PROTEIN PURIFICATION

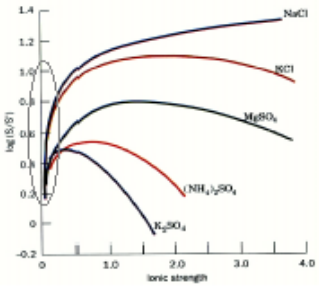
General strategy
Tissue ⟶ disrupt ⟶ crude fractionation ⟶ selected fractionation

<u>Proteins can be separated by:</u>
Solubility: **salting out**
Centrifugation
Dialysis
Chromatography
- ***Size/Mass:*** **Molecular Sieve - gel filtration**
- ***Charge:*** **ion-exchange chromatography**
- ***Hydrophobicity:*** **hydrophobic interaction chromatography, reverse phase chromatography**
- ***Binding affinity:*** **affinity chromatography, antibodies**

Electrophoresis: **SDS gel electrophoresis**

<u>Purified proteins can be analyzed by:</u>
Protein Assay: **Characteristic of protein**
Sequencing: **Edman degradation, (proteolysis)**
3D structure: **X-ray crystallography, NMR**
Synthesis: **automated solid phase**

Selective precipitation
- Ionic strength
- pH
- Organic solvents
- "molecular crowding" agents (polyethylene glycol)

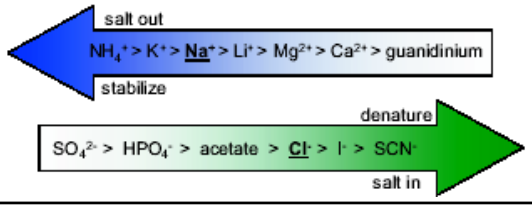


Salt fractionation

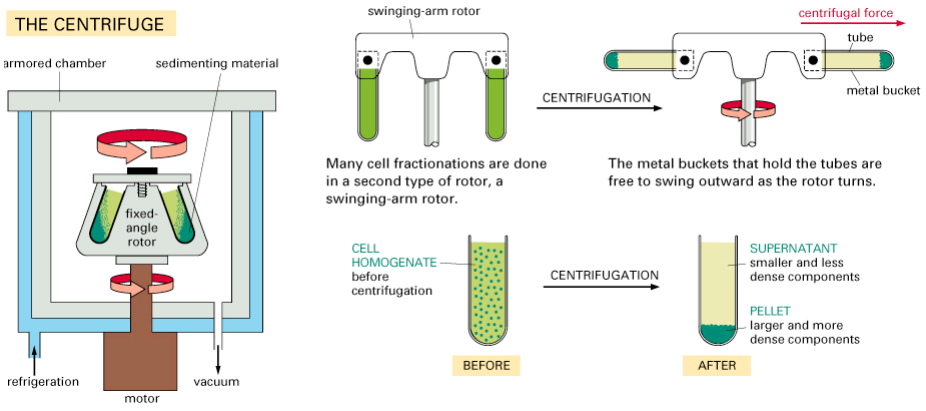
At low ionic strength, increasing salt concentrations tend to increase solubility- "**salting in**"

At some point, solubility begins to decrease as ionic strength increases- "**salting out**" the protein

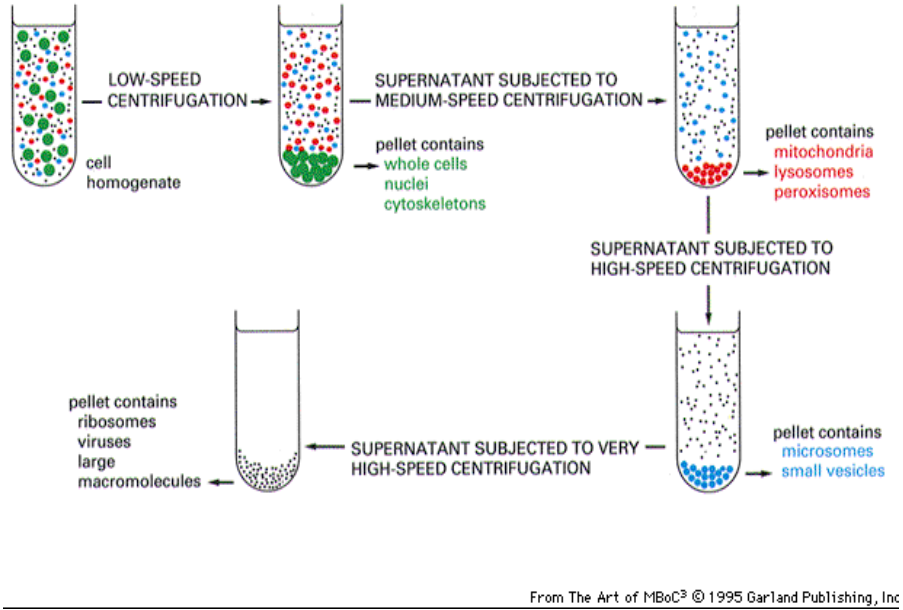
Some ions are more effective than others in affecting protein solubility (this ranking is known as the ***Hofmeister series***)



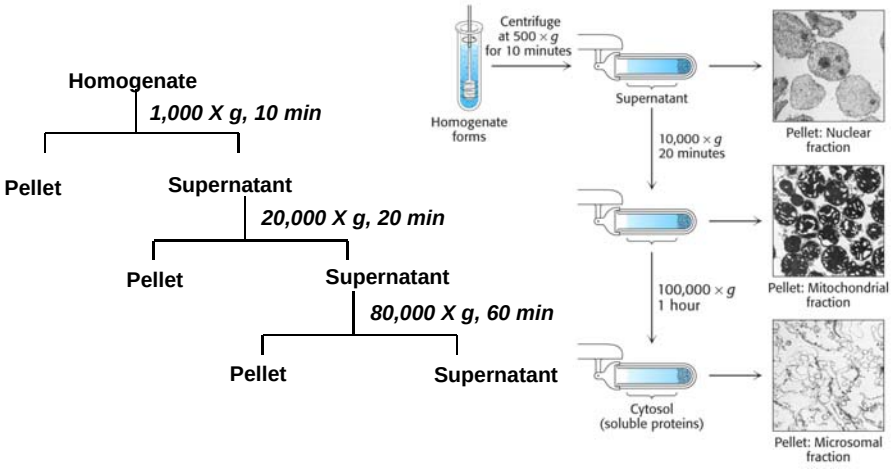
Centrifugation



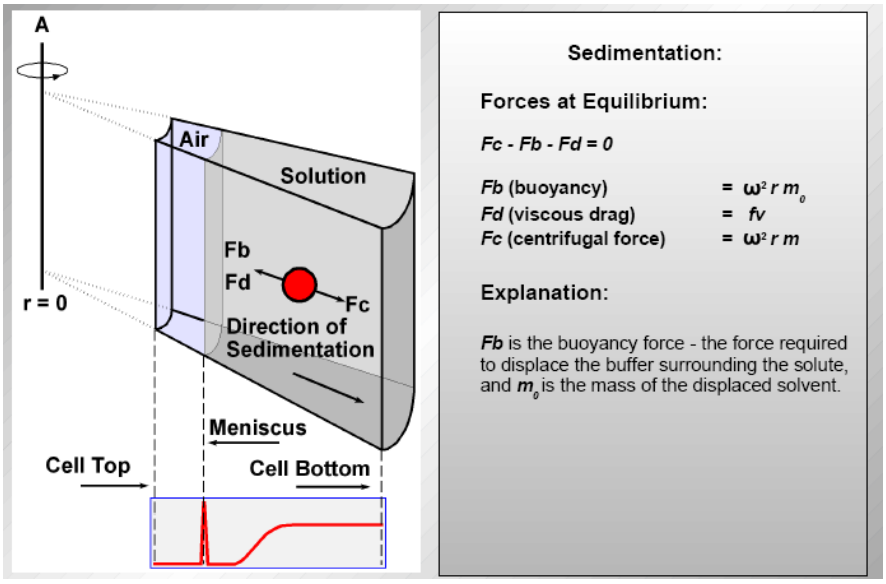
Centrifugation



Centrifugation



Reduce contamination by repetition



Sedimentation:

Forces at Equilibrium:

$Fc - Fb - Fd = 0$

Fb (buoyancy)	$= \omega^2 r m_0$
Fd (viscous drag)	$= fv$
Fc (centrifugal force)	$= \omega^2 r m$

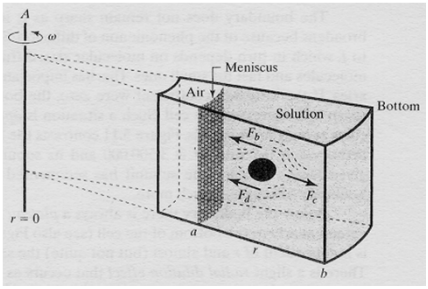
Explanation:

Fb is the buoyancy force - the force required to displace the buffer surrounding the solute, and m_0 is the mass of the displaced solvent.

