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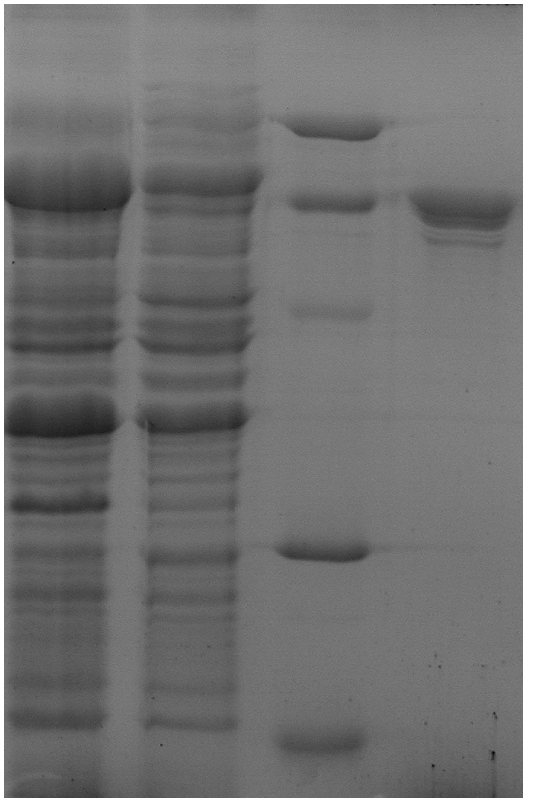
Chapter 6: Techniques of Protein and Nucleic Acid Purification



Voet & Voet: Pages 129-161

Any introductory Biochemistry textbook will cover some of these topics but few will cover these topics in the same detail

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Introduction

- **Major portion of most biochemical investigations is the purification of materials of interest**
  - **Formidable Task !**
    - **Typical cell contains thousands of different substances, many of which have closely related physical and chemical properties**
    - **Material may be unstable and/or present in vanishingly small quantities**
    - **Typical biochemical purification would be considered unreasonably difficult by most synthetic chemists**

Ability to purify materials of interest has largely driven Biochemical advances

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General Protein / Nucleic Acid Purification Strategies

Purification protocols involve multiple steps (typical) using different experimental procedures

Physical Property	Experimental Procedure	
Solubility	1. Salting in	2. Salting out
Ionic Charge	1. Ion exchange chromatography	2. Electrophoresis
	3. Isoelectric focusing	
Polarity	1. Adsorption chromatography	2. Paper chromatography
	3. Reverse-phase chromatography	4. Hydrophobic interaction chromatography
Molecular Size	1. Dialysis & Ultrafiltration	2. Gel electrophoresis
	3. Size exclusion chromatography	4. Ultrafiltration
Binding specificity	1. Affinity chromatography	

Affinity Chromatography is the most powerful technique but ... cannot be applied to all systems

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Solubility-based Purification

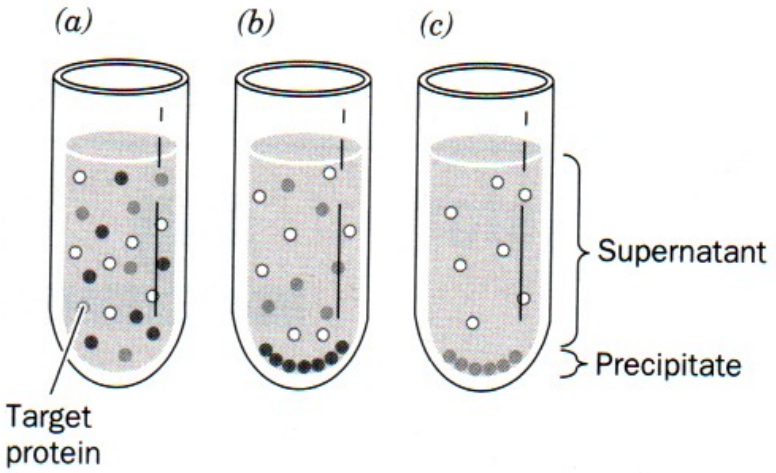
Solubilities of proteins are sensitive to ionic strength, organic solvents, pH, *etc.*

Adjust physiochemical property to just below the point at which the protein of interest precipitates

- **Contaminating proteins of lesser solubility will precipitate**

Separate soluble and insoluble material by centrifugation or filtration

Typically the first step (if used) in a protein purification



(a) mixture of 3 protein - white, gray, black

(b) solution altered – black protein precipitates

(c) solution altered again – gray protein precipitate (white protein remains in supernatant)

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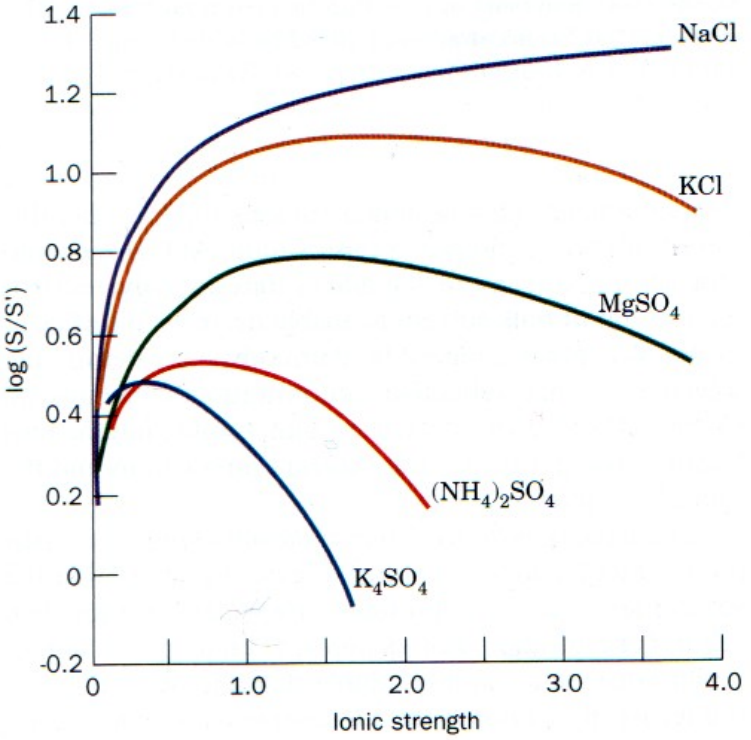
Protein Solubility and Ionic Strength

