CHAPTER 4

Techniques of protein
And
Nucleic Acid purification

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.

General Protein Purification Strategy

Procedure

❑ Solubility – Salting in
            - Salting out

❑ Ionic charge - Ion exchange chromatography
            - Electrophoresis
            - Isoelectric focusing

❑ Polarity - Adsorption chromatography
        - Paper chromatography
        - Reverse phase chromatography
   - Hydrophobic interaction chroma.

Cont.......

❑Molecular Size-Dialysis & Ultra filtration

-Gel electrophoresis

-Gel filtration chromatography

-Ultracentrifugation

❑ Binding Specificity

-Affinity chromatography

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

Solubility based purification

❑ Solubility 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.

- Precipitate proteins other than the protein of interest

- Separate soluble and insoluble materials by centrifugation or filtration

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Typically the first step in protein purification
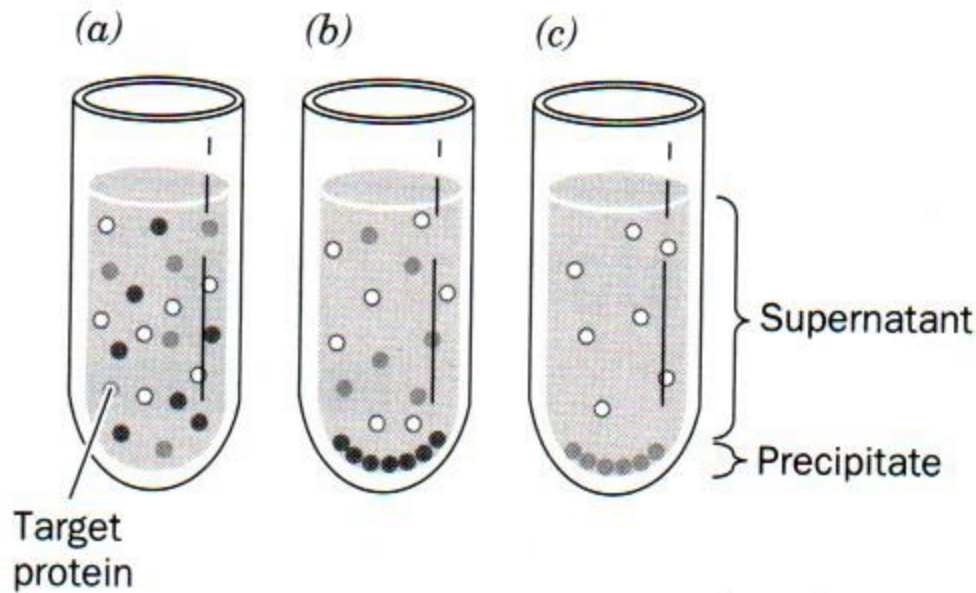
a) Mixture of 3 proteins- White , Grey , Black

b) Solution altered-Black protein precipitates (supernatant removed)

c) Solution altered again- Grey protein precipitates (white protein remains in supernatant)

A. Effect of salt concentration

❑The solubility of a protein in aqueous solution is a sensitive function of the concentrations of dissolved salts.

❑The salt concentration in is expressed in terms of the ionic strength (I), which is defined as

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

❑Where Ci=molar con. Of the ith ionic species and Zi is its ionic charge/valence electron.

❑*The solubility of a protein at low ionic strength generally increases with the salt concentration----Salting in*

Ionic Strength(I)

Ionic Strength (I) is a measure of the electrical intensity of a solution containing ions.

Mathematically,

$$I = \frac{1}{2}\sum_{i=1}^{i=n} c_i z_i^2$$

Where,

- I = Ionic strength of solution
- c_i = concentration of ion, i.
- z_i = charge on the ion, i.

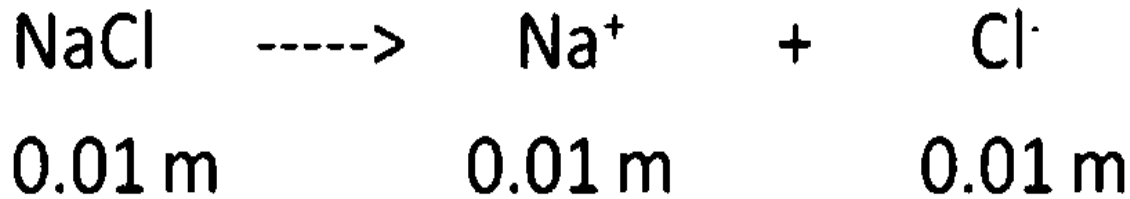For example, if a solutions contains three ions: A, B & C

The ionic strength can be expressed as:

$$I = \frac{1}{2}(c_A z_A^2 + c_B z_B^2 + c_C z_C^2)$$

Example

What is the ionic strength of 0.01 molal solution of NaCl ?
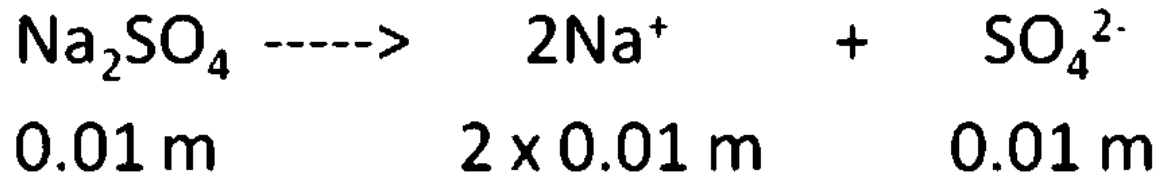
Since NaCl dissociates as follows

$$NaCl \quad -----> \quad Na^+ \quad + \quad Cl^-$$

$$0.01\ m \qquad\qquad 0.01\ m \qquad\qquad 0.01\ m$$

i.e. $c_{Na} = 0.01\ m$ & $c_{Cl} = 0.01\ m$

$$I = \frac{1}{2}\left[c_{Na} z_{Na}^2 + c_{Cl} z_{Cl}^2 \right] = \frac{1}{2}\left[0.01 \times 1^2 + 0.01 \times 1^2 \right] = 0.01$$

Example 2

What is the ionic strength of 0.01 molal solution of Na_2SO_4 ?

Since Na_2SO_4 dissociates as follows

$$Na_2SO_4 \ \text{----->} \quad 2Na^+ \quad + \quad SO_4^{2-}$$

0.01 m   2 x 0.01 m   0.01 m

i.e. $c_{Na} = 0.02$ m & $c_{SO4} = 0.01$ m

$$I = \frac{1}{2}\left[c_{Na}z_{Na}^2 + c_{SO_4}z_{SO_4}^2 \right] = \frac{1}{2}\left[0.02 \text{ x } 1^2 + 0.01 \text{ x } 2^2 \right]$$

$$= \frac{1}{2}\left[0.02 + 0.04 \right] = 0.030$$

Cont…..

❑**salting in** phenomenon is that as the salt concentration of the protein solution increases, the additional counter ions more effectively shield the protein molecules' multiple ionic charges and thereby increase the protein's solubility.

❑At high ionic strengths, the solubilities of proteins, as well as those of most other substances, decreases……Salting out

❑Salting out-results competition between the added salt ions and other dissolved solutes for solvation.

Cont……

☐ Salting out is the basis of one of the most commonly used protein purification procedures.

