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.

Principle

The level of Interaction (adsorption) packing material & sample A,B differs, resulting in different speeds of travel of A & B in a media (paper, column etc.)

Usually sample to be analysed is injected into a carrier (gas or liquid)

Carrier is usually inert (does not react with packing materials)

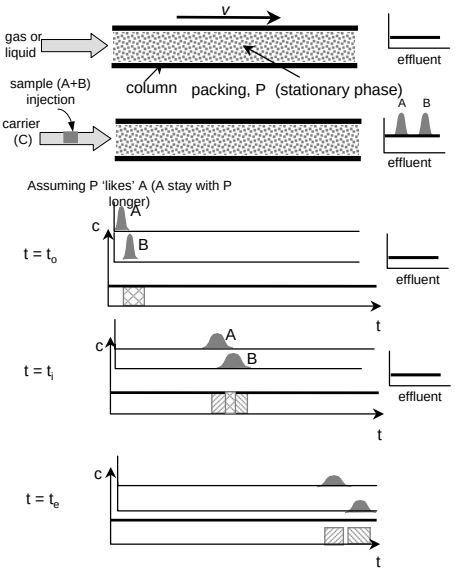
The components in sample, being separated after chromatography, are analyzed

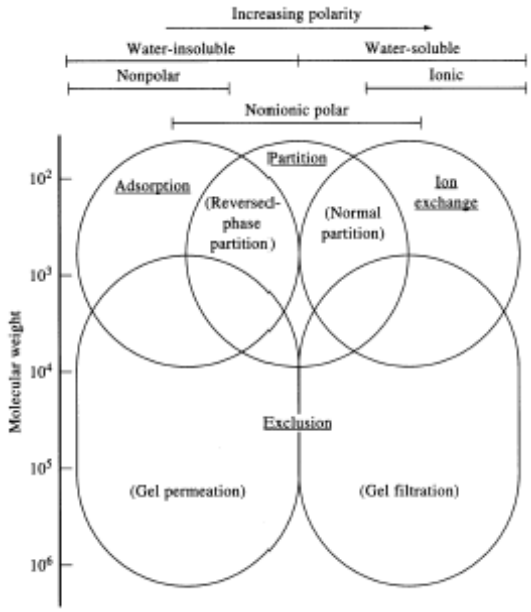
Types of chromatography

LC - Liquid (carrier & A,B) Chromatography

GC - Gas (carrier & A,B) Chromatography

HPLC - High Pressure Liquid Chromatography

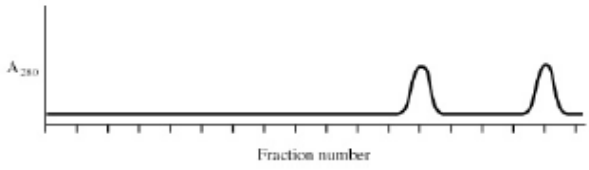




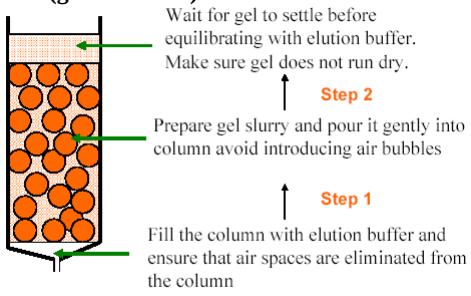
Gel-filtration chromatography: **proteins passed over a column filled with a hydrated porous beads made of a carbohydrate or polyacrylamide polymer [large molecules exit (elute) first]**

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)

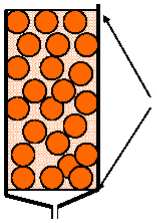


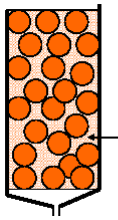
Sephadex G-50 1-30 kD
Sephadex G-100 4-150 kD
Sephadex G-200 5-600 kD
Bio-Gel P-10 1.5-20 kD
Bio-Gel P-30 2.4-40 kD
Bio-Gel P-100 5-100 kD
Bio-Gel P-300 60-400 kD
Sephadex is a trademark of Pharmacia.
Bio-Gel is a trademark of Bio-Rad.

