7. Separation by Precipitation

7.1. Protein Solubility

Separation by Precipitation

- **General concept**
  - Precipitate part of a mixture through alteration of some property of the solvent
  - Remove or collect precipitate by filtration or centrifugation
- **Types**
  - Salting out at high concentration
  - Precipitation with organic solvents
  - Precipitation with organic polymers and other materials
  - Affinity precipitation
  - Precipitation by selective denaturation

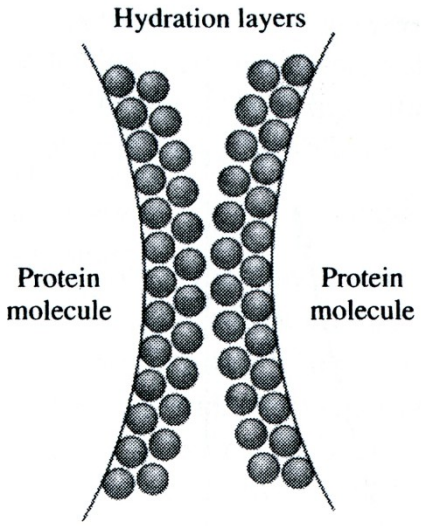
Solubility of Proteins at Low Salt Concentrations

- **Factors determining protein solubility**
  - Polar interactions with the aqueous solvent
  - Ionic interactions with the salts (0.15~0.2 M in cell)
  - Repulsive electrostatic forces between molecules or small aggregates

- **Factors affecting protein solubility**
  - Protein structure and size
    - Larger size → lower solubility
  - Protein charge
    - Higher charge → higher solubility
  - Solvent

Protein Structure and Size & Solubility

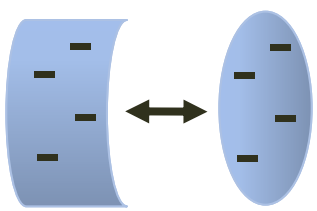
- **Protein structure**
  - Tendency during protein folding
    - Surface: polar groups
    - Core: hydrophobic groups
  - Still many nonpolar groups exist on the protein surface
    - 57% nonpolar, 25% polar, 19% charged (studies of 69 proteins)
  - Formation of hydration layer on the protein surface
    - Association of charged and polar groups
    - Immobilized by nonpolar groups
- **Larger size → lower solubility**
  - Solubility in the presence of the polymer
    - $lnS = \beta' - K'c_p$
      S; solubility
      β' : constant
      C_p : polymer concentration
    - $K' = V/ 2.303 \times [(r_s + r_r)/r_r)]^3$
      V : Partial specific volume of the polymer
      r_s : radius of the protein
      r_r : radius of the polymer rod



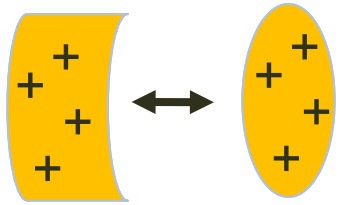
Protein Charge & Solubility

- **Higher charge → Higher solubility**
  - Greater interaction with dipolar water molecule
  - Repulsive reaction between protein molecules of like charge
- **Factors determining protein charge**
  - Ionizable residues and their *pKa*
  - Solvent accessibility of the ionizable residues
  - pH of the solution
  - Temperature, Chemical nature of the solvent
    - Dielectric constant
    - Ionic strength
- **Solubility depending on pH : Isoelectric precipitation**
  - Minimized electrostatic repulsion at pI→ hydrophobic interaction
  - Proteins have a minimum solubility around their isoelectric point
  - Lowering pH of tissue extract to pH 6.0 ~ 5.0
    - Generation of protein aggregates containing other materials (ribosome, membrane fragments etc.)