Sedimentation

- Forces
  - Centrifugal: $\omega^2 rm$
    - ω = angular velocity
      - radians per second
    - r = distance from axis
    - m = molecular mass
  - Buoyancy: $-\omega^2 r\, m_0$
    - m_0 = displaced mass
  - Friction: $-fv$
    - f = frictional coefficient
    - v = radial velocity
  - (Diffusion)

Unit: 1 Svedberg = 10^{-13} second



$$m_0 = m\bar{v}\rho$$

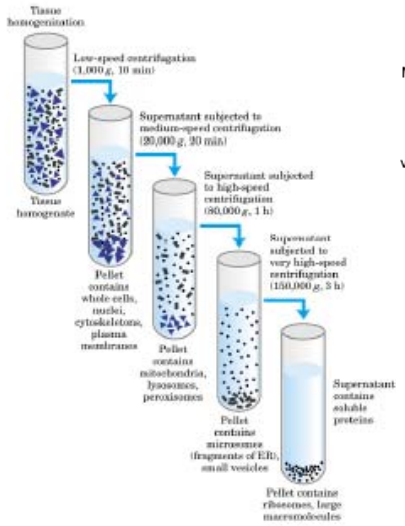
$\bar{v}$ = partial specific volume

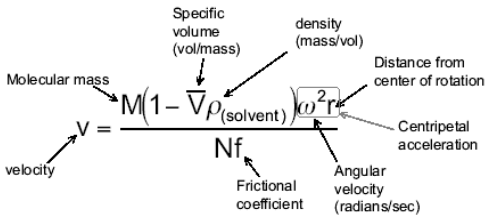
ρ = solution density

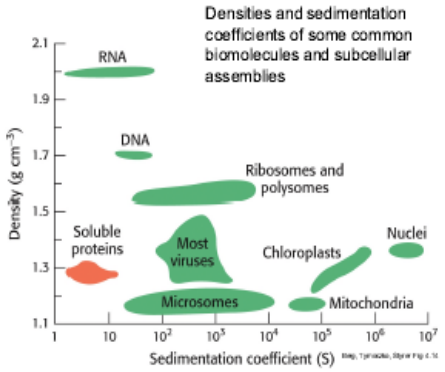
$$\omega^2 rm(1-\bar{v}\rho) - fv = 0$$

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

s = sedimentation coefficient







Unit: 1 Svedberg = 10^{-13} second

Diffusion and Sedimentation

- Molecular interpretation

$$D = \frac{k_B T}{f} = \frac{RT}{Nf}$$

k_B = Boltzmann constant

N = Avogadro's number

f = frictional coefficient

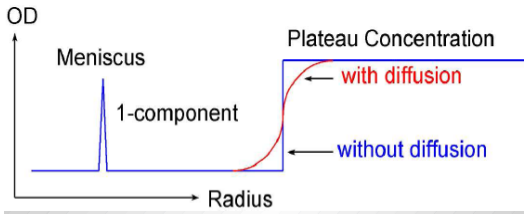
- Stokes' Law

  Sphere of radius a

  $f_0 = 6\pi\eta a$

  η = viscosity

- Non-spherical
  - $f/f_0 > 1$, where f_0 is frictional coefficient for sphere with equivalent volume



The frictional coefficient for an unsolvated, sphere of radius r, is given by the Stokes equation:

$$f = 6\pi\eta r$$

(η: Solution viscosity)

For a perfectly spherical molecule the Stokes radius can be calculated from the formula for the volume of a sphere and the molecular weight and specific volume of the molecule:

$$V_p = \frac{4}{3}\pi r^3 = \frac{M\bar{V}}{N} \Rightarrow r = \left(\frac{3M\bar{V}}{4\pi N}\right)^{1/3}$$

$$f_0 = 6\pi\eta\left(\frac{3M\bar{V}}{4\pi N}\right)^{1/3}$$

Interpreting s

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

- s is a function of
  - frictional coefficient, f:
    - Shape effects for anything other than an unhydrated sphere
  - partial specific volume, $\bar{v}$
    - Difficult/tedious to measure accurately
    - Can be estimated from AA composition in proteins

- Calculating M from s, D

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

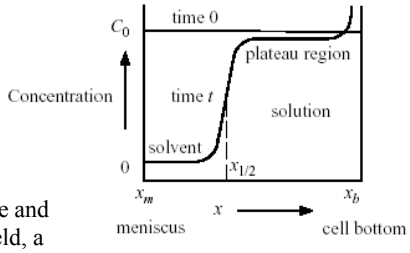
  - No shape effect

- Determining shape from s, M
  - f and f_0 can be determined from s and Stokes eqn
  - Correct for hydration
  - However, many shapes can give a given shape factor
  - Still useful for resolving multimer structures

Determination of Sedimentation Coefficient

Boundary Sedimentation

Boundary sedimentation is sedimentation of macromolecules in a homogeneous solution.



(a) Begin with a homogeneous solution of macromolecules.

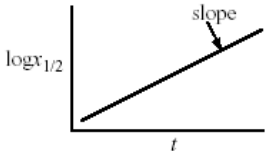
(b) As the solution is spun in the ultracentrifuge and macromolecules move down the centrifugal field, a solution-solvent boundary is generated. The boundary can be monitored by refractive index, color (absorption) etc.

(c) By following the boundary with time, the sedimentation coefficient can be determined.

$$S = \frac{v_t}{\omega^2 x} = \frac{dx_{1/2}/dt}{\omega^2 x_{1/2}} = \frac{1}{\omega^2}\frac{d\ln x_{1/2}}{dt}$$

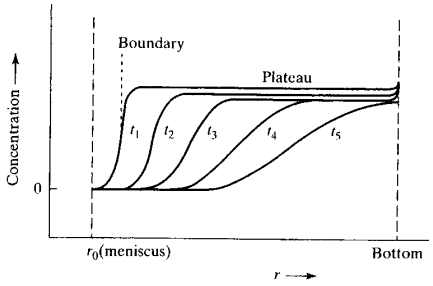
$$= \frac{2.303}{\omega^2}\frac{d\log x_{1/2}}{dt}$$

$$= \frac{2.303}{\omega^2}\,(\text{slope})$$



Moving boundary

- Method
  - Analytical ultracentrifuge
  - Molecules move to bottom
  - Follow boundary, r_b
    - Radial scanning of cell
    - Measure absorption
  - Determine s
  - Correct for concentration, temperature
- Limitations
  - Expensive
  - Analysis, not preparative
  - Cannot separate mixtures



$$\frac{dr_b}{dt} = v = s\omega^2 r_b$$

$$\ln\frac{r_b(t)}{r_b(t_0)} = s\omega^2(t - t_0)$$

Sedimentation equilibrium

- Method
  - Centrifuge at low speed until sedimentation flow equals reverse diffusion flow
  - Scan steady state profile
    - Analytical ultracentrifuge
  - Fit model
- Limitations
  - Excellent Mw determination over broad range: 100-10^7
  - Expensive
  - Not preparative

$$J_S = v_S C = \frac{M(1-\bar{v}\rho)\omega^2 r}{Nf} C$$

$$SS \Rightarrow J_S + J_D = 0:$$

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

$$\frac{1}{2rC}\frac{dC}{dr} = \frac{d\ln C}{dr^2} = \frac{M(1-\bar{v}\rho)\omega^2}{2RT}$$

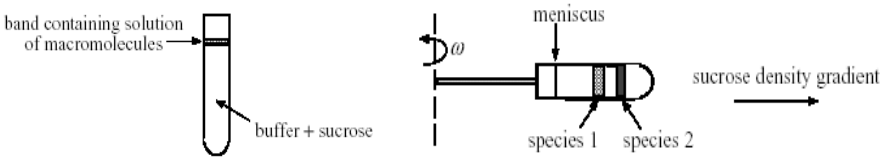
$$\ln\frac{C(r)}{C(r_0)} = \frac{M(1-\bar{v}\rho)\omega^2}{2RT}\left(r^2 - r_0^2\right)$$

Zone Sedimentation

(a) A thin layer of a solution of the macromolecule(s) is placed on top of a solvent containing sucrose (sucrose solution).

