Scientific Practise
MCBG 2036
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Consultation Hours: Thursday- Friday: 13H30- 14H00

LEARNING OUTCOMES

By the end of this lecture you will be able to:

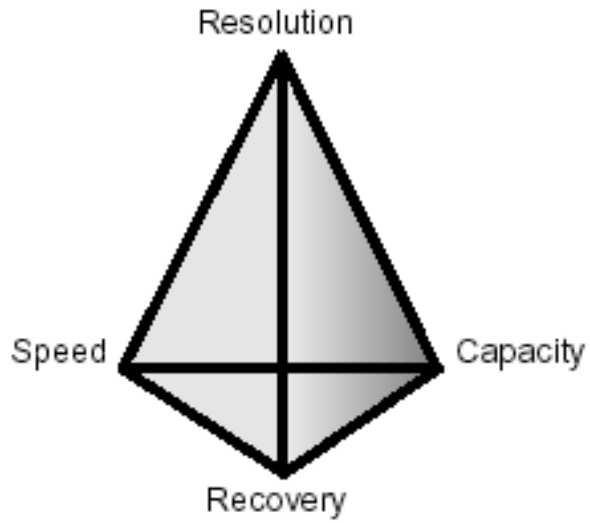
1.Describe most common methods of protein isolation and purification

2.Compare between different methods of protein purification

3.Construct a purification algorithm based on your knowledge in protein purification

4. Estimate percentage yield of the protein

Selection and Combination of Purification Techniques



Every technique offers a balance between resolution, capacity, speed and recovery.

Minimise sample handling
Minimise number of steps
Use different techniques at each step

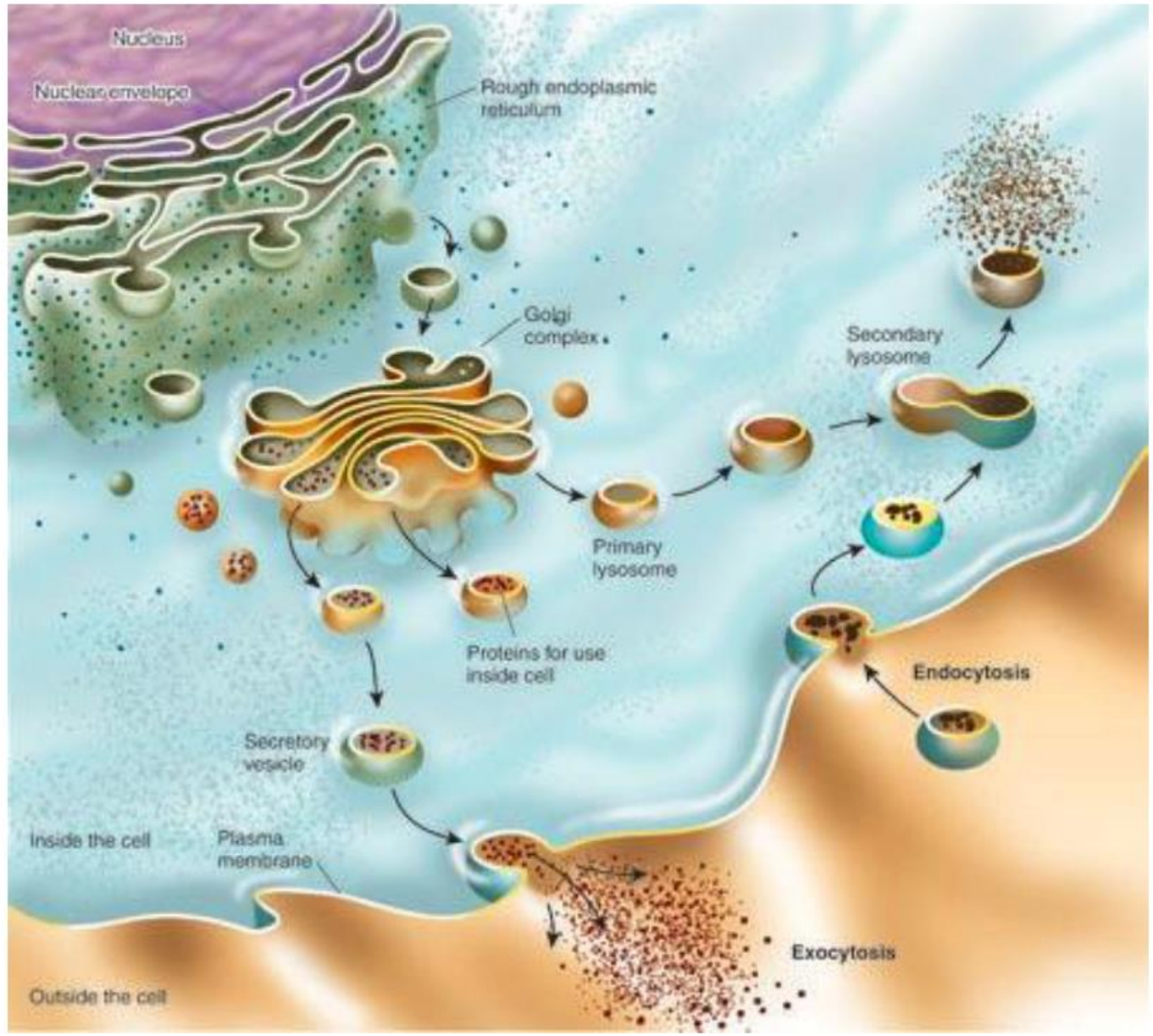
Goal: Fastest route to a product of required purity.

Protein Purification

- A process intended to isolate one or more proteins from a mixture, usually from cells or whole tissue extracts.
- Exploit protein properties for purification purposes.

1. Physio-chemical properties – charge or hydrophobicity
2. Binding affinity – Tags
3. Size – large or small
4. Biological activity – Substrate analogs

Intracellular and Extracellular

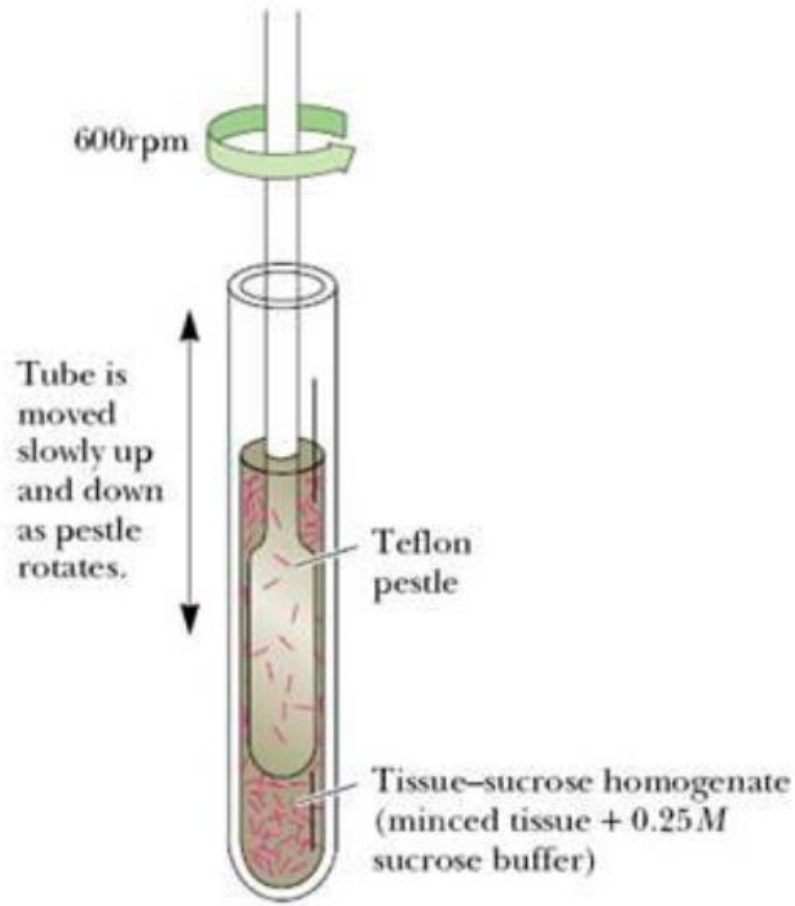


Isolation of Extracellular Proteins

- No need for cell disruption
- Secreted soluble proteins can be collected in the cell supernatant after centrifugation
- Membrane-bound proteins might be released from the cell simply using detergents

Isolation of Intracellular Proteins

- Needs cell disruption:
  - Detergents lysis
  - Enzymatic lysis
  - Osmotic lysis
  - Freeze-thaw cycles
  - Ultrasonication
  - Homogenization



Sample material

- Choice of a starting material is key to the design of a purification process.
- Use a convenient source where the protein is abundant.
- In a plant or animal, a particular protein usually isn't distributed homogeneously throughout the body; different organs or tissues have higher or lower concentrations of the protein. Use of only the tissues or organs with the highest concentration decreases the volumes needed to produce a given amount of purified protein.

