnetic torque required to maintain a known rotation velocity between two concentric cylinders have been devised for measuring the viscosity of such molecules at the necessarily low shear rate. As seen in Section 4, the orientation of nonspherical molecules due to forced flow provides a measure of the shape of the molecule.

7.C.2. Viscosity, Friction Coefficient, and Macromolecular Shape in Solution

If we express Eq. 7-44 in the form

$$F = fv = \left(\frac{A\eta}{d}\right) v \tag{7-53}$$

then the *frictional coefficient*, f, for the sliding of one *plate* of solution past another is simply

$$f = \frac{A\eta}{d} \tag{7-54}$$

where η = viscosity of solvent

It is straightforward, but tedious, to further modify the geometry of the shear problem to determine the frictional resistance offered by a *sphere* or macromolecule of more complex shape. Basically, all that is involved is a refinement in the definition of viscosity (Eq. 7-44) to limit the definition to the incremental velocity difference between two plates of infinitesimal area separated by an infinitesimal distance; then with the usual procedures of calculus (complicated only by the three-dimensional nature of the problem), integration of the refined Eq. 7-44 over all the surface elements of the object in question leads to an expression of the form, Force $= f \cdot$ velocity. The form of the frictional coefficient for some simple shapes is given in Table 7-3, where the nature of a prolate (cigar-shaped) and oblate (disk-shaped) ellipsoid is shown in Fig. 7-22.

The effect of molecular shape on frictional coefficient (Table 7-3) is most directly evident from comparison to the friction coefficient that would be expected for a sphere of the same volume, as shown in Fig. 7-23. Since the force of frictional drag is directly related to the *surface area* of the ob-

Table 7-3 Friction Coefficients Corresponding to Some Simple Shapes

Shape	Frictional Coefficient	Explanation
Sphere	$f = 6\pi\eta R$	R = sphere radius
Prolate ellipsoid	$f = 6\pi\eta R_0 \dfrac{(1 - b^2/a^2)^{1/2}}{(b/a)^{2/3} \ln\{[1 + (1 - b^2/a^2)^{1/2}]/(b/a)\}}$	a = major axis, b = minor axis, R_0 = radius of sphere of equal volume = $(ab^2)^{1/3}$
Oblate ellipsoid	$f = 6\pi\eta R_0 \dfrac{(a^2/b^2 - 1)^{1/2}}{(a/b)^{2/3} \tan^{-1}(a^2/b^2 - 1)^{1/2}}$	a = major axis, b = minor axis, R_0 = radius of sphere of equal volume = $(a^2b)^{1/3}$
Log rod	$f = 6\pi\eta R_0 \dfrac{(a/b)^{2/3}}{(3/2)^{1/3}\{2\ln[2(a/b)] - 0.11\}}$	a = half-length, b = radius, R_0 = radius of sphere of equal volume = $(3b^2a/2)^{1/3}$

ject in question, and since a spherical object has the *least* surface area of any object having the same volume (see Problems), it is expected that frictional coefficients for nonspherical objects will in general be *larger* than for the "equivalent" sphere of the same volume (Fig. 7-23).

Experimentally, the hydrodynamic *shape* of a macromolecule (i.e., its shape in solution) may be determined from any measurement that yields

