Crude Isolation using centrifugation and sedimentation

Sources of Material

- Natural Sources
- Molecular Biology - cloning of DNA examples: genes, DNA elements, etc
- Recombinant technology - protein expression
- Synthetic systems - Chemical synthesis of peptides and nucleic acids

Natural Sources

- Tissue or organ of interest - liver and pancreas
- Sample is in its native form
- Specific species - thermophilic microorganisms (*taq* DNA polymerase isolated from *Thermus aquaticus*)
- Human samples
  - Blood - purification of red and white blood cells
    microorganisms (viruses and bacteria)
  - tissue samples - tumors cells (HeLa cells - immortal cell line isolated from cervical cancer from Henrietta Lacks, who died from cancer in 1951.)
  - Adult and embryonic stem cells

Natural Sources II

- Advantages
  - Native form - posttranslational modifications
  - Natural environment
- Disadvantages
  - Quantity - Sperm Whale hemoglobin was the first protein structure
  - Seasonal quantity - plants
  - Storage
  - Microorganisms can be difficult to grow

Natural Sources III

- DNA and RNA isolation
- genomic DNA
- mRNA (messenger RNA)- molecules that encode genes with the introns removed. Exons are the regions that will be spliced together to form mRNA. Introns are the intervening sequences.
- mRNA converted into complementary DNA (cDNA)
- in order to isolate must be able to remove protein and other itmes from the nucleic acid. Need to exploit the properties specific to nucleic acids.

Recombinant technology

- Expression of protein of interest in a heterologous system
- Advantages
  - QUANTITY
  - Ease of expression - the use of strong promoters to drive expression
  - Not dependent on natural sources - Can make it when you need it
  - Production outside it normal environment (Can be an advantage and disadvantage)
- Disadvantages
  - Folding
  - Proper posttranslational modifications - glycosylation, phosphorylation, etc
  - May not be functional

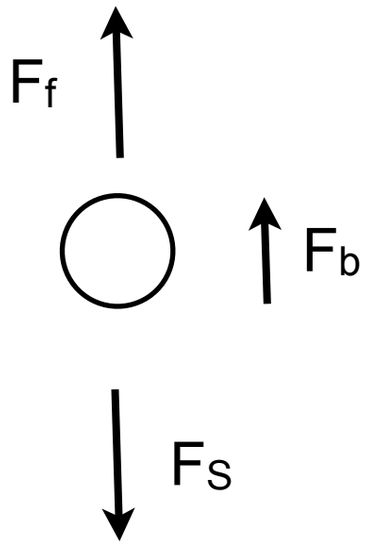
Sedimentation

- Sedimentation is used to separate cellular species based on size

Frictional force $F_f = -f\mu$

Constant velocity=μ
Frictional coefficient =f

f depends on size and shape of the particle. Bulky or elongated particles experience more drag



Buoyant force $F_b = -m_0(\omega^2 r)$

Mass of fluid displaced= m_0

$m_0 = m\,\nu\,\rho = (M/N)\,\nu\,\rho$

ν = partial specific volume (mL)

ρ =density of solvent (g/mL)

Angular velocity = ω

Axis of radius = r (in book x)

Gravitational or sedimentation force $F_S = m(\omega^2 r) = (M/N)(\omega^2 r)$

mass of a single particle = m
Molar weight of solute (g/mol) =M
Avogadro's number (6.0221×10^{23} molecules/mole) =N

G-force = $(\omega^2 r)/g$
g = gravity $9.8066 ms^{-2}$
G-force is independent of rotor