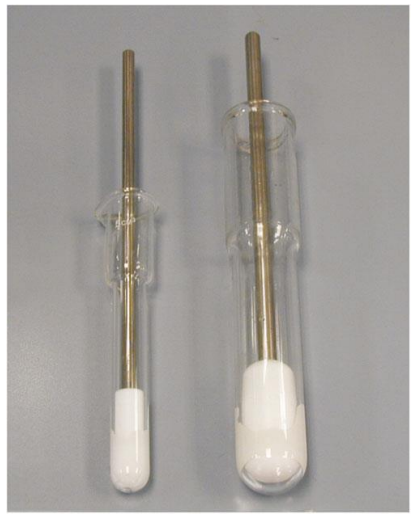
Protein has to be brought into solution by:

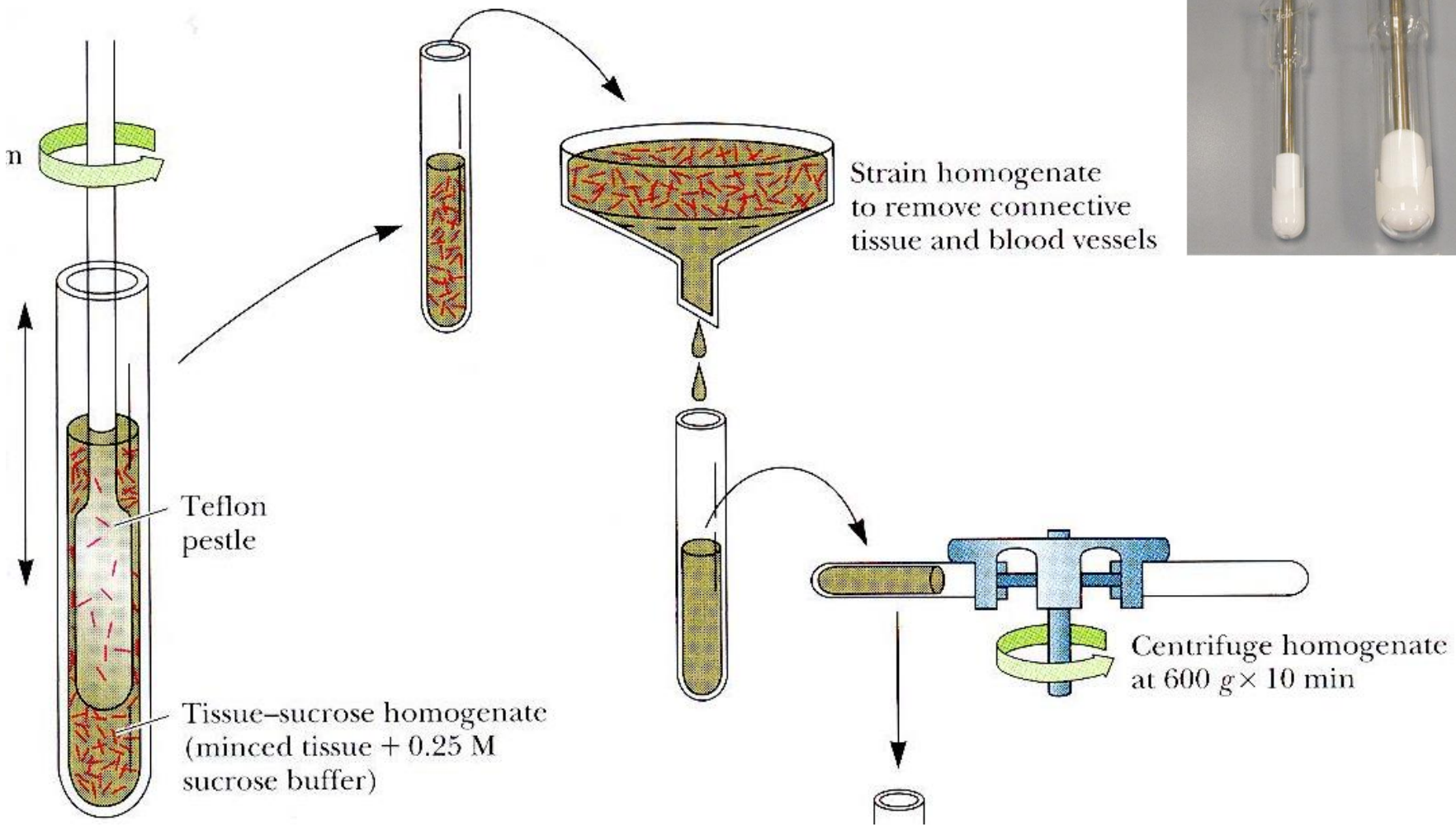
- Repeated freeing and thawing
- Sonication
- Homogenization
- Filtration
- Permeabilization by organic solvents.
- Soluble proteins can be separated by cell membranes, DNA etc by centrifugation.

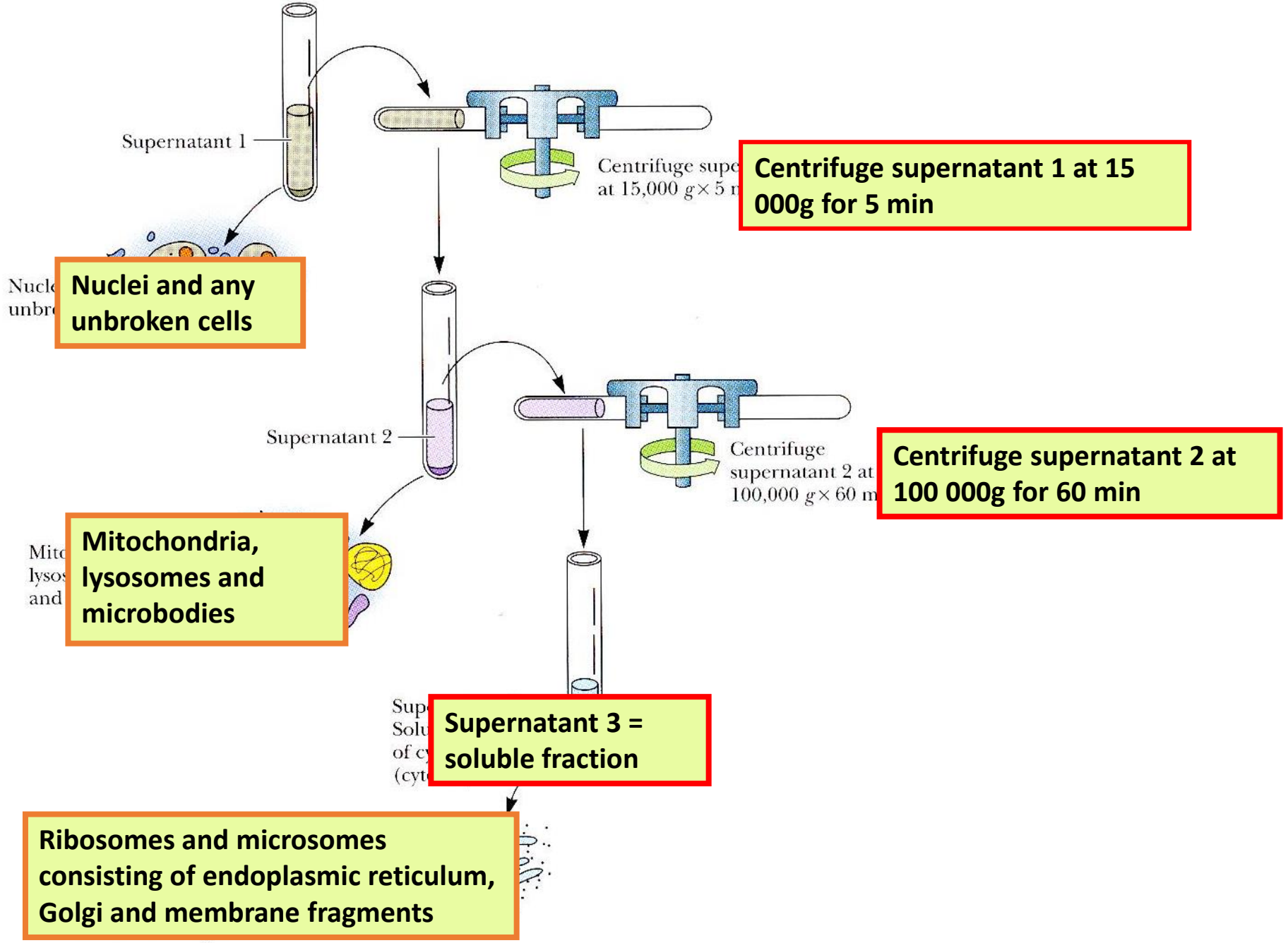
Preserve enzyme activity during purification

- Once protein / enzyme is released from its intracellular environment, conditions can be harsh and may result in denaturation.
- Counteract this by including in the buffers, reagents that can counteract these potentially damaging effects.
- Perform the procedures in cold conditions. Cold room or on ice.