Solubility of carboxy-hemoglobin at its isoelectric point as a function of ionic strength and ion type. Here *S and S'* are, respectively, the solubilities of the protein in the salt solution and in pure water. The logarithm of their ratios is plotted so that the solubility curves can be placed on a common scale.
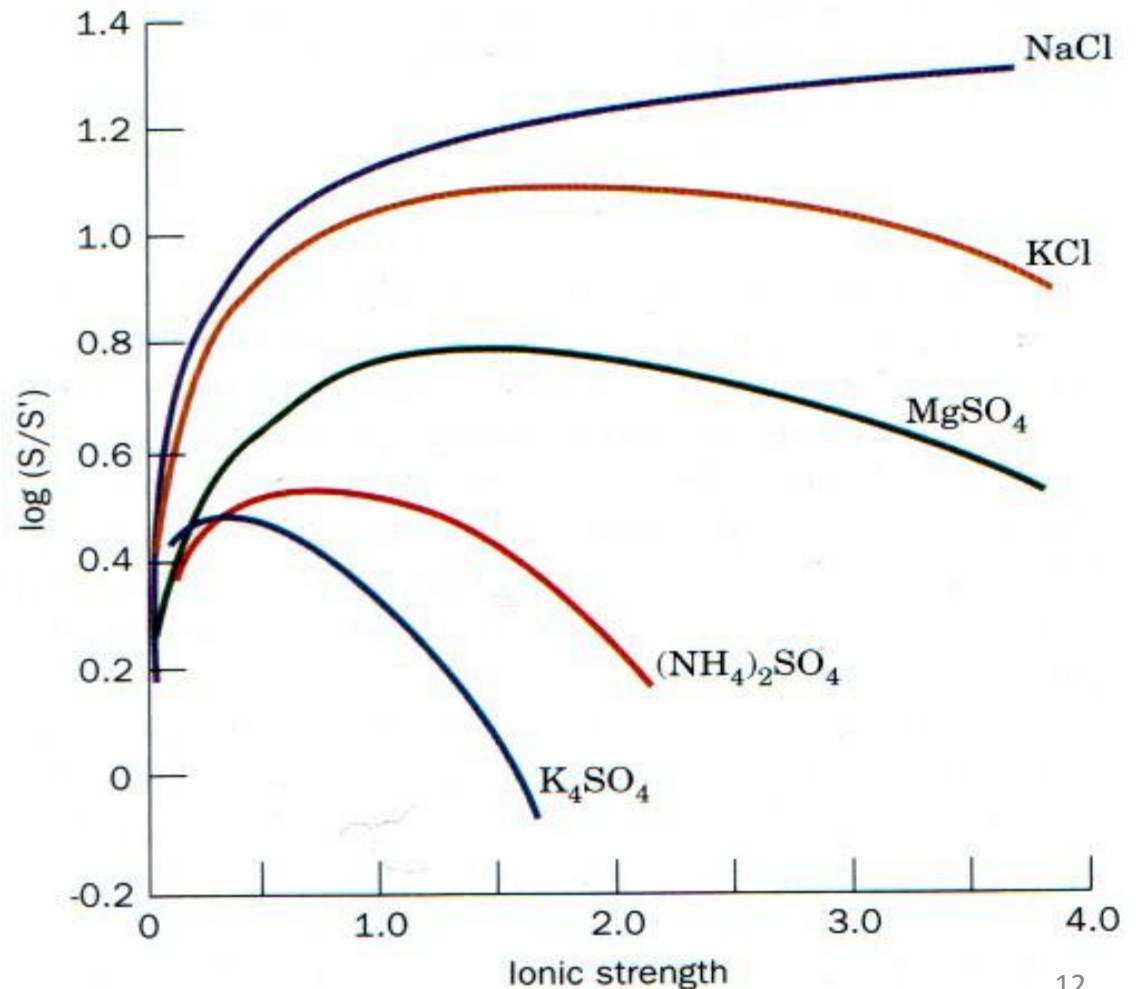
Cont….

❑By adjusting the salt concentration in a solution containing a mixture of proteins to just below the precipitation point of the protein to be purified, many unwanted proteins can be eliminated from the solution.

❑Then, after the precipitate is removed by filtration or centrifugation, the salt concentration of the remaining solution is increased so as to precipitate the desired protein.

Cont….

❑In this manner, a significant purification and concentration of large quantities of protein can be conveniently effected.

❑Consequently, salting out is often the initial step in protein purification procedures.

❑Ammonium sulfate is the most commonly used reagent for salting out proteins because its high solubility ($3.9M$ *in water at $0^{\circ}C$*) *permits the* achievement of solutions with high ionic strengths (up to 23.4 in water at $0^{\circ}C$).

Cont…..

❑ Certain ions, notably I^-, ClO_4^-, SCN^-, Li^+, Mg^{+2}, Ca^{+2}, and Ba^{+2}, increase the solubilities of proteins rather than salting them out. These ions also tend to denature proteins.

❑ Conversely, ions that decrease the solubilities of proteins stabilize their native structures, so that proteins which have been salted out are not denatured.

Exercise

❖Calculate the ionic strength of

a. a 0.02 M $BaSO_4$ solution

b. a solution containing 0.02 M NaCl and 0.02 M $BaSO_4$

B. Effects of organic solvents

❑ Water miscible organic solvents, such as acetone and ethanol, are generally good protein precipitants because their low dielctric constants reduce the solvating power of their aqueous solutions for dissolved ions such as proteins.

❑ The different solubilities of proteins in these mixed solvents from the basis of a useful fractionation technique. This procedure is normally used near 0°C or less because, @ higher T, organic solvents tend to denature proteins.

Cont……

❑ The lowering of the dielecectric constant by organic solvents---increases salting out bahavior of proteins.

❑ But, organic solvents such as dimethyl sulfoxide or N,N-dimethylformamide are rather good protein solvents because of their relatively high dielectric constants.

C. Effects of pH

❑ At isoelectric pH, pI, the protein molecule carries no net charge and is therefore immobile in an elcetric field. Thus, a protein at its pI should not be subjected to salting in.

❑ As pH is varied from a protein's pI,or as a protein's net charge increases, it subjected to salting in bcause the electrostatic interactions between neighboring molecules that promote aggregation and precipitation should likewise increase

❑ Hence, in solutions of moderate salt concentrations, the solubility of a protein as a function of pH is expected to be at a minimum at the protein's pI and to increase about this point with respect to pH.

❑A protein mixture is adjusted to the pI of protein's to be isolated so as to selectively minimize its solubility.