(b) As the sample is spun in the centrifuge, a band containing macromolecules will move down the centrifugal field. Also, a sucrose gradient will have developed. The sucrose gradient ensures that the density of the "solvent" is always greater than the density of the sedimenting zone. This ensures the stability of the band.

(c) *S* is determined from the displacement of the band(s) with time in the centrifuge tube.



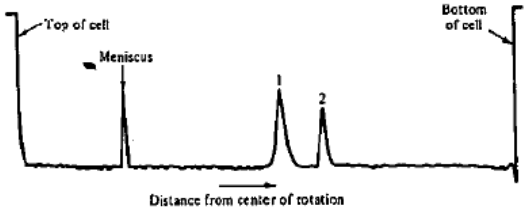
Another way to reach sedimentation equilibrium is when the macromolecule becomes "buoyant" in a density gradient. Under this condition,

$(1 - v^2\rho(x)) = 0$ and v_t or $S = 0$

(a) In these experiments, a density gradient is established by adding concentrated salt solution, e.g. CsCl, to the solution of macromolecule(s).

(b) The solution is then spun at high speeds (ω).

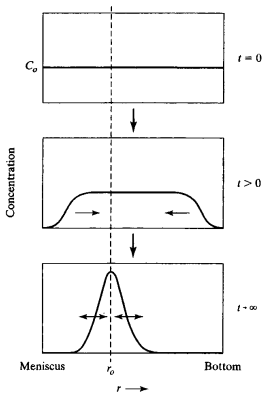
(c) The various macromolecular species will form bands at points in the salt gradient where the macromolecules become buoyant; i.e., at x's where $(1 - v^2\rho(x)) = 0$ for the species. Many biological macromolecules have "buoyant densities" sufficiently different that they can be separated or resolved by density-gradient centrifugation.



Sedimentation equilibrium in density gradient

- Method
  - Small amount of solute in dense salt (e.g., CsCl)
  - Allow salt to reach equilibrium, thus creating a gradient
  - Ensure that at some point in cell, r_0, $\rho(r_0)=1/\bar{v}$
  - Then $(1-\bar{v}\rho)$
    - >0: r<r_0 and <0: r>r_0
- Limitations
  - Exquisite sensitivity of band position to solute density, e.g., ^{14}N vs ^{15}N

$$\ln\frac{C(r)}{C(r_0)} = \frac{M(1-\bar{v}\rho)w^2}{2RT}\left(r^2 - r_0^2\right)$$

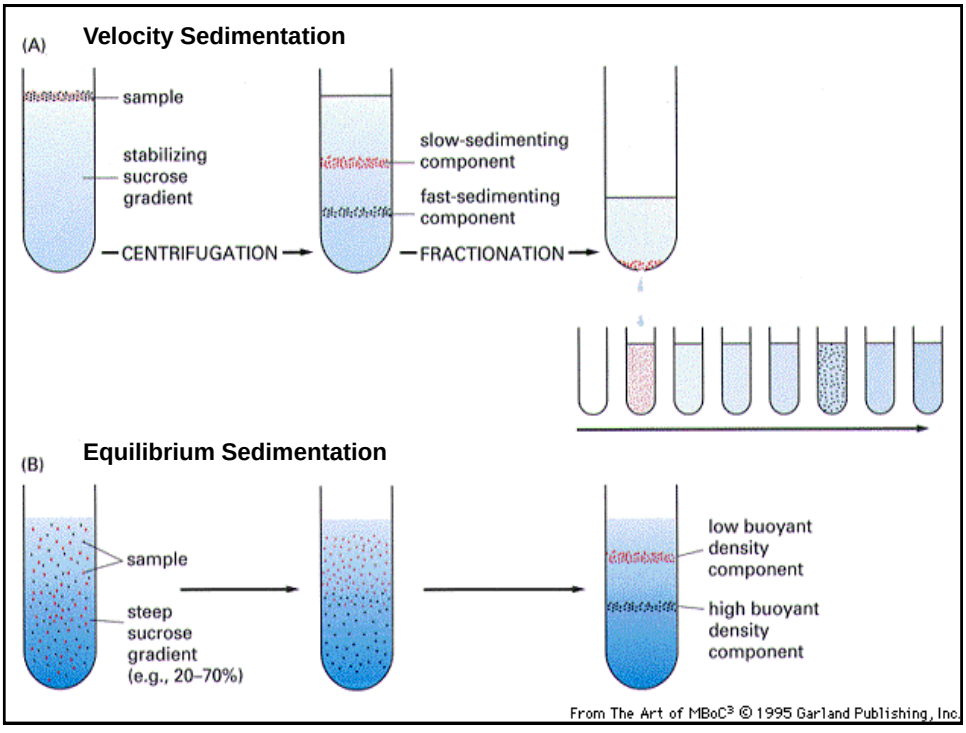


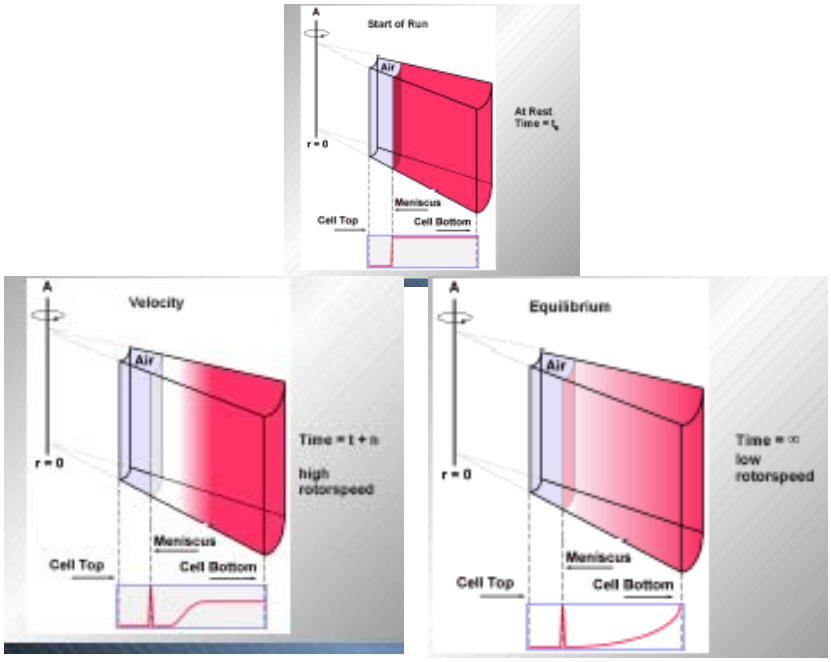
$$\frac{C(r)}{C(r_0)} = e^{-\frac{1}{2}\frac{(r-r_0)^2}{\sigma^2}}$$

$$\sigma^2 = \frac{RT}{w^2 r_0 M\bar{v}(d\rho/dr)}$$

Ultracentrifugation Techniques

- **Finer separation & less convective mixing using *Density Gradients* (sucrose, CsCl)**
- ***Velocity Sedimentation*: components sediment at different rates**
  - **Based on size & shape**
  - **Described by *sedimentation coefficient, S* (Svedberg)**
  - **Distinct bands, collect individually**
- ***Equilibrium Sedimentation*: sedimentation through step gradient to position of equal density**
  - **Based on *buoyant density*, independent of size or shape**
  - **Collect distinct bands of different densities**



