# *Precipitation*

# Introduction

- widely used for the recovery of bulk proteins
- can be applied to fractionate proteins (separate different types) or as a volume reduction method
- For example: all the proteins in a stream might be precipitated and redissolved in a smaller volume or a fractional precipitation might be carried out to precipitate the protein interest and leave many of contaminating proteins in the mother liqour
- Precipitation is usually induced by addition of a *salt* or an *organic solvent*, or by *changing the pH* to alter the nature of the solution.
- the primary advantages: relatively inexpensive, can be carried out with simple equipment, can be done continuously and leads to a form of the protein that is often stable in long-term storage

# Protein Solubility

- The most important factors affecting the solubility of proteins are structure and size, protein charge, and the solvent. Explanations follow for each of these factors.

## Structure and Size

- In the native state, a protein in an aqueous environment assumes a structure that

✓minimizes the contact of the hydrophobic amino acid residues with the water solvent molecules and

✓maximizes the contact of the polar and charged residues with the water.

- The major forces acting to stabilize a protein in its native state are hydrogen bonding, van derWaals interactions, and solvophobic interactions (driven forces of folding protein).
- In aqueous solution, these forces tend to push the hydrophobic residues into the interior of the protein and the polar and charged residues on the protein's surface.

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# Protein Solubility

- Thus, in spite of the forces operating to force hydrophobic residues to the proteins interior, the surface of proteins usually contains a significant fraction of non polar atoms. The forces acting on a protein lead to the achievement of a minimum Gibbs free energy.
- For a protein in its native configuration, the net Gibbs free energy is on the order of only 10 to 20 kcal/mol.
  - This is a relatively small net free energy, which means that the native structure is only marginally stable and can be destabilized by relatively small environmental changes
  - Water molecules bind to the surface of the protein molecule because of association of charged and polar groups and immobilization by nonpolar groups.

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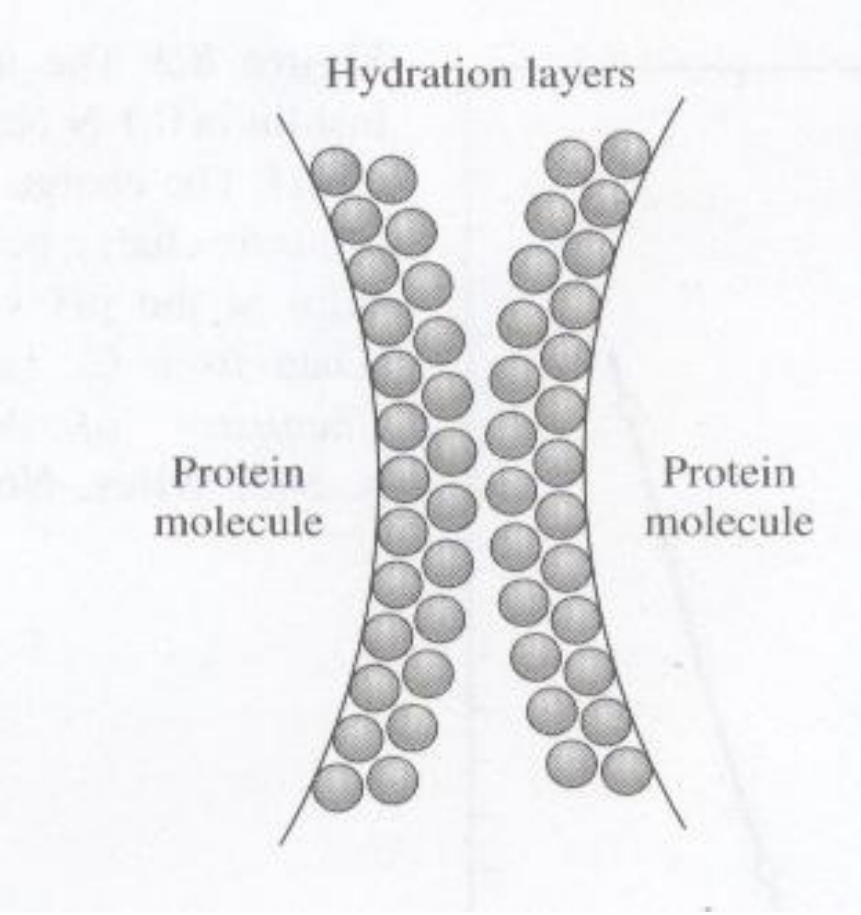
# Protein Solubility




- For example, a study of the hydration of human serum albumin found two layers of water around the protein.
- These hydration layers are thought to promote solubility of the protein by maintaining a distance between the surfaces of protein molecules. This phenomenon is illustrated in Figure 1.

