Particularly with synthetic polymers, random coil calculations permit direct conclusions regarding the effect of solvent. In a "good" solvent that attaches itself to the polymer at many places, the polymer will swell (larger root-mean-square end-to-end distance), because the polymer chain is no longer as free to fold back on itself. Similarly, a "poor" solvent effectively compresses the polymer because of repulsive forces between solvent and external polymer surface.

Similar effects arise for the biologically more common polymers that possess many electrical charges along the chain. These charges ordinarily act to stabilize the (usually rigid) structure of enzymes and nucleic acids, through electrostatic bonds between parts of the chain. The presence of highly polar or charged species, such as urea, guanidine, salt, or detergent disrupts these electrostatic bonds and allows the biopolymer to unfold. For example, the (charged) sodium carboxymethylcellulose polymer in the presence of high salt concentration exhibits the same dimensions in solution as does the (uncharged) cellulose polymer. Detergents have recently been found of great value in the selective dismembering of biological membrane components, to permit the isolation of membrane-bound protein enzymes and drug receptors. Another use for detergents devolves from their capacity to unfold and coat most proteins into an approximate rod shape, where the length of the rod is directly related to the molecular weight of the polypeptide. The great difficulty in determination of the molecular weight of a protein is that almost all determinations depend on the shape of the protein; the addition of detergent or guanidine hydrochloride unfolds most polypeptides to a common shape (rod or random coil), and the polypeptides can then be compared on the basis of size (molecular weight) alone.

6.B. TRANSLATIONAL DIFFUSION

Translational diffusion of a substance across a *boundary* is a problem or an advantage in a great number of biophysical measurements. Analysis of diffusion is required for understanding the patterns of movement and separation of components of mixtures in the *ultracentrifuge,* in *chromatography,* and *electrophoresis.* Diffusion is the basis for analysis of the spreading of antigen on an agar plate in *immuno-assays.* Diffusion rates may be used to calculate the *molecular weight* of large molecules, one of the most basic (and most difficult) problems in biochemistry. Finally, it is essential to understand *passive* diffusion before it is possible to treat *active* transport across membrane boundaries.

In this section, we first examine an intuitive derivation of diffusion behavior, in a way that provides for a direct comparison to the rigorously derived random walk solution. The utility of the intuitive result becomes clear in the ensuing treatments of electrophoresis and sedimentation, where

in addition to diffusion at a boundary, the boundary itself is forced to move under an electrical or centrifugal force.

Intuitive "Derivation" of the Diffusion Equation

Physical "laws" represent intuitive claims that cannot be derived from simpler claims, and whose consequences find no contradiction in experiment. Newton's laws of motion and the laws of thermodynamics are examples; so is the very important Fick's law of flow. Fick's intuition was that flow of substance from an area of one concentration to an area of different concentration should be proportional to the difference in concentration between the two regions, by analogy to the flow of heat, which is proportional to the difference in temperature between the two regions in question (an index finger freezes faster in liquid nitrogen than in ice water!):

$$\text{Flow} = -D\left(\frac{\partial c}{\partial y}\right) \cdot (\text{cross-sectional area across which flow occurs}) \qquad (6\text{-}51)$$

where D is the constant of proportionality, the minus sign indicates that the direction of flow is from larger to smaller concentration region, and the calculus notation is used to refine the statement of the law to incorporate an infinitesimal change in concentration in crossing the boundary. The form of Eq. 6-51 is much more general than the present applications would suggest: a similar equation holds for flow of heat proportional to a temperature gradient, flow of fluid proportional to a pressure gradient, or flow of electricity proportional to a voltage (electrical "pressure") gradient.

To test the intuitive claim of Eq. 6-51 we need merely look at a diffusion situation that compares directly to the random walk problem. Suppose, then, that at time zero, an infinitesimally thin layer of red dye is surrounded on both sides by solvent; at later times, the dye will spread out into the solvent, and the concentration of dye in any thin layer can be determined (for example) by scanning the absorption of light through a very thin slit that moves across the solution. Equation 6-51 is couched in terms of *flow* rather than *total amount* of substance, however, so we will want to consider the *rate of change* of concentration in any one thin layer (see Fig. 6-4).

