Chapter 5

Methods for the Separation and Characterization of Macromolecules

5.1 General Principles

Diversity, Complexity and Dynamics of macromolecular structure

1. Protein exit, under physiological conditions, as a simple polypeptide chain or as an aggregate of several chains?
2. Are the chains of one or several kinds chains? Approximate MW?
3. Approximate size and shape? A globular or fibrous protein?
4. What is its ionic character? Is it most rich in acidic or basic residues ?

Separate macromolecules on basis of mass, size, shape, charge, or some combination of these parameters.

The concept of flow

All transport processes can be described in term if a quantity called **flow (J)**

Flow (J) is defined as the # of mass unit (moles of grams) crossing 1 cm^2 of surface in 1 sec

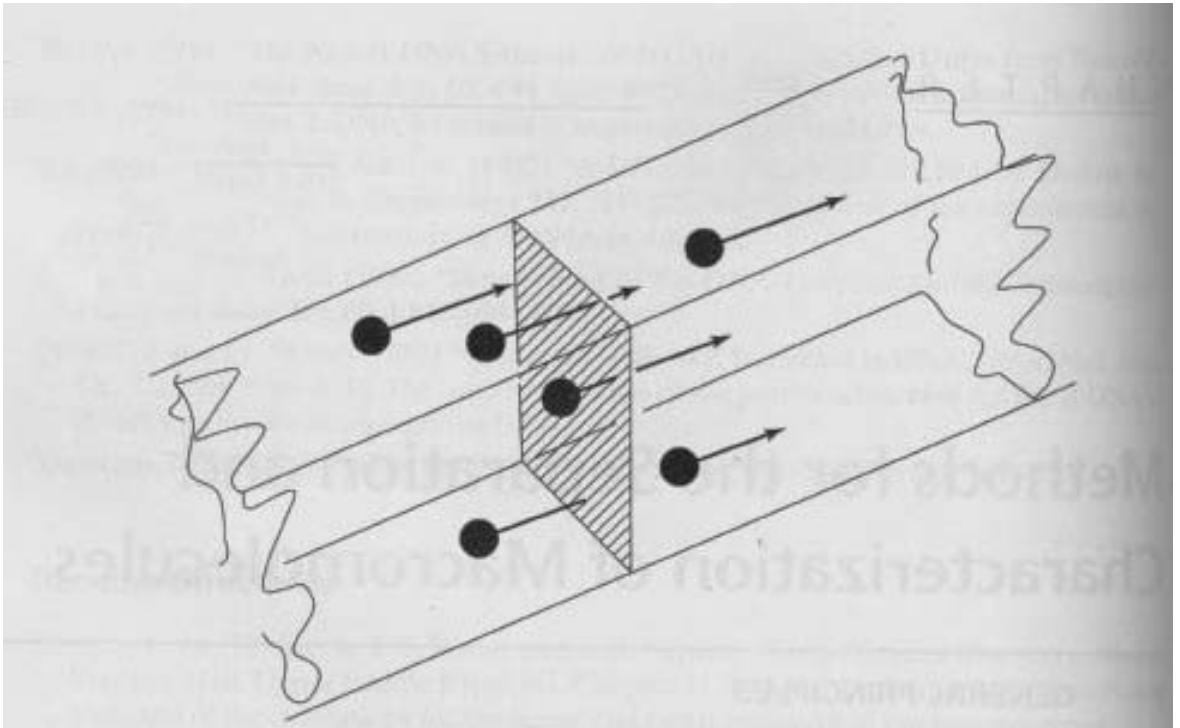


Figure 5.1 The concept of flow. Here we consider a case in which flow is occurring in one dimension, as will occur in sedimentation or electrophoresis experiments, or in diffusion from a planar boundary. We consider a 1 cm^2 area perpendicular to the direction of molecular transport, and define, J_i, the flow of a particular substance (i) as the net number of moles or grams of i that cross this surface in 1 second.

mol cm^{-2} s^{-1}

5.2 Diffusion

- Random *Brownian motion* of molecules in solution
- The random motion of solute molecules will ultimately make the **concentrations uniform** through out the system.
- From a thermodynamic viewpoint, the **entropy** of the system must **increase**,
- or the **free energy** **decrease**,
- accompanied by an evening of the concentration distribution of the solute.

Diffusion from an initially sharp boundary between solution & solvent

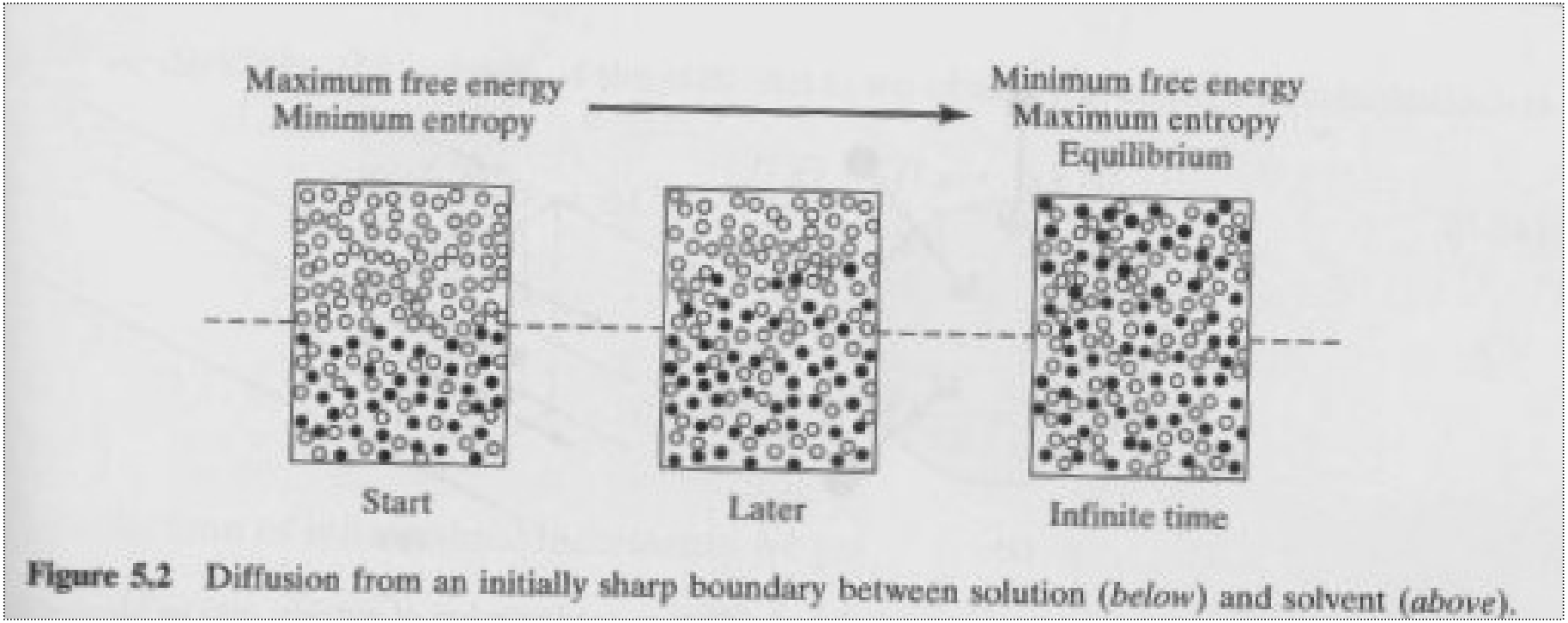


Figure 5.2 Diffusion from an initially sharp boundary between solution (*below*) and solvent (*above*).

- The random motion of solute molecules will ultimately make the **concentrations uniform** through out the system.

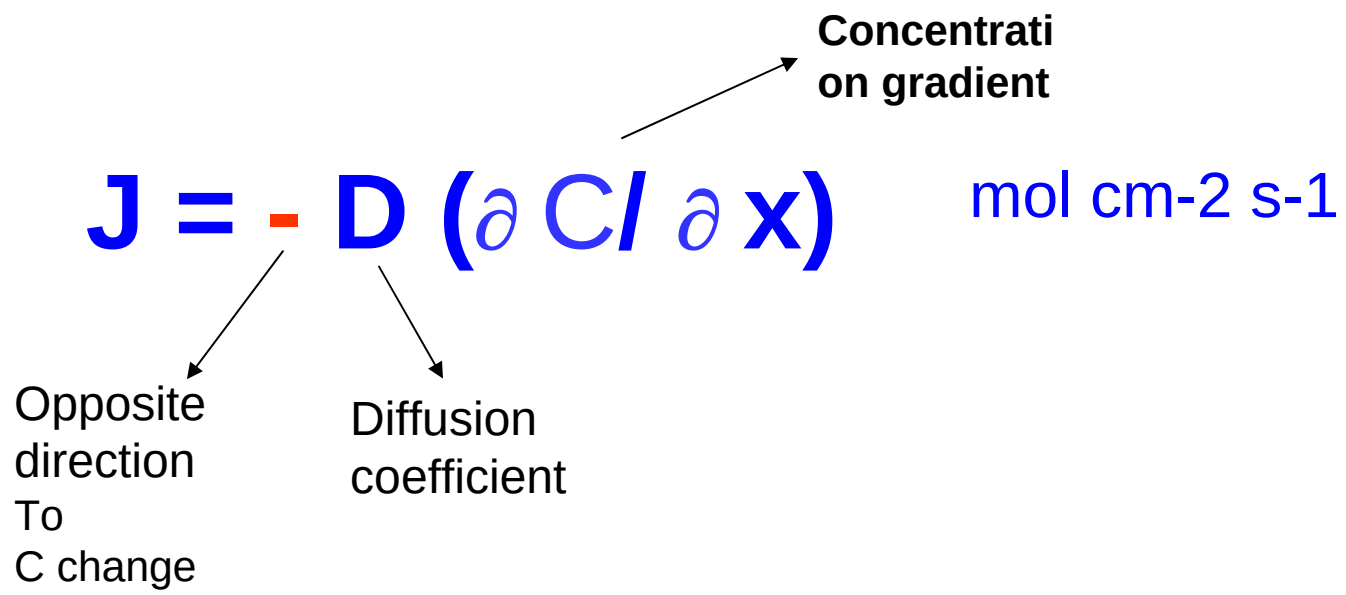
5.2.1 Description of Diffusion

- The transport process carrying out this **re-distribution** is the diffusion of solute, and net transport of matter by diffusion will occur whenever there is a **concentration gradient**.

- Once that concentration gradient is abolished, and concentration is every where uniform, **no further net flow** of matter will occur (although molecules are still moving to and fro)

Fick's First Law

- $J = -D\left(\partial C / \partial x\right)$ mol cm-2 s-1



Diffusion of substances into or out of a cell
Complicated
3D

diffusion from a very thin layer
1D, **Simple,**
Zonal Ultracentrifugation
Gel Electrophoresis

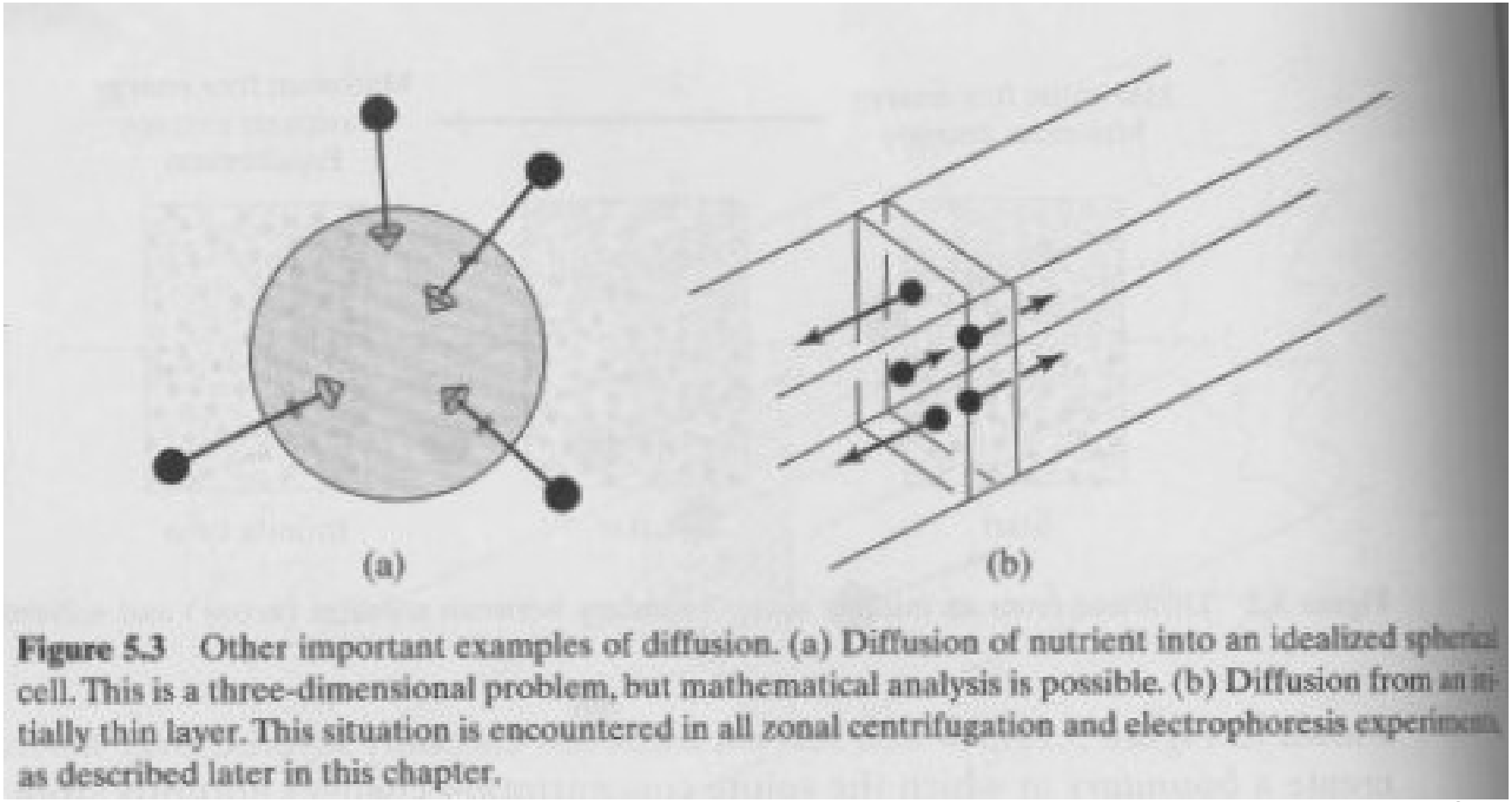


Figure 5.3 Other important examples of diffusion. (a) Diffusion of nutrient into an idealized spherical cell. This is a three-dimensional problem, but mathematical analysis is possible. (b) Diffusion from an initially thin layer. This situation is encountered in all zonal centrifugation and electrophoresis experiments as described later in this chapter.

Fick's First Law $J = -D\left(\frac{\partial C}{\partial x}\right)$

A continuity equation

It is not enough

Describes the concentration as a function of both spatial position & time

A thin slab thickness Δx,

the cross section A to the direction $\perp$ to flow

The mass flowing **in** during time Δt is $\mathbf{J(x)\ A\ \Delta t}$

The mass flowing **out** during time Δt is $\mathbf{J(x+\Delta x)\ A\ \Delta t}$

The change in mass $\mathbf{\Delta w = J(x)\ A\ \Delta t\ -\ J(x+\Delta x)\ A\ \Delta t}$

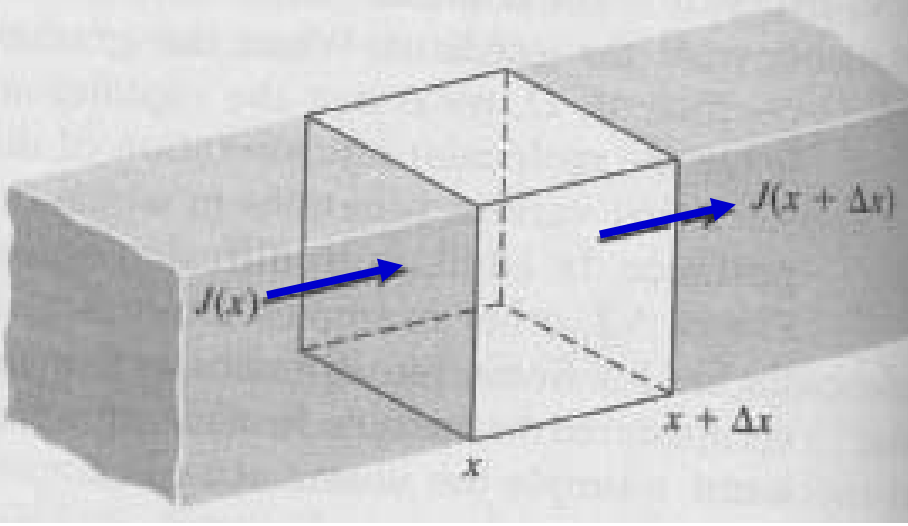


Figure 5.4 The continuity equation. The change of concentration within the slab is determined by differences in flow in and flow out.

The change in mass $\Delta w = [J(x)\, A\, \Delta t] - [J(x+\Delta x)\, A\, \Delta t]$

Divide by volume of the slab, $A\, \Delta x$

$$\Delta w / A\, \Delta x = \Delta C$$

$$[J(x) - J(x+\Delta x)]\, \Delta t / \Delta x = \Delta C$$

$$-\Delta J / \Delta x = \Delta C / \Delta t$$

In the limit of infinitesimal increment

$$-(\partial J / \partial x) = \partial C / \partial t$$

Continuity equation

$$\partial C / \partial t = -(\partial / \partial x)(-D\, \partial C / \partial x)$$

$$\partial C / \partial t = -D\, (\partial^2 C / \partial^2 x)$$

Fick's second law

Fick's Second Law $\partial C / \partial t = -D (\partial^2 C / \partial^2 x)$

- How the concentration in a gradient changes with time

- In a uniform concentration gradient, where ***dc/dx* =constant** for all values of *x*

- **left diffusing out to the right, $\partial J / \partial x < 0$, $\partial c / \partial t > 0$**

$$(\partial c / \partial t)_x = D (\partial^2 c / \partial x^2)_t$$

•Dep. on time, t & distance, x

A specific 1D example consider diffusion in the ± x direction from a thin slab of thickness δ

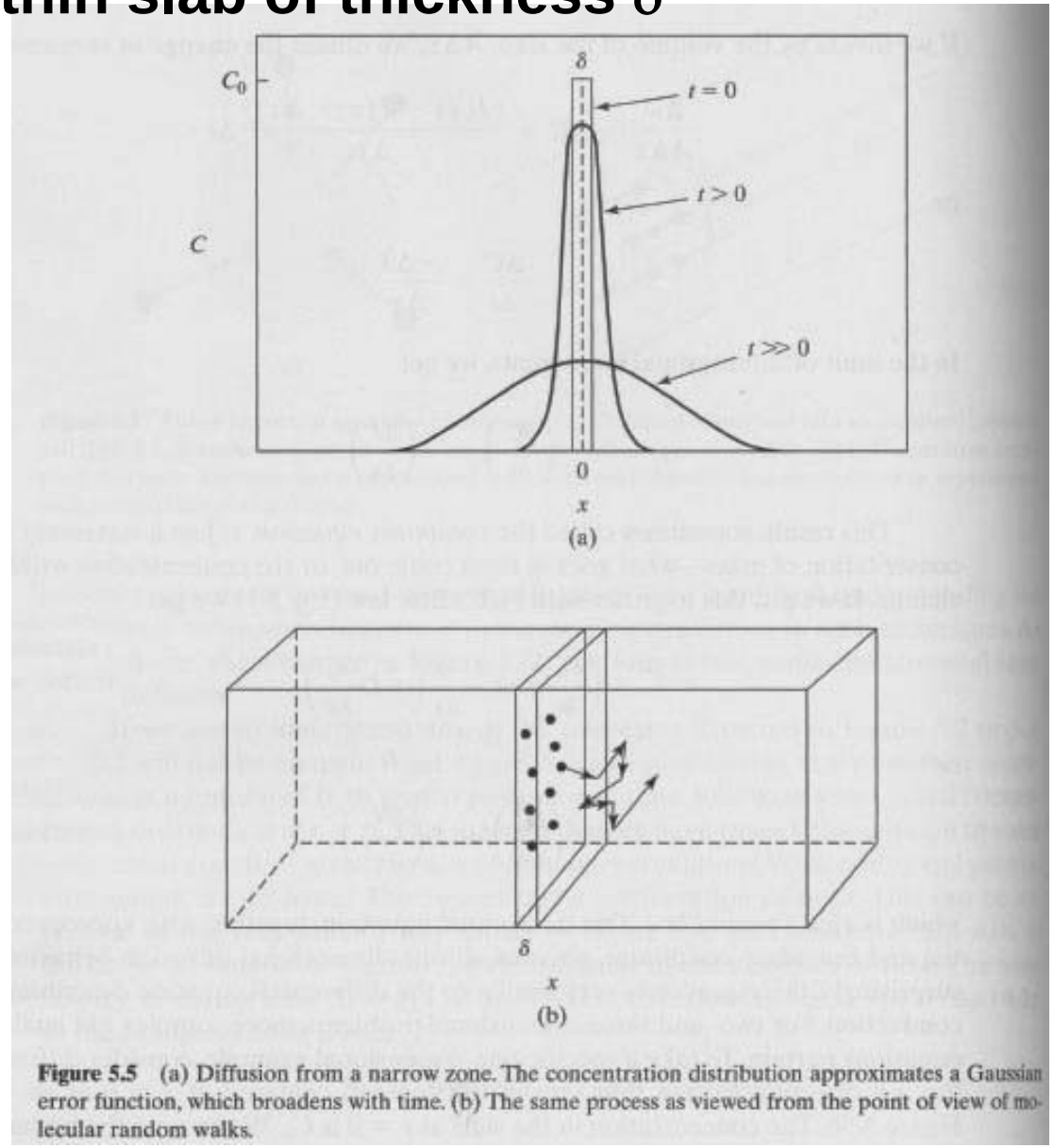


Figure 5.5 (a) Diffusion from a narrow zone. The concentration distribution approximates a Gaussian error function, which broadens with time. (b) The same process as viewed from the point of view of molecular random walks.

Diffusion from a narrow zone

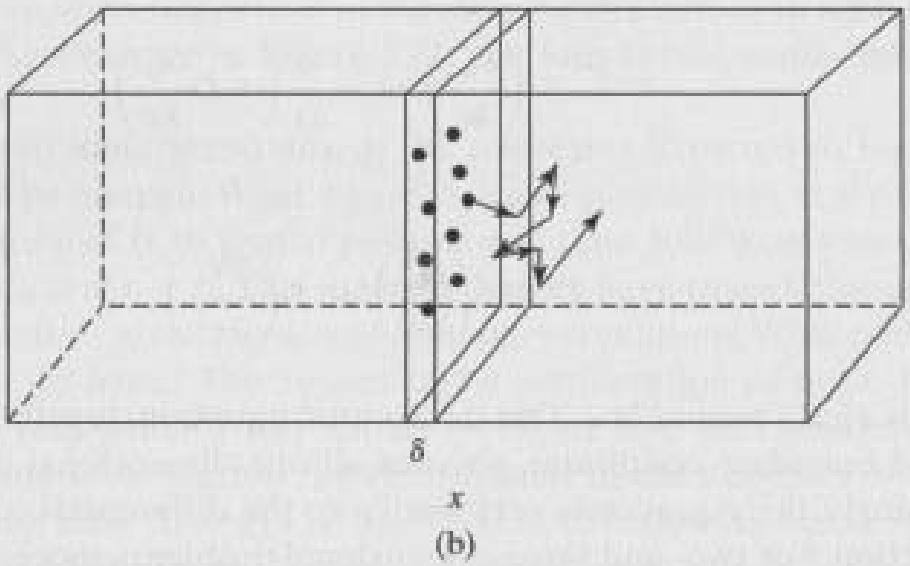


Figure 5.5 (a) Diffusion from a narrow zone. The concentration distribution approximates a Gaussian error function, which broadens with time. (b) The same process as viewed from the point of view of molecular random walks.

<x>=0 since + & - displacement

$\langle x^2 \rangle$=?

By evaluating the ration of the Integral

$\langle x^2 \rangle$= (5.7) & (5.8)

$\langle x^2 \rangle$= 2 Dt

Diffusion molecule is making random steps in solution.