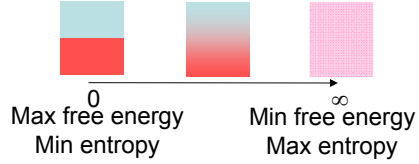


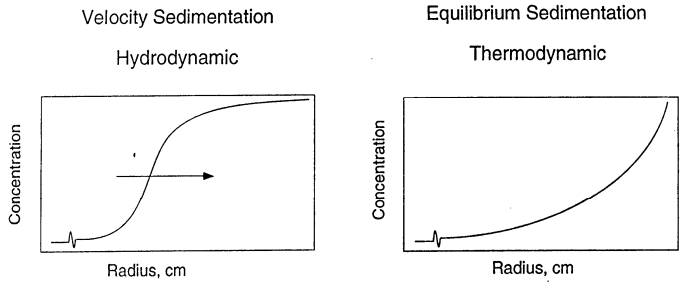
Diffusion and Centrifugation

Diffusion coefficient can be related to molecular parameters

Einstein's equation: $D=k_BT/f$ - valid for any shape

Stokes-Einstein equation: $D=k_BT/6\pi\eta r$





Net Transport -

Shape and Molecular Weight

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

No Net Transport -

Molecular Weight

$$M = \frac{2RT}{\omega^2(1-\bar{v}\rho)} \times \frac{d\ln c}{dr^2}$$

Interpretation of the sedimentation coefficient.
s depends on both size and shape.
Assume that *M* is spherical and unhydrated:

Then, $f_o = 6\pi\eta\, R_o$ (Stokes Law)

$$V_o = \frac{M\bar{v}}{\mathrm{N}} = \frac{4}{3}\pi R_o^3;\ \text{hence, } R_o = \left(\frac{3M\bar{v}}{4\pi\ \mathrm{N}}\right)^{1/3}$$

So, $f_o = 6\pi\eta(3M\bar{v}/4\pi\ \mathrm{N})^{1/3}$

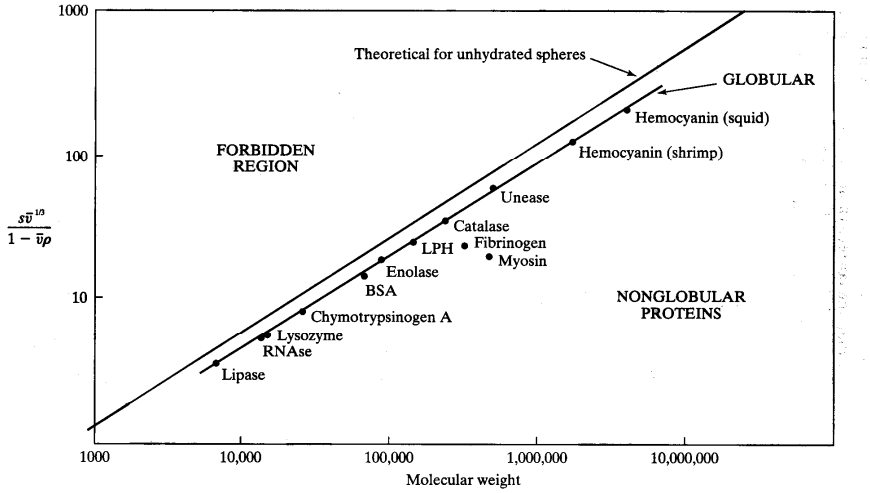
So, $s^o_{20,w} = \dfrac{M(1-\bar{v}\rho)}{\mathrm{N}f} = \dfrac{M(1-\bar{v}\rho)}{\mathrm{N}6\pi\eta(3M\bar{v}/4\pi\ \mathrm{N})^{1/3}}$

$$\therefore\ s^o_{20,w} = \frac{M^{2/3}(1-\bar{v}\rho)}{6\pi\eta\,\mathrm{N}^{2/3}(3/4\pi)^{1/3}\bar{v}^{1/3}}$$

The sedimentation coefficient for unhydrated, spherical macromolecules should be proportional to the 2/3 power of the molecular weight.
The above expression can be rearranged to:

$$s^* = \frac{s^o_{20,w}\bar{v}^{1/3}}{(1-\bar{v}\rho)} = \frac{M^{2/3}}{6\pi\eta\mathrm{N}^{2/3}(3/4\pi)^{1/3}}$$

The straight line in the plot below is the behavior predicted for anhydrous, spherical molecules. No real macromolecule can appear at a point above or to the left of the upper line. Moreover, the upper line indicates the lowest molecular weight possible for a molecule having a given value of *s**.



Globular proteins fall on a line parallel to the theoretical line, since they are approximately spherical and are hydrated to similar extent.
Highly asymmetric molecules fall far below the line.

If we know the molecular weight of the macromolecule, *s* can provide semi-quantitative information on its shape. We can calculate *f* from the relation:

$$s = \frac{M(1-\bar{v}\rho)}{Nf};\ \text{so},\ f = \frac{M(1-\bar{v}\rho)}{Ns}$$

Then, we can use f to calculate the Stokes radius (i.e., the effective radius) :

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

We can also calculate f_o, the frictional coefficient for an anhydrous sphere:

$$f_o = 6\pi\eta R_o, \text{ where } R_o = \left(\frac{3M\bar{v}}{4\pi N}\right)^{1/3}$$

The ratio f/f_o will be greater than 1.0. However, interpretation is complicated by the fact that this departure from unity can result from a combination of hydration and non-spherical symmetry. If we know the degree of hydration from some independent measurement, the analysis can be taken further.

Hydration of various biopolymers: A comparison of the results from different methods

Sample	NMR-freezing	Calorimetric	Hydrodynamic[‡]	Isopiestic	NMR-calculated[#]
Ovalbumin	0.33	0.32	0.14	0.30	0.37
Bovine serum albumin	0.40	~0.40	0.41	0.32	0.45
Hemoglobin	0.42	0.32	0.63	0.37	0.42
Lysozyme	0.34	~0.30	0.46	0.25	0.36
Myoglobin	0.42	—	0.45	0.32	0.45
DNA	0.59	0.61	—	0.84[¶]	—

In the "NMR freezing" method, the NMR spectrum of the water in the sample is obtained at -35 °C. Only the bound water, yields a sharp signal, which can be integrated.

Assume, then, that we know the extent of hydration. We rewrite f/f_o in terms of f_{sp}, the hypothetical frictional coefficient for a spherical molecule of a given hydration:

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

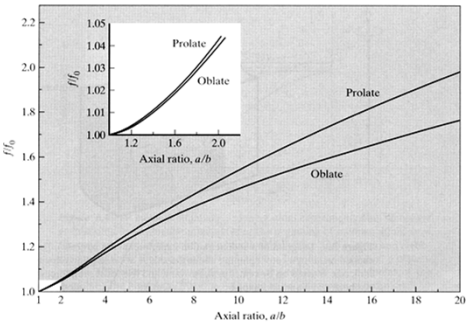
f_{sp} / f_o depends only on hydration : $f_{sp} / f_o = (1+\delta)^{1/3}$

f / f_{sp} is a pure shape factor and can be used to estimate the axial ratio.

TABLE 5.1 FRICTIONAL COEFFICIENT RATIOS

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

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



Zonal Sedimentation

1. Advantages:
   Much greater resolution of sample components.
   Much greater flexibility for detecting component (enzymatic activity, radiotracers, bioassays).
2. Requires a density gradient (typically sucrose, glycerol) to prevent convection.
3. Modest equipment requirements.

From the tube number in which a given component appears, the distance traveled can be estimated and, hence, s calculated.
The presence of the gradient causes some complications, however. Note that the velocity of sedimentation at point r is given by:

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

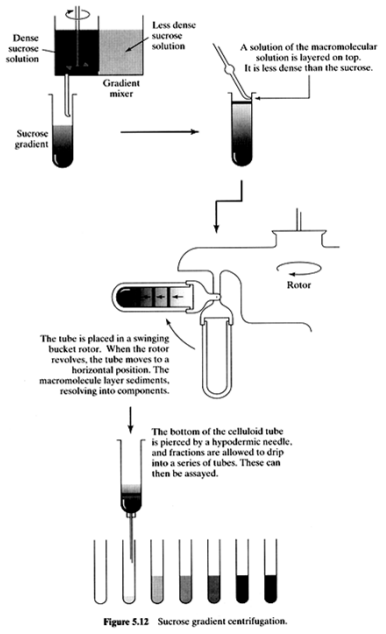


Figure 5.12 Sucrose gradient centrifugation.

