## Centrifugation

**1. Principles of Centrifugation / theory and key equations**

$$F_s = m\omega^2 r = \frac{M}{N}\omega^2 r$$

where ω = angular velocity (radians / sec)

r = radius of particle from axis of rotation

note: ω (1/sec) = rpm x (2π rad / rev) x (1 min / 60 sec)

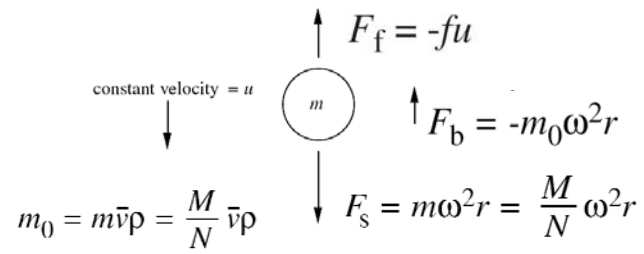
$$\text{RCF (Rel. Centrifugal Force)} = \frac{Fc}{Fg} = \frac{m\omega^2 r}{ma} = \frac{(2\pi\ \text{rpm}/60)^2 \times r}{980\ \text{cm/sec}^2}$$

$$= 1.119 \times 10^{-5}\ (\text{rpm})^2\ r$$

for r = 9.0 cm

| rpm | 1000 | 5000 | 10,000 | 20,000 | 40,000 |
|---|---|---|---|---|---|
| RCF | 100 | 2500 | 10,000 | 40,000 | 160,000 |

## Sedimentation of Particles in a Gravitational Field




$$F_s + F_b + F_f = 0$$

$$\frac{M}{N}\omega^2 r - \frac{M}{N}\bar{v}\rho\omega^2 r - fu = 0$$

$$\frac{M}{N}(1 - \bar{v}\rho)\omega^2 r - fu = 0$$

$$\frac{M(1 - \bar{v}\rho)}{Nf} = \frac{u}{\omega^2 r} \equiv s$$

**Table 1. Approximate Values of Partial Specific Volumes for Common Biological Macromolecules**

| Substance | $\bar{v}$ (mL/g) | |
|---|---|---|
| Proteins | 0.73 | (0.70-0.75) |
| Polysaccharides | 0.61 | (0.59-0.65) |
| RNA | 0.53 | (0.47-0.55) |
| DNA | 0.58 | (0.55-0.59) |

*Data from Beckman review article by* ***Greg Ralston.***

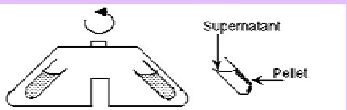
## Use of Centrifugation in Biochemistry

$$\frac{M(1 - \bar{v}\rho)}{Nf} = \frac{u}{\omega^2 r} \equiv s$$

1. **Preparative Centrifugation**
   - rotors
   - density gradient methods
     sucrose gradients / isopyncic methods (CsCl gradients)
2. **Analytical Ultracentrifugation**
   - instrument and **optic systems**
   - **sedimentation velocity** experiments
     sed. coefficient (s) (S = $10^{-13}$s)
   - **sedimentation equilibrium** exp.
     molecular weight
   - **diffusion constants** / $D = \frac{RT}{Nf}$

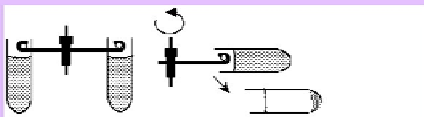
## Centrifuge Rotors

A. Fixed Angle Rotor




**Advantage:** Sedimenting particles have only short distance to travel before pelleting.
**Shorter run time.**
The most widely used rotor type.

B. Swinging Bucket Rotor

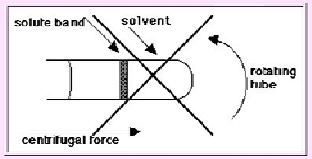


**Advantage:** Longer distance of travel may allow better separation
*eg* in density gradient centrifugation.
Easier to withdraw supernatant without disturbing pellet.

http://www.resonance-pub.com/centrifuge.htm

## 10.4 Density Gradient Centrifugation

In absence of a density gradient, separated **bands** of solute in the centrifuge are gravitationally unstable.




CAN'T OCCUR because layer of concentrated, dense solution overlaying less dense solvent would lead to mixing by convection and nullify the separation.
In absence of stabilising density gradient, can form **boundaries** *(cf electrophoresis 9.3)* but **not zones.** In **analytical ultracentrifuge**, moving boundaries and concentration distributions observed by optical device.