And the # of theses steps is proportional to the time of diffusion

The spreading of an initially sharp boundary by diffusion

$$C(x, t) = [C_o \delta / 2 (\pi D t)^{1/2}]\ e^{-x2 / 4Dt}$$

$$\langle x2 \rangle = 2Dt$$

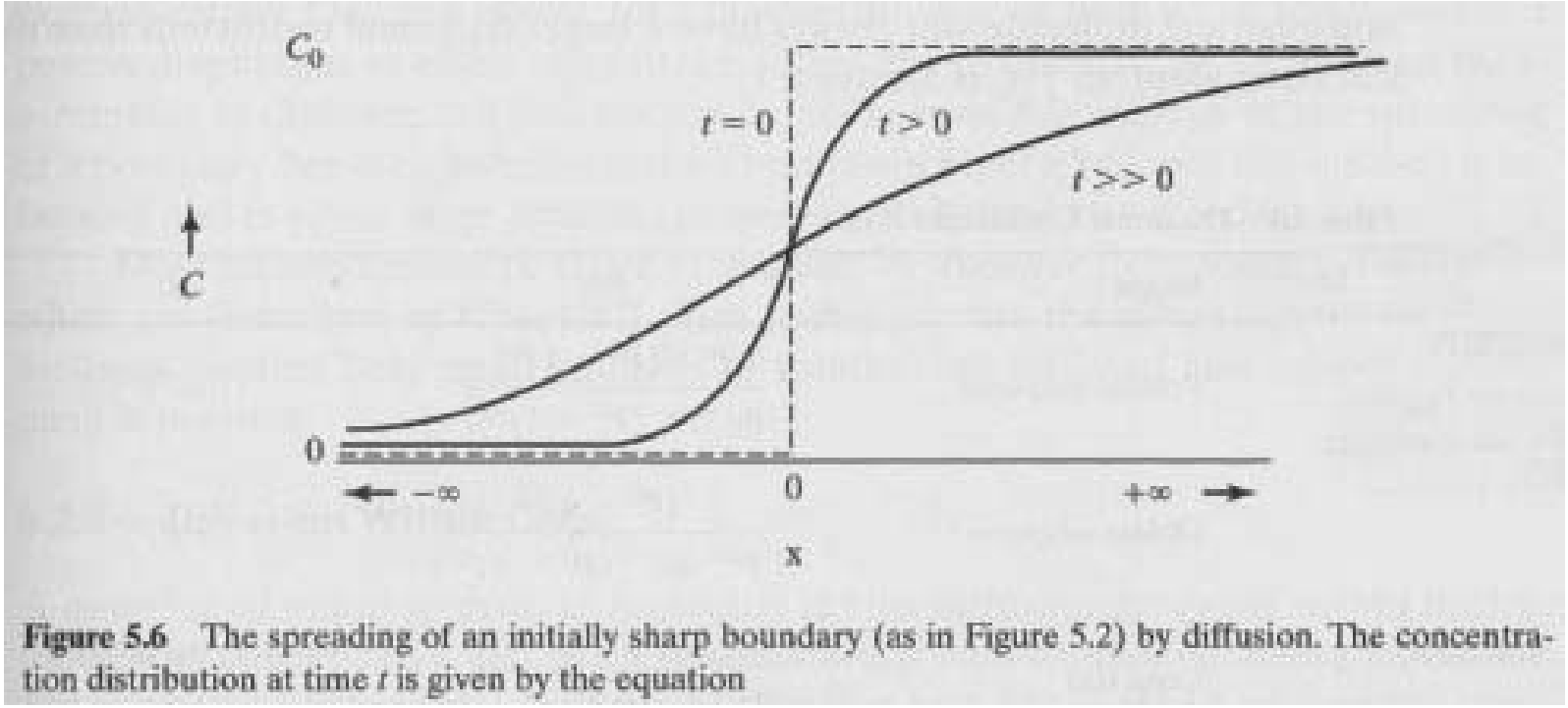


Figure 5.6 The spreading of an initially sharp boundary (as in Figure 5.2) by diffusion. The concentration distribution at time t is given by the equation

Diffusion molecule is making random steps in solution and that the number of thess steps is proportional to the time of diffusion

That diffusion from a thin layer is nothing different from a random walk from an origin

5.2.2 the Diffusion Coefficient (D) and the Frictional Coefficient (f)

$$D = kT/f = RT/\mathscr{R} f$$

K is the Boltzmann constant,
$\mathscr{R}$: Avogadro's constant
f : is a quantity frictional coefficient

$$D = kT/f = RT/\mathcal{R}f$$

K is the Boltzmann constant,
$\mathcal{R}$:Avogadro's constant
f : is a quantity frictional coefficient

Stokes' Law: $f_o = 6\pi\eta R_e$

"R_e" a sphere of radius

η: viscosity of the medium

f_o : the min. possible fractional coef.

$$D = RT/\mathcal{R}f = RT/6\pi\mathcal{R}\eta R_e$$

Frictional coefficient (*f*): depends on the size and shape of the molecule

Nonspherical particles

f (frictional coefficient)

depends on the size and shape of the molecule

$f / f_o \geq 1$: the fractional coef. ratio of the particle to that of a sphere of equal volume

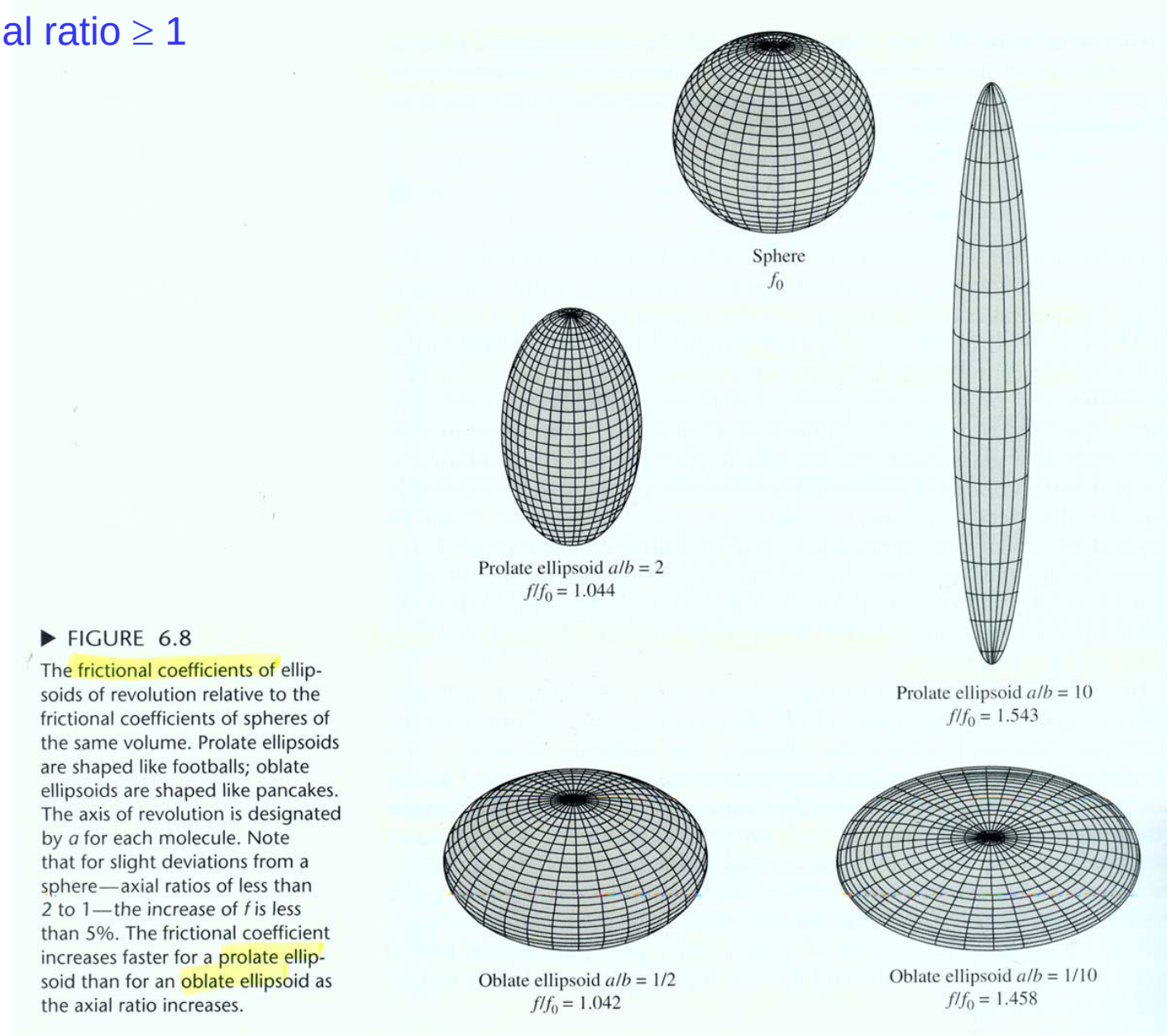
TABLE 5.1 FRICTIONAL COEFFICIENT RATIOS

Shape	f/f_0	R_e
Prolate ellipsoid	$\dfrac{P^{-1/3}(P^2-1)^{1/2}}{\ln[P+(P^2-1)^{1/2}]}$	$(ab^2)^{1/3}$
Oblate ellipsoid	$\dfrac{(P^2-1)^{1/2}}{P^{2/3}\tan^{-1}[(P^2-1)^{1/2}]}$	$(a^2b)^{1/3}$
Long rod	$\dfrac{(2/3)^{1/3}P^{2/3}}{\ln 2P-0.30}$	$\left(\dfrac{3b^2a}{2}\right)^{1/3}$

In these equations, $P = a/b$, where a is the semimajor axis (or the half-length for a rod) and b is the minor axis (or radius of a rod). R_e is the radius of a sphere equal in volume to the ellipsoid or rod, so $f_0 = 6\pi\eta R_e$.

f / f_o : the fractional coef. ratio of the particle to that of a sphere of equal volume

fractional ratio ≥ 1



▶ FIGURE 6.8

The frictional coefficients of ellipsoids of revolution relative to the frictional coefficients of spheres of the same volume. Prolate ellipsoids are shaped like footballs; oblate ellipsoids are shaped like pancakes. The axis of revolution is designated by *a* for each molecule. Note that for slight deviations from a sphere—axial ratios of less than 2 to 1—the increase of *f* is less than 5%. The frictional coefficient increases faster for a prolate ellipsoid than for an oblate ellipsoid as the axial ratio increases.

Prolate have bigger fractional coeff.

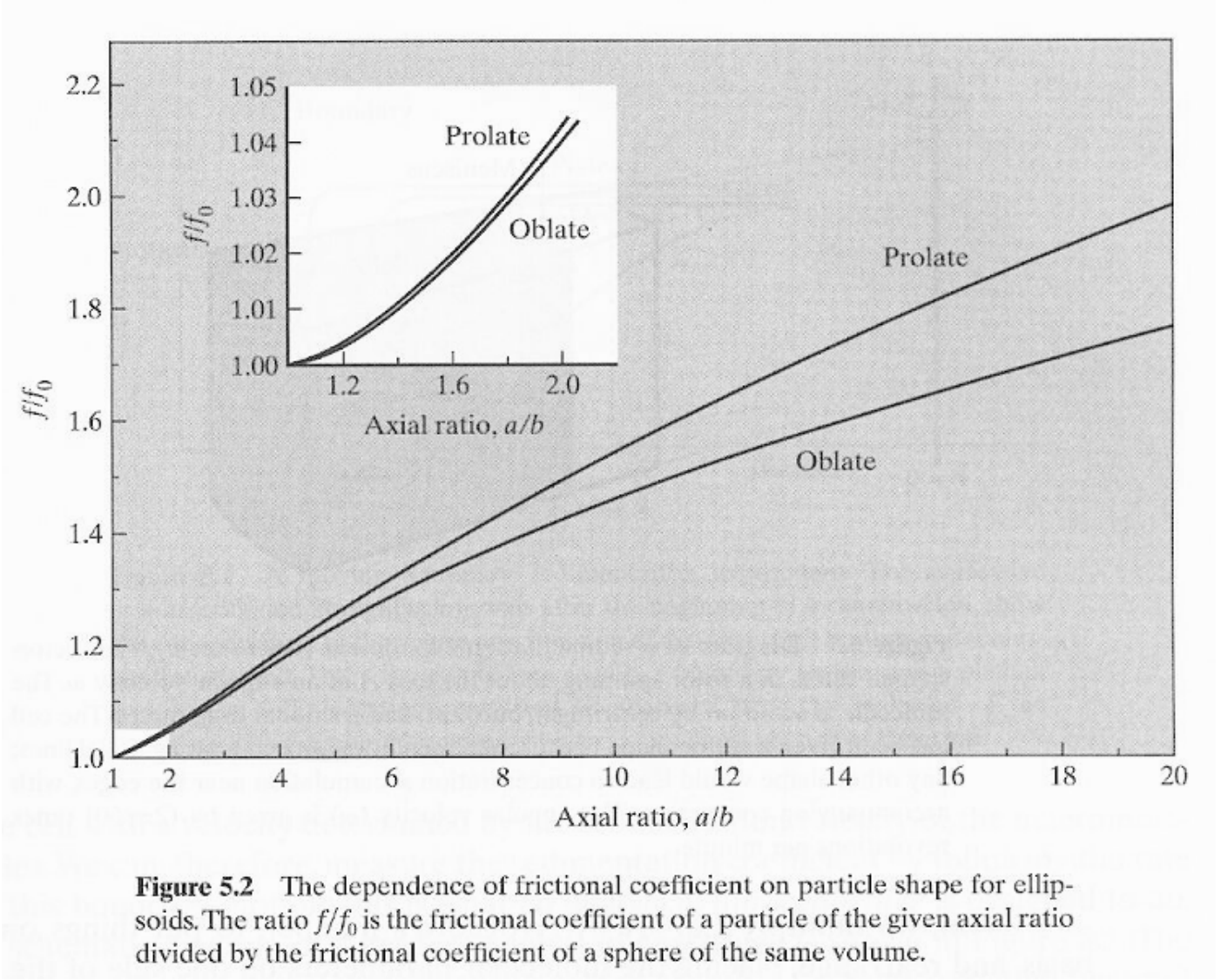


Figure 5.2 The dependence of frictional coefficient on particle shape for ellipsoids. The ratio f/f_0 is the frictional coefficient of a particle of the given axial ratio divided by the frictional coefficient of a sphere of the same volume.

5.2.3 Diffusion Within Cells

•How diffusion proceeds within the cytoplasm or nucleus of a cell?

•Photobleaching of dye label

•Molecule to be studied is labeled with fluorescent dye, and then injected into the cell

•The fluorescence recovers at a rate depending on D

•The half time for recovery is proportional to 1/D

Photobleaching Measurement of fluorescin-labeled DNA

DNA

Protein/dextran

cytoplasm nucleus

Diffusion of small DNA molecules in cells

DNA (21-6000bp)
Labeled with a fluorescent dye

Microinjected into either cytoplasm or nucleus

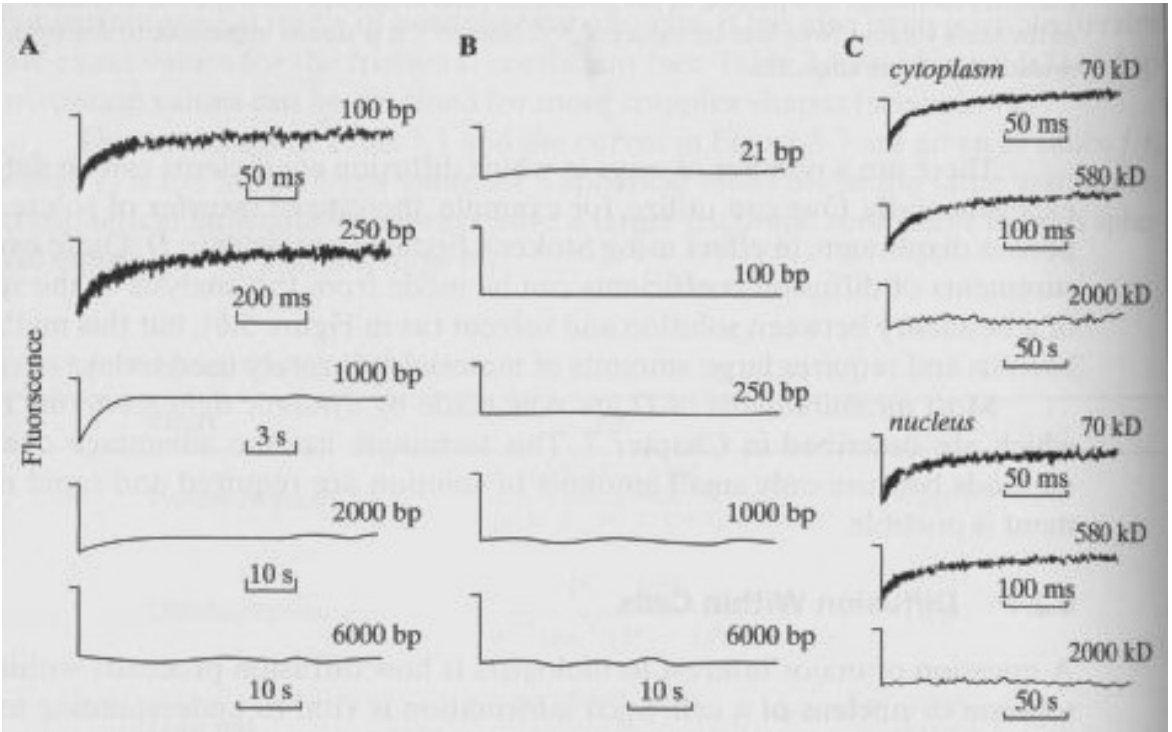


Figure A5.1 Photobleaching measurements of fluorescein-labeled DNA and dextran diffusion in HeLa cells. Recovery curves are for DNA in cytoplasm (A) and nucleus (B), and for dextran in cytoplasm and nucleus (C). Note that *none* of the DNA samples can diffuse at significant rates in the nucleus, whereas dextran molecules can. Thus, the hindrance in nucleus is specific for nucleic acid.

- **D_{cyto}/D_{w}** decreasing sharply with **DNA length**

In nucleus, the DNA did not diffuse at all
Other than simple viscous drag
most probably entanglement or binding to cellular constituents
Dextran molecules can diffuse In the nucleus

5.3 Sedimentation

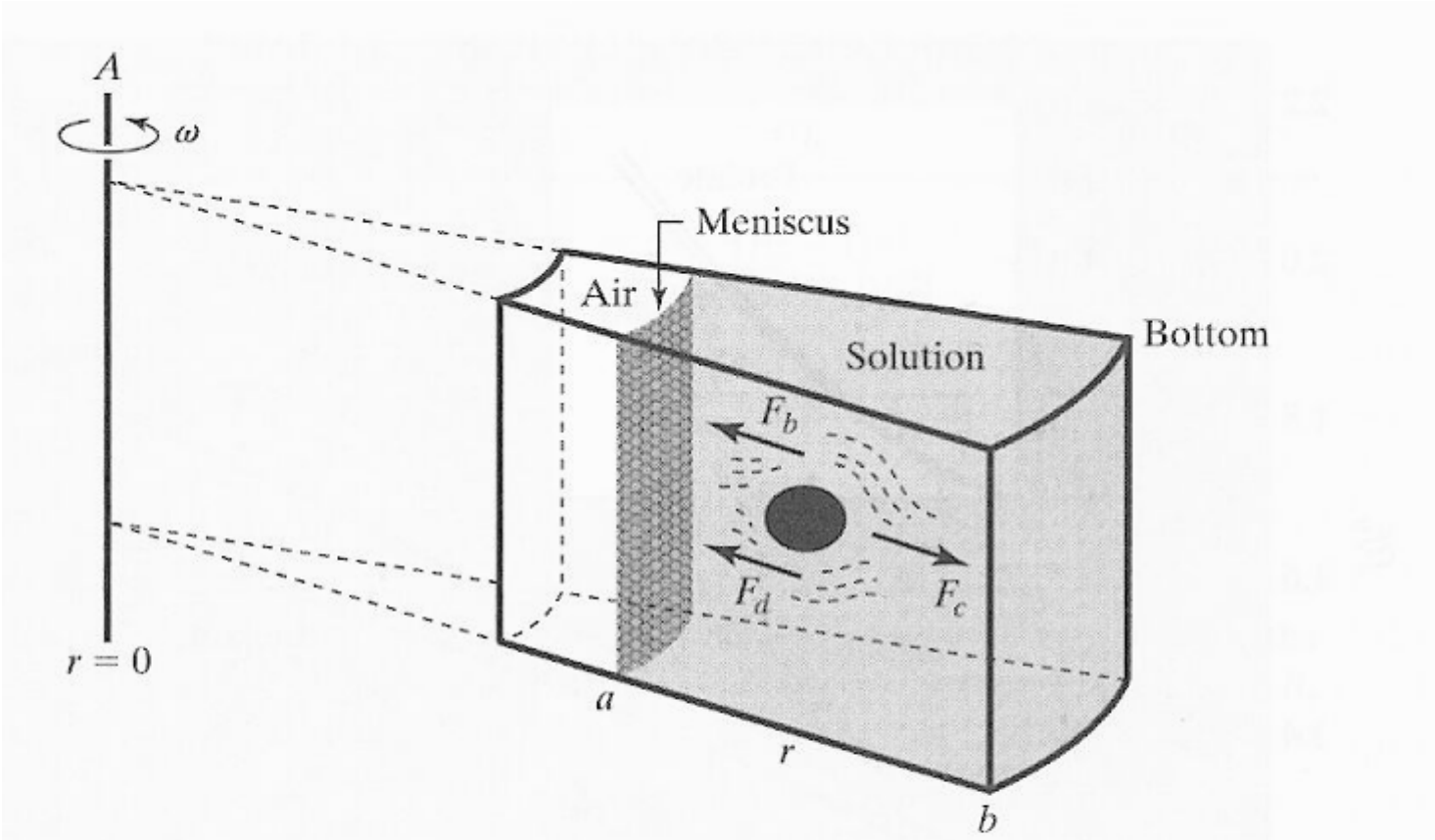


Figure 5.3 Diagram of a sedimentation experiment (not to scale). The sector-shaped cell is in a rotor spinning about the axis A at an angular velocity ω. The molecule is acted on by centrifugal, buoyant, and frictional drag forces. The cell has been given a sector shape because sedimentation proceeds along radial lines; any other shape would lead to concentration accumulation near the edges, with ing convection. The angular velocity (ω) is given by ($2\pi/60$) times

Fig. 5.8

Total force zero

$$F_c + F_b + F_d = 0$$

$$(\omega^2 r m) + (-\omega^2 r m_0) + (-f v) = 0$$

$m_0 = m \upsilon \rho$ (υ = **partial specific volume**)

$$\omega^2 r m (1 - \upsilon \rho) - f v = 0$$

$$\frac{M(1 - \upsilon \rho)}{\mathcal{R} f} = \frac{v}{\omega^2 r} = s$$

The velocity divided by the centrifugal field strength ($\omega^2 r$) is called sedimentation coefficient

Sedimentation coefficient, $s = v / \omega^2 r$

1 Sevedberg, 1S = 1×10^{-13} sec

Buoyancy factor: $(1 - \upsilon \rho)$

Buoyancy factor α $1/f$ (frictional coefficient)

5.3.1 Moving Boundary Sedimentation

Determined by the Sedimentation Coefficient

What happens when a centrifugal field is applied to a solution of large molecules?

All begin to move, and a region near the meniscus becomes entirely cleared of solute.

The moving boundary between solvent & solution

With a velocity determined by the sedimentation velocity of the macromolecules

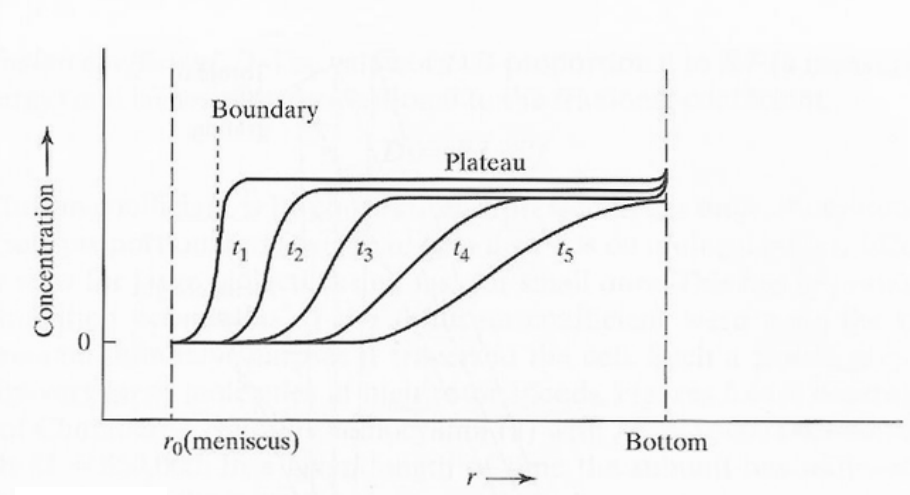


Fig. 5.9 A moving boundary sedimentation experiment. The successive graphs, obtained at regular intervals after the beginning of sedimentation, show the concentration of the solute as a function of distance. Cell bottom and meniscus are marked. There is a solute-free region, a boundary region, and a plateau region. The sedimentation coefficient is measured from the midpoint of the boundary. The boundary broadens with time as a consequence of diffusion.

