Chapter 3 Centrifugation

Biochemistry and Molecular Biology (BMB)

- 3.1 Introduction
- 3.2 Basic Principle of sedimentation
- 3.3 Types, care and safety of centrifuges
- 3.4 Preparative centrifugation
- 3.5 Analytical centrifugation

Analytical Biochemistry (AB)

- 3.4.3 Ultracentrifugation

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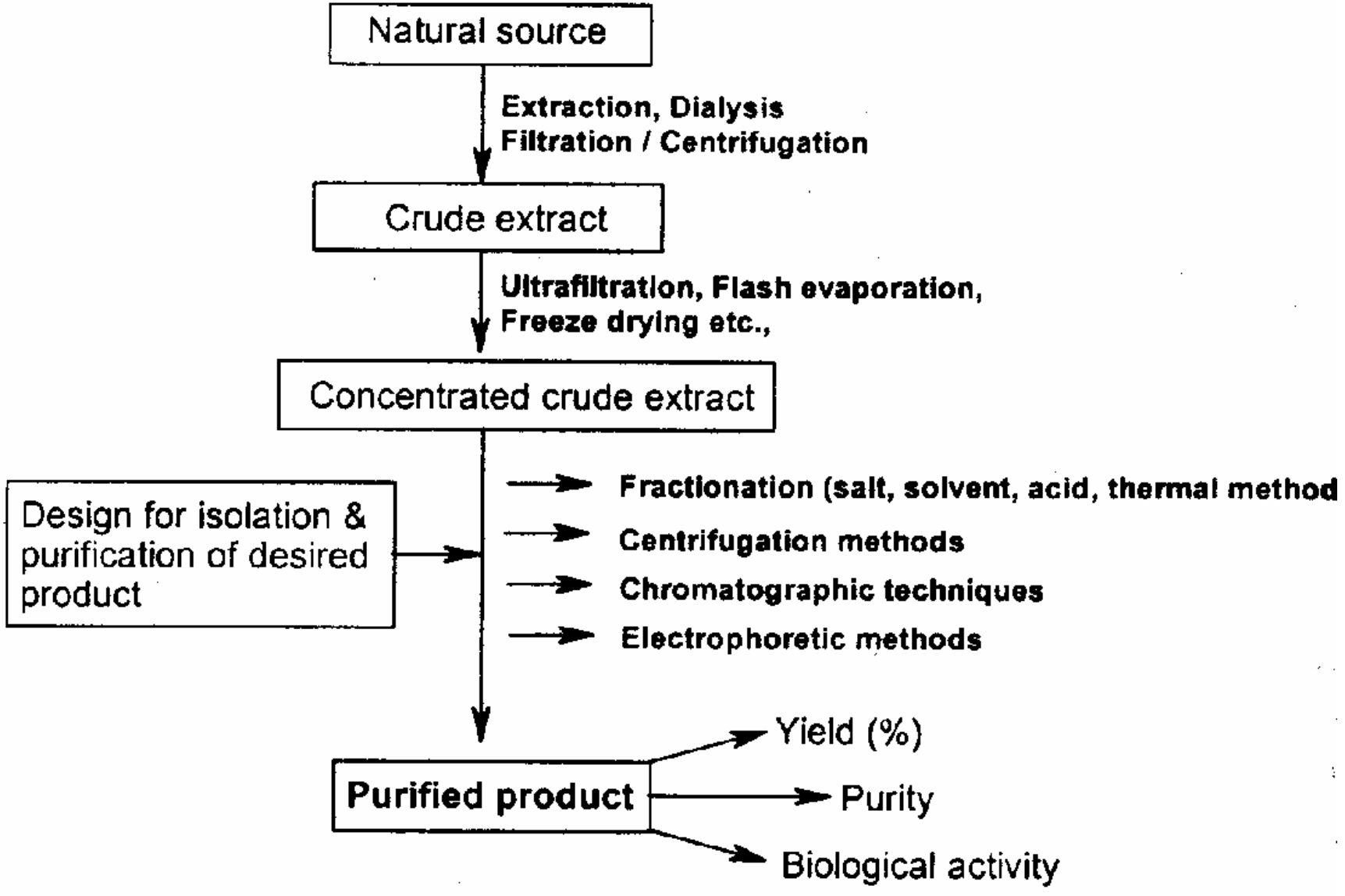
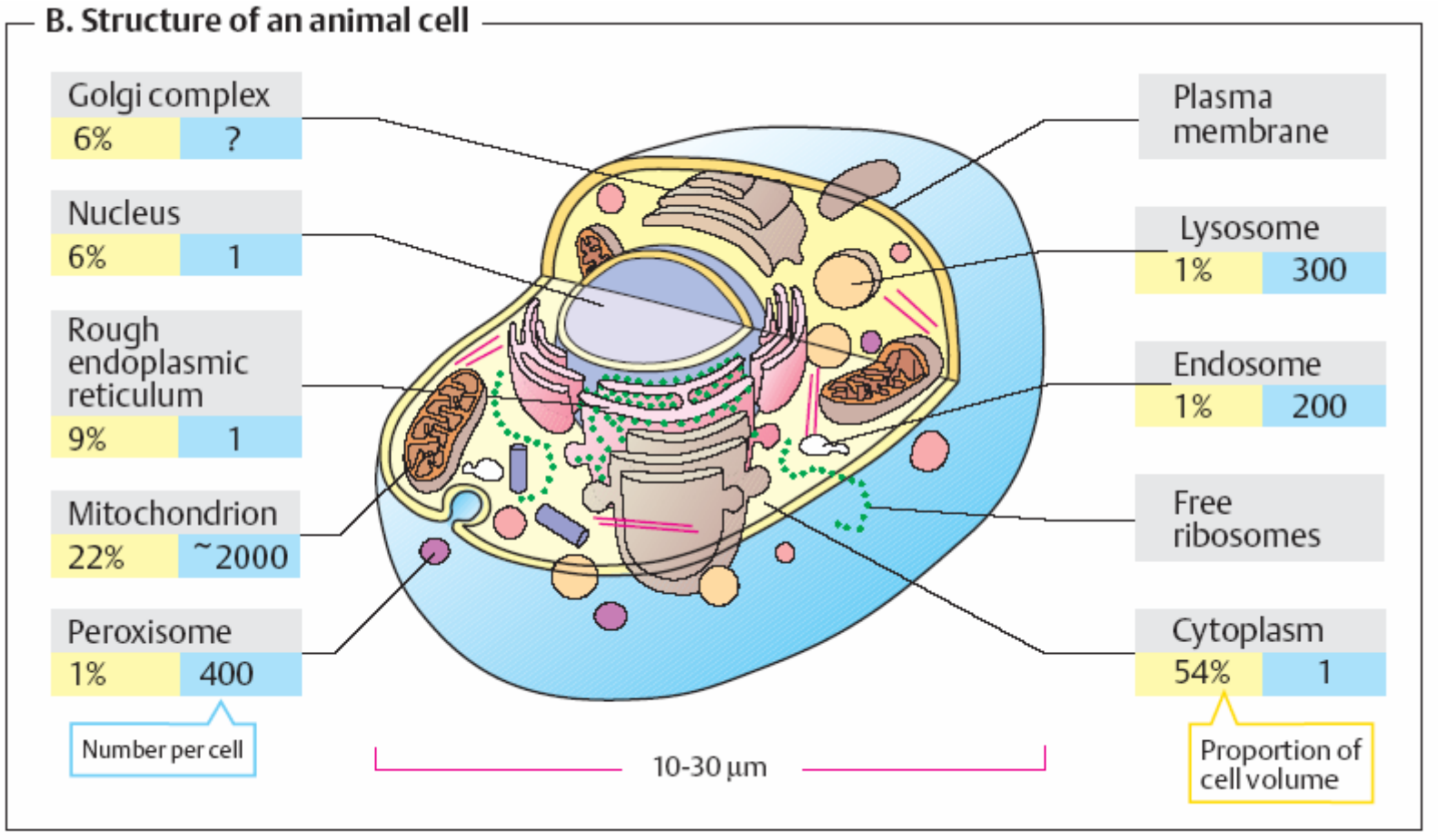
General Steps in Biochemical Separation



Separation of Macromolecules

- Chromatography, precipitation
- Electrophoresis, ultracentrifugation



Densities of biological material

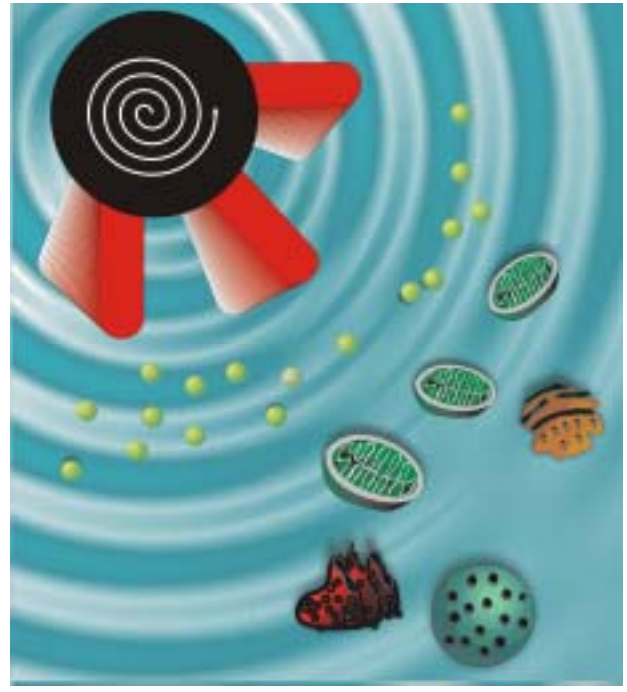
Material	Density (g/cm^3)
Microbial cells	1.05 - 1.15
Mammalian cells	1.04 - 1.10
Organelles	1.10 - 1.60
Proteins	1.30
DNA	1.70
RNA	2.00

Introduction (MBM 3.1)

Principles of centrifugation

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

In a solution, particles whose density is 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. If there is no difference in 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

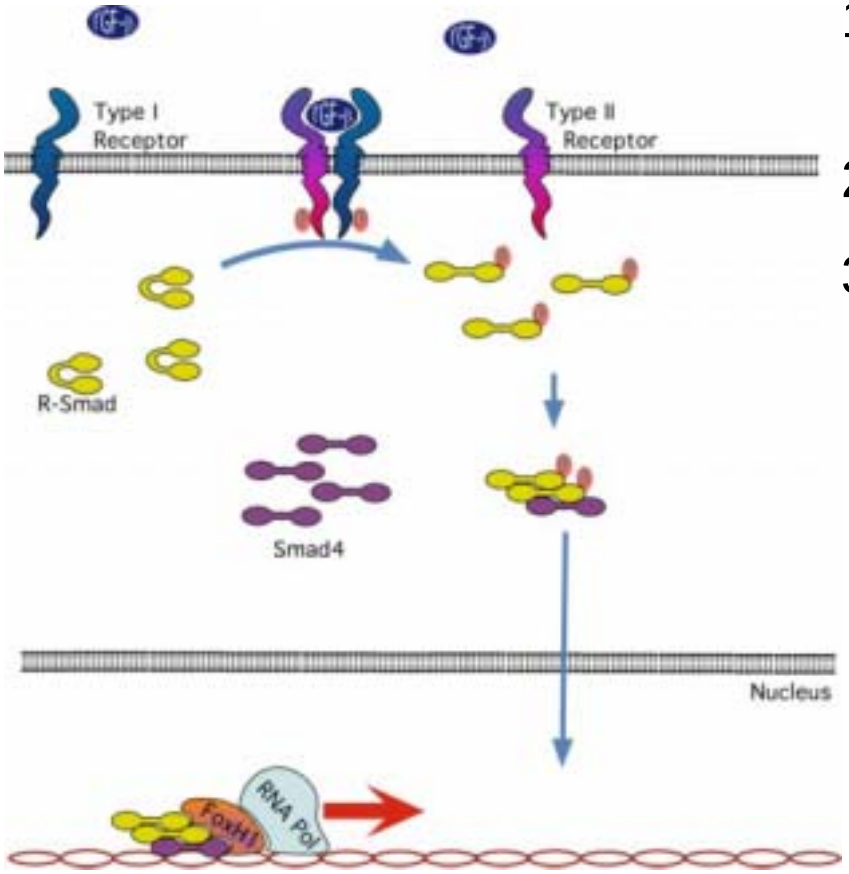
Methodology:

-Utilizes density difference between the particles/macromolecules and the medium in which these are dispersed

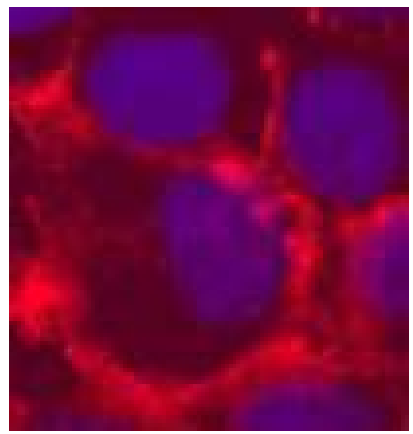
-Dispersed systems are subjected to artificially induced gravitational fields

Type 1– Preparative Centrifugation

- Collect (isolation) material:
  cell, subcellular structure, membrane vesicles



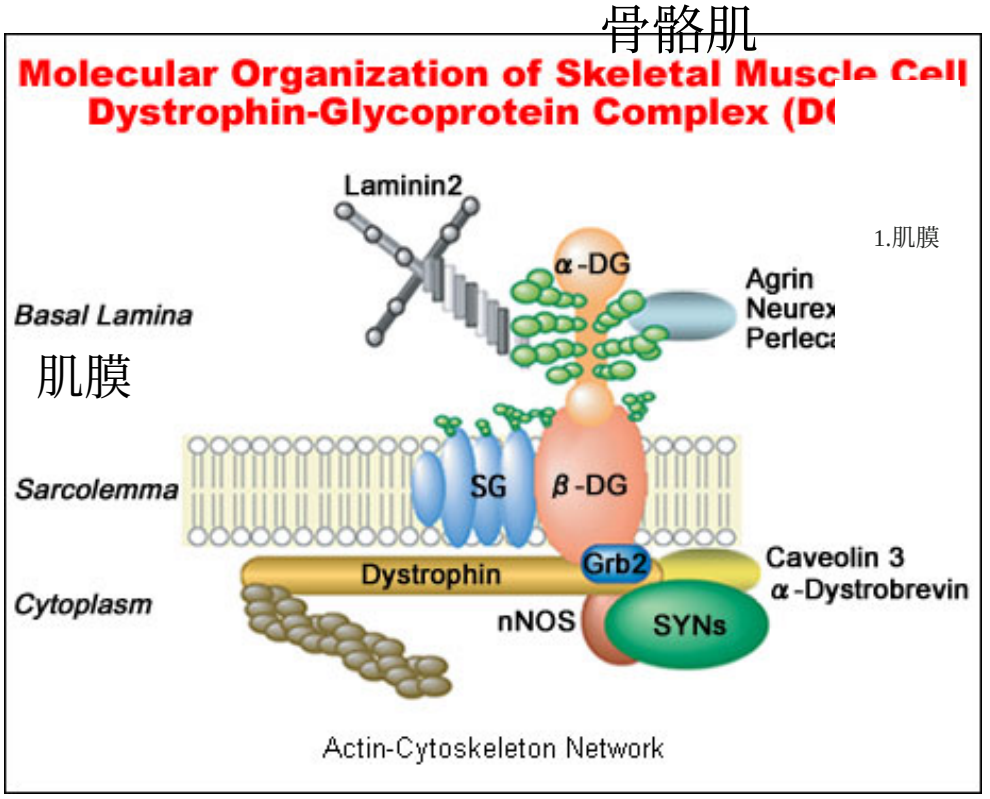
1. Handle larger liquid volumes (i.e. 1 to several thousand litres)
2. Range of designs
3. Typical rotating speed: 500 - 2000 rpm



Immunofluorescent imaging of human cells (U2OS) with pan Cadherin antibody

Type 2– Analytical Ultracentrifugation (AUC)

■ Determine the mass, shape and stoichiometry ratio of non-covalent association of macromolecules (protein-protein, small molecule-protein, quaternary structure)



1. Rotates at high speeds e.g. 30000 rpm
2. The high speeds used in such devices generate considerable amounts of heat
3. Therefore cooling arrangements are required in ultracentrifuges

http://www.cgmh.org.tw/chldhos/intr/c4a90/new_page_50.htm

3.2 Basic Principle of Sedimentation (AB 3.4.3)

Relative centrifugal force

$$F = M\omega^2 r$$

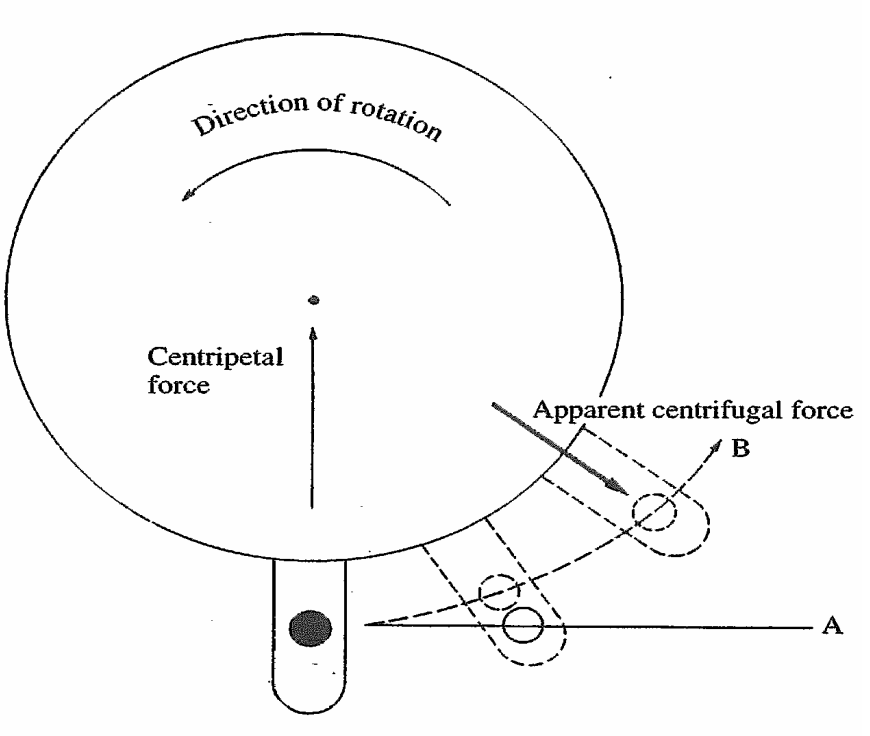


Figure 3.38 Centrifugal effects. 1

M: mass of particle

r: radius of rotation (cm) (*ie* distance of particle from axis of rotation)

ω :Average angular velocity (radians/sec)

$$\omega = \frac{2\pi \text{ rev min}^{-1}}{60}$$

Rev: revolution per minute (r.p.m.)

1 revolution = 2 π radians =360

Centrifugal Field

$G=r\omega^2$ depends on the radical distance of the particle from the rotation axis and the square of the angular velocity

$$G = \frac{4\pi^2 \left(\text{rev min}^{-1}\right)^2 r}{3600}$$

CALCULATION OF CENTRIFUGAL FIELD

What is the applied centrifugal field at a point equivalent to 5 cm from the centre of rotation and an angular velocity of 3000 rad s^{-1}?

The centrifugal field, G, at a point 5 cm from the centre of rotation may be calculated using the equation $G = \omega^2 r$

$G = (3000)^2 \times 5 \text{ cm s}^{-2} = 4.5 \times 10^7 \text{ cm s}^{-2}$

Angular Velocity

$$\omega = \frac{2\pi \text{ rev min}^{-1}}{60}$$

rev: revolution per minute (r.p.m.)

CALCULATION OF ANGULAR VELOCITY

For the pelleting of the microsomal fraction from a liver homogenate, an ultra-centrifuge is operated at a speed of 40 000 r.p.m. What is the angular velocity, ω, in radians per second?

The angular velocity, ω, may be calculated using the equation:

$$\omega = \frac{2\pi \text{ rev min}^{-1}}{60}$$

$\omega = 2 \times 3.1416 \times 40\,000/60 \text{ rad s}^{-1} = 4188.8 \text{ rad s}^{-1}$

Relative Centrifugal Force (RCF)

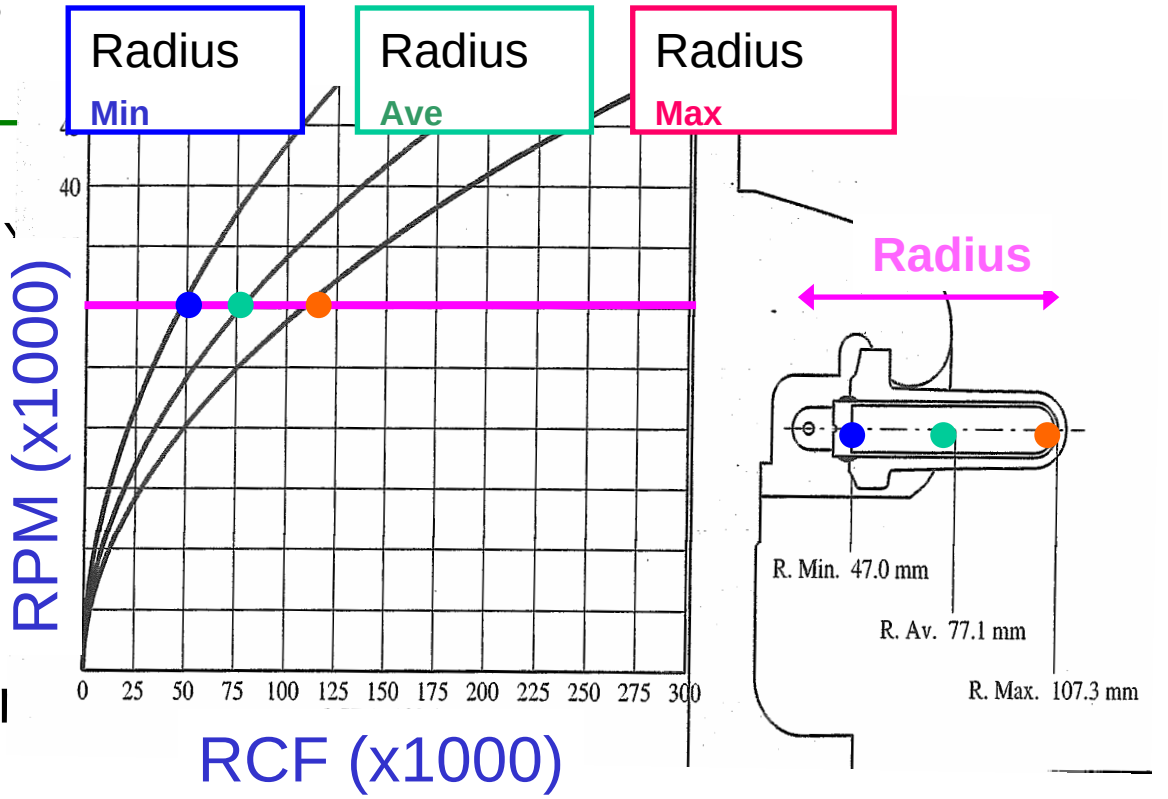
$$RCF = \frac{f_c}{f_g} = \frac{M\omega^2 r}{Mg} = \omega^2 r \times g^{-1}$$

RCF value "No. x g" (multiples of earth's gravitational force).

