

# Methods of Protein separation and purification

## Specific learning objectives

- Protein Fractionation:
  - Differential centrifugation
  - Chromatography
  - Salt Fractionation
- Clinical applications

# Protein Fractionation

- Protein fractionation required to separate and characterize protein
- Protein differ in their molecular size and charge, can be separated on basis of following properties:
  - Molecular size
  - Solubility
  - Electric charge
  - Binding affinity
  - Adsorption properties
  - Hydrophobicity

## Characteristics of proteins utilized in various separation procedures

Characteristic	Procedure
<b>Solubility</b>	1. Salting in 2. Salting out
<b>Ionic Charge</b>	1. Ion exchange chromatography 2. Electrophoresis 3. Isoelectric focusing
<b>Polarity</b>	1. Adsorption chromatography 2. Paper chromatography 3. Reverse-phase chromatography 4. Hydrophobic interaction chromatography
<b>Molecular Size</b>	1. Dialysis and ultrafiltration 2. Gel electrophoresis 3. Gel filtration chromatography 4. Ultracentrifugation
<b>Binding Specificity</b>	1. Affinity chromatography

# Differential Centrifugation

- First step in protein purification is to break and open these cells, releasing their proteins into a solution called a crude extract.
- Differential centrifugation used to prepare subcellular fractions or to isolate specific organelles.
- Once extract or organelle preparation is ready, various methods are available for purifying one or more of proteins.