Figure 1 Schematic diagram of the limit of approach of two protein molecules to each other because of the hydration layers on each molecular surface

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# Protein Solubility

- The size of a protein becomes important with respect to solubility when the protein is excluded from part of the solvent- happen when nonionic polymers - are added to the solution result in steric exclusion of protein molecules from the volume of solution occupied by the polymer.
  - Juckes developed a model for this phenomenon based on the protein molecule being in the form of a solid sphere and the polymer molecule in the form of a rod- gave the following equation for S, the solubility of the protein:

$$\ln S = \beta' - K' c_p \qquad \text{E.1}$$

$$K' = \frac{\bar{V}}{2.303}\left(\frac{r_s + r_r}{r_r}\right)^3 \qquad \text{E.2}$$

$r_s$ and $r_r$= the radius of the protein solute and polymer rod, respectively,

$\bar{V}$ = the partial specific volume of the polymer,

$c_p$ = the polymer concentration, and β' = a constant.

# Protein Solubility

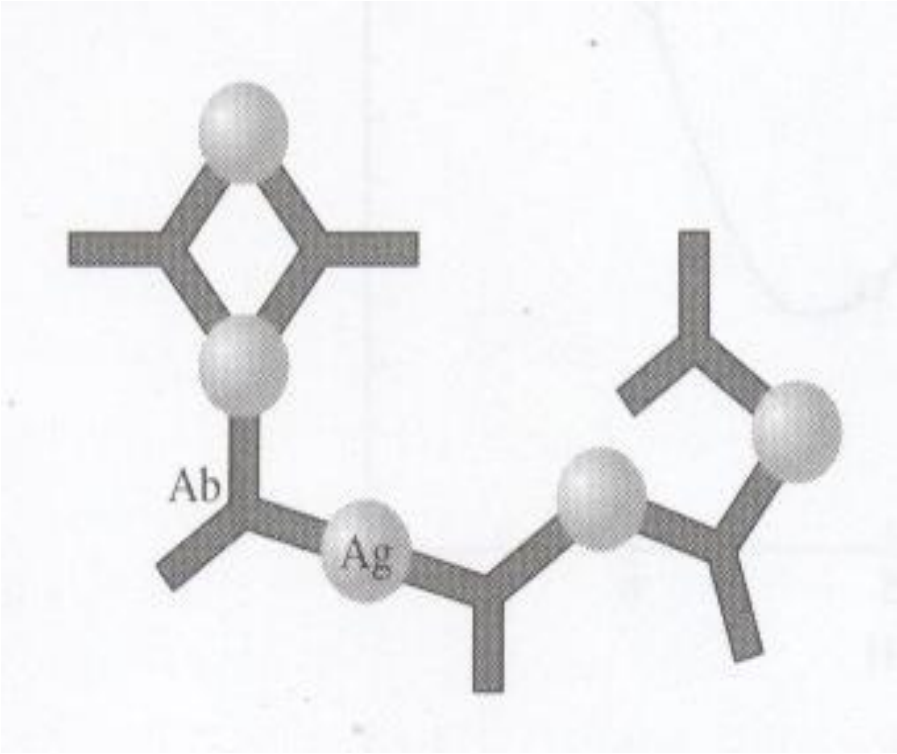


Figure 2 Schematic representation of antibody—antigen (Ab—Ag) interaction.

- Based on this model- can expect the lowest protein solubility for large proteins.
- Molecular size - predominant factor in a type of precipitation known as affinity precipitation. When affinity groups or antibodies to a specific biomolecule (antigen) are added to a solution, the antibody—antigen interaction can form large multimolecular complexes as shown in Figure 5.2.
- Such complexes are usually insoluble and cause selective precipitation of the antigen.

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# Protein Solubility

## Charge

- The net charge of a protein has a direct bearing upon the protein's solubility.
- The solubility of a protein increases as its net charge increases, a result of greater interaction with dipolar water molecules.
- A repulsive reaction between protein molecules of like charge further increases solubility.
- A simple way to vary the charge on a protein is by changing the pH of the solution. The pH of the solution in which a protein has zero net charge is called the isoelectric pH or isoelectric point.
- The solubility of a protein - minimum at the isoelectric point. A typical example is shown in Figure 3.
- Nonuniform charge distribution, however, results in a dipole moment on the molecule, which leads to an increase in solubility and a move in the minimum solubility away from the isoelectric point.

