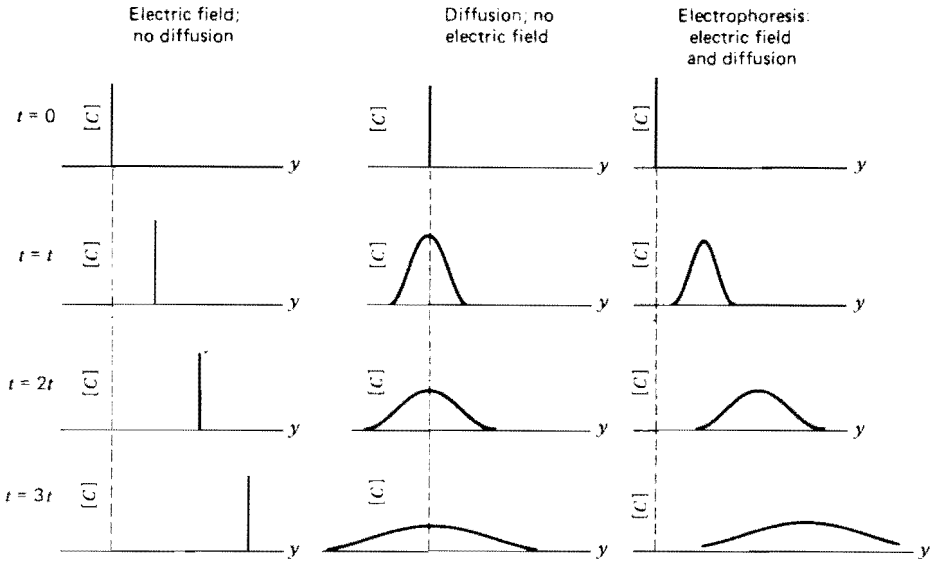

FIGURE 7-3. Plots of ion concentration versus distance under various conditions. Effect of electric field alone is given by Eq. 7-3; effect of diffusion alone is given by Equations 6-55 and 6-56; effect of diffusion plus electric field (electrophoresis) is given by Equations 7-17 and 7-19.

$$\frac{\partial[C]}{\partial t} = D\frac{\partial^2[C]}{\partial(y')^2} \tag{7-19}$$

Equation 7-19 thus shows that in a frame moving along with the ions in the electrophoresis experiment, diffusion proceeds as if the ions were stationary. In other words, diffusion and ion mobility are independent processes, as seen pictorially in Fig. 7-3.

Electrophoresis Techniques and Applications

Gel Electrophoresis The electrophoresis techniques provide some of the very best present means for separation, isolation, and analysis of mixtures of macromolecules, particularly proteins. Applications range from medical diagnostic information to criteria for purity of biochemical preparations, as shown below. The gel itself is most often agar, starch, cellulose acetate, or polyacrylamide, and the experiment consists simply of injection of the sample fluid into a small region of the tubular or strip-shaped gel, followed by immersion of the gel in a suitable buffer and application of an electric field by connection of electrodes at either end of the gel. The various macroions in the sample then migrate at different (constant) rates in the gel, and their location in the gel may be determined after the experiment by staining and/or measurement of light absorption at various positions along the

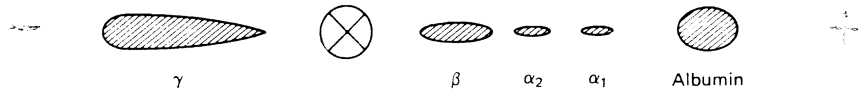

FIGURE 7-4. Electrophoretic pattern of normal human serum proteins for agar gel electrophoresis: anode at right, cathode at left. (After L. P. Cawley, *Electrophoresis and Immunoelectrophoresis,* Little, Brown & Co., Boston, 1969, p. 12.)

gel cylinder or strip. The result of a gel electrophoresis experiment on human serum using agar as the gel medium is shown in Fig. 7-4 (sample initially injected at point marked ⊗).