Moving Boundary Sedimentation Experiment

The concentration (**C**) of the solute is a function of distance (**r**)

Solute free region, a **boundary** region and a **plateau** region

The sedimentation coefficient is measured from the **midpoint of the boundary**

The boundary broadens with time as a consequence of **diffusion**

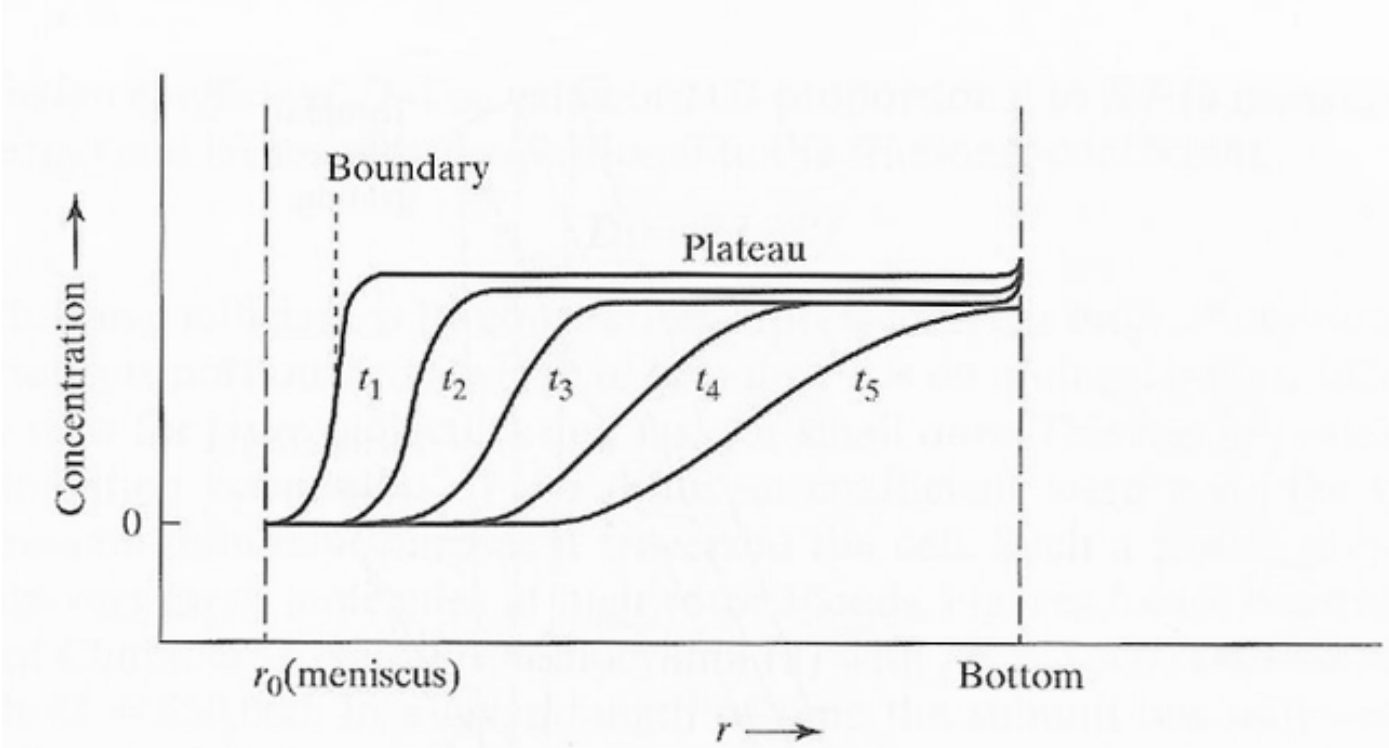


Fig. 5.10 A moving boundary sedimentation experiment. The successive graphs, obtained at regular intervals after the beginning of sedimentation, show the concentration of the solute as a function of distance. Cell bottom and menis-

Analytical ultracentrifuge

Measure the sedimentation coefficient by following the rate of this boundary motion

high speed up to 70,000 rpm

evacuated chamber

scanning absorption optical system

Double-beam spectrophotometer

Monchromator 200-800nm

At each point r, the absorbance difference between the soltion sector and the reference sector is recorded

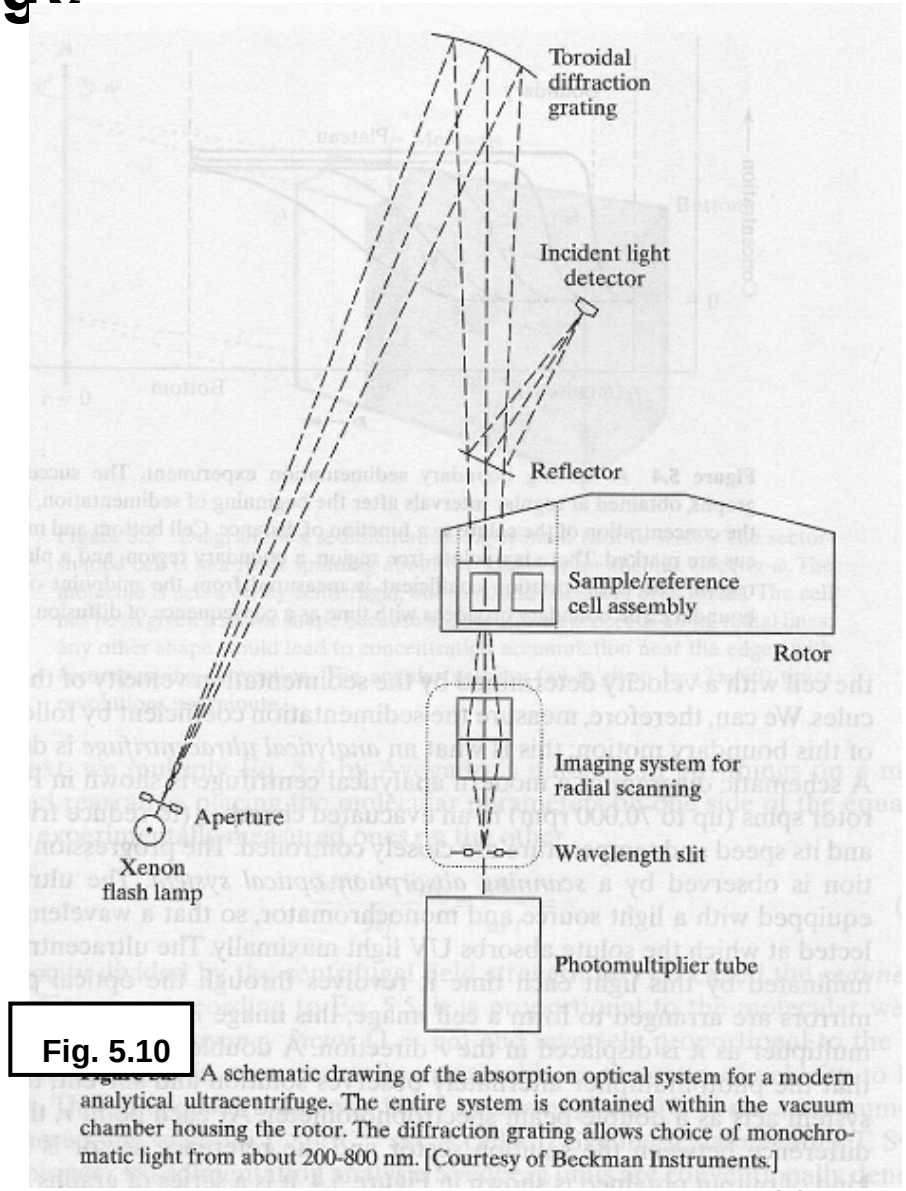


Fig. 5.10 A schematic drawing of the absorption optical system for a modern analytical ultracentrifuge. The entire system is contained within the vacuum chamber housing the rotor. The diffraction grating allows choice of monochromatic light from about 200-800 nm. [Courtesy of Beckman Instruments.]

Sedimentation velocity

The boundary position is usually defined as the midpoint of the absorbance step, and can be recorded at successive times during the experiment

Sedimentation velocity $v = dr_b / dt$

$$\frac{v}{\omega^2 r} = s$$

$$v = dr_b / dt$$
$$= r_b \omega^2 s$$

$$\int dr_b / r_b = \int \omega^2 s \, dt$$

$$\ln [r_b(t) / r_b(to)] = \omega^2 s (t - to)$$

ln r_b (t) versus t
will be a straight line with

slope = $\omega^2 s$, which to calculate "s"

Diffusion

Molecules are driven in random directions by thermal motion.

$$D = RT / \mathcal{N} f$$

Diffusion coefficient "**D**" (cm^2/sec)

- -Inversely proportional to f
- -Depends on the molecular size
- -Very slow for large molecules and fast for small ones
- -Important effects on sedimentation boundaries
- -diffusion coefficient were zero
- -If diffusion coefficient were zero, the boundary would remain infinitely sharp as it traversed the cell.

Sedimentation & Diffusion

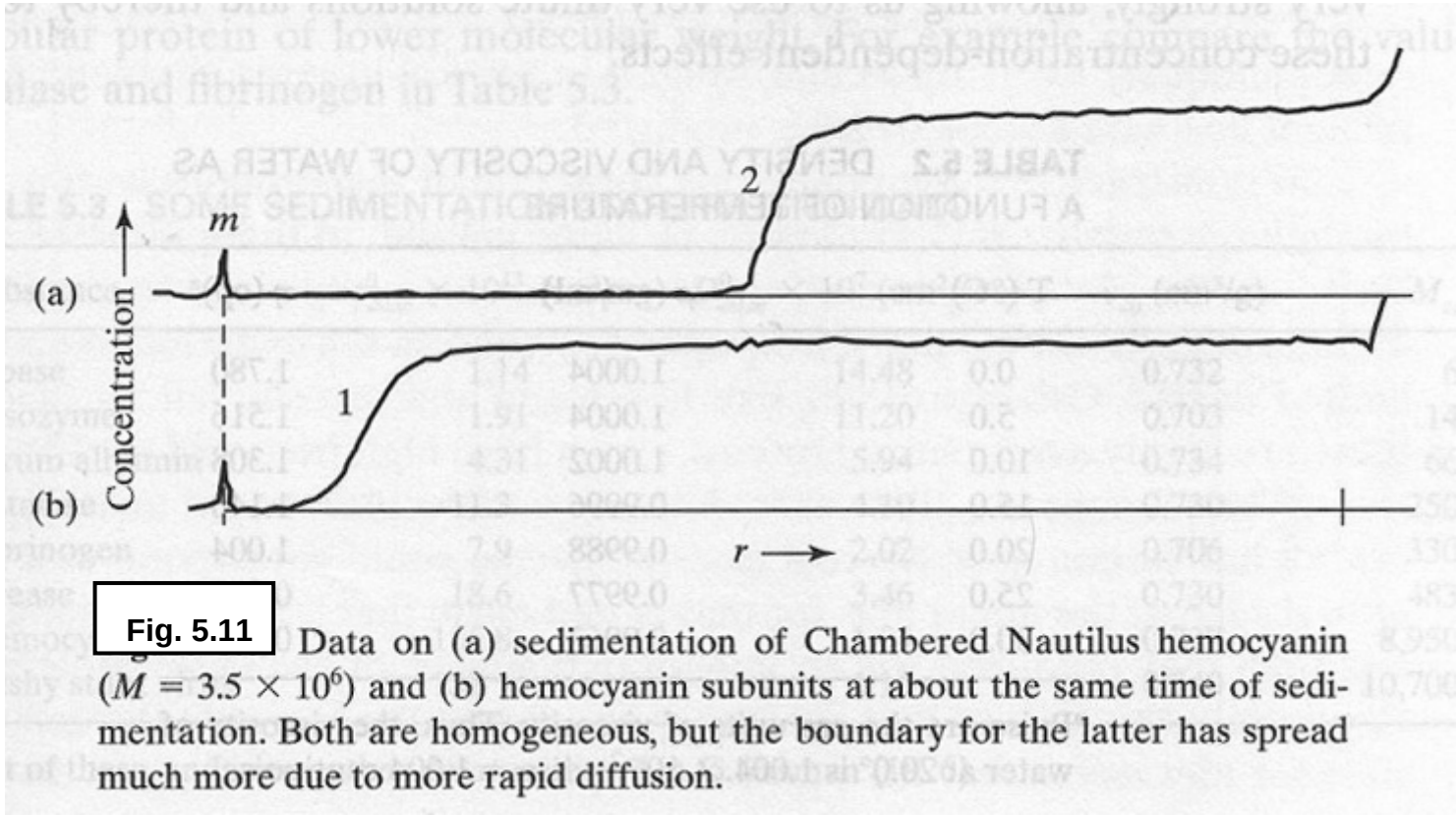


Fig. 5.11 Data on (a) sedimentation of Chambered Nautilus hemocyanin ($M = 3.5 \times 10^6$) and (b) hemocyanin subunits at about the same time of sedimentation. Both are homogeneous, but the boundary for the latter has spread much more due to more rapid diffusion.

Sedimentation coefficient (S)

a characteristic property of each biological macromolecule.

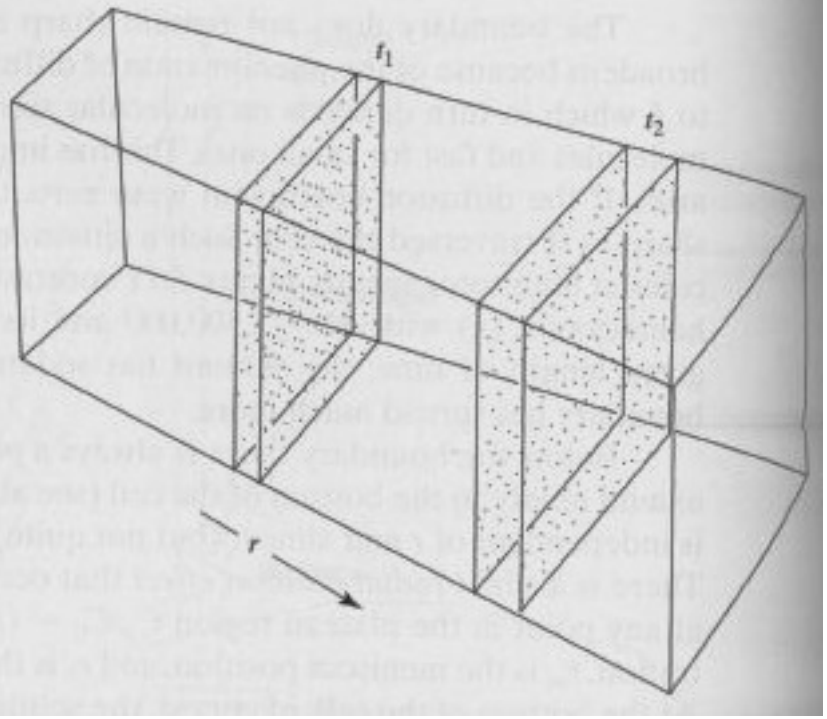
"S" depends on "υ", " ρ" & "f"

$$\frac{M(1-\upsilon\rho)}{\mathcal{R}f} = \frac{v}{\omega^2 r} = s$$

Depends on the temperature and the buffer solution in which the molecule is dissolved

Radial dilution effect

Figure 5.12 The radial dilution effect. A thin laminum containing a group of solute molecules, within the "plateau" region of the concentration gradient, is followed with time. The volume of the laminum increases as it moves with the particles, because (1) the cell is sector shaped with sides converging toward the axis of the rotor, and (2) the particles at the front edge see a slightly greater centrifugal field than those at the back. Because the volume increases, but the number of particles remains unchanged, the concentration of solute decreases with time.



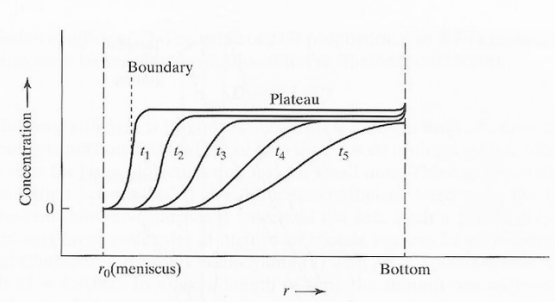


Figure 5.4 A moving boundary sedimentation experiment. The successive graphs, obtained at regular intervals after the beginning of sedimentation, show the concentration of the solute as a function of distance. Cell bottom and meniscus are marked. There is a solute-free region, a boundary region, and a plateau region. The sedimentation coefficient is measured from the midpoint of the boundary. The boundary broadens with time as a consequence of diffusion.

The cell is sector shaped with slides converging toward the axis of the rotor
The particles at the **front** edge see a slightly greater centrifugal field than those at the **back**

$$C_p/C_o = (r_m/ r_b)^2$$

At the bottom of the cell, of course, the solute "piles up" in a dense layer

t_2 dilute than t_1

Standard conditions

In pure water at 20°C

$$S_{20,w} = S_{T,b} \frac{(1 - v\rho)_{20,w}\ \eta_{T,b}}{(1 - v\rho)_{T,b}\ \eta_{20,w}}$$

$S_{20,w}$: the value expected at the hypothetical standard condition.
$S_{T,b}$: the value measured under experimental condition.

ρ:density, η:viscosity

$$S_{20,w} \approx S_{T,b} \frac{(1 - v\,\rho_{20,w})}{(1 - v\,\rho_{T,b})} \frac{\eta_{T,b}}{\eta_{T,w}} \frac{\eta_{T,w}}{\eta_{20,w}}$$

Standard conditions

ρ:density & η:viscosity

TABLE 5.2 DENSITY AND VISCOSITY OF WATER AS A FUNCTION OF TEMPERATURE

T (°C)	ρ (gm/ml)	η (cp)[a]
0.0	1.0004	1.780
5.0	1.0004	1.516
10.0	1.0002	1.308
15.0	0.9996	1.140
20.0	0.9988	1.004
25.0	0.9977	0.891
30.0	0.9963	0.7978

[a]Poise are the cgs units of viscosity. Thus, the viscosity of water at 20.0° is 1.004×10^{-2} poise = 1.004 centipoise.

Frictional coefficient α concentration

"**S**" may depend upon macromolecule concentration

Interaction between the sedimentation molecules will alter the sedimentation behavior

Frictional coefficient increase with **concnetration**

$$f = f_0 (1 + KC + \ldots)$$

$$f = f_0 (1 + KC + \ldots)$$

Sedimentation α Concentration

"S" may depend upon macromolecule concentration

f^o , is the value of f at C=0

$$s = s^\circ / 1 + KC$$

K usually > 0;
C, increase;
s, decrease

Interaction between the sedimentation molecules will alter the sedimentation behavior

Concentration dependence of the sedimentation
s = s° / 1+ KC

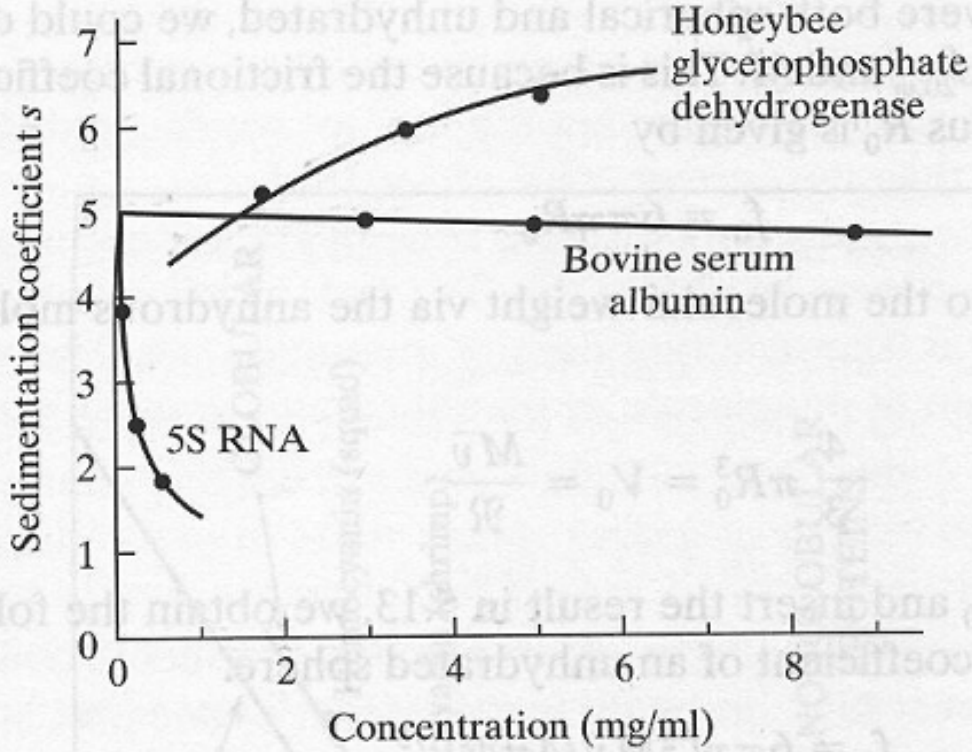


Figure 5.7 Concentration dependence of the sedimentation coefficient. Bovine serum albumin (BSA) and the ribosomal nucleic acid (5S RNA) exhibit behavior typical of compact and extended macromolecules, respectively. The behavior of honeybee glycerophosphate dehydrogenase is that to be expected for a reversibly associating substance. [Data from R. L. Baldwin (1957), *Biochem. J.* **65**, 503; D. G. Comb and Zehavi-Willmer (1967), *J. Mol. Biol.*, **23**, 441; and R. R. Marquardt and R. W. Brosemer (1966), *Biochem. Biophys. Acta*, **128**, 454.]

Might be expected, the effect is most pronounced with highly extended macromolecules

DNA & RNA absorb UV light very strongly, very dilute solution and thereby to escape from these concentration-dependent effects

Interpreting the Sedimentation Coefficient "s"

$$\frac{M(1-\nu r)}{\mathcal{R} f} = \frac{\nu}{w2\, r} = s$$

MW ↑

s ↑

"**s**" dep. on. "***f***" dep. on the **size**, **shape** and **hydration** of the macromolecule.

Highly **asymmetric** molecule might have a lower sedimentation coefficient than a globular protein of lower MW

Sedimentation & Diffusion

TABLE 5.3 SOME SEDIMENTATION AND DIFFUSION DATA

Substance	$s^0_{20,w} \times 10^{13}$ (sec)	$D^0_{20,w} \times 10^7$ (cm²/sec)	$\bar{v}_{20}$ (cm³/g)	$M_{s,D}$
Lipase	1.14	14.48	0.732	6,667
Lysozyme	1.91	11.20	0.703	14,400
Serum albumin	4.31	5.94	0.734	66,000
Catalase	11.3	4.10	0.730	250,000
Fibrinogen	7.9	2.02	0.706	330,000
Urease	18.6	3.46	0.730	483,000
Hemocyanin (snail)	105.8	1.04	0.727	8,950,000
Bushy stunt virus	132	1.15	0.740	10,700,000

Most of these, and many other data, are listed in G. Fasman (1976).

Highly **asymmetric** molecule might have a lower sedimentation coefficient than a globular protein of lower MW

Interpreting the Sedimentation Coefficient

I. Spherical and Unhydrated macromolecule

$$f_o = 6 \pi \eta R_o$$ (R_o,:unhydrated sphere of radius)

Molecular weight & the anhydrous molecular volume V_o

$$3/4 \pi R_o^3 = V_o = M \upsilon / \mathcal{N}$$

$$f_o = 6 \pi \eta (3M \upsilon / 4\pi \mathcal{N})^{1/3}$$

$$\frac{M (1 - \upsilon \rho)}{\mathcal{N} f} = \frac{\upsilon}{\omega^2 r} = s$$

$$S^0 = \frac{M (1 - \upsilon \rho)}{\mathcal{N} 6 \pi \eta (3M \upsilon / 4\pi \mathcal{N})^{1/3}} = \frac{M^{2/3} (1 - \upsilon \rho)}{6 \pi \eta \mathcal{N}^{2/3} (3/4\pi)^{1/3} \upsilon^{1/3}}$$

II. Spherical and Anhydrous Macromolecule

$$S^0 = \frac{M^{2/3}(1 - v\rho)}{6\pi\eta\mathscr{R}^{2/3}(3/4\pi)^{1/3} v^{1/3}}$$

s* :for any anhydrous and spherical macromolecule

$$S^* = \frac{S^0_{20,w} v^{1/3}}{(1 - v\rho)} = \frac{M^{2/3}}{6\pi\eta\mathscr{R}^{2/3}(3/4\pi)^{1/3}}$$

Highly asymmetric proteins/high frictional coefficient, smaller s
ex:fibrous protein/ myosin

Predicted Mw ~ 100,000 , correct MW=540,000

Anhydrous and Spherical Macromolecule

$S^* = \frac{s\bar{v}^{1/3}}{1 - \bar{v}\rho}$

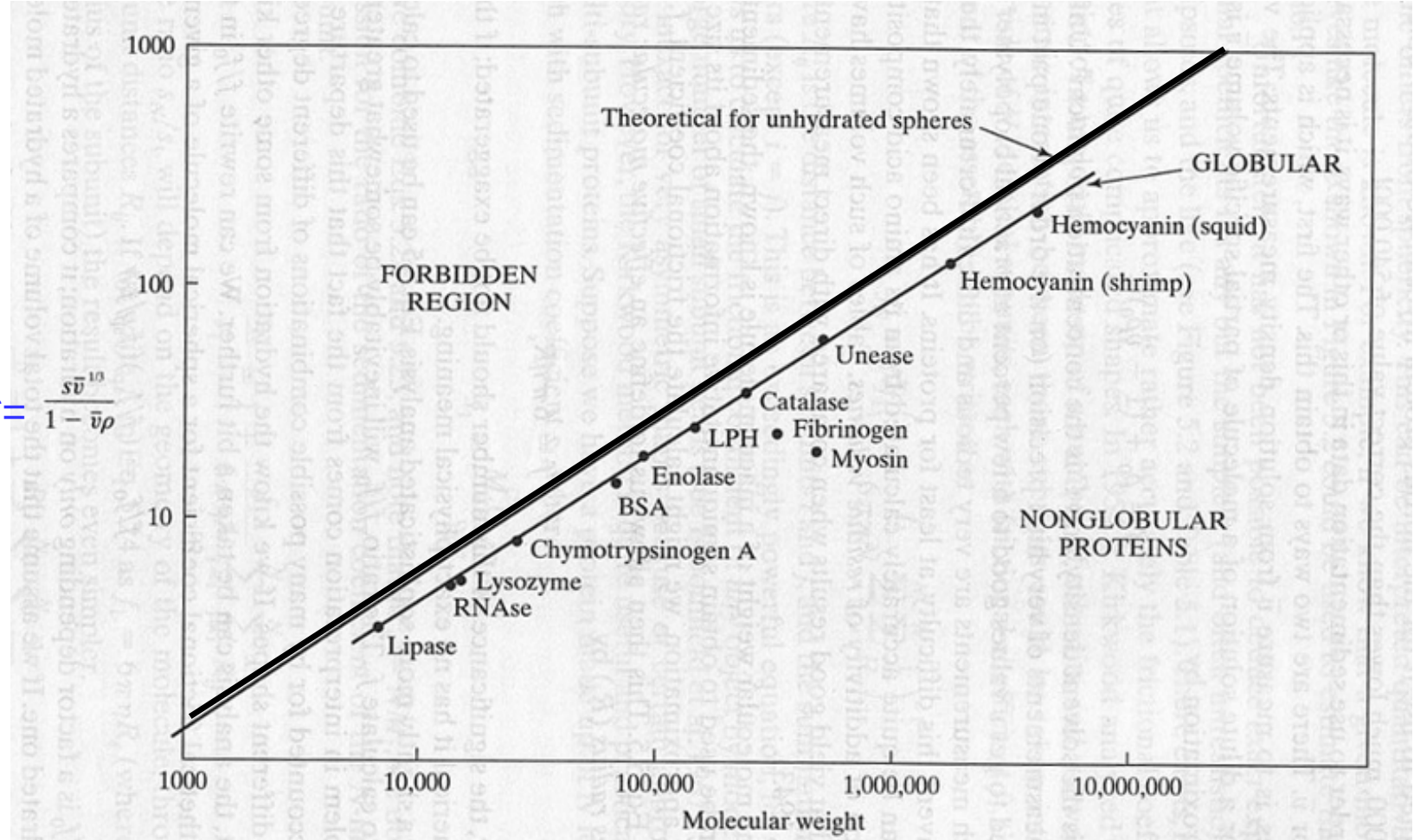


Figure 5.8 Graphs of $s\bar{v}^{1/3}/(1 - \bar{v}\rho)$ vs. molecular weight on a log-log scale. The upper line corresponds to Eq. 5.17. Data for various proteins are also plotted on this graph; those for *globular* proteins fall on or close to an empirical line of correct slope (lower line on graph). Note that some *fibrous* proteins deviate markedly from this relationship.

partial specific volume "ν"

$$\rho = \rho_o + (1 - \nu \rho) C$$ (ρ_o is the solvent density)

residue volumes: calc. from its aa composition

effective molecular radius / Stockes radius, R_s

$$f = 6 \pi \eta R_s$$

Different degree of hydration and different shapes

f_{sp} : the hypothetical frictional coefficient for a spherical molecule of a given hydration

$$f / f_0 = (f / f_{sp})(f_{sp} / f_0)$$

(f_{sp} / f_0): dep. on hydration

$$f_{sp} / f_0 = (1+\delta)^{1/3}$$

δ : The total volume of a hydrated molecule is the sum of the anhydrous volume and the volume of hydrating water.