- At low ionic strength (*I*), protein solubility typically increases with increasing ionic strength (salting in)
  - Due to shielding of protein charges
- At high ionic strength, protein solubility typically decreases with increasing ionic strength (salting out)
  - Due to competition for molecules of solvation

Salting out is the basis of one of the most common purification protocols

$$I = \frac{1}{2}\sum c_i Z_i^2$$



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Chromatography

Chromatography – separation technique in which solute in the mobile phase are selectively retarded by a stationary phase

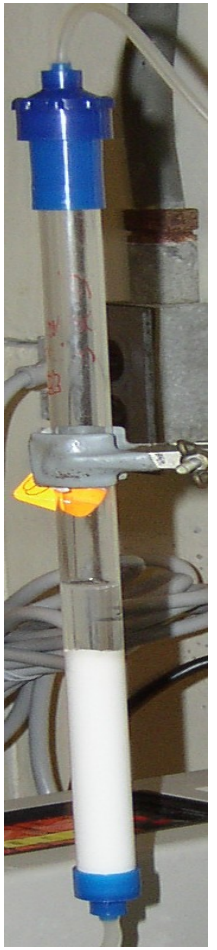
- **Solute in "mobile" phase is percolated through a column containing a "stationary" phase that exerts a retarding force**

  Mobile Phase – contains solute and percolates through stationary phase

  Stationary Phase – inert material that exerts retarding force on solutes in mobile phase

Continuous process in which sample is subject to repeated, identical separations

- **Chromatographic methods are classified according to their mobile and stationary phases (*eg.* liquid-liquid, gas-liquid)**
  - **Further classified according to retarding force exerted by stationary phase (*eg.* ion exchange, affinity)**



Chromatography column

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Ion Exchange Chromatography (IEC)

- Stationary phase contains insoluble, inert matrix (beads) uniformly coated with charged groups
  - Ions (including proteins) in the mobile phase reversibly bind to stationary phase through electrostatic interactions
  - Strength of binding depends upon pH and both the type and concentration of ions in solution
    - Competition for available binding sites on stationary phase
- **Anion exchange** – anions (mobile phase) bind cationic stationary phase
- **Cation exchange** – cations (mobile phase) bind anionic stationary phase

Typically the first chromatography step in a purification

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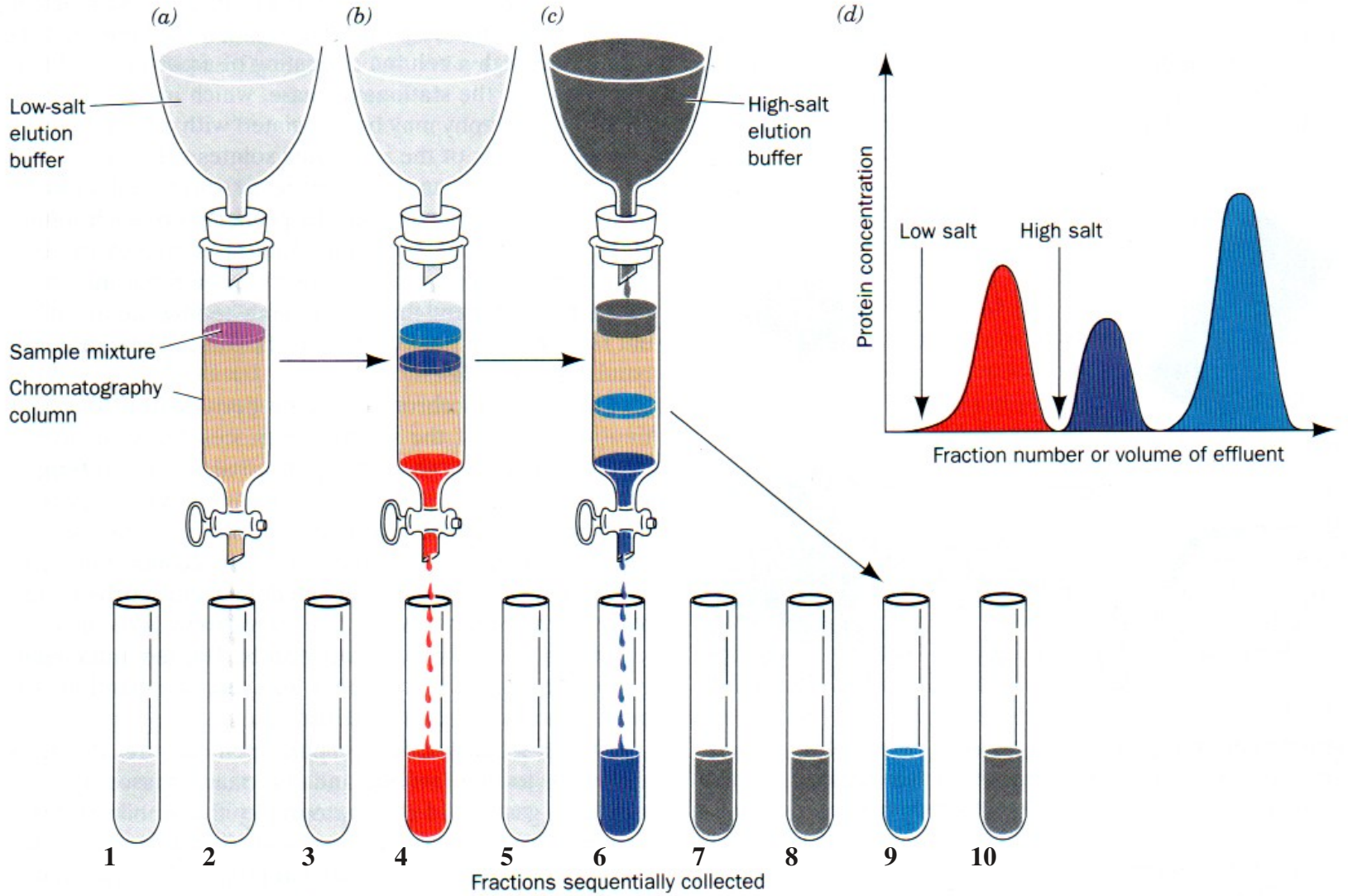
IEC and Stepwise Elution



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Size Exclusion Chromatography (SEC)