## Differential Centrifugation

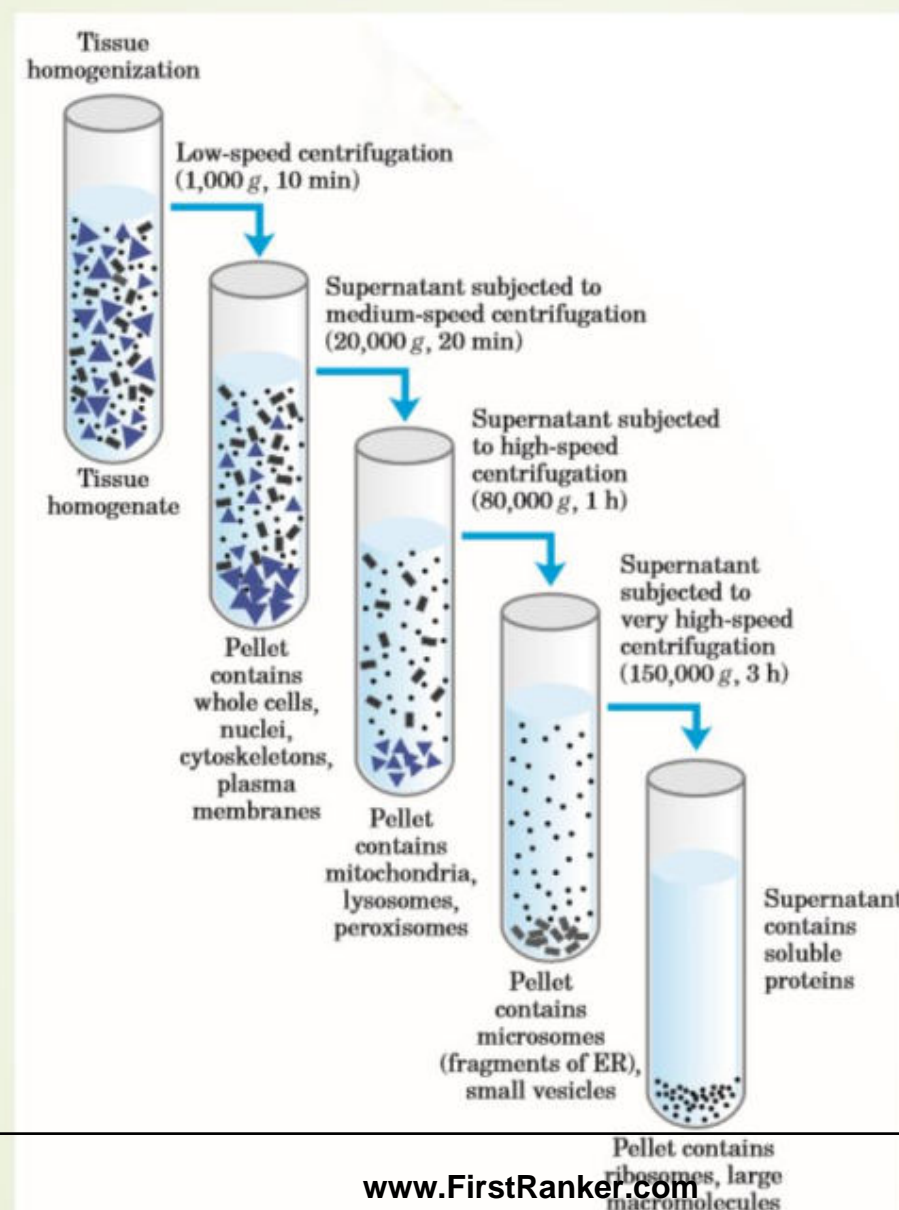


Fig1.8 a: Lehninger Principles of Biochemistry by David L Nelson



# Chromatography

- Sample to be examined called solute or analyte allowed to interact with two immiscible phases-mobile and stationary phase
- These two phases could be solid and liquid, gas and liquid, liquid and liquid
- Stationary phase may be solid or liquid supported on solid, does not move.
- Mobile phase moves sample through stationary phase. Mobile phase may be liquid or gas

## Cont--

Chromatography techniques separate one protein from another based upon difference in their:

- Based on shape of chromatographic bed:
  - Planar (Thin layer and paper chromatography) and Column Chromatography
- Based on physical state of mobile and stationary phase:
  - Gas, Liquid, High-Pressure Liquid Chromatography

## Cont--

- Based on mechanism of separation:
  - Size (size exclusion chromatography)
  - Charge (ion-exchange chromatography)
  - Ability to bind a specific ligand (affinity chromatography)
  - Gel-Filtration/Molecular Exclusion Chromatography
  - Hydrophobicity (hydrophobic interaction chromatography)
  - Adsorption Chromatography
  - Partition Chromatography