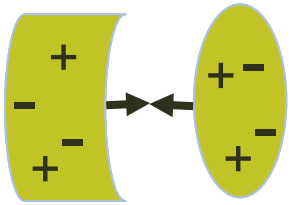
Isoelectric Precipitation



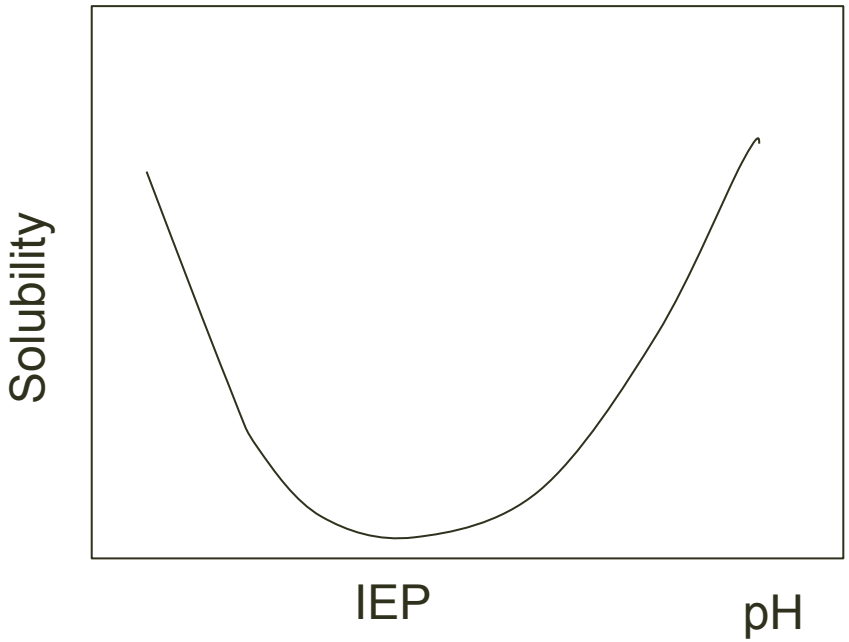
pH > IEP

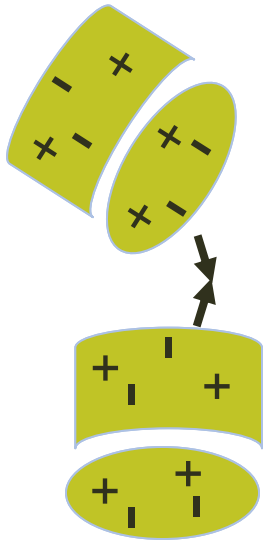


pH < IEP



pH = IEP





Solvent & Solubility

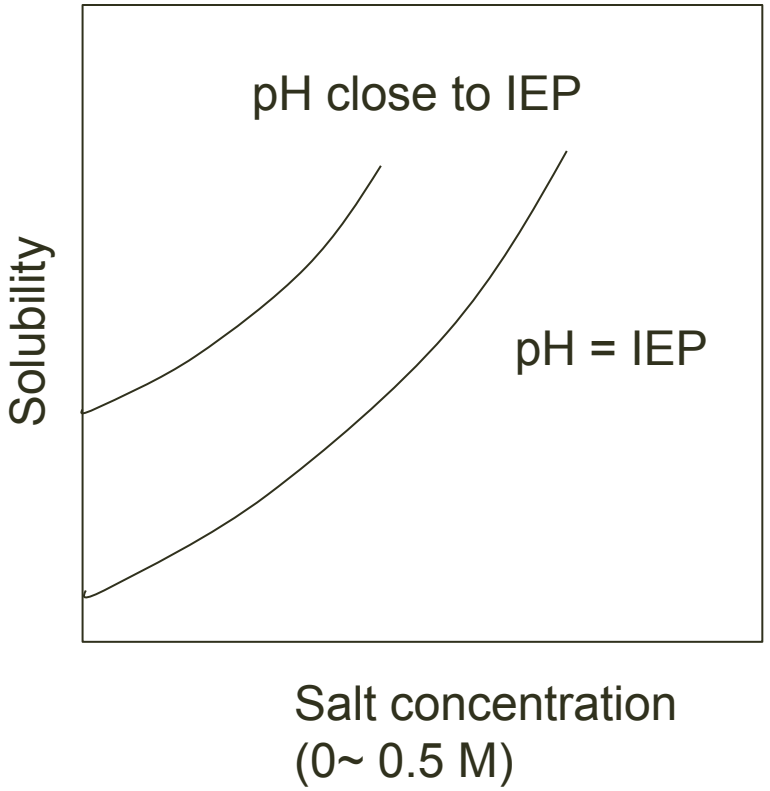
- **Effects of solvent**
  - Ionic strength of solvent
    - Salting in
      - Increase in solubility by increasing salt concentration
    - Salting out
      - Decrease in solubility at high salt concentration
  - Hydrophobicity
    - Monohydric alcohol
      - At room tempertaure
        - » Protein denaturation by binding to hydrophobic region
      - At low temperature
        - » Reduction of water activity
        - » Precipitation with organic solvent

Kirkwood's model

- $\mathbf{Ln(S_p/S_0) = K_iI - K_sI}$
  - S_p : solubility of the dipolar ion at ionic strength I
  - S_0 : solubility of the dipolar ion in the absence of salt
  - K_i : salting-in constant
  - K_s : salting-out constant
  - $I = \frac{1}{2} \sum c_i z_i^2$
    - c_i : molar concentration of any ions
    - z_i : charge of ions
- $\boldsymbol{K_i}$ α $\boldsymbol{(u/\varepsilon T)^2}$
- $\boldsymbol{K_s}$ α $\boldsymbol{V_e/\varepsilon T}$
  - ε : dielectric constant of the solvent
  - u : dipole moment
  - V_e : exclusive volume of the dipolar ion

Salting-In

- **High salting-in effect (K_i)**
  - Proteins with high dipole moments (u)
    - Globulins
      - Insoluble in the absence of salt
      - Higher dipole moment than the albumins
    - Albumin
      - Soluble in water and in high salt solution
  - Solvent with low dielectric constant
    - Relatively nonpolar solvents



