LECTURE 5:

Isolation and Purification of Enzymes

Isolation and Purification of Enzymes

The Aim of a purification procedure:

- Maximum possible yield- recovered activity
- Maximum catalytic activity – no degraded or other inactivated enzymes
- Maximum possible purity – no other enzymes or large molecules

Choice of Source

❖ A source in which the target enzyme is abundant

❖ A suitable expression system for the recombinant enzyme

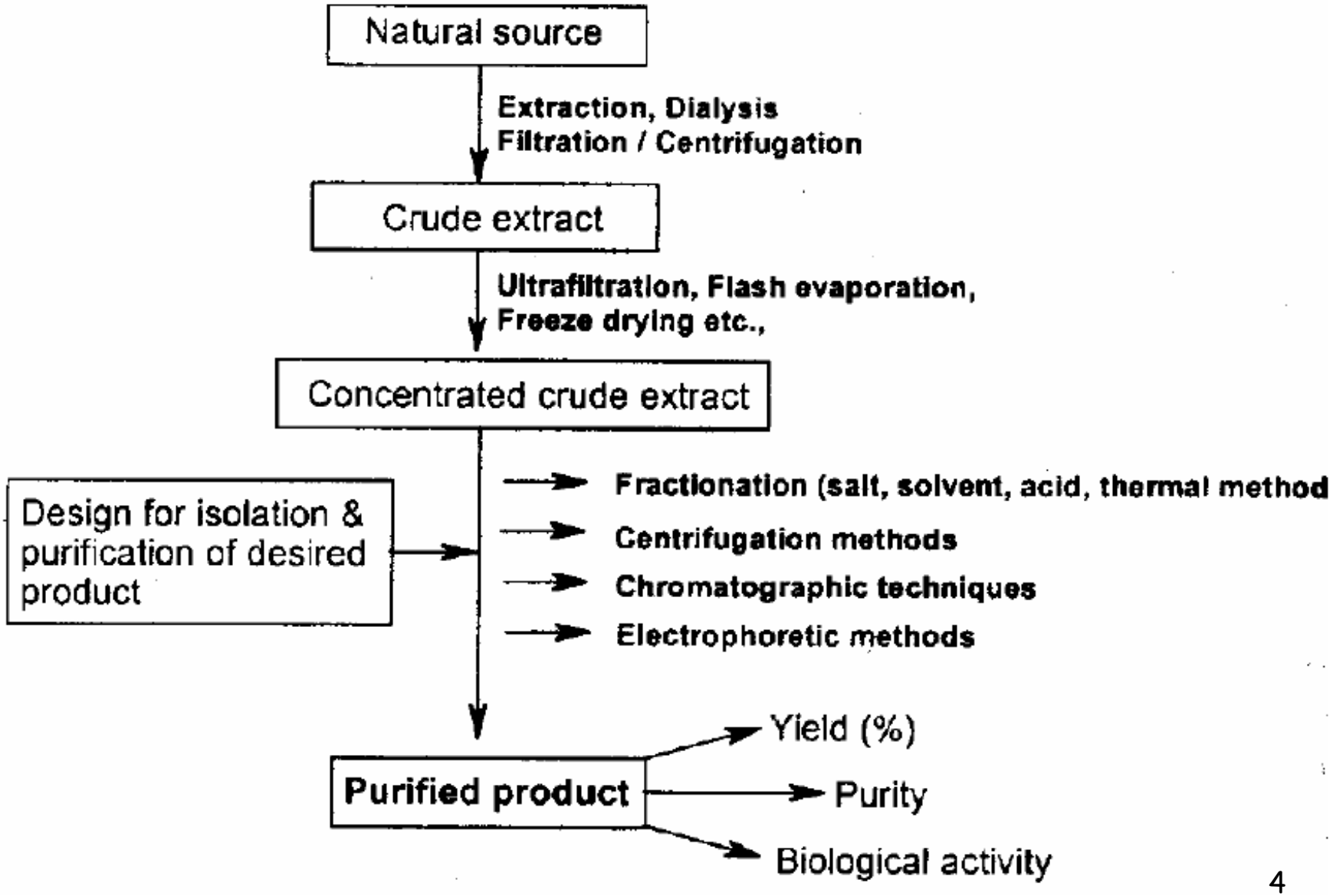
– Prokaryotes, Eukaryotes, insect cells

Enzyme source:

1. Tissue samples
2. Plant materials

3. Cultured
    - Mammalian or plant cells
    - Yeast
    - Bacteria

General Steps in Biochemical Separation



Methods of solubilization of animal cells

- **Cells can be lysed by hypotonic shock**
  - Cells with high salt inside and no salt outside will swell and rupture

- **Bacteria outer membranes must be digested**
  - Hen egg white lysozyme digests β (1-4) linkages in the (glycosidic bonds) of polysaccharides

- **Mechanical breakage blenders homogenizers**
  - French press - high pressure 20,000 lbs/in^2 forced through a small hole disrupts cells
  - Ultrasound or sonication disrupts cells

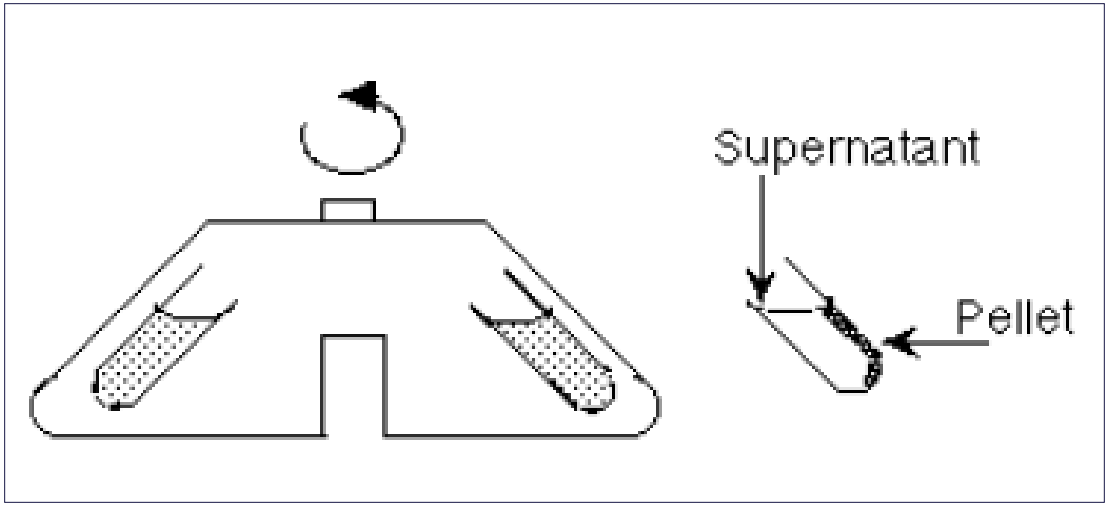
Centerifugation

A centrifuge is used to separate particles or macromolecules:

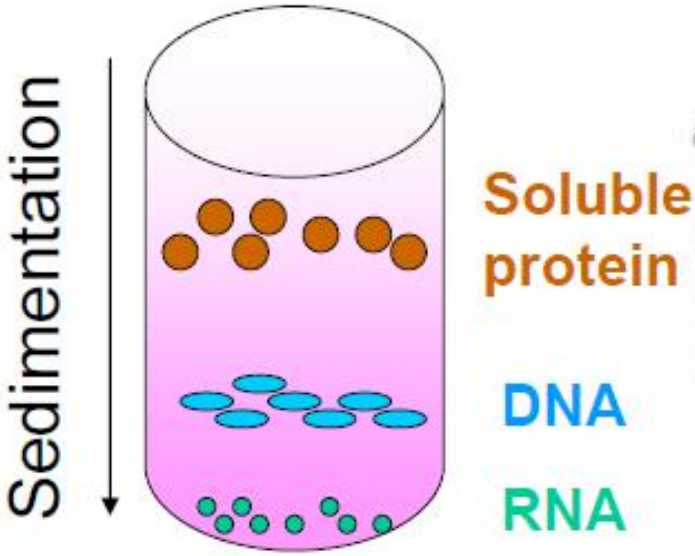
- Cells
- Sub-cellular components
- Proteins
- Nucleic acids

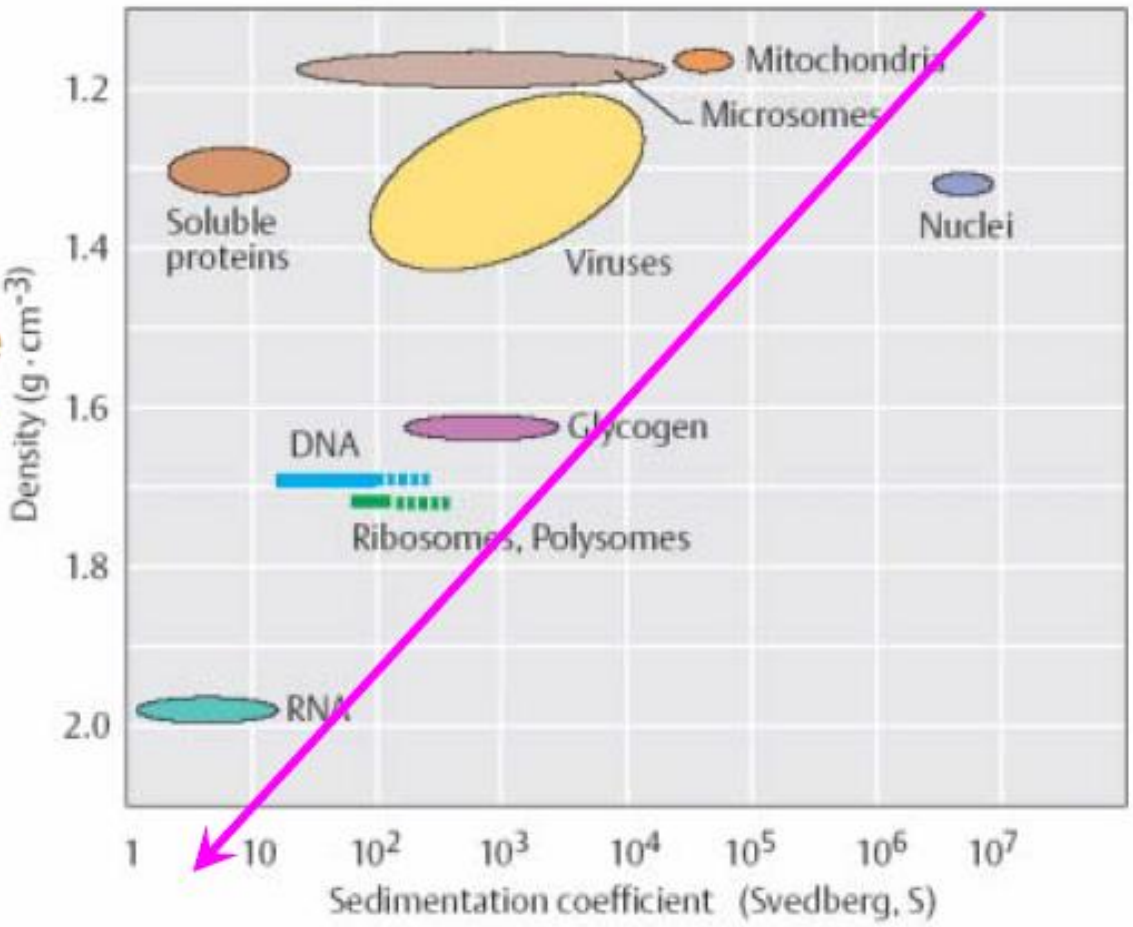
Basis of separation:

- Size
- Shape
- Density



Sedimentation coefficient values (Svedberg, **S**) of different biomolecules during centrifugation





A Summary of Centrifuge Techniques and Applications

Centrifuge Techniques	Applications
Preparative	
Velocity sedimentation centrifugation (pelleting)	Separation and isolation of particles in a solution. May be applied to precipitates, cell organelles, cells, or biomolecules.
Fractional centrifugation	Isolation of particles, based on size, by successive centrifugation at increasing rotor speeds. May be used to separate cell organelles (Figure 4.11).
Analytical	
Differential centrifugation	Sedimentation of particles in a medium of homogeneous density. Used to measure the sedimentation coefficient, *s*, and MW of a particle (Figure 4.12A).
Sedimentation equilibrium centrifugation	Used to determine MW of a macromolecule or other particle.
Density gradient centrifugation	
Zonal	Gradient is present in the tube before centrifugation and sample is layered on top. Used to isolate purified molecules and determine *s*.
Isopycnic	Gradient is formed during centrifugation. Used to isolate purified molecules and determine *s*.