(f / f_{sp}): shape factor, how much the molecule differs from sphericity.

The frictional behavior of a molecule made up of a number of subunits

N identical subunits, each with frictional coefficient f_1,

The frictional coef. of the **assembly**

$$f_N = N f_1 \left[1 + f_1 / 6 \pi \eta N_1 \left(\sum \sum 1/R_{ij} \right)\right]^{-1}$$

R_{ij} is the distance between subunits i and j

The frictional behavior of a molecule made up of a number of subunits

A protein made up of N identical subunits , each with s_1

$$S_1 = \frac{M\,(1 - \upsilon\,\rho)}{\mathcal{R}\,f}$$

$$S_N \;/\; S_1 = 1 + [\, f_1 \,/\, 6\,\pi\,\eta\, N_1\, (\Sigma\,\Sigma\; 1/R_{ij})]$$

The ratio S_N / S_1 will depend on the geometry of the molecule, through the subunit-subunit distance R_{ij}

$$f_1 = 6\,\pi\,\eta\, Rs$$

$$S_N \;/\; S_1 = 1 + [R_s / N\, (\Sigma\,\Sigma\; 1/R_{ij})]$$

All us to test various postulated geometries of subunit arrangement, provided that S_N, S_1, M_1 and N are known

Predicted values for S_N/S_1 for various possible quaternary arrangements of a tetramer

TABLE 5.4 PREDICTED SEDIMENTATION COEFFICIENT RATIOS FOR DIFFERENT TETRAMER STRUCTURES

Structure	s_4/s_1
Linear	2.208
Square-planar	2.353
Tetrahedral	2.500

Calculated assuming spherical subunits in contact, Eq. 5.25.

$$S_N / S_1 = 1 + [R_s / N (\Sigma \Sigma 1/R_{ij})]$$

Calculation of "M" from s and D

$$\frac{M\,(1-\upsilon\rho)}{\mathcal{R}\,f} = \frac{v}{\omega^2 r} = s$$

$$D = RT/\mathcal{R}f$$

Svedberg equation

$$s/D = \frac{M\,(1-\upsilon\rho)/\mathcal{R}f}{RT/\mathcal{R}f} = \frac{M\,(1-\upsilon\rho)}{RT}$$

Analysis of Mixtures in Moving Boundary Sedimentation

Multiple compounds present

Determine both “s”

The enemy of resolution is “**Diffusion**”

The rate of boundary separation depends on the actual velocities of boundary motion

Higher rotor speeds will in general lead to greater resolution

Analysis of Mixtures in Moving Boundary Sedimentation

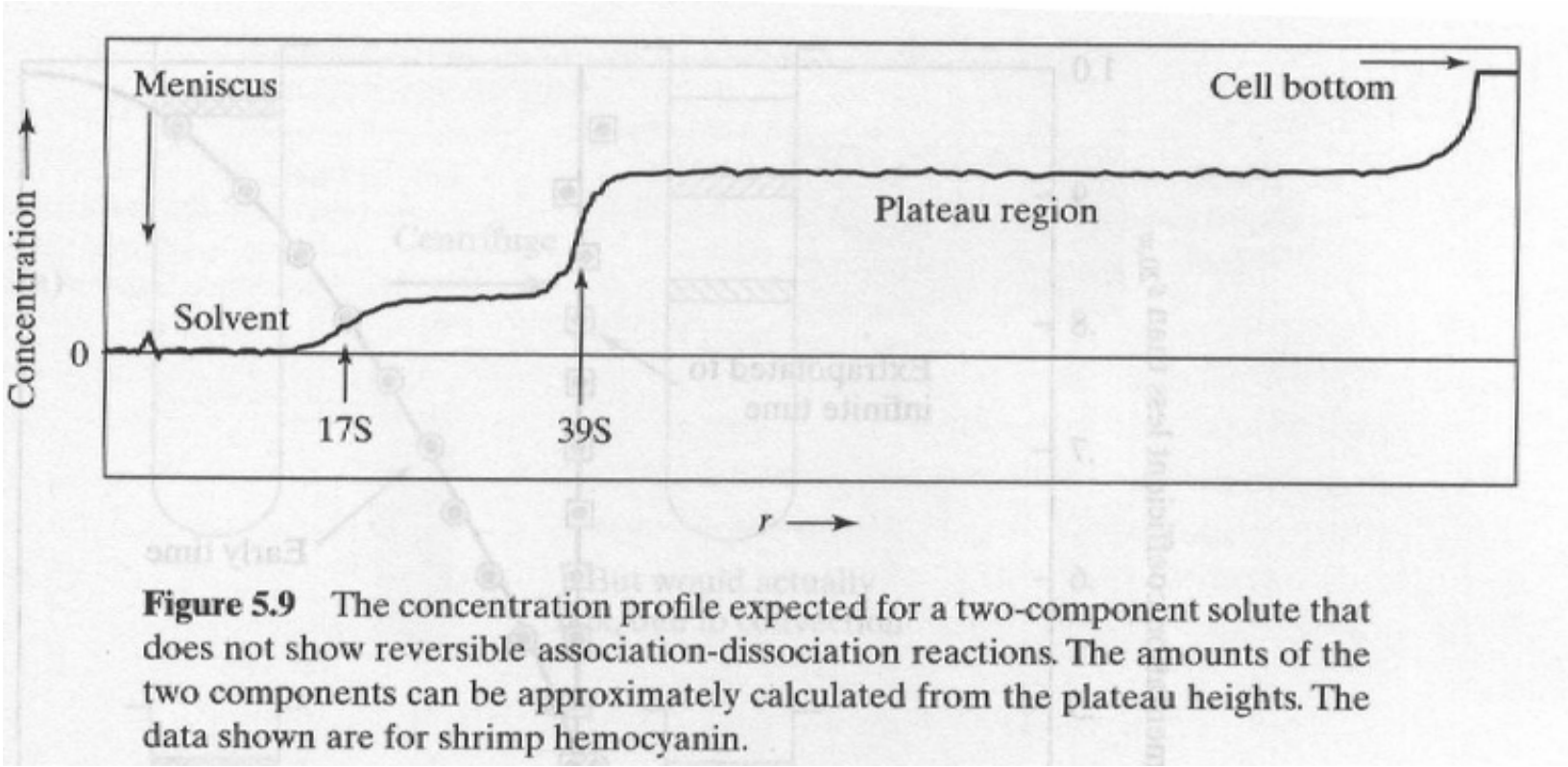


Figure 5.9 The concentration profile expected for a two-component solute that does not show reversible association-dissociation reactions. The amounts of the two components can be approximately calculated from the plateau heights. The data shown are for shrimp hemocyanin.

5.3.2 Zonal Sedimentation

moving boundary sedimentation

an expensive and specialized instrument is required

high conc. (1 mg/ml) is required

has difficulty in resolving complex mixtures, only lowest component is resolved from others, due to convection.

Density gradient- sucrose or glycerol

How the Diffusion-Smeared boundary produced

As sedimentation proceeds for longer and longer times, the separation between different components becomes more and more dominant over the spreading of any one boundary.

Van Holde and weischet have utilized this fact to extrapolate out the diffusion smearing

Diffusion-smeared boundary produced be a single, homogeneous substance can be corrected so as to demonstrate this homogeneity

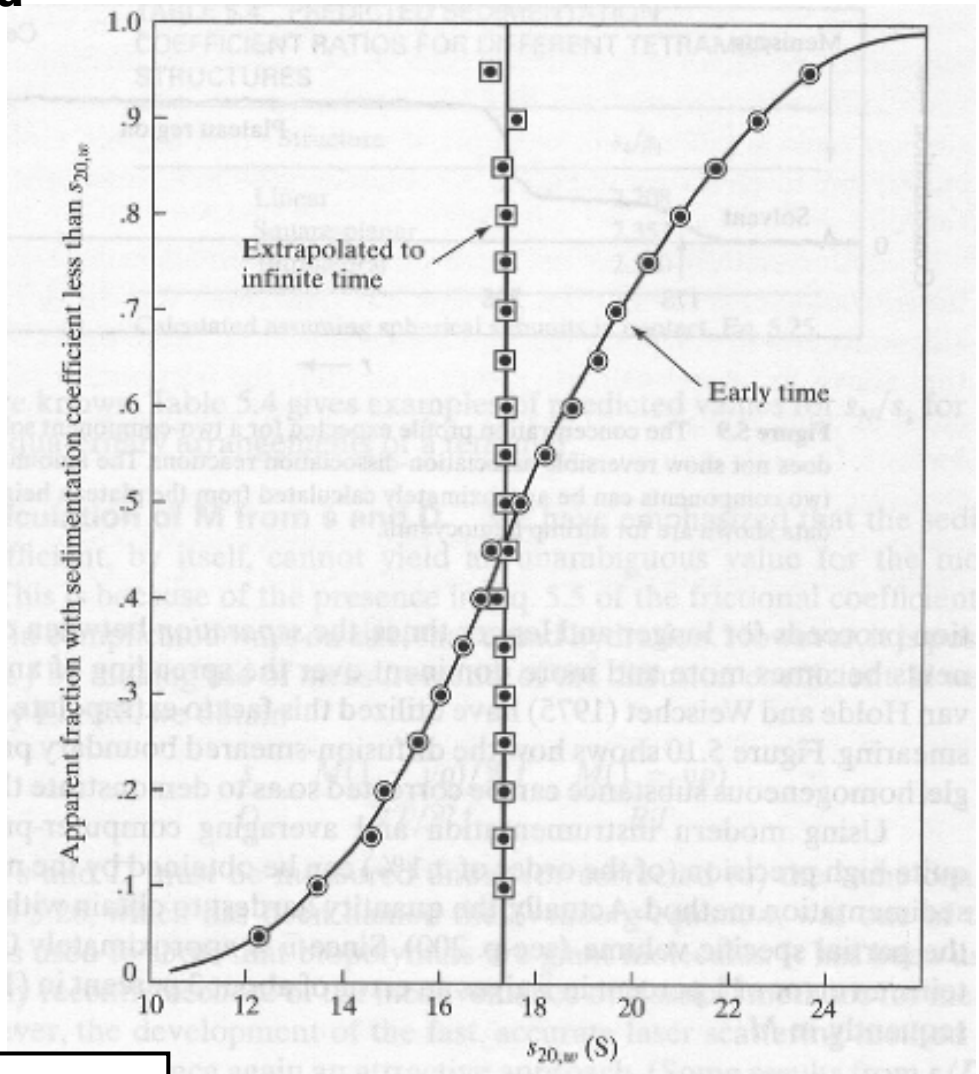


Fig. 5.16 Removing the deleterious effect of diffusion smearing on boundary resolution. Data are from a study of squid hemocyanin. The curve with the circles depicts the apparent distribution of sedimentation coefficients at an early time during an experiment. The line through the squares shows the apparent distribution when extrapolated to infinite time. The fact that it is a vertical "step" function shows that the sample is homogeneous, with $s_{20,w}$ = 17.2 *S*.

Analysis of Sedimentation Velocity

An extreme case is the fitting of data, by interactive methods, to the differential equation governing sedimentation and diffusion

Lamm equation

$$\frac{dc}{dt} = \frac{1}{r}\frac{d}{dr}\left[rD\frac{dc}{dr} - s\omega^2 r^2 c\right]$$

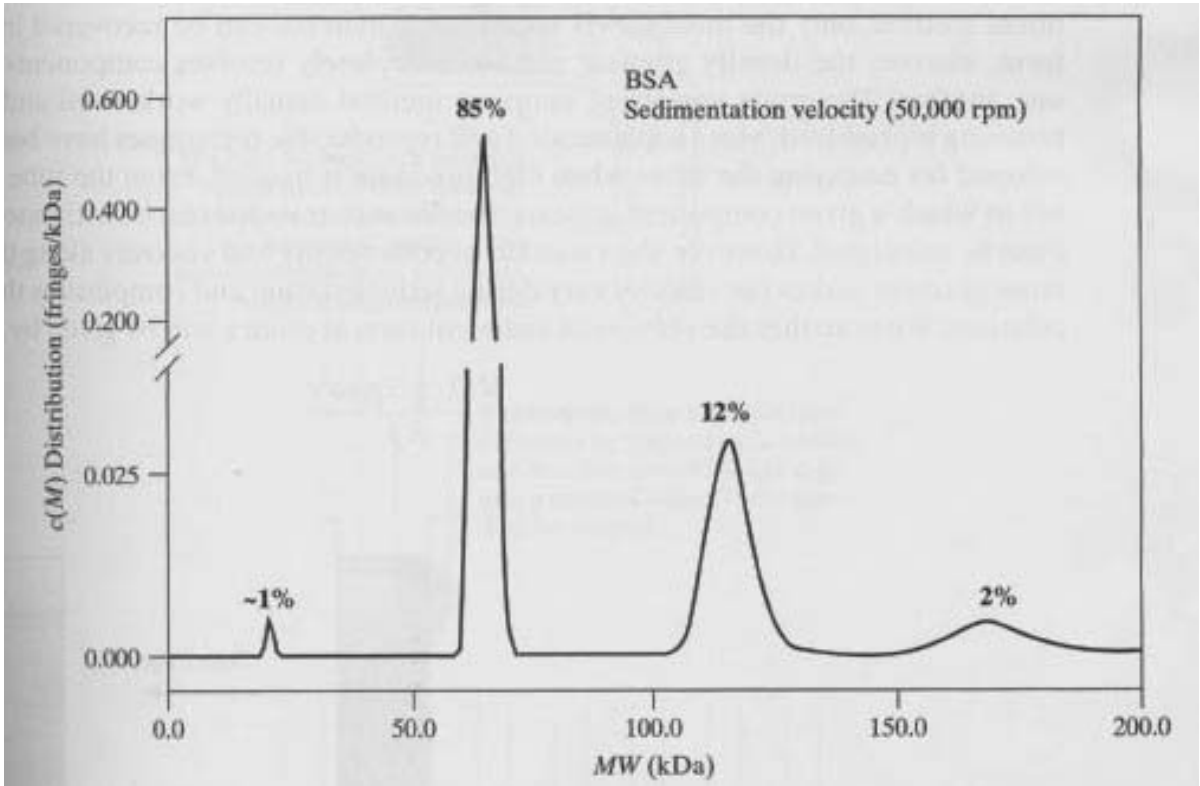


Figure 5.17 Analysis of sedimentation velocity data via fitting to the Lamm equation (Eq. 5.35). A serum albumin sample is shown to consist mostly of monomer, but small amounts of dimer and trimer were also present. [Courtesy of Drs. S. Anderson and D. Malencik.] The analysis is according to methods devised by P. Shuck (see, for example, Lebowitz, et al, 2002).

5.3.2 Zonal Sedimentaiton

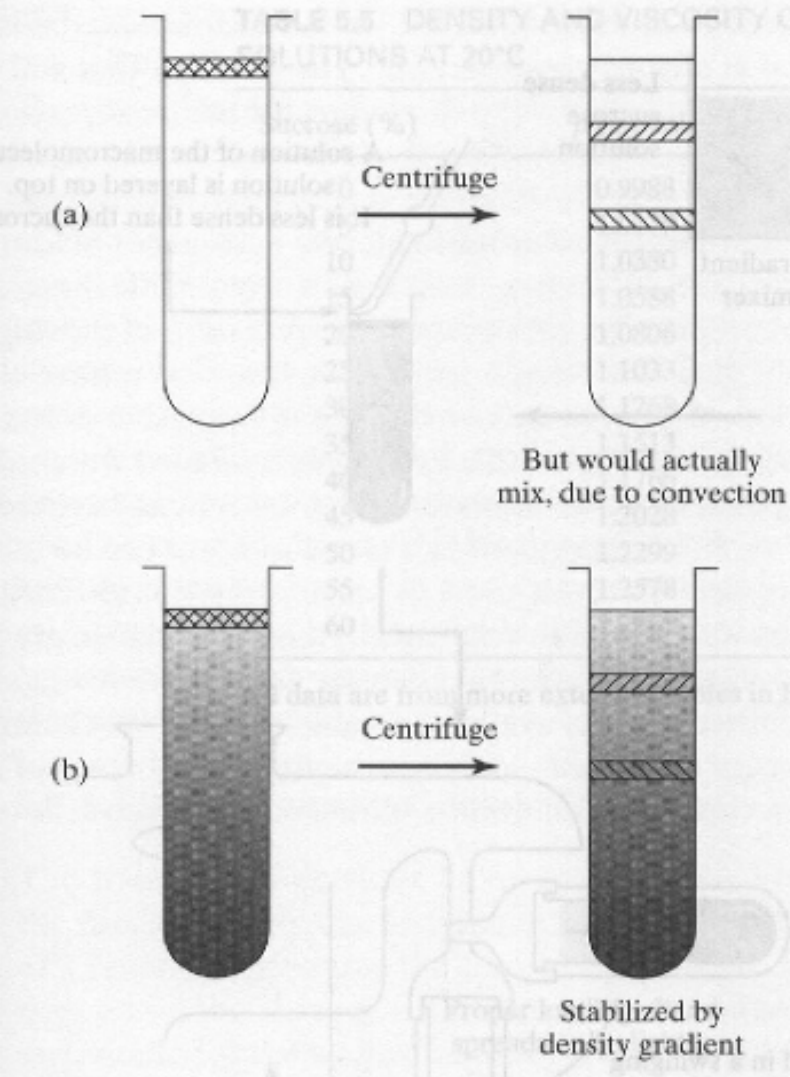


Fig. 5.18 The principle of Zonal Sedimentation. (a) The concept of zonal separation. A thin layer containing two different components will separate into two zones after transport. (b) The imposition of a density gradient will allow the zones to be stable against convection.

Sucrose Gradient Centrifugation

High % load first

Gradient keep in few hrs

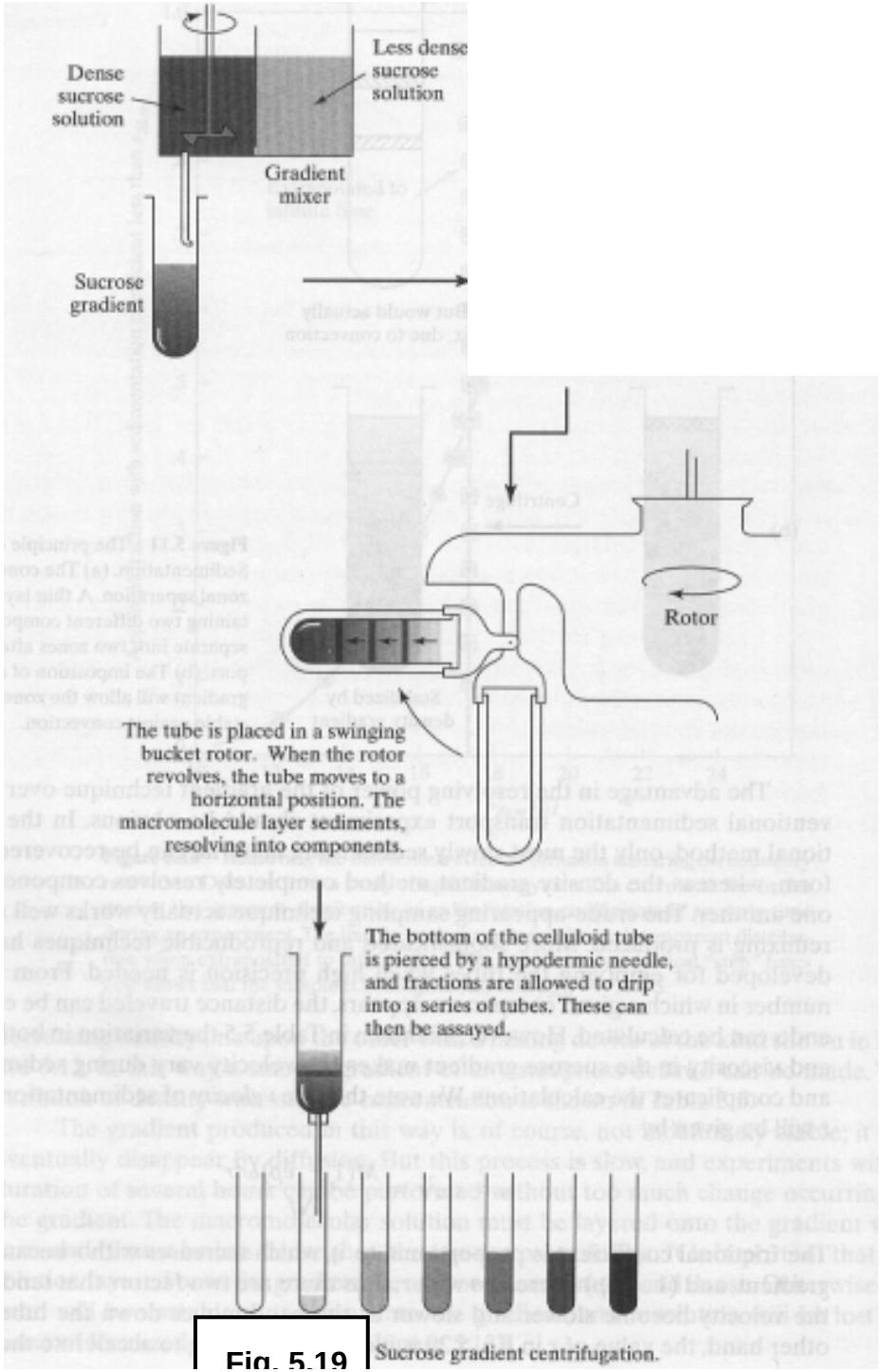


Fig. 5.19 Sucrose gradient centrifugation.

Zonal Sedimentation

a density gradient, sucrose or glycerol density gradient

no convection

r is increasing & accelerate the motion (υ)

However, 2 factors that tend to make the velocity become slower and slower as the band moves down the tube

f is proportional to η & increases with r

$(1- \upsilon r)$ decreases, increases with r

If the form of the gradient is chosen correctly, these factors will balance and sedimentation will proceed at a nearly constant rate The gradient that will produce this effect is called an "**Isokinetic gradient**"

TABLE 5.5 DENSITY AND VISCOSITY OF SUCROSE SOLUTIONS AT 20°C

Sucrose (%)	ρ (gm/ml)	η (cp)
0	0.9988	1.004
5	1.0179	1.148
10	1.0380	1.337
15	1.0588	1.592
20	1.0806	1.946
25	1.1033	2.449
30	1.1268	3.189
35	1.1513	4.323
40	1.1766	6.163
45	1.2028	9.376
50	1.2299	15.42
55	1.2578	28.07
60	1.2867	58.50

All data are from more extensive tables in Fasman (1976).