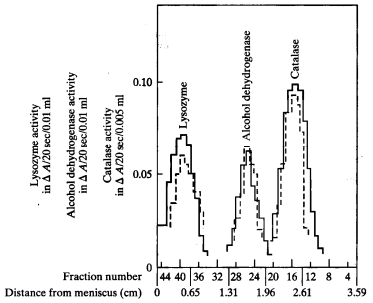
$$v = s\omega^2 r = \frac{M(1-\bar{v}\rho)\omega^2 r}{Nf}$$

The frictional coefficient (proportional to η) increase with r (due to the gradient) and (1-vρ) decrease with r; these two factors cause the velocity to decrease as the band moves down the tube.
However, $\omega^2 r$ is increasing, tending to increase the velocity.
These two factors balance when the density gradient is chosen correctly and sedimentation proceeds at a nearly constant rate.
Such gradients are called *isokinetic.*
Even with isokinetic gradients it is common to include marker substances of known sedimentation coefficient to provide internal calibration.

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).



Determination of the partial specific volume, $\bar{v}$.

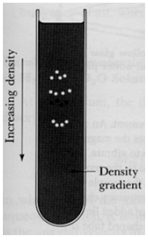
The partial specific volume is the volume increment produced in a solution when when unit mass of solute is added - - i.e., $\bar{v} = dv/dm$.

It is sometimes approximated as the reciprocal of the density of the solute.

Because $\bar{v} = dv/dm$, it can be determined from the change in the density of a solution with solute concentration.

1. Pycnometry: A pycnometer is a container that can be filled with great precision. The volume of the pycnometer is determined by filling with water and weighing, since the density of water is accurately known. The pycnometer is then filled with the solution of interest (solute concentration must be high: 10 - 50 mg/mL) and reweighed.

2. Linderstrom-Lang density gradient columns: A density gradient of bromobenzene and kerosene (both immiscible with water) is prepared in a narrow cylinder. When placed on the surface of the gradient, a small droplet of aqueous solution will fall through column of liquid until it reaches a point at which its density matches that of the density gradient. The gradient can be calibrated with solutions of known density.



3. $\bar{v}$ may also be calculated from the amino acid compositio n or sequence because the partial specific volumes of the individual residues are well known.

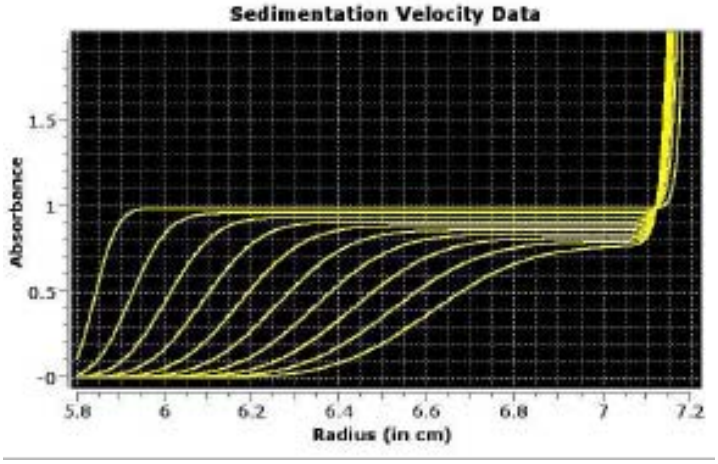
Although this approach may ignore contributions to the partial specific volume arising from conformational effects (e.g., cavities in the structure or exceptionally close packing), the values calculated for most proteins agree with the experimental value to within 1%.
A 1% error in the partial specific volume leads to a 3% error in the (1-$v\rho$) term and, hence, a 3% error in *M*.

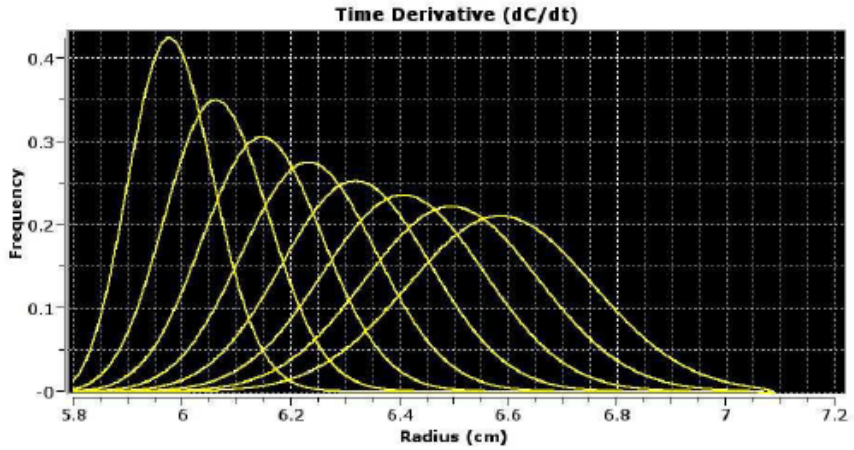
Amino acid	M_r	$\bar{v}$ (ml g^{-1})	H_i mol-H_2O mol-aa (pH 6-8)	H_i mol-H_2O mol-aa (pH 4)
Ala	71.1	0.74	1.5	1.5
Arg	156.2	0.70	3	3
Asn	114.1	0.62	2	2
Asp	115.1	0.60	6	2
Asx[c]	114.6	0.61	4	2
Cys	103.2	0.63[b]	1	1
2Cys	204.3	0.63	-	-
Gln	128.1	0.67	2	2
Glu	129.1	0.66	7.5	2
Glx[c]	128.6	0.665	4.8	2
Gly	57.1	0.64	1	1
His	137.2	0.67	4	4
Ile	113.2	0.90	1	1
Leu	113.2	0.90	1	1
Lys	128.2	0.82	4.5	4.5
Met	131.2	0.75	1	1
Phe	147.2	0.77	0	0
Pro	97.1	0.76	3	3
Ser	87.1	0.63	2	2
Thr	101.1	0.70	2	2
Trp	186.2	0.74	2	2
Tyr	163.2	0.71	3	3
Unk[c]	119.0	0.72	2.4	2
Val	99.1	0.86	1	1

4. Parallel sedimentation Equilibrium Measurement in H_2O and D_2O

$$M_H(1-\bar{v}\rho_H)=\frac{2RT}{\omega^2}\left(\frac{d\ln c}{dr^2}\right)_{H_2O};\ M_D(1-\bar{v}\rho)=\frac{2RT}{\omega^2}\left(\frac{d\ln c}{dr^2}\right)_{D_2O}$$

$$\frac{M_H(1-\bar{v}\rho_H)}{M_D(1-\bar{v}\rho_D)}=\left(\frac{d\ln c}{dr^2}\right)_H\Big/\left(\frac{d\ln c}{dr^2}\right)_D$$




Time Derivative (dC/dt)
Frequency
0.4
0.3
0.2
0.1
-0
5.8
6
6.2
6.4
6.6
6.8
7
7.2
Radius (cm)

Chromatography

Physical separation method based on the differential migration of analytes in a mobile phase as they move along a stationary phase.

Mechanisms of Separation:

- **Partitioning**
- **Adsorption**
- **Exclusion**
- **Ion Exchange**
- **Affinity**

Chromatographic Separations

Based on the distribution (partitioning) of the solutes between the mobile and stationary phases, described by a partition coefficient, K:

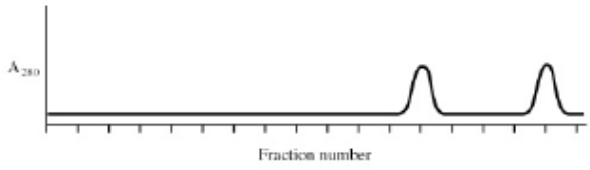
K = Cs/Cm

where Cs is the solute concentration in the stationary phase and Cm is its concentration in the mobile phase.