Just as photographs are made more useful by use of fine-grain film to make possible an image of greater detail and variety, both biochemical research and medical diagnosis have profited from the use of electrophoresis to separate components of biological fluids so that individual components can be analyzed separately for a better "fingerprint." The most studied and most interesting biological fluid is blood: after the cells are removed the remaining fluid is called *plasma*, and if the blood is allowed to clot, the fluid extruded is called *serum* (serum is the same as plasma, but lacks fibrinogen —see Table 7-1).

$$\text{Blood} \xrightarrow[\text{cells}]{\text{Remove}} \text{Plasma} \xrightarrow[\text{(removes fibrinogen)}]{\text{Allow to clot}} \text{Serum}$$

Table 7-1 Average Distribution of Normal Human Plasma Proteins

Albumin	50%
α_1-globulin	4
α_2-globulin	12
β-globulin	13
Fibrinogen	8
γ-globulin	13

URINE, CSF
SIMILAR BUT
100x LOWER LEVELS

The names of these plasma proteins derive from their position following an electrophoresis experiment; the functions of the components are described in any biochemistry text.

EXAMPLE *Gel Electrophoresis in Diagnosis of Disease*

Figure 7-5 shows the normal electrophoretic pattern for serum proteins, along with the characteristic changes that appear in three abnormal conditions. Because these experiments were conducted with cellulose acetate as the gel, the final gel strip is transparent and the proteins may be located according to their absorption of light as shown in the figure. Besides the conditions described in the figure, there have been several recent attempts to correlate the serum glycoprotein level with incidence of atherosclerosis (arteriosclerosis);

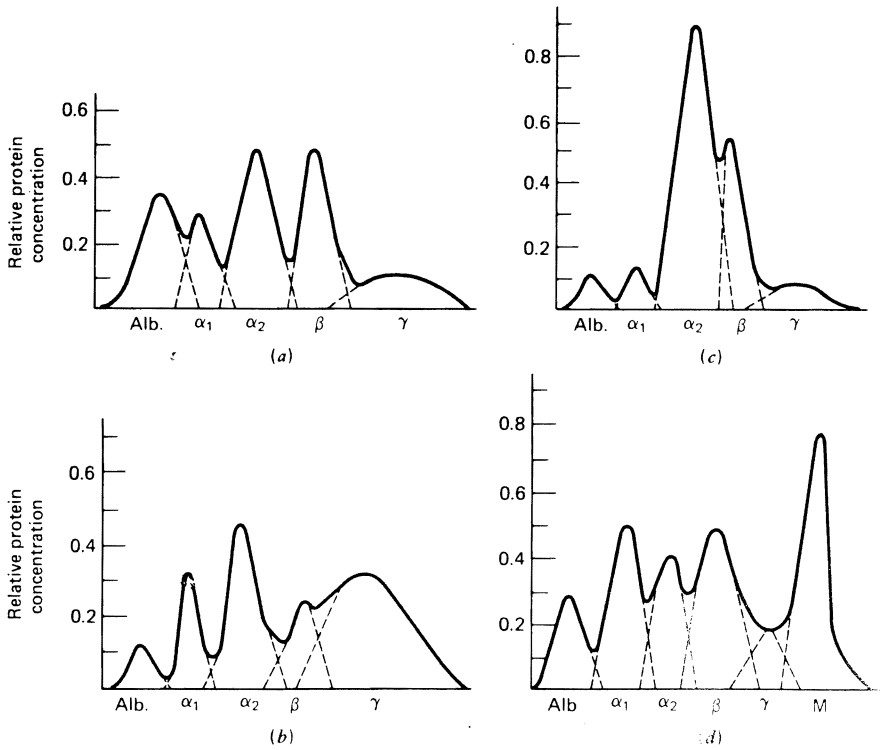

FIGURE 7-5. Cellulose acetate gel electrophoresis patterns for (*a*) normal serum, (*b*) infectious hepatitis, (*c*) lipid nephrosis, and (*d*) gamma myeloma. Protein fractions are listed in Table 7-1. Protein concentration is determined from optical absorption (see Section 4). [From *Clinica Chimica Acta*, 672 (1960).]

in this connection, it is interesting to note that Australian aborigines have a much lower incidence of arteriosclerosis and also lower serum glycoprotein level than do urban populations. With the much more selective separative capacity rendered by the recent disc-electrophoresis (see below), the diagnostic value of electrophoresis is bound to improve.