Convection

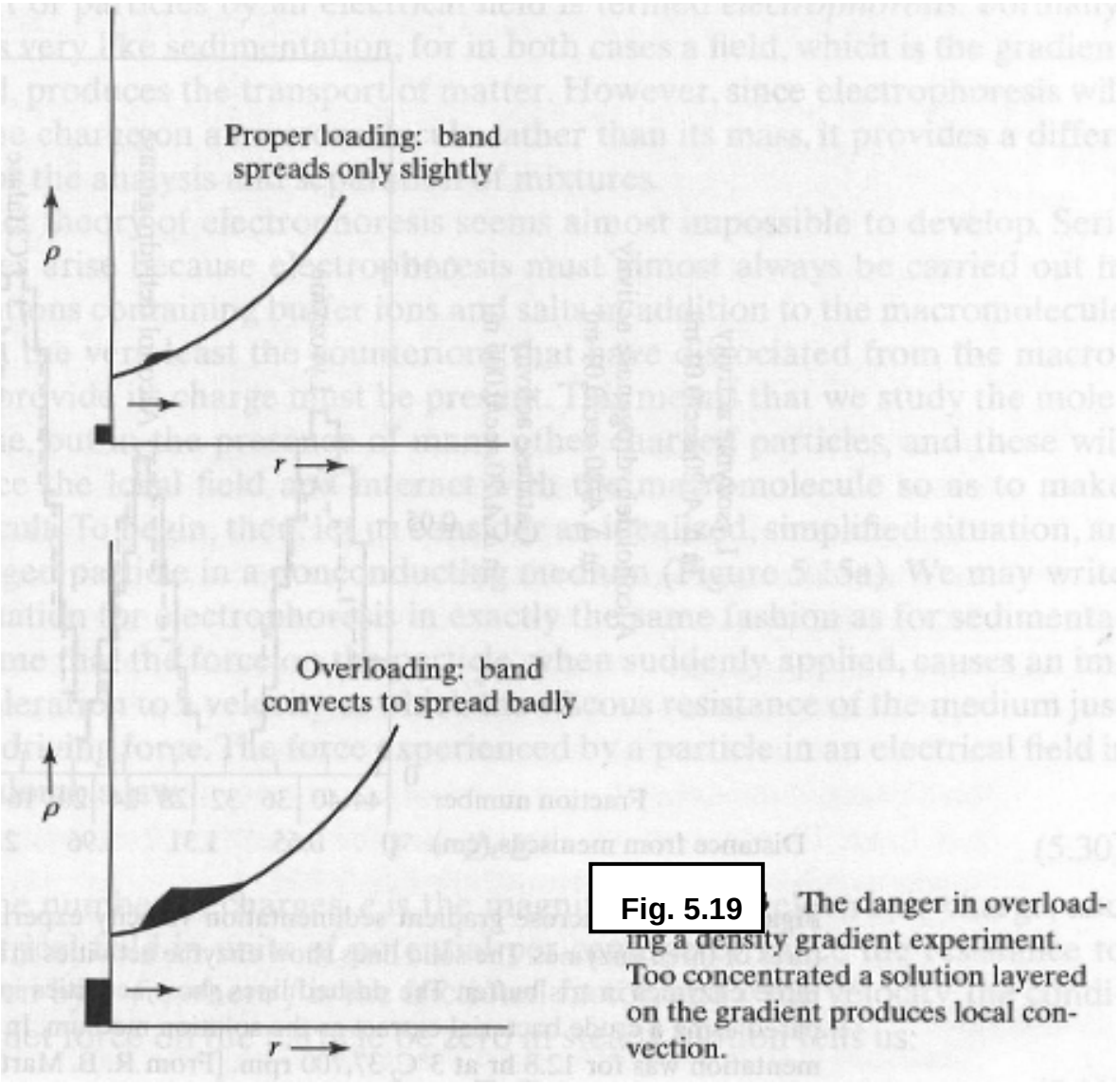


Fig. 5.19 The danger in overloading a density gradient experiment. Too concentrated a solution layered on the gradient produces local convection.

Sucrose Gradient Sedimentation

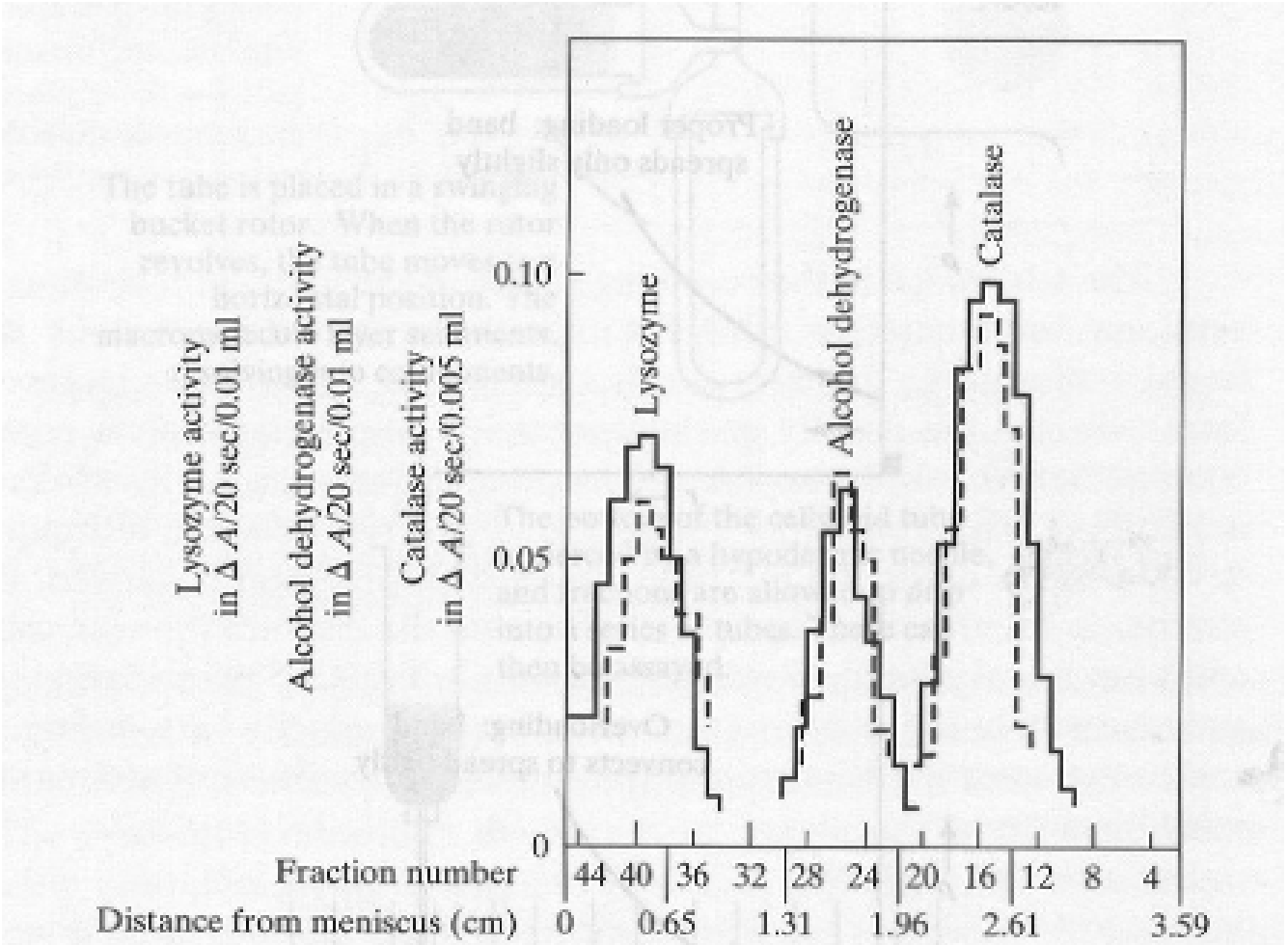


Figure 5.14 A sucrose gradient sedimentation velocity experiment with mixtures of three enzymes. The solid lines show enzyme activities in a mixture of the three enzymes in tris buffer. The dashed lines show activities in a mixture prepared using a crude bacterial extract as the solution medium. In each case, sedimentation was for 12.8 hr at 3°C, 37,700 rpm. [From R. B. Martin and B. Ames (1961), *J. Biol. Chem.*, **236**, 1372.]

5.3.3 Sedimentation Equilibrium

Smaller centrifugal force

Concentration gradient

Becomes steeper & steeperBackflow due to diffusion becomes more and more pronounced

Backflow & outward flow balance

Sedimentation Equalibrium

Fick's first law

$$J_D = - D \left(\partial C / \partial x\right)$$

Sedimentation Equilibrium

Fick's first law $J_D = - D (\partial C / \partial x)$

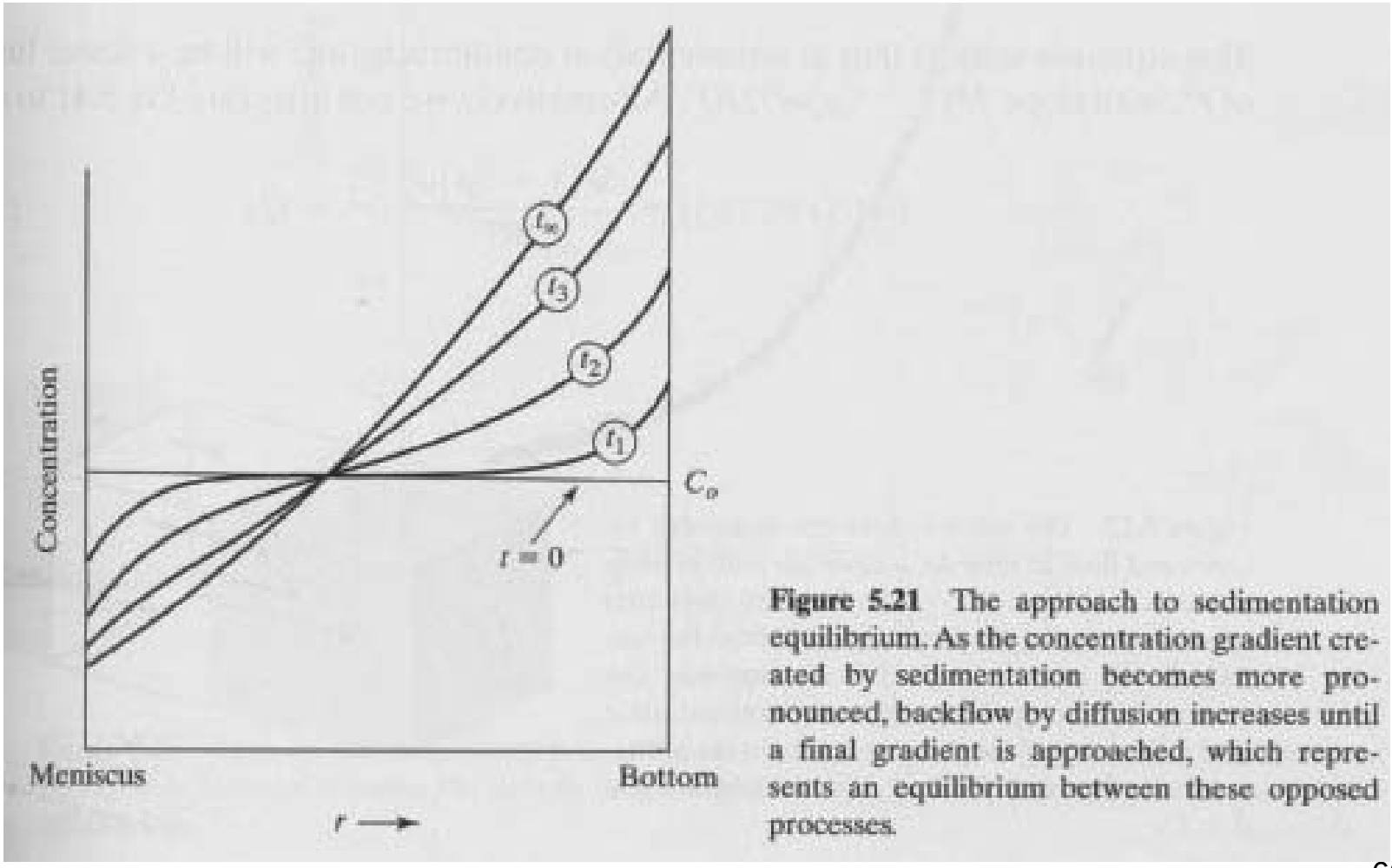


Figure 5.21 The approach to sedimentation equilibrium. As the concentration gradient created by sedimentation becomes more pronounced, backflow by diffusion increases until a final gradient is approached, which represents an equilibrium between these opposed processes.

Relation between molecular velocity & flow

moves a distance $\Delta x = v \Delta t$

Surface “s”

The amount of solute passing $\Delta w = C \; S\Delta x = C \; S \; v\Delta t$

$J = \Delta w / S\Delta t = C \; v$

Sedimentation flow $\mathbf{J_s = C \; v_s}$

The product of **the velocity** with which molecules sediment and their local concentration “**C**”

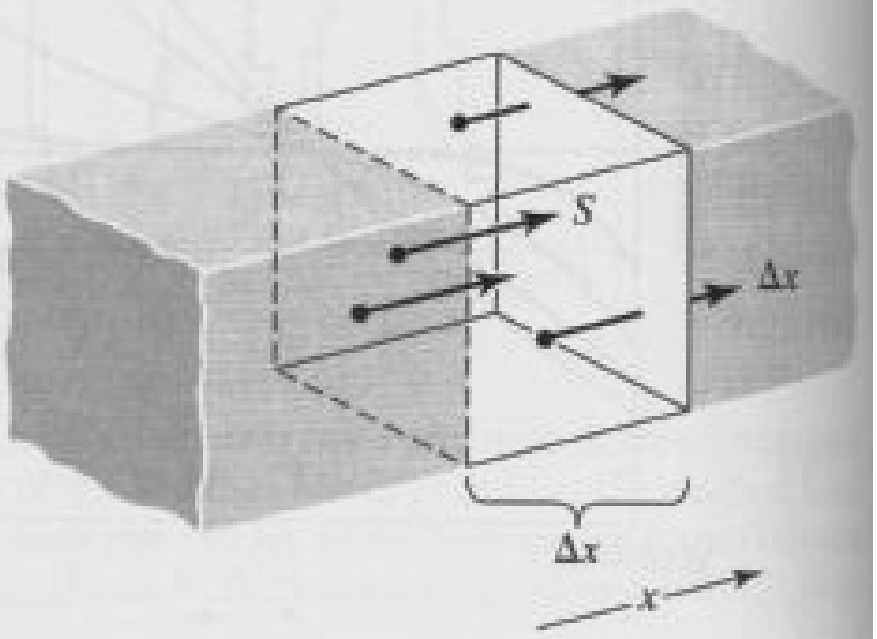


Figure 5.22 The relation between molecular velocity and flow. In time Δt, a molecule with velocity v moves a distance $\Delta x = v\Delta t$. Thus, all molecules in a slab extending back this distance from the surface S will pass through S in Δt seconds. The amount of solute passing will be the concentration times the slab volume: $\Delta w = CS\Delta x = CSv\Delta t$. Because the flow is defined as $J = \Delta w/S\Delta t$, we find $J = Cv$.

Sedimentation Equilibrium

When sedimentation equilibrium is attend the net flow at every point in the cell will be "0"

$$J = J_s + J_D = 0$$

$$\ln \frac{C(r)}{C(r_o)} = M(1-\upsilon\rho)\omega^2/2RT\ (r^2-r_o^2)$$

C is a functional only of "r", not also of "t".

At sedimentation equilibrium, **lnC** will be a linear function of r^2 with a slop of "**$M(1-\upsilon\rho)\omega 2/2RT$**"

$$\frac{C(r)}{C(r_o)} = e^{M(1-\upsilon 2\rho)\omega 2/2RT\ (r^2-r_o^2)}$$

Sedimentation Equilibrium

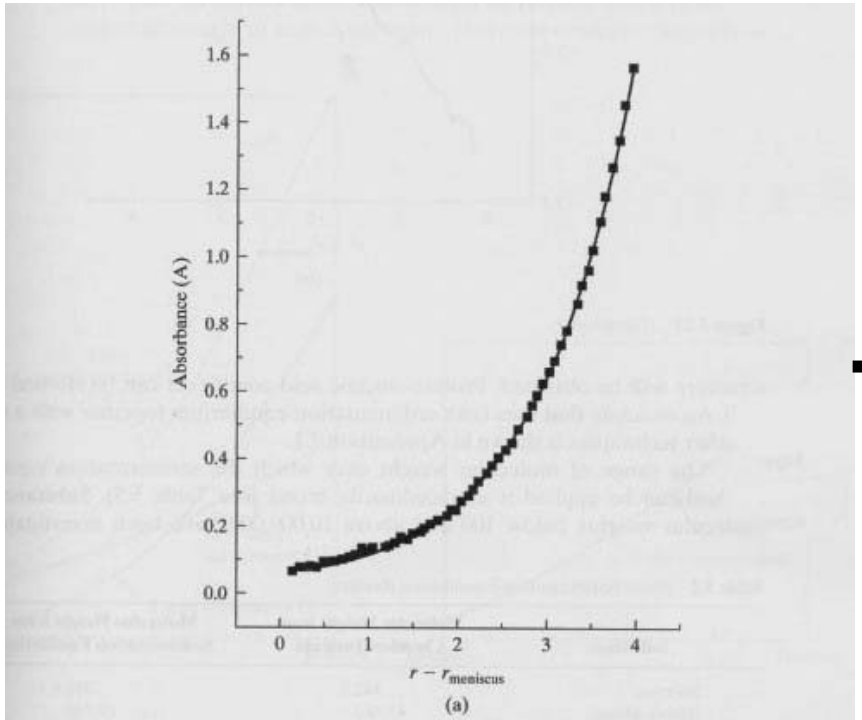


Figure 5.23 Data on the sedimentation equilibrium of monomeric squid hemocyanin. (a) C versus r curve, (b) ln C versus r^2 curve. The data fit the curve predicted for an ideal, homogeneous component of 382,000 Da.

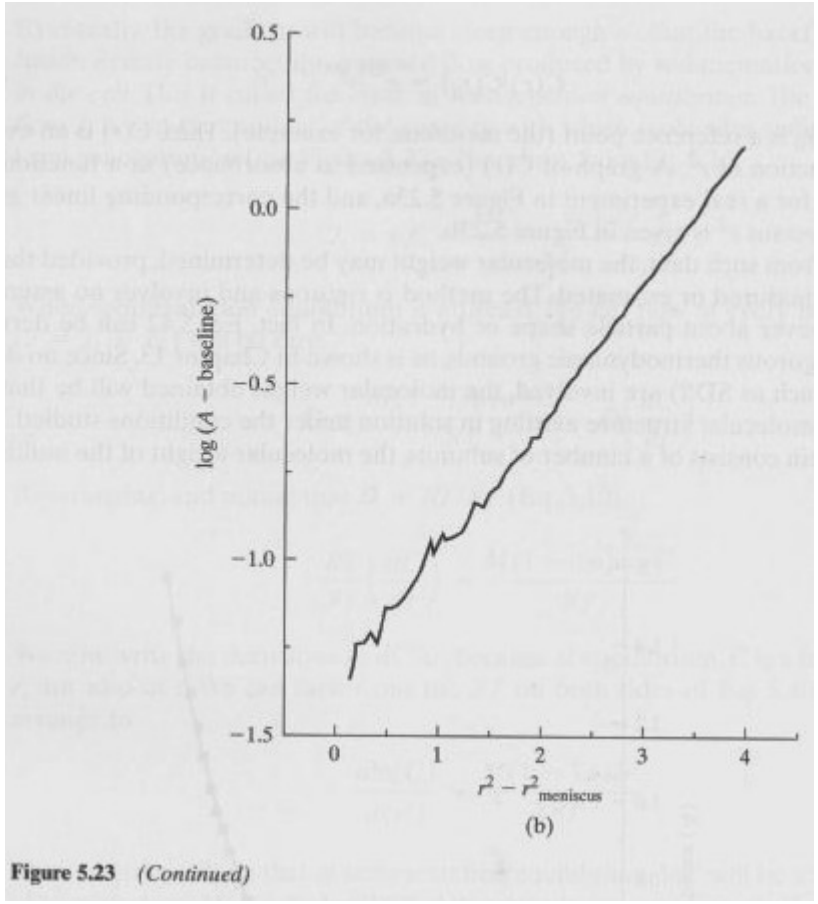


Figure 5.23 *(Continued)*

C(r) & r^2

ln C(r) & r^2

Sedimentation Equilibrium

- Multisubunit & Protein-nucleic acid complexes

- The range of MW over which the sedimentation equilibrium method can be applied 100 – 10,000,000
- by rotor speed can be adjusted

- Table 5.5

Sedimentation Equilibrium

If the macromolecular solute is not homogeneous

but instead consist of several components (for example, different aggregation states of a given protein subunit

2 methods

Multiexponential curve fitting

Use of average MW

A mixture of two components

The steepness of each concentration gradient will depend on the MW

The **sum** of the component concentrations will not be a simple exponential function and will exhibit curvature on an **ln C_r** versus **r^2** plot

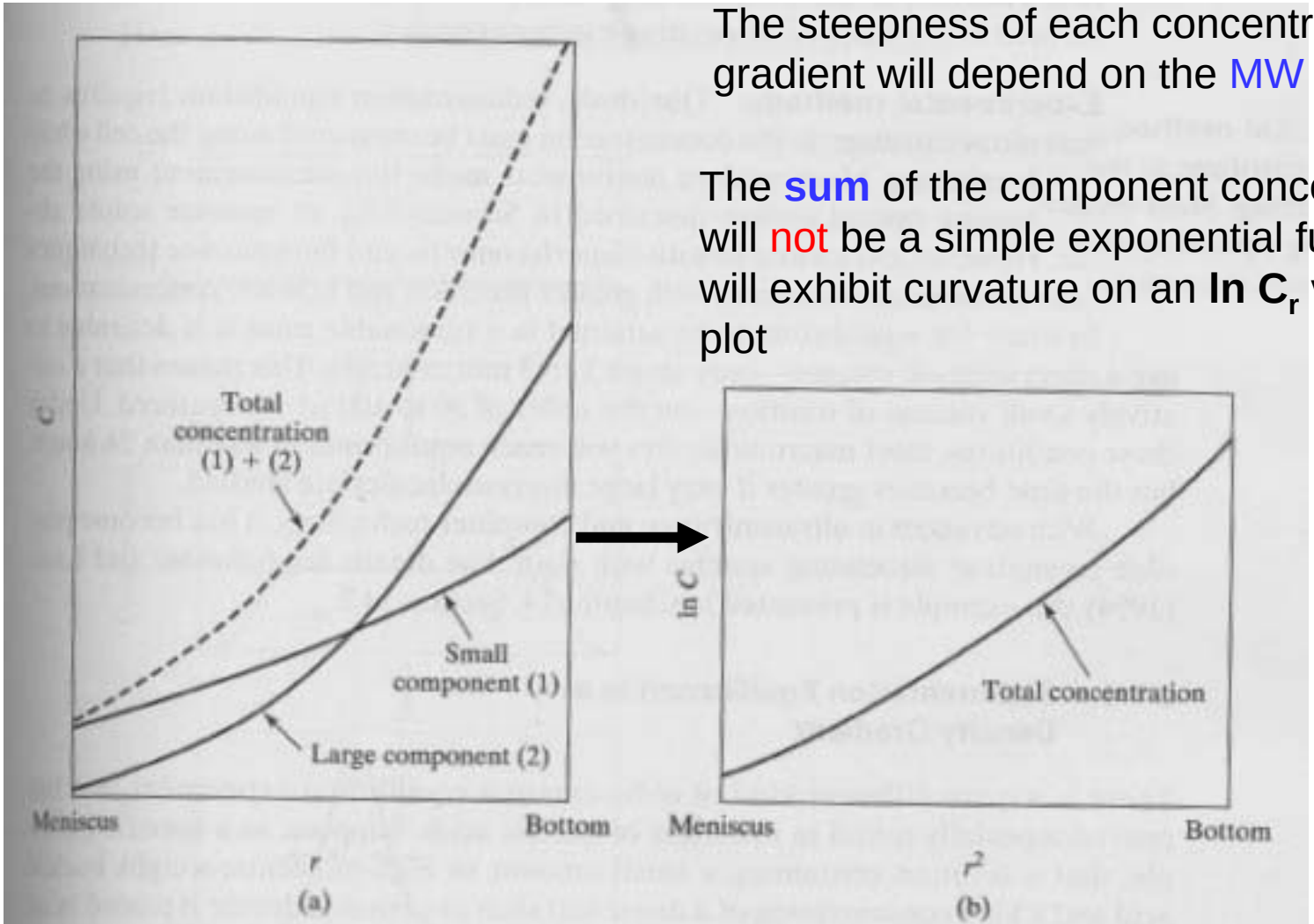


Figure 5.24 (a) The concentration curves at sedimentation equilibrium for a mixture of two components of different molecular weight (curves 1 and 2). Each is an exponential in r^2. The total concentration $(C_1 + C_2)$ is *not* a simple exponential. (b) The $\ln C_i$ versus r^2 plot corresponding to the curve in (a).