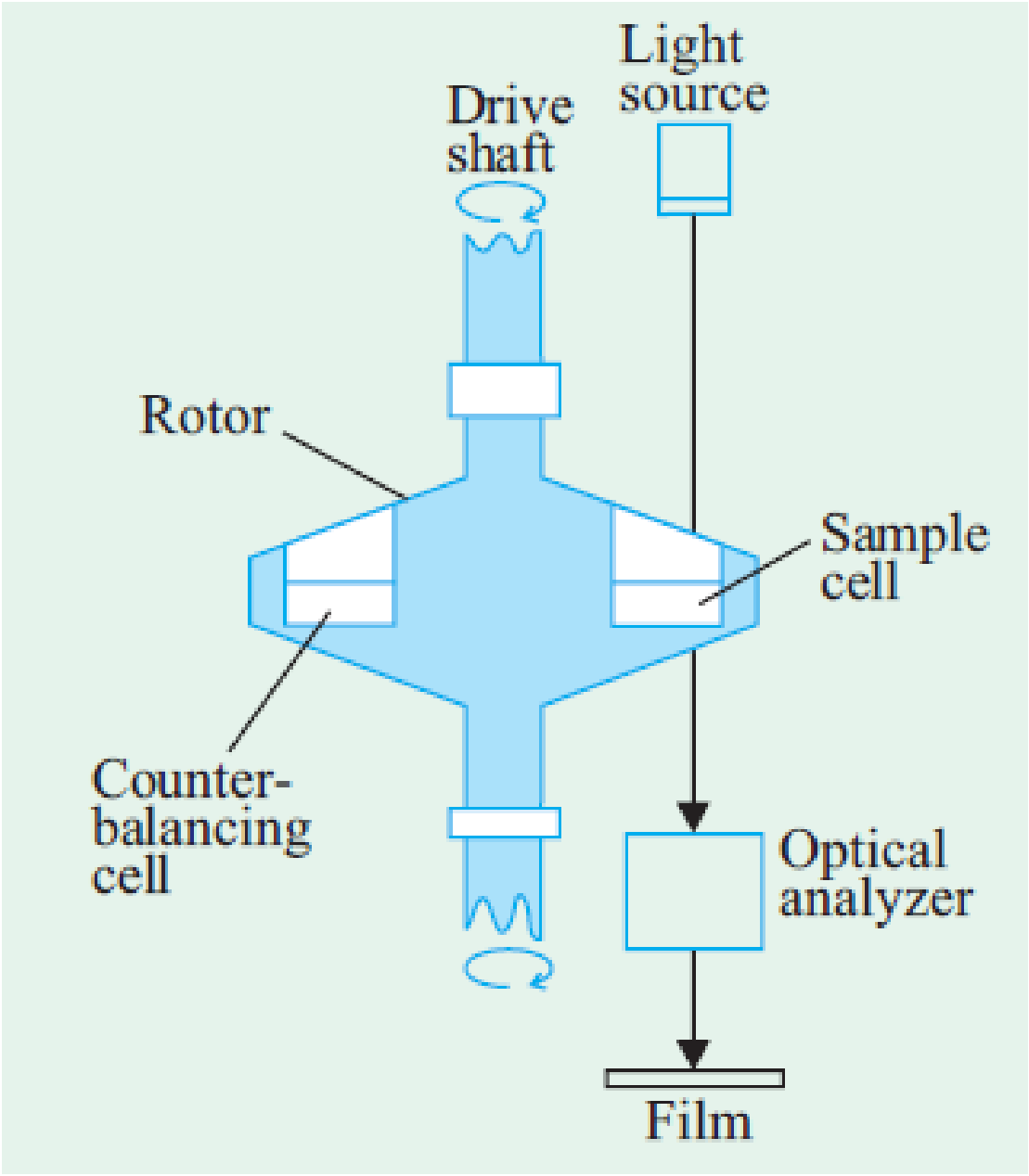
Analytical Ultracentrifugation

- An analytical ultracentrifuge spins a rotor at an accurately controlled speed and temperature
- The concentration distribution of the sample is determined at known times using absorbance measurements.

It can determine:

- Purity of macromole
- Relative molecular mass of solute
- Change in relative molecular mass of supermolecular complexes
- Conformational change of protein
- Ligand-binding study

The centrifuge rotor and method of making optical measurements



Protein Precipitation

- Once the protein is precipitated – can separate by centrifugation to form a pellet
- The pellet can be re-dissolved in buffer for further purification

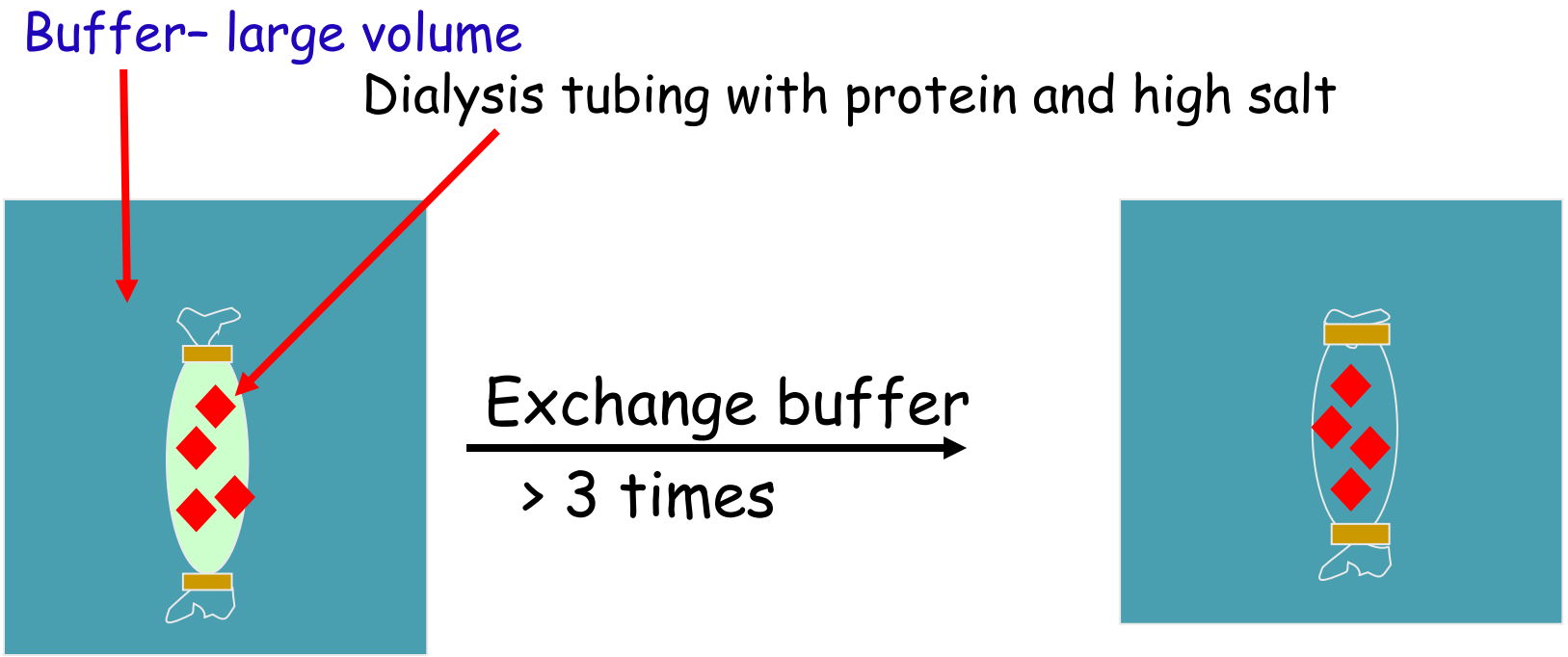
(ii) Solvent precipitation:

- Use of sovents like ethanol, acetone, Polyethyleneglycol (PEG)
- Mechanism: reduction of water for protein solvation which normally occur at pI
- Disadvantages: flammable (acetone); easily denatures proteins when conducted at temperatures above $0^{o}C$

iii) Selective denaturation of contaminant proteins heat, extremes of pH, and organic solvents

Dialysis/ Ultrafiltration

- Passage of solutes through a semi-permeable membrane.
- Pores in the dialysis membrane are of specific molecular weight cut-off that allows smaller molecules (salt) to pass