Solubility of Hemoglobin

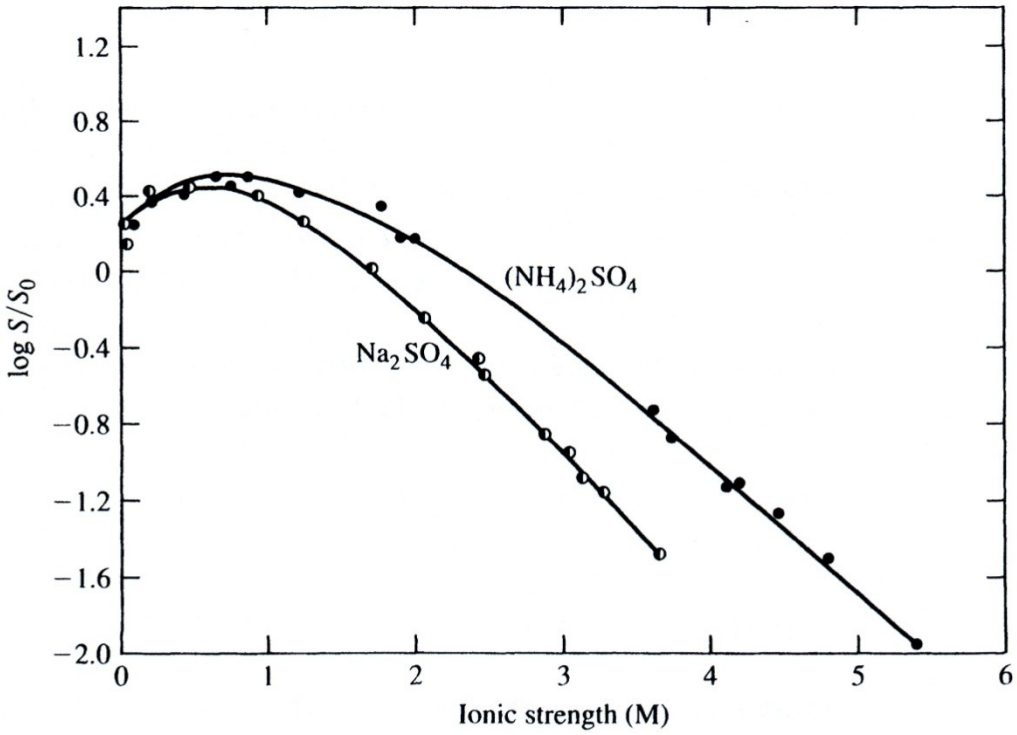
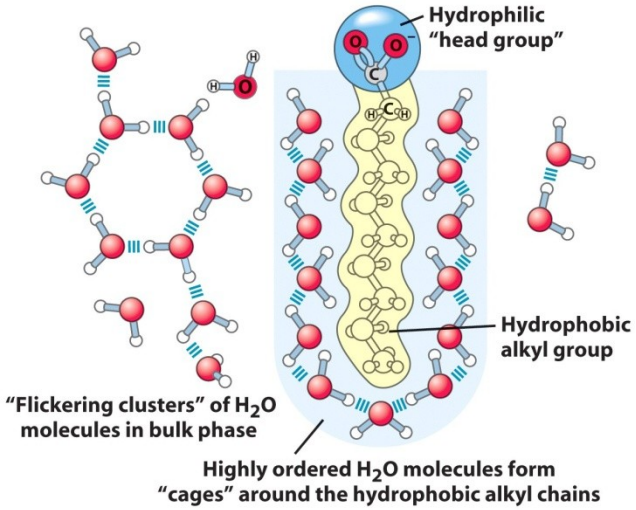


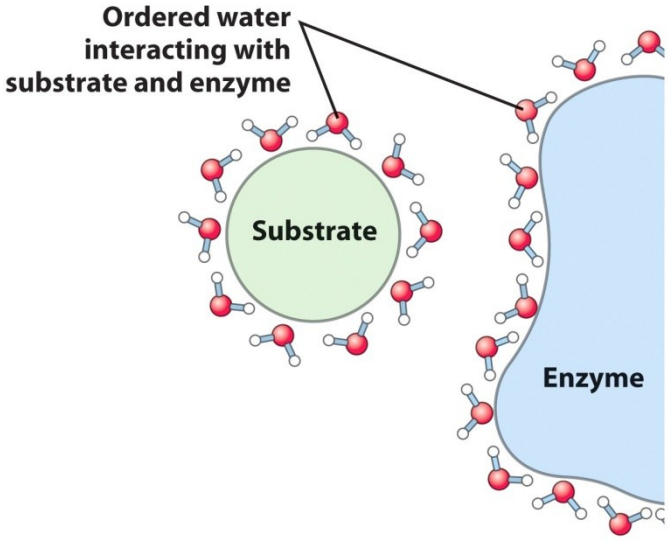
Figure 8.4 The effect of $(NH_4)_2SO_4$ and Na_2SO_4 on the solubility of hemoglobin: S_0 is the solubility in pure water, and S is the solubility in the salt solution. (Data from C. Tanford, *Physical Chemistry of Macromolecules*, p. 244, Wiley, New York, 1961.)