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# Protein Solubility

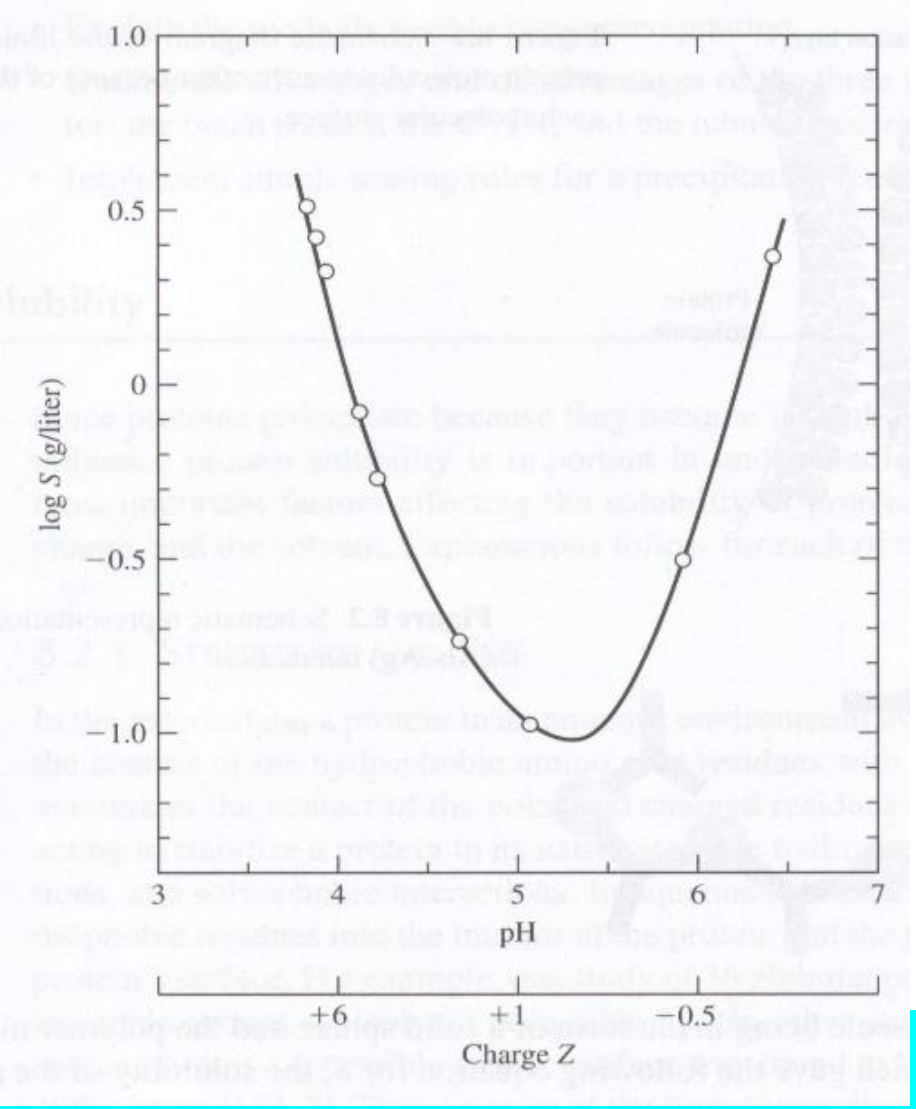


Figure 3 The solubility (S) of insulin in 0.1 N NaCl as a function of pH. The charge Z is the average protonic charge per 12,000 g of insulin at the pH values indicated.

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# Protein Solubility

- The net charge of a protein is determined by the following factors:
  - the total number of ionizable residues,
  - the accessibility of the ionizable residues to the solvent,
  - the dissociation constants (or $pK_a$ values) of the ionizable groups, and
  - the pH of the solution
- Besides the chemical makeup of the ionizable groups, factors that can influence the $pK_a$ values are
  - the chemical nature of the neighboring groups (e.g.. inductive effects),
  - the temperature,
  - the chemical nature of the solvent as partially reflected by its dielectric constant, and
  - the ionic strength of the solvent.