Sedimentation

- within a short period of time the forces balance

$$F_S + F_f + F_b = 0$$

$$(M/N)(\omega^2 r) - (M/N)\,\nu\,\rho\,(\omega^2 r) - f\mu = 0$$

$$(M/N)\,(\omega^2 r)(1 - \nu\,\rho) - f\mu = 0$$

Rearranging terms and collecting terms related to particle on one side and the experiment on the other

$$M\,(1 - \nu\,\rho)/Nf = \mu/(\omega^2 r)$$

- $\mu/(\omega^2 r)$ is the sedimentation coefficient (velocity per unit gravitational acceleration) and this depends on the properties of the particle
- proportional to molecular weight corrected for the effects of buoyancy
- indirectly proportional to friction coefficient
- Molecules with different sizes will move with different velocities
- Molecules with same MW but different shapes will move at different velocities (related to f)

Sedimentation

- Sedimentation depends on the effective MW corrected for buoyancy
- If the density of solute is greater than that of solvent, the solute will move to the bottom of the cell
- If the density of solute is less than that of the solvent, the solute will move to the top of the cell - lipoproteins
- Density gradient sedimentation - a gradient of concentration of sucrose or cesium chloride from higher concentrations at the bottom to lower concentration at the top .
- Macromolecules will settle in a region of the cell where solvent density equals their own buoyant density.

Manipulation of solubility

- Examples of manipulating conditions to isolate various cellular components
  - Cytoplasmic extract of rabbit Reticulocyte
  - Purification of DNA
  - Selective precipitation of proteins
  - Membrane floatation

Example - Rabbit Reticulocyte Lysate

- Cell free system for studying eukaryotic translation
- reticulocytes are highly specialized cells primarily responsible for the synthesis of hemoglobin, which represents more than 90% of the protein made in the reticulocyte. These immature red cells have already lost their nuclei, but contain adequate mRNA, as well as complete translation machinery, for extensive globin synthesis.
- Ribosomes and associated factors are very large. Differential centrifugation exploits the size and mass of large complexes.
- Make adult rabbits anemic by subcutaneous injections of 1.2% Acetylphenylhydrazine. Increases the number of red blood cells
- Bleed rabbits by cardiac puncture. Collect blood
- Centrifuge blood 2,000xg to isolate red blood cells
- Lyse in hypotonic buffer - low salt buffer will cause cells to rupture
- Centrifuge at 37,000xg - pellet large organells, cellular debris, hydrophic lipids,

Purification of DNA

- Differential precipitation
- ion-exchange chromatography
- Gel filtration chromatography

Differential precipitation

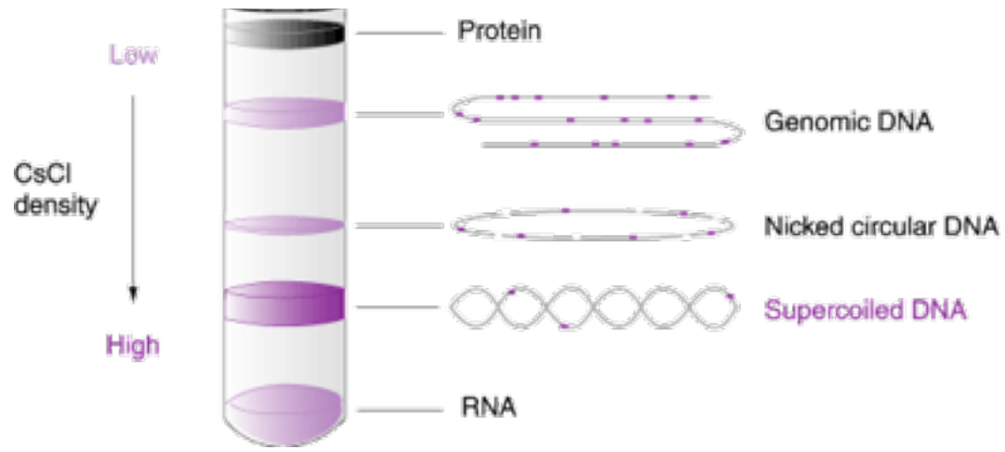
- Alkaline lysis in the presence of Detergent - Sodium Dodecyl Sulfate (SDS)
- This treatment will disrupt cell wall, denatures bacteria chromosomal DNA and protein
- small circular DNA (plasmids) will denature at high pH but will renature when the pH is brought lower
- plasmids are intertwined so base pairing will return