❑In practice, this technique is combined with salting out so that the protein being purified is usually salted out near its pI.
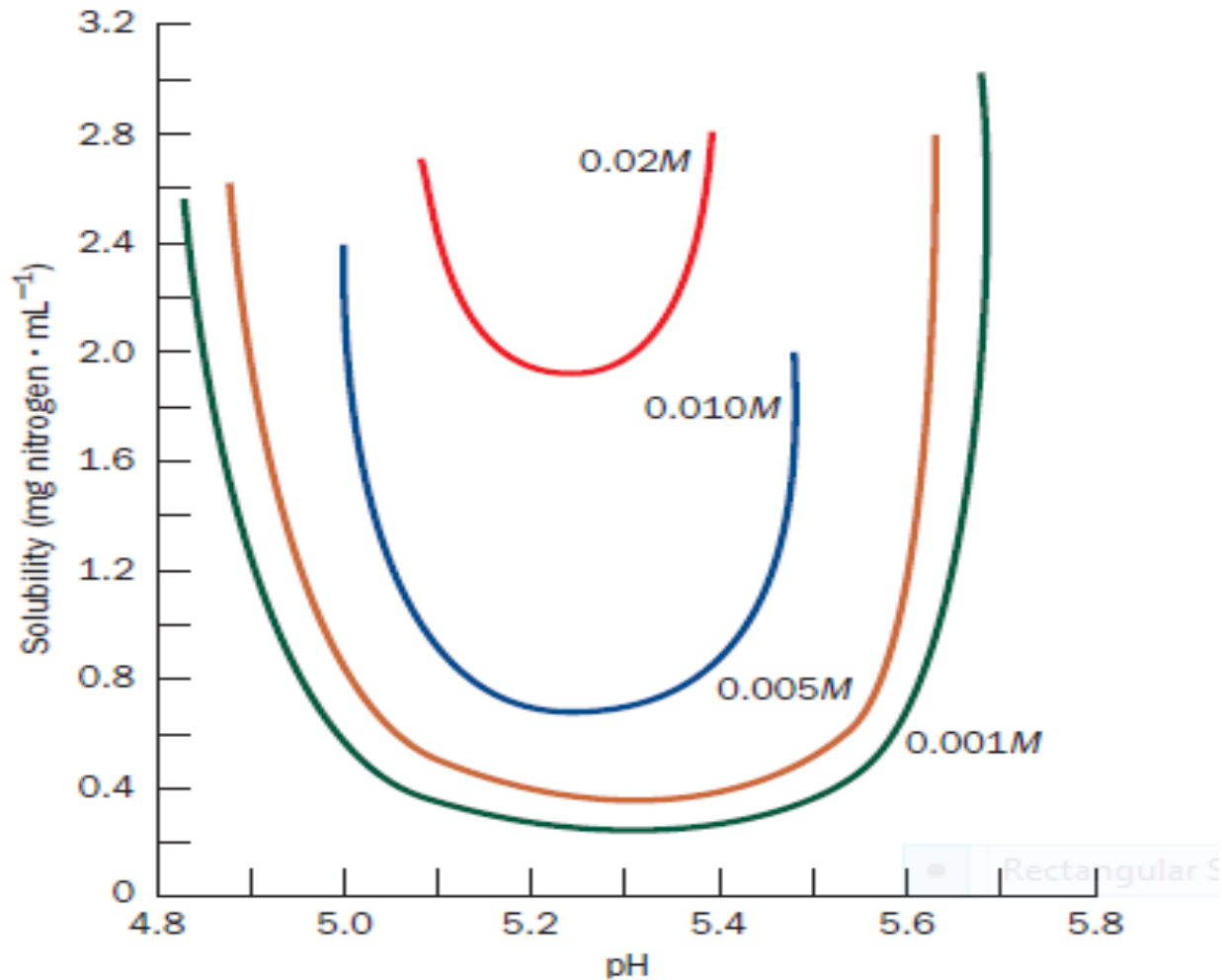
Cot....

Figure shows the solubility of β-lactoglobulin as a function of pH at several NaCl concentrations.

D. Crystallization

❑ Crystallization is the final step in protein purification.

❑ Crystallization is done by bringing the protein solution just past its saturation point with the types of precipitating agents.

❑ An increment of precipitating agent leds the protein to precipitate from the solution in crystalline form.

❑ The range of crystallization is usually from microscopic to 1mm or more across.

Chromatographic Separations

❑**Chromatography** (from Greek word for *chromos* for colour) is the collective term for a family of laboratory techniques for the separation of mixtures.

❑It involves passing a mixture which contains the analyte through a stationary phase, which separates it from other molecules in the mixture and allows it to be isolated.

Cont……

❑Chromatography is the **physical separation** of a mixture into its **individual components**.

❑chromatography uses to separate the components of

-**inks** and **dyes**

-candy shells

-**pigments** in **plants chemical composition** of many substances.

Cont……..

❑Chromatography is the most powerful separation technique in Biochemistry.

❑Modern separation methods rely heavily on Chromatogrphic procedures.

❑The separation of a mixture by distribution of its components between a mobile and stationary phase over time

– mobile phase = solvent

– stationary phase = column packing material

Cont.....

❑ If the mixture being fractionated starts its journey through the column in a narrow band, the different retarding forces on each component that cause them to migrate at different rates will eventually cause the mixture to separate into bands of pure substances.

❑ The power of chromatography derives from the continuous nature of the separation processes.

Classification of Chromatography

| General Classification	Specific Method	Stationary Phase	Type of Equilibrium
Liquid chromatography (LC) (mobile phase: liquid)	Liquid-liquid, or partition	Liquid adsorbed on a solid	Partition between immiscible liquids
	Liquid-bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Liquid-solid, or adsorption	Solid	Adsorption
	Ion exchange	Ion-exchange resin	Ion exchange
	Size exclusion	Liquid in interstices of a polymeric solid	Partition/sieving
Gas chromatography (GC) (mobile phase: gas)	Gas-liquid	Liquid adsorbed on a solid	Partition between gas and liquid
	Gas-bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Gas-solid	Solid	Adsorption
Supercritical-fluid chromatography (SFC) (mobile phase: supercritical fluid)		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

Basics of Chromatography
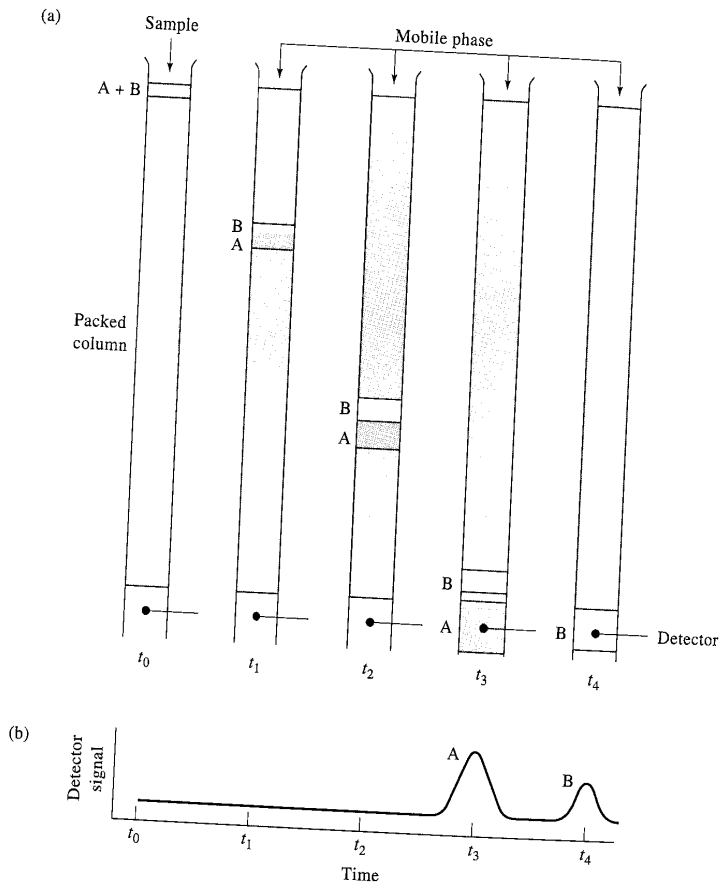
Figure 26-1 (a) Diagram showing the separation of a mixture of components A and B by column elution chromatography. (b) The output of the signal detector at the various stages of elution shown in (a).