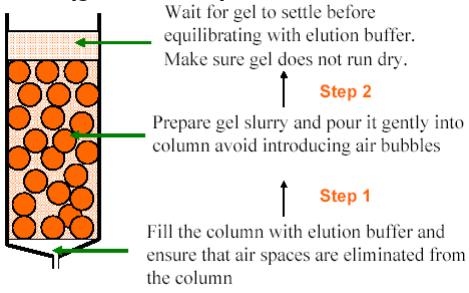
Gel-filtration chromatography: **proteins passed over a column filled with a hydrated porous beads made of a carbohydrate or polyacrylamide polymer [large molecules exit (elute) first]**

Ion-exchange chromatography: **separation of proteins over a column filled with charged polymer beads (bead +charge = anion-exchange; bead -charge = cation exchange). Positively charged proteins bind to beads of negative charge & vice versa. Bound proteins are eluted with salt. Least charged proteins will elute first.**

Affinity chromatography: **proteins are passed through a column of beads containing a covalently bound high affinity group for the protein of interest. Bound protein is eluted by free high affinity group.**



Size Exclusion (gel filtration)

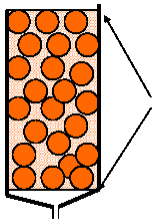


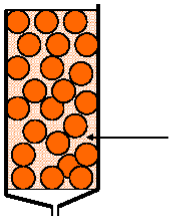
Sephadex G-50 1-30 kD
Sephadex G-100 4-150 kD
Sephadex G-200 5-600 kD
Bio-Gel P-10 1.5-20 kD
Bio-Gel P-30 2.4-40 kD
Bio-Gel P-100 5-100 kD
Bio-Gel P-300 60-400 kD
Sephadex is a trademark of Pharmacia.
Bio-Gel is a trademark of Bio-Rad.

Biochemists refer to a protein's size in terms of its molecular weight, in kDa (a kilodalton, kD or kDa, is 1000 times the molecular mass of hydrogen)
Each amino acid residue counts for about 110 daltons, that is, about 0.11 kDa.

Size Exclusion (gel filtration)

Vt or total column volume
Refers to total volume occupied by the gel in the column, and not the size of the column

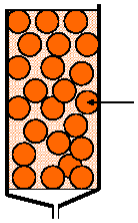




Vo or void volume

Void volume = space outside the granules

Rule: Vo = 1/3 column volume



Vt – Vo = volume occupied by gel space, including gel matrix (Vgel)

Stationary phase (Vs) = Vt-Vo-Vgel
This is difficult to measure

Vs also labeled as Vi

$$Kd = \frac{Ve - Vo}{Vs}$$

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Ve = elution volume of solute
Vo = void volume of column
Vs = volume of stationary phase (= Vi)
Vi = Vt-Vo-Vgel matrix.

For convenience, expression Kav is used

Size Exclusion (gel filtration)

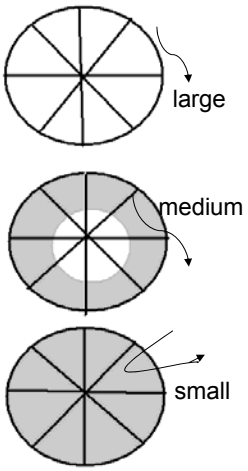
- Column matrix and solvent are selected to minimize adsorption
- Isocratic elution (same buffer throughout)
- Particle size determines determines the void volume- Vo
- Pore size determines the resolving range

– Molecules larger than the largest pore are excluded, elute at Vo

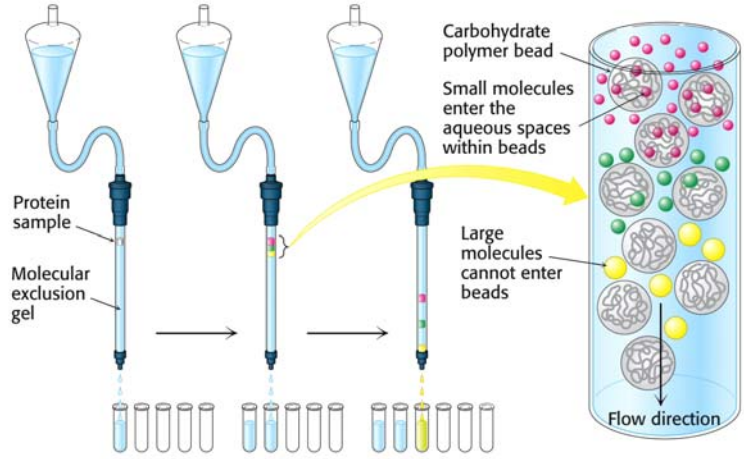
– Molecules that are smaller than the smallest pore are included they sample both Vi and Vo elute at Vt=Vi + Vo

– Molecules that can occupy some but not all of the pores elute at an intermediate volume Ve

- Partition coefficient Kav = (Ve-Vo)/(Vt-Vo)
- Kav is proportional to ln (MW) in the resolving range



Gel-Filtration Chromatography – Separation based on size

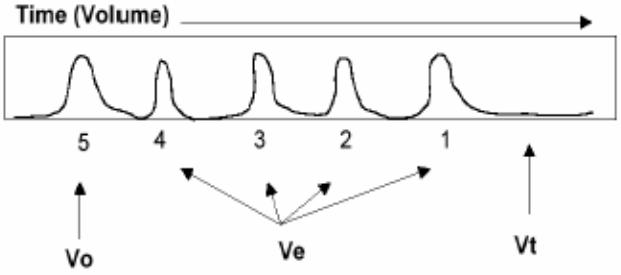


•***Determination of Molecular Weight***

Initially a mixture of known proteins is run through the gel filtration column
1) Ribonuclease A: 13,700
2) Chymotrypsinogen A: 25,000
3) Ovalbumin: 43,000
4) Bovine Serum Albumin: 67,000
5) Blue Dextran: 2,000,000
•Vo is determined using the Blue Dextran as a marker.

•Ve is determined for each of proteins 1-4.

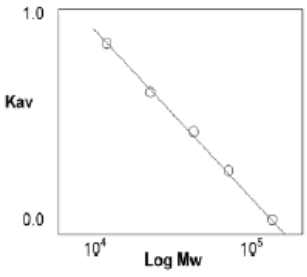
•Vt is calculated from the formula $\pi r2 \times h$ (or from low Mw compound such as riboflavin)



c) Kav is calculated for each known protein by substituting the experimentally determined Vo, Vt and Ve values into the formula:

Kav = (Ve-Vo)/(Vt-Vo)

d) The *Kav values* are then plotted versus the known *molecular weights of the related proteins on a log scale* to make a standard curve for the column.

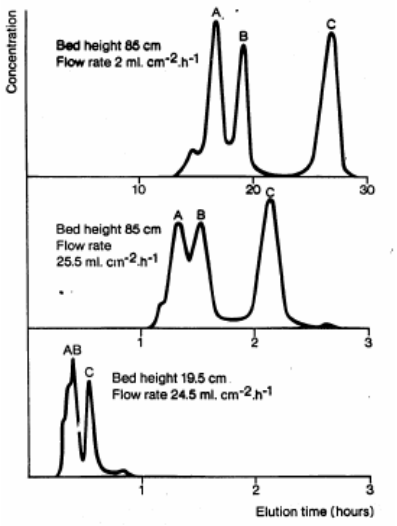


e) Now the protein of unknown molecular weight is loaded and eluted from the same column.

f) The Ve for the unknown protein is marked and used to calculate it's Kav.

g) The experimentally derived Kav is then used to determine the molecular weight of the unknown protein from the standard curve.

- Three proteins (A, B, C) may be well separated under Bed height 85 cm and buffer flow rate of 2 ml/cm²/h
- If flow rate is increased by about 10 fold, 25 ml/cm²/h, proteins A and B may become closer together and its separation affected
- If the flow rate is maintained at 25 ml/cm²/h and the Bed height is reduced, separation may become poor.