Alkaline lysis method of plasmid DNA purification

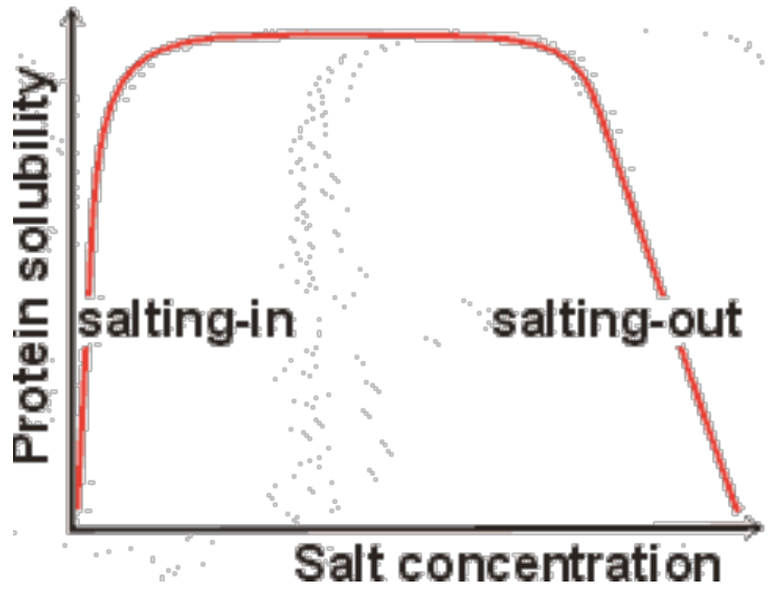
- Manipulating the conditions to purify DNA
- lyse bacteria with NaOH and SDS
- Neutralize with NaAcetate - precipitate forms
- Centrifuge 16,000xg.
- Take supernatant and extract with phenol:chloroform mixture (50:50) - more dense than aqueous solution
- Centrifuge (16,000xg) to assist extraction - more dense on the bottom of the tube. DNA is very soluble in aqueous solution
- specifically precipitate DNA by adding ethanol
- Pellet precipitated DNA by centrifugation (16,000xg)

CsCl preparation of DNA

- CsCl solution has a density of 1.55g/mL
- Under high centrifugal force, a solution of Cesium Chloride (CsCl) molecules will dissociate and the heavy Cs^+ atoms will be forced towards the outer end of the tube
- **DNA** molecules will migrate to the point where they have the same density as the gradient (**neutral buoyancy**, or **isopycnic centrifugation**)
- The gradient is sufficient to separate types of **DNA** with slight differences in density due to differing **G+C** content, or physical form (linear *versus* circular molecules)