Consider then the region between y and $(y + dy)$ in Fig. 6-4. For convenience, assume that the thin layer has unit cross-sectional area, so that the volume of the layer is just $(dy \cdot 1 \cdot 1) = dy$. The time rate of change in concentration in the layer is simply given by the difference between the flow of dye into the region and flow of dye out of the region:

$$\frac{\partial c}{\partial t} = \frac{\text{Flow in (moles/sec)} - \text{Flow out (moles/sec)}}{\text{Volume of region (cc)}} \text{ in moles cc}^{-1}\text{sec}^{-1}$$

$$\frac{\partial c}{\partial t} = \frac{-D(\partial c/\partial y)_{\text{at } y \text{ (entrance to region)}} + D(\partial c/\partial y)_{\text{at } y+dy \text{ (exit)}}}{dy \cdot 1 \cdot 1} \qquad (6\text{-}52)$$

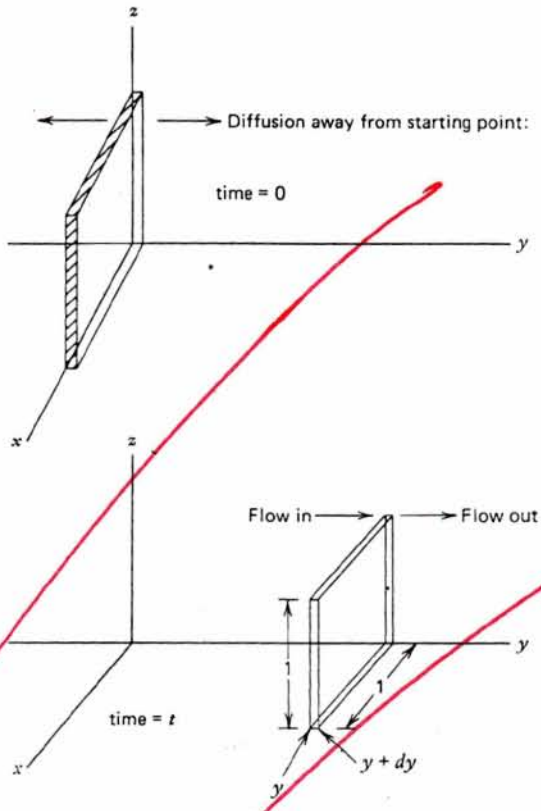

FIGURE 6-4. Progress of a diffusion experiment, starting with all the substance in a thin layer at $y = 0$ at time zero; Eq. 6-52 gives the rate of change in concentration of substance in region, y, $y + dy$ at later time, t. The layer is taken to have unit cross-sectional area.

Equation 6-52 poses the same problem that was faced early in the random walk case: the result is precise, but inconveniently phrased. The choice of an infinitesimal region may now be turned to advantage as follows. If concentration at time, t, is a function of distance, then concentration *gradient*, $(\partial c/\partial y)$, is also a function of distance, say $f(y)$. Now over any small interval, a function may be evaluated in terms of its value and the value of its derivatives at a nearby point, using a Taylor series (see Appendix):

$$f(y)_{\text{at } y} = f(y_0) + f'(y)_{\text{at } y_0}(y - y_0) + \frac{f''(y)}{2!}_{\text{at } y_0}(y - y_0)^2 + \cdots \qquad (6\text{-}53)$$

Equation 6-53 may now be used to evaluate $(\partial c/\partial y)$ at $(y + dy)$, from $(\partial c/\partial y)$ at y:

$$\left[\frac{\partial c}{\partial y}\right]_{\text{at } y+dy} \cong \left[\frac{\partial c}{\partial y}\right]_{\text{at } y} + \frac{\partial}{\partial y}\left[\frac{\partial c}{\partial y}\right]_{\text{at } y} \cdot (y + dy - y) \qquad (6\text{-}54)$$

Substituting the right-hand side of Eq. 6-54 for $(\partial c/\partial y)_{\text{at } y+dy}$ in Eq. 6-52 gives

$$\boxed{\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial y^2}} \quad \text{Diffusion Equation} \tag{6-55}$$

Equation 6-55 is the famous *diffusion equation,* which is one of the most important equations in physical science; most of the others are encountered later in this book. Solution of Eq. 6-55 entails specialized methods pioneered by Fourier, and would detract from the simplicity of the result, which is

$$\boxed{\frac{c(y)}{c(0)} = \frac{1}{2\sqrt{\pi Dt}} e^{-y^2/4Dt}} \tag{6-56}$$

where $c(y)$ is the concentration of substance at distance y at time t, and $c(0)$ is the concentration of substance initially at the origin at time zero.