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

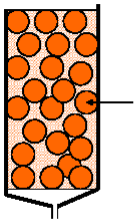




Vo or void volume

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

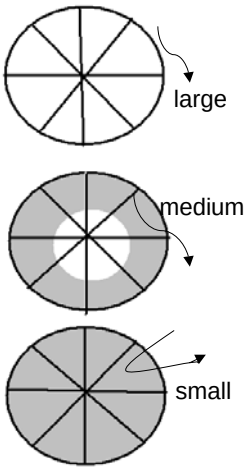
– Molecules larger than the largest pore are excluded, elute at Vo

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

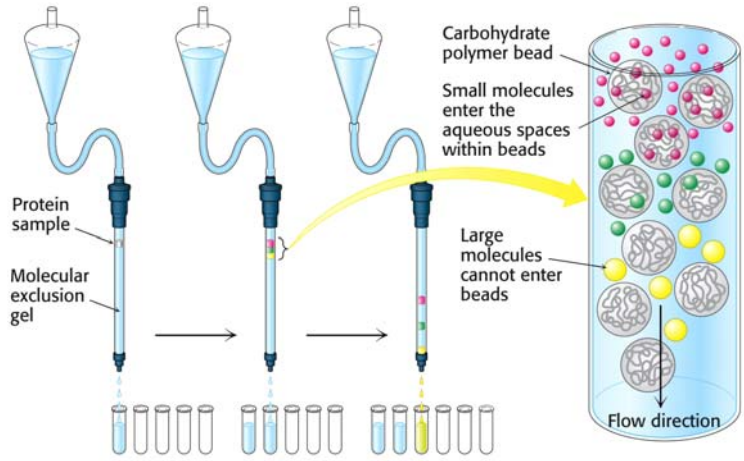
– Molecules that can occupy some but not all of the pores elute at an intermediate volume Ve

• Partition coefficient Kav = (Ve-Vo)/(Vt-Vo)

• Kav is proportional to ln (MW) in the resolving range



Gel-Filtration Chromatography – Separation based on size



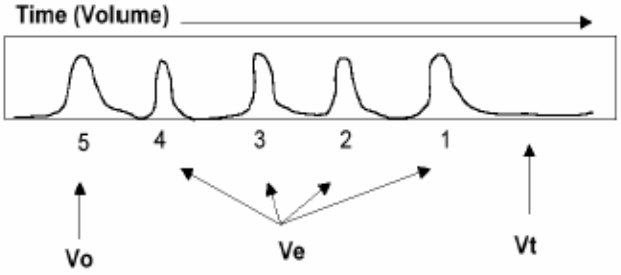
•***Determination of Molecular Weight***

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.

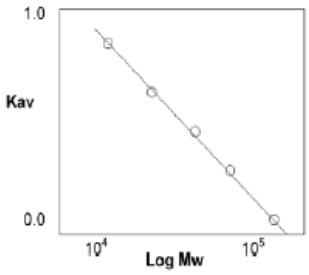
•Vt is calculated from the formula $\pi r2$ x h (or from low Mw compound such as riboflavin)



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.

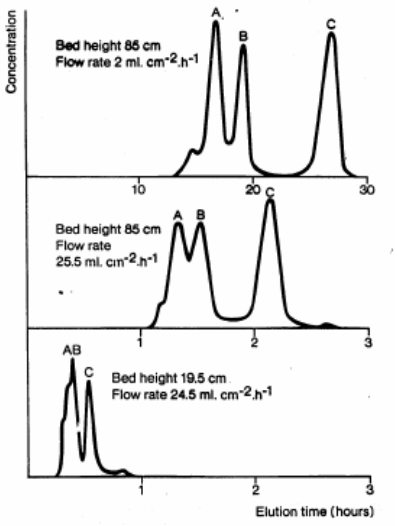


e) Now the protein of unknown molecular weight is loaded and eluted from the same column.