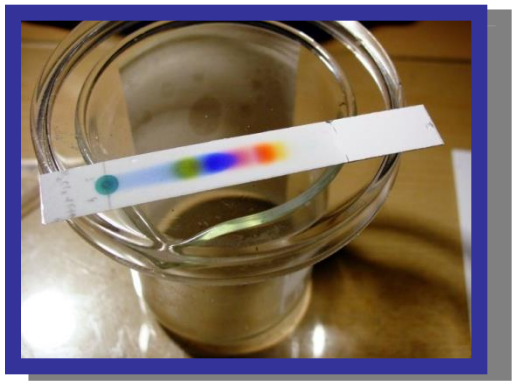
Chromatography

Planar Chromatography – the stationary phase is supported on a flat plate or paper and the mobile phase moves through the stationary phase by capillary action or by gravity

Column Chromatography – the stationary phase is held in a narrow tube through which the mobile phase is forced under pressure or by gravity

Types of Chromatography…

Paper



Thin layer

HPLC

Gas

Column

Liquid Chromatography

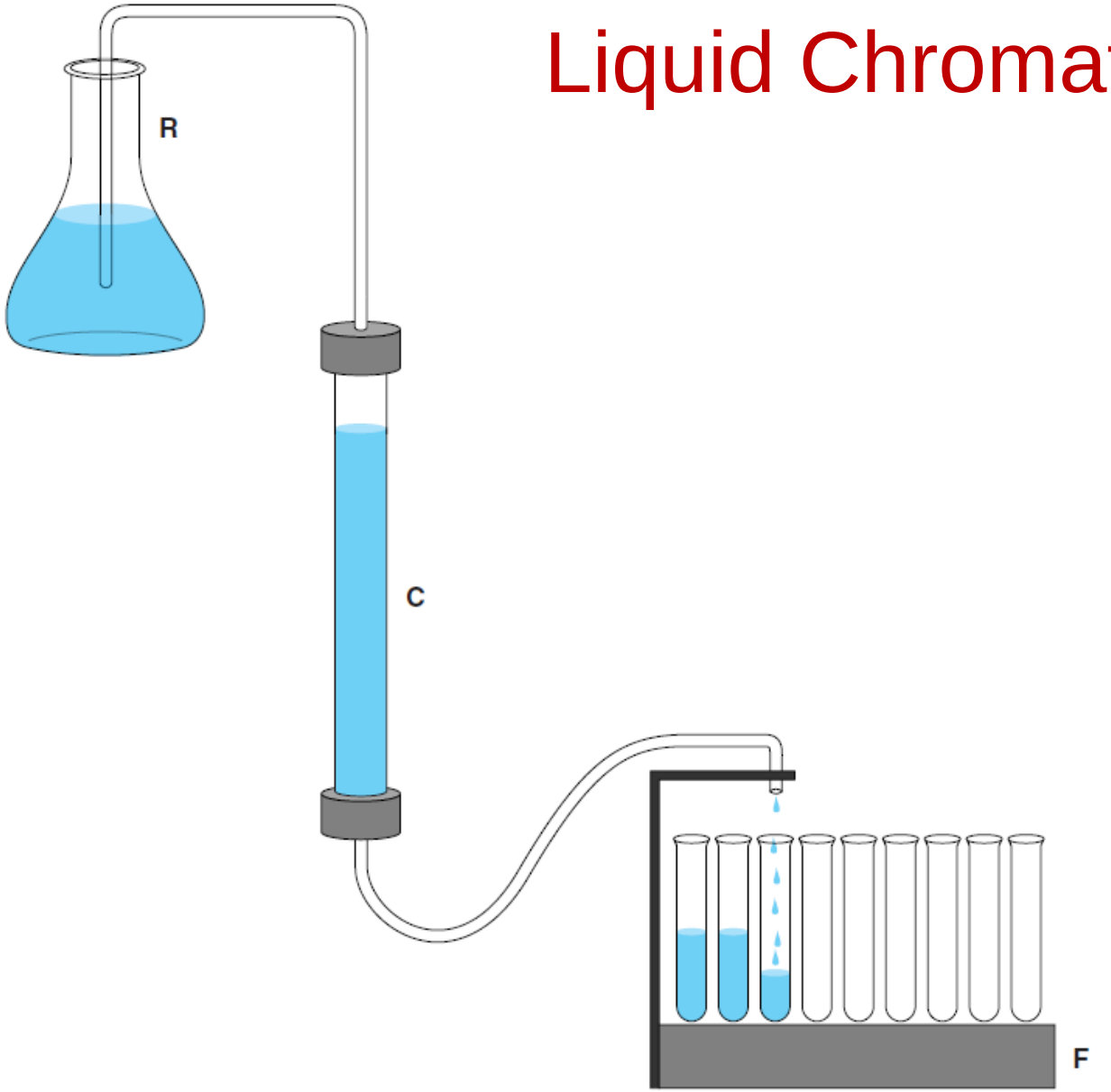


Figure 4–1. Components of a simple liquid chromatography apparatus. **R:** Reservoir of mobile phase liquid, delivered either by gravity or using a pump. **C:** Glass or plastic column containing stationary phase. **F:** Fraction collector for collecting portions, called fractions, of the eluant liquid in separate test tubes.

Mechanisms of Liquid Chromatographic Separation:

- Partitioning
- Adsorption
- Exclusion
- Ion Exchange
- Affinity

1. Gel filtration (Size Exclusion) Chromatography

- Molecules separated on the basis of size.
- The resins contain small pores; small molecules enter the pores, while larger molecules cannot
- Therefore, large molecules migrate more rapidly through a resin than small molecules

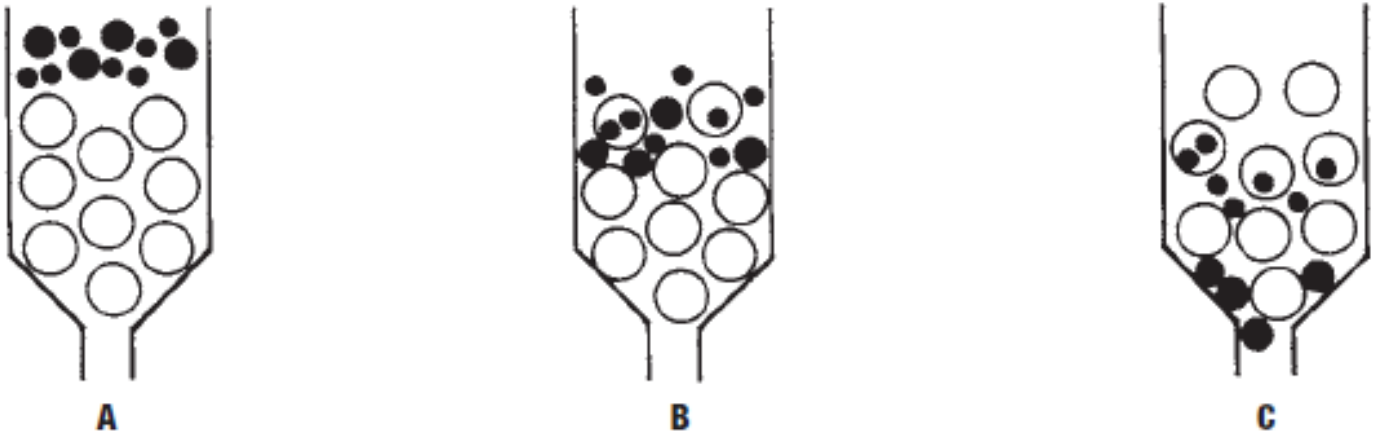


FIGURE 5.7 Separation of molecules by gel filtration. **A** Application of sample containing large and small molecules. **B** Large molecules cannot enter gel matrix, so they move rapidly through the column. **C** Elution of the large molecules first and then smaller molecules.