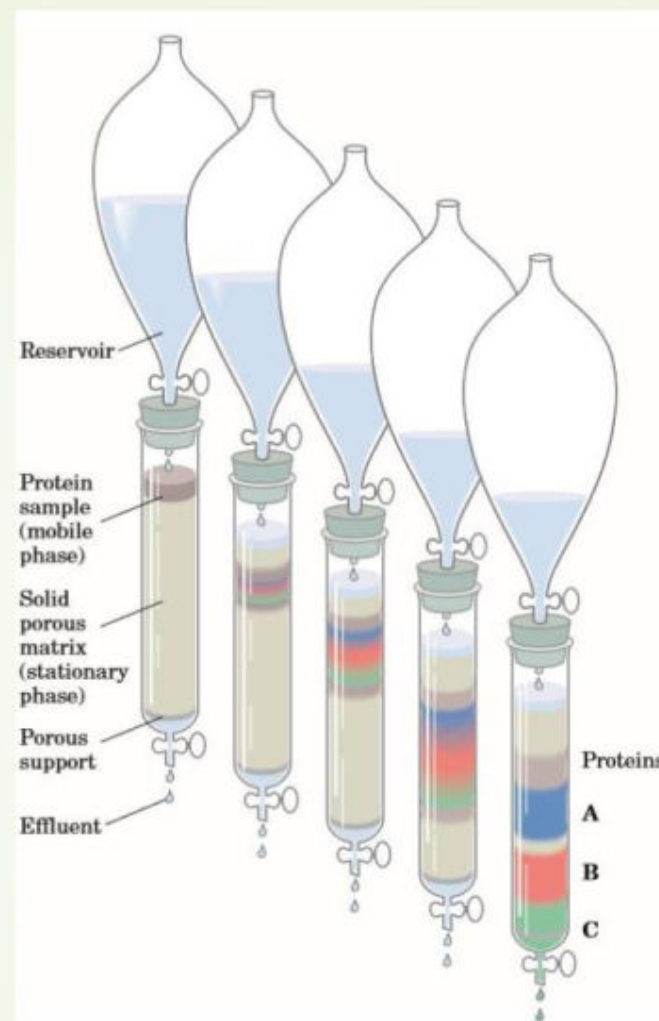
## Planar Chromatography

- Stationary phase present as or on plane
- Plane can be paper impregnated by substance acting as a stationary phase called paper chromatography
- If substance acting as stationary phase spread on glass, metal or plastic plate called thin layer chromatography (TLC)
- In TLC, stationary phase is thin layer of silica gel or alumina on glass, metal or plastic plate
- TLC detect aminoacidopathies

# Column Chromatography

- Porous solid material with appropriate chemical properties (stationary phase) held in a column, and a buffered solution (mobile phase) pass through it.
- Protein-containing solution, layered on the top of column, pass through solid matrix as an ever-expanding band within larger mobile phase.
- As mobile-phase liquid emerges from column, it is automatically collected in a series of small portions called fractions.

## Column Chromatography



Column chromatography: The standard elements of a chromatographic column include a solid, porous material supported inside a column, generally made of plastic or glass. The solid material (matrix) makes up the stationary phase through which flows a solution, the mobile phase. The solution that passes out of the column at the bottom (the effluent) is constantly replaced by solution supplied from a reservoir at the top. The protein solution to be separated is layered on top of the column and allowed to percolate into the solid matrix. Additional solution is added on top. The protein solution forms a band within the mobile phase that is initially the depth of the protein solution applied to the column. As proteins migrate through the column, they are retarded to different degrees by their different interactions with the matrix material. The overall protein band thus widens as it moves through the column. Individual types of proteins (such as A, B, and C, shown in blue, red, and green) gradually separate from each other, forming bands within the broader protein band. Separation improves (resolution increases) as the length of the column increases. However, each individual protein band also broadens with time due to diffusional spreading, a process that decreases resolution. In this example, protein A is well separated from B and C, but diffusional spreading prevents complete separation of B and C under these conditions. Fig 3.17: Lehninger Principles of Biochemistry by David L Nelson



# High-Pressure Liquid Chromatography (HPLC)

- It is liquid chromatography tech i.e; mobile phase is liquid, stationary phase may be solid or liquid
- Instead of solvent (mobile phase) being allowed to drip through a column under gravity, it is forced through under high pressure
- Column materials are very finely divided and, as a consequence, more interaction sites and greater resolving power.
- Column is made of finer material, high-pressure applied to column to obtain adequate flow rates.
- Net result is both high resolution and rapid separation.

## Cont--

- In a typical HPLC setup, a detector that monitors absorbance of eluate at a particular wavelength is placed after column.
- In sample HPLC elution profile, proteins are detected by setting detector to 220 nm (characteristic absorbance wavelength of peptide bond).
- In a short span of 10 minutes, a number of sharp peaks representing individual proteins can be readily identified.

## Cont--

Gel filtration by HPLC clearly defines individual proteins because of its greater resolving power:

1. Thyroglobulin (669 kd): Large protein
2. Catalase (232 kd),
3. Bovine serum albumin (67 kd),
4. Ovalbumin (43 kd), and
5. Ribonuclease (13.4 kd).

