Molecular Weight Determination

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.

Centrifugation / Light scattering / Mass Spec / X-ray diffraction / Osmometry

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).**

Centrifugation - Goals for this unit:

1. **Understand essential theoretical concepts of movement of a particle under a centrifugal force.** Terms & Units

$$F_s + F_b + F_f = 0$$

2. **Know differences between "preparative" and "analytical" types of centrifugation.** RCF = Relative Centrifugal Force

3. **Analytical Centrifugation**

Instrument: Optic systems - general principles / interpretation

Schlieren / Interference / Absorption optics

Common Applications (transport vs. equilibrium experiments)

Sedimentation Coefficient - "s" vs. "S"

Diffusion Coefficient D = RT/Nf

Frictional Coefficient / frictional coeff. ratio $f = 6\pi\eta R$

Sedimentation Equilibrium

Centrifugation: Terms and Units

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

($g\ cm\ /\ sec^2$)

Energy: force x distance Joule = $Kg\ m^2/sec^2$

($R = 8.314 \times 10^7\ g\text{-}cm^2/(sec^2\text{-}mol\text{-}K)$) erg = $g\ cm^2/sec^2$

Partial specific volume $\bar{v}$ (cm^3/g)

Viscosity: η (~0.01 g /(cm-sec))

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

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

Diffusion Constant: $D = \frac{RT}{Nf}$ (~ 10^{-7} cm^2/s)

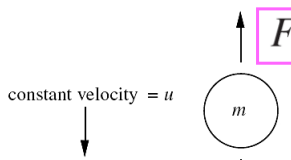
Refractive Index : $n = c/v$

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.***

Sedimentation of Particles in a Gravitational Field



$F_f = -fu$

$F_b = -m_0\omega^2 r$

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

$m_0 = m\bar{v}\rho = \frac{M}{N}\bar{v}\rho$

$F_s + F_b + F_f = 0$

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

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

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

Molecular *Experimental*

Preparative 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

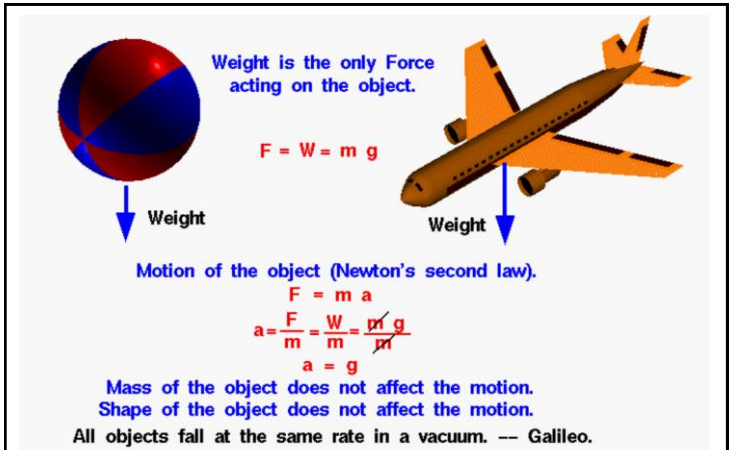
note: ω (1/sec) = rpm x (2π rad / rev) x (1 min / 60 sec)

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

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

for r = 9.0 cm

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



Falling objects in vacuum: $\text{vel} = \text{vel}_o + \text{“g” x time}$

$\text{dist} = \text{vel}_o \text{ x time} + 0.5 \text{ x “g” x time}^2$

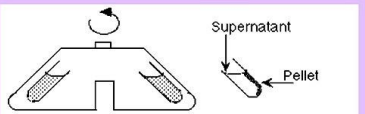
Use of Centrifugation in Biochemistry

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

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

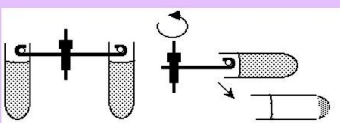
Centrifuge Rotors

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

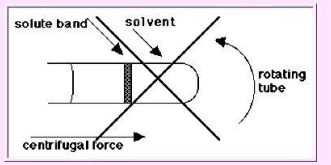


Advantage: Longer distance of travel may allow better separation *eg* in density gradient centrifugation.
Easier to withdraw supernatant without disturbing pellet.