- Ability to dissolve in mobile phase

- Interaction with stationary phase

- Time on X axis

- Response on Y

❖ Ion Exchange Chromatography

❑ In the process of ion exchange , ions that are elctrostatically bound to an insoluble and chemically inert matrix are reversibly replaced by ions in solution.

✓ $R^+A^- + B^- \longleftrightarrow R^+B^- + A^-$

Here, R^+A^- is an anion exchanger in the A- form and B- represents anions in solution.

❑ Cation Exchangers similarly bear negatively charged groups that reversibly bind cations.

Cont….

❑ **Anion Exchange**-anions in mobile phase bind cationic stationary phase

❑ **Cationic Exchange**-cations in mobile phase bind anionic stationary phase

❑ The affinity with which a particular polyelectrolyte binds to a given ion exchanger depends on the identities and concentrations of the other ions in solution.

Cont………

❑ The binding affinities of polyelectrolyte bearing acid-base groups are also highly pH dependent because of the variation of their net charges with pH. These principles are used to great advantage in isolating biological molecles by ion exchange chromatography (see the figure of IEC and step wise elusion on the next slide).

IEC And Stepwise Elusion
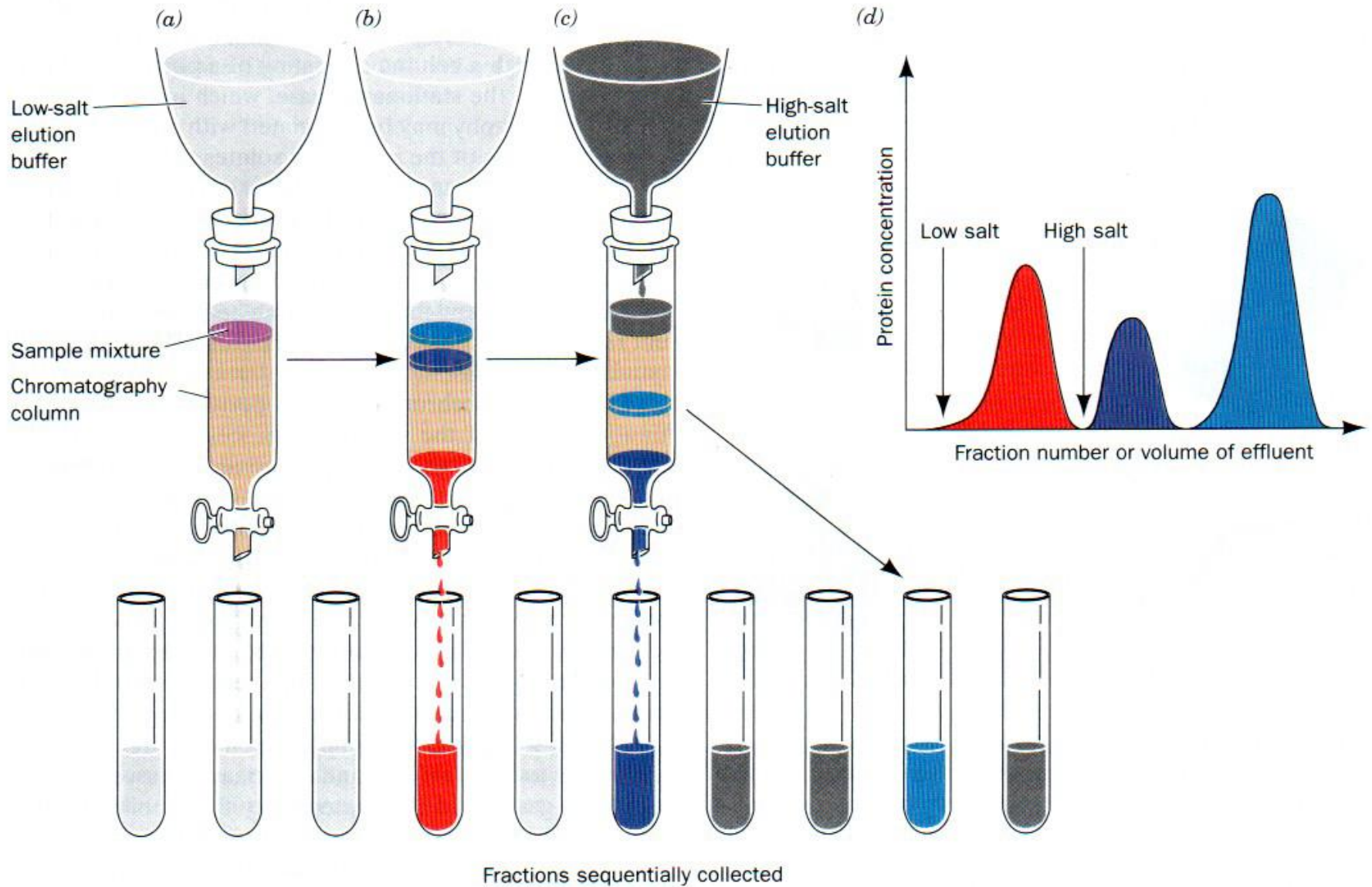
❑In IEC the tan region of the column represents the ion exchanger and the colored bands represen the various proteins.

❑(a) the protein mixture is bound to the topmost portion of the ion exchanger in the chromatography column

❑(b) As the elution progresses, the various proteins separate into discrete bands as a consequence of their different affinities for the ion exchanger under the prevailing solution conditions.

✓ Here the 1ˢᵗ band of protein (red) has passed through the column and is being isolated as a separate fraction, whereas the other, less mobile, bands remain near the top of the column

❑(c) The salt concentration in the elusion buffer is increased to increase the mobility of and thus elute the remaining bands