- Also known as *gel filtration chromatography* or *molecular sieve chromatography*
- Separation is based upon molecular size (and shape)
- Stationary phase contains pores that span a narrow size range
  - Large molecules cannot enter small pores and flow rapidly through column
  - Smaller molecules enter some or all pores (depending upon their size) and traverse the column more slowly

    (Note: unlike IEC, SEC slows but does not stop solute in mobile phase)
- Behavior of molecules on a particular size exclusion column can be quantitatively characterized

Typically the last chromatography step in a purification

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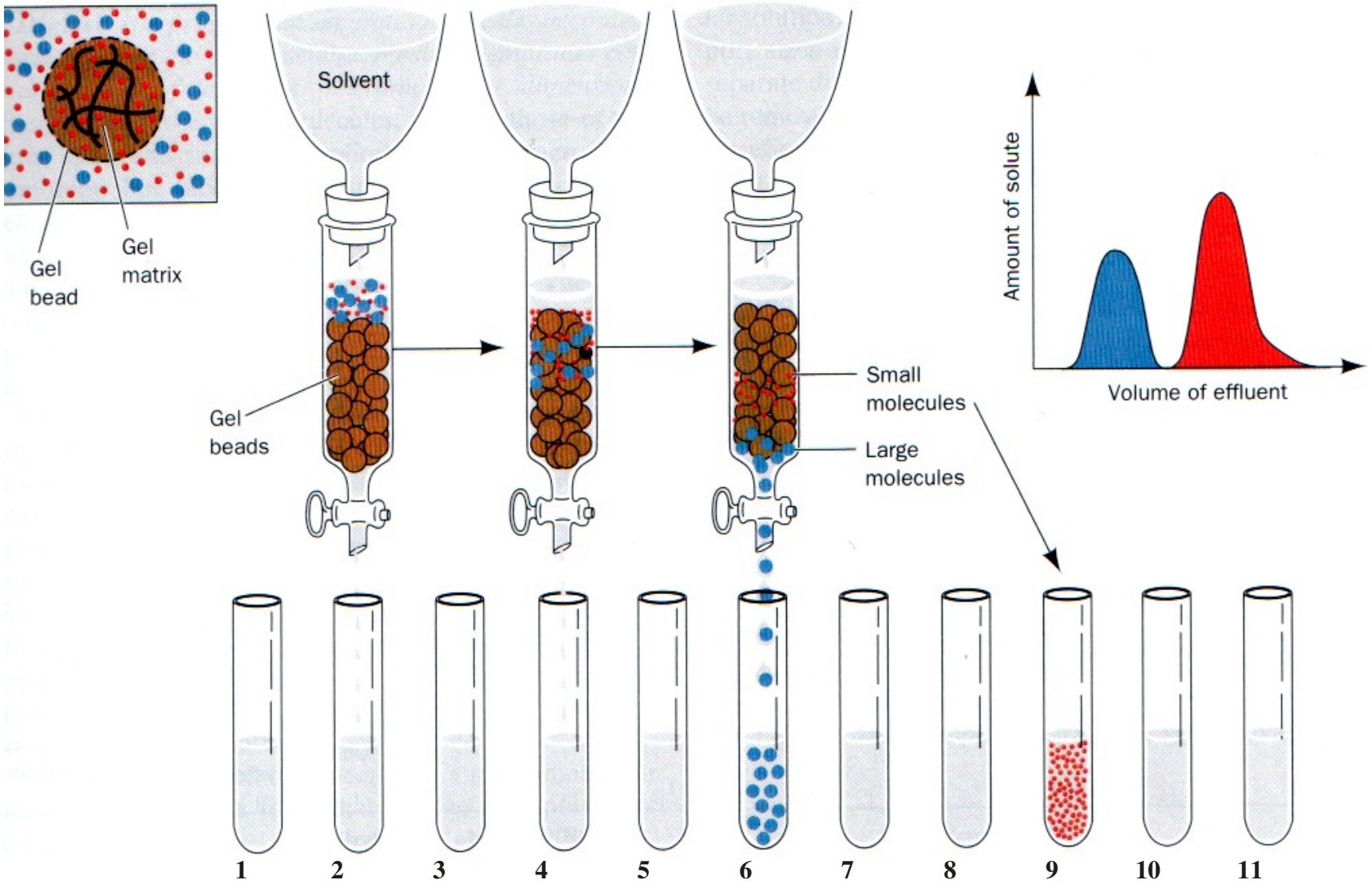
SEC



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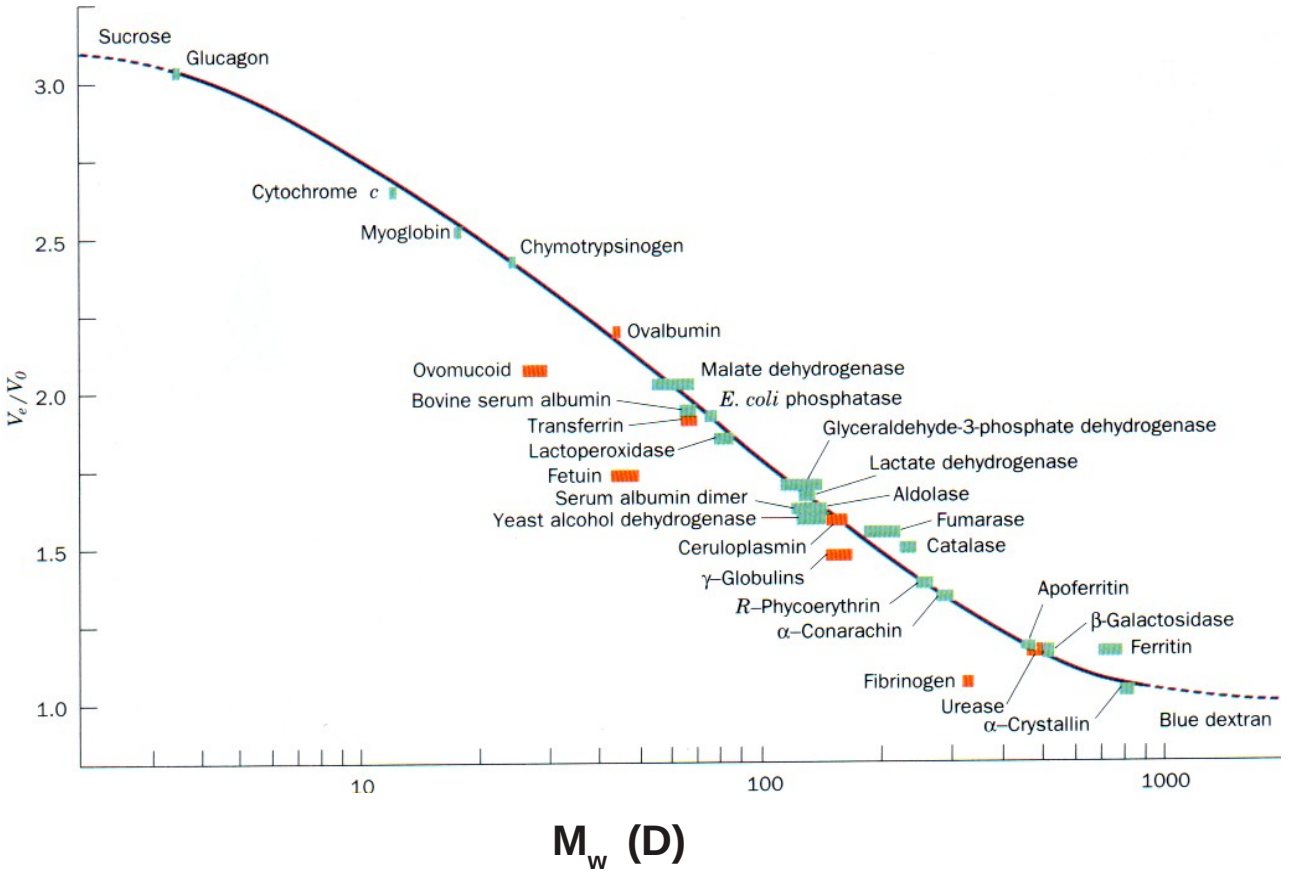
SEC and M_w