Because **rotors** are different from various manufactures, we use **RCF** to represent the **centrifugation force.**

$$RCF = \left(\frac{2\pi\ rmp}{60}\right)^2 r \times g^{-1}$$

RCF =
1.12 x **10^{-5}** x **$(rpm)^2$** x



rpm:
revolution per mi
r: radius of rotor

Relative centrifugal force

RCF =
$1.12 \times 10^{-5} \times (\text{rpm})^2 \times r$

CALCULATION OF RELATIVE CENTRIFUGAL FIELD

A fixed-angle rotor exhibits a minimum radius, r_{min}, at the top of the centrifuge tube of 3.5 cm, and a maximum radius, r_{max}, at the bottom of the tube of 7.0 cm. See Fig. 3.2a for a cross-sectional diagram of a fixed-angle rotor illustrating the position of the minimum and maximum radius. If the rotor is operated at a speed of 20 000 r.p.m., what is the relative centrifugal field (RCF) at the top and bottom of the centrifuge tube?

The relative centrifugal field may be calculated using

$$RCF = 1.12 \times 10^{-5}\ \text{r.p.m.}^2\ r$$

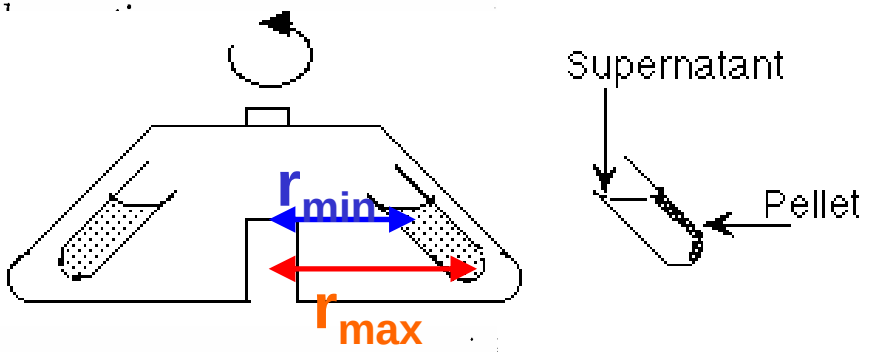
Top of centrifuge tube:

$$RCF = 1.12 \times 10^{-5} \times (20\,000)^2 \times 3.5\ g = 15\,680\ g$$

Bottom of centrifuge tube:

$$RCF = 1.12 \times 10^{-5} \times (20\,000)^2 \times 7.0\ g = 31\,360\ g$$

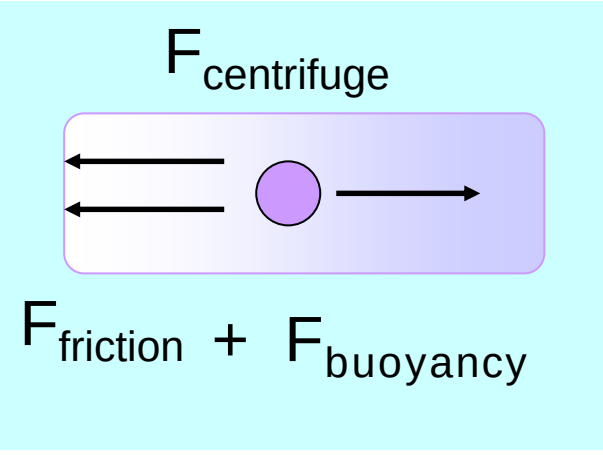
This calculation illustrates that, with fixed-angle rotors, the centrifugal field at the top and bottom of the centrifuge tube might differ considerably, in this case approximately two-fold.



Interacting Forces in Centrifugation

Sedimenting force, $m_p\omega^2 r$, is opposed by...

m_p = the mass of equal volume of solvent



. **Frictional Resistance against particle moving through fluid.**

= f.v

f = frictional coefficient of particle in the solvent

v = particle velocity

. **Flotation Force** $F=m_s\, r\omega^2$

BALANCE between the **sedmenting force** and **counteracting force**

Net force = $(m_p - m_s)r\omega^2 - fv$

Sedimentation Coefficient (s), 沉降係數

When the frictional force balances the driving force, $\frac{dv}{dt} = 0$

$$w^2 r(m_p - m_s) - f \cdot v = 0$$

$$S = \frac{v_t}{\omega^2 x} = \frac{m(1 - \bar{v}_2 \rho)}{f}$$

where $S \equiv$ terminal velocity / unit acceleration

Sedimentation coefficients have units of sec. 10^{-13} sec is called 1 svedberg (or 1 *S*). T. Svedberg pioneered research on sedimentation in an ultracentrifuge.

$$1\ S \equiv 10^{-13}\ \text{sec}$$

Theodor Svedberg (1884-1971),
Chemist from Sweden

1926 Nobel prize

1908. He described a new method (ultracentrifuge) of producing colloid particles and gave convincing evidence of the validity of the theory on the Brownian movements

$$S = \frac{v_t}{\omega^2 x} = \frac{m(1-\bar{v}_2\rho)}{f}$$

S Can be considered "Sedimentation Rate" of a particle under centrifugation force $=(dr/dt)/(1/ r\omega^2)$

m= particle mass

f = frictional coefficient of the particle in the solvent

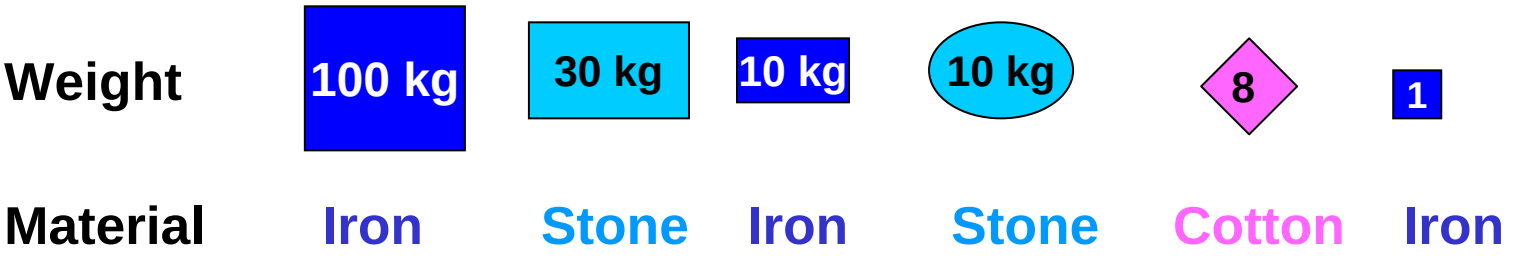
ρ= density of solution

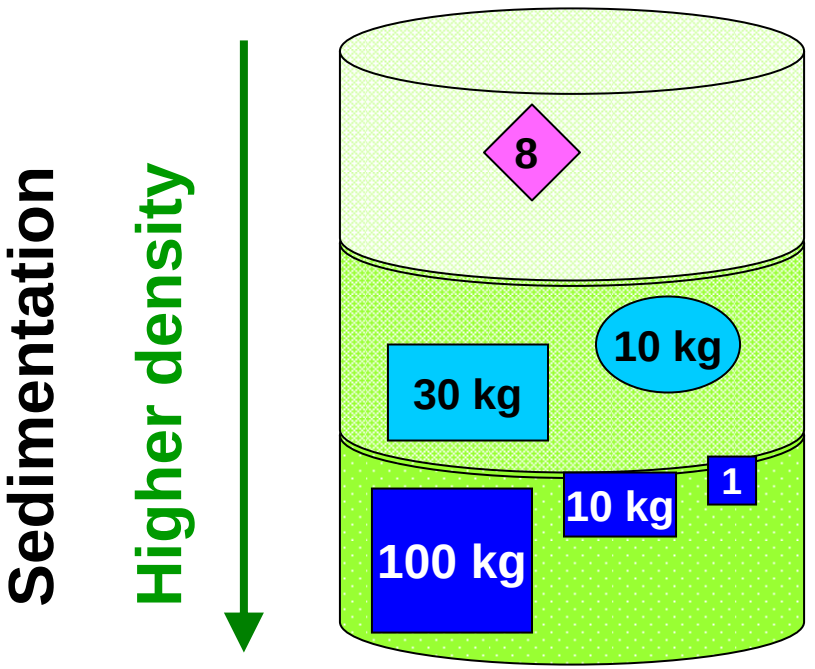
v = particle velocity

- S is increased for particle of larger mass (because sedimenting force a m(1-*vr*)
- S is increased for particle of larger density (equal volume)
- S is increased for more compact structures (Shape) of equal particle mass (frictional coefficient is less)
- S is increased with rotational speed

Mild, non-denaturing procedure, useful for protein purification, and for intact cells and organelles

Separation by Sedimentation

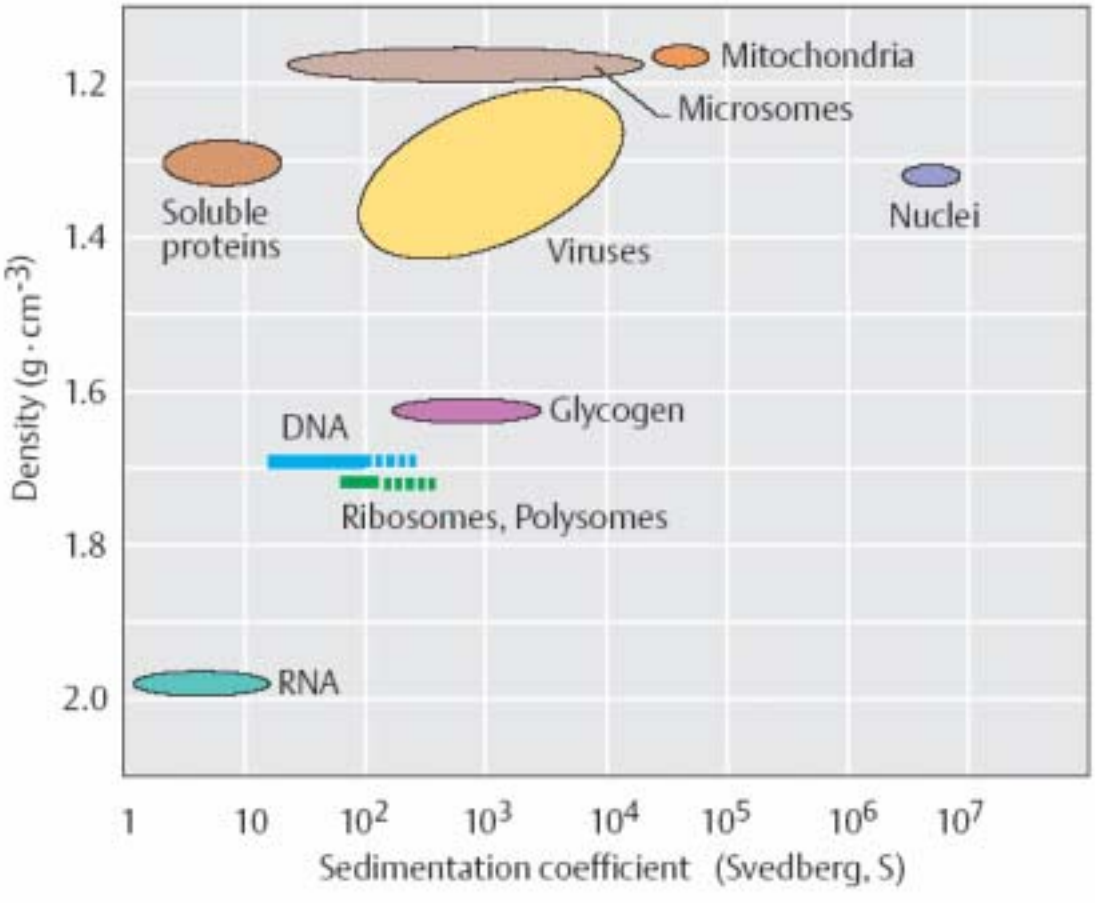




- Mass
- Density
- Shape

Subcellular Fractionation

Densities and sedimentation coefficients for biomolecules, cell organelles, and viruses.