**Create DENSITY GRADIENT in tube**
Use a non-interacting, low M.Wt solute in continuously increasing concentration from meniscus to bottom of tube.

Important technique for purifying proteins and particularly nucleic acids.

**Two different types of density gradient** centrifugation, for two different purposes are.

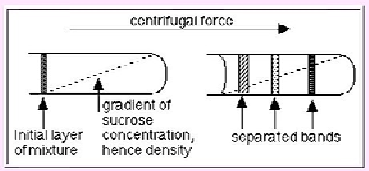
- **Zonal (or Rate Zonal) Centrifugation**
  (Sucrose density gradient centrifugation)
- **Isopycnic Centrifugation**
  (Caesium chloride density gradient centrifugation)

http://www.resonance-pub.com/centrifuge.htm

## Zonal Centrifugation

http://www.resonance-pub.com/centrifuge.htm

Mixture to be separated is layered on top of a
**SUCROSE, or FICOLL, GRADIENT**
(increasing concentration down the tube)
- provides gravitational stability as different species move down tube at different rates forming separate bands.




Species are separated by differences in **SEDIMENTATION COEFFICIENT (S)**

$$= \frac{\text{Rate of movement down tube}}{\text{Centrifugal force}}$$

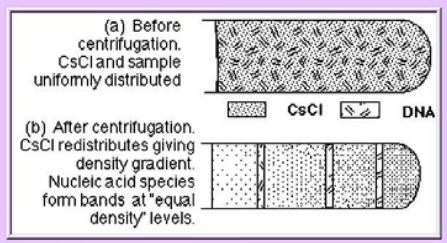
S is increased for particle of LARGER MASS
**(because sedimenting force** $\alpha M(1-\nu\rho)$

S is also increased for MORE COMPACT STRUCTURES of equal particle mass (frictional coefficient is less)

Mild, non-denaturing procedure, useful for protein purification, and for intact cells and organelles.

## Isopyncic Centrifugation

Molecules separated on EQUILIBRIUM POSITION, NOT by RATES of sedimentation.
Each molecule floats or sinks to position where density equals density of CsCl solution. Then no net sedimenting force on molecules.
**Isopycnic = Equal density**
and separation is on basis of DIFFERENT DENSITIES of the particles.




Very useful for purifying nucleic acid species of different density; also in separating proteoglycans extracted from cartilage.

http://www.resonance-pub.com/centrifuge.htm

## Analytical Ultracentrifuge: The sorts of questions for which answers are sought

(1) Is the sample homogeneous? Is it pure?
(2) If there is a single component, what is the molecular weight?
(3) If more than one type present, can the molecular weight distribution of the sample be obtained?
(4) Can an estimate be obtained of the size and shape of the particles? Are the molecules compact and spherical (globular) or long and thin (rod-like)?
(5) Can the macromolecules be distinguished on the basis of density?
(6) Can interactions between solute molecules be detected? Aggregation between molecules changes molecular weight, changes in molecular weight as a function of the concentrations of the components can illuminate the type of reaction (*e.g.*, reversible or nonreversible?), the stoichiometry, and the strength of binding.
(7) Can changes in conformation or shape of the particles be measured?

## Molecular Weight Determination

**Light scattering / Centrifugation / Osmometry / X-ray diffraction**

**Electrophoresis and chromatographic** methods are popular for rapid estimation of molecular weights of proteins and nucleic acids. However, such methods, though rapid and sensitive, have **no rigorous theoretical base**; **they are empirical techniques** that **require calibration and assumptions** that may be invalid.

The **analytical ultracentrifuge** enables the **direct measurement of molecular weights** of solutes in the **native state** and as they exist in solution, **without calibrations or assumptions concerning shape**. The method is applicable to molecules with molecular weights ranging from several hundreds (sucrose) up to many millions (virus particles).