Electrophoresis is of diagnostic value in analysis of proteins of two other body fluids: cerebrospinal fluid and urine. The only difficulty is that while the protein constituents of both fluids are similar to those in serum, the respective concentrations are down by a factor of 100 and 1000. The most specific changes in the relative amounts of cerebrospinal fluid proteins are found for multiple sclerosis, meningitis, and encephalitis, with some changes also observed in degenerative diseases or neoplastic (cancerous) infiltration. Appearance of proteins in urine is a sensitive indicator of renal (kidney) malfunction—ordinarily, the kidneys filter out almost all serum protein in formation of urine, but in kidney disease, proteins can leak through into the urine. Since electrophoretic mobility is a function of molecular weight, the severity of the dysfunction can be judged in part from the size of the proteins that escape from the

kidney. Detection of the relatively small "Bence Jones proteins," which correspond to the "light" chains of the immunoglobins (gamma globulins), is diagnostic for plasmocytoma and to a lesser extent primary macroglobulinemia. In all these tests, the procedure is as for serum proteins, except that electrophoresis is preceded by a 100-fold or 1000-fold concentration by dialysis under pressure (see Chapter 2.C).

The rate at which a protein can move through a solution (sedimentation, diffusion) or through a gel (chromatography, electrophoresis) is greatly affected by the macromolecular *shape*. Determination of the molecular weight of an unknown protein (with unknown shape) is thus a difficult matter. Two general methods are now available for inducing virtually any protein to take on the same shape: (1) 6M Guanidine HCl unfolds the tertiary structure of proteins so that they are to a good approximation random coils, or (2) sodium dodecyl sulfate (a common detergent) at 5×10^{-4} M or greater binds to proteins all along their length and causes them to behave as rods of constant diameter whose long axis is proportional to the molecular weight of the polypeptide chain. [In both methods, the —S—S— bonds that hold various peptide chains together must be reduced (broken) before treatment with guanidine or detergent.] Following treatment with detergent, it is found that the electrophoretic mobility correlates smoothly with molecular weight of the unraveled protein (see Fig. 7-6), in marked contrast to the irregular correlation of diffu-

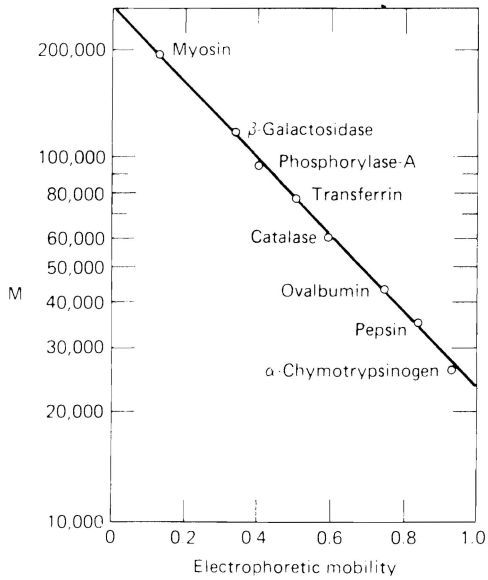

FIGURE 7-6. Correlation between molecular weight (log scale) and electrophoretic mobility for proteins that have been previously unraveled by reduction of disulfide linkages and treatment with sodium dodecyl sulfate detergent. [From H. R. Trayer et al., *J. Biol. Chem. 246,* 4486 (1971).]