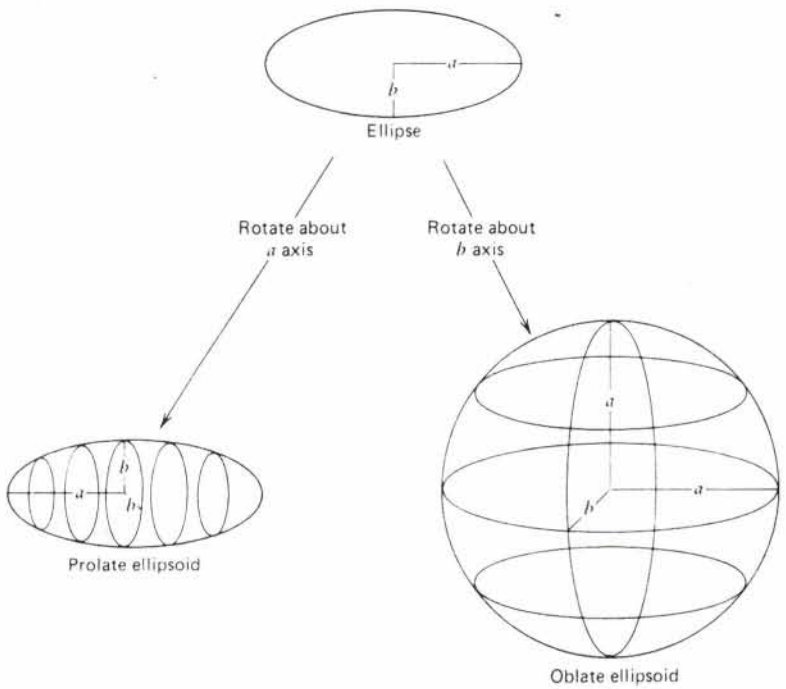


FIGURE 7-22. Prolate and oblate ellipsoids of revolution. (From C. Tanford, *Physical Chemistry of Macromolecules*, Wiley, 1961.)

to Some Simple Shapes

Explanation
R = sphere radius
a = major axis, b = minor axis, R_v = radius of sphere of equal volume = $(ab^2)^{1/3}$
a = major axis, b = minor axis, R_v = radius of sphere of equal volume = $(a^2b)^{1/3}$
a = half-length, b = radius, R_v = radius of sphere of equal volume = $(3b^2a/2)^{1/3}$

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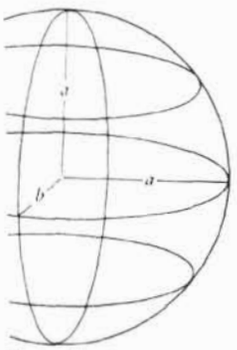

blate ellipsoid

olution. (From C. Tanford,

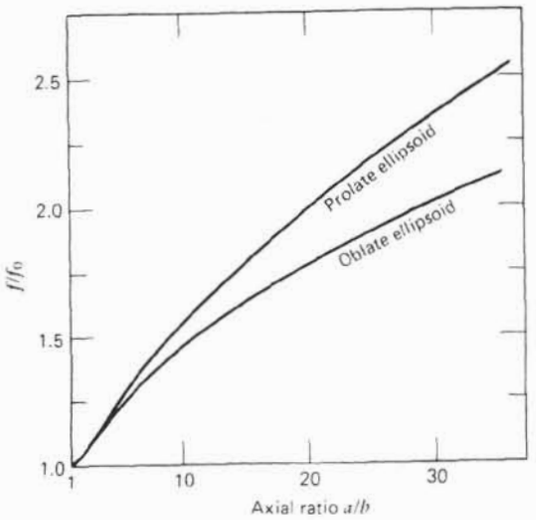

FIGURE 7-23. Frictional coefficient as a function of molecular asymmetry for a range of ellipsoids of revolution. f_0 is the frictional coefficient that would correspond to a sphere of the same volume as the ellipsoid in question. (From C. Tanford, *Physical Chemistry of Macromolecules*, Wiley, 1961.)

the *frictional coefficient*, combined with an independent measurement that yields the effective *radius* of the molecule so that f_0 may be computed from

$$\boxed{f_0 = 6\pi\eta R} \qquad \text{Stokes Law} \tag{7-55}$$

where R is the radius of the macromolecule considered as a sphere.

The molecular radius is generally obtained from (a) light scattering or low-angle X-ray scattering experiments (see Section 4), or (b) the partial specific volume of the molecule and its molecular weight. If the molecule had no water of hydration adhering to its surface, its partial specific volume would be given by

$$\bar{v}_{\text{solute}} = \frac{(\text{volume per molecule})(\text{molecules/mole})}{\text{grams per mole of molecules}} = \frac{\text{volume per gram}}{\text{of solute}}$$

$$\bar{v}_{\text{solute}} = \frac{4\pi R^3 N_0}{3M}, \quad R = \left[\frac{3M}{4\pi N_0}\,\bar{v}_{\text{solute}}\right]^{(1/3)} \tag{7-56}$$

Any real macromolecule will have some amount, δ (grams solvent per gram dry solute), of solvent (with partial specific volume, $\bar{v}_{\text{solvent}}$) bound to the macromolecule and thus increases its effective size according to

$$\boxed{R = \left[\frac{3M}{4\pi N_0}\left[\bar{v}_{\text{solute}} + \delta\,\bar{v}_{\text{solvent}}\right]\right]^{(1/3)}} \tag{7-57}$$