$$S = \frac{v_t}{\omega^2 x} = \frac{m\left(1 - \bar{v}_2 \rho\right)}{f}$$

Require high density media

High concentrated CsCl

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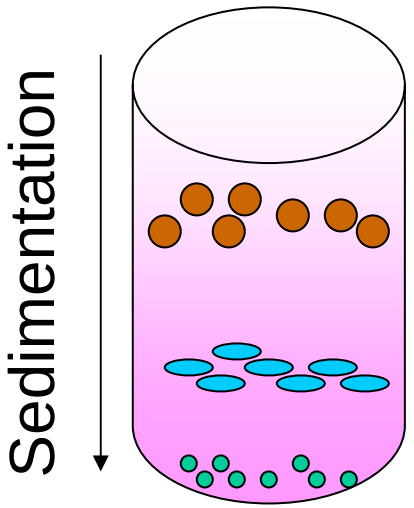
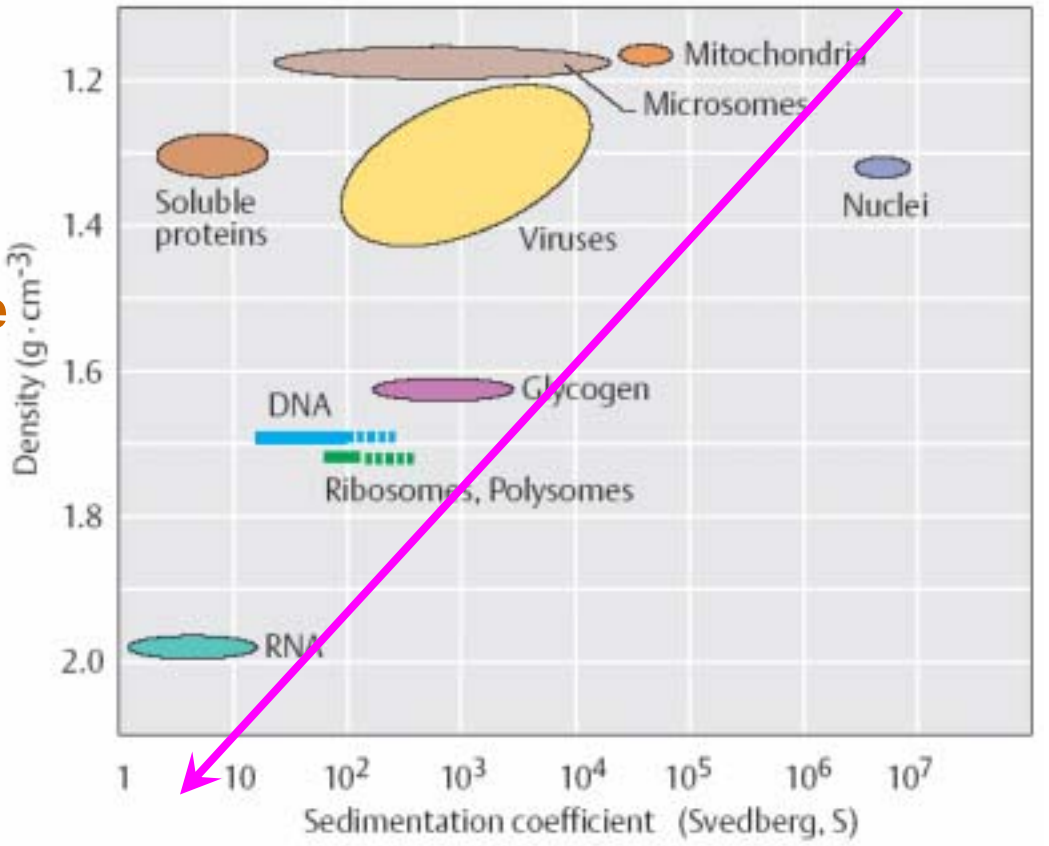
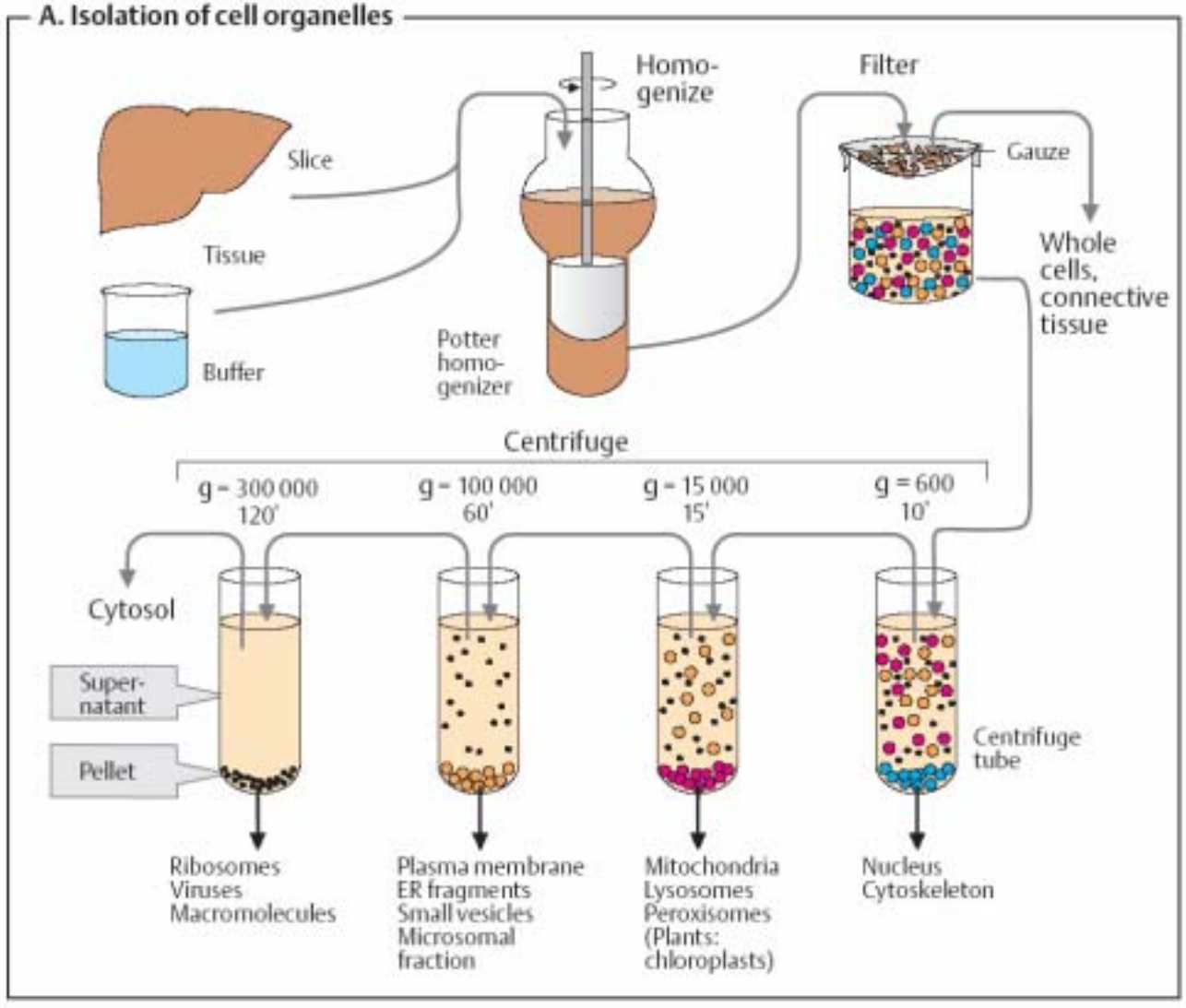


Soluble protein

DNA

RNA





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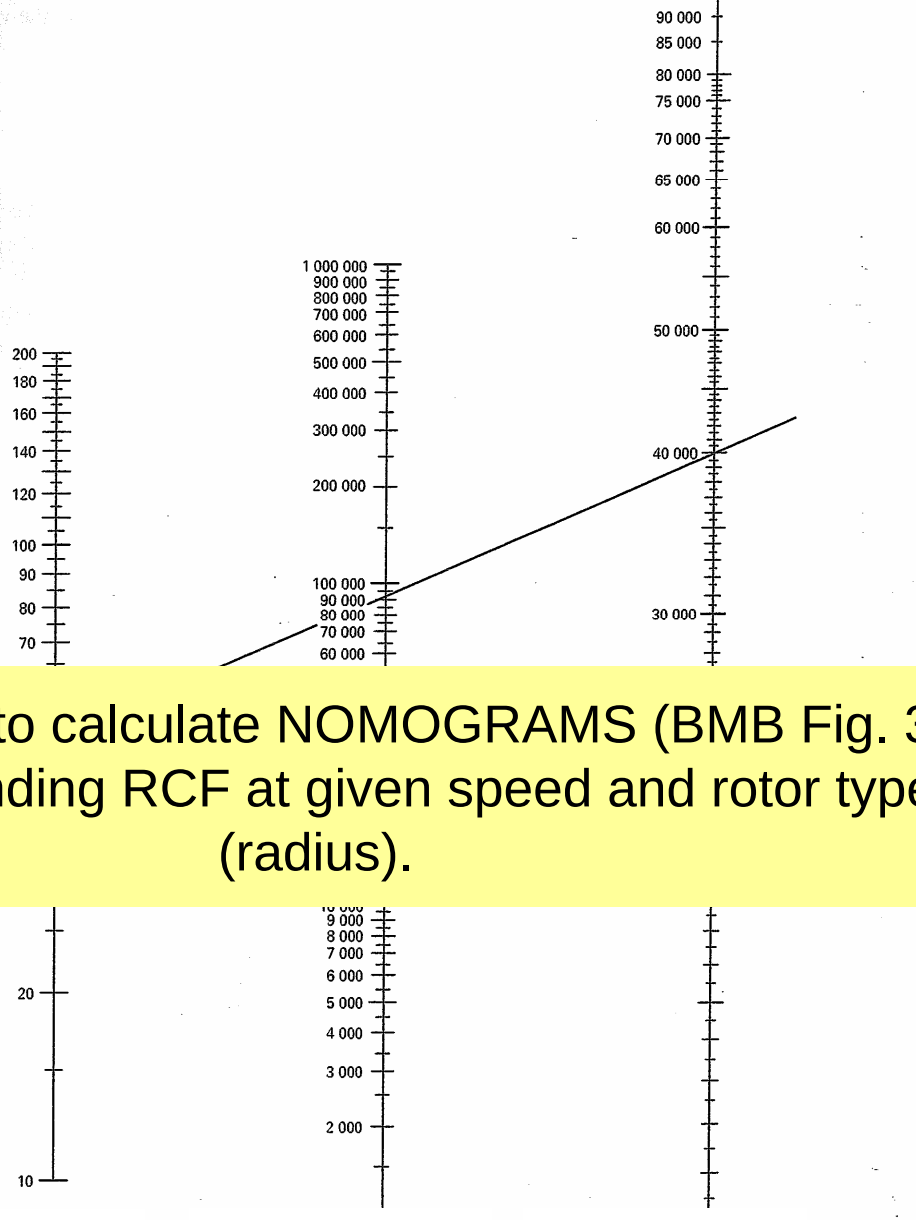
NOMOGRAMS

Conversion between relative centrifugal force



Equation used to calculate NOMOGRAMS (BMB Fig. 3.1) for quickly finding RCF at given speed and rotor type (radius).