An example of the success of Eq. 6-56 in fitting actual experimental diffusion results is shown in Fig. 6-5. It is readily shown (see Problems) that the distance between the inflection points of the theoretical Eq. 6-56 is $2\sqrt{2Dt}$. Furthermore, since the left-hand side of Eq. 6-56 simply represents the probability of finding a given dye molecule at point y, the average absolute distance away from the origin after time t may be obtained from

$$< y^2 > = \frac{1}{2\sqrt{\pi Dt}} \int_{-\infty}^{+\infty} y^2 e^{-y^2/4Dt}\, dy \tag{6-57}$$

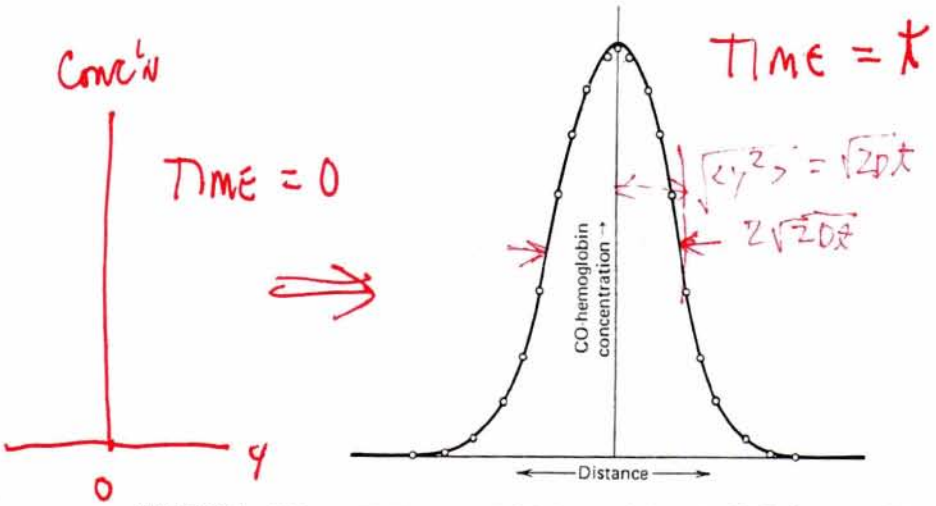

FIGURE 6-5. Diffusion of CO-hemoglobin in H_2O from a thin initial layer [O. Lamm and A. Polson, *Biochem. J. 30,* 528 (1936).] The solid line is the theoretical prediction from Eq. 6-56.

or just

$$\boxed{\sqrt{< y^2 >} = \sqrt{2Dt}} \tag{6-58}$$

But the situation described by Eq. 6-56 and shown in Fig. 6-5 is identical to that treated in the random walk analysis that led to the result

$$\boxed{\sqrt{< y^2 >} = \sqrt{Nl^2} = \sqrt{\nu t\, l^2}} \tag{6-59}$$

where

N = total number of steps in the walk
l = length of one "step"
ν = number of steps per unit time
t = duration of the "walk"

Correspondence of Eqs. 6-58 and 6-59 leads immediately to the important result

$$\boxed{D = \frac{1}{2}\nu\, l^2 \quad \text{in one dimension}} \tag{6-60}$$

A treatment with more algebra but the same intellectual content (see Problems) shows that similar results obtain when the treatment is extended to two or three dimensions:

$$\boxed{D = (1/4)\nu l^2 \quad \text{for diffusion in two dimensions}} \tag{6-60b}$$

$$\boxed{D = (1/6)\nu l^2 \quad \text{for diffusion in three dimensions}} \tag{6-60c}$$

The significance of Eqs. 6-60 is that they give the relation between the intuitive (empirical, physical) model based on continuous flow and the deductive (microscopic, statistical) model based on jerky progress by means of many small random steps. The reason physical scientists are so concerned with statistics is that almost all physical models are based on the behavior of one or two particles at a time, and some sort of averaging process is always required (Equations 6-49, 6-57) to apply the model calculation to the macroscopic experiment involving huge numbers of particles: the diffusion problem is one of the few for which a rigorous correspondence between the (microscopic) model and (macroscopic) reality can be made.