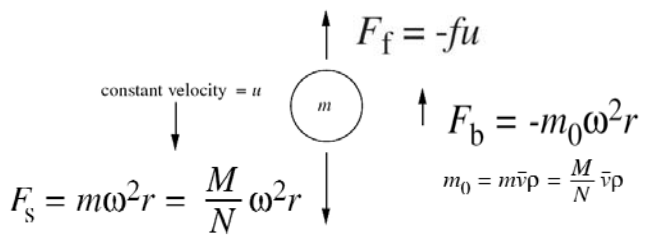
**Sedimentation equilibrium** methods require only **small sample sizes (20-120 µL)** and **low concentrations (0.01-1 g/L).**

## Conformational Changes

**X-ray diffraction and NMR techniques are currently the only techniques available that are capable of providing structural details at atomic resolution.**

**Nevertheless, the overall size and shape of a macromolecule or complex in solution can be obtained through measurement of the rate of movement of the particles through the solution. Sedimentation velocity experiments in the analytical ultracentrifuge provide *sedimentation and diffusion coefficients* that contain information concerning the size and shape of macromolecules and the interactions between them. Sedimentation coefficients are particularly useful for monitoring *changes* in conformation in proteins.**

## Sedimentation of Particles in a Gravitational Field




$$\frac{M(1 - \bar{v}\rho)}{Nf} = \frac{u}{\omega^2 r} \equiv s$$

$$D = \frac{RT}{Nf}$$

$$M = \frac{s^0 RT}{D^0(1 - \bar{v}\rho)}$$

## Centrifugation: Terms and Units

**Force:** mass x acceleration ($F = ma = m\omega r^2$)

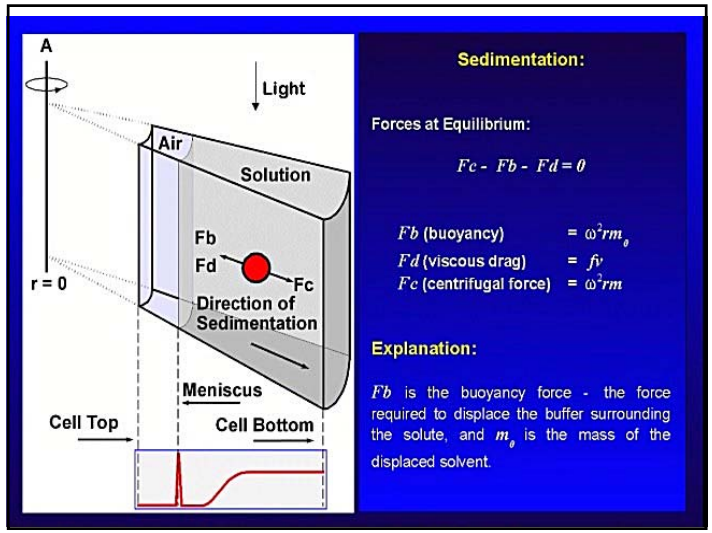
**Energy:** force x distance

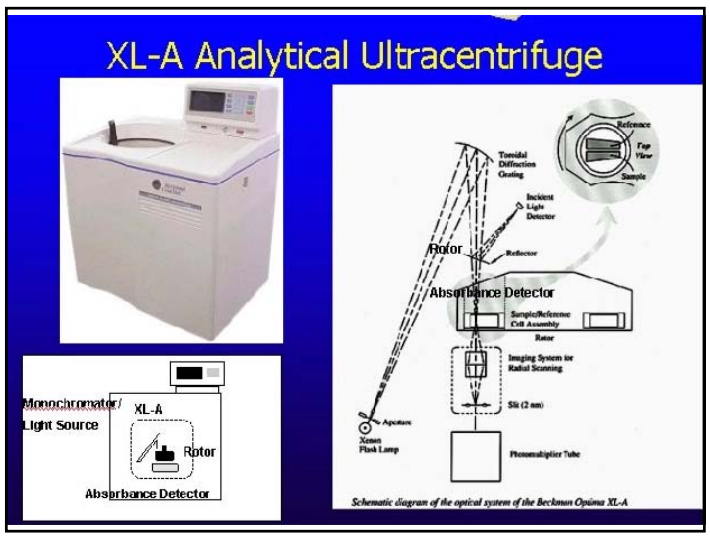
**Viscosity:** $\eta$ (~0.01 g (cm-sec)