Use of average MW

A mixture of n solute components of M_i

$$\frac{d \ln C_i(r)}{d(r^2)} = [\omega^2 (1 - \upsilon \rho) / 2RT] M_{wr}$$

M_{wr} is the weight average molecular weight of the material at point r in the cell

$$M_{wr} = \Sigma C_i M_i / \Sigma C_i = \Sigma C_i M_i / C_i$$

is the weight average molecular weight of the mixture at point r in the cell

Experimental Methods

A short solution column, 1-3 mm
Small volume of solution 30-100 μl
Reach equilibrium in less than 24 hrs

5.3.4 Sedimentation Equilibrium in a Density Gradient

Especially useful in the study of nucleic acids

a small of high-MW nucleic acid & a high concentration of a dense salt such CsCl etc

Spun for a long time at high speed

The salt will eventually reach sedimentation equilibrium, giving a concentration gradient like the

$$\ln \frac{C(r)}{C(r_o)} = M(1-\upsilon\rho)\omega^2/2RT\ (r^2-r_o^2)$$

5.3.4 Sedimentation Equilibrium in a Density Gradient

at some point, $\mathbf{r_o}$ in the cell,
$\rho (r_o) = 1/ \upsilon$

υ : specific volume of the macromolecule

$(1-\upsilon \rho)$: Buoyancy factor
α the solvent density ρ
Positive, above this point
Negative, below it

Eventually, equilibrium will be established and **diffuse** away from $\mathbf{r_o}$
But, the effect of **sedimentation** drives them back

The distribution of solute concentration is approximately Qaussian eq

5.3.4 Sedimendation Equilibrium in a Density Gradient

Gaussian eq: $C(r) = C(r_o)\, \mathbf{e}^{-(r-r_o)^2 / 2\sigma^2}$

Stand deviation σ

$$\sigma = (RT / \omega^2 r_o M \upsilon (d\rho/dr))^{1/2}$$

(dr/dr) is the density gradient established be the salt

If the MW is large, the band will be narrow (σ is smaller) , because s will be large and D small

5.3.4 Sedimentation Equilibrium in a Density Gradient

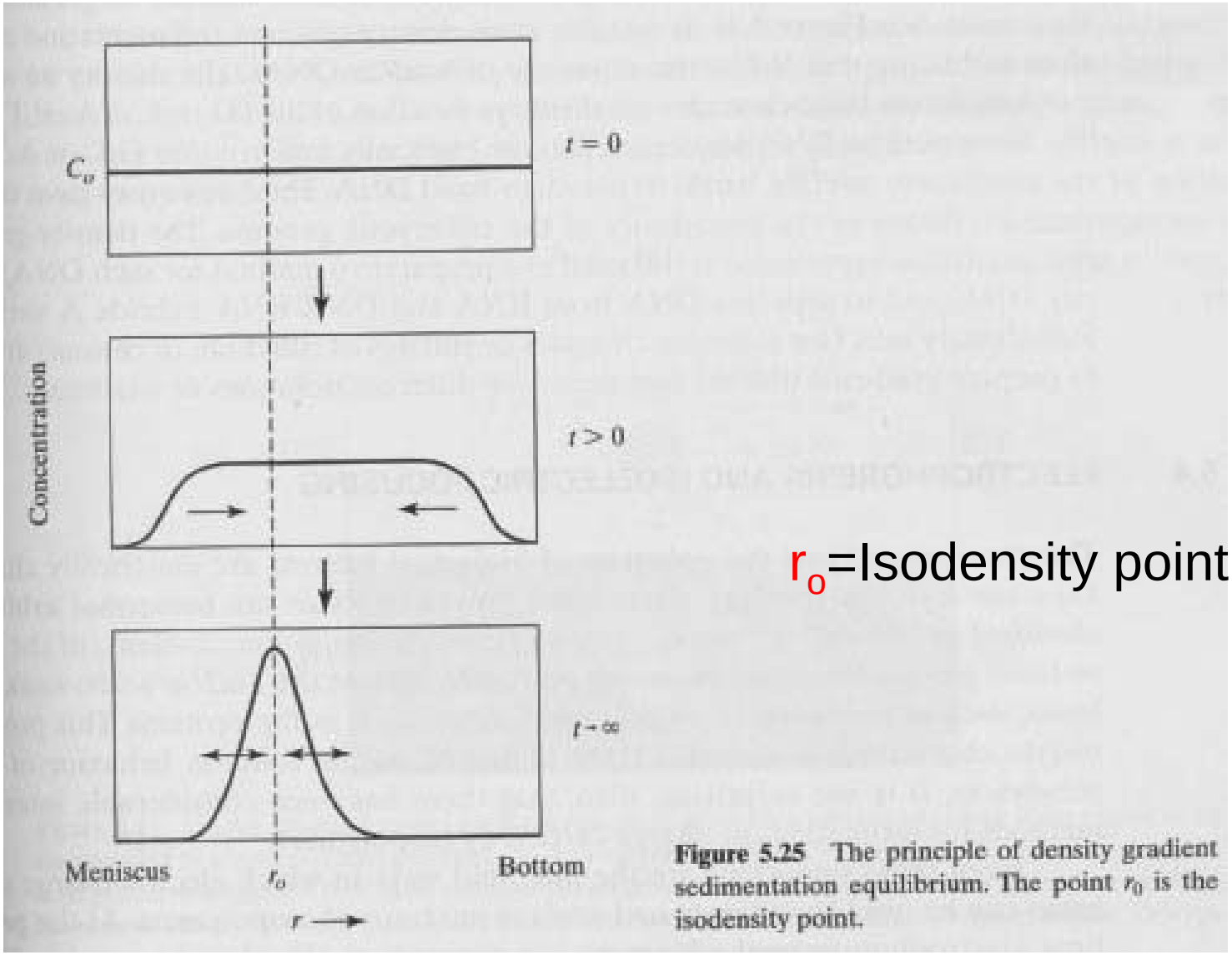
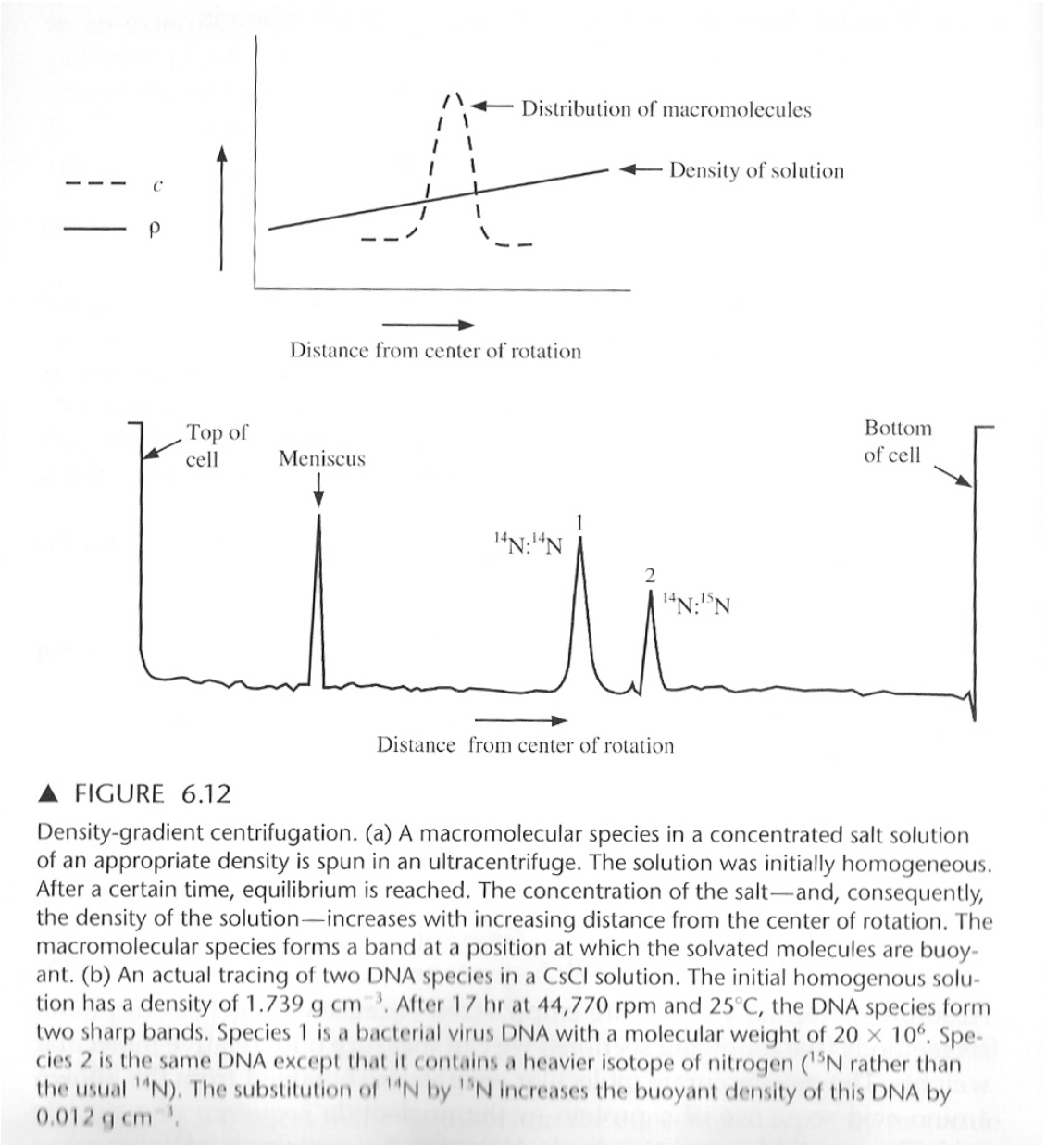


Figure 5.25 The principle of density gradient sedimentation equilibrium. The point r_0 is the isodensity point.

r_o=Isodensity point

Density Gradient sedimentation equilibrium of a mixture of DNA



▲ FIGURE 6.12

Density-gradient centrifugation. (a) A macromolecular species in a concentrated salt solution of an appropriate density is spun in an ultracentrifuge. The solution was initially homogeneous. After a certain time, equilibrium is reached. The concentration of the salt—and, consequently, the density of the solution—increases with increasing distance from the center of rotation. The macromolecular species forms a band at a position at which the solvated molecules are buoyant. (b) An actual tracing of two DNA species in a CsCl solution. The initial homogenous solution has a density of 1.739 g cm^{-3}. After 17 hr at 44,770 rpm and 25°C, the DNA species form two sharp bands. Species 1 is a bacterial virus DNA with a molecular weight of 20×10^6. Species 2 is the same DNA except that it contains a heavier isotope of nitrogen (^{15}N rather than the usual ^{14}N). The substitution of ^{14}N by ^{15}N increases the buoyant density of this DNA by 0.012 g cm^{-3}.

CsCl gradient

CsCl conc. ↑ ,
the density of CsCl ↑

5.4 Electrophoresis and Isoelectric Foucing

electrically charged

Ionization constants

Strong polyacids, such as nucleic acids

Weak polybases, such as poly-l-Lysing; or polyampholytes, such as the protein

5.4.1 Electrophoresis: General Principle

Electrophoresis : transport of particles by an **electrical field**

Dep. on the **charge** on a macromolecule rather than its mass.

Analysis and separation of mixture

Analysis difficulty: in aq. solution containing buffer ions, salts and macromolecule

Coulomb's Law	$F = Z e E$

(Z: the # of charge , e: the magnitude of the electronic charge,
E: the electrical field in units of potential per centimeter)

Electrophoresis

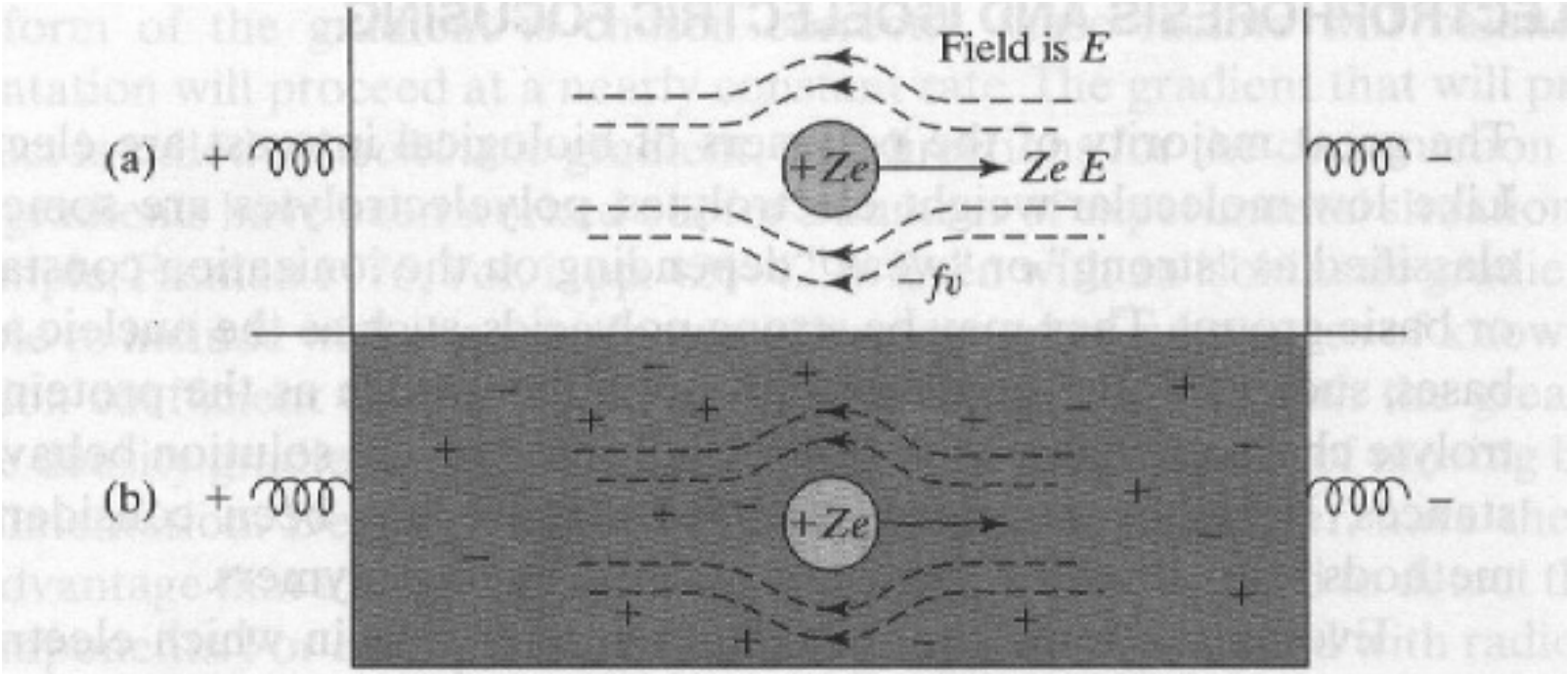


Fig. 5.27 (a) An idealized model for electrophoresis in which a particle of charge Ze is placed in an electric field in a nonconducting solvent. (b) A more realistic model, in which a charged macromolecule is subjected to an electric field in an aqueous salt solution. The small ions form an ion atmosphere around the macromolecule in which ions of charge opposite to that of the macromolecule predominate. This ion atmosphere is distorted by the field and by the motion of the macromolecule.

Electrophoretic mobility

Since the resistance to motion is given by " -fv "

Net force on the particle be "0" in steady motion/frictional force

$$f v = Z e E$$

Electrophoretic mobility, U

$$U = v/E = Z e/f$$

$$U = Z e / 6 \pi \eta R$$

Macromolecule is surrounded by a counterion atmosphere

The asymmetric atmosphere
complication
correction/realistic model

Isoelectric point
has "0" charge/ "0" mobility
pH>pI, protein will be "+" and move toward the "-" electrode
pH<pI, protein will be "-" and move toward the "+" electrode

moving boundary electrophoresis/free electrophoresis
~moving boundary sedimentation

zonal techniques /zonal electrophoresis ~ gel electrophoresis

Through some kind of **matrix**, which provides stability against convention, as does a sucrose gradient in zonal sedimentation

TABLE 5.6 SOME MEDIA FOR ZONAL ELECTROPHORESIS

Medium	Conditions	Principal Uses
Paper	Filter paper moistened with buffer, placed between electrodes	Small molecules: amino acids or nucleotides
Polyacrylamide gel	Cast in tubes or slabs; cross-linked	Proteins and nucleic acids
Agarose gel	As polyacrylamide, but no cross-linking	Very large proteins, nucleic acids, nucleoproteins, etc.

[3]A new technique, called *steady-state electrophoresis* may solve this problem. It resembles sedimentation equilibrium, and therefore is described in Chapter 13.

Electrophoresis

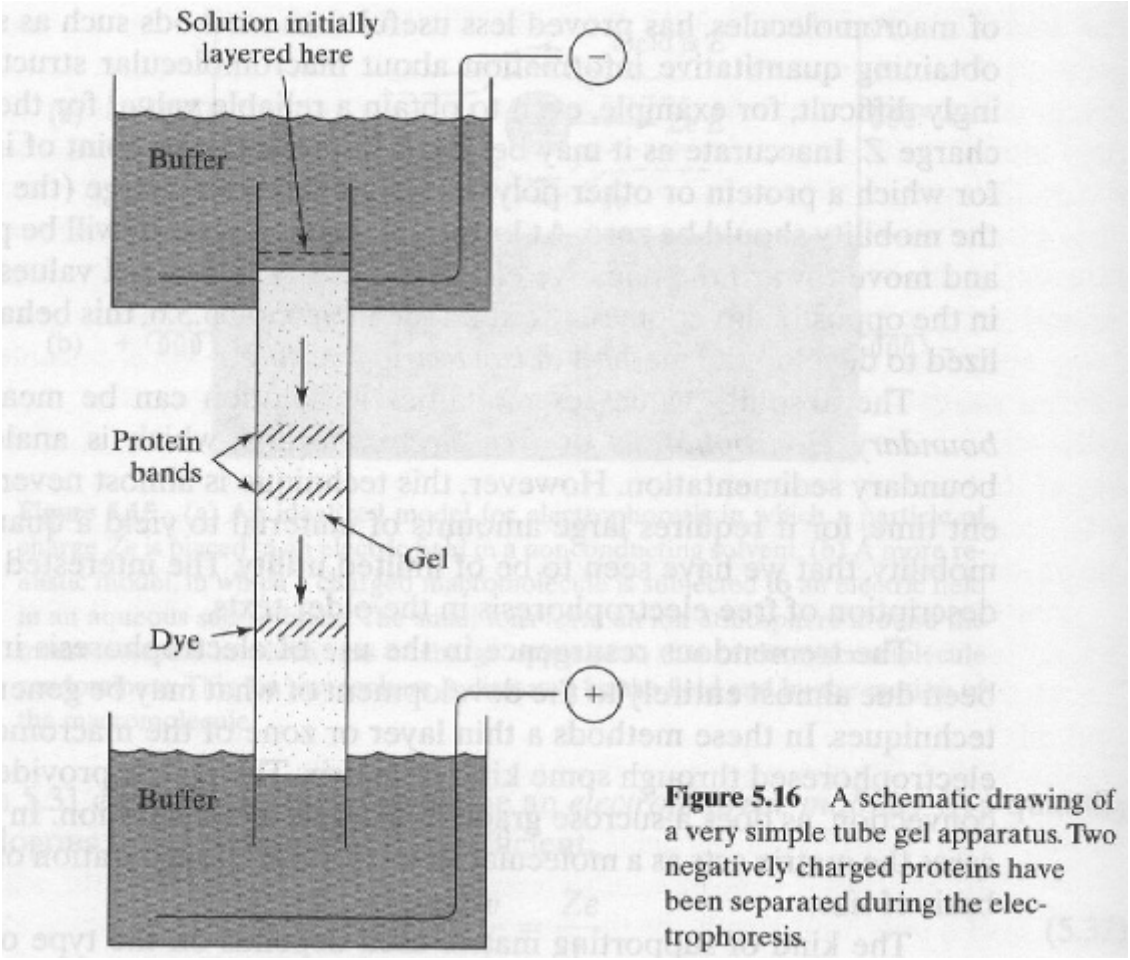


Figure 5.16 A schematic drawing of a very simple tube gel apparatus. Two negatively charged proteins have been separated during the electrophoresis.

Parallel lanes & under identical conditions, increasing the accuracy of comparison

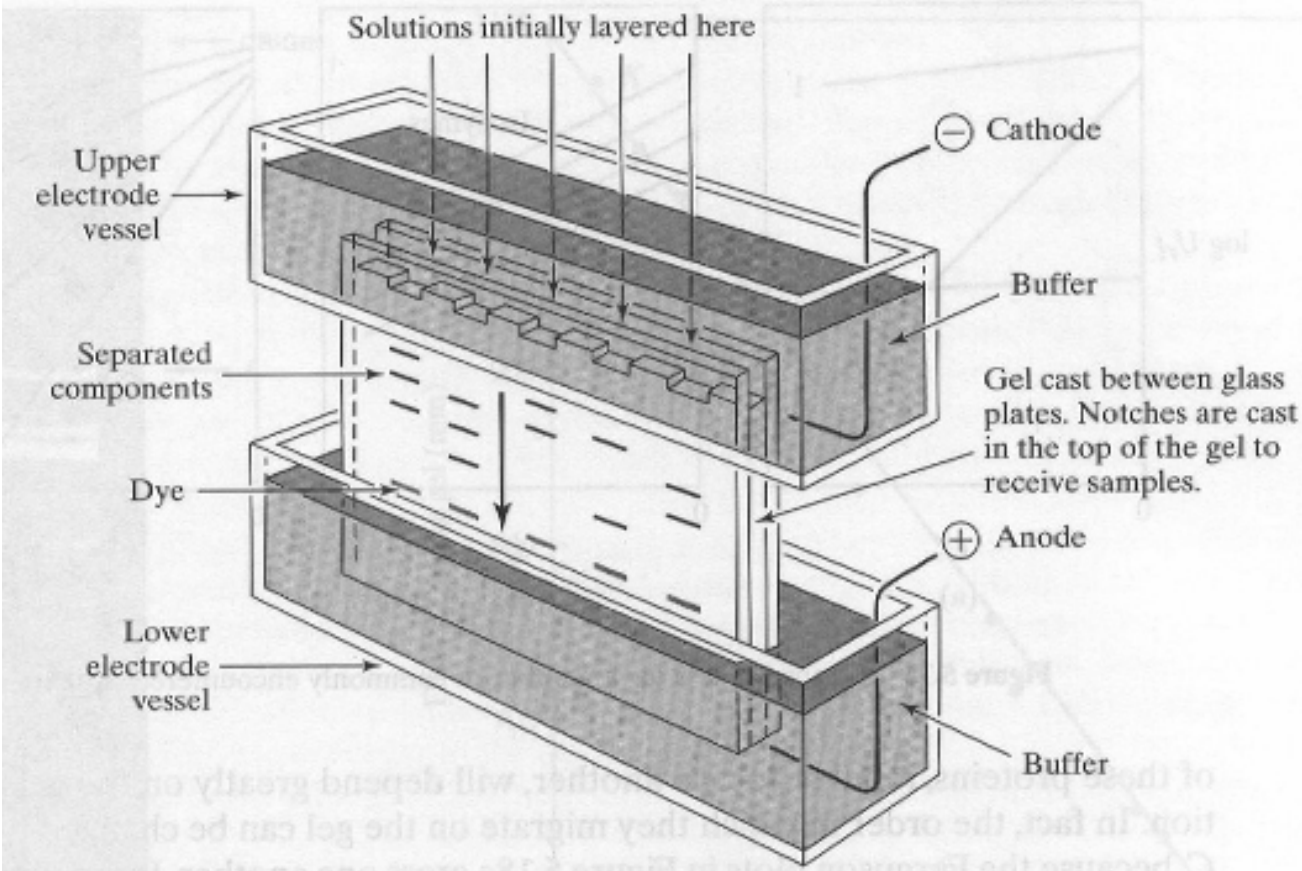
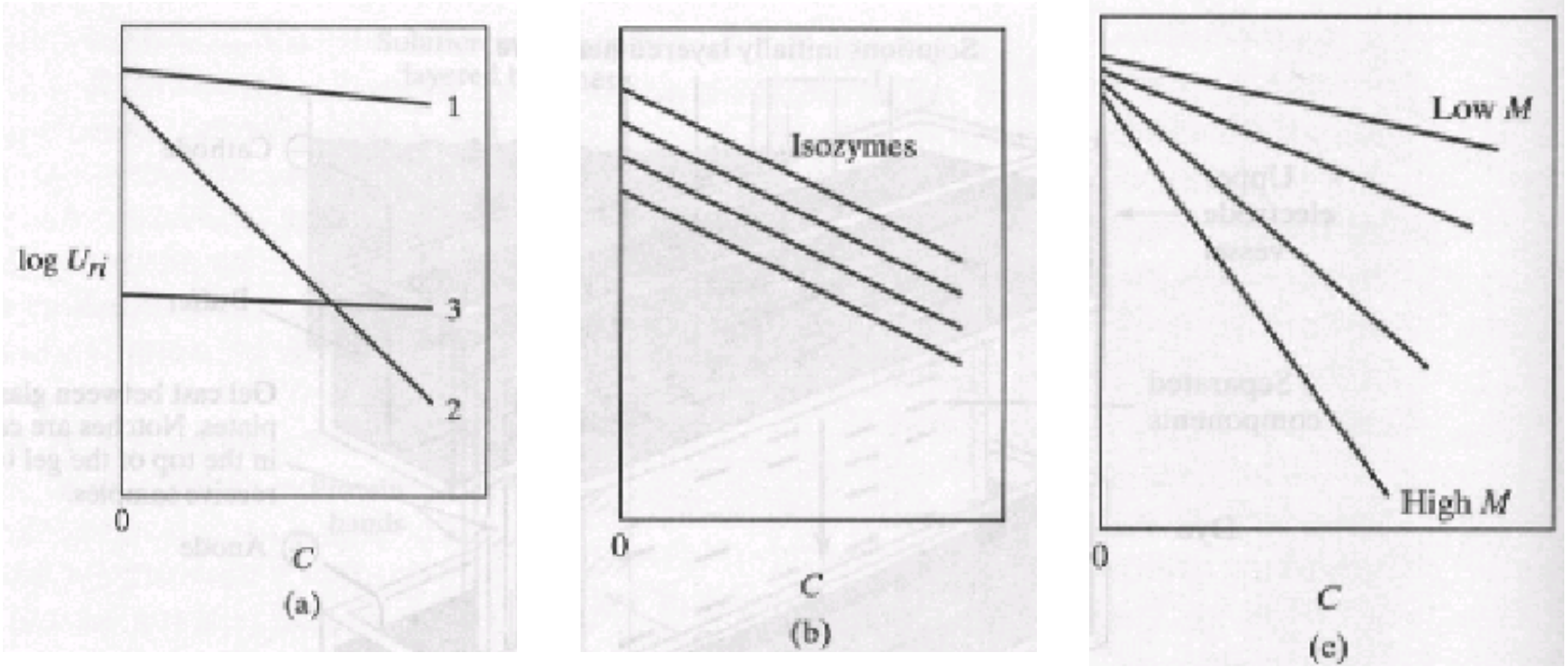


Figure 5.17 A typical slab gel electrophoresis apparatus. This apparatus is designed for small (8.3 cm by 10.3 cm) gel slabs, which can be 0.5 mm or 1 mm thick. Multiple samples are run on each gel.