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.



CANT 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:

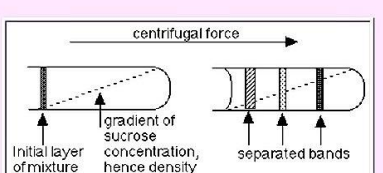
- **Zonal (or Rate Zonal) Centrifugation**
  (Sucrose density gradient centrifugation)
- **Isopycnic Centrifugation**
  (Caesium chloride density gradient centrifugation)

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)**

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

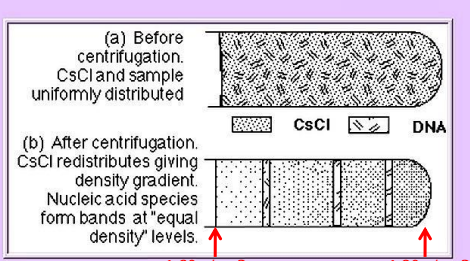
S is increased for particle of LARGER MASS
(because sedimenting force $\propto M(1-v\rho)$

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)?

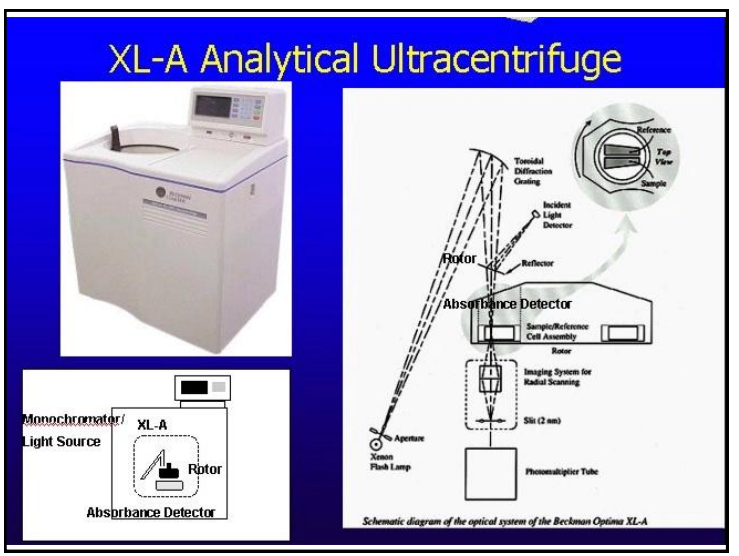
(5) Can interactions between solute molecules be detected?

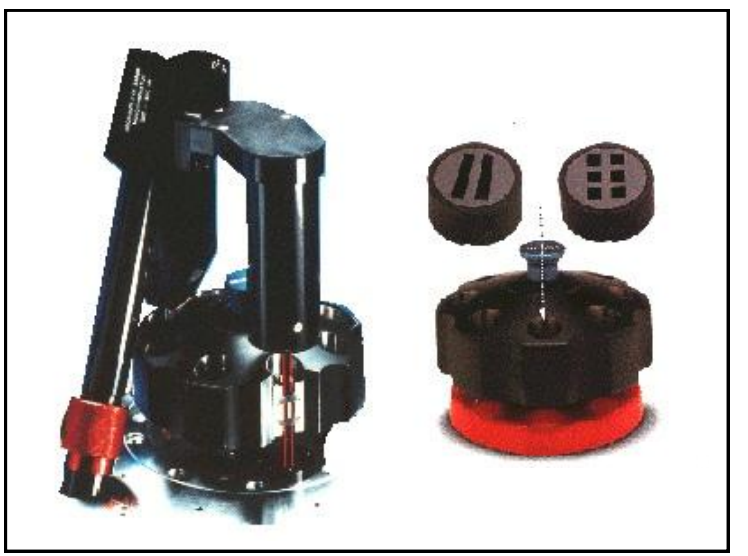
(6) Can changes in conformation or shape of the particles be measured?