Proteins purified by Gel filtration

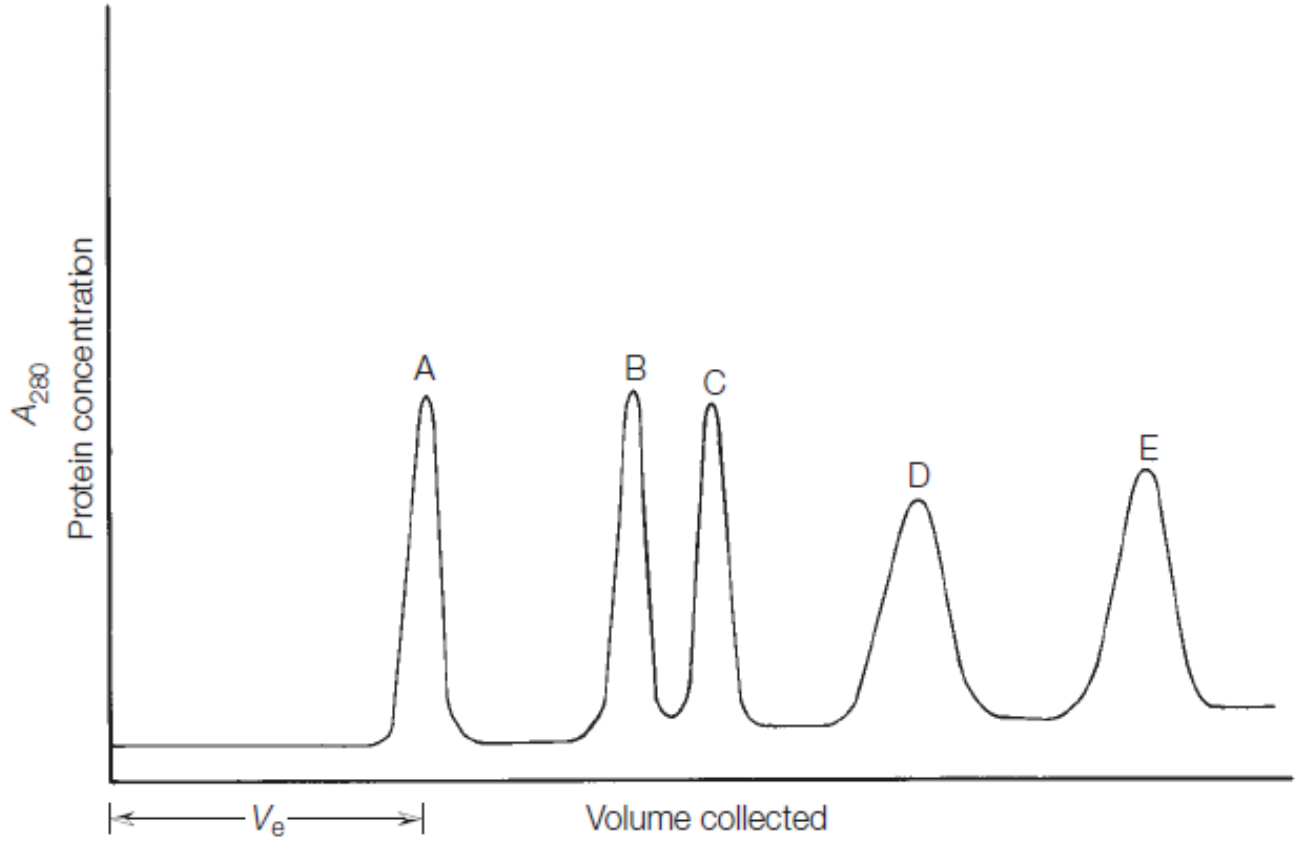


FIGURE 5.8
Elution curve for a mixture of several proteins using gel-filtration chromatography.
A = hemoglobin;
B = egg albumin;
C = chymotrypsinogen;
D = myoglobin;
E = cytochrome *c*.

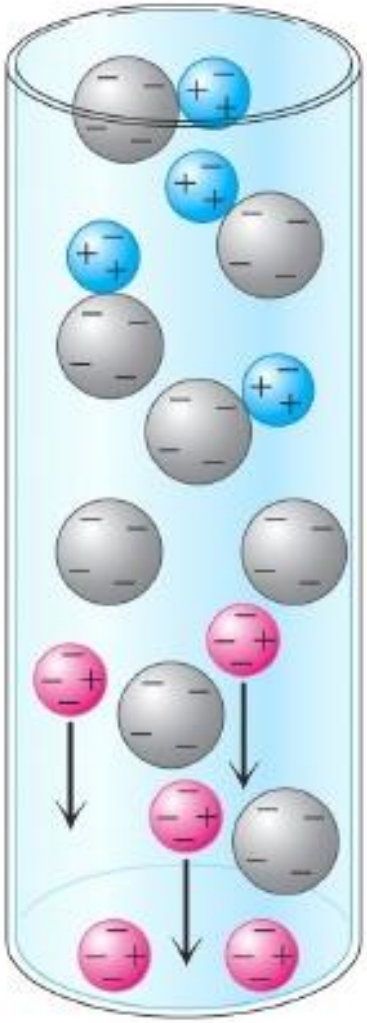
2. Ion Exchange Chromatography

- Column packing beads have covalently attached (either positively or negatively) charged groups
- **Cation exchange** matrix has negatively charged groups (e.g., carboxymethyl (CM) groups)
- **Anion exchange** matrix has positively charged groups (e.g., DEAE (diethylaminoethyl) groups)

Anion exchangers	**Functional group**
diethyl-aminoethyl (DEAE)	$-OCH_2CH_2N^+H(CH_2CH_3)_2$
quaternary aminoethyl (QAE)	$-OCH_2CH_2N^+(C_2H_5)_2CH_2CH(OH)CH_3$
quaternary ammonium (Q)	$-CH_2N^+(CH_3)_3$
Cation exchangers	**Functional group**
carboxymethyl (CM)	$-OCH_2COO^-$
sulfopropyl (SP)	$-CH_2CH_2CH_2SO_3^-$
methylsulfonate (S)	$-CH_2SO_3^-$