Ion Exchange Chromatography

Two common examples of ion exchangers are:

Anion exchanger:

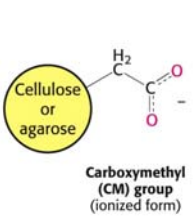
Inert Matrix—CH_2—CH_2—$NH(CH_2CH_3)^{2+}$ **die**thyl**a**mino**e**thyl (DEAE) group

Cation exchanger:

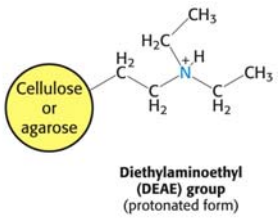
Inert Matrix —CH_2—COO^- **c**arboxy**m**ethyl (CM) grou

The inert (uncharged) matrix is

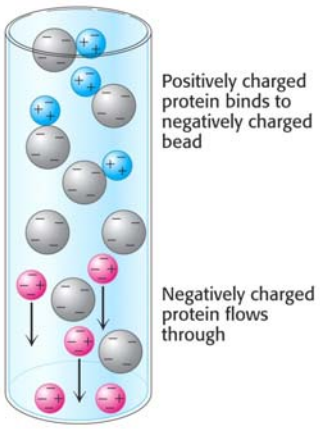
most commonly cellulose or agarose



Carboxymethyl (CM) group
(ionized form)



Diethylaminoethyl (DEAE) group
(protonated form)



❖ The surface of a protein has both positive and negative charges, and therefore can bind to both cation and anion exchangers.

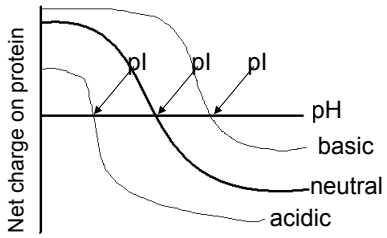
❖ The binding affinity of a protein depends on:

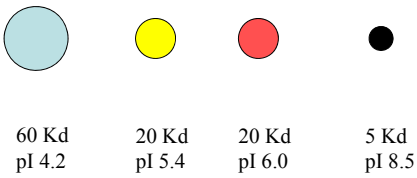
a. the concentration of salt ions in the mobile phase that compete with the protein for binding to the ion exchanger.

b. the pH of the mobile phase, which influences the ionization (and therefore the charge) properties of the protein.

❖ A protein can be eluted from the matrix by applying a buffer at higher salt concentration (or different pH) that reduces the protein's affinity for the matrix.

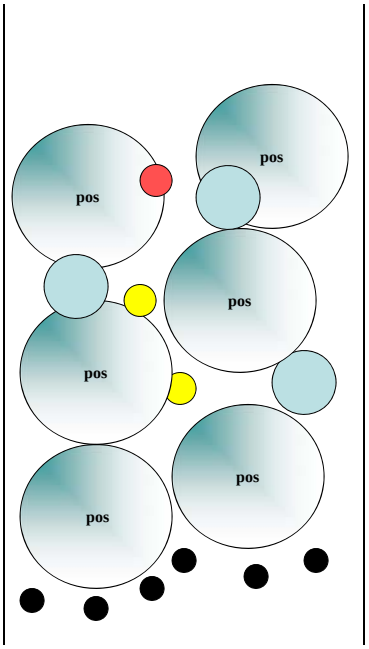
pH vs pI





60 Kd pI 4.2 | 20 Kd pI 5.4 | 20 Kd pI 6.0 | 5 Kd pI 8.5

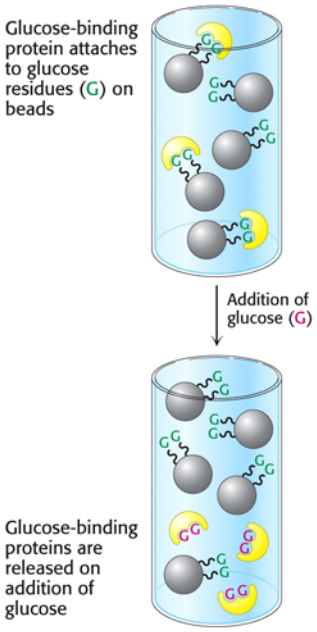
- Ion-exchange column chromatography separates proteins on the basis of charge.
- pH 7.2
- positively charged column



Affinity Chromatography

Small molecules are attached to beads and complex protein mixtures are applied.

Bound proteins can be eluted with the small molecule or with denaturing reagents (urea, guanidine, etc.)



Hydrophobic Interaction Chromatography

Stationary phase: Non-polar (octyl or phenyl) groups attached to an inert matrix

Exposed hydrophobic regions on proteins will bind to similar groups on the resin

Possible elution strategies:

1. Decreasing salt concentration (since higher salt augments hydrophobic interactions)

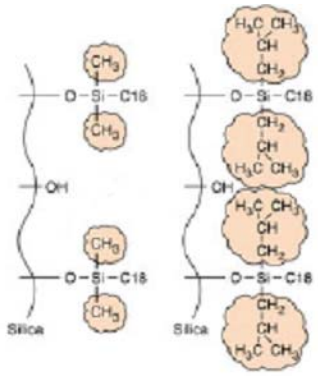
2. Increasing concentrations organic solvents

High Pressure Liquid Chromatography (HPLC)

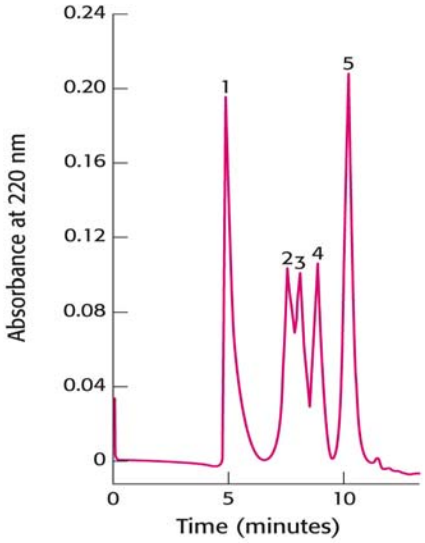
•sample is vaporized and injected;

•moves through a column containing stationary phase under high pressure;

•separates mixture into compounds according to their affinity for the stationary phase



High Pressure Liquid Chromatography



High pressure limits diffusion and increases interactions with chromatography media

HPLC gives very high resolution of protein components

UV Absorption

- **A_{max} of Tyr and Trp ~ 280 nm**
- **Tyr and Trp distribution ~ constant**
  - **A_{280} of 1.0 ≅ 1 mg/ml protein**
  - **sensitivity ~ 5-10 μg/ml**
- **sample recovery is possible**
- **interfering substances (eg., nucleic acids have A_{max} of 260 nm**
  - **correction factors possible**
  - **eg., mg/ml protein = $(A_{235} - A_{280})/2.51$**

Bradford (Coomassie-blue G-250)

- **A_{max} of CB G-250 shifts from 465 t0 595 nm when bound to protein**
  - **dye reacts primarily with Arg**
  - **lesser extent with His, Lys, Tyr, Trp, Phe**
- **sensitivity is 1-100 μg/ml depending on circumstances**
- **single step and few interfering substances**
- **protein concentration extrapolated from standard curve**
- **sample not recoverable**

Membrane based filtration methods

Ultrafiltration

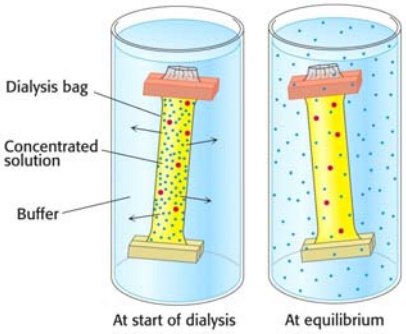
• Molecules migrate through a semipermeable membrane under pressure or centrifugal force

• Typically used to concentrate macromolecules but can be used for crude size fractionation and buffer exchange

Dialysis

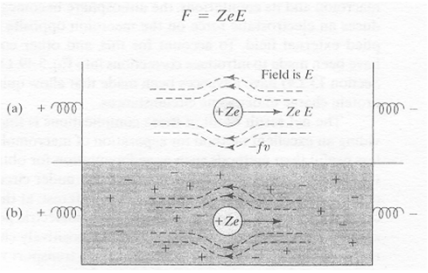
• Molecules diffuse through a semipermeable membrane if smaller than the pore size

• Commonly used to remove low molecular weight compounds and change the buffer composition



Electrophoresis

- Principle
  - Most macromolecules charged
    - NA: strong polyacids
    - Protein: polyampholytes
  - Will move in electric field
- Theory
  - In non-conducting medium similar to sedimentation
  - In reality, aqueous solution of buffer and counter-ions confounds any analysis
- Limitation
  - Not quantitative
  - Used for qualitative analysis and preparatively