7.2. Salting Out

Salting Out

- **Salting out**
  - Protein precipitation at high salt concentration
  - Depending on hydrophobicity of the protein
    - cf. Salting in : depending on surface charge distribution and polar interactions with the solvent
- **Principles**
  - Ordering of water molecules around hydrophobic residues on surface of a protein
  - Solvation of salt ion “pull off” the ordered water molecules from the hydrophobic side chains → increasing hydrophobic interaction between proteins





Salting Out

- **Dissolving protein**
  - $P + nH_2O \rightarrow P\ nH_2O$
    - nH_2O : ordered water around hydrophobic residues
  - $\Delta G^0 = \Delta H^0 - T\Delta S^0$
    - $\Delta S^0 < 0$, $\Delta G^0 < 0$, $\Delta H^0 < 0$

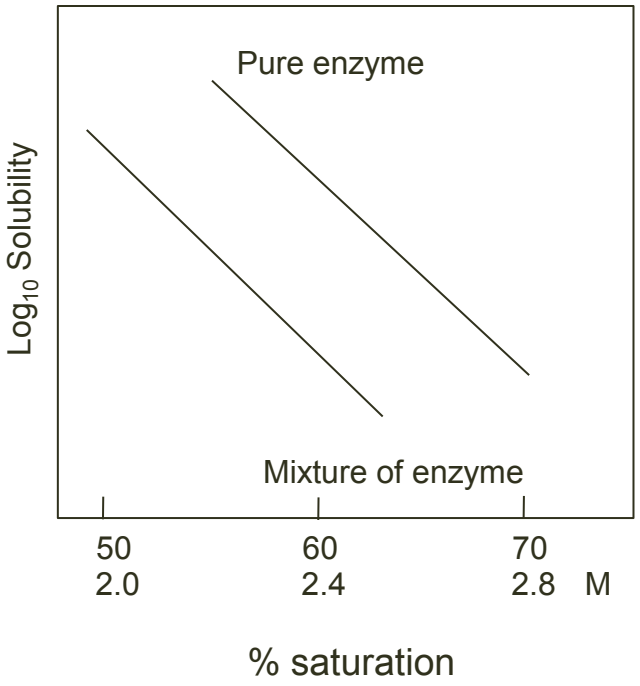
- **Interaction of hydrophobic molecules**
  - $P\ nH_2O + P'\ mH_2O \rightarrow P\text{-}P' + (n+m)\ H_2O$
    - $\Delta S^0 > 0$, $\Delta H^0 > 0$
    - ΔG^0 is depending on the relative magnitudes of ΔS^0 *and* ΔH^0
      - At low salt concentration : $\Delta G^0 > 0$, no aggregation
      - At high salt concentration : salt ions trap liberated water : $\Delta G^0 < 0$, aggregation
  - More aggregation at high temperature

Factors Affecting Salting Out

- **Solubility of pure protein**
  - Cohn equation (empirical)
    - $lnS = \beta - K'_s I$
    - $lnS_p = lnS_0 - (K_s - K_i)\, I$ (from Kierwood equation)

      K'_s : salting-out constant
      - Characteristic of the specific protein and salt
      - Independent of T or pH

      β : hypothetical solubility at zero ionic strength
      - Depending on T and pH
      - Minimum at the IEP
  - Decrease in solubility in the presence of other proteins
- **pH**
  - Higher precipitation close to protein's IEP
- **Temperature**
  - Decrease solubility with increasing temperature



Salts

- **Catastrophic salt**
  - Direct interaction and destabilization of protein

- **Salts for salting out**
  - Hydration of polar regions on the protein without direct interaction
    - Related with molar surface tension increments of the salts
    - Salts increasing surface tension of the solvent → enhance interaction between proteins
    - Multivalent anions
      - $Acetate^- < SO_4^{2-} < PO_4^{3-}$
    - Innocuous cations
      - $Na^+ < K^+ < NH_4^+$
  - Salts with high solubility
    - Require high concentration of salt for salting out
  - Low cost
  - Low ΔH
    - No heating while dissolving in the salt
    - Little variation in solubility depending on temperature
  - Density of concentrated solution
    - Should not be too high to separate precipitate by centrifugation

→ Ammonium sulfate it the best choice !

Salting Out with Ammonium Sulfate

- **Advantage of using ammonium sulfate**
  - Little variation in solubility depending on temperature (0-30 ^{o}C)
  - High solubility ; 4 M in pure water
  - Density of saturated solution : 1.235 g/cm^{-3}, not too high
- **Considering factors**
  - Contamination of heavy metals (esp. iron) is detrimental to sensitive enzymes → Add EDTA
  - Best to use at a neutral pH (pH 6-7.5)
    - Slight acidifying action → add ~ 50 mM buffer (phosphate)

Calculation of the Amount of Ammonium Sulfate

- Solubility : 533g/L at 20 °C, 4.05 M at 100% saturation
- 761g to 1L water to make saturated solution
  - Specific volume of ammonium sulfate : 0.54 ml/g
  - Density : 1761/1425 = 1.235g/cm^{-3}
- Gram ammonium sulfate to be added to 1L of solution
  - At M_1 molar, to take it to M_2 molar

    $g=[533(M_2-M_1)]/(4.05-0.3M_2)$
  - At S_1% saturation, to take it to S_2% saturation