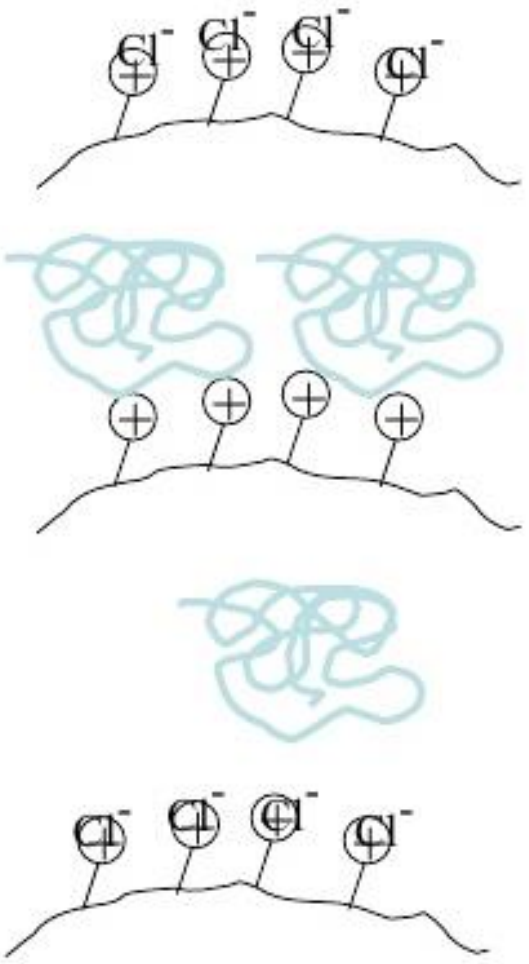
Table 6.I. Functional groups of ion exchangers

Ion exchange chromatography

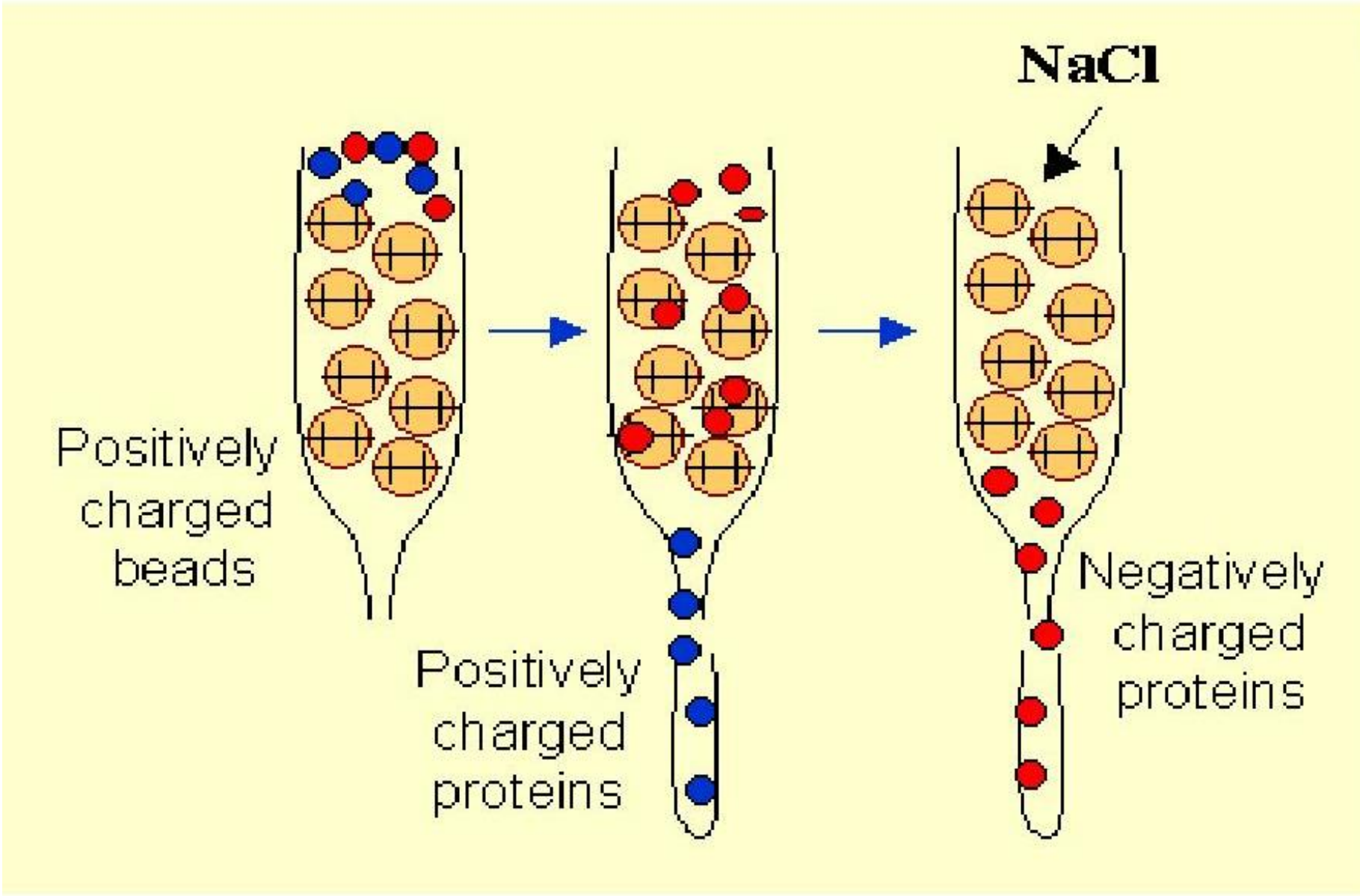


Positively charged protein binds to negatively charged bead

Negatively charged protein flows through



Ion – exchange Chromatography



3. Hydrophobic interaction chromatography

• Biomolecules separated based on their tendency to associate with a stationary phase matrix coated with **hydrophobic groups** (eg, phenyl Sepharose, octyl Sepharose).

•Proteins with exposed hydrophobic surfaces adhere to the matrix via hydrophobic interactions that are enhanced by a mobile phase of high ionic strength.

• A high salt concentration (1-1.5 M neutral salt) used facilitates the interaction between the hydrophobic chromatographic medium and the hydrophobic patches present on protein molecules

• During separation, the decrease in salt concentration will lead to the elution of bound molecules

4. Affinity Chromatography

- A high-selectivity separation of biomolecules achieved through their specific interactions

- Based on the biological function or the unique chemical structure of a given biomolecule

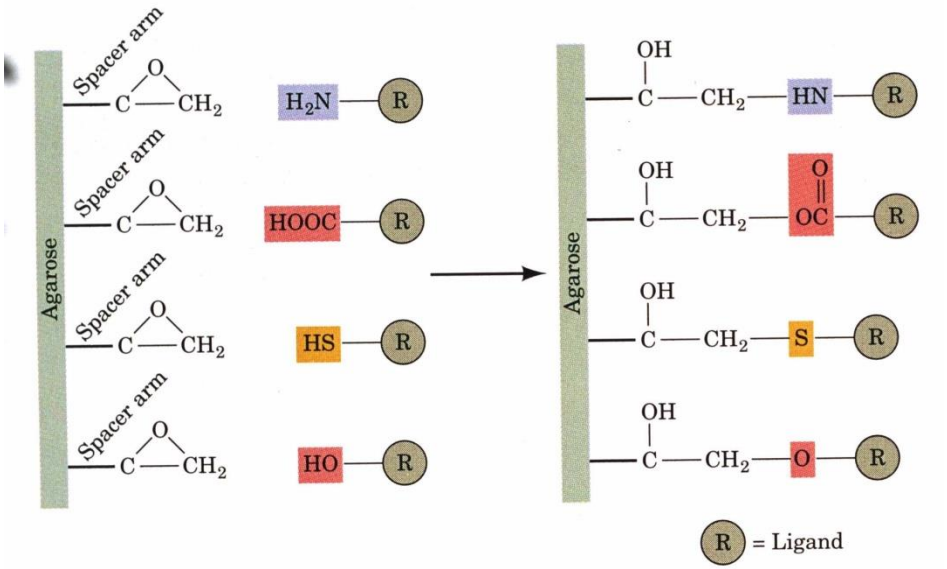
- During affininty chromatography, the interacting partner of the biomolecule is immobilised on a chromatographic resin

- The ligand, fixed to the stationary phase, reversibly binds the desired biomolecule present in the multi-component mobile phase

- The materials can be eluted from the column by changing the composition of the mobile phase.