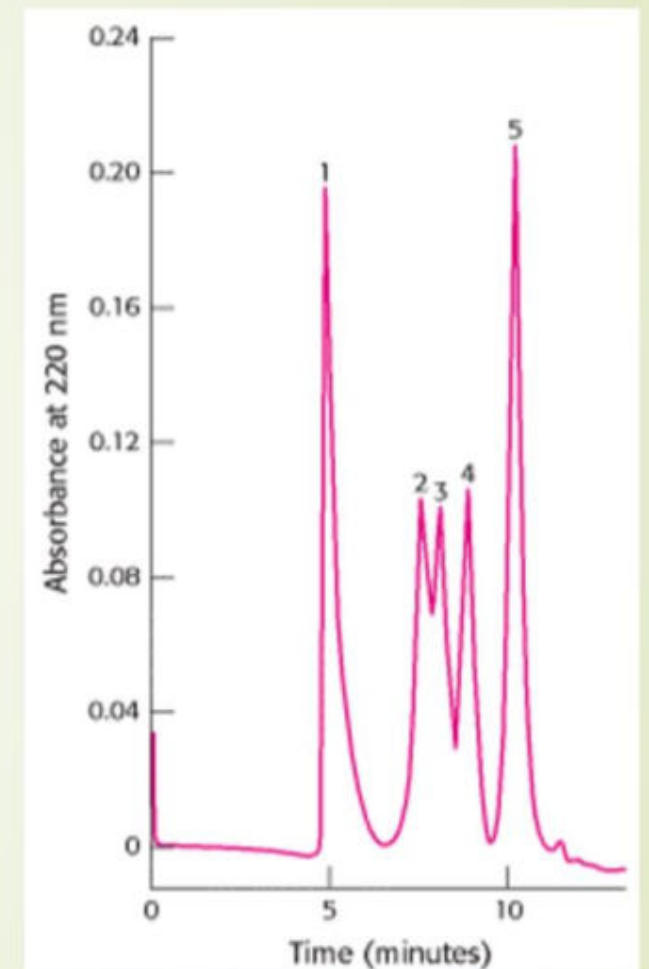


Fig. 4.6. High-Pressure Liquid Chromatography (HPLC): Biochemistry 7<sup>th</sup> edition by Jeremy M. Berg, John L. Tymoczko and Lubert Stryer.

## Ion-Exchange Chromatography

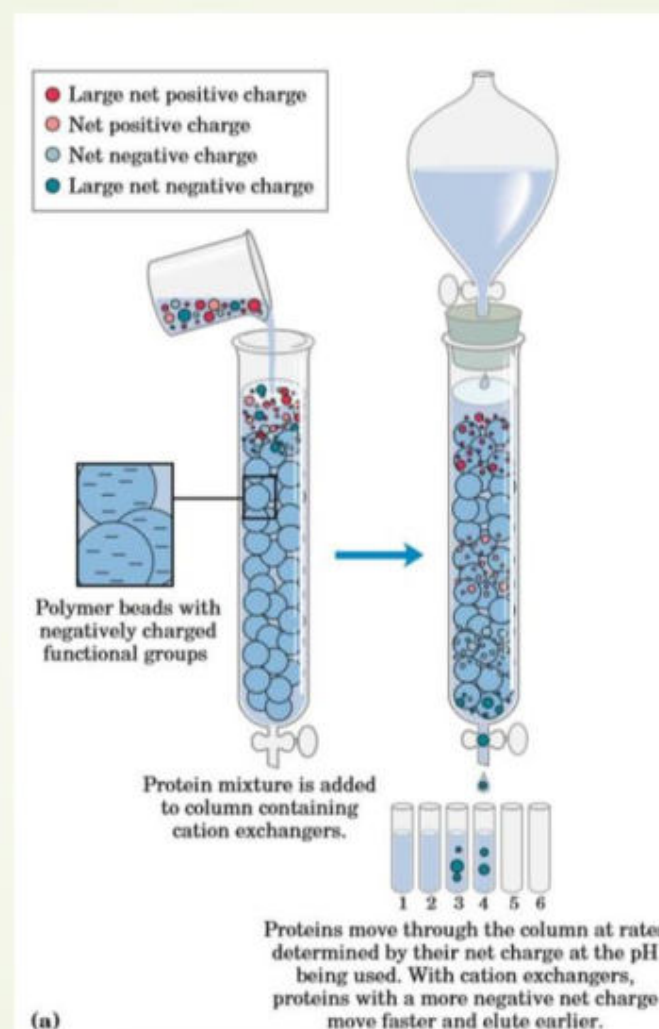
- In mobile phase, proteins with a net positive charge migrate through matrix more slowly than those with a net negative charge, because migration of former is retarded more by interaction with stationary phase.
- Expansion of protein band in mobile phase caused both by separation of proteins with different properties and by diffusional spreading.