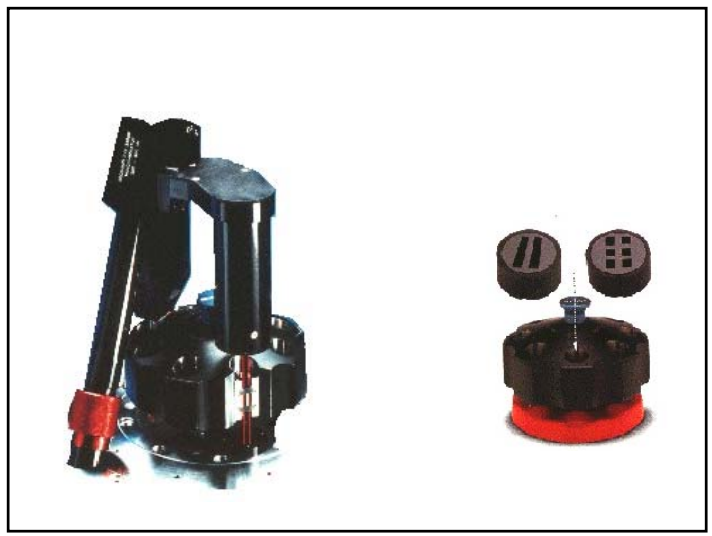
**Frictional Coefficient:** $f = 6 \pi \eta R$ (~ $10^{-8}$ g/sec)

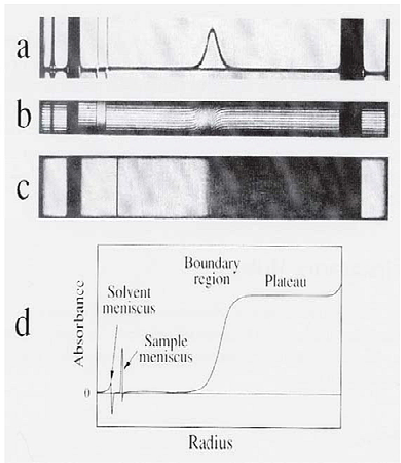
**Sedimentation Coefficient:** s (sec) [ $1S = 10^{-13}$ s]