Sample preparation







Centrifuge supernatant 1 at 15 000g for 5 min

Nuclei and any unbroken cells

Centrifuge supernatant 2 at 100 000g for 60 min

Mitochondria, lysosomes and microbodies

Supernatant 3 = soluble fraction

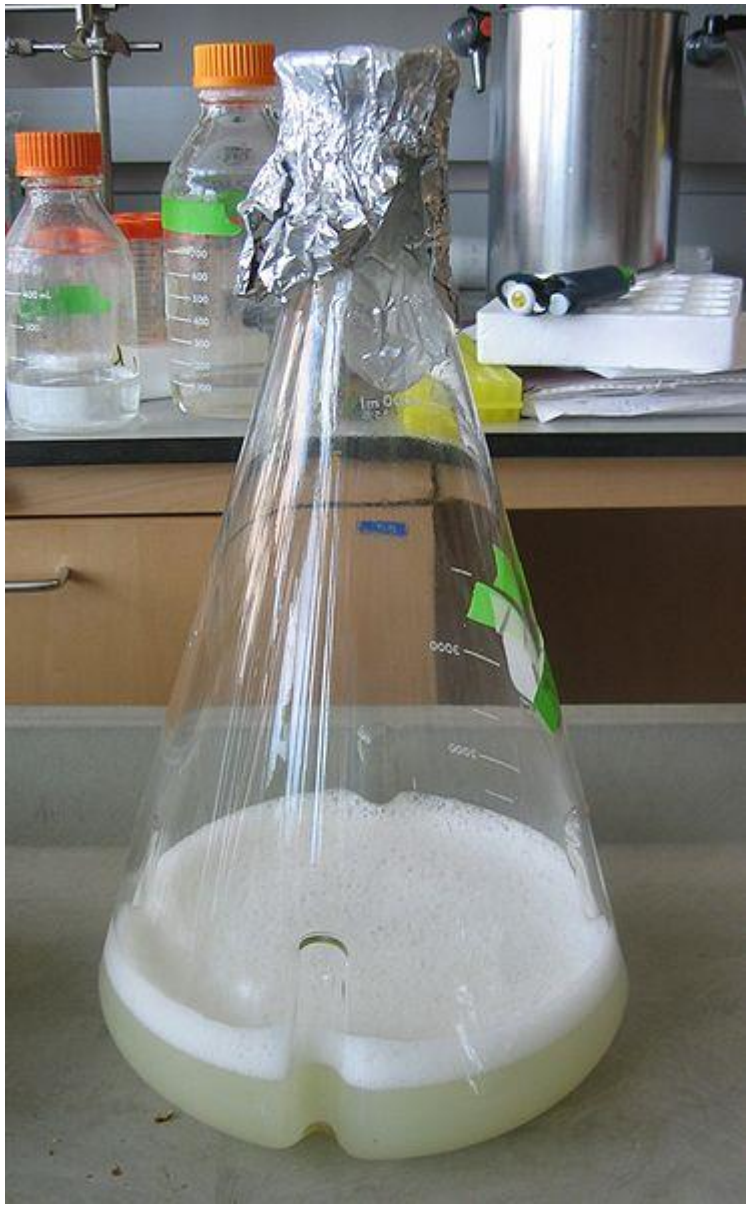
Ribosomes and microsomes consisting of endoplasmic reticulum, Golgi and membrane fragments

Use of recombinant DNA technology

If the protein is present in low abundance, or if it has a high value, recombinant DNA technology may be used to develop cells that will produce large quantities of the desired protein (known as an **expression system**).

Recombinant expression **allows the protein to be tagged**, e.g. by a His-tag, to facilitate purification, which means that the purification can be done in fewer steps.

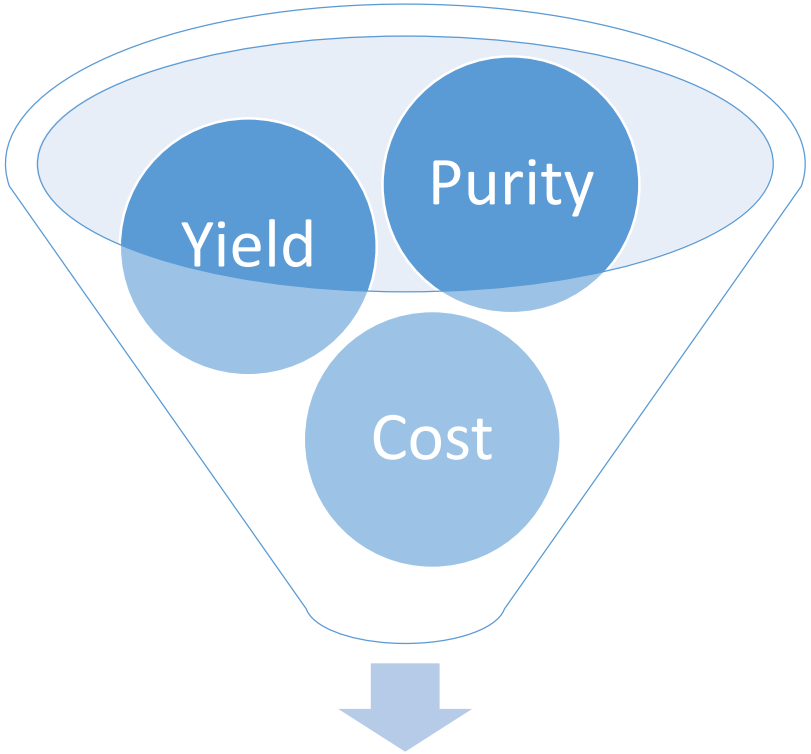
Recombinant expression usually starts with a higher fraction of the desired protein than is present in a natural source.



Recombinant bacteria can be grown in a flask containing growth media. Optimum conditions must be established.

Protein purification- deciding on the objectives

Structural studies- milligram quantity but pure compound



Biological activity of enzyme- microgram quantity but 100% purity

Industrial applications- grams or kilograms quantity but purity not essential

Purification of Proteins

- Differential centrifugation
- Differential salt precipitation
- Differential solvent precipitation
- Preparative electrophoresis
- Column chromatography

More than one approach may be required

Monitoring Purification

- At each step **collect** a series of “fractions”
- Assay each for the protein of interest, and **pool** those containing it prior to the next step
- The **protein conc. and the volume** of the pooled fraction must be determined and these are used to **determine the purity and yield after each step.**
- Enzymes – calculate **specific activity**

How Are Proteins Isolated and Purified from Cells?

TABLE 5.1 Example of a Protein Purification Scheme: Purification of an Enzyme from a Cell Extract

Fraction	Volume (mL)	Total Protein (mg)	Total Activity* (U)	Specific Activity†	Percent Recovery‡
1. Crude extract	3,800	22,800	2,460	0.108	100
2. Salt precipitate	165	2,800	1,190	0.425	48
3. Ion exchange chromatography	65	100	720	7.2	29
4. Molecular sieve chromatography	40	14.5	555	38.3	23
5. Immunoaffinity chromatography§	6	1.8	275	152	11

A typical protein purification scheme uses a series of separation methods. Note the dramatic increase in specific activity* of the enzyme through a series of five different purification procedures.

*The term “specific activity” refers to the activity of the enzyme per mg of protein (Units/mg)

Purification Table

TABLE 2-3 Summary of steps in endoglucanase purification

Step	Volume (ml)	Protein (mg)	Activity[a] (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Cell-free spnt.	12,000	6,720	20,300	3.0	1	100
Ultrafiltration	1,000	4,750	17,800	3.7	1.2	87.6
Ammonium sulfate ppt.	400	3,370	13,800	4.2	1.4	68.3
DEAE						
Peak I	400	624	6,400	10.3	3.4	31.6
Peak II	60	119	2,750	23	7.6	13.6
SP-Sephadex	25	21	975	45.9	15.2	4.8
Prep. PAGE	10	7.9	514	65.1	21.5	2.5

[a] Units represent micromoles of reducing sugar formed per minute at 60°C.

Purification fold= Specific activity at a particular step/Specific activity of initial step

Yield= Total activity at a particular step/ total activity of initial step

How do I calculate the total activity and the specific activity?

Total Activity – this is a measure of how many units of protein you have in a sample. Simply **multiply the activity in the sample by the total volume.**

Specific Activity – specific activity is a way to measure how much of a measured protein there is with all of the other contaminating proteins**. Divide the Activity by the mg of protein**. The higher this value, the higher purity.

How do I calculate the % recovery and the fold purification?

- When calculating the **% recovery (yield),** use the **total activity**. Thus you have the total starting amount and the final amount to give you the % yield.
- The **specific activity** values of the starting and final preparation give you a value for the **fold purification**.
- However, this number along with the percent yield, indicates if a step was worthwhile or not.
- A poor fold purification with a low yield is a step to avoid in the future, while a high fold purification **and** high yield is ideal.