Effects of salts on the solubility of proteins

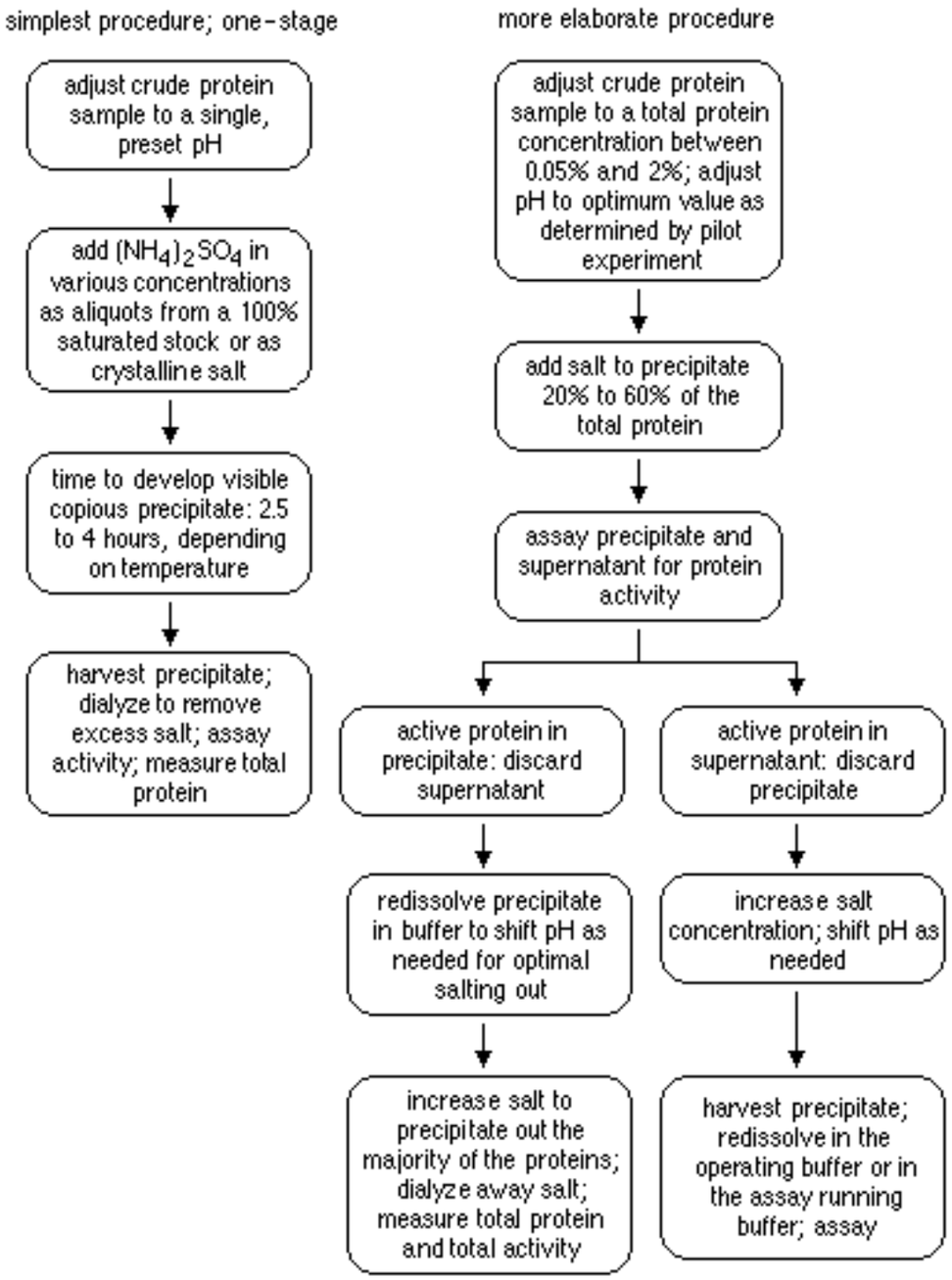


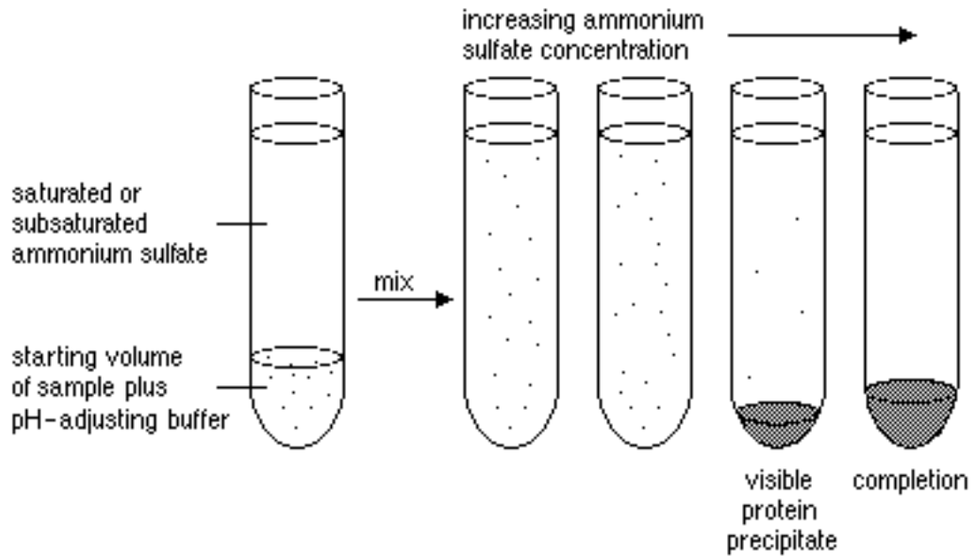
- “Salting-in” results from protein-counter ion binding and the consequent higher net protein charge and solvation
- “Salting-out” results form strongly hydrated molecules near the protein’s surface, removing water and dehydrating the protein