## Cont--

- ▶ As length of column increases, resolution of two types of protein with different net charges generally improves.
- ▶ Rate at which protein solution can flow through column decreases with column.

## Ion-Exchange Chromatography

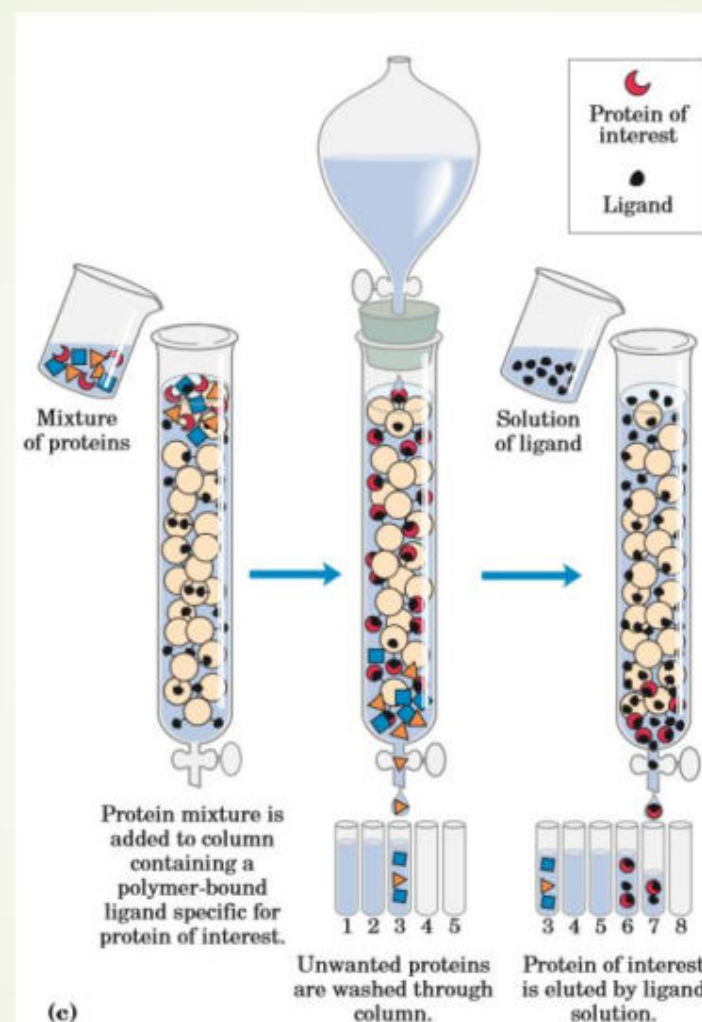


Ion-exchange chromatography exploits differences in the sign and magnitude of the net electric charges of proteins at a given pH. The column matrix is a synthetic polymer containing bound charged groups; those with bound anionic groups are called cation exchangers, and those with bound cationic groups are called anion exchangers. Ion-exchange chromatography on a cation exchanger is shown here. The affinity of each protein for the charged groups on the column is affected by the pH (which determines the ionization state of the molecule) and the concentration of competing free salt ions in the surrounding solution. Separation can be optimized by gradually changing the pH and/or salt concentration of the mobile phase so as to create a pH or salt gradient. Fig 3.18 a: Lehninger Principles of Biochemistry by David L Nelson.