Ferguson plots

$$\log U_{ri} = \log U^{o}_{ri} - k_i C$$

C: gel conc.
U_{ri}^{0} : the relative mobility of i / U_{ri} (C=0), free electrophoresis
k_i : dep on molecule size of i, large molecule/large k



Charge proportional to its length

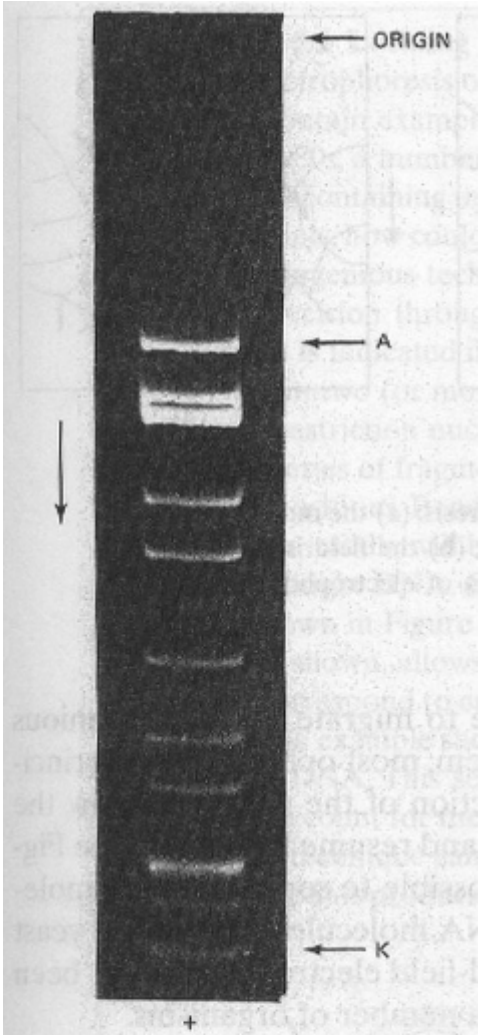


Figure 5.19 Gel electrophoresis of defined fragments of DNA produced by the action of the restriction endonuclease CfoI on the bacterial plasmid pBR 322.

logMi & Uri are approximately **linearly** related
In 100-1000 bp

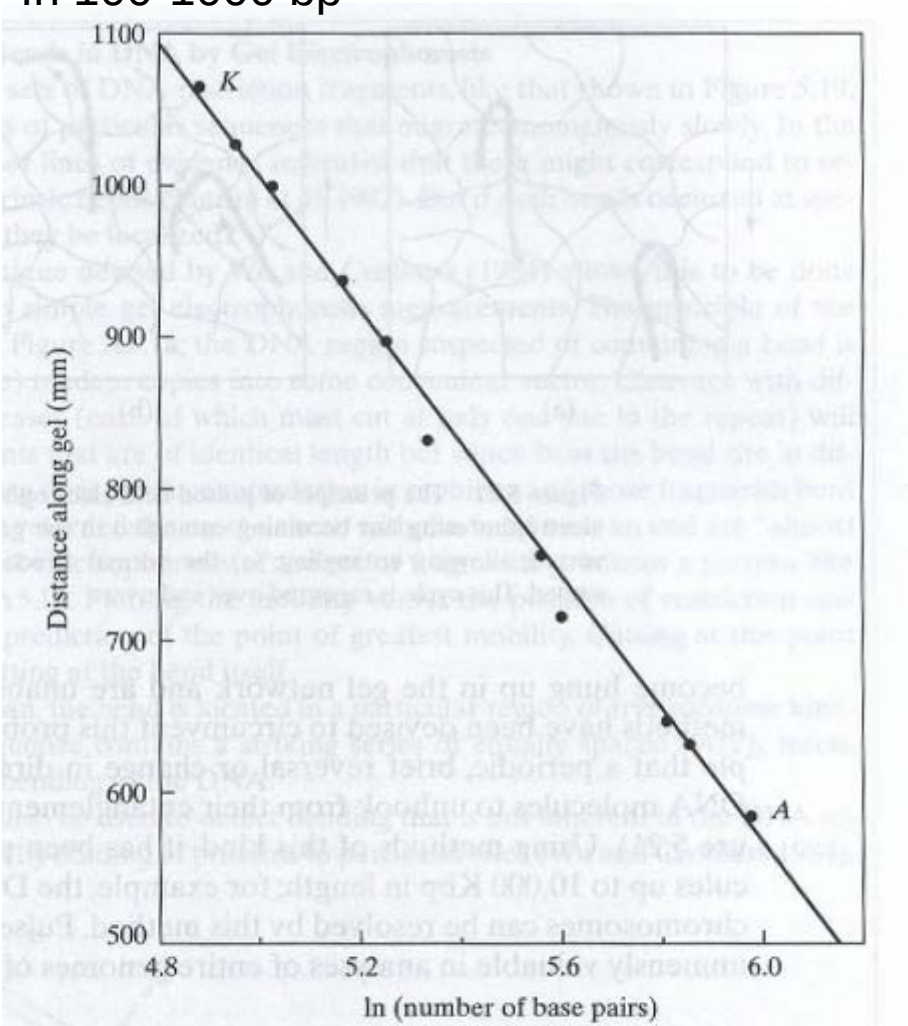


Figure 5.20 A graph of log M_i versus mobility for some of the fragments shown in Figure 5.19. The molecular weight, M, is expressed in base pairs; it is precisely known for each fragment. It will be noted that two fragments migrate anomolously. This is not unusual; there *are* sequence effects on mobilities, often associated with bending of specific sequences.

Pulsed Field Electrophoresis

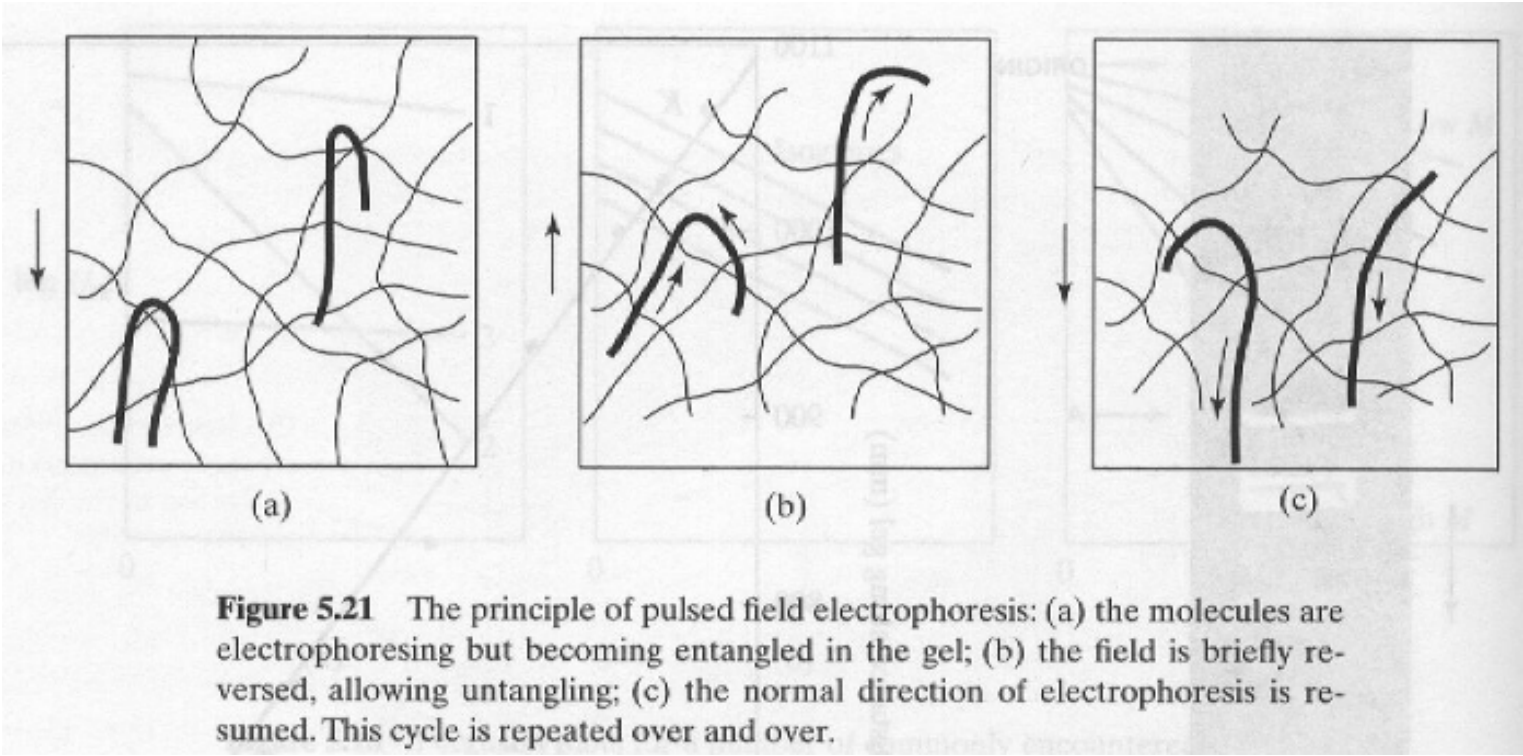


Figure 5.21 The principle of pulsed field electrophoresis: (a) the molecules are electrophoresing but becoming entangled in the gel; (b) the field is briefly reversed, allowing untangling; (c) the normal direction of electrophoresis is resumed. This cycle is repeated over and over.

Effects of DNA tertiary Structure on Electrophoretic Mobility

The migration of DNA in gel dep. on molecular dimensions

Use electrophoresis to measure DNA **bending** and **supercoiling**

Bent, short piece of DNA will migrate **more slowly** than a linear, rod-like DNA of the same overall length

A series of restriction sites are used to produced different permuted fragments, all of the same length, **relative mobilities** verse **cutting position** locates the site of bend

Bending does produce **retardation** in mobility, and those fragments bent near the middle will be retarded the most.

Locating Bends in DNA by Gel Electrophoresis

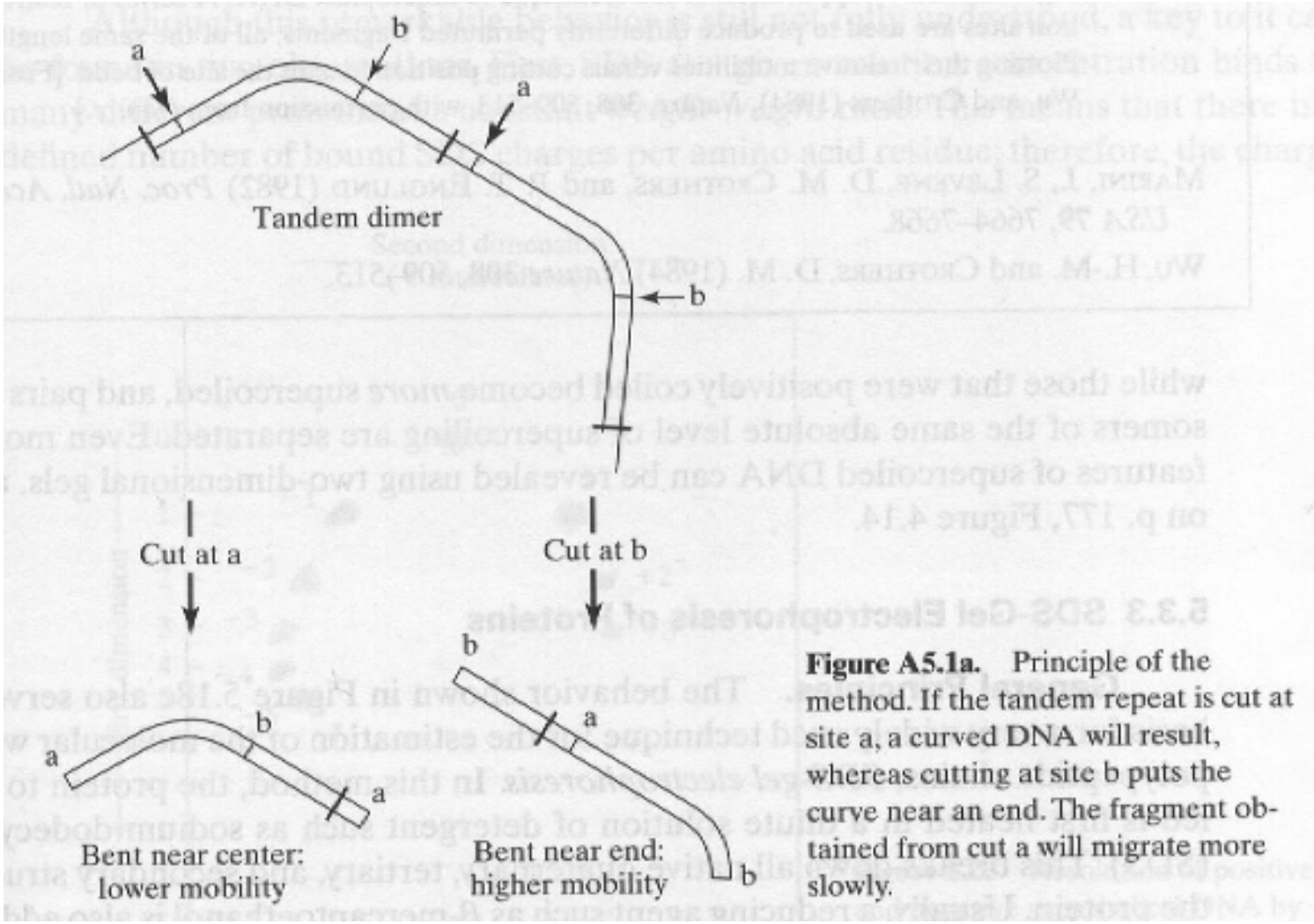


Figure A5.1a. Principle of the method. If the tandem repeat is cut at site a, a curved DNA will result, whereas cutting at site b puts the curve near an end. The fragment obtained from cut a will migrate more slowly.

Bending does produce **retardation** In mobility
Those fragments bent **near the middle** will be **retarded the most**

Kinetoplast DNA

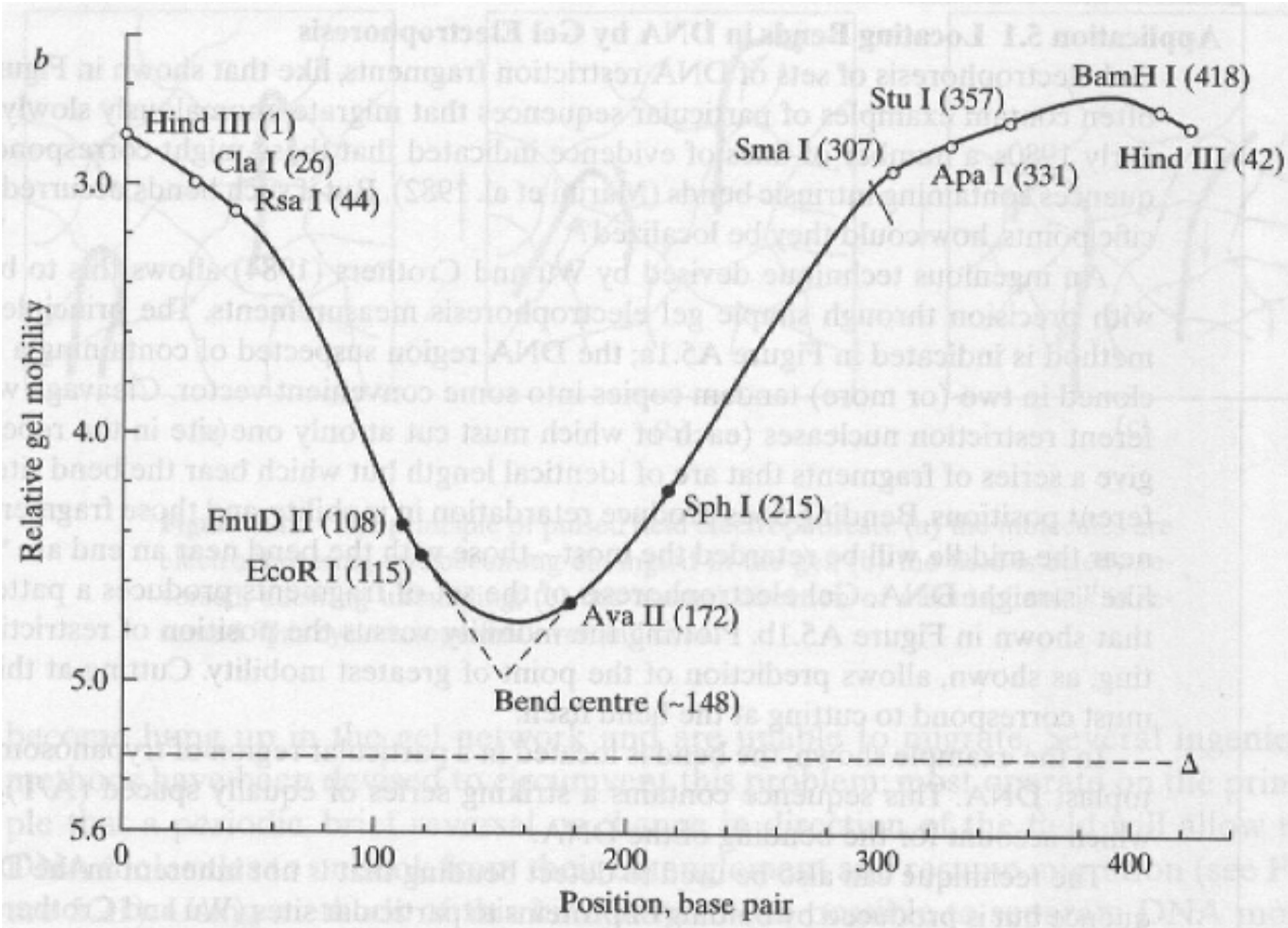


Figure A5.1b. Application of the technique to kinetoplast DNA. A series of restriction sites are used to produce differently permuted fragments, all of the same length. Plotting their relative mobilities versus cutting position locates the site of bend. [From Wu and Crothers (1984), *Nature*, **308**, 509–513, with permission from *Nature*.]

One dimensional gel-topisomerisomers

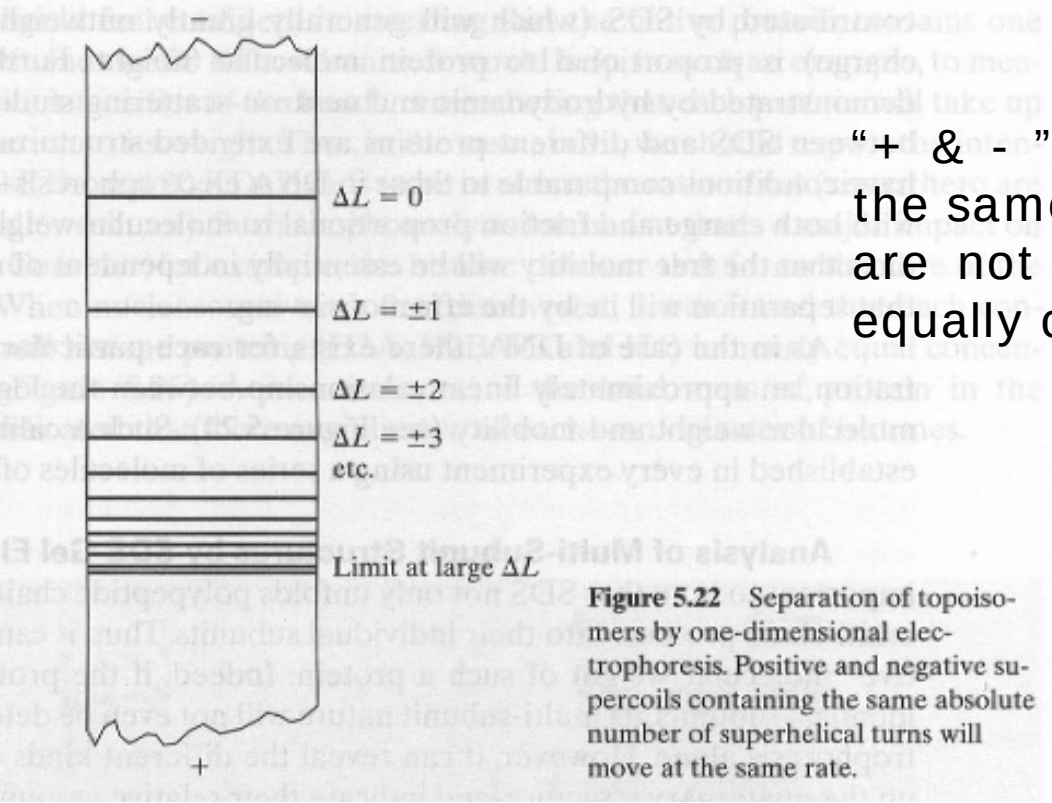


Figure 5.22 Separation of topoisomers by one-dimensional electrophoresis. Positive and negative supercoils containing the same absolute number of superhelical turns will move at the same rate.

"+ & - " topisomerisomers with the same degree of supercoiling are not separated for they are equally compact.

Two Dimensional Gel

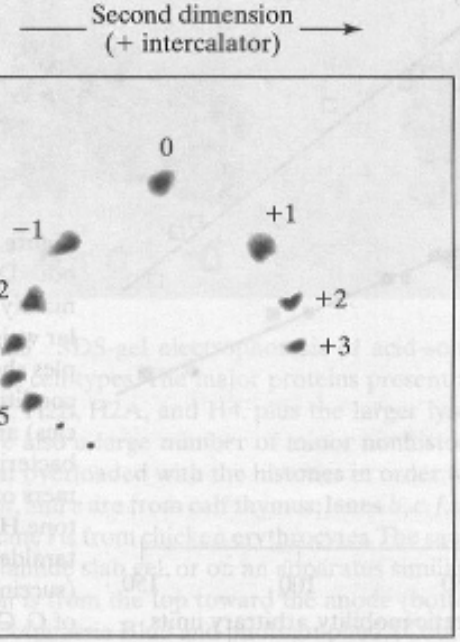


Figure 5.23 Resolution of positive and negative superhelical DNA by two-dimensional electrophoresis.

5.4.3 SDS-Gel Electrophoresis of Protein

Sodium dodecyl sulfate (SDS)
breaks down all native 4°, 3° and 2° structures in the protein

The charge contribute by SDS is α to the protein MW indep. on the charge

β-mercaptethanol / dithorthreitol (DDT)
reduce s-s bonds

stained by the dye Coomassie Blue

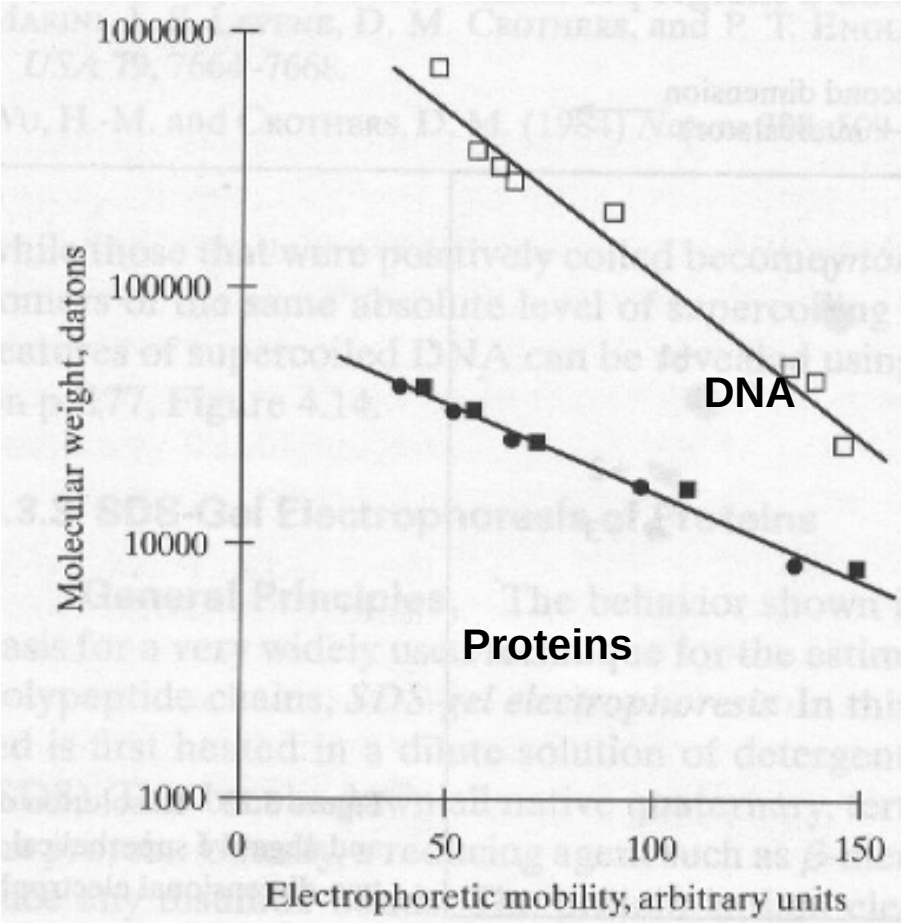


Figure 5.24 Demonstrating that both DNA and proteins give approximately linear graphs of log (molecular weight) versus mobility. The samples shown were run under identical conditions. DNA samples (open circles) are restriction fragments from a bacterial plasmid. Proteins are polymers of the globular domain of histone H5, crosslinked with either glutaraldehyde (squares) or dithiobis (succinimidyl propionate). [Courtesy of G. Carter.]