    $g=[533(S_2-S_1)]/(100-0.3S_2)$

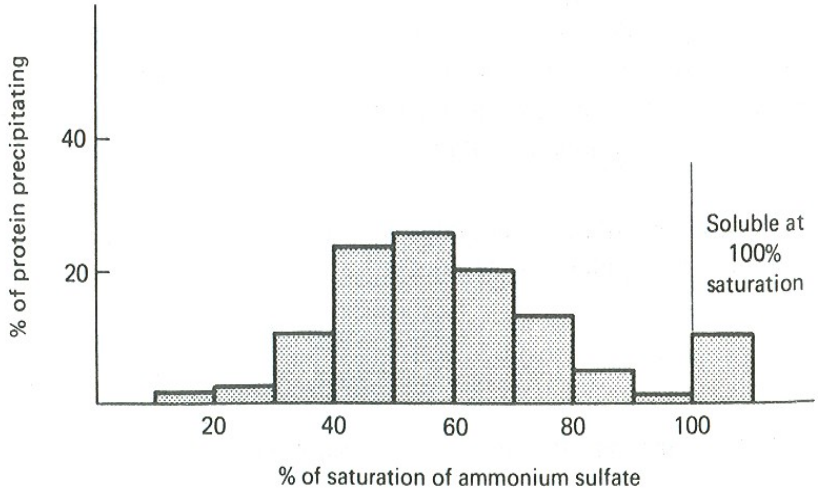
Appendix A: Precipitation Tables

Table I. Ammonium Sulfate, Grams to be Added to 1 Liter

From S_1% \ To S_2% →	5	10	15	20	25	30	35	40	45
0	27	55	84	113	144	176	208	242	277
5		27	56	85	115	146	179	212	246
10			28	57	86	117	149	182	216
15				28	58	88	119	151	185
20					29	59	89	121	154
25						29	60	91	123
30							30	61	92
35								30	62
40									31
45									
50									

Applications of Salting Out with Ammonium Sulfate

- **Protein purification by ammonium sulfate fractionation**
  - Proteins are stable for years as a 2-3M ammonium sulfate suspension
  - Prevention from proteolysis and bacterial action
  - Determining proper concentration of ammonium sulfate
    - Try serial saturation range
    - e.g. 0-40, 40-60,60-80
    - Can be changed depending on the protein concentration and properties of other proteins
- **Concentration of protein**



Procedure of Salting Out with Ammonium Sulfate

- Slow addition of finely ground powder with stirring
  - If proteins precipitate below 50%, saturate ammonium sulfate solution can be used
- Continue stirring for 10-30 min after complete dissolving
- Centrifuge 10,000g for 10 min or 3,000g for 30 min
- Add more solid ammonium sulfate to make next concentration, repeat stirring and centrifugation
- Dissolve the pellet with no more than 1-2 times of the volume of the precipitate
- Remover insoluble material by further centrifugation

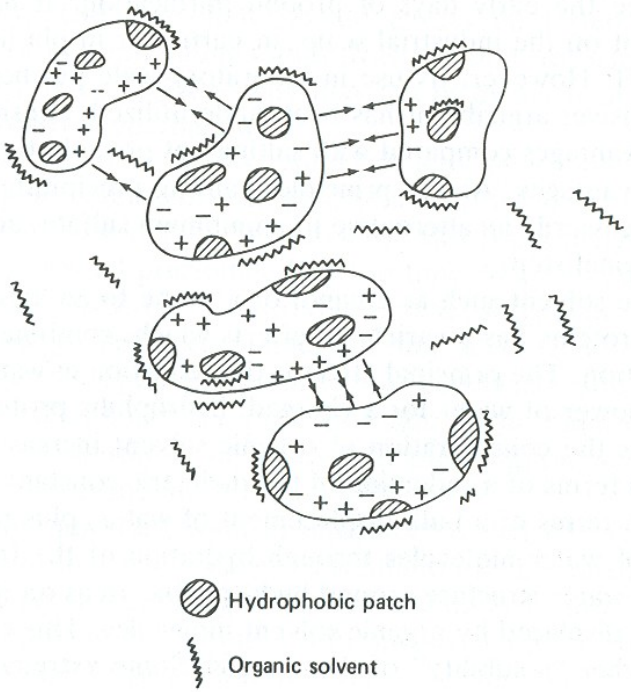
7.3. Precipitation with Organic Solvents