# Affinity Chromatography

- Based on binding affinity of a protein
- Beads in column covalently attached to chemical group.
- Protein with affinity for this particular chemical group bind to beads in column, and its migration retarded.
- Enabling purification of a biomolecules w.r.t. biological function
- Substance to be purified is specifically and reversibly adsorbed to a ligand, immobilized by a covalent bond to a chromatography bed material
- Substances of interest bound to ligand while unbound substance washed away

## Affinity Chromatography



# Gel-Filtration/Molecular Exclusion Chromatography

- More-discriminating separations on basis of size and shape
- Sample is applied to top of a column consisting of porous beads made of an insoluble but highly hydrated polymer such as dextran or agarose or polyacrylamide (act as stationary phase)
- Small molecules can enter these beads, but large ones cannot
- Gel filtration uses aqueous mobile solvent to separate and characterize molecules

## Cont--

- Small molecules distributed in aqueous solution both inside beads and between them, whereas large molecules located only in solution between beads.
- Large molecules flow more rapidly through this column and emerge first because a smaller volume is accessible to them.
- Molecules enter a bead will flow from column at an intermediate position, and small molecules, which take a longer path, exit last.



# Gel-Filtration Chromatography

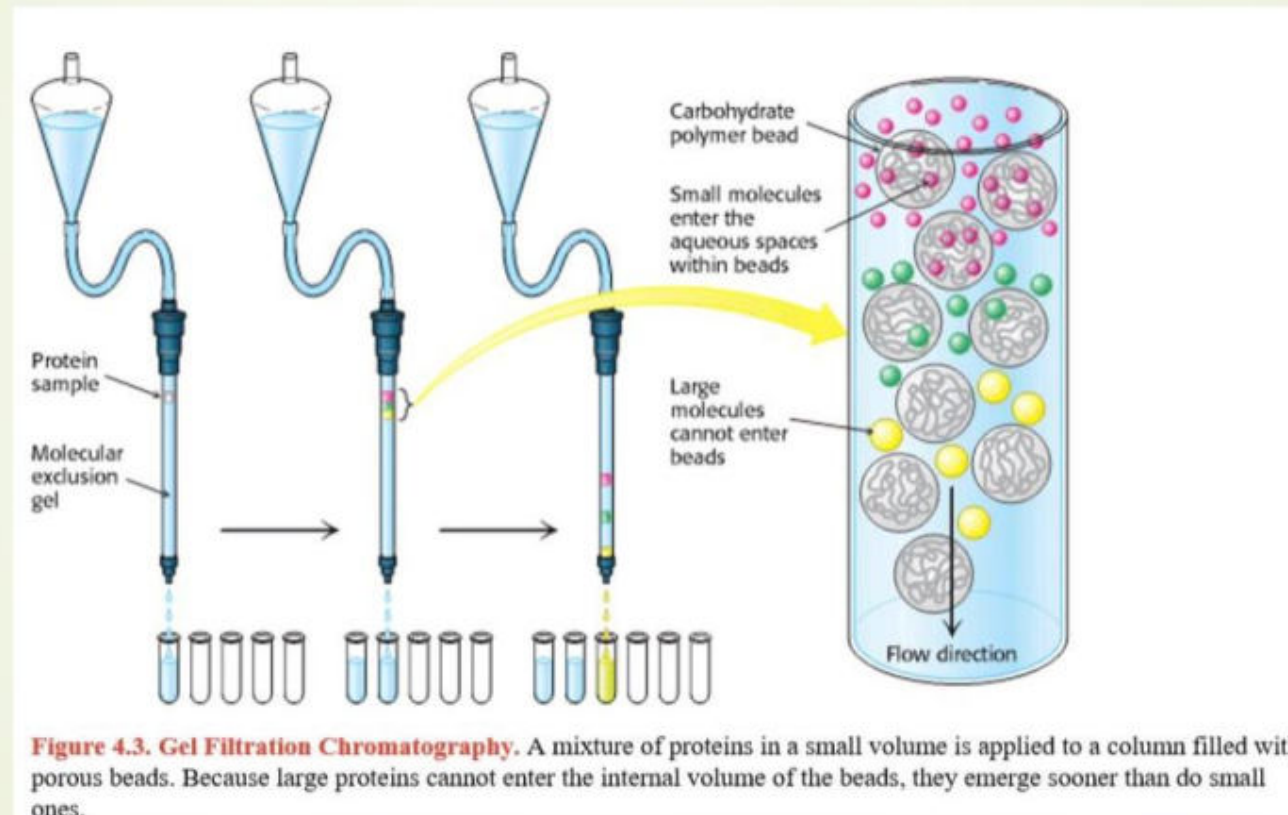


Fig. 4.3. Biochemistry 7<sup>th</sup> edition by Jeremy M. Berg, John L. Tymoczko and Lubert Stryer.