Nucleosomal Core Histones

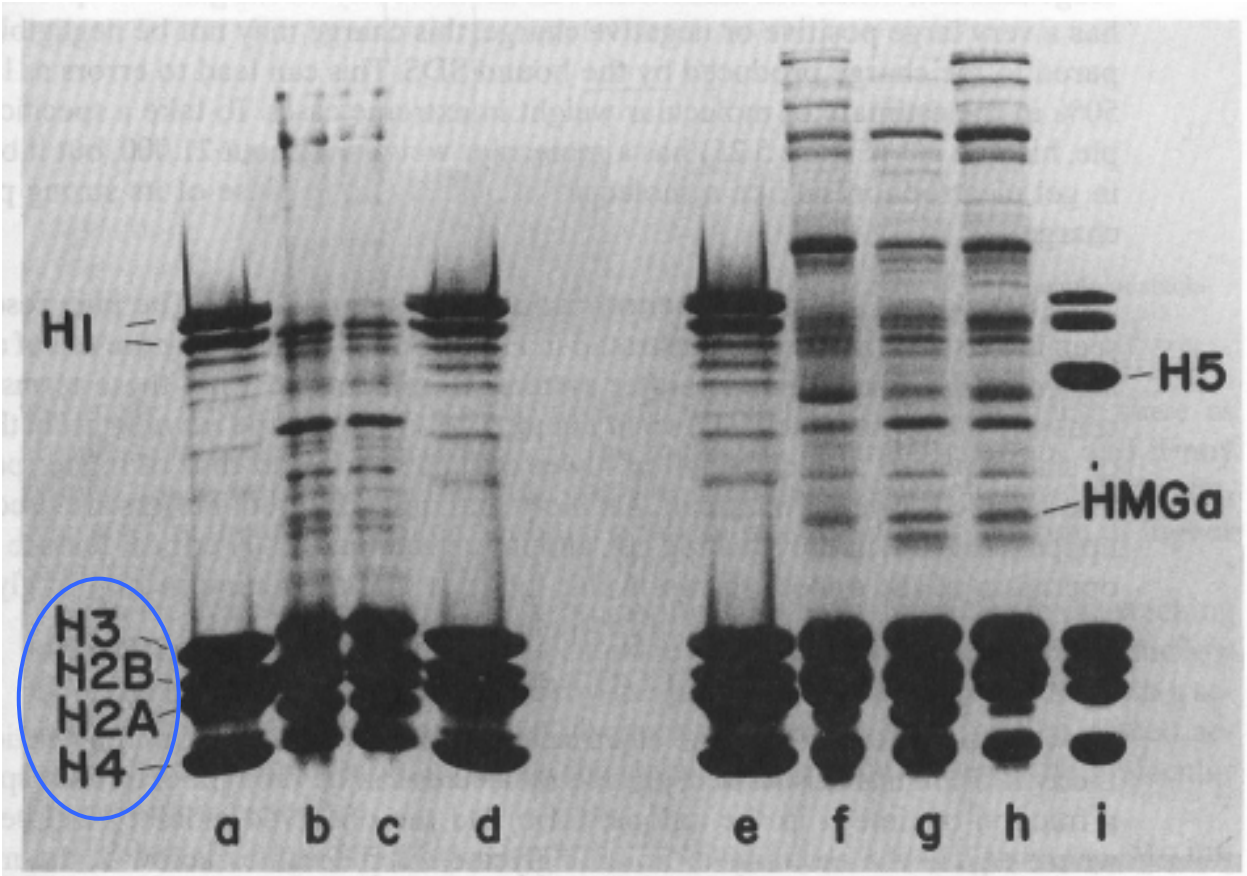


Figure 5.25 SDS-gel electrophoresis of acid-soluble nuclear proteins from a number of cell types. The major proteins present are the nucleosomal core histones, H3, H2B, H2A, and H4, plus the larger lysine-rich histones, H1 and H5. There are also a large number of minor nonhistone proteins. The gel has been somewhat overloaded with the histones in order to reveal these minor proteins. Lanes *a*, *d*, and *e* are from calf thymus; lanes *b*, *c*, *f*, *g*, and *h* are from various yeast strains. Lane *i* is from chicken erythrocytes. The samples were run on a 15 percent polyacrylamide slab gel, or on an apparatus similar to that shown in Figure 5.18. Migration is from the top toward the anode (bottom). The gel was stained with the dye Coomassie Blue and photographed. [Courtesy of Dr. J. Davie.]

Histone octamer that forms the core of the nucleosome

nucleosome containing 4 small histone proteins (H2A, H2B, H3 and H4)

Two of each - octamer of histone.

Stacking Gels and Discontinuous Buffer Systems

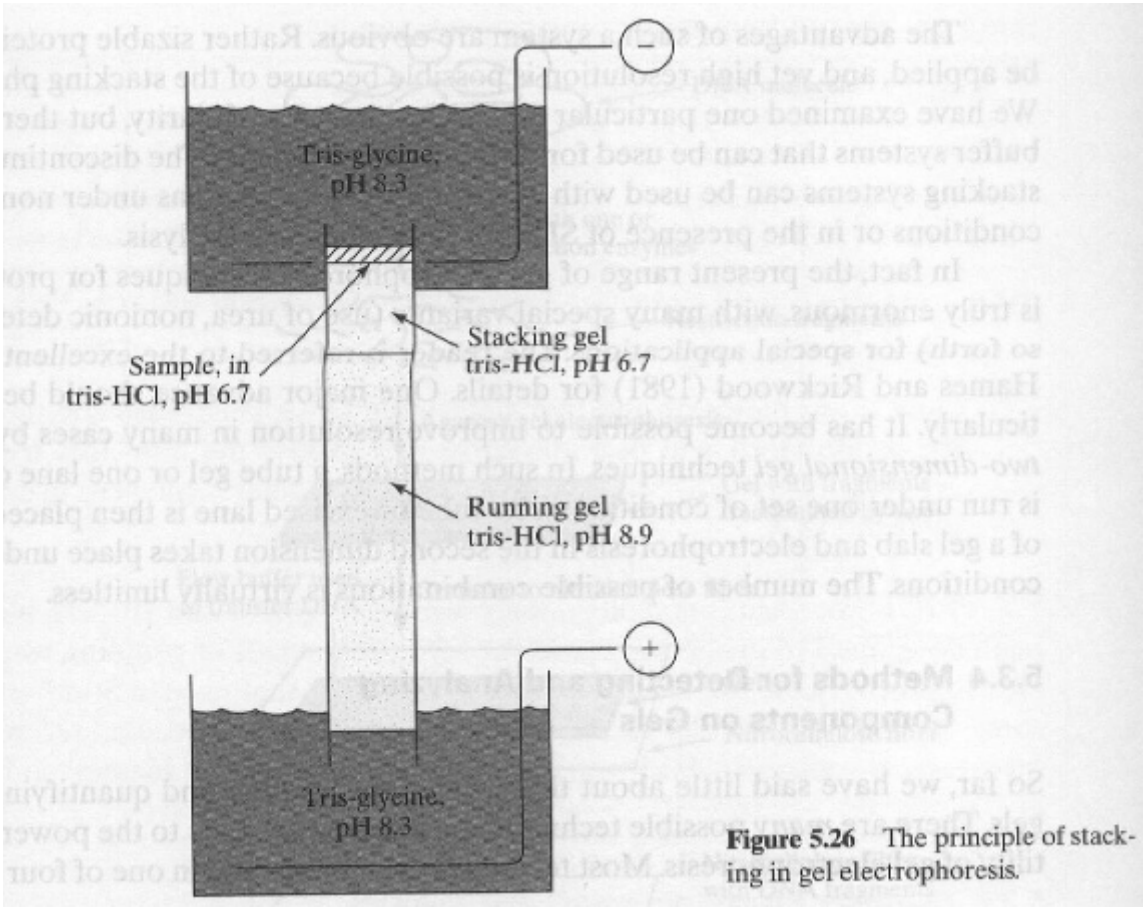


Figure 5.26 The principle of stacking in gel electrophoresis.

5.4.4 Methods for Detecting and Analyzing Components on Gel

Staining:
nucleic acids: ethidium bromide, fluorescence
Protein:dye coomassie blue /greater sensitivity, silver salt

Autoradiography
Photographic film

Direct radioactivity scanning
phosphoimagers

Blotting –Table 5.7
Southern blotting-DNA
Northern bloting-RNA
Western blotting-Proteins

Southern blotting

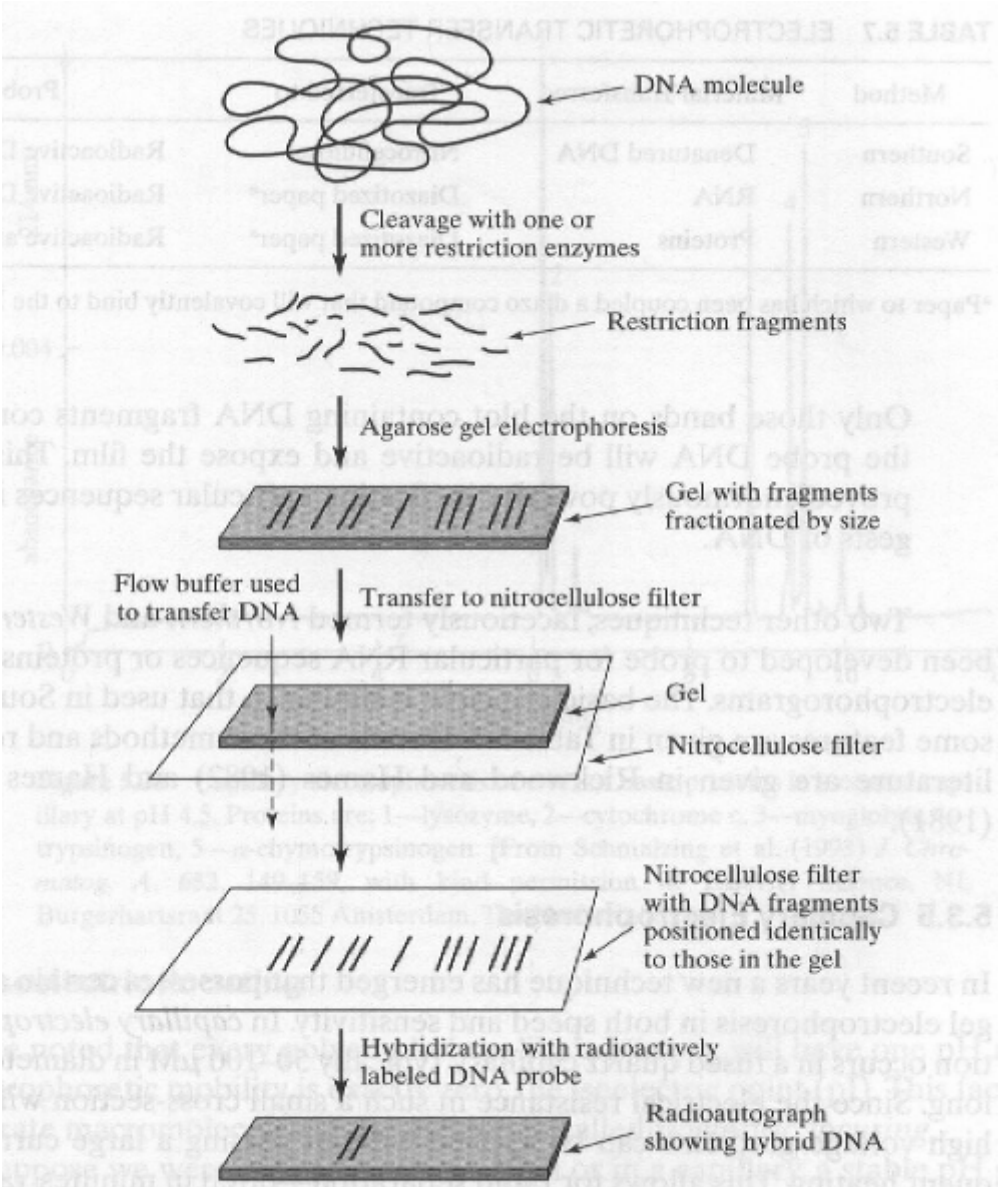


Figure 5.27 The principle of Southern blotting of DNA fragments. From Watson, *Molecular Biology of the Gene*, 4th ed. Copyright 1987 by J. D. Watson, reprinted by permission of Benjamin-Cummings Publishers.

5.4.5 Capillary Electrophoresis

quartz capillary

a small cross section, 50-100 uM in diameter and 20-60 cm long
high voltage

rapidly separation/in min

detection by UV/absorbance or Fluorescence

small sample volumes (nl)

extremely small ammount of material can be analyzed.
Femto mole (10^{-15} mol) – UV
Zepto mole (10^{-12} mol) – Fluorescence

5.4.5 Capillary Electrophoresis

Limitation:

Electroosmotic flow-because of the negatively charged groups on the walls of the capillary

protein may be absorbed or denatured by interaction with the surface

Coating the capillary by a polymeric material

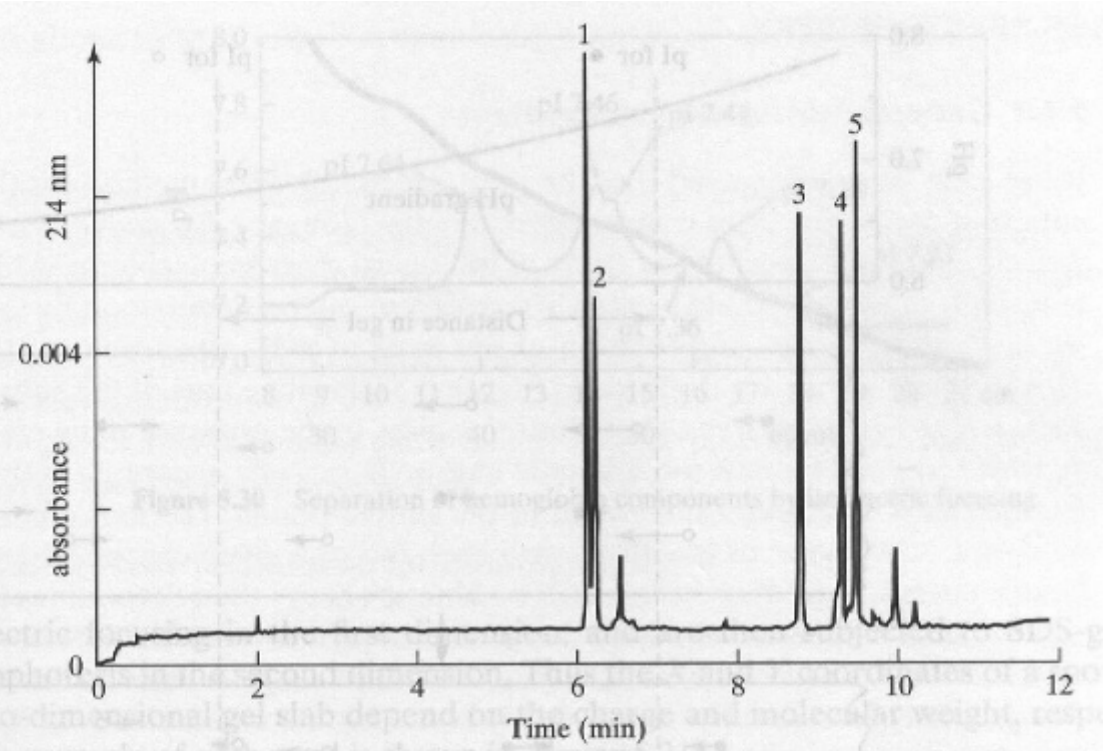


Figure 5.28 Capillary electrophoresis of a set of basic proteins in a coated capillary at pH 4.5. Proteins are: 1—lysozyme, 2—cytochrome c, 3—myoglobin, 4—trypsinogen, 5—α-chymotrypsinogen. [From Schmalzing et al. (1993) *J. Chromatog. A.* **652**, 149–159, with kind permission of Elsevier Science, NL Burgerhartsraat 25, 1055 Amsterdam, The Netherlands.]

5.4.6. Isoelectric Focusing

A stable pH gradient between the anode and cathode

molecule would migrate until each reached the point at which the pH equaled its PI

cathode become basic/ox. : $2H^+ + 2\ e^- \rightarrow H_2$

anode become acidic /red. : $H_2O \rightarrow 4\ H^+ + 4e^- + O_2$

5.4.6. Isoelectric Focusing (IEF)

pI smaller, more acidic, with more “-”, migrate to “+” anode

pI bigger, more basic, with more “+”, migrate to “-” cathode

2D-gel
isoelectric focusing & SDS gel electrophoresis
analytical or preparative purpose

Isoelectric Foucing

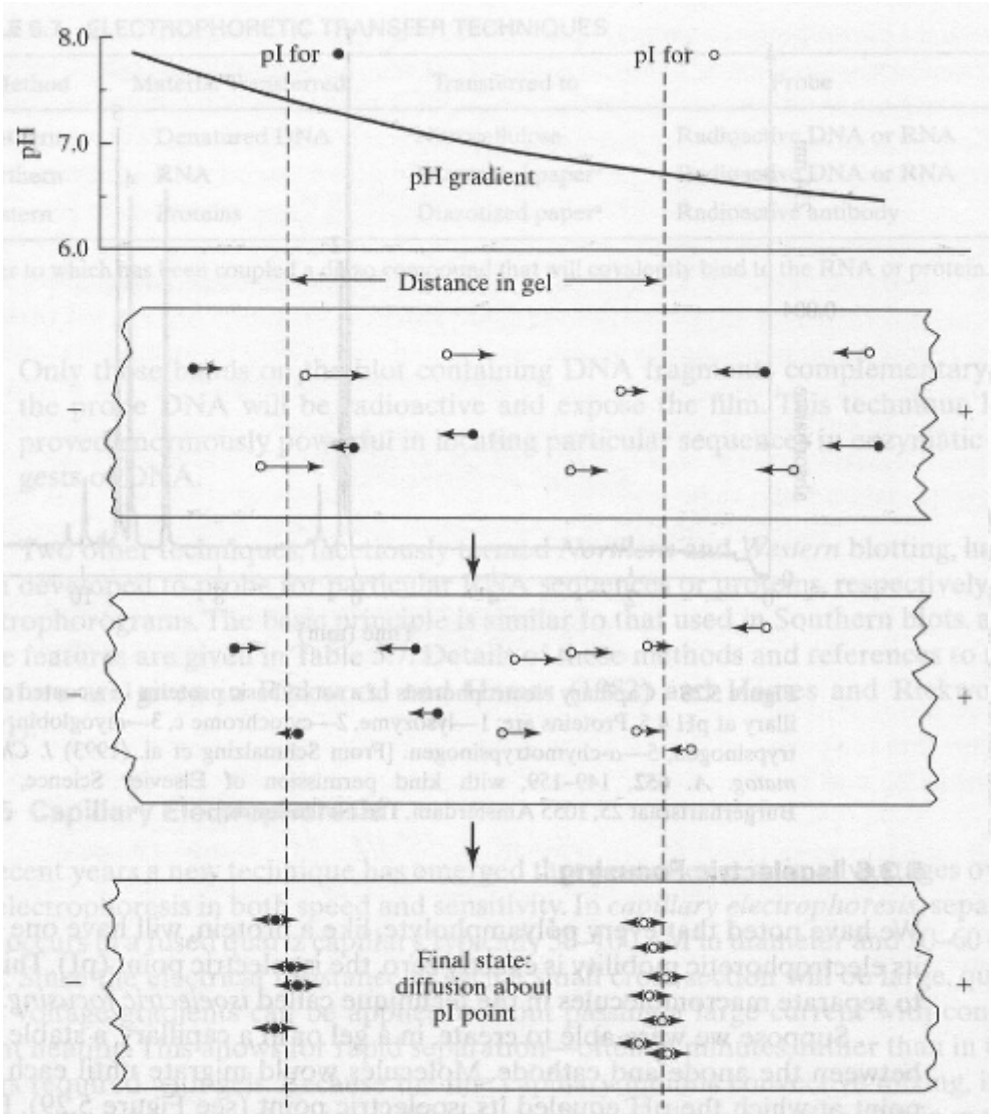


Figure 5.29 The principle of isoelectric focusing. If a stable pH gradient exists in a gel or capillary, each protein molecule will migrate toward the position where it is isoelectric. Here, two kinds of protein molecules (open and filled circles), initially uniformly distributed in the system, migrate toward their respective pI values.

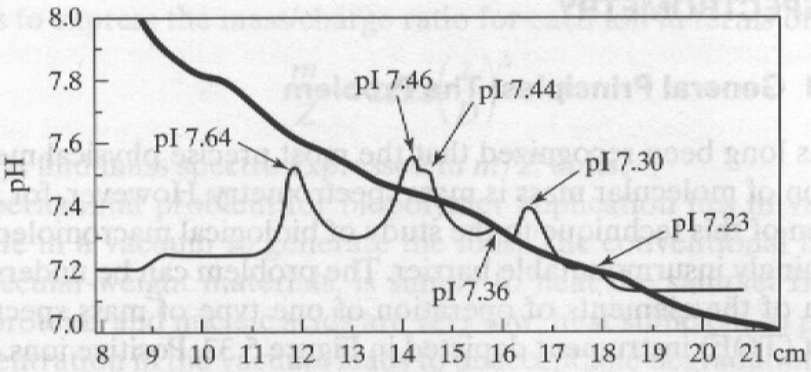


Figure 5.30 Separation of hemoglobin components by isoelectric focusing.

Two-dimensional gel

Isoelectric focusing & SDS gel electrophoresis

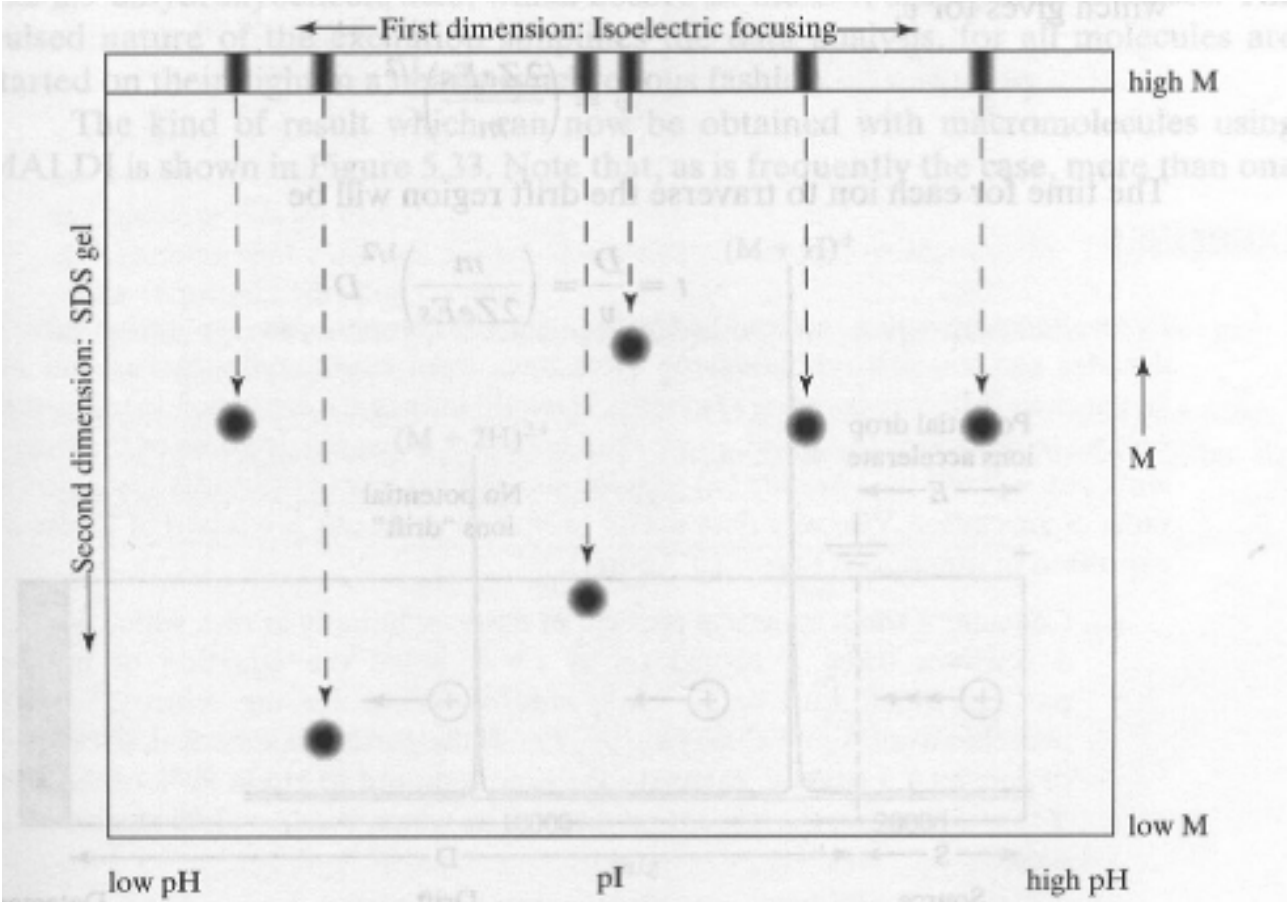


Figure 5.31 An O'Farrell gel, utilizing isoelectric focusing in one dimension and SDS-gel electrophoresis in the second.