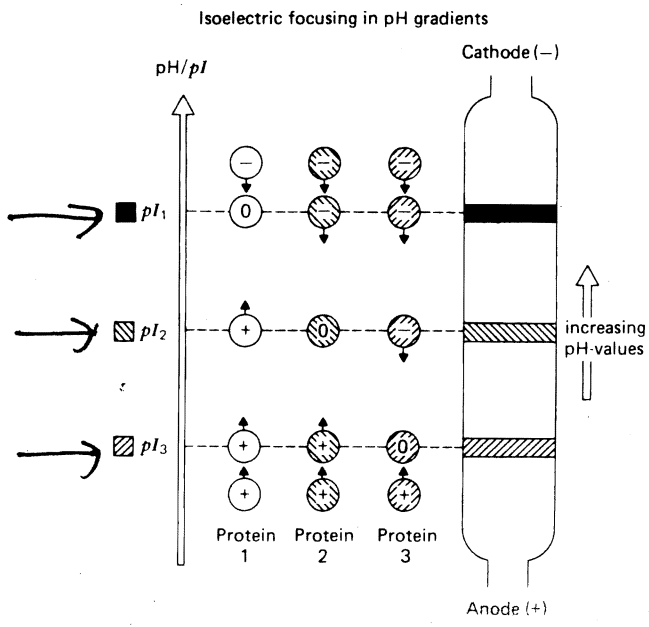

FIGURE 7-7. Three proteins of respective isoelectric points, pI_1, pI_2, and pI_3, in a schematic electrofocusing experiment. Each protein carries a negative charge above its *pI* (and thus is forced down the column) and a positive charge below its *pI* (and then is forced up the column). Each protein therefore moves in the electric field until it reaches that level in the column where the pH of the gel matches the *pI* of the protein. [From H. Haglund, *Methods of Biochemical Analysis 19*, 1 (1970).]

sion constant (or mobility) with molecular weight shown in Table 6-1. Confronted with an unknown protein, one need merely inject a sample containing the protein and several standard proteins of known molecular weight into a polyacrylamide gel (after prior treatment with detergent) and interpolate to find the molecular weight of the unknown protein—this is rapidly becoming the method of choice for finding the approximate molecular weight of proteins.

Isoelectric Focusing in pH Gradients The principle of isoelectric focusing is illustrated in Fig. 7-7. The technique is based on the fact that any ampholyte (a molecule with two or more dissociable protons) is positively charged at a pH below its isoelectric point and negatively charged at a pH above the isoelectric point; at the isoelectric pH, the ampholyte will be neutral and thus stationary in an electric field (see Chapter 3.C for detailed discussion of these terms). Thus, by preparing a gel (again the gel serves to minimize diffusion) with a pH-gradient throughout its length, a given protein on application of an electric field will migrate to the pH matching its isoelectric point, *pI*, as shown in Figures 7-7 and 7-8.

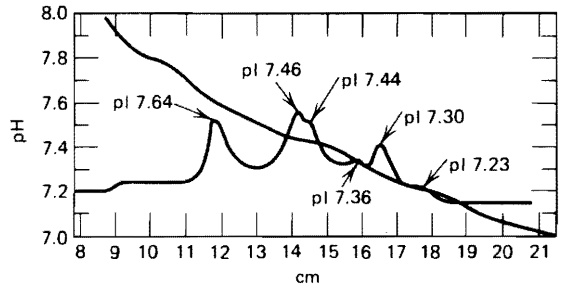

FIGURE 7-8. Separation of hemoglobin components by isoelectric focusing. The smooth curve represents the pH as a function of distance along the column; the curve with peaks indicates the appearance of various proteins (measured from optical absorbance). (From H. Haglund, *Science Tools 14*, 17 (1967), reproduced by permission from LKB Producter AB.)

EXAMPLE *Separation of Hemoglobin Components by Isoelectric Focusing*

One of the most spectacular accomplishments in isoelectric focusing is shown in Fig. 7-8. In that example, it proved possible to separate two proteins whose isoelectric points differed by only 0.02 pH unit!

Immunoelectrophoresis In this technique, electrophoresis is conducted as usual in a strip of agar (negatively charged) or agarose (neutral) gel; the strip is then removed, and trenches are placed on either side of the strip so that the edges of the strip are immersed in a solution containing various antibodies. The antibodies are chosen to be specific to certain of the proteins (antigens) that have been separated in the electrophoresis; thus a precipitin band will form for each antigen/antibody reaction. As seen in Fig. 7-9, a group of proteins with the *same* electrophoretic mobility may be resolved by reaction with antibody, because the *concentrations* of the components of the group are different, and thus their rates of radial diffusion differ.

By now it is clear that the term "gamma globulin" encompasses a large number of proteins, which are now classified as immunoglobulins IgG, IgA, and IgM, as described in any recent immunology text. The utility of immunoelectrophoresis lies in the detection of decreases or increases in the concentrations of these immunoglobulins: each immunoglobulin is thought to be produced by a group of cells (clone) deriving from a single ancestral cell, and assay of individual immunoglobulins is of great value in differential diagnosis of types of bone marrow tumors as well as deciding whether such tumors are benign (immunoglobulin level is relatively constant with time, over several months) or malignant (immunoglobulin level increases with time).