## Adsorption Chromatography

- Separation of components present in mixture based on relative differences in adsorption of components to stationary phase present in chromatography column
- Components of mixture travel with different rates due to differences in their non-covalent interactions with stationary phase
- Adsorption involves weak non-covalent interactions bet components of mixture and stationary phase

# Partition Chromatography

- Separation based on solute partitioning between two liquid phases
- Paper chromatography: End of paper dipped into solvent mixture consists of aqueous and organic components.
  - Solvent soaks into paper by capillary action because of fibrous nature of paper
  - Aqueous component of solvent binds to cellulose of paper and form stationary phase with it.
  - Organic component of solvent continuous migrating forming mobile phase

## Hydrophobic Interaction Chromatography

- It separates proteins based on their tendency to associate with a stationary phase matrix coated with hydrophobic groups (eg, phenyl Sepharose, octyl Sephadex).
- Proteins with exposed hydrophobic surfaces adhere to matrix via hydrophobic interactions enhanced by employing a mobile phase of high ionic strength.

## Cont--

- Clinical applications:
- Screening and diagnosis of inherited disorders of aa metabolism e.g. phenylketonuria and Tyrosinemia
- Peptide fingerprinting for diagnosis of disease like sickle cell anemia

## Cont--

- After non-adherent proteins are washed away, polarity of mobile phase is decreased by gradually lowering its salt concentration.
- If interaction between protein and stationary phase is strong, ethanol or glycerol added to mobile phase to decrease its polarity and weaken hydrophobic interactions.



# Salt Fractionation

- Protein show variation in solubility depends on conc of salts in solution.
- Used to separate serum proteins into albumins and globulins, albumins are soluble and globulins are not soluble in water
- Salting in: Globulins are soluble in weak salt solutions, going into solution at salt conc 0.1mol/L
- Due to electrostatic attraction between salt ion and charged group on protein, which decrease intermolecular electrostatic attraction of proteins and increases interaction of proteins molecules with water, polar solvent making them soluble

## Cont--

- Salting Out: Solubility of proteins lowered at high salt concentrations, an effect called “salting out.”
- As the salt concentration increased, salt ions compete for water molecules of hydration of hydrated groups of proteins, resulting in decreased solubility and precipitation of protein out of solution
- Ammonium sulfate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) used for salting out proteins, albumin precipitated on fully saturating solution
- Useful to isolate a protein of interest from a mixture of different proteins.

# Summary

- Proteins are separated and purified based on their properties.
- Proteins precipitated by addition of certain salts.
- Chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties.
- These include ion-exchange, size-exclusion, affinity, and high-performance liquid chromatography.

## Cont--

- Polyacrylamide gels (PAG) provide a porous matrix for separating proteins on basis of their mobility in an applied direct current electrical field.
- Constant ratio at which anionic detergent SDS binds proteins enables SDS-PAGE to separate polypeptides on basis of relative size.

# Reference Books

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- 1) Harper's Illustrated Biochemistry-30<sup>th</sup> Ed
- 2) Biochemistry 7<sup>th</sup> edition by Jeremy M. Berg, John L. Tymoczko and Lubert Stryer.
- 3) Lehninger Principles of Biochemistry by David L Nelson, 6<sup>th</sup> Ed
- 4) Fundamentals and techniques of biophysics and molecular biology 2<sup>nd</sup> Ed. by Pranav Kumar
- 5) Principles and Techniques of Biochemistry and Molecular Biology 7<sup>th</sup> Ed. By Keith Wilson and John Walker



Thank you