❑(d) The elution diagram of the protein mixture from the column
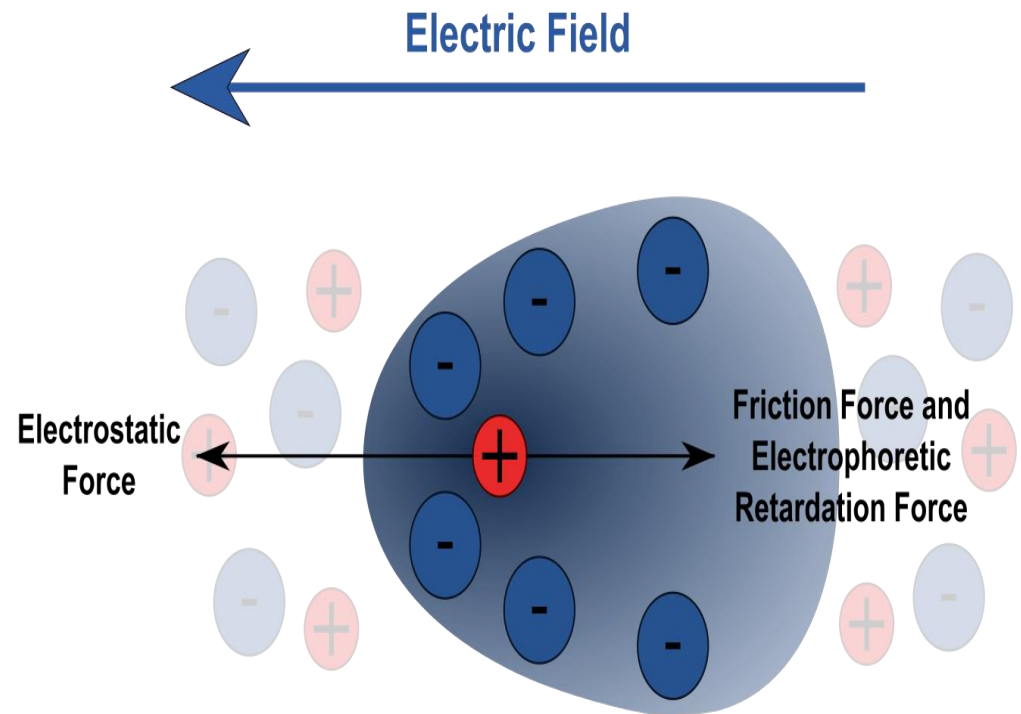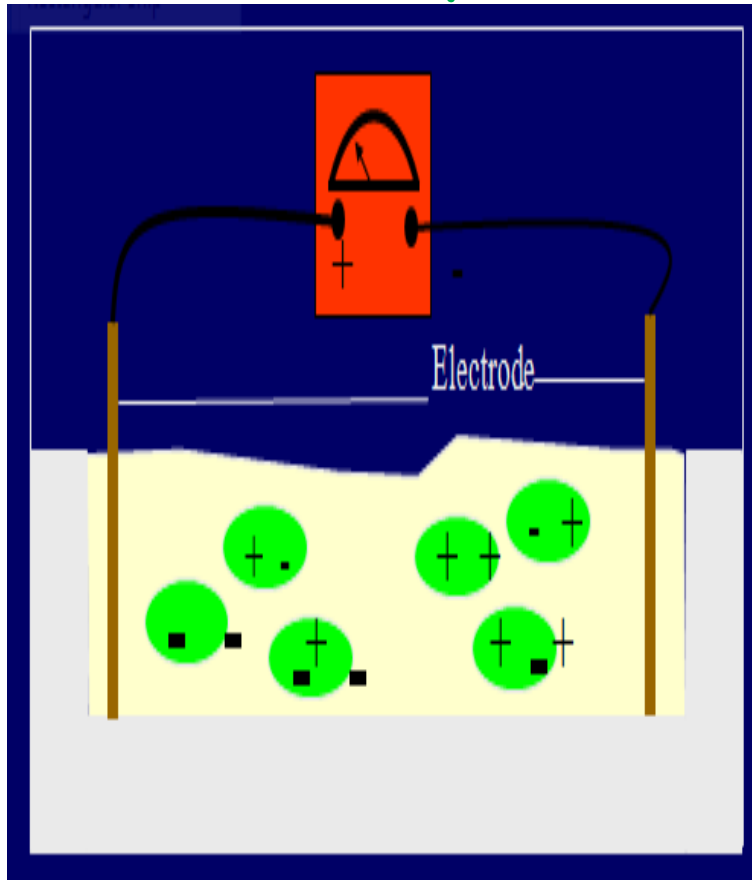
Cont...

❑Various proteins bind to the ion exchanger with different affinities.

❑As the column is washed with the buffer, a process known as elution.

-Proteins with lower affinity for IE-moves faster through the column than higher affinity.

❑This occurs because the progress of a given protein through the column is retarded relative to that of the solvent due to interactions between the protein molecules and the ion exchanger.
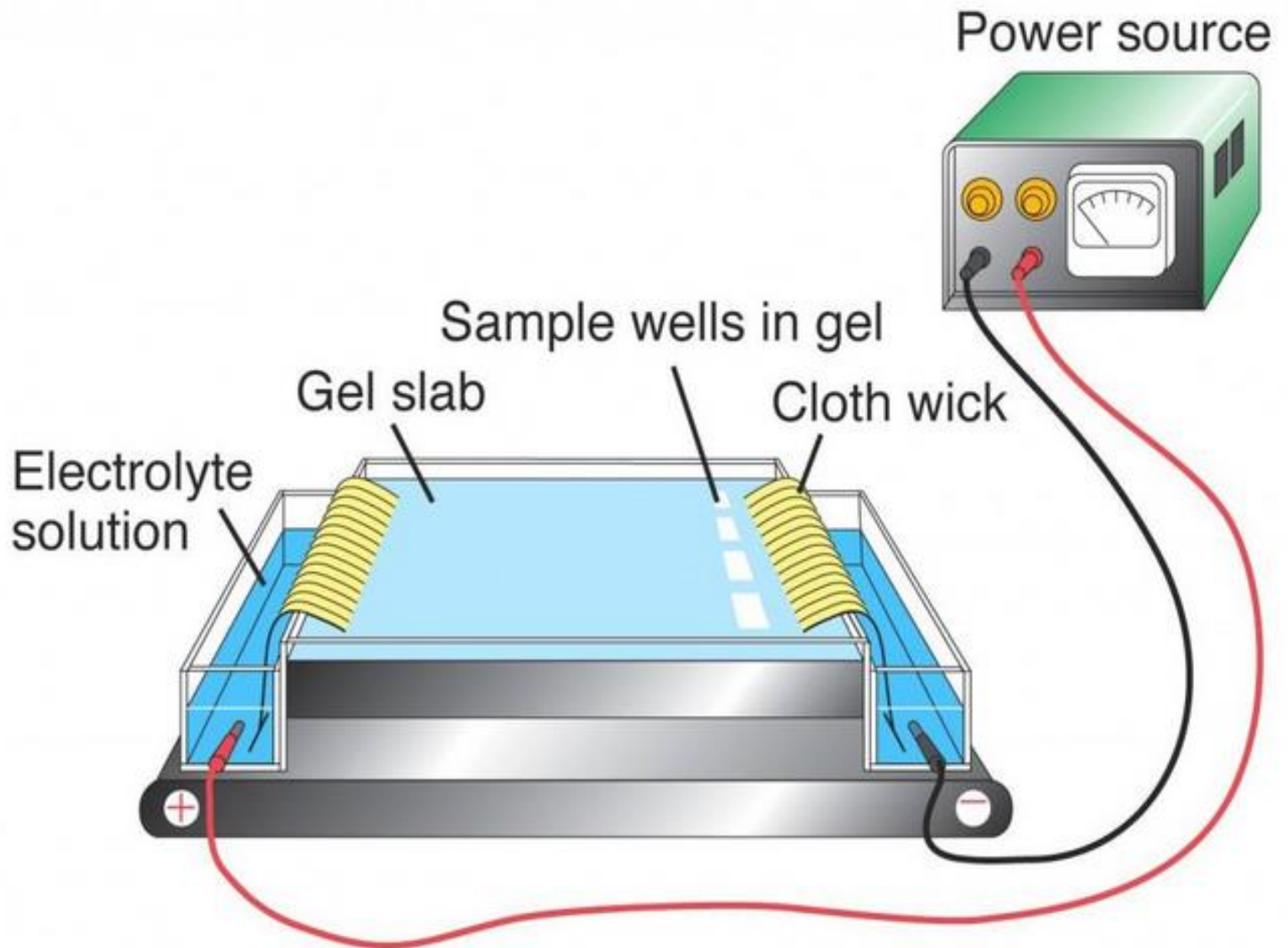
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❑ The greater the binding affinity of a protein for the ion exchanger, the more it will be retarded. Thus, proteins that bind tightly to the ion exchanger can be eluted by changing the elution buffer to one with a higher salt concentration (and/or a different pH), a process called **stepwise elution.**

Electrophoresis

☐ **Electrophoresis** is the process of moving charged molecule in solution by applying an electric field across the mixture.