Discontinuous ("Disc") Electrophoresis With any conventional electrophoresis experiment, the success in resolving separate components depends

not justify more detailed treatment here, but the principles are treated at length in any discussion of "transference numbers" in conventional physical chemistry texts. Figure 7-13 shows a typical experimental isotachopherogram for a mixture of several biologically interesting anions. Similar procedures have been used to analyze quantitatively for potassium, sodium, calcium, magnesium, and lithium in serum, based on differences in the ionic mobility of the component ions.

7.B. SEDIMENTATION

Suspensions of very large particles in water will eventually settle out under the force of gravity, if the density of the particle is greater than the density of the solution. For biological macromolecules, however, the suspension will never settle out of its own accord, because the (randomly oriented) thermal velocity of the macromolecules is much larger than the (uniformly directed) velocity due to the force of gravity. For example, for a macromolecule of molecular weight, 100,000, the gravitational (potential) energy between two points which are 1.0 cm apart amounts to

$$E_{\text{gravity}} = \text{mgh} \cong (100{,}000/6 \times 10^{23})\text{kg} \cdot (10\ \text{m sec}^{-2}) \cdot (10^{-2}\ \text{m})$$
$$= 1.7 \times 10^{-23}\ \text{joule} \qquad (7\text{-}23)$$

where the acceleration of gravity has been rounded off to 10 m sec^{-2}. For the same molecule, the thermal energy (see Section 5) is of the order of

$$kT = (1.38 \times 10^{-23}\ \text{joule}\ ^\circ\text{K}^{-1}) \cdot (2.93 \times 10^{2}\ ^\circ\text{K}) = 4 \times 10^{-21}\ \text{joule} \qquad (7\text{-}24)$$

or about 200 times bigger. The ultracentrifuge is simply a device for "amplifying" gravity to the point where the "gravitational" energy exceeds the thermal energy so that the macromolecules will settle to the bottom of the container.

Sedimentation (settling-out) *rates* allow for separation of mixtures of macromolecules according to their *size* and *density*. The sedimentation pattern at *equilibrium* furnishes an absolute determination of *molecular weight* and an indication of the heterogeneity of the mixture. Use of both methods in concert provides a definite value for macromolecular weight, and mixed information about the *shape* and *extent of hydration* (water-binding) for the macromolecule. Although newer chromatographic and electrophoretic techniques have largely supplanted the use of the ultracentrifuge as a means for preparative *separation* of proteins in solution, the great majority of protein molecular weights in the literature are determined from sedimentation methods, and sedimentation techniques are still the method of choice for characterization of nucleic acid size and shape; for study of subunit:multimer equilibria for proteins and nucleic acids; and other polymers; and for separation of biological membrane components.

7.B.1 Sedimentation Rate

Analysis of electrophoresis experiments is relatively simple because the driving force (the electric field) is *constant* in time throughout the process. In contrast, the driving force (centrifugal force) in sedimentation studies *varies* with the distance away from the center of rotation, so that the force on a macromolecule increases as the molecule sediments toward the bottom of its solution—this complication in fact renders impossible any analytical solution (i.e., an exact solution expressible in a finite number of terms) of the macromolecular motion. There are, however, two simplifying possibilities for which the mathematical solution is compact: either when there is *zero net force* on the molecule, or when there is *zero net flow* of molecules