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:

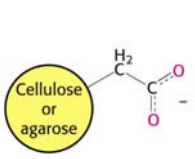
Inert Matrix—CH_2—CH_2—$NH(CH_2CH_3)^{2+}$ **di**e**t**hyl**a**mino**e**thyl (DEAE) group

Cation exchanger:

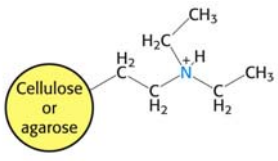
Inert Matrix —CH_2—COO^- **c**arboxy**m**ethyl (CM) group

The inert (uncharged) matrix is

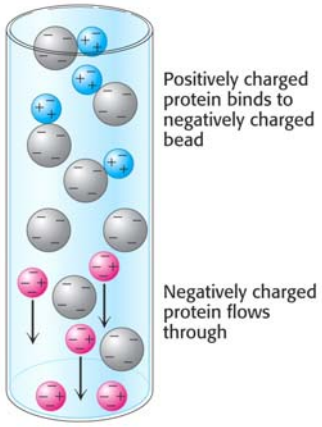
most commonly cellulose or agarose



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.

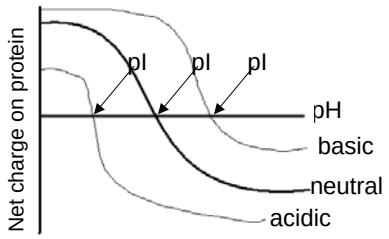
❖ The binding affinity of a protein depends on:

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.

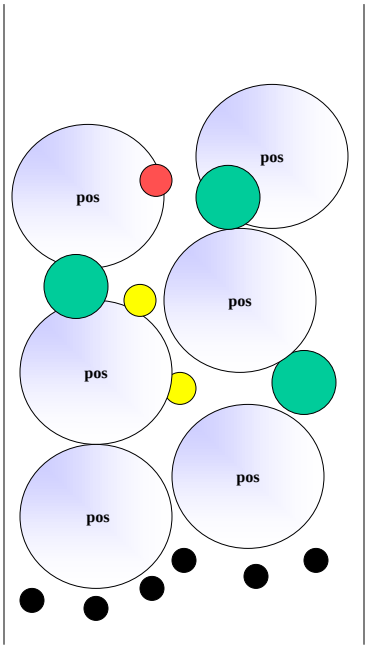
❖ A protein can be eluted from the matrix by applying a buffer at higher salt concentration (or different pH) that reduces the protein's affinity for the matrix.

pH vs pI



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

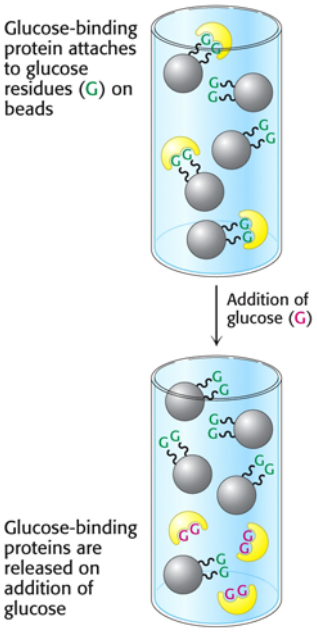
- Ion-exchange column chromatography separates proteins on the basis of charge.
- pH 7.2
- positively charged column



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:

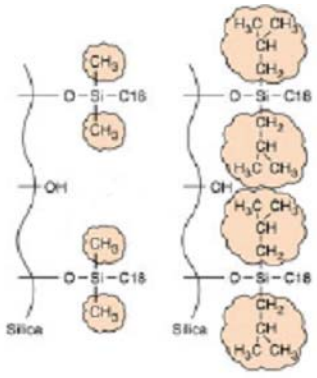
1. Decreasing salt concentration (since higher salt augments hydrophobic interactions)
2. Increasing concentrations organic solvents