Electrode

Electric Field

Electrostatic Force

Friction Force and Electrophoretic Retardation Force

Power source

Sample wells in gel

Gel slab

Cloth wick

Electrolyte solution

credit:mun.ca: The gel electrophoresis instrumentation

Cont……

❑Is widely used for the analytical separation of biological molecules.

❑The laws of electrostatics state that the electrical force, $F_{electric}$, on an ion with charge q in an electric field of strength E is expressed by

$$F_{electric} = q*E…………1$$

❑The resulting elctrophoretic migration of the ion through the solution is opposed by a fractional force

$$F_{friction} = vf \ ……2$$

Cont…..

❑where v is the rate of migration (velocity) of the ion and f is its **frictional coefficient**.

❑**The frictional coefficient is** a measure of the drag that the solution exerts on the moving ion and is dependent on the size, shape, and state of solvation of the ion as well as on the viscosity of the solution.In a constant electric field, the forces on the ion balance each other

$$q*e=v*f$$…………………………….3

Cont…..

❑ Each ion moves with a constant characteristic velocity.

❑ An ion's electrophoretic mobility,μ, is defined

$$\mu = V/E = q/f \ \text{……………….}4$$

❑ Equations (1-4) applies only to ions at infinite dilution in a non-conducting solvent.

❑ Molecules at isoelctric points, pI, have zero electrophoretic mobility. Where as for proteins and other polyelectrolytes that have acid –base properties, the ionic charge, and hence the electrophoretic mobility is a finction of pH.

Driving force of migration

❑Resultant of the electrostatic force of attraction between the electric field and the charged molecule, and the retarding forces due to friction and electrostatic repulsion from molecules of the transport medium.
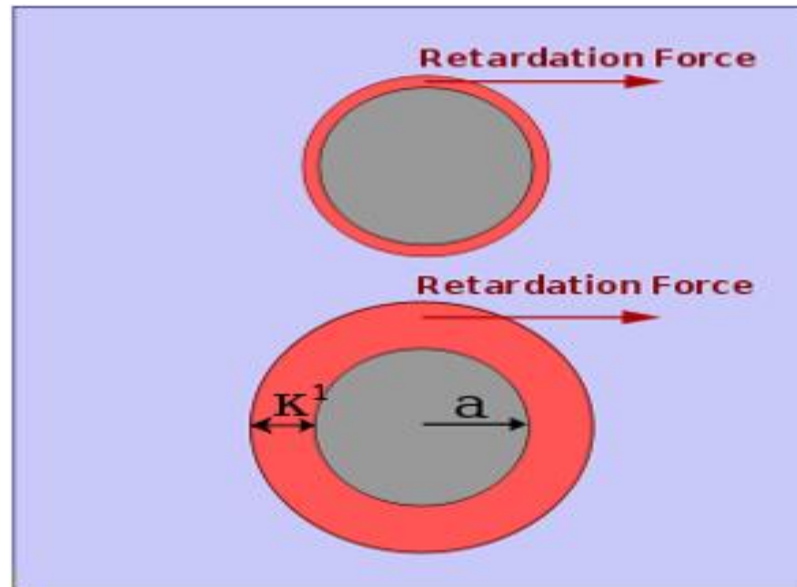

Retardation Force

Retardation Force

K^1 a

Illustration of electrophoresis retardation

Supporting Media for Electrophoresis

❑ Paper

- filter paper such as Whatman no.1 and no.3MM

- Used to good effect

❑ Cellulose acetate

- containing 2 to 3 acetyl groups

- to give sharper bands

- more easily rendered transparent

- low solvent capacity

- enhancing the resolution

❑ Gels

- 3 dimensional semisolid colloids

- resolving power enhanced due to sieve effect operating

- prepared from starch, agar, or polyacrylamide

General Procedure for Electrophoresis

Immersion of two electrodes in two separate buffer chambers but not fully isolated from each other

Migration of charged particles from one chamber to the other by using an electric field