At this stage, it is reasonable to wonder why molecules should behave as if they were bouncing around very rapidly. To make the question less hypothetical, one can consider the motion of pollen particles (Brownian motion), which is readily apparent in an ordinary microscope. Detailed experiments have shown that the random motion of such particles is *not*

ascribable to uneven evaporation of solvent, capillary action at the edge of the solution, cavitation (formation of small bubbles), uneven heating, attractive forces between particles, or in fact *any external* influence. The explanation for the phenomenon is that any one particle is being bombarded on all sides by solvent molecules, and while the *average* of many such collisions is to leave the particle where it started, a given particle at any one instant "feels" a net force that causes it to move. This random motion is responsible for all chemical reactions (molecules must collide before they can react), and when we consider the action of enzyme catalysts in the enhancement (speeding up) of reaction rates, we will return to a short diffusion argument to find out how often the reactants collide.

Applications The principal applications for the diffusion equation, Eq. 6-55, involve (1) diffusion from a point, (2) diffusion from one layer to another, and (3) diffusion across a membrane. Diffusion between layers occurs in chromatography, electrophoresis, and sedimentation, and is discussed in those sections as well as later in this section. Diffusion through a membrane is discussed in our examination of the Donnan equilibrium (Chapter 2), and we now look at two examples of diffusion from a point, as they are regularly applied in immunology.

EXAMPLE *Immuno-diffusion*

The immune system of the human body relies on a number of large proteins (antibodies) that circulate in the blood and may tend to localize in certain organs. The purpose of these antibodies is to recognize any strange (and presumably harmful) toxins, or antigens, and remove them from circulation by forming a very strong antigen:antibody complex. Bacteria, for example, may be recognized by characteristic oligosaccharide groups that project from the bacterial surface; protein antigens are recognized from a characteristic oligopeptide region of the protein, and so forth. Most immuno-chemistry is centered around formation, often followed by precipitation, of the antigen: antibody complex, as in the usual tests for blood "type."

To understand how the immuno-diffusion methods work, one must be aware of the dependence of the solubility of the antigen:antibody complex on the concentration ratio of the two reactants (see Fig. 6-6). As seen in Fig. 6-6, very little precipitate results when antibody *or* antigen is in excess.

One simple explanation for the behavior shown in Fig. 6-6 is illustrated graphically in Fig. 6-7. At small antibody (Ab) to antigen (Ag) ratios, soluble Ab:Ag complexes are formed, with small numbers of Ab and Ag per complex. At large Ab:Ag ratios, the polyfunctional nature of the antibody permits formation of extended networks (precipitation), with the most compact network at the largest Ab:Ag ratios.

The purpose of this introductory discussion is to show that when a solution of antibody is brought into contact with a solution of antigen, there will be a

The immuno-diffusion methods are extraordinarily sensitive: radial immunodiffusion can detect as little as 3 micrograms/cc of antigen in serum. The techniques determine the number of antigens in a mixture and the identity of antigens in different preparations. The tests provide a criterion for purity of vaccines or other biologically active antigens, including enzymes and hormones. The gel-precipitation methods are used in sero-diagnosis of such infectious diseases as histoplasmosis, blastomycosis, and coccidiomycosis. Recently, rabbit serum containing myoglobin antibodies has been used to detect myoglobin in urine; thus, myoglobin released by a damaged heart may be detected a few hours after a heart attack to provide early diagnosis of the attack, using immunodiffusion methods.

EXAMPLE *Diffusion at a Boundary*

Diffusion at a boundary is of interest for two reasons: (1) it provides a means for determination of the diffusion constant of a macromolecule, from which we will shortly be able to find the molecular weight, and (2) it occurs in most of the preparative and analytical methods for separation of mixtures of macromolecules in the purification that must precede any study of function on a molecular level.