- $V_t = V_x + V_o$, where V_t is the total column volume, V_x is volume occupied by the stationary phase and V_o is void (remaining) volume
- The ratio of the elution and void volumes (V_e/V_o) varies approximately linearly with log M_w
  - Outliers on above plot tend to have highly asymmetric shapes (non-spherical)

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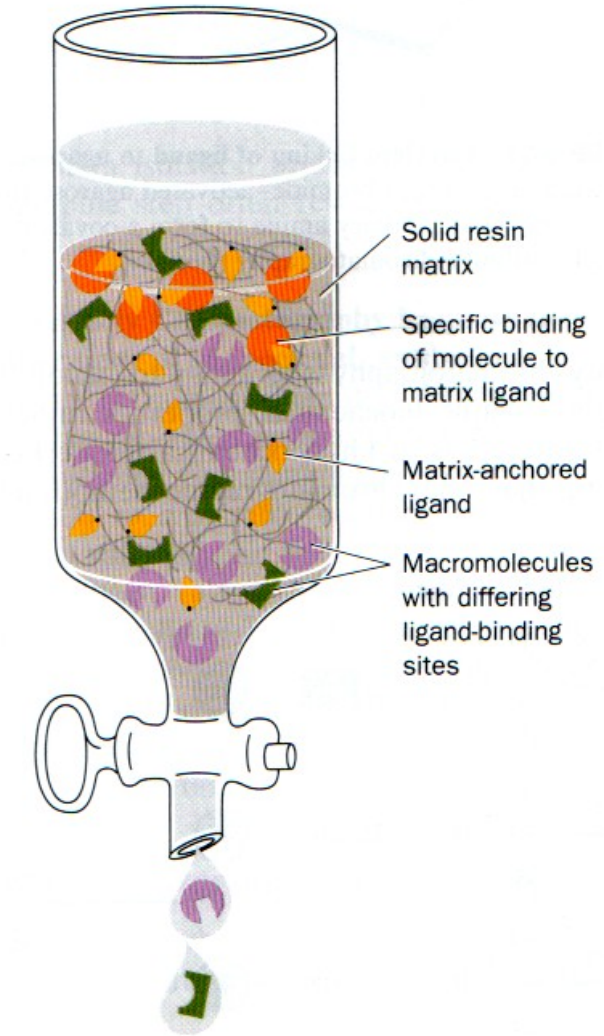
Affinity Chromatography

- Characteristic property of many proteins is their ability to tightly bind specific molecules (ligands) non-covalently
- Attaching (covalently) the ligand to an inert and porous matrix allows this property to be exploited as a purification technique
  - Only the desired protein(s) in an impure mixture will bind to the matrix
  - Desired protein can be recovered in pure form by altering the elution conditions such that the protein is released

Single-step purification in favourable cases !!



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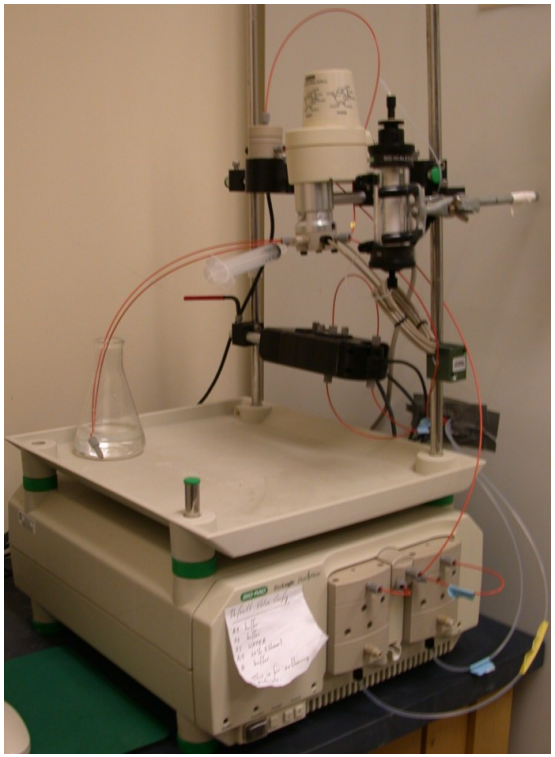
HPLC



- **High Performance Liquid Chromatography (HPLC) is an improvement to column based chromatographic techniques**
  - **Applies to all discussed chromatographic methods**
- **Improves separations using more and smaller beads of stationary phase**
  - **Increases column back-pressure necessitates the use of pumps to force the mobile phase through the column (up to 5000 psi)**
  - **Increases sensitivity by generating narrower elution "peaks"**
- **HPLC is amenable to automation and is often faster than non HPLC separations**