**Diffusion Constant:** $D = \frac{RT}{Nf}$ ($cm^2/s$)



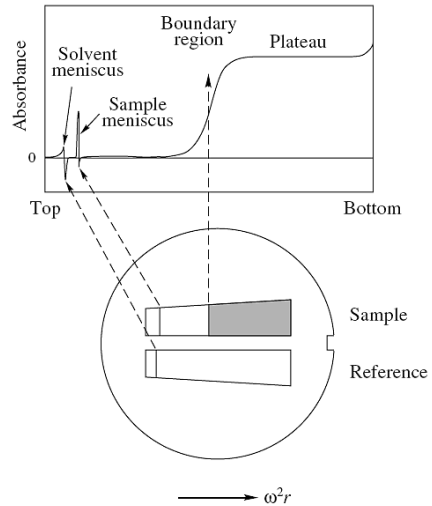



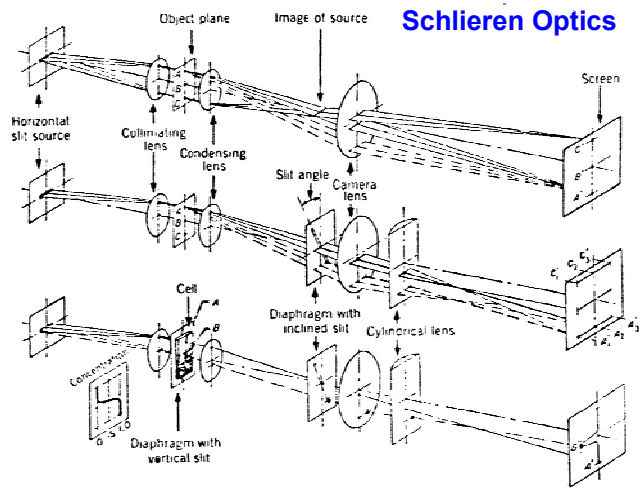


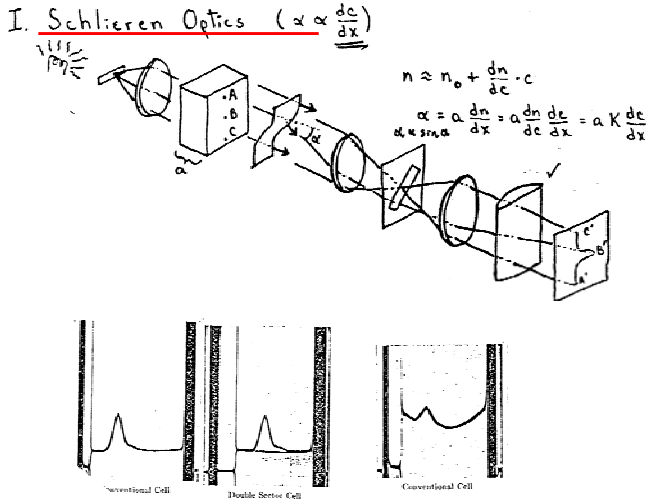

**Schlieren Optics:** peak at max. in conc. gradient

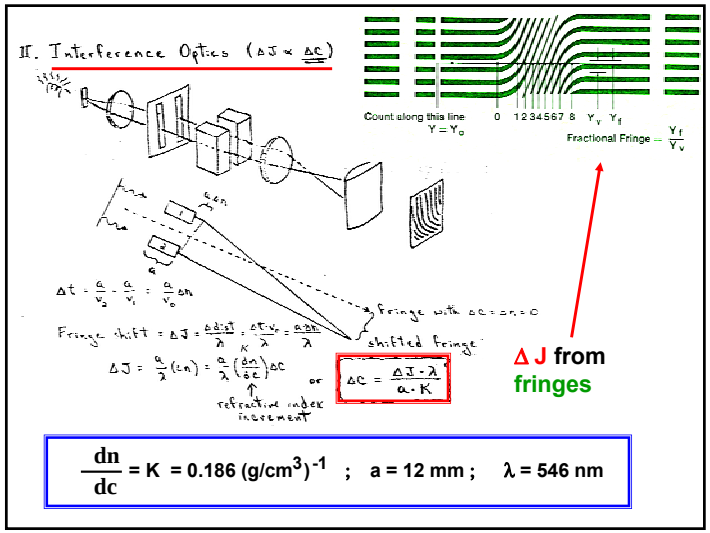
**Interference Optics:** fringe shift proportional to concentration

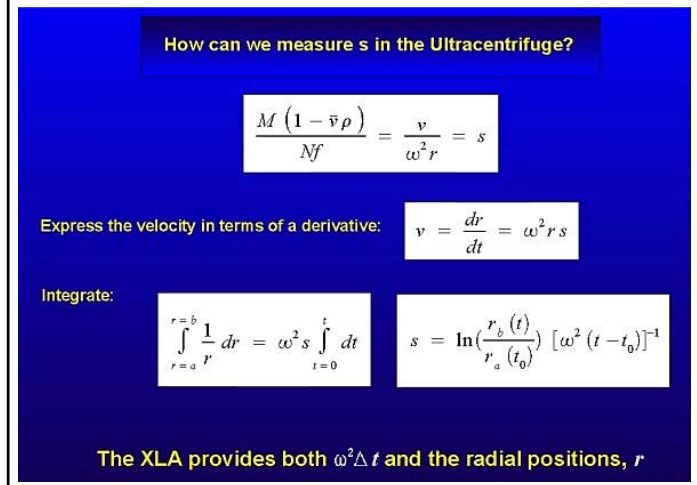
**Absorption Optics:** optical density dependent of extinction coeff. and concentration