Separation of different ions migrating at different speeds
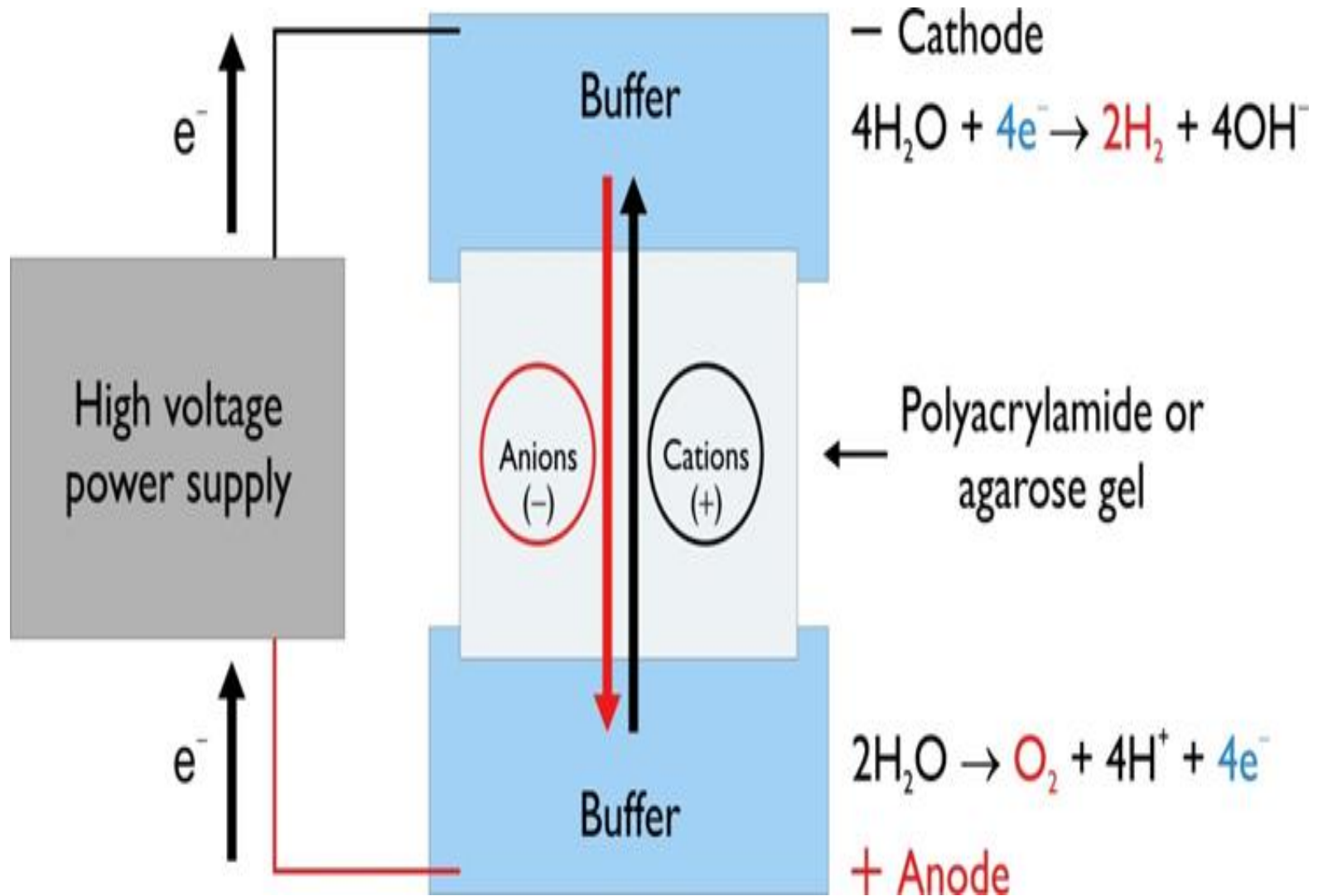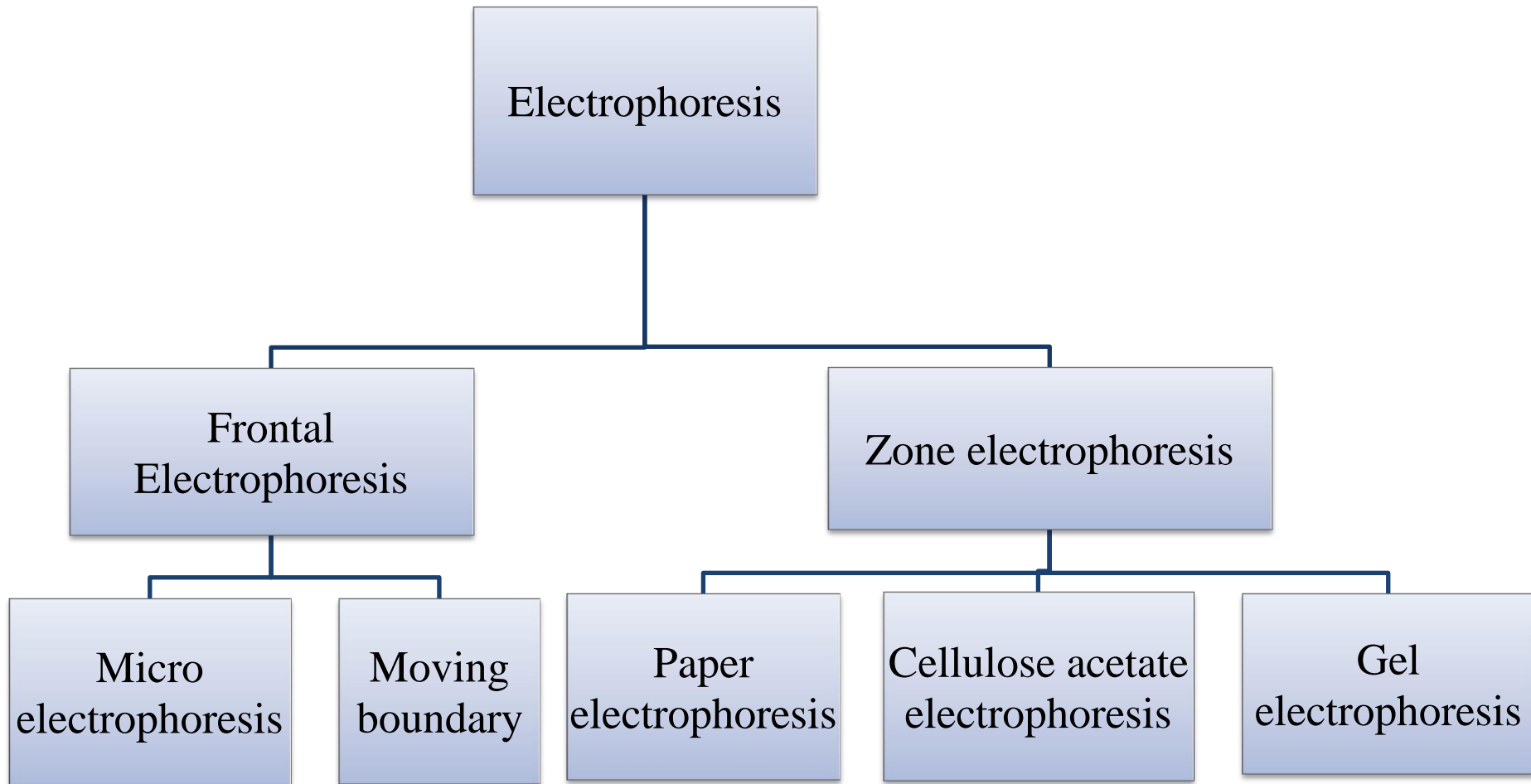
Cont….