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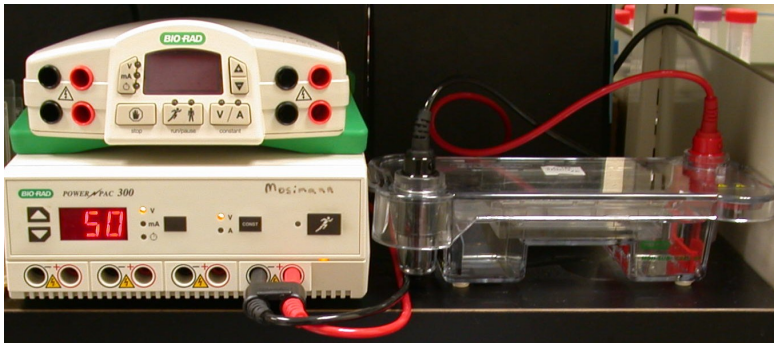
Electrophoresis

- **Electrophoresis refers to the migration of ions in an electric field**
  - **Fast, easy and cheap separation method**
  - **Not generally usable for large scale separation and recovery of samples**
- **Migration of ions including proteins depends upon both charge and the frictional coefficient, *f***
  - ***f* is size, shape and solution viscosity dependent**



$$F_{electric} = qE$$

$$F_{friction} = vf$$

$$qE = vf$$

$$\mu = \frac{v}{E} = \frac{q}{f}$$

q – molecule charge
E – electric field
v – velocity
f – frictional coeff.

μ – electrophoretic mobility

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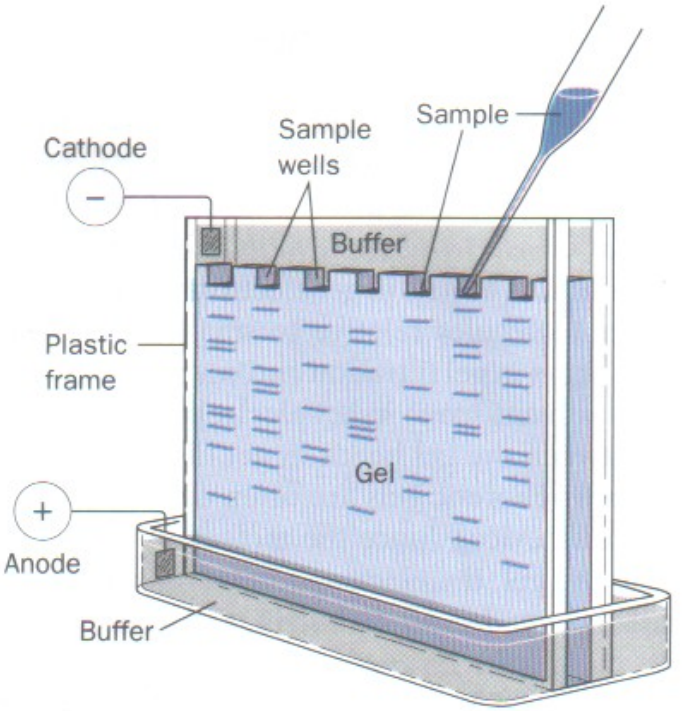
Gel Electrophoresis



Largely replaced Paper chromatography

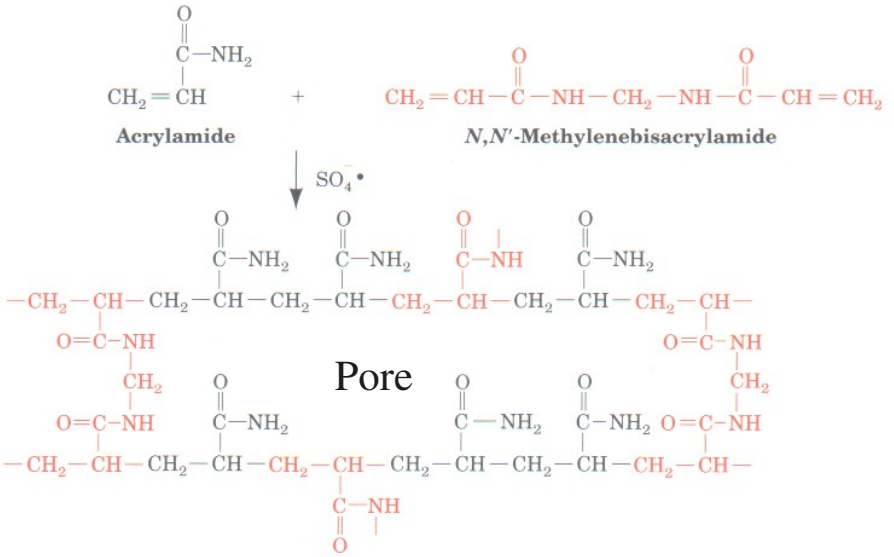
- Gel is porous and typically made of polyacrylamide (< 200 kD) or agarose (<10000 kD)

Separation is based upon both

a) Electrophoretic mobility

b) Filtration

- Since the gel is a continuous slab, large proteins are retarded relative to small (opposite of SEC)



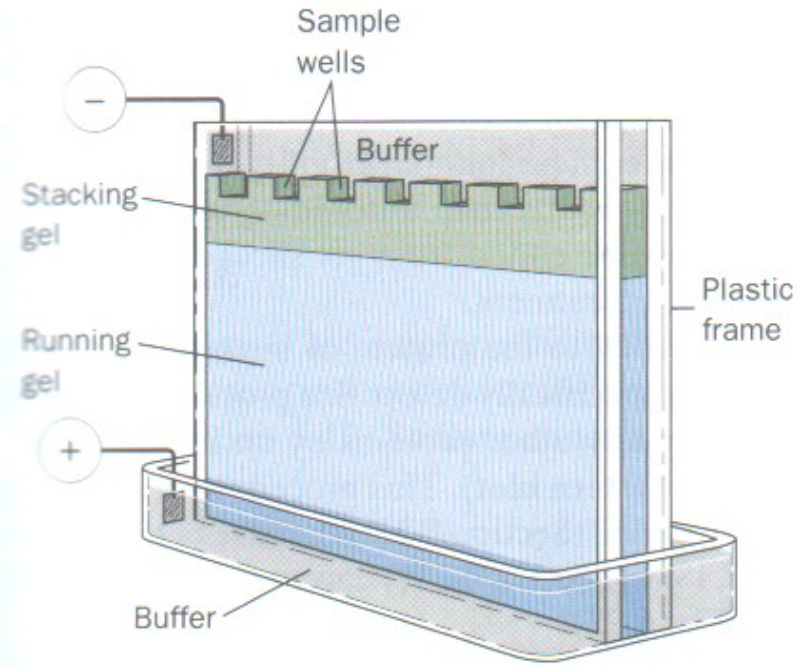
Discontinuous Gel Electrophoresis

Discontinuous gel electrophoresis is the current standard

- Utilizes second large pore “stacking” gel and buffer to generate narrow sharp “bands”