Calculation of Yield and purification factor

Purification Factor (fold purification) =

$$\frac{\text{Specific activity after a particular step}}{\text{Specific activity of initial step}}$$

%Yield =

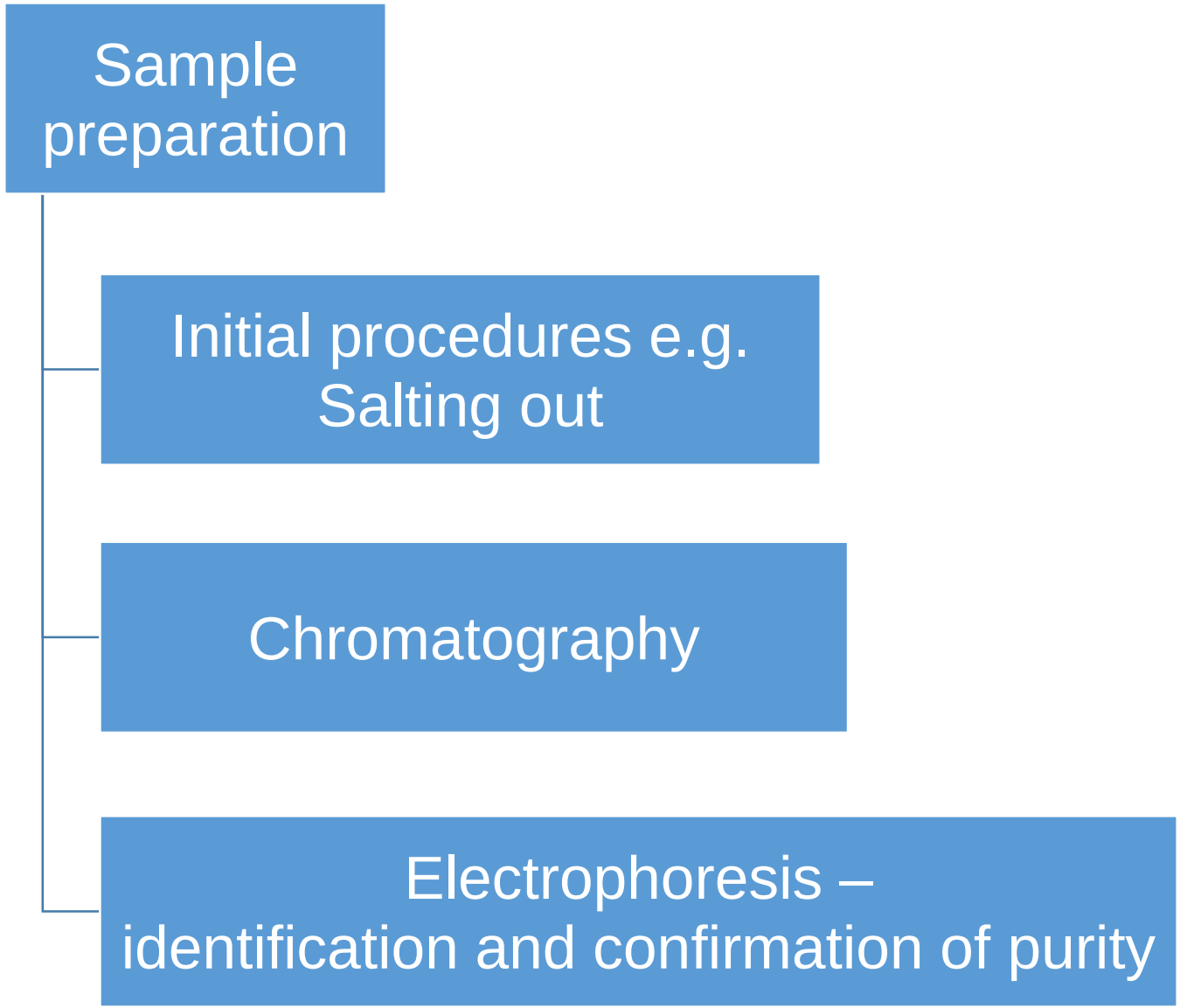
$$\frac{\text{total enzyme activity after a particular step}}{\text{Total enzyme activity in initial sample}} \times 100\ (\%)$$

Purification table

- Example of a Protein purification scheme: Purification of an Enzyme from a cell Extract

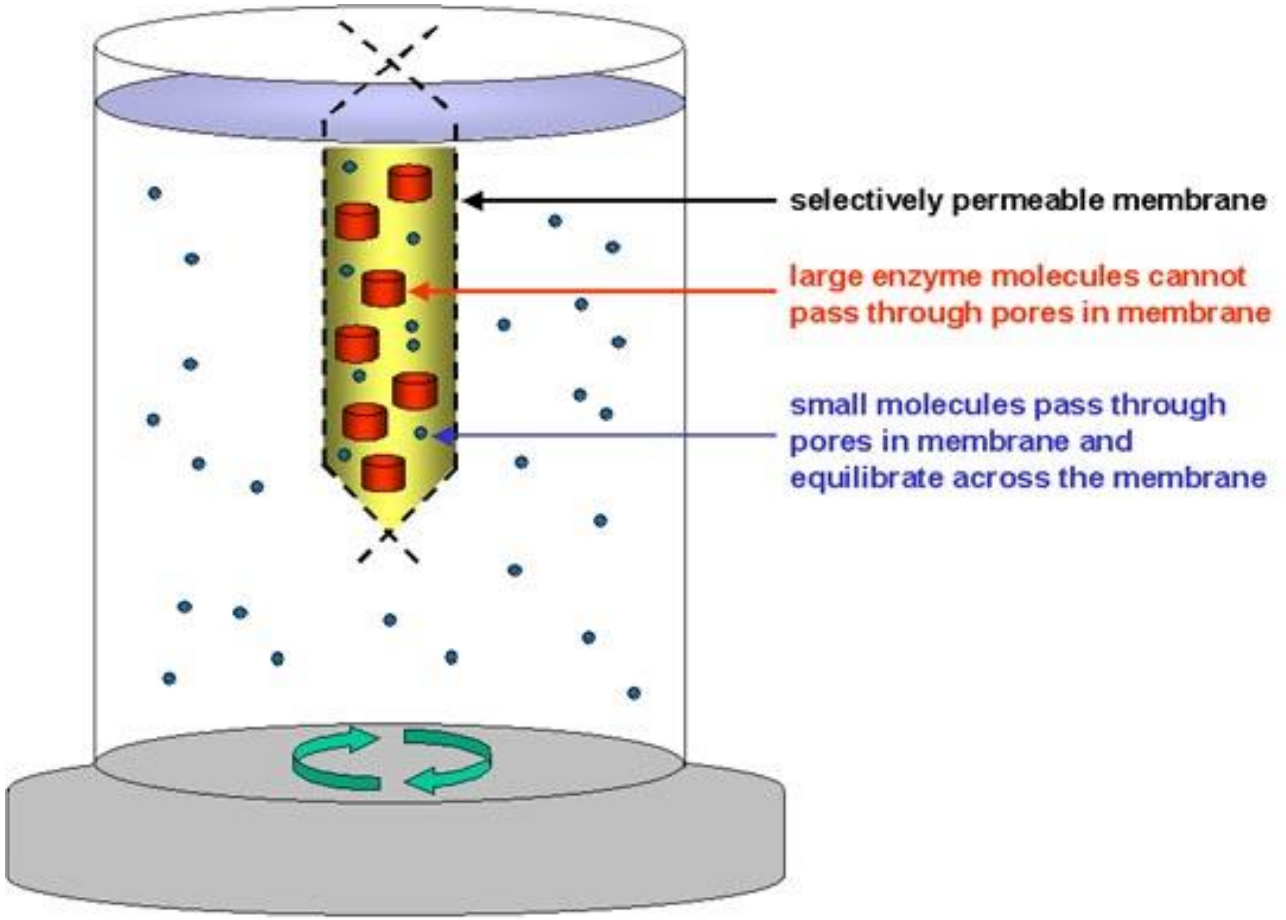
Fraction	Volume (mL)	Total Protein (mg)	Total activity	Specific activity	Percent recovery	Fold purification
Crude extract	3800	22800	2460	0.108	100	1
Salt precipitate	165	2800	1190	0.425	48	
Ion exchange chromatography	65	100	720	7.2	29	
Gel filteration chromatography	40	14.5	555	38.3	23	
Immunoaffinity chromatography	6	1.8	275	152	11	

Stages involved in purification of a protein



Initial Procedures

- **Dialysis** is useful for removing small molecules from macromolecule solutions –