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# Protein Solubility

## Solvent

The solvent affects the solubility of proteins primarily through two parameters, hydrophobicity and ionic strength

*Hydrophobicity*

- observations of single-phase solutions of water and monohydric alcohols - cause protein denaturation at room temperature - can be avoided at sufficiently low temperatures.
- Studies of monohydric alcohols have shown that denaturing efficiency is as follows:

  methanol < ethanol < propanol <butanol
- conclusion : alcohols with longer alkyl chains - binding more effectively to apolar groups on the protein, weakening intraprotein hydrophobic interactions and thus leading to denaturation.
- when the temperature is low, the monohydric alcohols compete for the water of hydration on the protein and cause the protein molecules to approach more closely, so that van der Waals interactions lead to aggregation.

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# Protein Solubility

*Ionic strength*

- The ionic strength of the solvent can have both solubilizing and precipitating effects.
- The solubilizing effects - referred to as salting in, while the precipitating actions are called salting out.
- The addition of small quantities of neutral salts to a protein solution often increases protein solubility; the 'salting in' effect.
- However, increasing salt concentrations above an optimal level leads to destabilization of proteins in solution and eventually promotes their precipitation- known as 'salting out'
- salting-in effects by considering the solute size, solute shape, solute dipole moment, solvent dielectric constant, solution ionic strength, and temperature.

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# Protein Solubility

- Kirkwood's models describe the interactions which as follows:

$$\ln\left(\frac{S_p}{S_0}\right) = K_i I - K_S I \qquad \text{E3}$$

- where $S_p$ = the solubility of the dipolar ion at ionic strength I,
  $S_o$ = the solubility of the dipolar ion in the absence of salt,
  $K_i$ = the salting-in constant, and $K_s$ = the salting-out constant.

Ionic strength is defined by

$$I = \frac{1}{2}\sum_i c_i z_i^2 \qquad \text{E4}$$

where $c_i$, is the molar concentration of any ion and $z_i$ is its charge.

The salting-in and salting-out constants can be related to other variables as follows:

$$K_i \alpha \left(\frac{u}{\varepsilon T}\right)^2 \qquad \text{E5}$$

$$K_s \alpha \frac{V_e}{\varepsilon T} \qquad \text{E6}$$

where $\varepsilon$ = the dielectric constant of the solvent, $T$ = temperature,
$V_e$ = the excluded volume of the dipolar ion, $u$ = dipole moment (C cm)

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# Protein Solubility

- the salting-in term increases more than the salting-out term as the of dielectric constant decreases.
- The dielectric constant decreases as the polarity of the solvent decreases. Therefore, the salting-in effect tends to predominate in relatively nonpolar solvents, while the salting-out effect is more dominant in aqueous solvents.
- At high ionic strength, the salting-out effect becomes predominant and can be described empirically by the Cohn equation

$$\ln S = \beta - K_s' I \qquad \text{E7}$$

- $K_s'$ is a salting-out constant characteristic of the specific protein and salt that is independent of temperature and pH above the isoelectric point.
- The constant β, the hypothetical solubility of the protein at zero ionic strength, depends only on temperature and pH for a given protein and is a minimum at the isoelectric point

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# Protein Solubility

the Kirkwood equation for the solubility of dipolar ions [E3] can be arranged to give

$$\ln S_p = \ln S_0 - (K_s - K_i)I \qquad \text{E8}$$

which is also identical in form to the Cohn equation, with

$$\beta = \ln S_o \qquad \text{E9} \qquad K_s' = K_s - K_i \qquad \text{E10}$$

- Both salting in and salting out are illustrated in Figure 4 for hemoglobin with ammonium sulfate or sodium sulfate being added.

- From zero ionic strength, the solubility of the protein increases to a maximum as salt is added and then continuously decreases as even more salt is added.