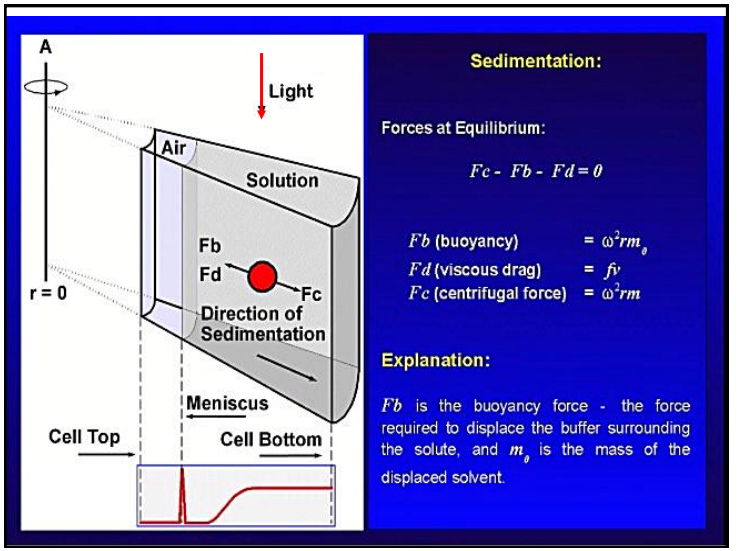
Use of Centrifugation in Biochemistry

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

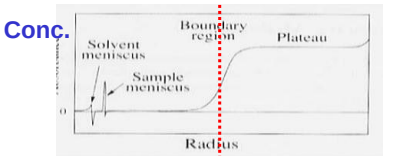
1. **Preparative Centrifugation**
   - **rotors**
   - **density gradient methods**
     sucrose gradients / isopyncic methods (CsCl gradients)
2. **Analytical Ultracentrifugation**
   - instrument and **optic systems**
   - **sedimentation velocity** experiments
     sed. coefficient (s) ($S = 10^{-13}$s)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





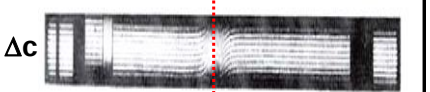


Optic systems:

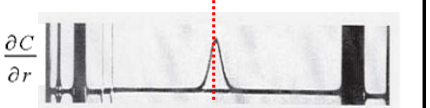


Absorption Optics: optical density dependent of extinction coeff. and concentration

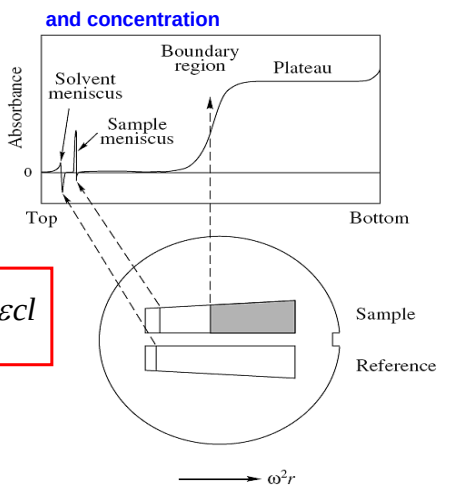
Interference Optics: fringe shift proportional to concentration



Schlieren Optics: peak at max. in conc. gradient

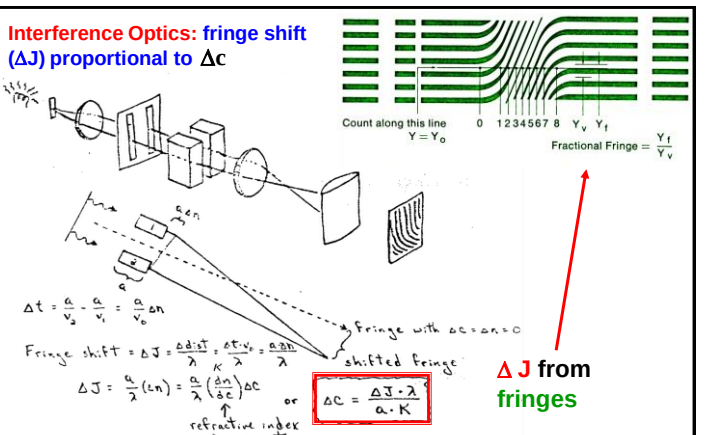


Absorption Optics: optical density dependent of extinction coeff. and concentration