Dialysis

Could also use gel filtration to remove the salt

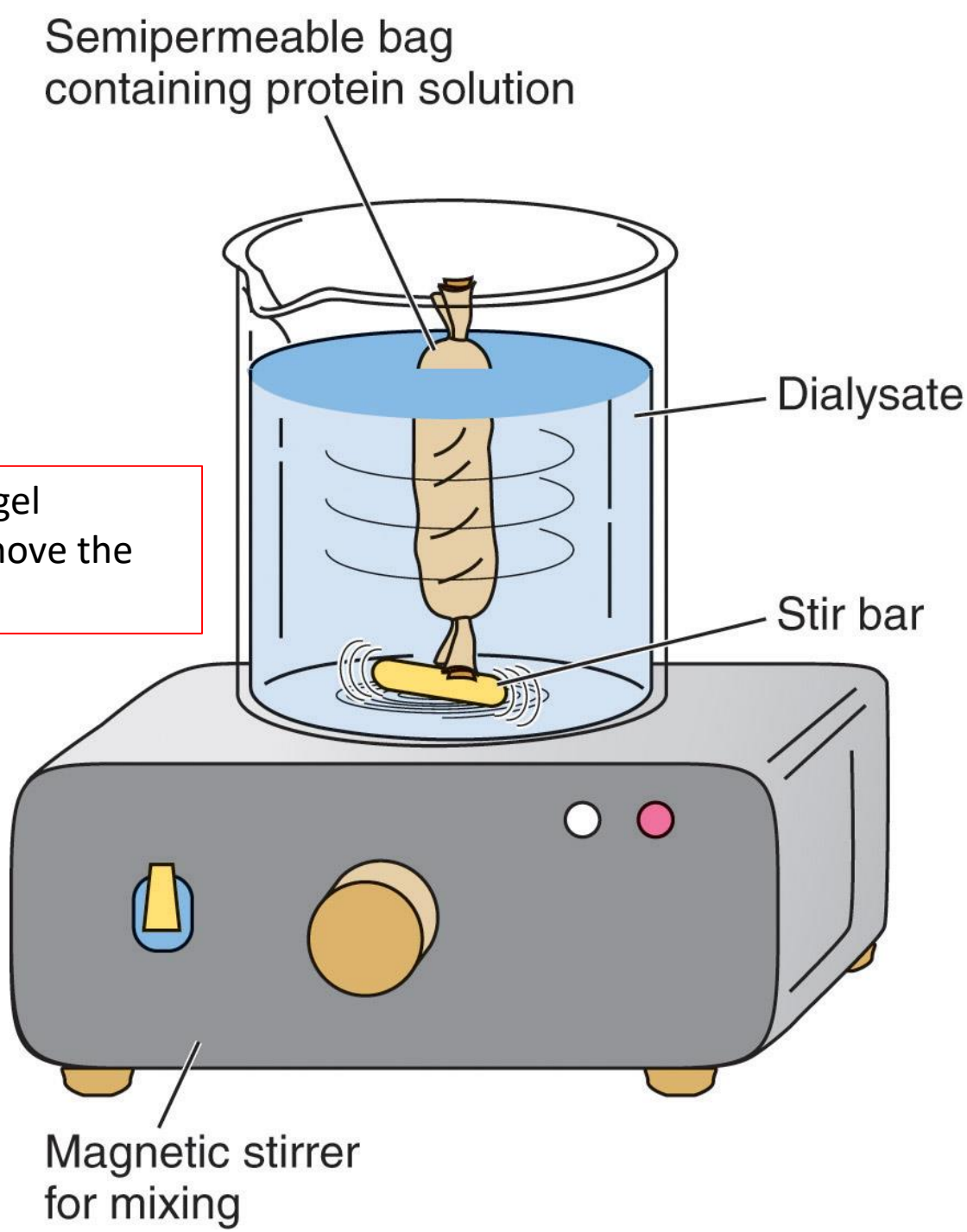
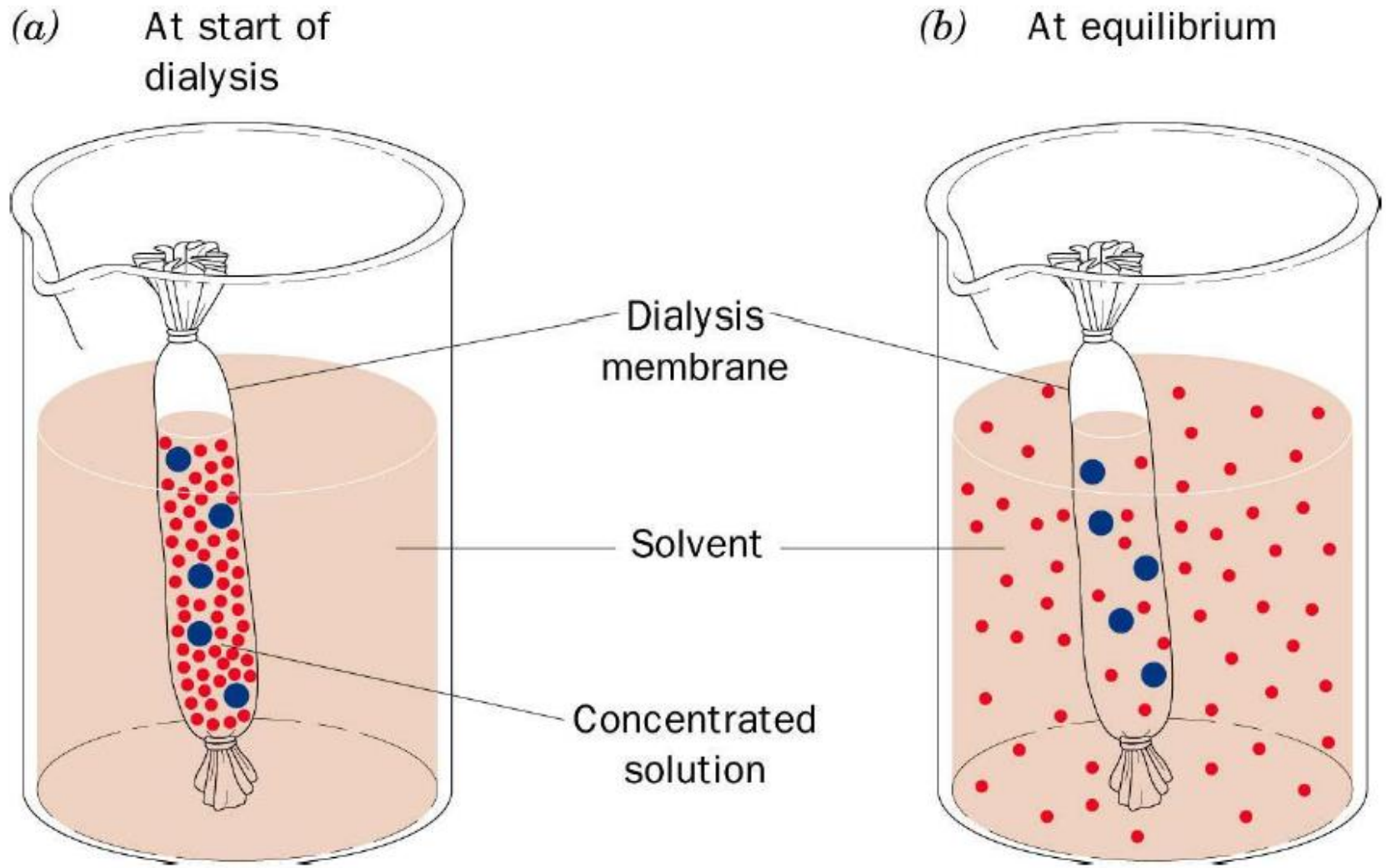


Fig. 5-A1, p. 127

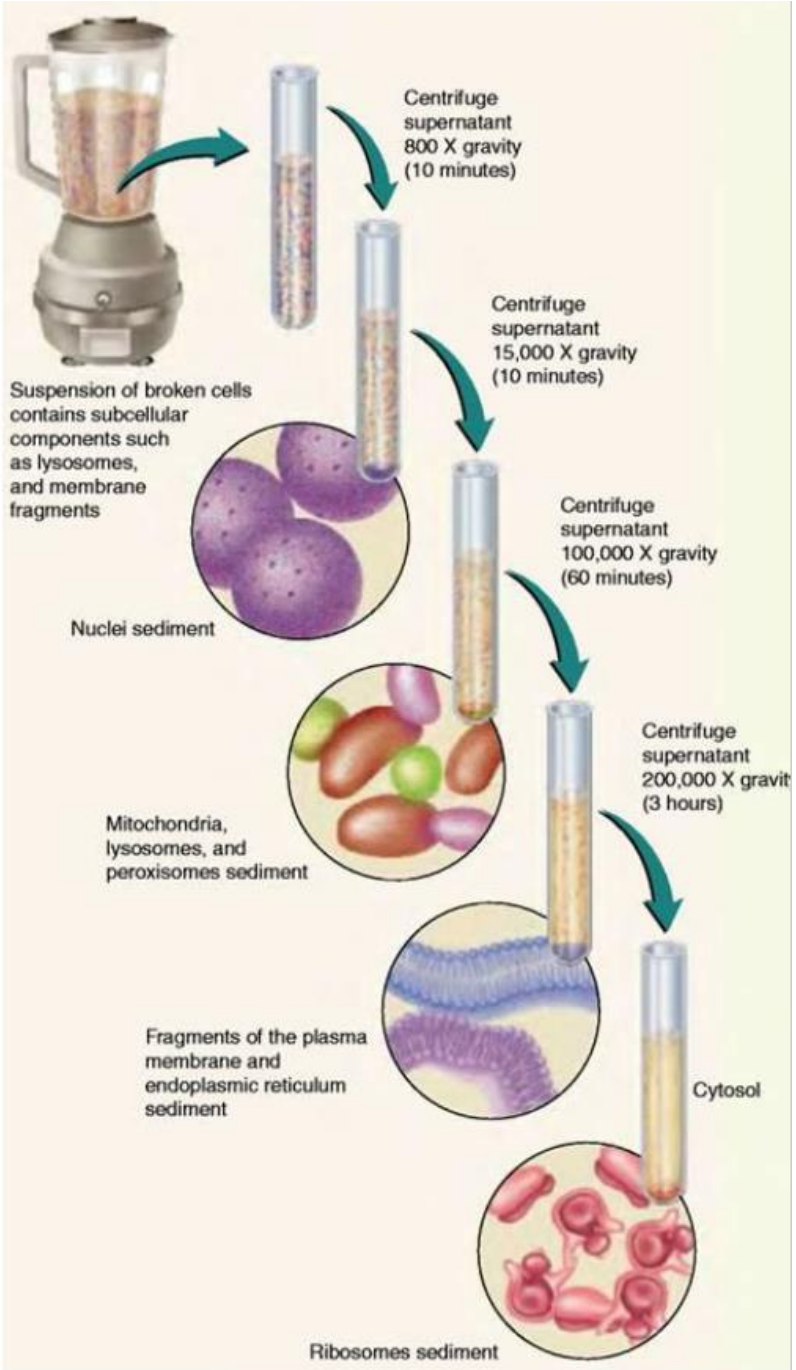
Dialysis


(a) At start of dialysis
(b) At equilibrium
Dialysis membrane
Solvent
Concentrated solution