Radical distance (mm)

Relative centrifugal field (xg)

Rotor speed (r.p.m)

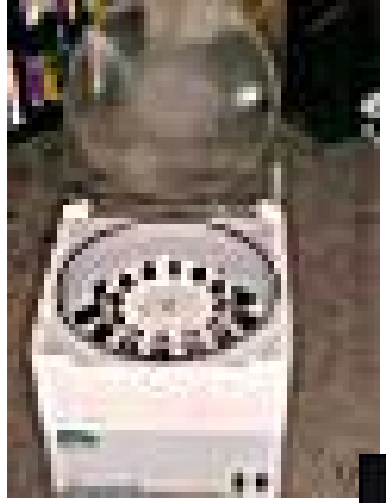
Types of Centrifuge BMB 3.3.1

- Maximum speed of sedimentation
- Presence /absence of vacuum
- Temperature control refrigeration)
- Volume of sample and capacity of centrifugation tubes

- Microfuge

0.5-1.5 cm^3, 10,000 g

Concentration of protein samples



- Large-capacity preparative centrifuge

5-250 cm^3, 3,000-7,000 g

H2500R-2

- High-speed refrigerated centrifuge

  5-250 cm^3, 100,000 g

Differentiation separation of nucleus, mitochondrial, protein precipitate, large intact organelle, cellular debris

- Ultracentrifugation

  5-250 cm^3, 600,000 g

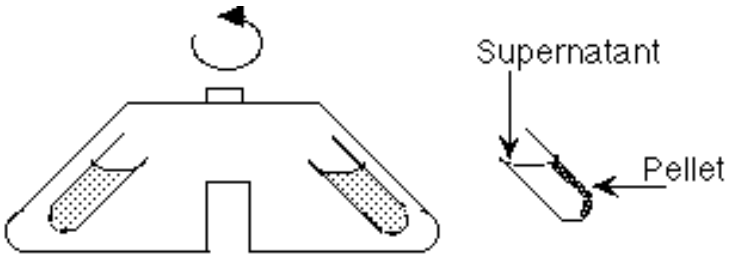
Microsomal vesicles, ribosome

Has to reduce excessive rotor temperature generated by **frictional resistance**

→ **sealed chamber, evacuated, cooling**

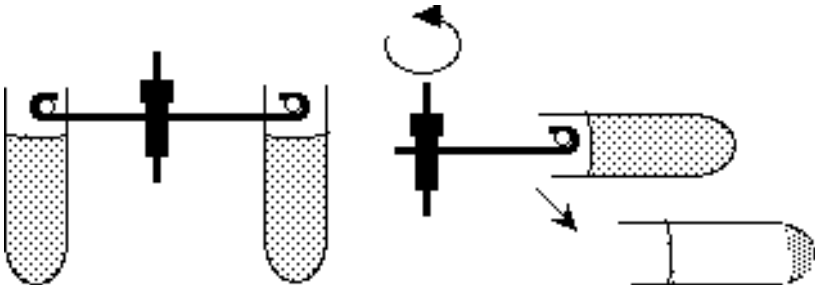
Centrifuge Rotors (MBM3.3.2)

Fixed Angle Rotor



Sedimenting particles have only short distance to travel before pelleting. **Shorter run time.** The most widely used rotor type.

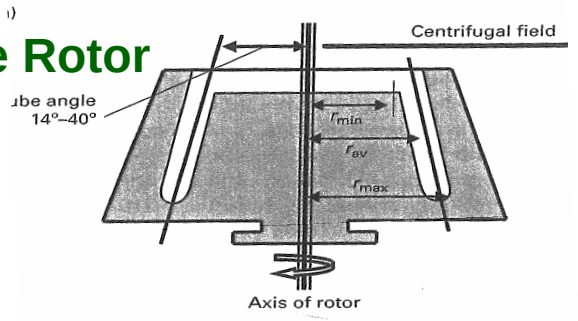
Swinging Bucket Rotor

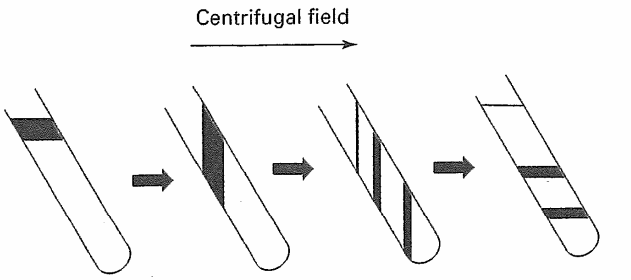


Longer distance of travel may allow better separation, such as in density gradient centrifugation. Easier to withdraw supernatant without disturbing pellet.

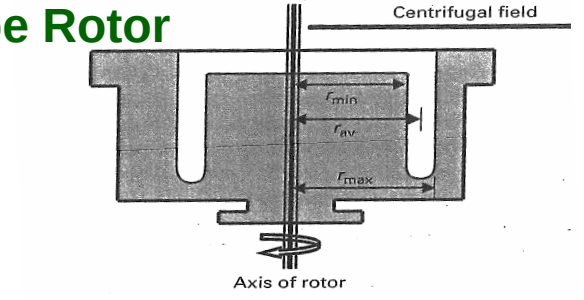
Centrifuge Rotors (MBM3.3.2)

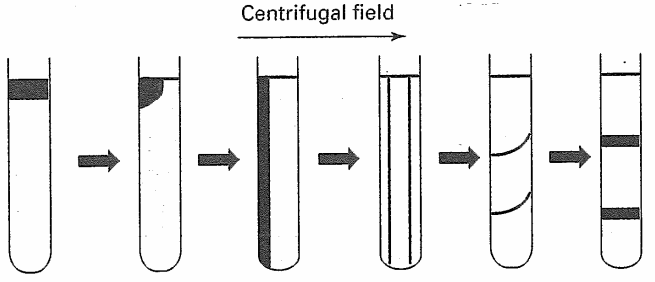
- **Fixed Angle Rotor**



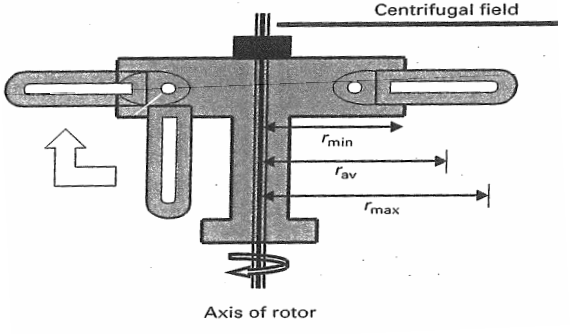


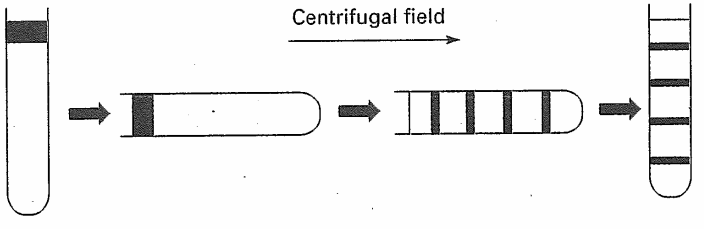
- **Vertical Tube Rotor**





- **Swinging Bucket Rotor**





Centrifuge Its Use and Safety (BMB 3.3.4)

On December 16, 1998, milk samples were running in a Beckman L2-65B ultracentrifuge using a large aluminum rotor . The rotor failed due to excessive mechanical stress

Mechanical stress

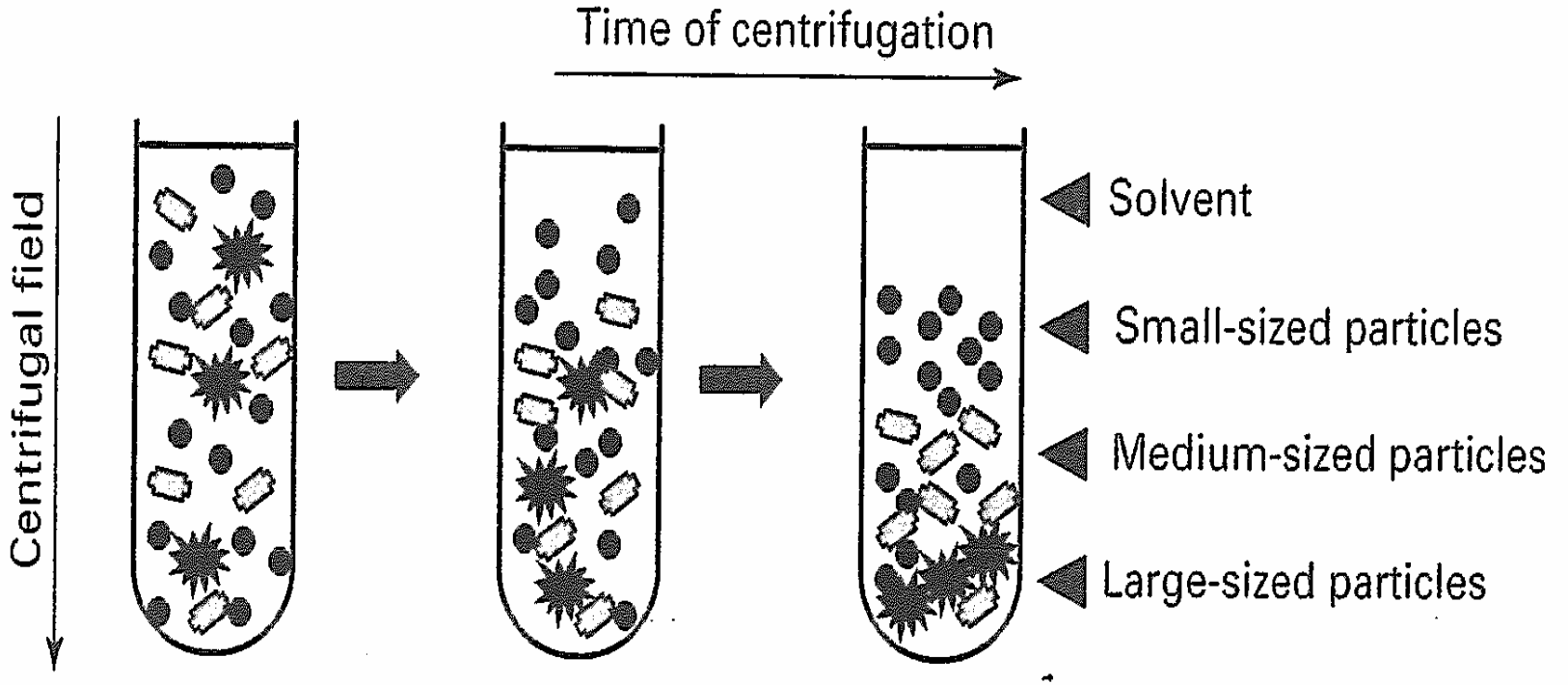
- Always ensure that loads are evenly balanced before a run.
- Always observe the manufacturers maximum speed and sample density ratings.
- Always observe speed reductions when running high density solutions, plastic adapters, or stainless steel tubes.