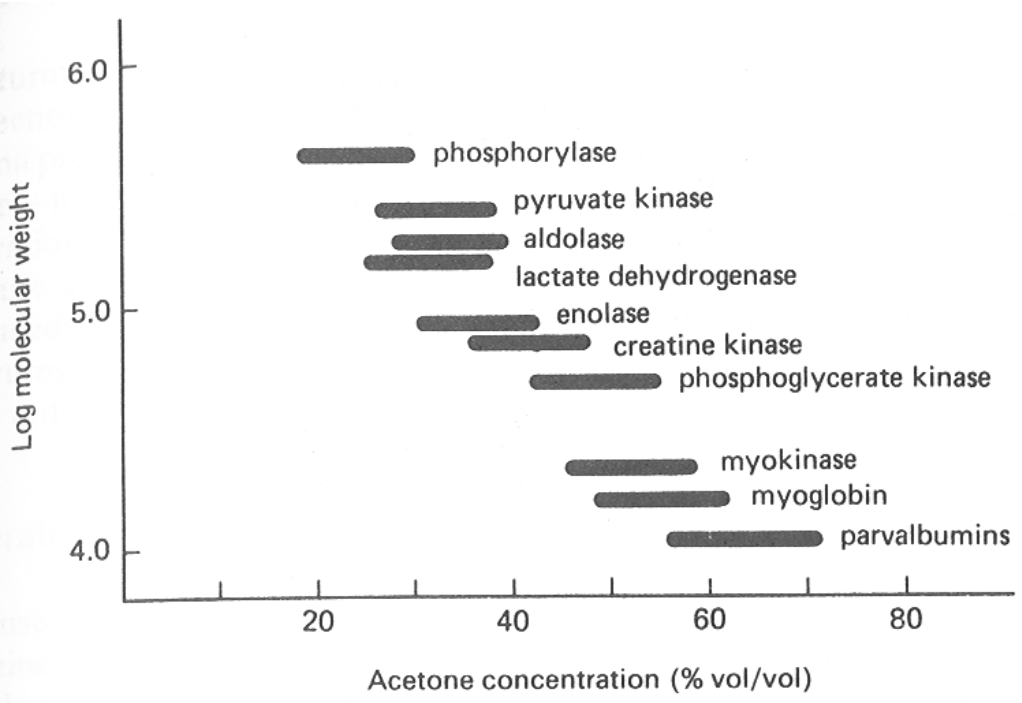
General Theory

- **Precipitation with Organic solvents**
  - Protein precipitation by water-miscible organic solvents
  - Important on the industrial scale
- **Principle**
  - Addition of miscible solvent (ethanol, acetone)
    - Reduction in water activity
    - Decrease in solvation power of water for a charged, hydrophilic proteins
      - * Reduction of the dielectric constant of the solvent
      - * $F=Q_1Q_2/(\varepsilon r^2)$: Force of ionic interaction in a solution
    - Increase in solubility of hydrophobic area
  - Net effect
    - Decrease in protein solubility → aggregation and precipitation

Precipitation with Organic Solvent

- Protein precipitation by electrostatic and dipolar forces
  - Hydrophobic interactions are less involved
- Large molecules precipitate at lower solvent concentration
  - At 50% solvent only <20,000 proteins far from their IEP remain in solution
- Precipitation at lower organic solvent concentration at around the IEP



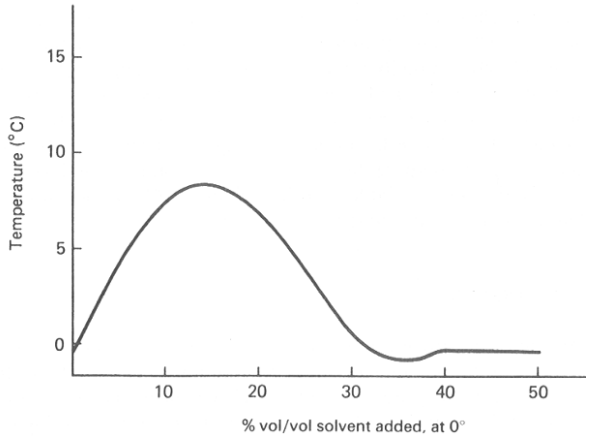


Choice of Solvent

- **Water-miscible and safe solvent**
  - Ethanol, acetone
  - Other solvents : Methanol, n-propanol, i-propanol, dioxan, ethers, ketones
- **Denaturation by solvent**
  - At higher temperature
    - Penetration inside of the protein and hydrophobic interaction with internal nonpalr residues
    - Longer chain alcohols are more denaturing than short-chain ones

Operating Conditions

- **Low temperature : 0°C**
  - Increase in temperature until addition of 20% v/v solvent
  - More efficient precipitation at low temperature



- **Proper salt concentration : 0.05-0.2 ionic strength**
  - Too high : inhibition of electrostatic aggregation
    - Need high conc. of organic solvent → denaturation pron
  - Too low : Fine precipate which is difficult to sediment
- **Assuming additive volume**
  - Addition of acetone to 50% v/v
    - 50 ml acetone + 50 ml water = 95 ml mixture

Operating Procedure

- **Incubation with solvent for 10-15 min**
- **Centrifugation and resuspend the pellet**
- **Serial increase in organic solvent**
  - Volume to add to 1 L to take % from x to y
  - [1000 (y-x)]/(100-y) ml
- **Removal of organic solvent**
  - Up to 5% v/v do not affect other fractionation methods (exception: hydrophobic chromatography)
  - Evaporation of organic solvent at 25-30^{o}C using Buchner flask
  - Gel filtration column