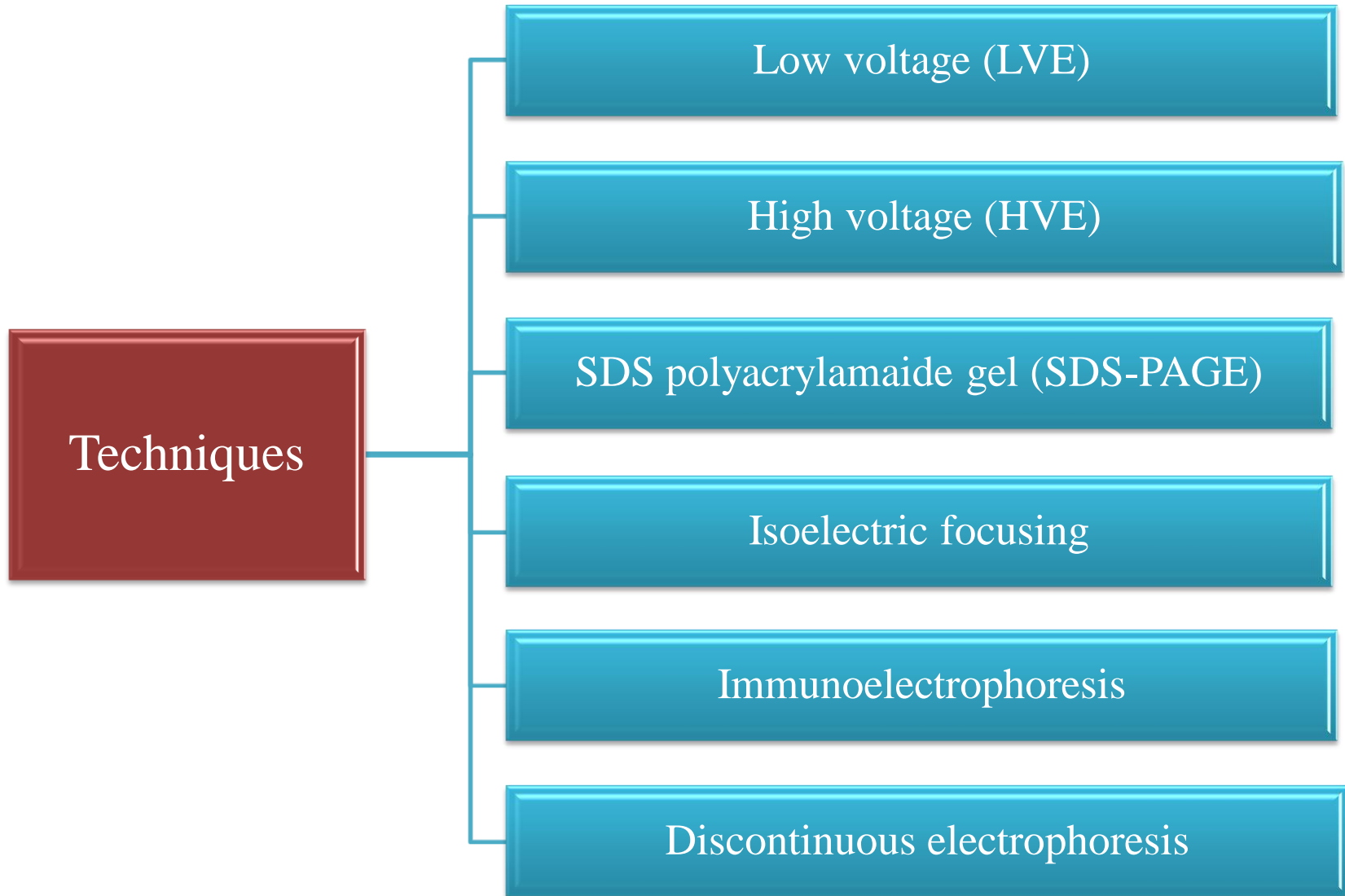Figure: Fundamentals of Electrophoresis

Factors Affecting Electrophoretic Mobility

❑*Charge* – higher the charge greater the mobility

❑*Size* – bigger the molecule greater the frictional and electrostatic forces exerted on it by the medium i.e. larger particles have smaller electrophoretic mobility compared to smaller particles

❑*Electric field* – increase of migration with the increase of voltage gradient

❑*Buffer* – dependence of migration on pH of the buffer

❑*Ionic strength* – greater the ionic strength of the buffer solution higher proportion of the current hence electrophoretic mobility

Types of electrophoresis

Techniques of electrophoresis

Techniques

- Low voltage (LVE)
- High voltage (HVE)
- SDS polyacrylamaide gel (SDS-PAGE)
- Isoelectric focusing
- Immunoelectrophoresis
- Discontinuous electrophoresis

Ultracentrifugation

❑Principles of centrifugation

➢ **A centrifuge is a device for separating particles from a solution according to size, shape and density, viscosity of the medium and rotor speed.**

➢ **In a solution , particles whose density higher than that of the solvent sink(sediment) and particles that are lighter than it float to the top.**

➢ **The greater the difference in density, the faster they move.**

Cont…..

❑ If there is no difference in the density (isopyknic conditions), the particles stay steady.

❑ To take advantage of even tiny differences in density to separate various particles in a solution, gravity can be replaced with the much more powerful---centrifugal force provided by a centrifuge.

Centrifugation

❑ A centrifuge is used to separate particles or macromolecules:

✓ cells
✓ Sub-cellular components
✓ Proteins
✓ Nucleic acids

❑ Basis of separation:

✓ Size
✓ Shape
✓ density

Ultracentrifugation

❑ Proteins are separated ultracentrifugation –very high speed spinning; with possibility of appropriate photography of the protein layers as they form in the centrifugal field, it is possible to determine the molecular weights of proteins.

❑ The ultracentrifuge is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating acceleration as high as 19600km/s2 ...around 50000rpm.

Cont….

❑ Modern ultracentrifuges can attain rotational speeds as high as 150,000 rpm (revolutions per minute) so as to generate centrifugal fields in excess of 1 million *g*.

✓ Sedimentation

✓ Preparative ultracentrifugation

Sedimentation

- The rate at which a particle sediments in the ultracentrifuge is related to its mass.

- The force, $F_{sedimentation}$, acting to sediment a particle of mass located at a distance r with angular velocity (rads/s).

- The $F_{centrifugal} = m\omega^2 r$, acts on the particle and $F_{buoyant} = V_p \rho \omega^2$ exerted by the solution.

- $F_{sedimentation} = m\omega^2 r - V_p \rho r \omega^2$ ....(1) where

  - V_p -particle volume
  - ρ- density of the solution
  - m- molar mass
  - ω- angular velocity

Cont…….

❑ The Motion of the particle through the solution is opposed by the fractional force:

$$- F_{friction} = vf \quad \text{..........(2)}$$

❑ where $v = dr/dt$ is the rate of migration of the sedimenting particle and f is its frictional coefficient. The particle's frictional coefficient can be determined from measurements of its rate of diffusion.

Cot……..

❑ Under the influence of gravitational force (centrifugal) force; the particle accelerates until the forces on it exactly balance:

$$-m\omega^2 r - V_p\rho r\omega^2 = vf \ldots\ldots\ldots(3)$$

✓ The mass of one mol of particle , is

-M=mN…….(4)   ,(N-Avogadro's no. 6.022*10^23)

❑ Thus, the particle's volume is expressed in terms of its molar mass:

-V_p= Ū*m =Ū*M/N …..(5) where Ū-particle's partial specific volume.

Sedimentation Rate

❑ Substituting equations (4)&(5) into equation (3) yields

$$vf = \frac{M\omega^2 r(1 - \bar{U} * \rho)}{N} \quad \ldots\ldots.(6)$$

❑ The sedimentation coefficient ,s, is defined as.

❑ The **sedimentation coefficient** *(s)* of a particle characterizes its <u>sedimentation</u> during <u>centrifugation</u>. It is defined as the ratio of a particle's sedimentation <u>velocity</u> to the applied <u>acceleration</u> causing the sedimentation

$$s = \frac{v}{r\omega^2} = \frac{1}{\omega^2}\frac{(d\ln r)}{dt} = \frac{M(1 - \bar{U} * \rho)}{Nf} \quad \ldots\ldots.(7)$$

❑ Sedimentation coefficient ,s, is usually expressed in units of second and known as svedbergs (S).

Stockes Equation

❑The fractional coefficient of unsolvated spherical particles with radius r, is determined according to Stockes equation as:

$$f = 6\pi\eta r \ \ldots\ldots..(8)$$

❑Where as the minimal fractional coefficient of a particle, f_o is calculated by assuming the particle as spherical ($V = 4/3 \eta r^3$)

$$f_o = 6\pi\eta(3M\bar{U}/4\pi N)^{1/3}\ldots\ldots\ldots(9)$$

Cont….

❑ If the fractional ratio, f/fo >1, implies that the particle is highly solvated and significantly elongated.

Example

❑ In a dilute buffer solution at 20℃ ,rabbit muscle aldolase has a fractional coefficient of $8.74*10^{-8}$ g/s, a sedimentation coefficient of 7.35 S, and a partial specific volume of 0.742cm3/g. Calculate the molecular mass of aldolase

➢ Assuming the density & viscosity of the solution to be 0.998 g/cm3 and $2*10^{-5}$, respectively.

Solution

- **Given**:
- -f = 8.74*10^-8 g/s
- -S =7.35 s
- -V (partial specific volume) = 0.742 cm^3/g
- -Density, ρ = 0.998g/cm3
- -Viscosity, η =2*10^-5
- **<u>Required</u>**: Molar mass

Solution

- The molar mass of the aldolase enzyme is calculated using sedimentation coefficient formula,

- $$\frac{S = M(1 - V*\rho)}{Nf}$$

- $(S*f = m(1 - V*\rho)$

- $m = \frac{S*f = 6.42*10^{\wedge}-7}{1 - v*\rho = 0.259} = 2.47 \mu g$