Measurement of diffusion constant. Consider a system consisting of an initially sharp boundary that separates a layer of solvent from a layer of solution of macromolecular concentration, $[C]_0$, as shown in Fig. 6-10. If the column of solution on either side of the boundary is sufficiently long, then the macromolecular concentration may be assumed to remain at either $[C]_0$ or zero far from the boundary—under these conditions, Eq. 6-55 may be solved to yield the concentration of macromolecule as a function of distance in the vicinity of the boundary:

$$[C]_{\text{at } y} = \frac{[C]_0}{2}\left[1 - \frac{2}{\sqrt{\pi}}\int_{u=0}^{u=(y/2\sqrt{Dt})} e^{-u^2}\,du\right] \tag{6-61}$$

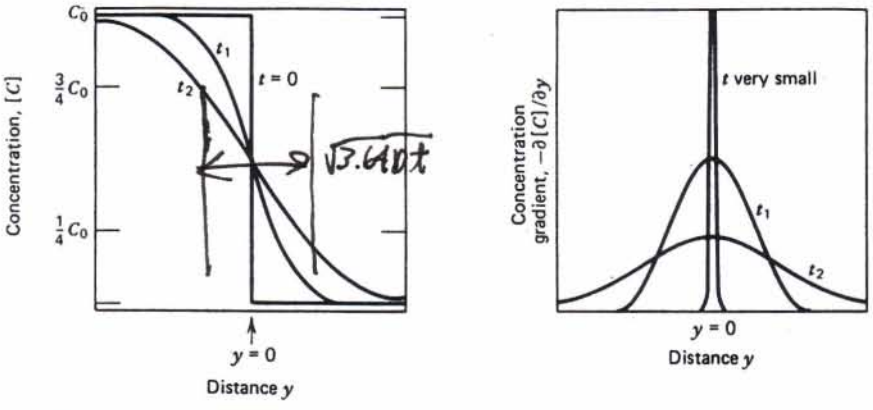

FIGURE 6-10. Progress of a diffusion experiment from an initially sharp boundary at $t = 0$. (From C. Tanford, *Physical Chemistry of Macromolecules,* John Wiley & Sons, 1961, p. 354.)

Plots of concentration near the boundary at later times are calculated from Eq. 6-6 and shown in Fig. 6-10. (The definite integral in Eq. 6-61 is the famous Gaussian or error-function, and is tabulated in many math tables.)

Both the algebra and the experiment are simplified by examination of the concentration *gradient*, $\partial[C]/\partial y$, rather than the concentration itself.

$$\left[\frac{\partial[C]}{\partial y}\right]_{\substack{\text{at } y,\\ \text{at } t}} = -\frac{[C]_0}{2\sqrt{\pi Dt}}\, e^{-y^2/4Dt} \tag{6-62}$$

The form of the concentration gradient as a function of distance at particular times is also shown in Figure 6-10.

The diffusion constant may be determined either from concentration (as measured by light absorption at a suitable wavelength, for example) or from concentration gradient (measured by refractive index methods—see Chapter 14.A.

(a) *Diffusion constant from concentration measurements.* From Eq. 6-61 it may be shown (see Problems) that the square of the distance between the points at which $[C] = [C]_0/4$ and $[C] = 3[C]_0/4$ is proportional to the time since the boundary was initially created:

$$\left(\Delta y_{\substack{\text{from } [C]_0/4\\ \text{to } 3[C]_0/4}}\right)^2 = 3.64\, Dt \tag{6-63}$$

Thus, D may be determined from the slope of an experimental plot of $(\Delta y)^2$ versus time.

(b) *Diffusion constant from concentration gradient.* From Eq. 6-62 one can readily show that the area under the plot of concentration gradient versus distance is

$$\text{Area}_{(\text{concentration gradient versus } y)} = -[C]_0 \tag{6-64}$$

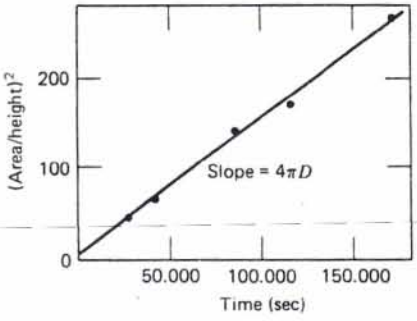

FIGURE 6-11. Determination of diffusion coefficient for ovalbumin from area and height of a plot of concentration gradient versus distance at particular times (see text). Since the boundary was not perfectly sharp at $t = 0$, the line in the graph does not pass through the origin. [From O. Lamm and A. Polson, *Biochem. J. 30,* 528 (1936).]