$$A = \log \frac{I_0}{I_t} = \varepsilon c l$$

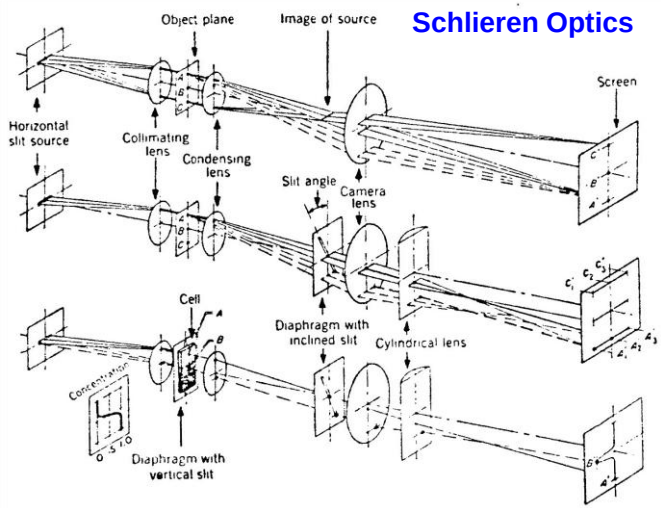
Interference Optics: fringe shift (ΔJ) proportional to Δc



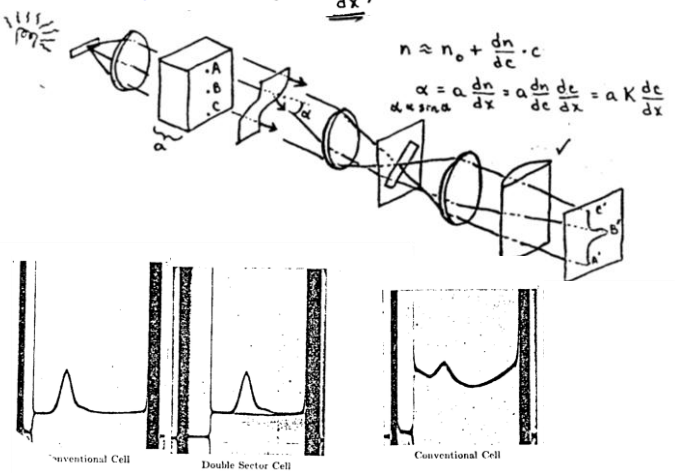
ΔJ from fringes

$$\frac{dn}{dc} = K = 0.186\ (g/cm^3)^{-1} \quad ; \quad a = 12\ mm\ ; \quad \lambda = 546\ nm$$

Schlieren Optics



Schlieren Optics: peak at max. in conc. gradient $\left(\alpha \propto \frac{dc}{dx}\right)$



How can we measure s in the Ultracentrifuge?

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

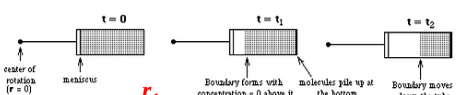
Express the velocity in terms of a derivative: $v = \frac{dr}{dt} = \omega^2 r s$

Integrate:

$$\int_{r=a}^{r=b} \frac{1}{r}\, dr = \omega^2 s \int_{t=0}^{t} dt \qquad s = \ln\left(\frac{r_b(t)}{r_a(t_0)}\right)\left[\omega^2 (t - t_0)\right]^{-1}$$

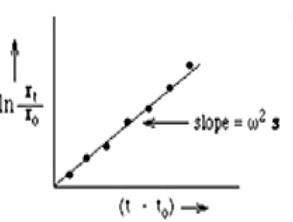
The XLA provides both $\omega^2 \Delta t$ and the radial positions, r