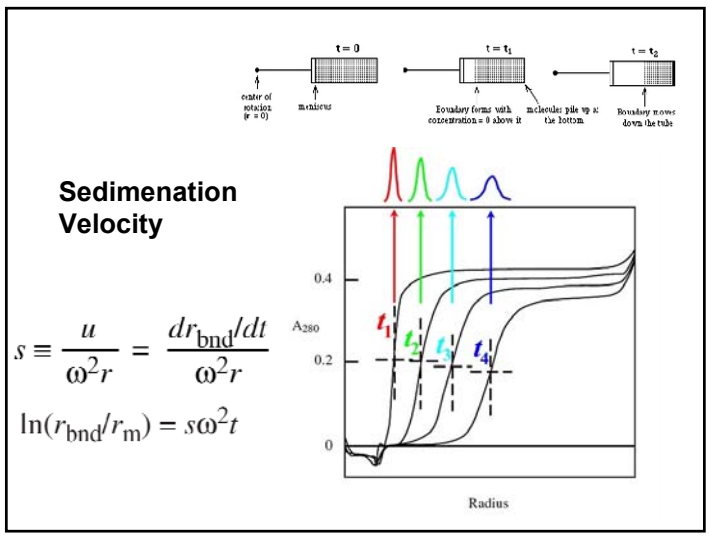


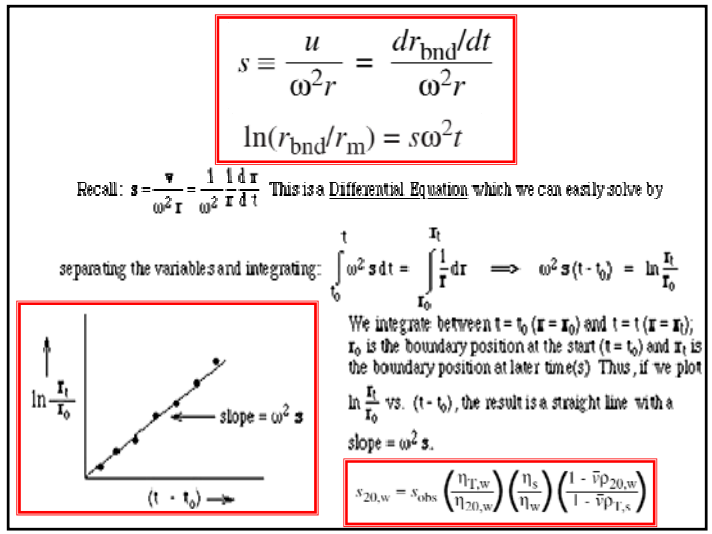




II. Interference Optics (ΔJ ∝ Δc)
Count along this line Y = Y₀
0 1 2 3 4 5 6 7 8 Yv Yf
Fractional Fringe = Yf/Yv
Δt = a/v₂ − a/v₁ = a/v₀ Δn
Fringe shift = ΔJ = a·Δn/λ = a·Δc·K/λ
ΔJ = a/λ (Δn) = a/λ (∂n/∂c) Δc
or
Δc = ΔJ·λ / a·K
refractive index increment
fringe with Δc = Δn = 0
shifted fringe
ΔJ from fringes
dn/dc = K = 0.186 (g/cm³)⁻¹ ; a = 12 mm ; λ = 546 nm


How can we measure s in the Ultracentrifuge?
M(1 − v̄ρ) / Nf = v/ω²r = s
Express the velocity in terms of a derivative:
v = dr/dt = ω²rs
Integrate:
∫ from r=a to r=b (1/r) dr = ω²s ∫ from t=0 to t dt
s = ln(r_b(t)/r_a(t_0)) [ω²(t − t_0)]⁻¹
The XLA provides both ω²Δt and the radial positions, r


t = 0
t = t₁
t = t₂
center of rotation (r = 0)
meniscus
Boundary forms with concentration = 0 above it
molecules pile up at the bottom
Boundary moves down the tube
Sedimenation Velocity
s ≡ u/ω²r = dr_bnd/dt / ω²r
ln(r_bnd/r_m) = sω²t
A280
0.4
0.2
0
t1
t2
t3
t4
Radius


s ≡ u/ω²r = dr_bnd/dt / ω²r
ln(r_bnd/r_m) = sω²t
Recall: s = v/ω²r = 1/ω² · 1/r · dr/dt This is a Differential Equation which we can easily solve by
separating the variables and integrating: ∫ from t0 to t ω² s dt = ∫ from r0 to rt (1/r) dr ⟹ ω² s (t − t0) = ln rt/r0
We integrate between t = t0 (r = r0) and t = t (r = rt); r0 is the boundary position at the start (t = t0) and rt is the boundary position at later time(s) Thus, if we plot ln rt/r0 vs. (t − t0), the result is a straight line with a slope = ω² s.
ln rt/r0
slope = ω² s
(t − t0)
s20,w = sobs (ηT,w/η20,w) (ηs/ηw) (1 − v̄ρ20,w)/(1 − v̄ρT,s)