Corrosion

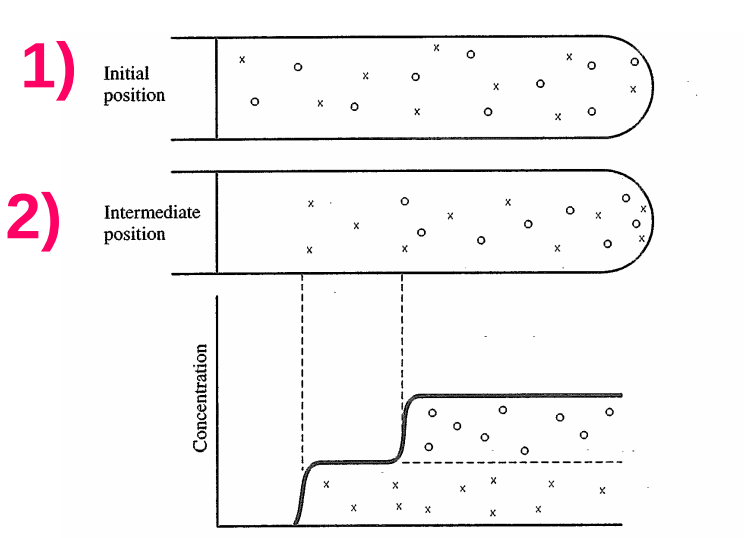
- Many rotors are made from either titanium or aluminum alloy, chosen for their advantageous mechanical properties. While titanium alloys are quite corrosion-resistant, aluminum alloys are not. When **corrosion** occurs, the metal is weakened and less able to bear the stress from the centrifugal force exerted during operation. The combination of stress and corrosion causes the rotor to fail more quickly and at lower stress levels than an uncorroded rotor

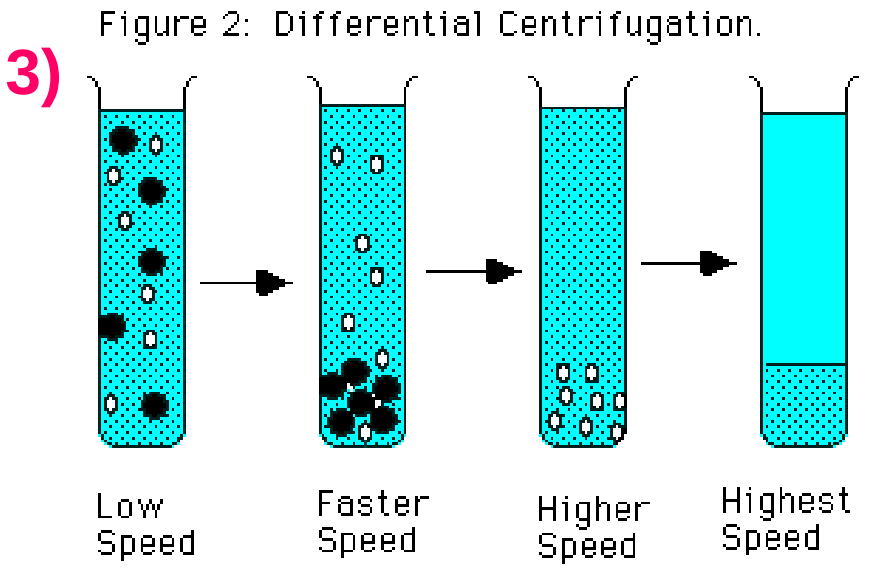
Differential Centrifugation BMB 3.4.1

- Based on the differences in the **sedimentation rate** of the biological particles of different size, shape and density



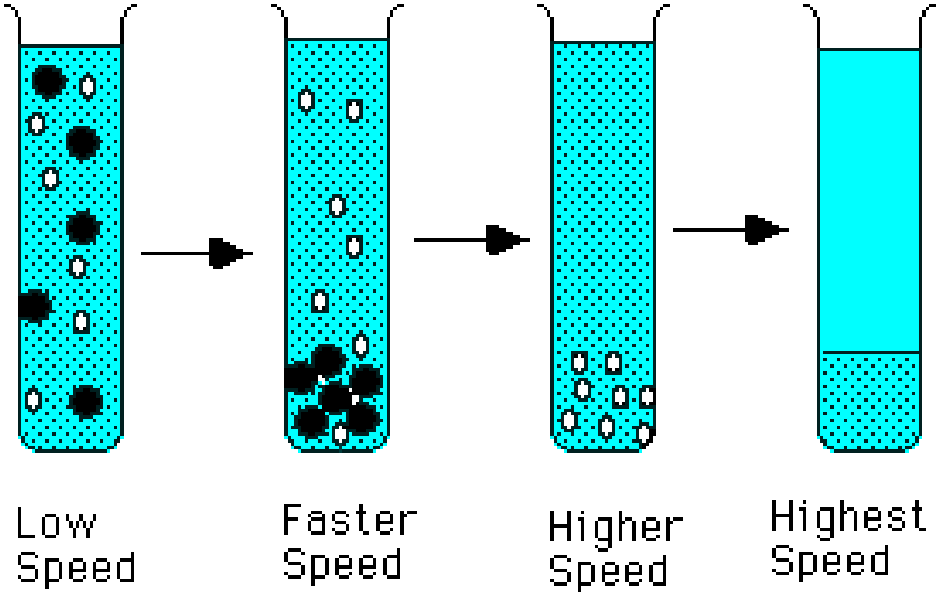
Moving Boundary (differential velocity) Centrifugation



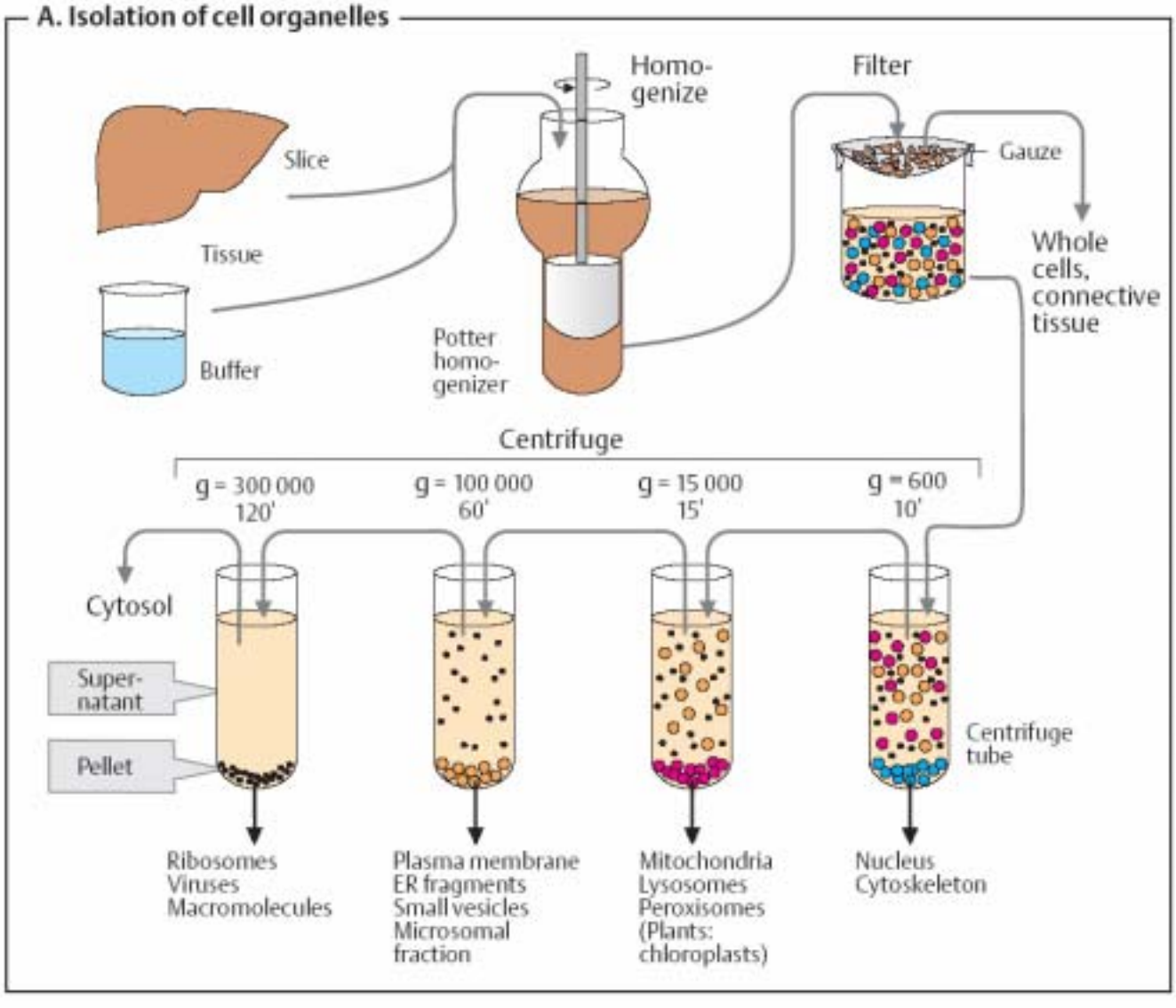


1) The entire tube is **filled with sample** and centrifuged
2) Through centrifugation, one obtains a separation of two particles but any particle in the mixture may end up in the **supernatant** or in the **pellet** or it may be distributed in both fractions, depending upon its **size, shape, density, and conditions of centrifugation**
3) Repeat sedimentation at **different speed**

Differential Velocity Centrifugation –cont.



- Medium: same density
- The sedimentation speed is determined mainly on the size, shape of particle.
- Application: low resolution separation such as preparation of nucleus



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Density Gradient Centrifugation (BMB 3.4.2)

- Important technique for purifying **proteins** and particularly **nucleic acids**.

Two different types of density gradient centrifugation, for two different purposes are:

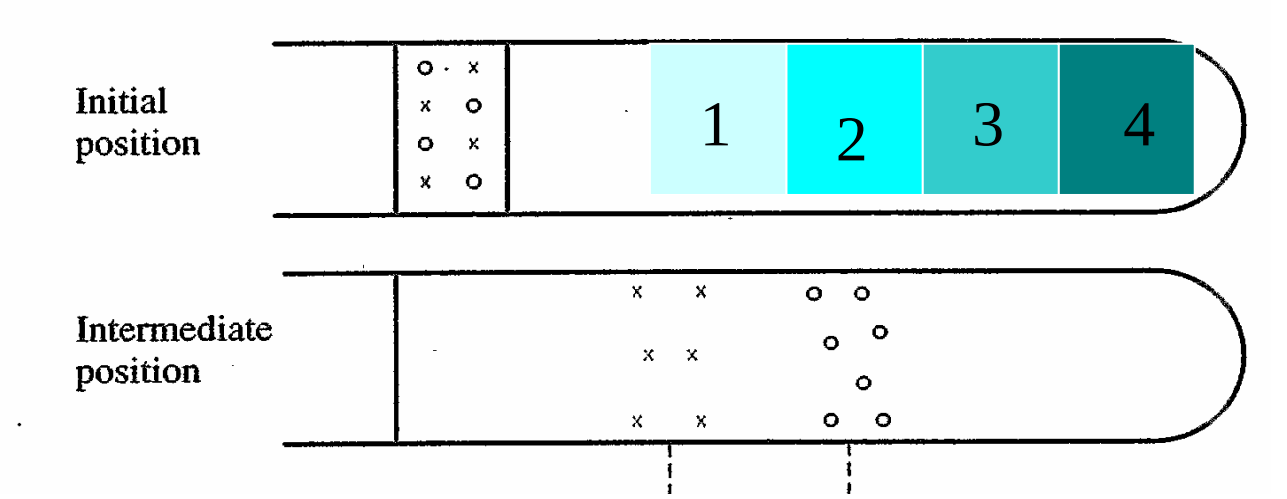
- **Zonal (or Rate Zonal) Centrifugation**

(Sucrose density gradient centrifugation)

- **Iso-density (Isopycnic) Centrifugation**

(Caesium chloride density gradient centrifugation)

Moving Zone Centrifugation



1. Preparation of **gradient sucrose density** for centrifugation medium

$$Density_1 < Density_2 < Density_3 < Density_4 < Density_{Analyte}$$

2.Sample is applied in a thin zone at the **top of the centrifuge tube** on a **density gradient**

Moving Zone (differential) Centrifugation –cont.