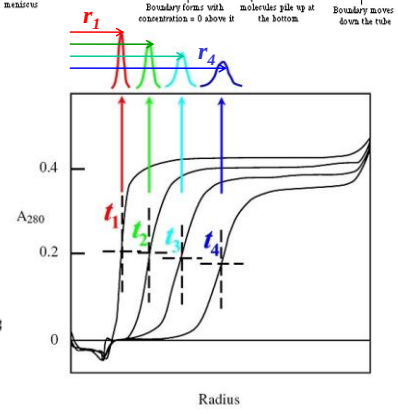
Sedimentation Velocity



$$s \equiv \frac{u}{\omega^2 r} = \frac{dr_{bnd}/dt}{\omega^2 r}$$

$$\ln(r_{bnd}/r_m) = s\omega^2 t$$

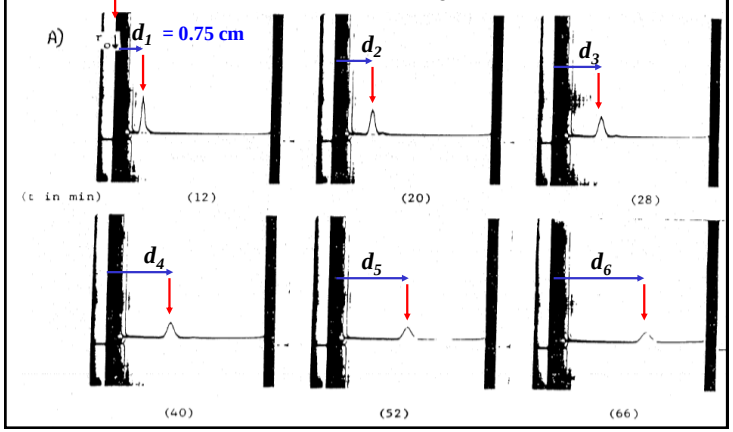




Sedimentation Velocity (s) Using Schlieren Optics

ω= 40,000 rpm, T = 20 ^{O}C, ρ = 1.00 g/cc, v-bar = 0.73 cc/g

magnification factor =2.57, r_o = 5.72 cm



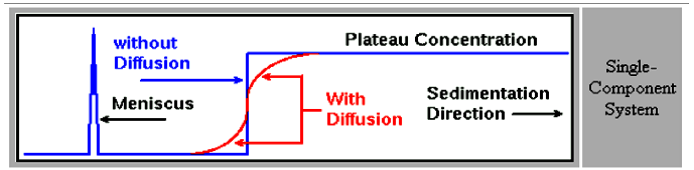


Figure 1: A single-component system shown without diffusion (in blue) and with diffusion (in red). The boundary spreads due to diffusion and gives a sigmopidal shape to the boundary.

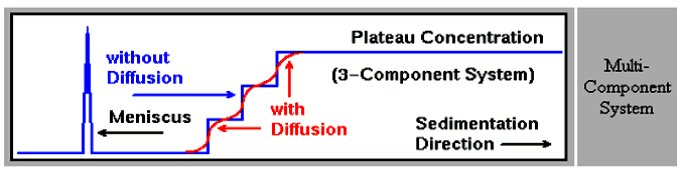


Figure 2: A multi-component system shown without diffusion (in blue) and with diffusion (in red). The step functions defining the boundary profiles of each component can lose definition as diffusion increases and overlays on top of sedimentation separation.

Flow in the Ultracentrifuge Cell:

Sedimentation:

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

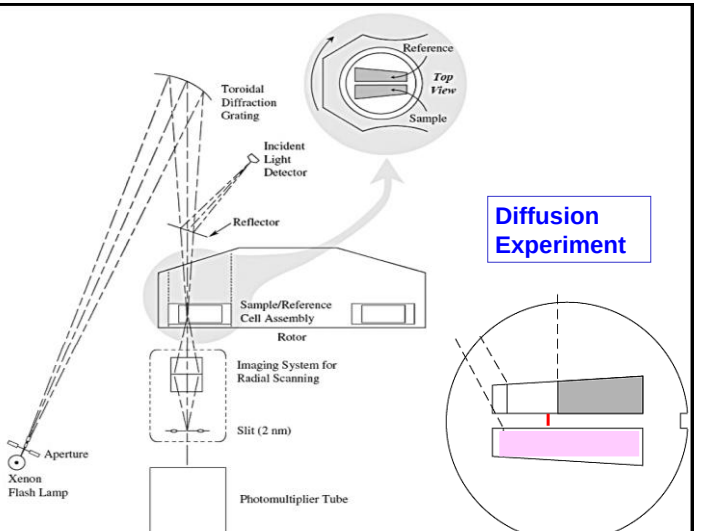
Diffusion:

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

Molecular Weight:

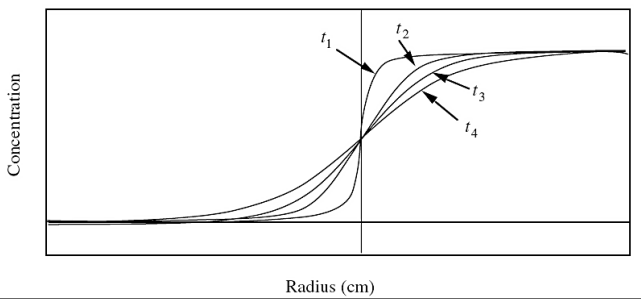
$$\frac{s}{D} = \frac{M\left(1-\bar{v}\rho\right)}{RT}$$

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



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

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



Diffusion Coefficient (D) using Schlieren Optics

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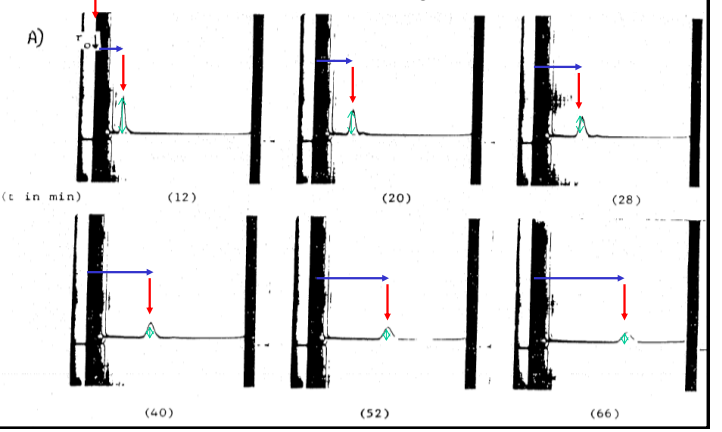
magnification factor =2.57, r_0 = 5.72 cm



$$\left(\frac{A}{I_H}\right)^2$$

$$\left(\frac{c_p}{(dc/dr)}\right)^2$$

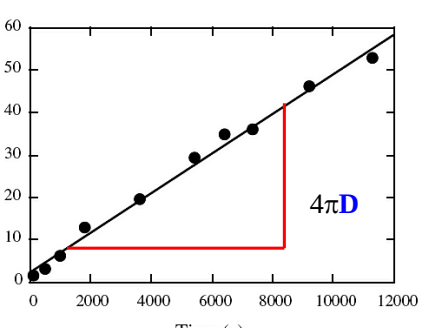
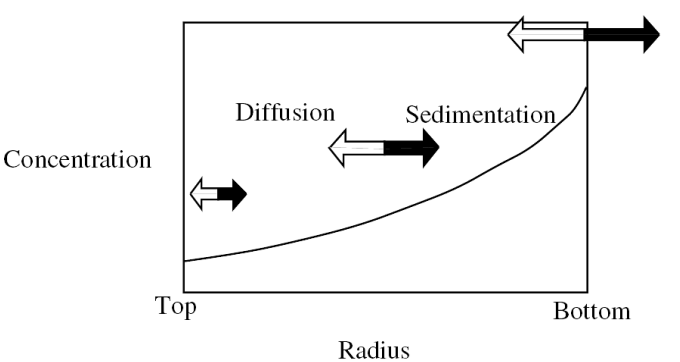


Figure 13. *Determination of the diffusion coefficient. The spreading of an initially sharp boundary of human spectrin was followed with time. The slope of the plot of* $[c_p/(dc/dr)]^2$ *versus time is* 4π *times the diffusion coefficient.*

Diffusion vs. **Sedimentation** at low speeds



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

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

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

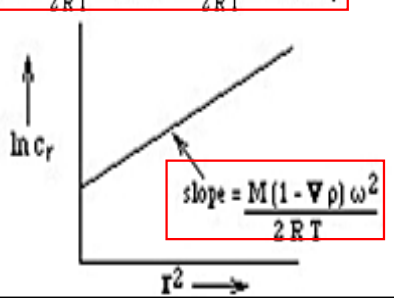
The equilibrium gradient is dependent on rotor speed, temperature, an on the molecular weight and buoyancy of the solute. This equation can be fitted by nonlinear least squares.