Table 6-1 Diffusion Coefficients for Macromolecules in Aqueous Solution

Macromolecule	Molecular Weight	$D_{20°,\text{water}} \times 10^7$ cm^{2}sec^{-1}
Ribonuclease	13,683	11.9
Chymotrypsinogen	23,200	9.5
Ovalbumin	45,000	7.76
Hemoglobin	68,000	6.9
Tropomyosin	93,000	2.24
Fibrinogen	330,000	2.02
Collagen	345,000	0.69
DNA	6,000,000	0.13
Tobacco mosaic virus	40,000,000	0.53

[a] *Diffusion coefficients have been corrected to the value that would be observed at 20°C in pure water for the same size and shape.* [*Data from C. Tanford,* Physical Chemistry of Macromolecules (*John Wiley, N. Y., 1961*), *pp. 358, 361*].

while the height of the curve at $y = 0$ is just

$$\left(\text{height at } y = 0\right)_{\text{concentration gradient versus } y} = \frac{+[C]_0}{2\sqrt{\pi D t}} \tag{6-65}$$

Thus the ratio of area to height is given by

$$\frac{\text{Area}}{\text{Height}} = 2\sqrt{\pi D t} \tag{6-66}$$

and a plot of (Area/Height)^2 versus t will give a straight line of slope, $4\pi D$, as shown in Fig. 6-11.

As evident from intuition (big molecules move more slowly) and as we will shortly show, diffusion coefficients are inversely related to molecular weight. Examples are shown in Table 6-1. The correlation between molecular weight and diffusion constant is irregular, because we have not yet accounted for the effect of macromolecular shape on the diffusion rate. This matter is explored after a discussion of viscosity.

PROBLEMS

1. Stirling's approximation, $\ln N! = N \ln N - N$, is fundamental to many statistical problems, including the Gaussian (normal) distribution on which diffusion-related phenomena are based. Derive this approximation (valid for large N) from the definition, $N! = N(N-1)(N-2)\cdots 3\cdot 2\cdot 1$. (*Hint:* consider a plot of $\ln N$ versus N.)

2. Show that the root-mean-square *number of steps* away from the origin in a one-dimensional random walk (Eq. 6-44), after N steps, is

itation occurs visibly at one par-
. Next, suppose that at time, t_1,
cm away from the origin. Finally,
ing a different concentration, c_2,
adius of the precipitin ring as a
substance at the origin, where all
oceed for the same length of time,

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5) that form the basis for these

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CHAPTER 7 Forced March

7.A. ELECTROPHORESIS

Most large biomolecules are ampholytes; that is, the molecule has a large number of labile protons, and each proton exhibits a characteristic pK_a. From the individual pK_as, it is possible (see Chapter 3) to compute the "isoelectric point," *pI*, as the pH at which the macromolecule possesses an equal number of positive and negative charges, and thus behaves as a net neutral species. If the solution pH is *below* the isoelectric pH, the macromolecule will have net *positive* charge; *above pI* the molecule will have net *negative* charge. The point of this discussion is that unless the solution pH happens to coincide exactly with the unique *pI*, the macromolecule will be electrically charged, and will move under the influence of an electric field. Analysis of this motion proceeds in three stages. First, we must explain why a charged molecule in solution moves at a constant *velocity* proportional to the applied electric field—the applied electric field represents a constant *force* on the molecule, so one would intuitively expect the molecule to *accelerate* along the applied field. Second, we will establish the important relation between ion motion and diffusion constant, required for understanding of the effect of molecular shape on transport phenomena (diffusion, sedimentation, electrophoresis). Finally, the empirical continuous-flow model will be applied to the ion motion, to predict the result of an electrophoresis experiment.