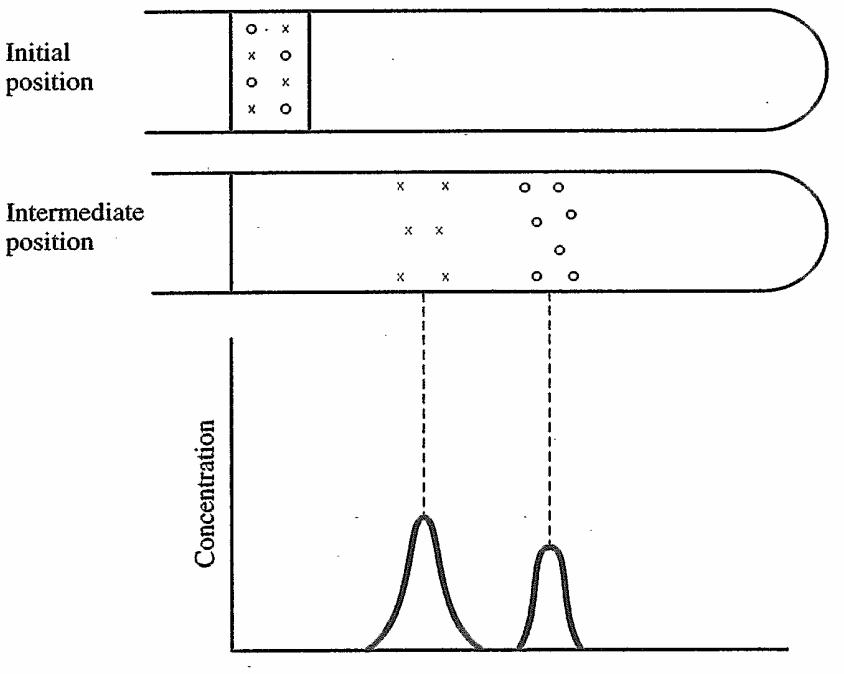
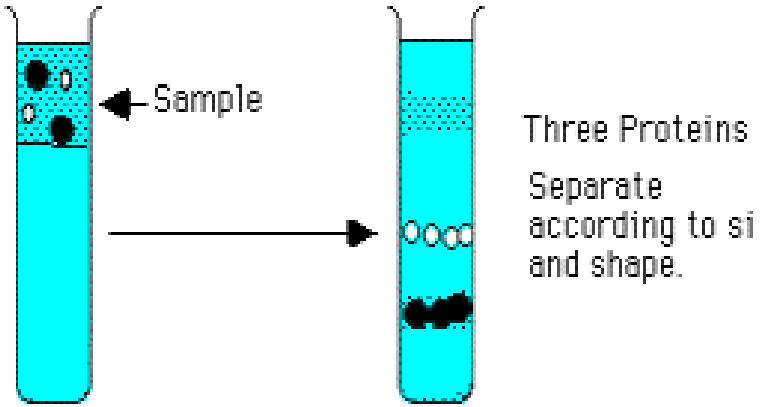


Figure 3b: Rate zonal centrifugation.



3. Under centrifugal force, the particles will begin sedimenting through the gradient in separate zones **according to their size shape and density**

Insufficient time--------- Incomplete separation

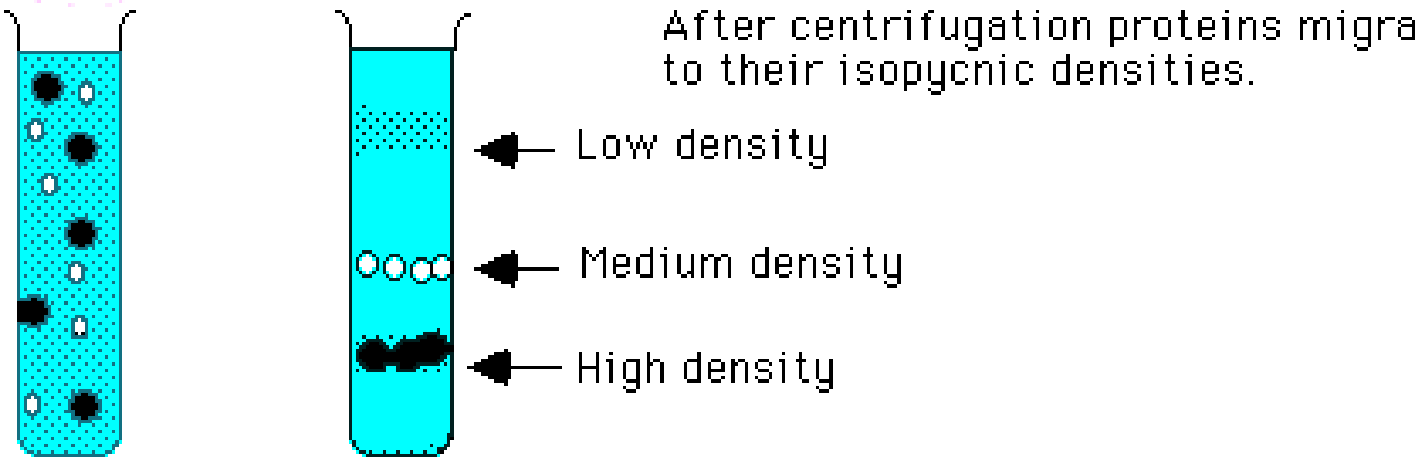
Overtime----------co precipitation of all analytes

Iso-density (Isopyncic) Centrifugation (AB3.4.3)

1. Preparation of **gradient sucrose density** for centrifugation medium

The gradient density has to cover the range of different densities of analytes

Figure 4: Isopycnic separation with a self-generating gradient



The sample is evenly distributed through-out the centrifuge tube centrifugation.

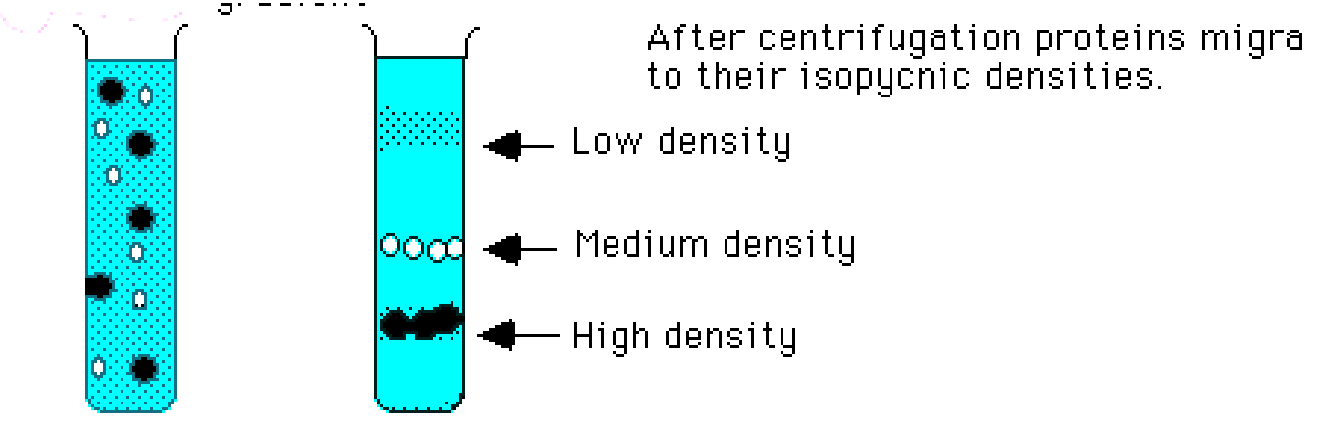
Iso-density (Isopyncic) Centrifugation (AB3.4.3)

等密度平衡離心法-equilibrium

Isopycnic = Equal density

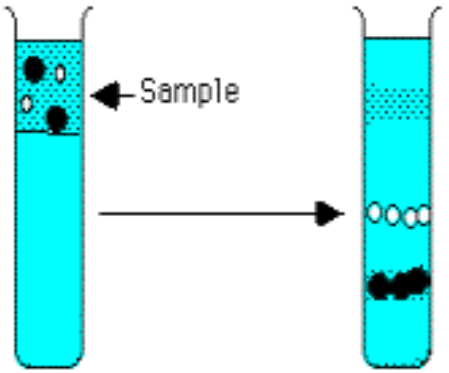
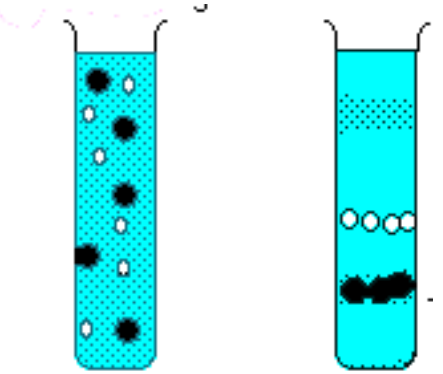
- Molecules separated on **equilibrium position**, NOT by **rates of sedimentation.**

After centrifugation, each molecule floats or sinks (=r**e-distribution**) to position where density equals density of CsC (or sucrose)l solution. Then no net sedimenting force on molecules and separation is on basis of **different densities** of the particles.

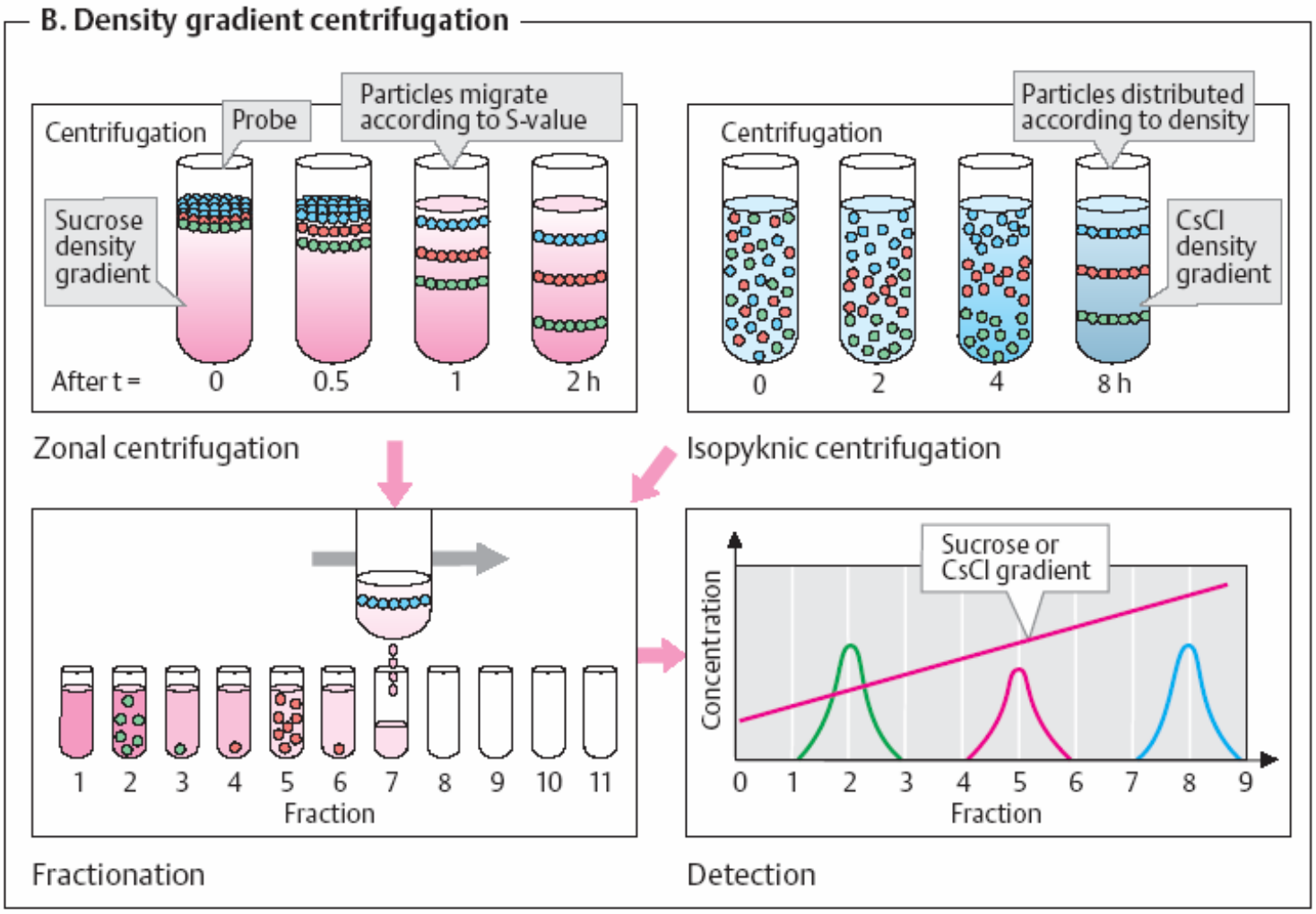


The sample is evenly distributed through-out the centrifuge tube
centrifugation.