Stacking gel buffer has lower pH than Running (resolving) gel buffer

- Apply current and ions of stacking gel move into the resolving gel
- Ions of buffer are neutralized increasing the local electric field and concentrating sample
- Once in running gel the electric field is constant



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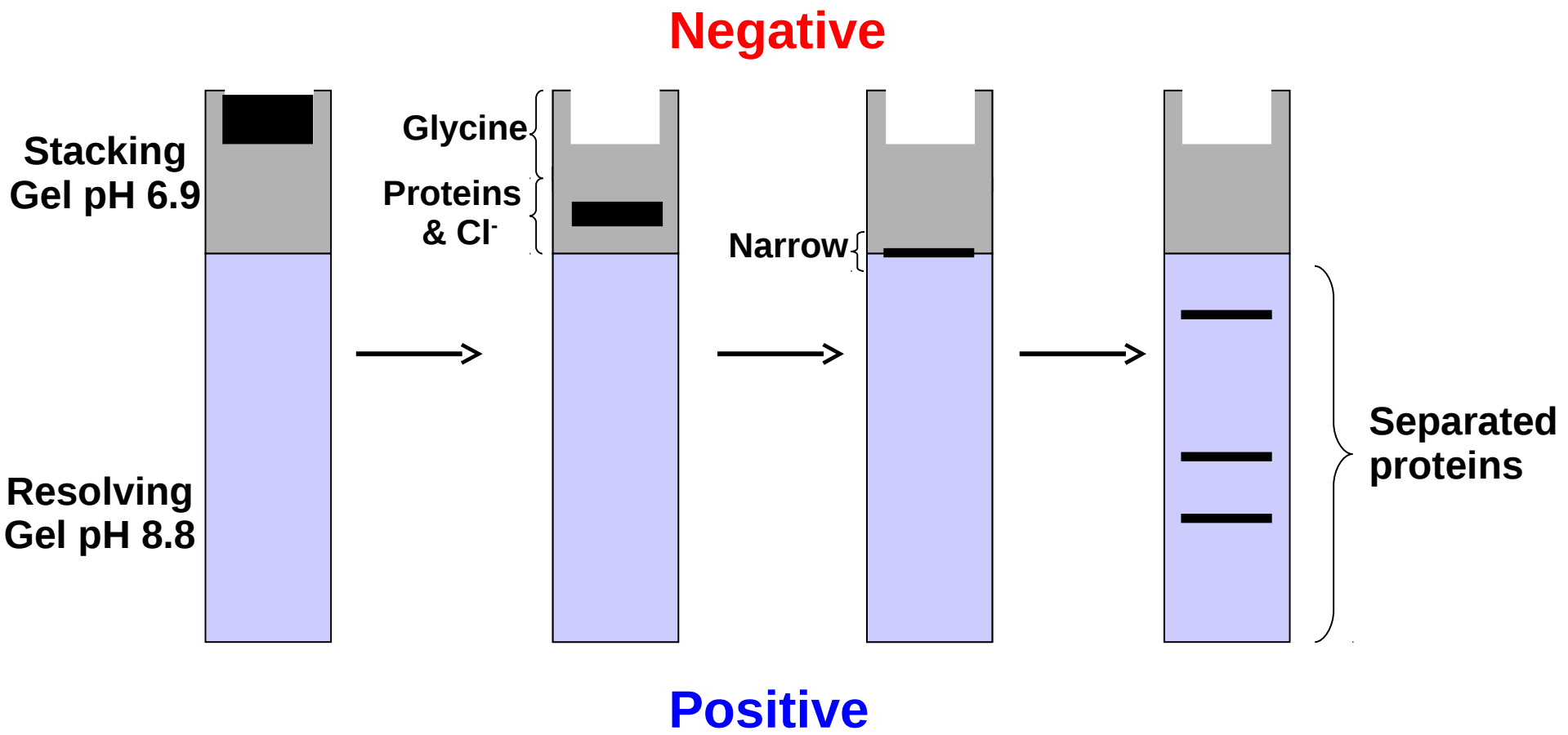
Discontinuous Gel Electrophoresis