## Flow in the Ultracentrifuge Cell:

**Sedimentation:** $\frac{M\left(1-\bar{v}\rho\right)}{Nf} = \frac{v}{\omega^2 r} = s$

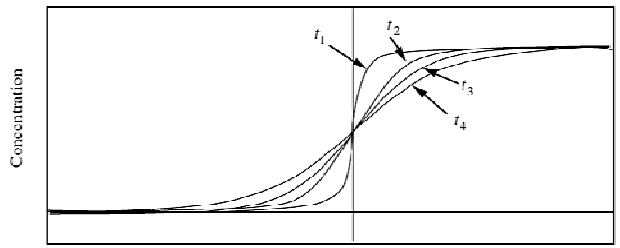
**Diffusion:** $D = \frac{R\,T}{N\,f}$

**Molecular Weight:** $\frac{s}{D} = \frac{M\,(1-\bar{v}\rho)}{RT}$

The ratio of s/D is proportional to the Molecular weight

$$f = 6\pi\eta R \qquad D = \frac{RT}{Nf}$$

$$M = \frac{s^0 RT}{D^0(1 - \bar{v}\rho)}$$



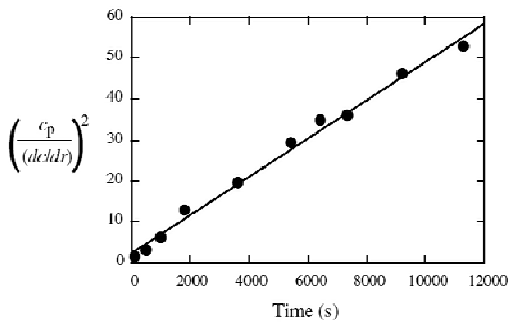




***Figure 13.*** *Determination of the diffusion coefficient. The spreading of an initially sharp boundary of human spectrin was followed with time. The slope of the plot of $[c_p/(dc/dr)]^2$ versus time is $4\pi$ times the diffusion coefficient.*

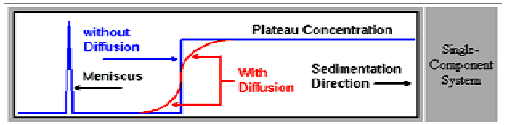



**Figure 1**: A single-component system shown without diffusion (in blue) and with diffusion (in red). The boundary spreads due to diffusion and gives a sigmoidal shape to the boundary.

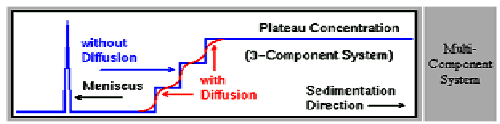
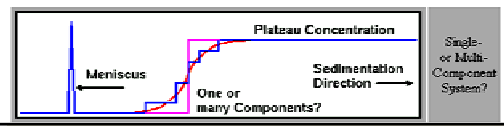
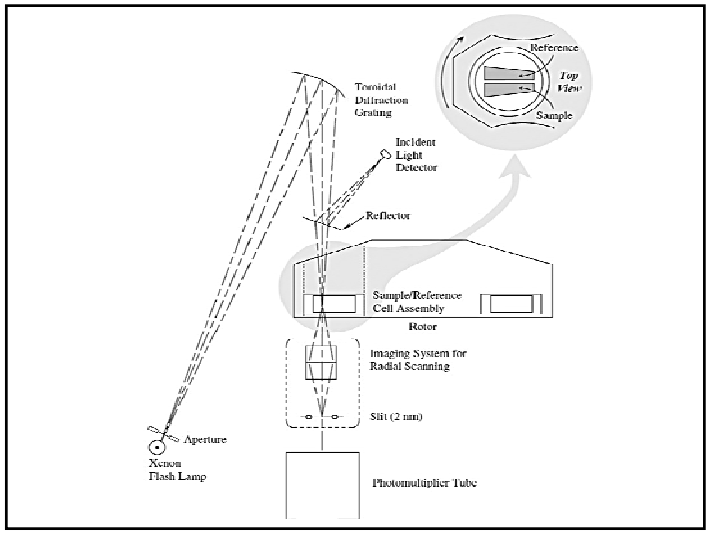
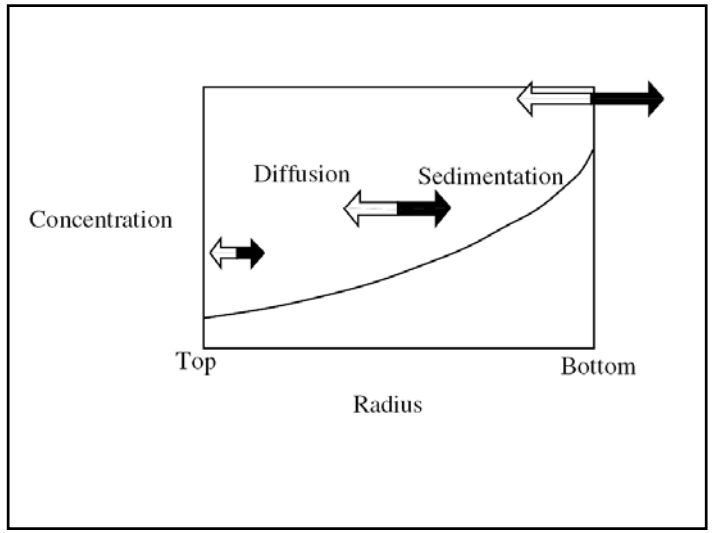