Dialysis

- **Based on osmotic pressure**
- **It allows you to get rid of most salt ions**
- **It is impossible to remove the salts completely**

Differential Centrifugation



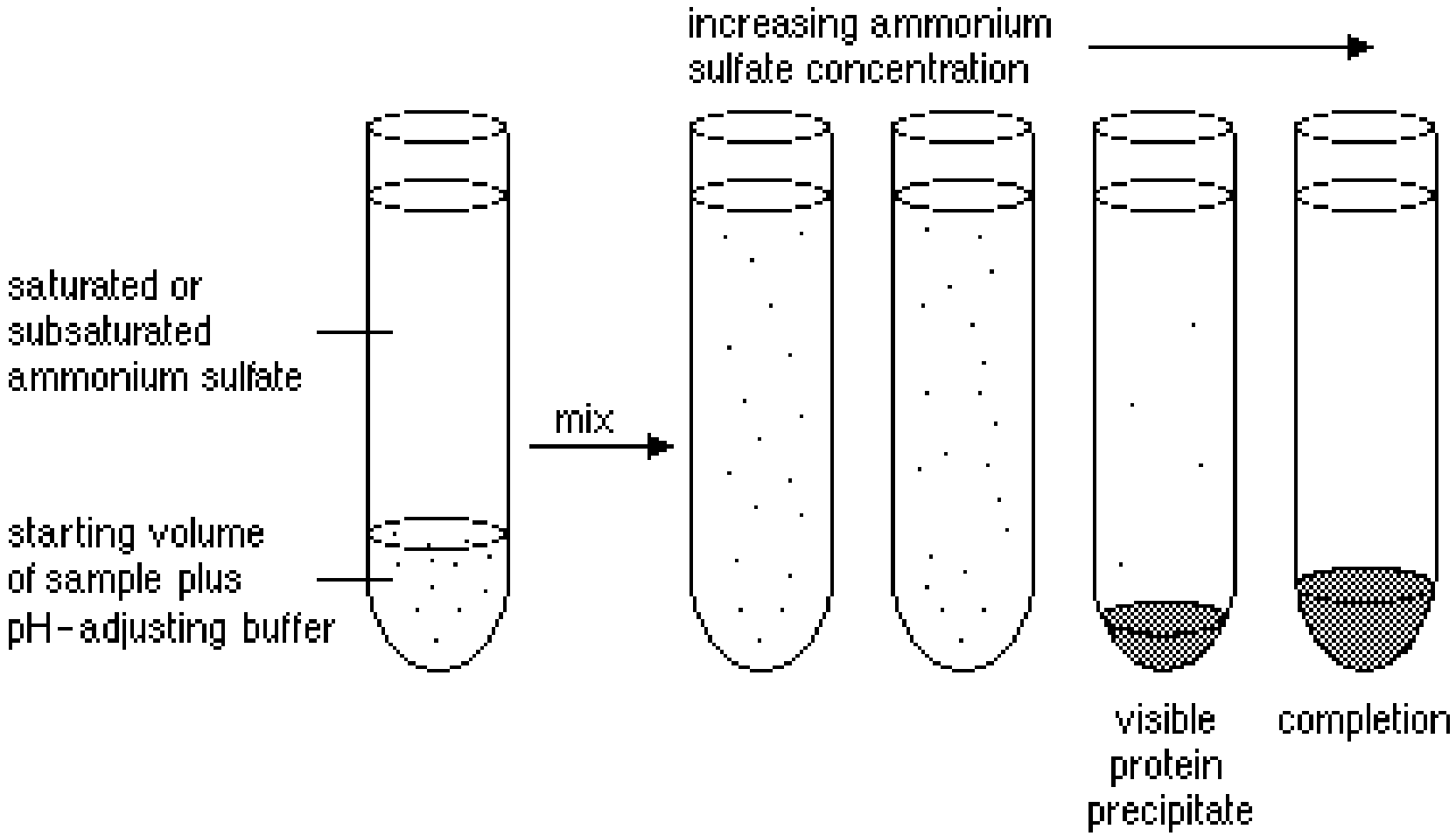
Differential Centrifugation

- **Separation of proteins (or any material) on the basis of their size, mass, and density**
- **It is a function of the size of the protein and the speed if centrifugation**
- **It is gives us a rough separation. It is basically fractioning!**

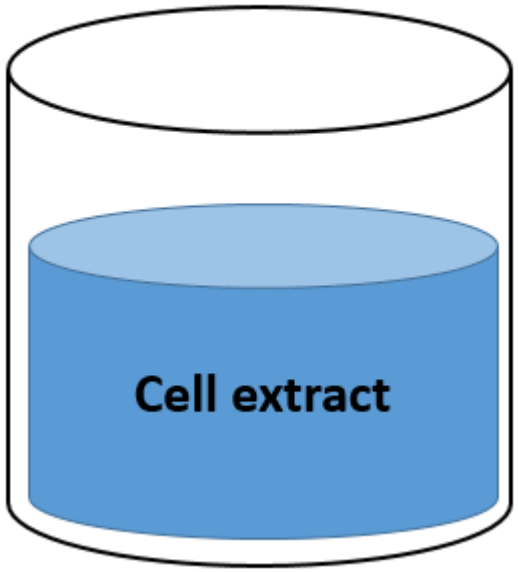
Ammonium Sulfate Precipitation

- Common 1st step in protein purification.
- Add increasing amounts of ammonium sulfate to protein extract.
- Exposes hydrophobic groups on the protein.
- Hydrophobic groups attract other protein hydrophobic groups.
- Results in aggregation of protein.
- Protein precipitates from solution.
- Inexpensive.
- Reversible aggregation!!

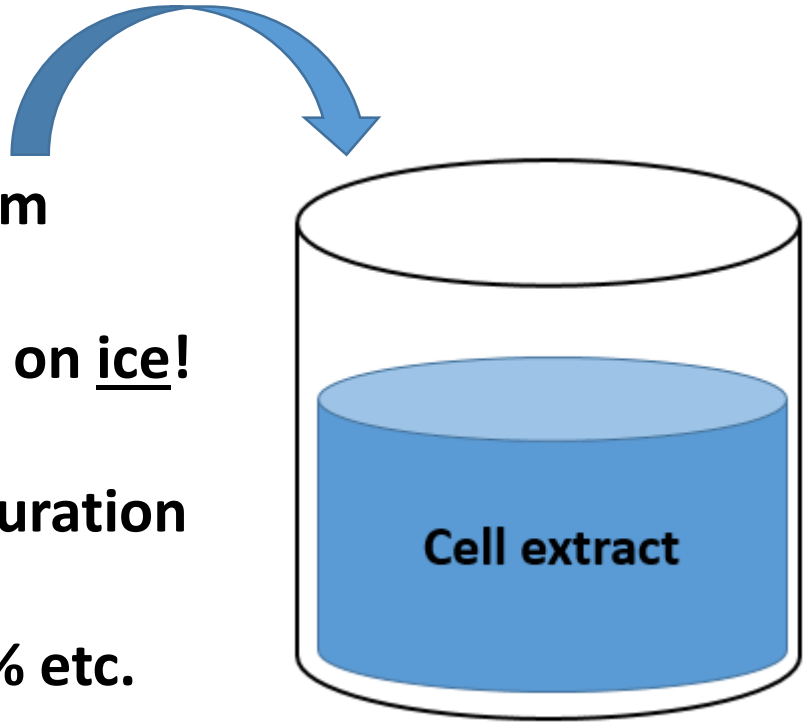
Salting out


increasing ammonium
sulfate concentration
saturated or
subsaturated
ammonium sulfate
starting volume
of sample plus
pH-adjusting buffer
mix
visible
protein
precipitate
completion