Affinity Chromatography

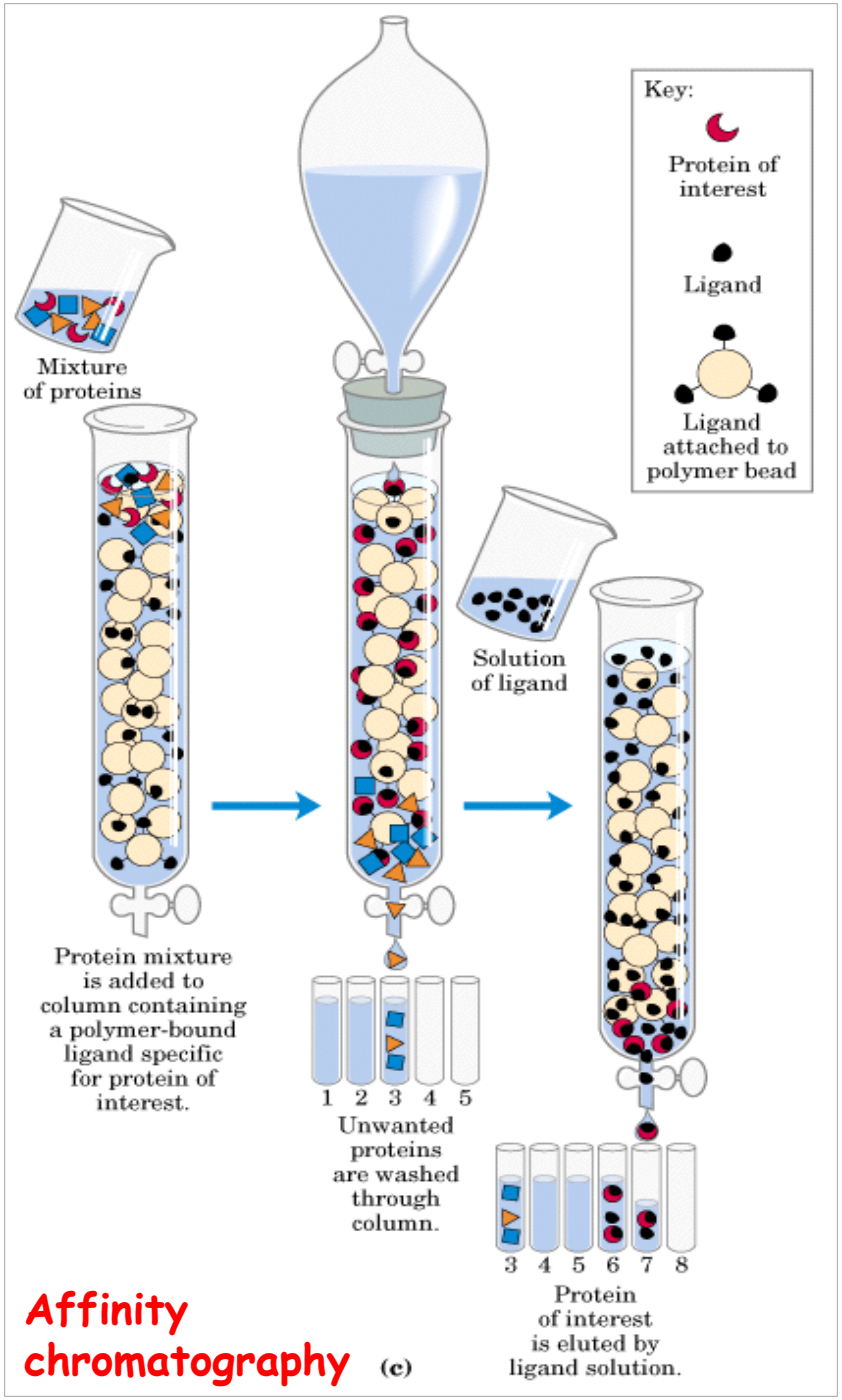
- Based on molecular complementary between an enzyme and substrate
- Substrate (R) is linked to a matrix with a spacer arm



Only protein that binds R will stick to column. put citrate on column citrate dehydrogenase will specifically bind. Add excess citrate and the enzyme will be released.

Commonly used interactions in affinity chromatography

Enzyme	Substrate analogue or inhibitor
Antibody	Antigen (virus, cells)
Nucleic acid	Complementary nucleic acid
Nucleic acid	Histone or other nucleic acid binding protein
Hormone	Hormone receptor
Glutathione	Glutathione S-transferase (GST) fusion protein
Metal chelate	His-tag fusion protein



Affinity chromatography

- A more specific adsorbent in which a **ligand specifically recognized by the protein of interest** is covalently attached to the column material
- When a mixture of proteins is passed through the column, only those few that bind strongly to the ligand stick, while the others pass through the column.
- Protein of interest is eluted with a **buffer** containing the **free ligand**, which competes with the column ligand to bind to the protein, and protein washes off (with bound ligand)

N/B

All the above column chromatography methods can be automated by
HPLC - **H**igh **P**erformance **L**iquid **C**hromatography