**Figure 2**: A multi-component system shown without diffusion (in blue) and with diffusion (in red). The step functions defining the boundary profiles of each component can lose definition as diffusion increases and overlays on top of sedimentation separation.





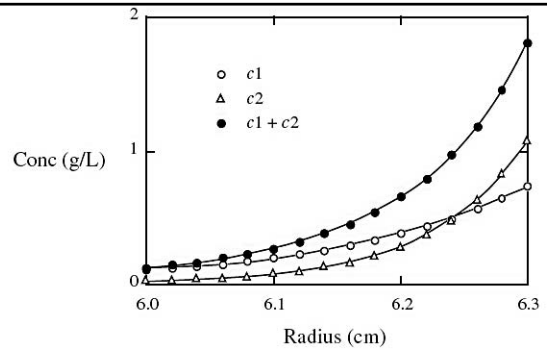




***Figure 16.*** *Sedimentation equilibrium distribution of two different solutes. Data were simulated for two species: (o) $M_r$ = 40,000; (Δ) $M_r$ = 80,000. The angular velocity was 15,000 rpm, and a partial specific volume of 0.73 was assigned to both species. The distribution of total solute concentration in the cell is also shown (●).*

## Sedimentation Equilibrium

At Equilibrium, the total flow is zero, and diffusion and sedimentation exactly balance out:

$$J = s\omega^2 rC - D\frac{\partial C}{\partial r} = 0$$

Therefore:

$$s\omega^2 rC = D\frac{\partial C}{\partial r}$$

Since:

$$\frac{s}{D} = \frac{M(1-\bar{v}\rho)}{RT}$$

$$\frac{\partial C}{C} = \frac{M\omega^2 r(1-\bar{v}\rho)}{RT}\partial r$$

## Sedimentation Equilibrium

$$\int_{C_0}^{C_r} \frac{1}{C}\,dC = \frac{M\omega^2(1-\bar{v}\rho)}{RT}\int_{r_0}^{r_s} r\,dr$$

After Integration:

$$C = C_0 \exp^{\frac{M\omega^2(1-\bar{v}\rho)}{RT}(r^2-r_0^2)} + Baseline$$

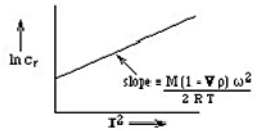
The equilibrium gradient is dependent on rotor speed, temperature, an on the molecular weight and buoyancy of the solute. This equation can be fitted by nonlinear least squares.

$$\omega^2 r\, c\, \frac{M(1-\bar{\nu}\rho)}{N_0 f} = \frac{kT}{f}\left(\frac{dc}{dr}\right) \Longrightarrow \omega^2 r\, c\, M(1-\bar{\nu}\rho) = N_0 kT\left(\frac{dc}{dr}\right)$$

Separate the variables and integrate the differential equation over $r_0$ to $r$ and from $c_0$ to $c_r$:

$$M(1-\bar{\nu}\rho)\omega^2 \int_{r_0}^{r} r\,dr = RT \int_{c_0}^{c_r} \frac{1}{c}\,dc \Longrightarrow M(1-\bar{\nu}\rho)\omega^2 \tfrac{1}{2}(r^2-r_0^2) = RT \ln\frac{c_r}{c_0}$$

$$\Longrightarrow \ln c_r = \frac{M(1-\bar{\nu}\rho)\omega^2}{2RT} r^2 - \frac{M(1-\bar{\nu}\rho)\omega^2 r_0^2}{2RT} + \ln c_0$$




This equation has the form of the equation of a straight line, $y = m\,x + b$, where $\ln c_r = y$ and $r^2 = x$.
If we plot $\ln c_r$ vs. $r^2$, the result should be a straight line with a slope $= \frac{M(1-\bar{\nu}\rho)\omega^2}{2RT}$. Concentration, c, can be measured at each radius, r, using optical systems built into analytical ultracentrifuges.