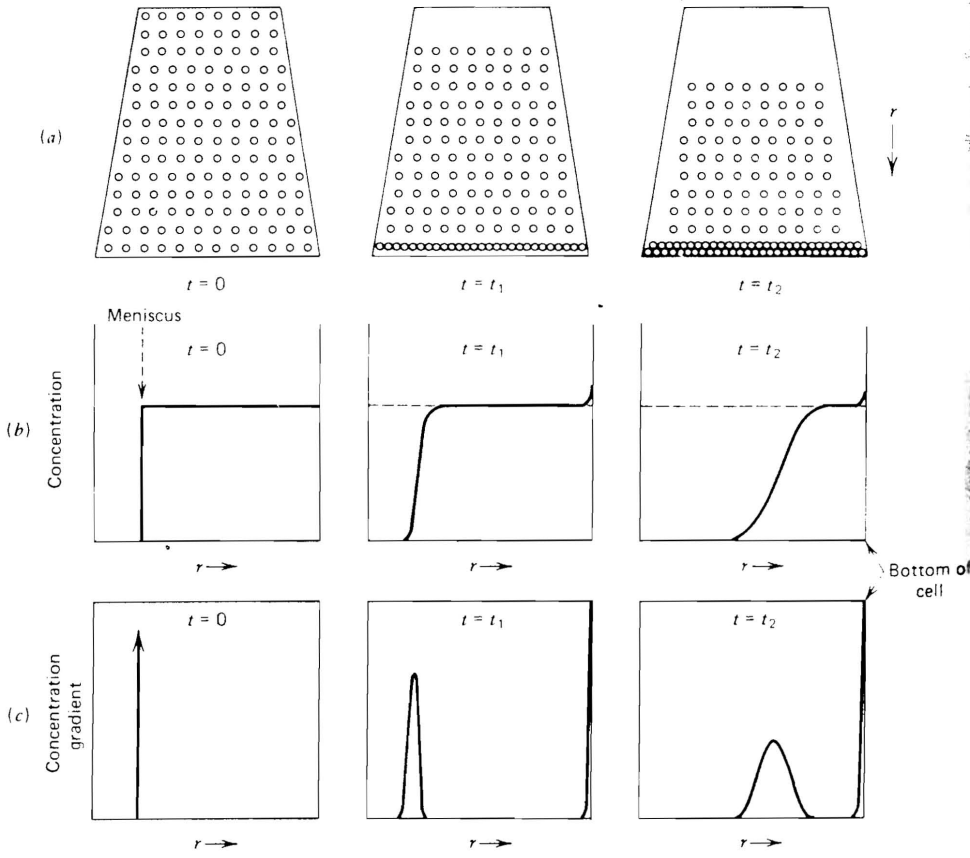

FIGURE 7-14. Progress of a sedimentation experiment at very high centrifugal force. (*a*) Distribution of macromolecules at three separate stages of the experiment. (*b*) and (*c*) show the concentration and the concentration gradient as a function of distance from the center of rotation, *r*, including the effect of diffusion at the boundary, for three separate times during the experiment. (After C. Tanford, *Physical Chemistry of Macromolecules*, Wiley, 1961, p. 366.)

across a particular region of the solution. All sedimentation techniques are designed to take advantage of one or the other of these two situations; we will consider first the case of zero net force on the macromolecule.

The progress of a sedimentation *rate* experiment is shown schematically in Fig. 7-14 for (the usual case of) a macromolecule more dense that the solution around it.

The net centrifugal force acting on a mole of macromolecules of molecular weight, M, is given by the product of the effective mass of the macromolecules and the acceleration, $\omega^2 r$, where ω is the angular velocity of the rotor:

$$F_{\text{centrifugal}} = \left[\begin{matrix}\text{mass of one mole} \\ \text{of macromolecules}\end{matrix} - \begin{matrix}\text{mass of solution displaced} \\ \text{by one mole of macromolecules}\end{matrix}\right]\omega^2 r \tag{7-25}$$

In other words, the macromolecule only tends to settle out if its density is greater than that of the solution around it. Now the mass of one mole of macromolecules is just the molecular weight, M, and

$$\begin{matrix}\text{Mass of solution displaced} \\ \text{by a mole of macromolecules}\end{matrix} = \frac{\text{g solution}}{\text{cc sol'n}} \cdot \frac{\text{g solute}}{\text{mole solute}} \cdot \frac{\text{cc solution displaced}}{\text{by 1 g solute}}$$

$$= \rho_{\text{solution}}\, M\, \bar{v}_{\text{solute}} \tag{7-26}$$

where ρ_{solution} is just the solution density, and $\bar{v}_{\text{solute}}$ (partial specific volume of solute)* is the volume increase of a very large volume of solution resulting from addition of one gram of solute (the large volume of solution is just to ensure that the concentration of macromolecule will be the same before and after addition of a little more macromolecule—see Chapter 2). The net centrifugal force may thus be written more compactly

$$F_{\text{centrifugal}} = \omega^2 r[M - M\bar{v}_{\text{solute}}\rho_{\text{solution}}] = M\omega^2 r\left(1 - \bar{v}_2\rho_1\right) \tag{7-27}$$