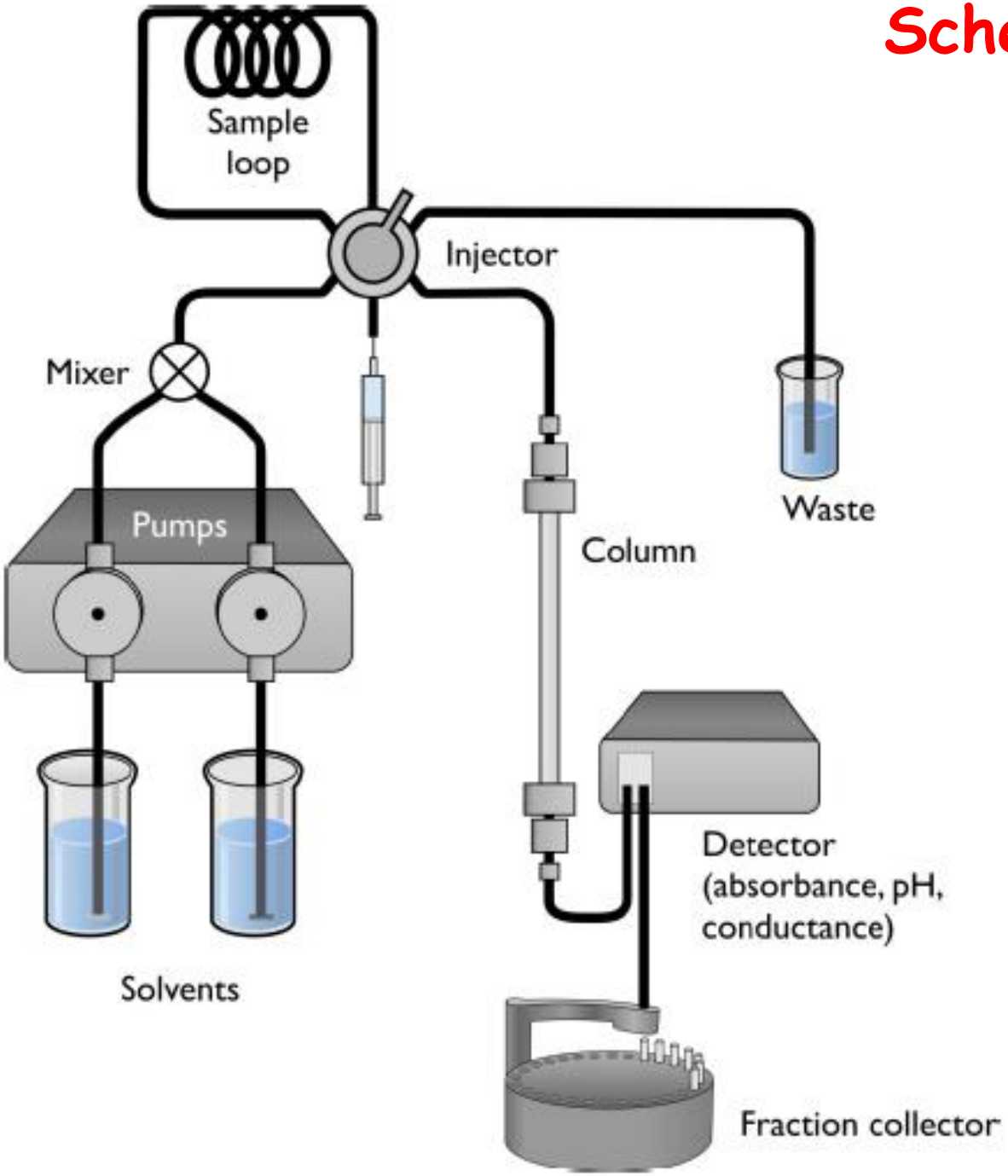
HPLC (High Performance Liquid Chromatography)

- High-pressure pumps are used to move the liquid phase through the column. HPLC pumps are capable of generating pressure of up to 50 megaPascals

- HPLC columns (stainless steel) are designed to withstand high pressures

- FPLC (fast protein liquid chromatography) system was developed for the separation of biopolymers. Column resins are specially-treated dextranbased or synthetic polymeric materials (Superdex, Superose, Sephacryl etc.)

Schematic diagram for HPLC



Several amino acids separated by HPLC

Column: Microsorb Cyano, 5 μm, 4.6 mm ID × 25 cm L
Mobile phase: 15.5% THF and 17.1% acetonitrile in 6 m*M* phosphate, pH 3.2
Flow: 1 mL/min
Temperature: 35°C
Detection: UV, 254 nm

Peak identification

1. Cys-A
2. Asn
3. Gln
4. Ser
5. Thr
6. Asp
7. Gly
8. His
9. Glu
10. Ala
11. Arg
12. Cys
13. Tyr
14. Pro
15. Val
16. Met
17. Ile
18. Phe
19. Leu
20. nor-Leu
21. Trp
22. Lys

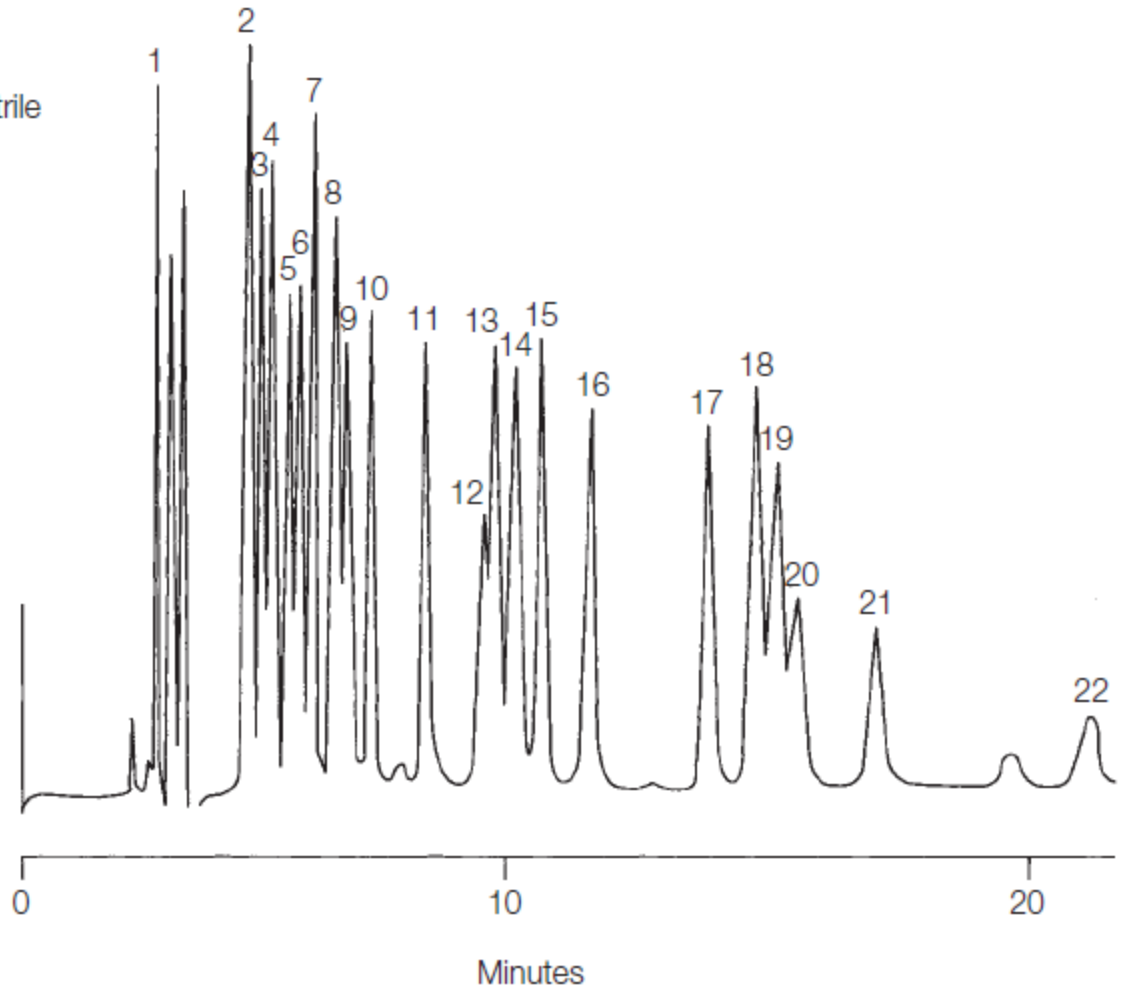


FIGURE 5.11 The separation of several amino acid phenylhydantoins by HPLC. *Courtesy of Rainin Instrument Co., Woburn, MA;* www.rainin.com.

Next lecture

How are pure enzymes analyzed?