High Pressure Liquid Chromatography (HPLC)

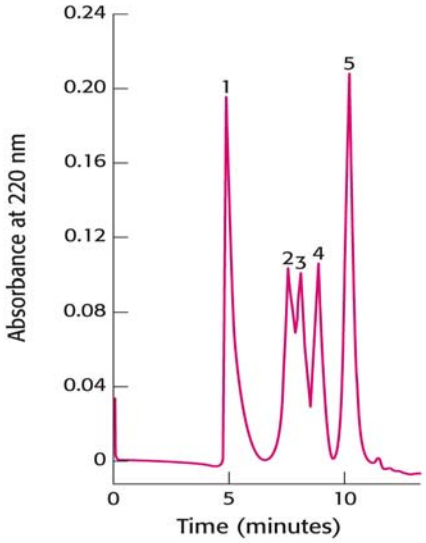
•sample is vaporized and injected;

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

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



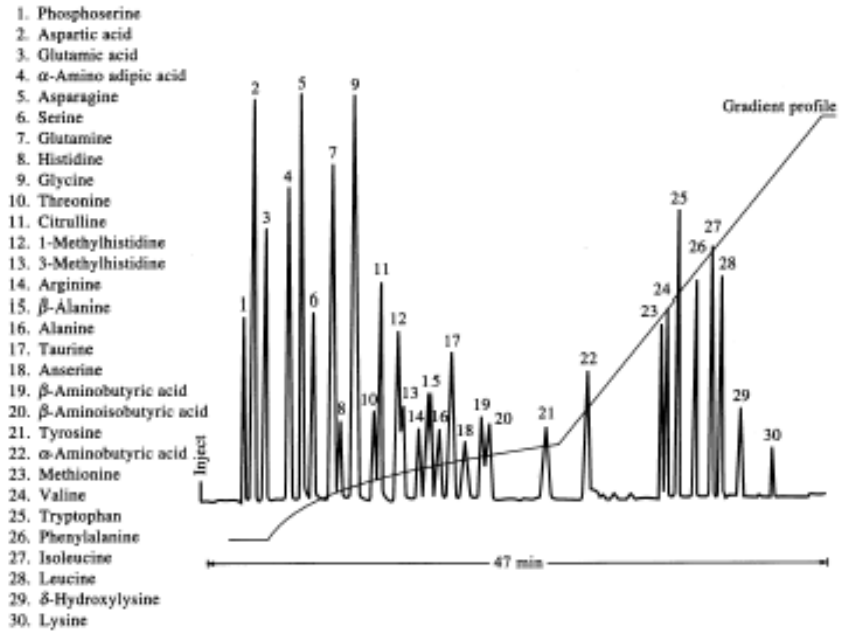
High Pressure Liquid Chromatography



High pressure limits diffusion and increases interactions with chromatography media

HPLC gives very high resolution of protein components

- HPLC Columns:
  - Stainless steel
  - 10-30 cm long
  - 4-10 mm internal diameter
  - 1-10 mm particle size - 40,000-60,000 plates/m



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

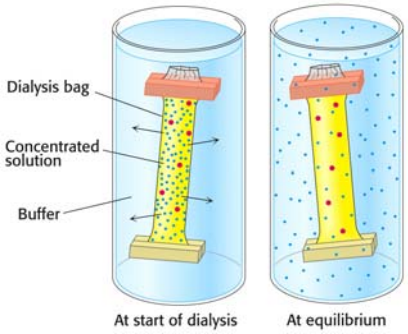
• Molecules migrate through a semipermeable membrane under pressure or centrifugal force