Ammonium Sulfate Precipitation Steps



Ammonium Sulfate Precipitation – Step 1

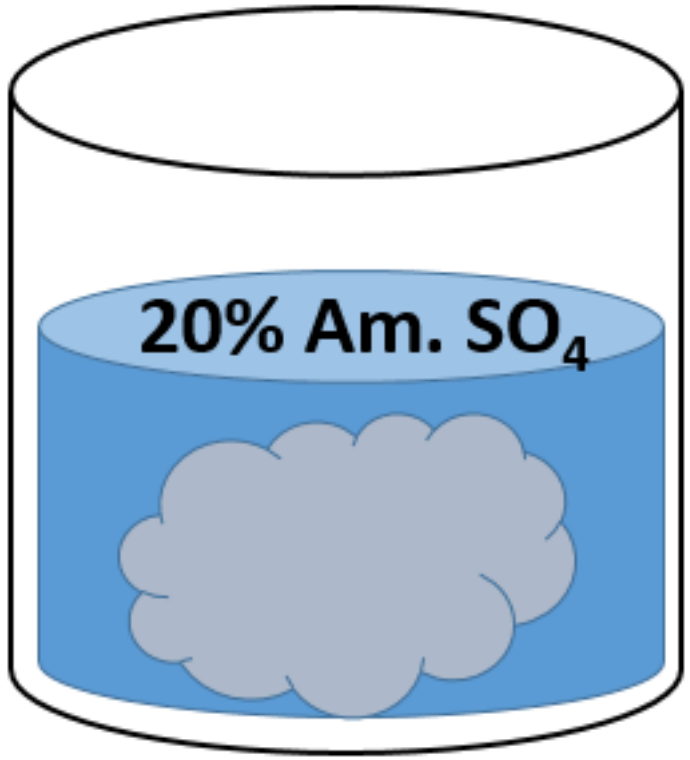


1. Add ammonium sulfate

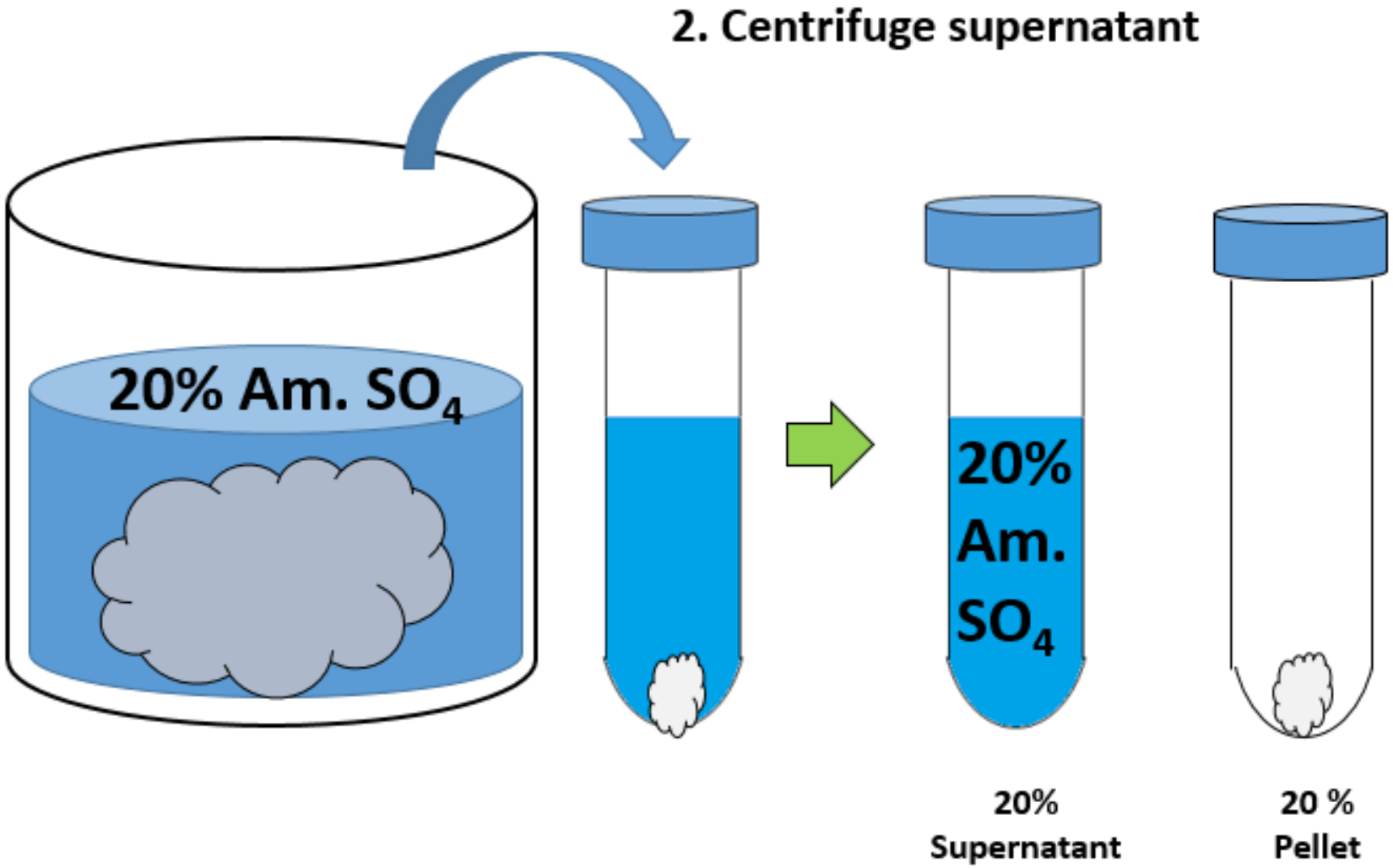
- **Must perform on ice!**
- **Increments of percent of saturation example)**
- **10%, 20%, 30% etc.**

Ammonium Sulfate Precipitation – Step 1 Cont.

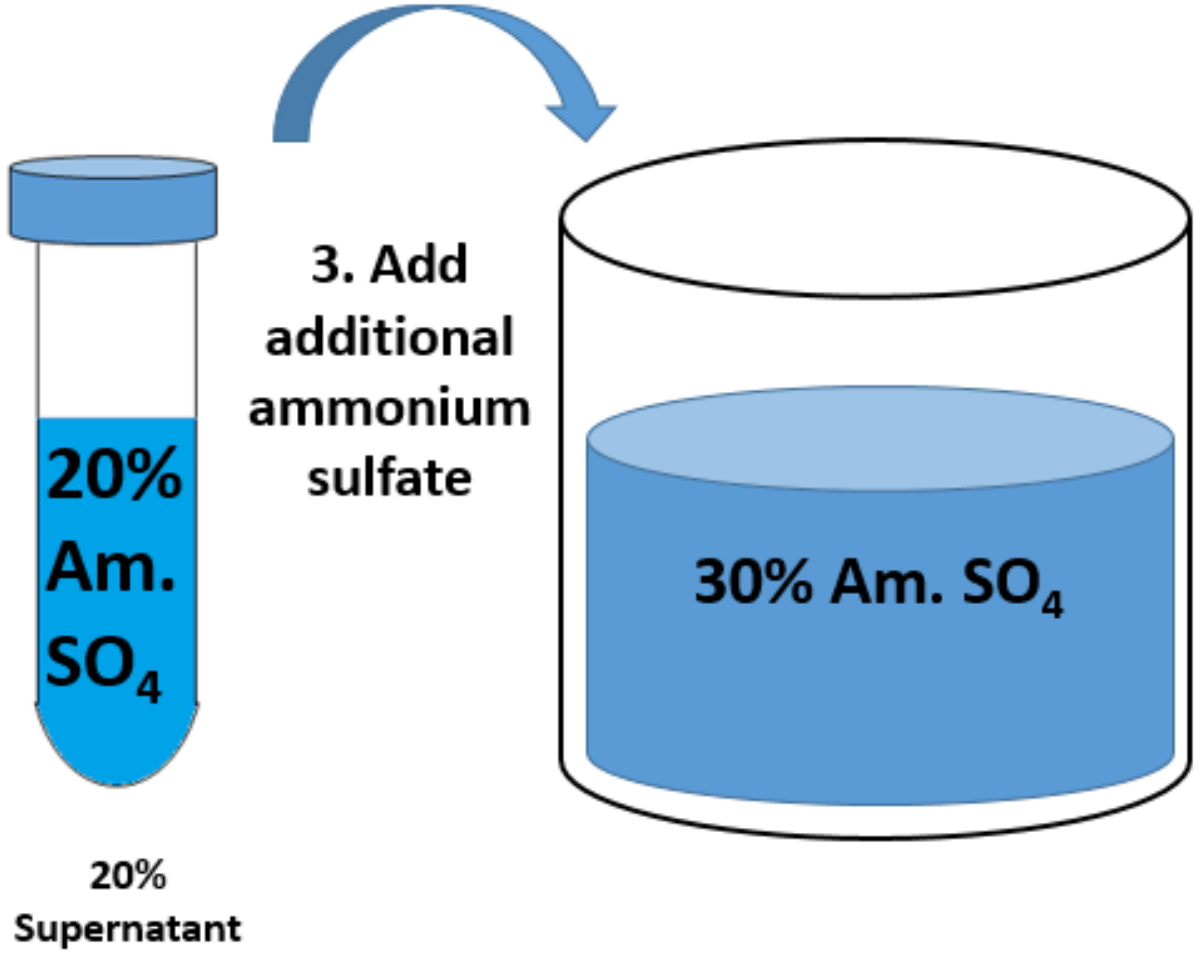
Protein aggregates and precipitates out from solution