Salting out of protein

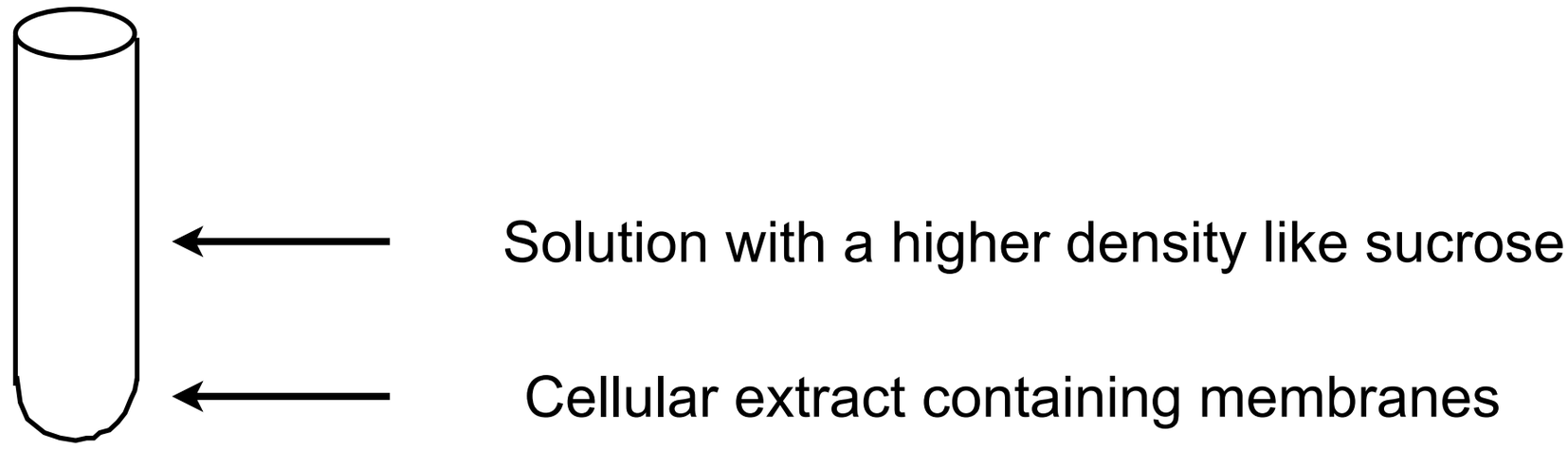
- Dissolving salt into a solution containing proteins
- Nature of the salt is extremely important
- Multiple charged anions are most effective
- Follows the Hofmeister series
  - SO_4^{2-} > HPO_4^{2-} -> acetate$^-$ > Cl^- > NO_3^-
  - $NH4^+$>K^+>Na^+>Li^+
- Water molecules will bind to salt, removing water from the protein
- This usually requires a high concentration of salt

Protocol for $(NH_4)_2SO_4$ Precipitation





Membrane Floatation



- Density of particles are lower than the density of the solution
- Membranes and associated factors will “float” to the top of the centrifuge tube
- This can seperate the more dense items (proteins, etc) from the less dense membranes
- Media used can be viscous and have high osmolarities
- Can be a serious problem for certain applications