Sedimentation Equilibrium (net flow = 0)

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

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

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

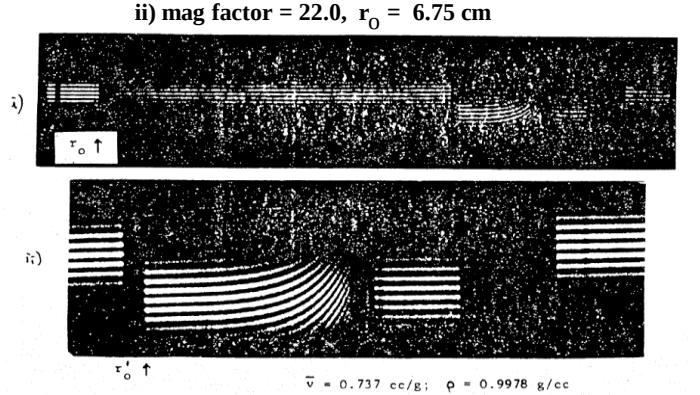


Sedimentation Equilibrium Using Interference Optics

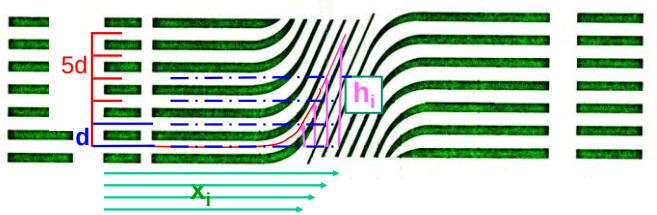
ω= 5,200 rpm, T = 20 °C, ρ = 1.00 g/cc, v-bar = 0.73 cc/g

i) mag factor = 8.90, r_o = 5.70 cm

ii) mag factor = 22.0, r_o = 6.75 cm



Interference Optics (Δc)



$$\Delta c_i = \frac{\Delta J_i \cdot \lambda}{a \cdot K}$$

where $\Delta J_i = h_i / d$

a = 12 mm ; λ = 546 nm

$\frac{dn}{dc} = K = 0.186\ (g/cm^3)^{-1}$

Since $c = c_m + \Delta c$

$c \sim \Delta c$

when $c_m \sim 0$ (Yphantis high speed condition)

Heterogeneous Samples

Binding ?

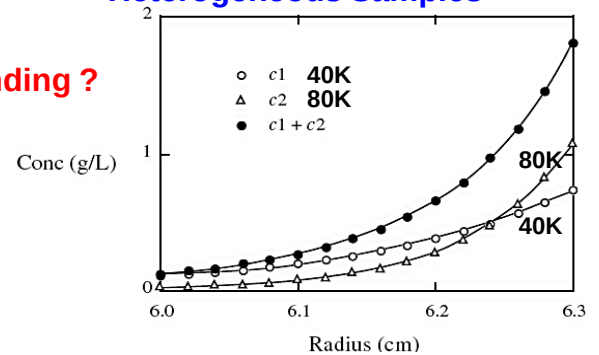
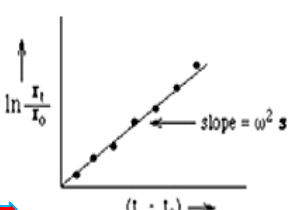


Figure 16. *Sedimentation equilibrium distribution of two different solutes. Data were simulated for two species: (o) M_r = 40,000; (Δ) M_r = 80,000. The angular velocity was 15,000 rpm, and a partial specific volume of 0.73 was assigned to both species. The distribution of total solute concentration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

KEY EQUATIONS

Sedimentation Velocity

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



Sedimentation Velocity + Diffusion

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

Sedimentation Equilibrium

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