As for electrophoresis, the forced motion will be opposed by a frictional force (in the opposite direction) proportional to the macromolecular velocity:

$$F_{\text{friction}} = -Nf\frac{dr}{dt} \tag{7-28}$$

where Avogadro's number, N, has been inserted into Eq. 7-28 because we are now dealing with a mole of particles rather than just one particle. A macromolecule in the ultracentrifuge will thus accelerate under the driving

In Chapter 2, we encountered the related quantity, partial molal *volume, $\bar{V}$, where $\bar{V} = M\bar{v}$ = volume of solution displaced by one* mole *of solute.*

centrifugal force. Until that force is exactly balanced by friction; thereafter, sedimentation will proceed at a *constant* rate, obtained by setting net force $= F_{\text{friction}} + F_{\text{centrifugal}} = 0$:

$$Nf\frac{dr}{dt} = M\omega^2 r[1 - \bar{v}_{\text{solute}}\rho_{\text{solution}}] \tag{7-29}$$

Defining a sedimentation coefficient, s, as the sedimentation rate per unit centrifugal force, and rearranging, we obtain

$$M = \frac{Nf\, dr/dt}{(1 - \bar{v}_{\text{solute}}\rho_{\text{solution}})\omega^2 r} = \left(\frac{Nf}{1 - \bar{v}_2\rho}\right)s$$

where

$$\boxed{s \equiv \frac{dr/dt}{\omega^2 r}} \qquad = \frac{1}{\omega^2}\frac{(dr/r)}{dt} = \frac{1}{\omega^2}\frac{d\ln r}{dt} \tag{7-30}$$

Finally, recalling that $f = (kT/D)$ (Eq. 7-13) and $(Nk) = R$, the desired result appears:

$$\boxed{M = \frac{RTs}{D(1 - \bar{v}_{\text{solute}}\rho_{\text{solution}})}} \tag{7-31}$$

Equation 7-31 represents one of the most direct determinations of molecular weight, and thus deserves some scrutiny. Experimental determination of D has been discussed in Chapter 6.B (see also section 6) and measurement of density and temperature poses no special problems. Sedimentation coefficient is obtained by first rearranging Eq. 7-30

$$\frac{dr/r}{dt} = \frac{d\ \log_e r}{dt} = \omega^2 s \tag{7-32}$$

Thus, a plot of log (distance from center of rotation to center of boundary) versus time gives a straight line whose slope is proportional to s, as shown in Fig. 7-15 for a glycoprotein; since s values are typically of the order of 10^{-13} sec, they are typically reported in units of Svedberg, or S, where

$$1 \text{ Svedberg} = \text{S} = 10^{-13} \text{ sec} \tag{7-33}$$

A final convention is to report all values for s and D as if they were determined at 20°C using distilled water solvent. The appropriate compensations for changes in solution density, partial specific volume, and viscosity, η, (Chapter 7.C) are given in Equations 7-34 and 7-35, assuming that f is proportional to η (Chapter 7.C). [In using these equations, it is implicitly assumed that the molecular properties of the *solute* (such as con-

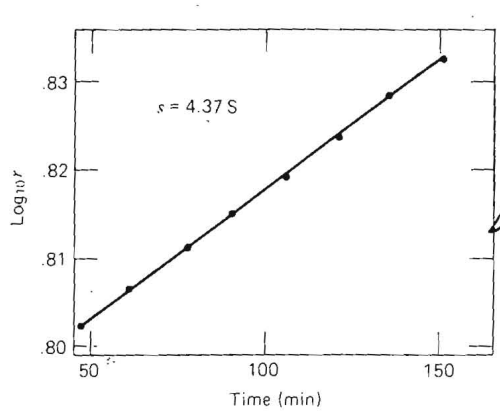

FIGURE 7-15. Determination of sedimentation coefficient from a plot of log (distance from center of rotation to middle of boundary) versus time in a sedimentation rate experiment, for a cellobiosylhydrolase enzyme from the wood-degrading fungus, *Trichoderma viride*. Rotor speed was 48,400 revolutions/min.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