Glycine: $H_3N^+CH_2COO^-$ $\rightleftharpoons$ $H_2NCH_2COO^-$ + H^+

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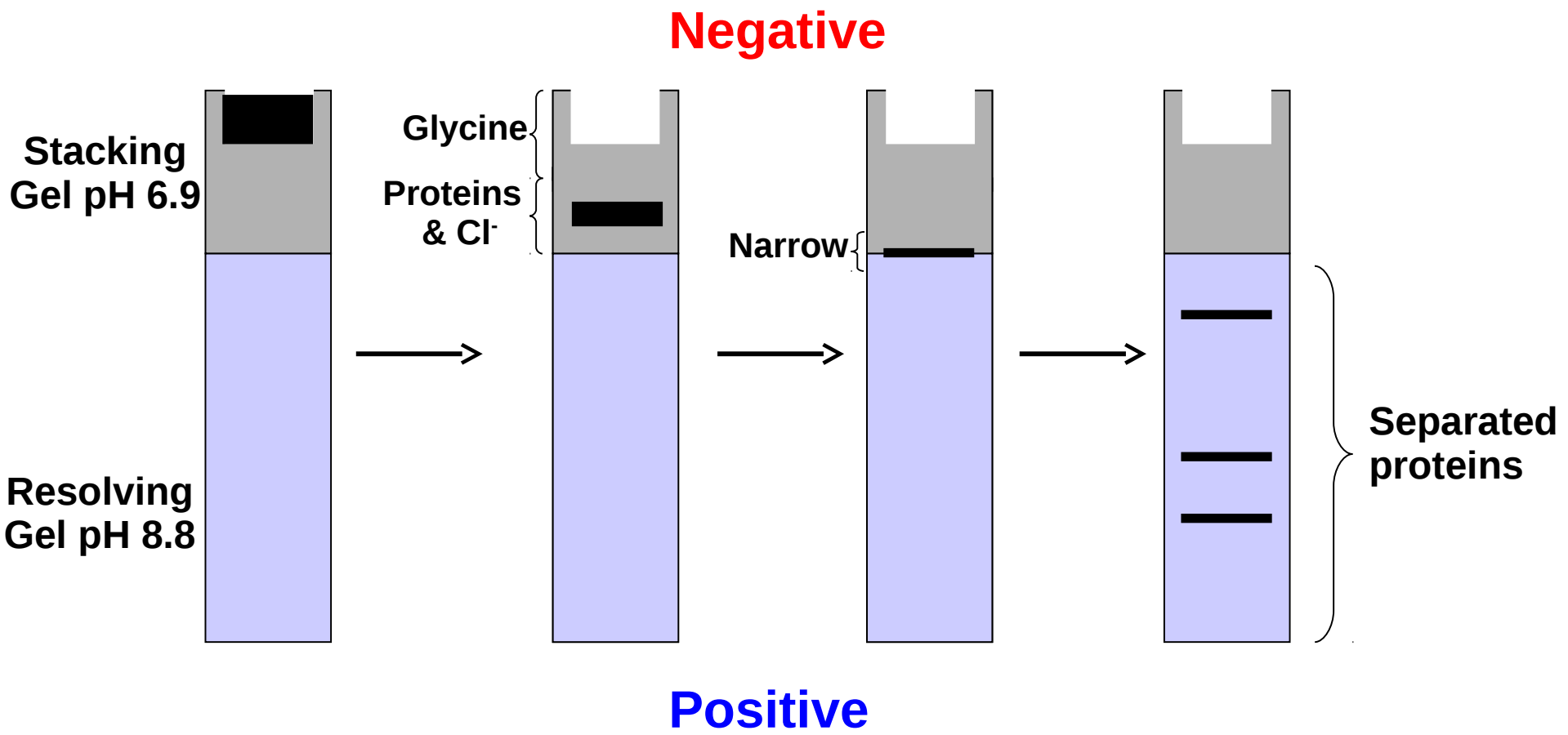
Discontinuous Gel Electrophoresis



Glycine: $H_3N^+CH_2COO^-$ $\rightleftharpoons$ $H_2NCH_2COO^-$ + H^+

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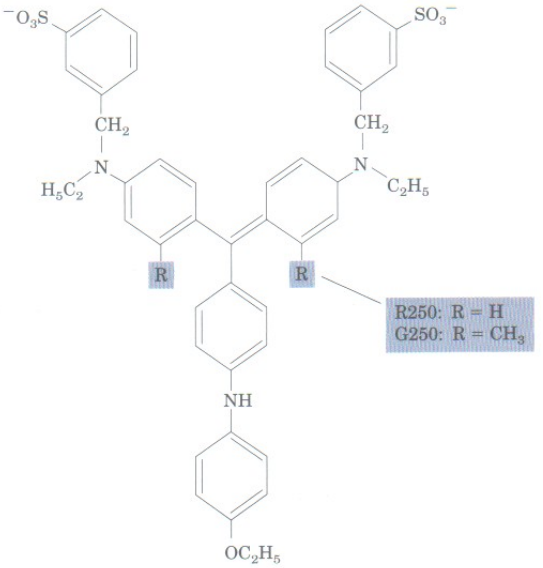
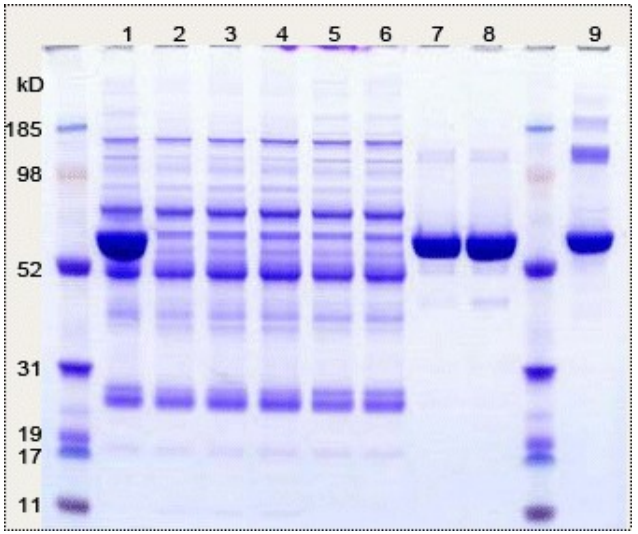
Detection Methods

- **Coomassie Stains are used to detect protein bands**
  - **Denatures protein and binds to hydrophobic core**
  - **Excess can be washed away**
  - **Detection limit is ~0.1 µg**
- **Silver Stain is up to 50x more sensitive but more expensive and difficult to apply**



Coomassie brilliant blue



Coomassie stained SDS-PAGE of Affinity Chromatography protein purification

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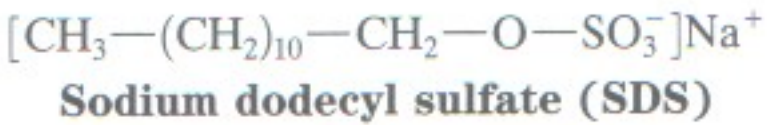
SDS-PAGE