7.4. Precipitation with Organic Polymers

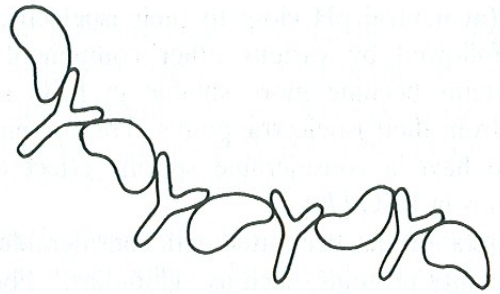
Organic Polymers

- **Polyethylene Glycol (PEG)**
  - High molecular weight neutral, water soluble polymer
    - MW 4,000, 6,000, 20,000
  - Similar to precipitation by organic solvent
- Removal of PEG
  - Not removed easily by dialysis or desalting column
  - Residual low levels of PEG is not detrimental to many procedures (salting out, ion exchange, affinity, gel filtration chromatography)
- **Charged polymers (polyelectrolytes)**
- Precipitation at very low percentage (0.05-0.1 w/v)
  - Cheap, no waste disposal → industrial application
- Formation of electrostatic complex between polyacrylic acid salts and positively charged proteins at low pH (3-6)
  - Efficient for basic proteins: lysozyme, cytochrome C, trypsin

7.5. Affinity Precipitation

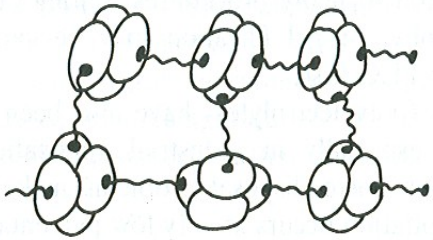
Affinity Precipitation

- **Principle**
  - Use of protein's specific and selective interaction with ligand
  - Ligands
    - Substrate or inhibitor for enzymes
      - Rare event of precipitation by binding
    - Antibody
      - Divalent, polyclonal
      - Can induced precipitation of target protein
    - Bis-ligand



Polyclonal antibody + monomer

Poly(mono)clonal antibody + dimer



Bis-ligand or antibody + dimer

7.6. Precipitation by Selective Denaturation

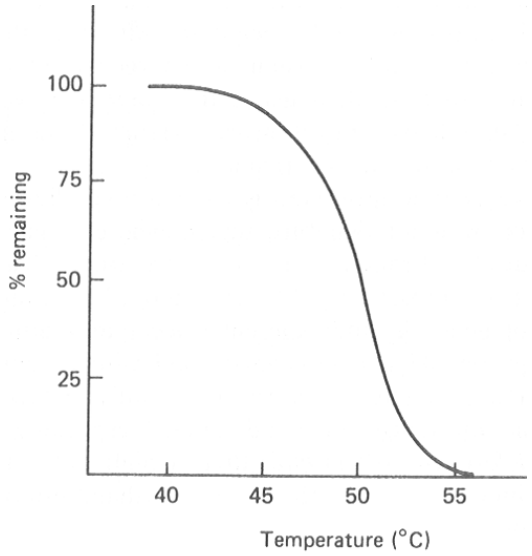
Precipitation by Selective Denaturation

- **General Principles**
  - Selective denaturation of unnecessary proteins
  - Denaturing method
    - Temperature
    - pH
    - Organic solvents
  - Denaturing conditions
    - Inter-dependent
    - Affected by ion concentration

Temperature Denaturation

- **Rate constant or heat denaturation of protein**
  - First-order process
  - $d(\ln k_{den})/dT = E_{act}/(RT^2)$
  - E_{act} = energy of activation

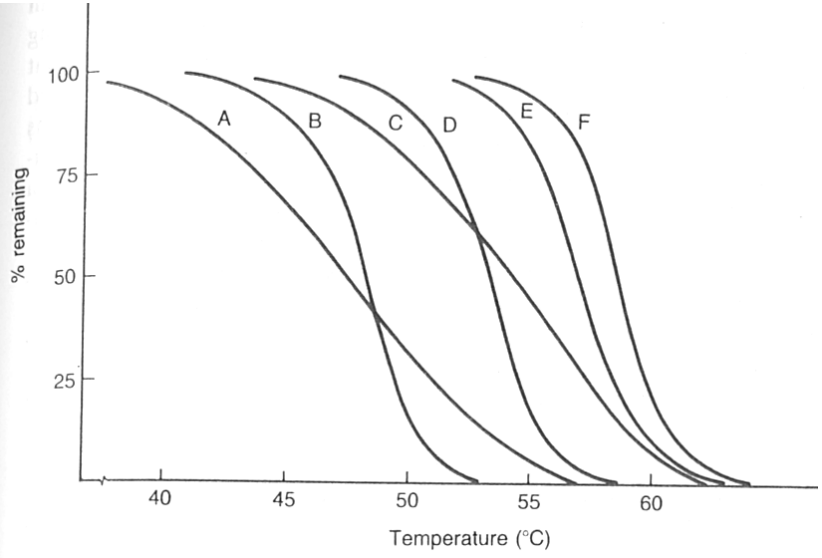
  $= \Delta H^{\ddagger} + RT$
    - Higher (200-400 kJ/mol) than normal chemical reaction (40-70 kJ/mol) because of high entropy change