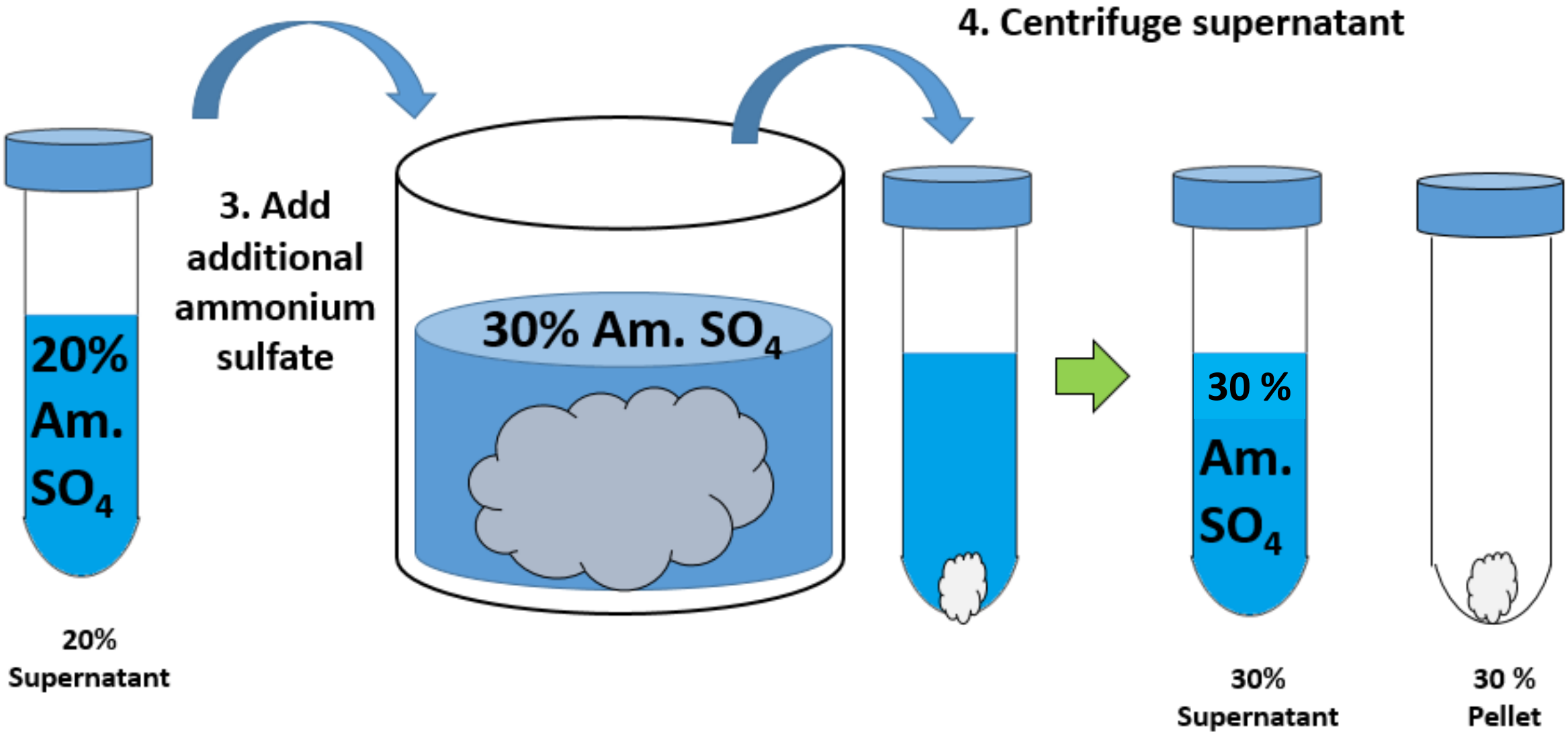
Ammonium Sulfate Precipitation – Step 2



Ammonium Sulfate Precipitation – Step 3



Ammonium Sulfate Precipitation – Step 4 (repeat 1-3)



Continue process: 40%, 50%, 60%...etc.

Ammonium Sulfate Precipitation Example

Step	Pellet - total protein (mg)	Supernatant - total protein (mg)	Pellet - total enzyme activity	Supernatant - total enzyme activity
Crude extract		6000	-	2000
10% (NH4)2SO4	100	5900	0	2000
20% (NH4)2SO4	1000	4900	100	1900
30% (NH4)2SO4	1000	3900	500	1400
50% (NH4)2SO4	800	3100	800	600
70% (NH4)2SO4	800	2300	500	100
80% (NH4)2SO4	1000	1300	100	0

- **Simple**
- **Pellets can be added together**
- **Removes non-protein molecules not seen in table**

Why does addition of ammonium sulfate precipitate proteins?

- Precipitation: hydrophobic patches on the surface of the protein
- Addition of ammonium sulfate: this dissolves = fewer water molecules in association with the protein
- So hydrophobic patches become “exposed”

Analytical purification utilizes 3 properties to separate proteins

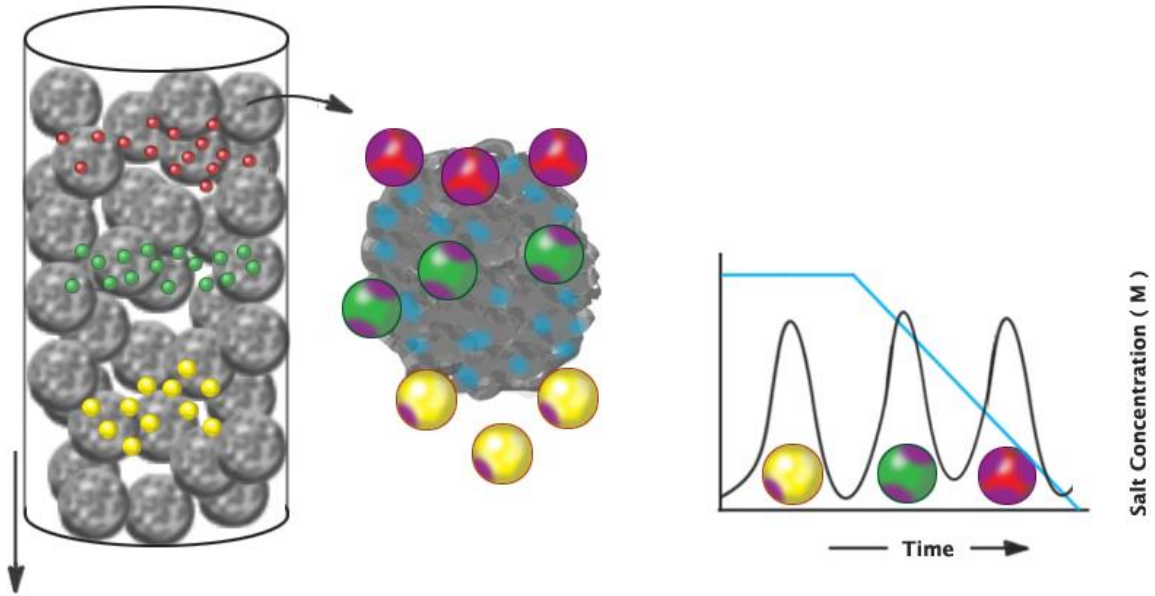
1. Proteins may be purified according to their isoelectric points by running them through a pH graded gel or an ion exchange column.
2. Proteins can be separated according to their size or molecular weight via size exclusion chromatography
3. Proteins may be separated by polarity/hydrophobicity via high performance liquid chromatography or reversed-phase chromatography.

Chromatography

- Most common and best approach to purifying larger amounts of proteins
- Achieves Highest level of purity and largest amount
- Requires less effort
- Lowest likelihood to damage the protein product
- Standard method for pharmaceutical industry

Protein Purification

- Hydrophobic Interaction Chromatography
  - Separates proteins based on repulsion to water
    - Proteins are eluted by decreasing the salt concentration of the buffer
    - The least hydrophobic proteins are eluted first



In a nutshell, HIC (also known as 'salting out') separates protein molecules using the properties of hydrophobicity. In this method, proteins containing both hydrophilic and hydrophobic regions are applied to an HIC column under high salt buffer conditions.

The salt in the buffer (usually ammonium sulfate) reduces the solvation of sample solutes and exposes the hydrophobic regions along the surface of the protein molecule. This facilitates the adsorption of these hydrophobic regions to the hydrophobic areas on the solid support and precipitates (crystallizes) proteins out of the solution.

https://info.gbiosciences.com/blog/the-basics-of-hydrophobic-interaction-chromatography

www.austincc.edu/awheeler/Files/.../Powerpoints/_chapter%204.ppt_.ppt

Postpurification Analysis

- Protein Sequencing
  - Determining the order of amino acids
- X-ray Crystallography
  - Determining tertiary and quaternary structure of protein

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RECOMBINANT PROTEINS

Protein Purification