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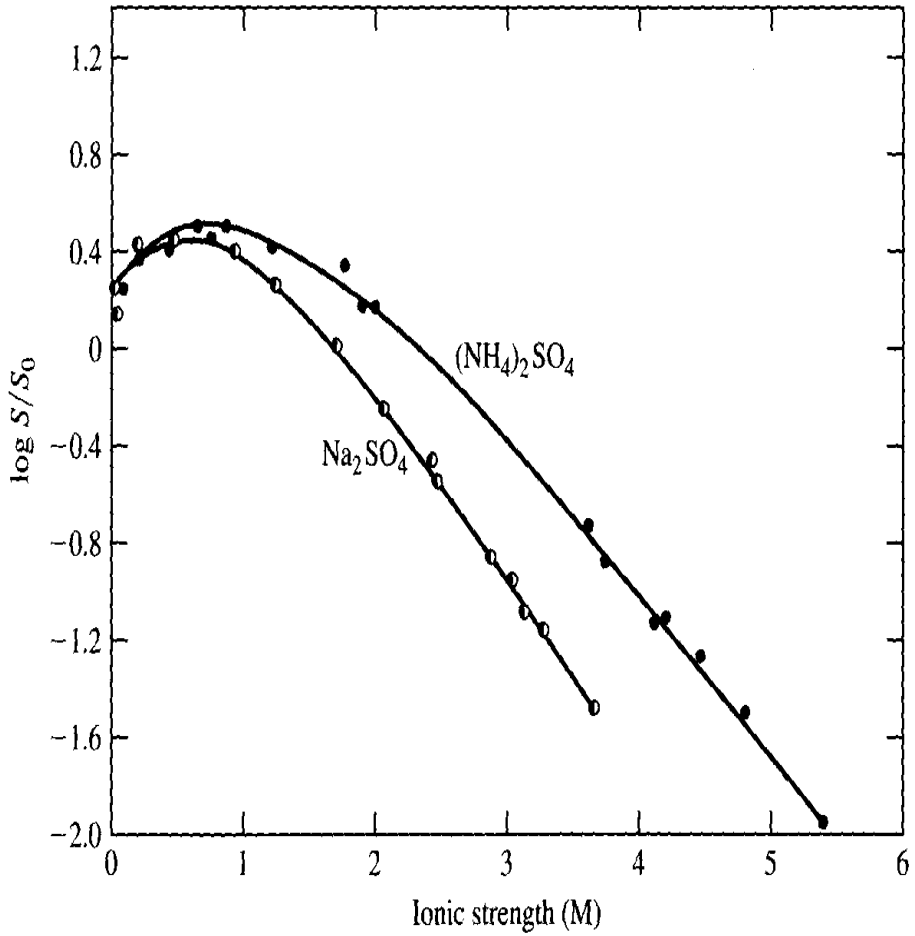
# Protein Solubility




- Figure 4: The effect of $(NH_4)\ SO_2$ and $Na_2\ SO_4$ on the solubility of hemoglobin: $S_0$ is the solubility in pure water, and S is the solubility in the salt solution.

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# Example 1

## Salting Out of a Protein with Ammonium Sulfate

Data were obtained on the precipitation of a protein by the addition of ammonium sulfate. The initial concentration of the protein was 15 g/liter. At ammonium sulfate concentrations of 0.5 and 1.0 M, the concentrations of the protein remaining in the mother liquor at equilibrium were 13.5 and 5 g/liter, respectively. From this information, estimate the ammonium sulfate concentration to give 95% recovery of the protein as precipitate.

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# Example 1

## Solution

**We can use the Cohn equation [Equation (7)], to solve this problem if we can determine the constants in the equation. Since ionic strength is directly proportional to concentration c for a given salt [Equation (4)], we can rewrite the Cohn equation as**

$$\ln S = \beta - K_s'' c$$

**Substituting the experimental data into this equation gives**

$$\ln 13.5 = \beta - 0.5 K_s''$$
$$\ln 5.0 = \beta - 1.0 K_s''$$

**Solving these equations for the constants yields**

$$\beta = 3.60$$
$$K_s'' = 1.99\ \mathrm{M}^{-1}$$

**For 95% recovery, the protein solubility in the mother liquor at equilibrium is 5% of the initial protein concentration. At this solubility, from the Cohn equation**

$$c = \frac{\beta - \ln S}{K_s''} = \frac{3.60 - \ln(0.05 \times 15)}{1.99} = 1.95\ \mathrm{M}$$

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# Precipitate Formation Phenomena

- important characteristics of protein precipitation are the particle size distribution, density and mechanical strength
- protein precipitates that consist largely of particle sizes with small particle sizes can be difficult to filter or centrifuge
- low particle densities also can lead to filtration or centrifugation problems and can give excessive bulk volumes of the final dried precipitate
- particles with low mechanical strength can give problem with excessive attrition when the dry particles are moved
- low strength can also be interpreted as gel formation, which leads to major problems in filtration and centrifugation
- precipitates form by a series of steps that occur in sequence; initial mixing, nucleation, growth governed by diffusion and growth governed by fluid motion
- The completion of the growth by fluid motion step can be followed by an "aging" step, where the particles are mixed until reaching a stable size

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# Precipitate Formation Phenomena

## Initial Mixing

- initial mixing – the mixing required to achieve homogenity after the addition of a component to cause precipitation
- important to bring precipitant and product molecules into collision as soon as possible
- important to know the mean length of eddies, also known as the "Kolmogoroff length", $l_e$

$$l_e = \left( \frac{\rho v^3}{P/V} \right)^{1/4} \qquad \text{E11}$$

where $\rho$ = the liquid density, $v$ = the liquid kinematic viscosity and $P/V$ = the agitator power input per unit volume of liquid