Theoretical denaturation curve
E_{act} = 400 kJ/mol , 10 min incubation

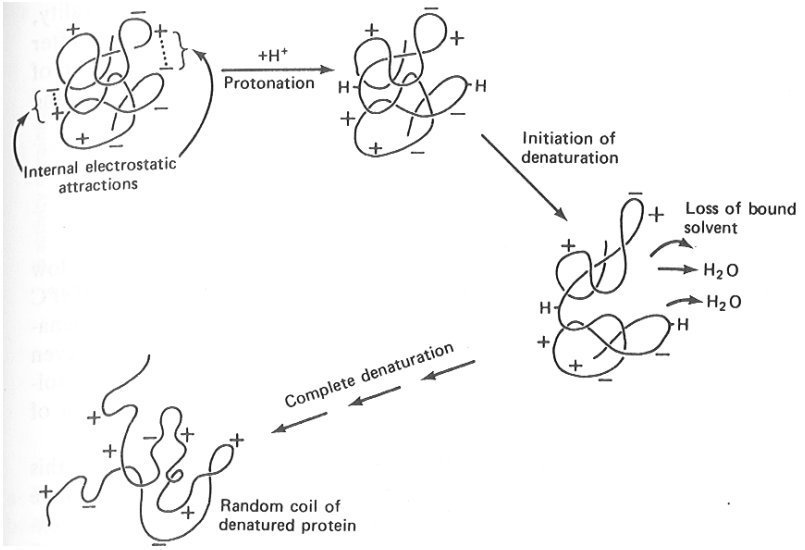
Purification using heat denaturation

- **Purification of protein D**
  - Heating for 10 min at 52°C → Denaturation of A,B, and C
- **Prevention of protease attack**
  - Proteases are heat resistant and more active at higher temperature
  - Addition of ammonium sulfate to inhibit protease activity
- **Sometimes the presence of substrate can stabilize the enzyme**
- **The result is pH-dependent**



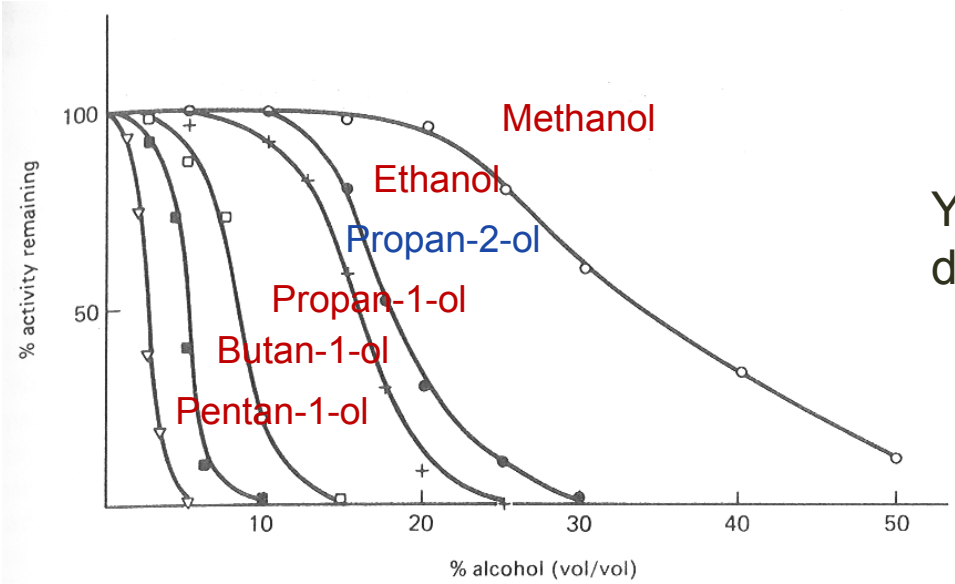
pH Denaturation

- **Optimal pH for enzyme stability**
  - Correlation with its physiological environmental pH
- **Effect of pH change in denaturation**
  - Disturbance of charge-charge interactions in proteins
  - Rate-limiting step
    - Opening-up step
    - Depending on temperature
- **Acids and bases for pH change**
  - Weak acid or base
    - Tris, acetic acid : pH 4.5-8.5
    - Lactic acid : , pH 3.4
    - Diethanolamine (pH 9)
    - Sodium carbonate (pH 10.5)
  - Strong acid or base (avoid in general)
    - Phosphric acid, sulfuric acid (pH 2)
    - NaOH, KOH (> pH 11)



Denaturation by Organic Solvents

- **Principles**
  - Denaturation at 20-30°C
  - Lower organic solvent concentration than for precipitation of native proteins
  - Differential sensitivity of proteins
    - E.g. rabbit muscle creatine kinase : stable to 60% v/v ethanol at 25°C
- **Effect of chain length of alcohols**
  - Longer chains are more denaturing
    - Addition of one methylen group → 2 fold decrease in alcohol concentration for 50% denaturation
  - Branched chains are less denaturing
    - Hydrophobicity is an important factor



Yeast glyceraldehyde phosphate dehydrogenase (30 °C, 30 min)