Why Does an Ion Subjected to a Constant Force Move at Constant Velocity?

A particle with charge, Q, when subjected to an electric field (voltage gradient! electrical "pressure" gradient), E, experiences a constant net force

$$\begin{aligned} m\,a &= QE \\ m &= \text{mass} \\ a &= \text{acceleration} \end{aligned} \qquad (7\text{-}1)$$

An *isolated* particle (e.g., in vacuum) would thus *accelerate* at a constant rate under the influence of such a force. However, an ion in aqueous *solution* is not free to move, but is held back by a frictional force proportional to its velocity:

$$m\,a = QE - f\frac{dy}{dt} \tag{7-2}$$

where f is the frictional coefficient. As soon as the electric field is turned on, the particle will accelerate until it reaches a limiting velocity (similar to the limiting velocity of a parachute) for which the frictional force just balances the (driving) electrical force, to give a net force of zero (i.e., *constant* velocity):

$$QE = f\frac{dy}{dt}$$

or

$$\text{limiting velocity} = \frac{dy}{dt} = \frac{QE}{f} \tag{7-3}$$

Microscopic Origin of Friction: Relation between Electrophoresis and Translational Diffusion

The simplest approach to this question begins from a microscopic model in which the molecule is assumed to undergo many collisions per second (random walk model); molecular motion between collisions is then calculated. To find out the average length of time between collisions, τ, use is made of a result employed in first-year chemistry courses in derivation of the ideal gas law:

$$\text{average translational kinetic energy} = \frac{1}{2}\,m\,v^2_{\text{thermal}} = \frac{3}{2}\,kT \tag{7-4}$$

where k is the Boltzmann constant, m is the mass of the particle, T is the absolute temperature, and v_{thermal} represents the average velocity of a gas molecule. Although the remaining argument is thus based on a property of gases, the result is nevertheless general, as is evident shortly.
Collecting definitions

τ = average time between collisions (sec/collision)
ν = frequency of collisions (collisions/sec)
l = average distance between collisions (distance/collision)
v_{th} = thermal velocity (distance/sec)

where

$$\tau = \frac{1}{\nu} \tag{7-5}$$

Finally substituting for v_{th} from Eq. 7-4 and using the result (Eq. 6-60c) from the three-dimensional random walk, $D = (\nu\, l^2/6)$, the desired result appears:

$$\mu = \frac{qD}{kT} \tag{7-12}$$

The result, Eq. 7-12 relating ionic mobility in an electric field to diffusion in the absence of an electric field, is susceptible to experimental test, since μ can be studied as a function of temperature and the D value then compared with a direct measurement of D from, say, diffusion at a boundary. Equation 7-12 turns out to be a *general* result, although it was arrived at by a *specialized* derivation. This is a frequent occurrence in physical science, and furnishes additional relevance to the study of simple models.

Another general result is now available, from comparison of the continuous-flow model, Eq. 7-3, with the microscopic model, Eq. 7-12:

$$f = \frac{kT}{D} \tag{7-13}$$

Equation 7-13 is valid for molecules of *any shape*, and since the dependence of f on molecular shape is well-known (see viscosity section), we will soon be in a position to account for diffusion or electrophoresis behavior of neutral or charged molecules of any shape.

What Does an Electrophoresis Experiment Look Like?

Up to now, both macroscopic and microscopic treatments agree that a charged molecule in solution (or in a gel) will move at a constant velocity, given by its ionic mobility (ion velocity per unit electric field). In addition, the relations between ion mobility, diffusion constant, and friction coefficient have been derived. Experimentally, electrophoresis was first carried out by carefully layering a dilute salt solution over a solution containing the charged macromolecule of interest and observing the steady motion of the boundary on application of an electric field (Tiselius apparatus), as shown in Fig. 7-1. In the diagram, it is supposed that there are two migrating macromolecules, both negatively charged, but having different ionic mobilities.

The main problem with the apparatus of Fig. 7-1 is that it is difficult to form and maintain the sharp initial boundary required for good resolution. Modern methods therefore rely almost exclusively on use of a medium such as paper or gel to reduce convective mixing, while still permitting almost unimpeded forced motion from the electric field. These methods consist of injection of a thin band of macromolecule-containing solution into