- necessary to mix until all molecules have diffused across all eddies
- this time can be estimated from the Einstein diffusion relationship

$$t = \frac{\delta^2}{2\mathcal{D}} \qquad \text{E12}$$

where δ is the diffusion distance and $\mathcal{D}$ is the diffusion coefficient for the molecule being mixed

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# Precipitate Formation Phenomena

- for spherical eddies of diameter $l_e$, this becomes

$$t = \frac{l_e^2}{8\mathcal{D}} \qquad \text{E13}$$

- thus precipitation is initiated in a well-stirred tank for a period of time determined on the basis of isotropic turbulence (turbulence in which the products and squares of the velocity components and their derivatives are independent of direction, or, more precisely, invariant with respect to rotation and reflection of the coordinate axes in a coordinate system moving with the mean motion of the fluid.)

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# Precipitate Formation Phenomena

## Nucleation

- is the generation of particles of ultramicroscopic size
- for particles of a given solute to form, the solution must be supersaturated with respect to the solute
- in a supersaturated solution the concentration of the solute in solution is greater than the normal equilibrium solubility of the solute
- the difference between the actual concentration in solution and the equilibrium solubility is called the degree of supersaturation or supersaturation
- the rate of nucleation increases exponentially up to the maximum level of supersaturation or supersaturation limit which is illustrated in Figure 5
- the rate of nucleation increases to a very high value at the supersaturation limit.
- High supersaturations - have negative consequences in carrying out precipitation - the precipitate tends to be in the form of a colloid, a gel, or a highly solvated precipitate
- to obtain precipitate particles having desirable characteristics, the supersaturation should be kept relatively low



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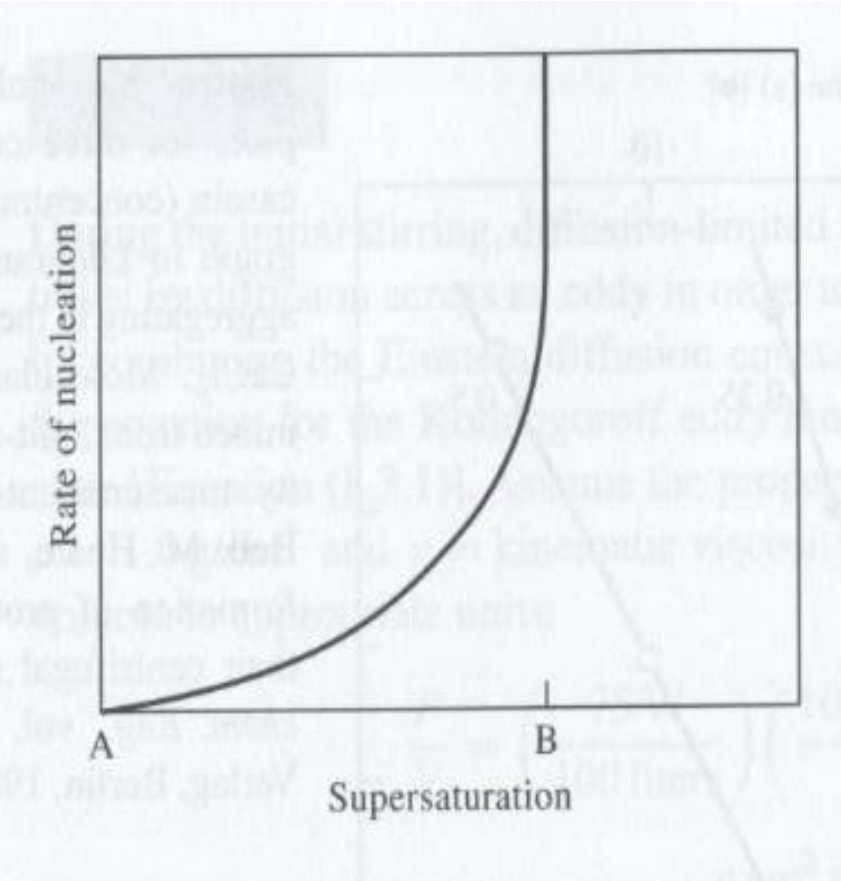
# Precipitate Formation Phenomena




- **Figure 5: Nucleation rate as a function of degree of supersaturation. The normal equilibrium solubility is at A and the supersaturation limit is at B**

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