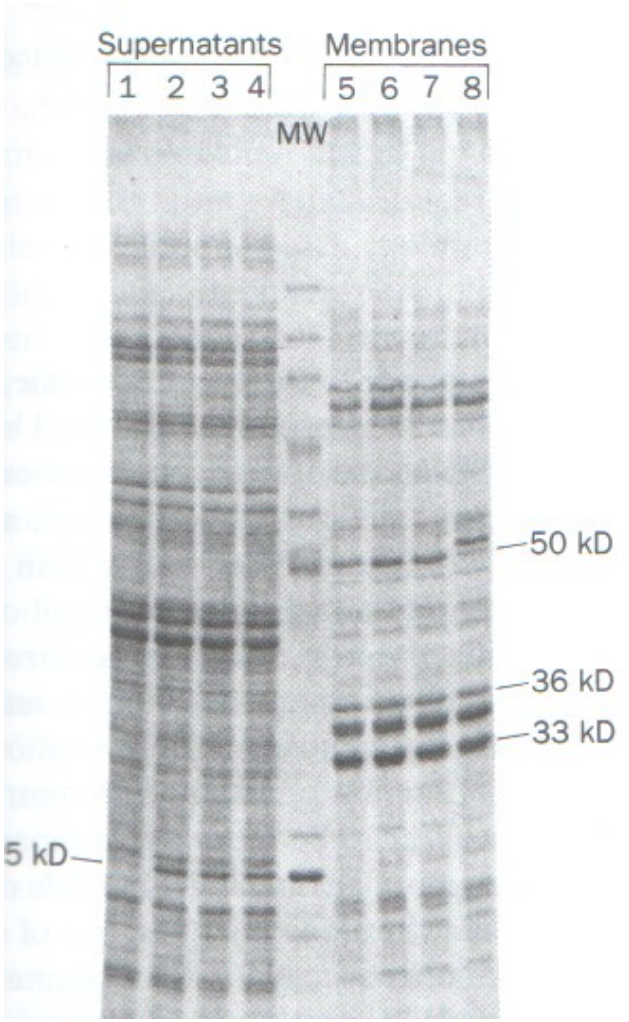
SDS is a detergent that denatures proteins and binds strongly to proteins

- **Most proteins bind SDS at a constant ratio (~ 1 SDS molecule per 2 residues)**
- **Swamps native charge of protein**
- **Results in ~ constant charge density AND similar shape for all proteins**

Separates based upon size only



Sodium dodecyl sulfate (SDS)



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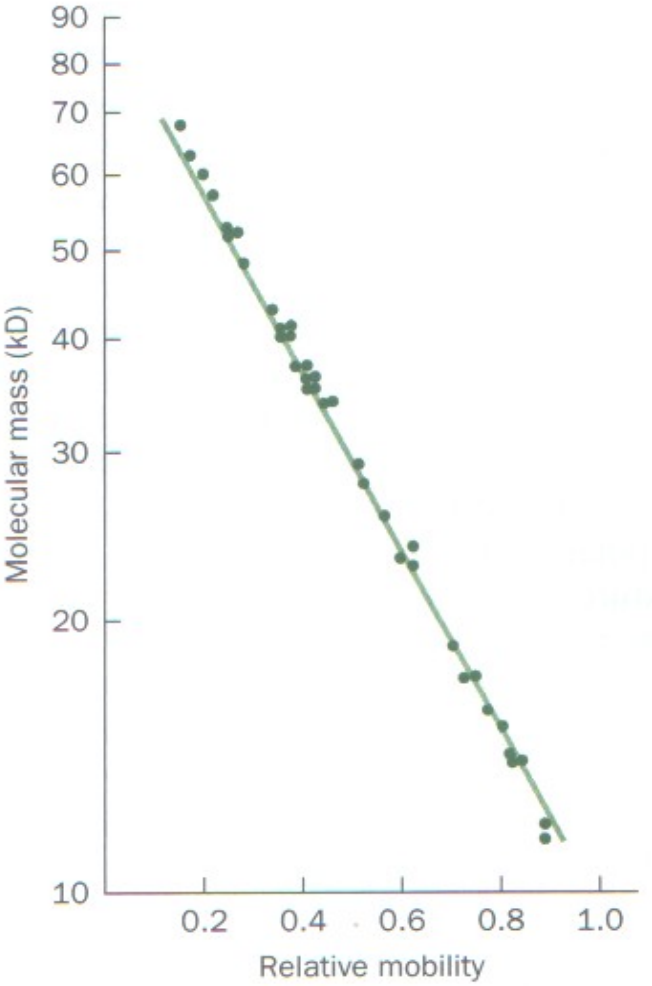
SDS-PAGE

- **Size estimates with an error of 5-10 %**
  - **Requires standard curve of relative mobility vs. Log M_w**
  - **Compare mobility of desired protein to standard curve to obtain M_w**
- **Many protein are made of more than one subunit**
  - **Subunit molecular weights since SDS is a denaturant (disrupts structure)**
- **Typically reduce disulfide bridges with β-mercaptoethanol**



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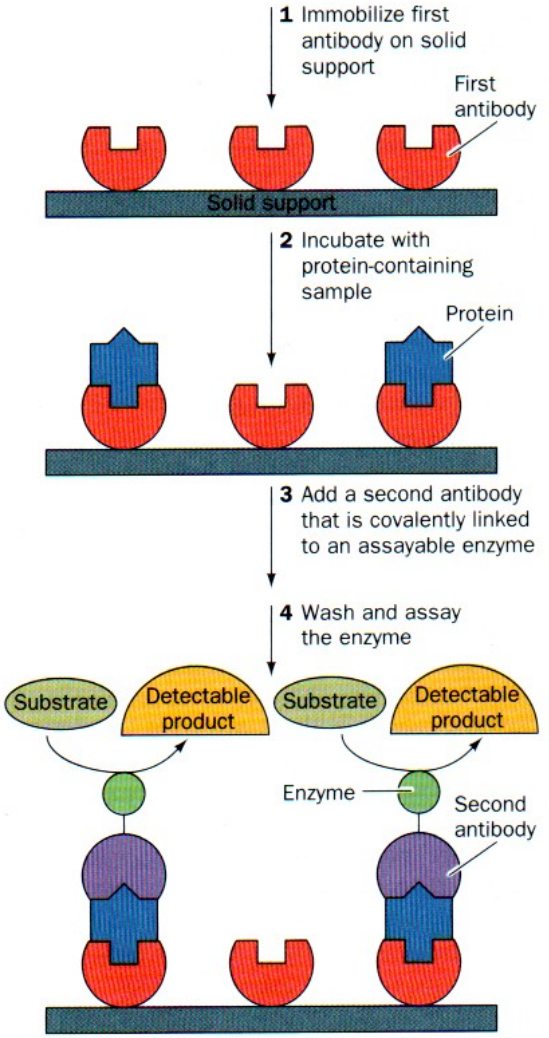
Assays

- All purification protocols require a means to quantitatively detect the macromolecules
  - Assay must be specific as many macromolecules have closely similar properties
- Functional assays are most common
  - Enzymatic activity, specific binding, observed biological activity, immunochemistry, …….



Steps 1 & 2 – Specific binding of protein of interest to known antibody (assay)

Steps 3 & 4 – Detection of binding using second known antibody