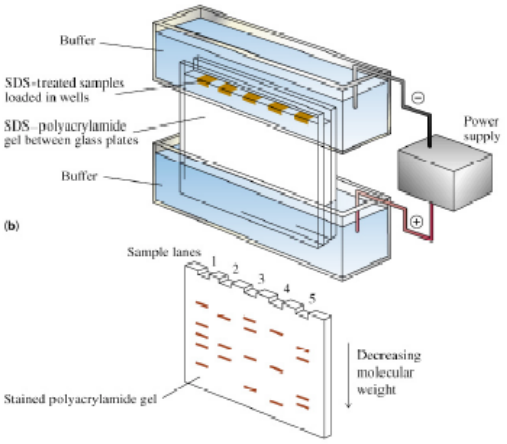


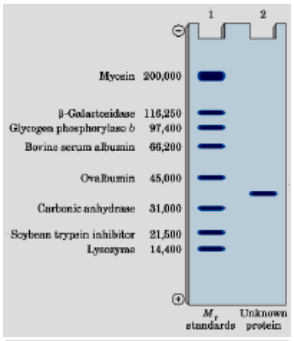
Steady motion :

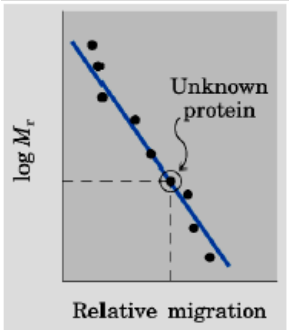
$$fv = ZeE$$

Electrophoretic mobility, U :

$$U = \frac{v}{E} = \frac{Ze}{f}$$





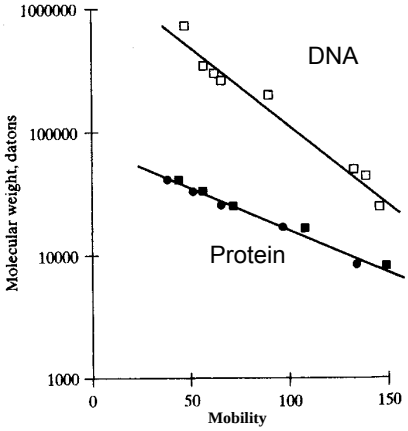


$$U_{ri} = \frac{U_i}{U_{dye}} = \frac{d_i}{d_{dye}}$$

d = distance moved at end of experiment

Size separation gels

- DNA gels
  - Charge ~ length or Mw
  - f ~ length or Mw
    - Extended coil
    - "freely draining" coil
  - U_{ri}^0 independent of Mw
- SDS-PAGE
  - SDS binds in w/w ratio to protein
    - Charge ~ length or Mw
  - SDS uncoils protein
    - f ~ length or Mw
  - U_{ri}^0 independent of Mw



$\log M = a - b\, U_{ri}$

Isoelectric focussing - pI

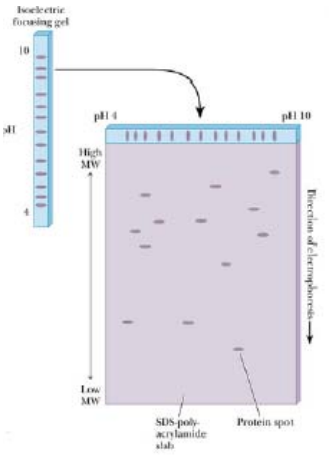
• Electrophoresis occurs through a stable pH gradient

• Proteins move through the gel until they reach the point in the pH gradient where the pH = pI

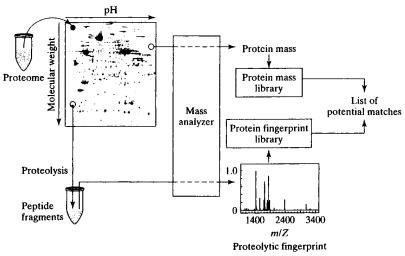
–molecules have zero net charge and don't move

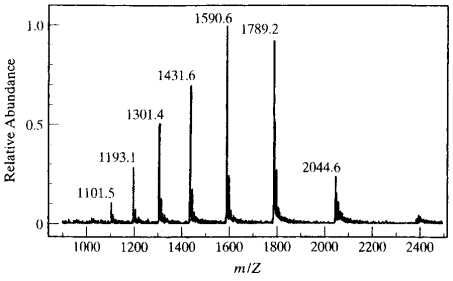
• The isoelectric point of a protein depends critically on the presence of amino acid side chains that can be

protonated/deprotonated – Asp, Glu, Lys, Arg etc



2D PAGE-MS





Mass spec measure m/Z:
Multiple charged states

$$1590.6 = \frac{(MW + nH)}{n} = \frac{(MW + n1.008)}{n}$$

$$1789.2 = \frac{(MW + [n-1]H)}{n-1} = \frac{(MW + [n-1]1.008)}{n-1}$$

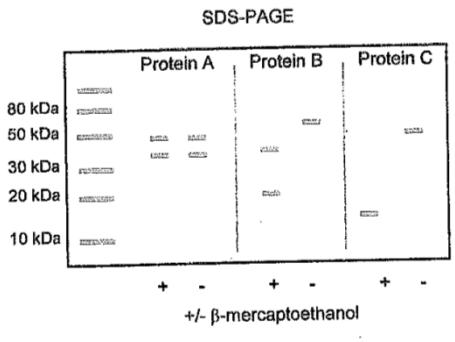
$$n = 9$$

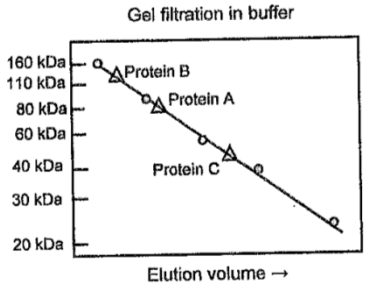
$$MW = 14{,}306 \text{ Da}$$

Draw the elution profile of the mixture from a gel filtration column.
What is the order in which you would observe the bands in an SDS PAGE gel?

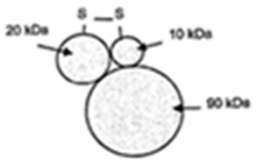
Protein	Molecular Weight (Da)	pI	glucose binding	Number of subunits
A	12,000	8.4	no	1
B	18,000	8.0	yes	1
C	32,000	4.8	no	1
D	30,000	5.2	yes	2

A solution contains a mixture five different proteins (named **ProP**, **ProQ**, **ProR**, **ProS** and **ProT**), with concentrations sufficient for 2D IEF-SDS-PAGE. Characteristics of the proteins are:
ProP – 210 amino acids total (R=7, K=4, D=8, E=12, H=1, P=9, N=2)
ProQ – 380 amino acids total(R=9, K=5, D=3, E=1, H=4, P=4, N=5)
ProR – 70 amino acids total (R=3, K=3, D=6, E=7, H=2, P=0, N=2)
ProS – 440 amino acids total (R=7, K=4, D=8, E=3, H=0, P=9, N=2)
ProT – 210 amino acids total (R=2, K=3, D=3, E=9, H=2, P=3, N=1)
The information in parentheses refers to the number of amino acid types R,K,D,E,H,P and N in each protein.
2D-gel electrophoresis (IEF & SDS-PAGE) is carried out to separate the proteins. Indicate the approximate relative final position of the 5 proteins at the completion of the 2D IEF SDG PAGE experiment on a rough sketch of a gel. You can assume that the protein mixture is loaded at the pH 7 position of the IEF gel. (On your gel indicate the direction of increasing pH for the IEF gel and the direction of migration for the SDS PAGE gel)





Protein	Mol wt from GF	Mol wt. from SDS -BME	Mol wt. from SDS +BME	S-S bonds
A				
B				
C				
Sample	120	30 and 90	10, 20 and 90	



Protein	Mol wt from GF	Mol wt. from SDS -BME	Mol wt. from SDS +BME	S-S bonds
A	90	40 and 50	40 and 50	None
B	120	60	20 and 40	Subunits connected
C	48	48	16	Interconnected
Sample	120	30 and 90	10, 20 and 90