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

Dialysis

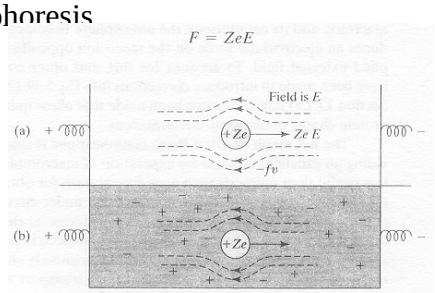
• Molecules diffuse through a semipermeable membrane if smaller than the pore size

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



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

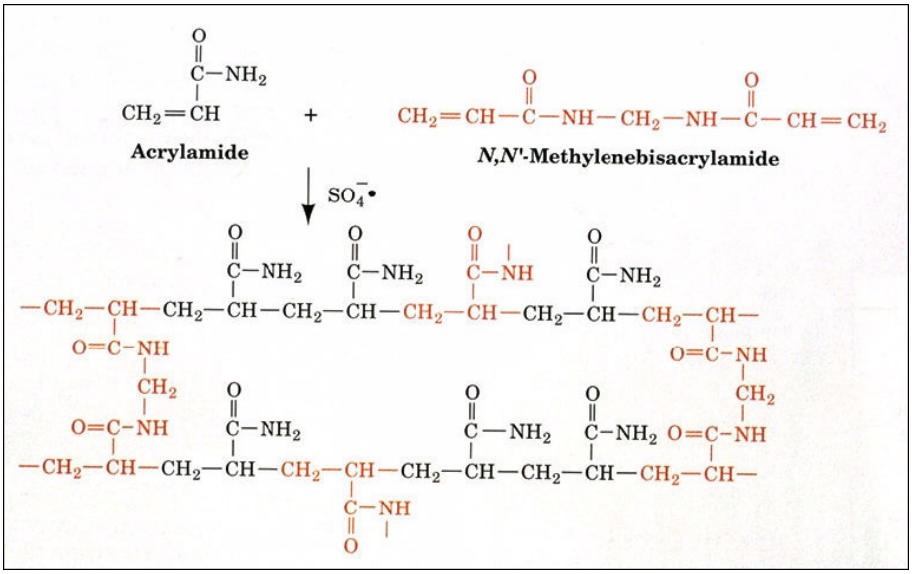


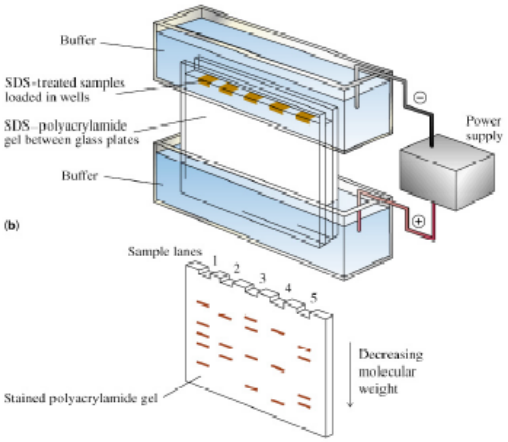
Steady motion :

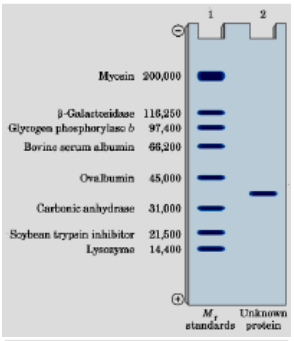
$$fv = ZeE$$

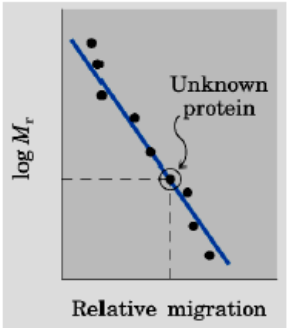
Electrophoretic mobility, U :

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



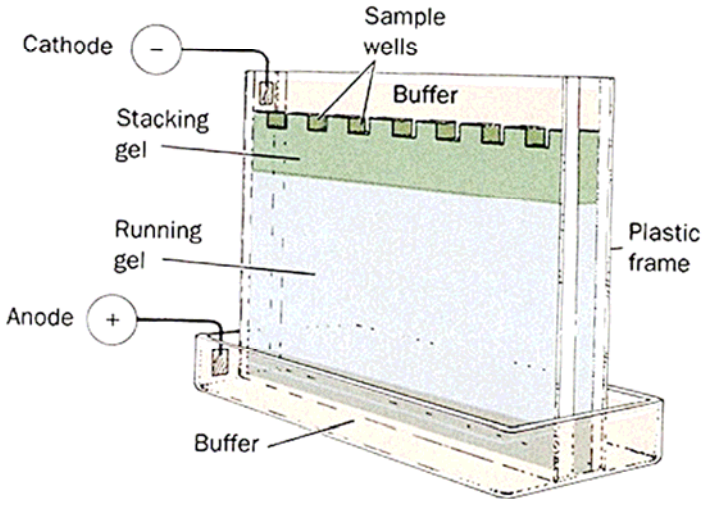






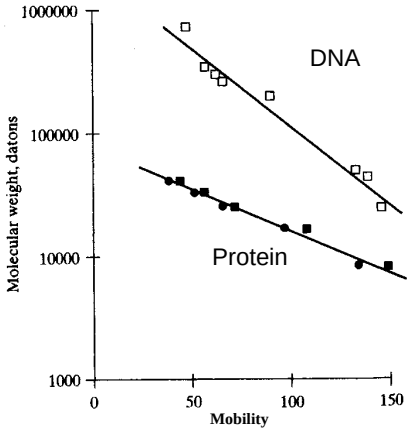
$$U_{ri} = \frac{U_i}{U_{dye}} = \frac{d_i}{d_{dye}}$$

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

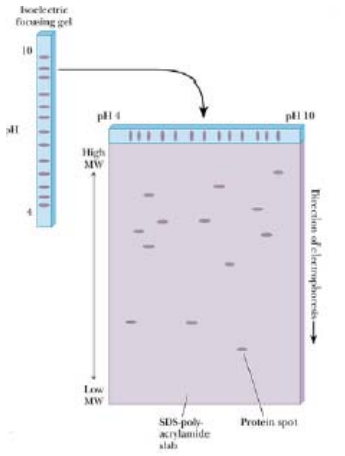
• Electrophoresis occurs through a stable pH gradient

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

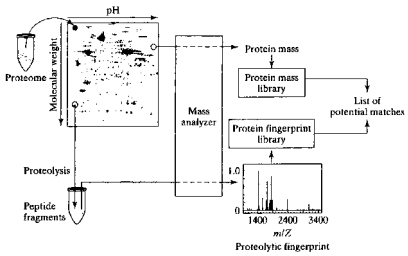
–molecules have zero net charge and don't move

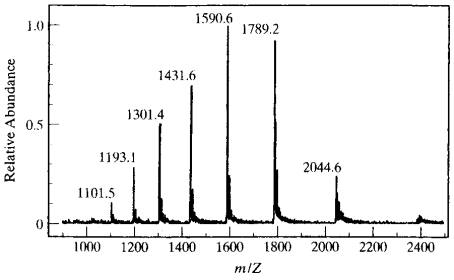
• The isoelectric point of a protein depends critically on the presence of amino acid side chains that can be

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)

Name	Monomer MW (kDa)	pI	Oligomer state
A	14	5.5	dimer
B	19	6.0	monomer
C	24	8.0	monomer
D	29	5.2	monomer
E	29	9.5	dimer
F	35	4.3	monomer
G	47	6.5	tetramer
H	47	7.5	dimer
I	62	6.0	monomer

1. Which protein would run through a Sephadex G200 column the first?
2. Which would come off second to the last?
3. Which protein would run fastest on an SDS acrylamide gel?
4. Which would run the slowest?