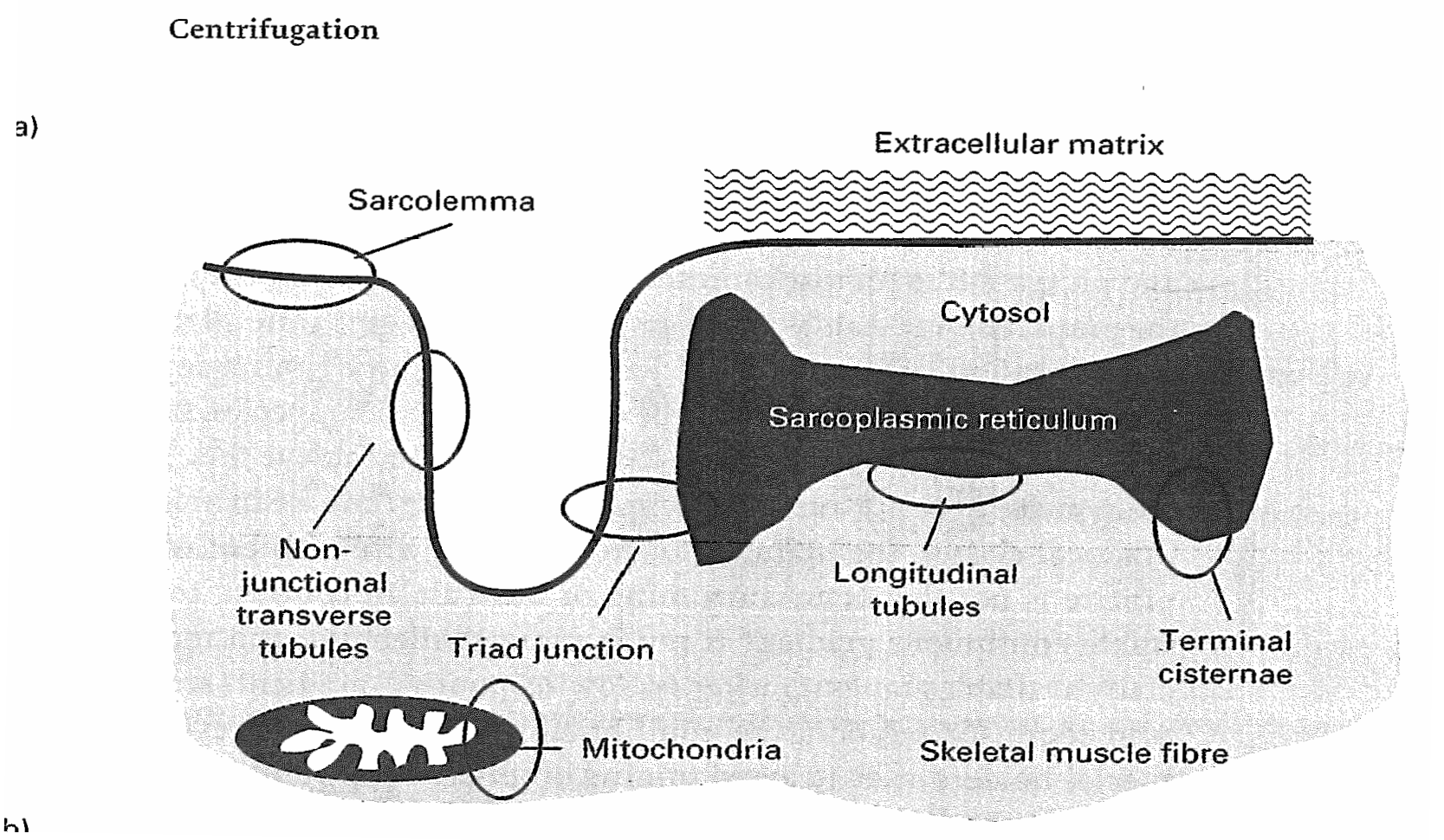
Comparison of Two Methods

	Moving Zone Centrifugation	Isopynic centrifugation
		
Centrifugation:	**Lower speed**, not complete sedimented, stop at proper time	Completely sediment to where the density is equilibrated, **high speed**, long running time
	Sedimentation Rate	**Sedimentation equilibrium**
Sample:	**Similar density, different MW**	**Similar MW, different density**
	Nucleic acid / cell organelle	Protein (similar density, but different in MW)

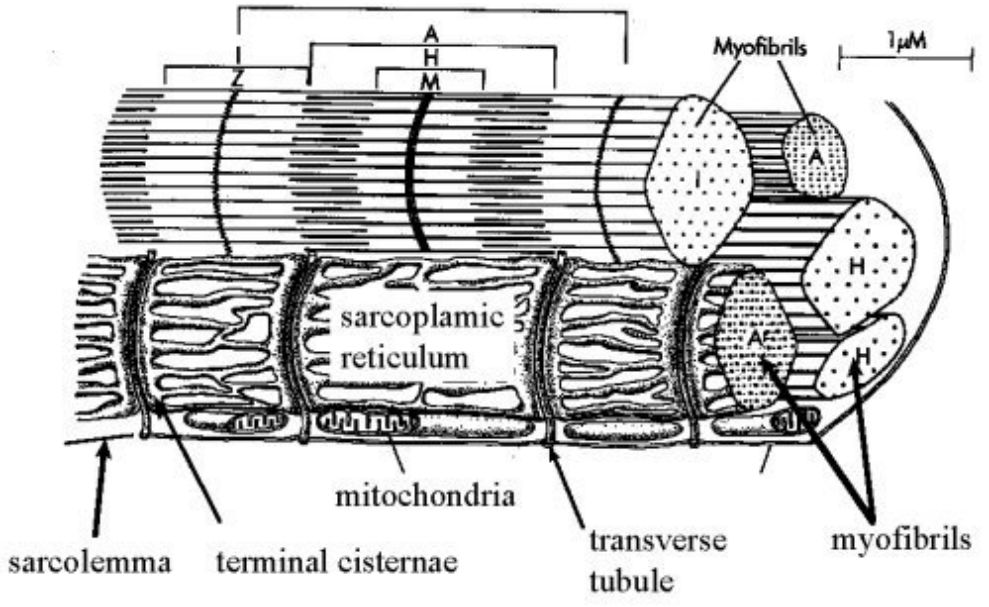
Density Gradient Centrifugation

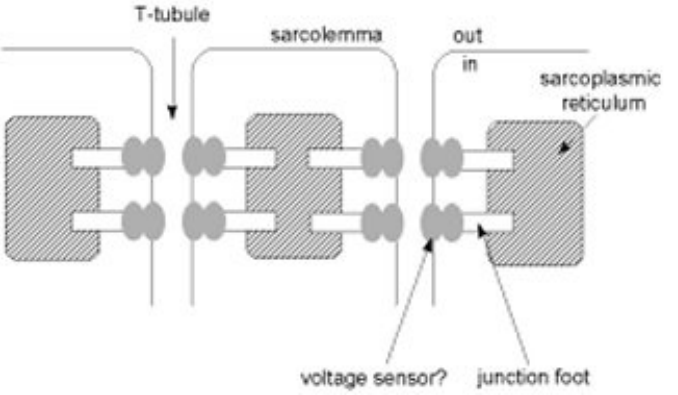


Subcellular Fractionation (BMB 3.4.4)



Skeletal Muscle





Sarcolemma :It is the surface membrane of the entire fiber

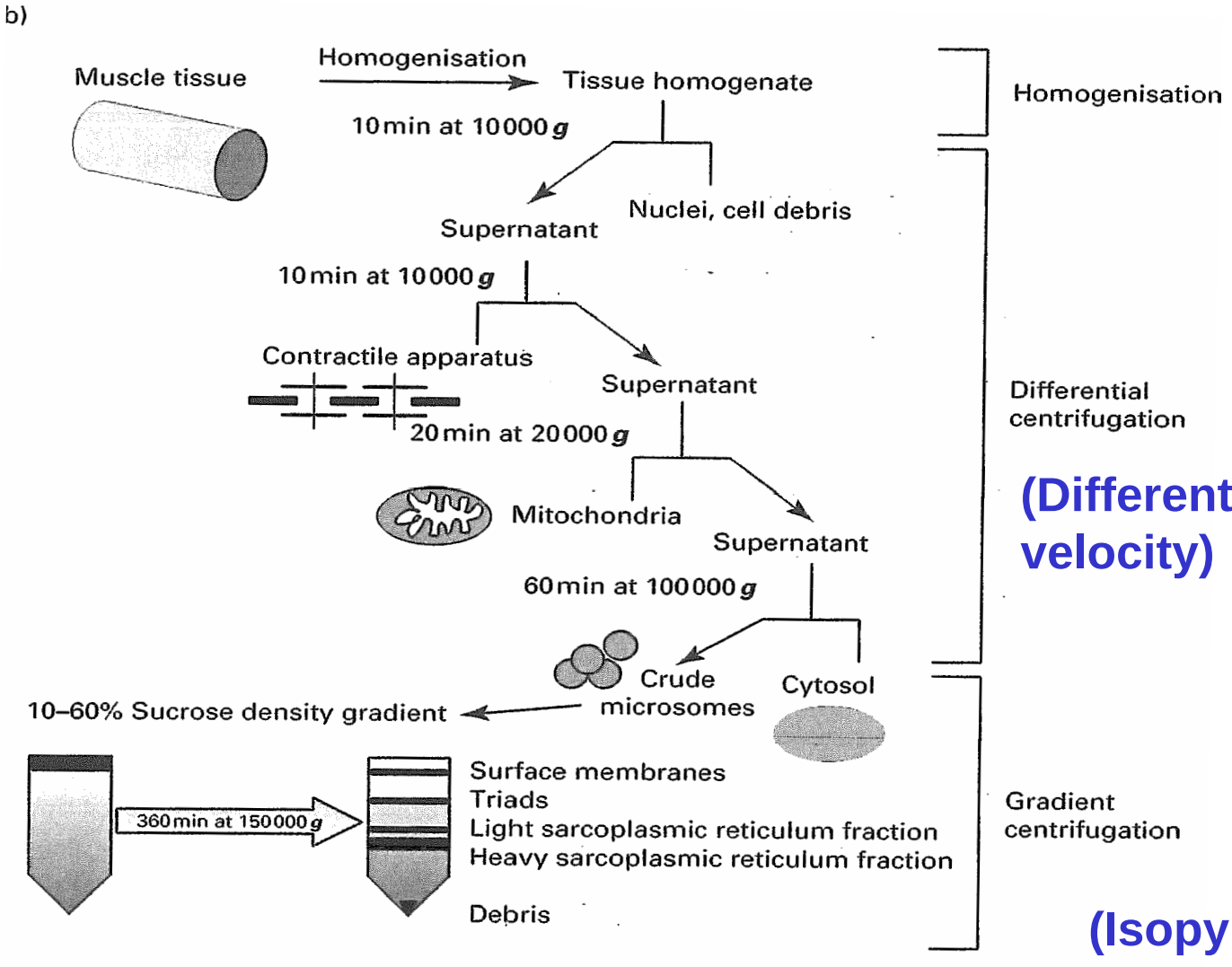
T-tubular membranes

They contain extracellular fluid (high in Ca and Na ions)

They are continuous tubes of sarcolemmal membrane that run through (transversely) the muscle fiber.

Sarcoplasmic reticulum The sarcoplasmic reticlum (SR) is the Ca store. It is a diffuse membrane structure that surrounds the sarcomere

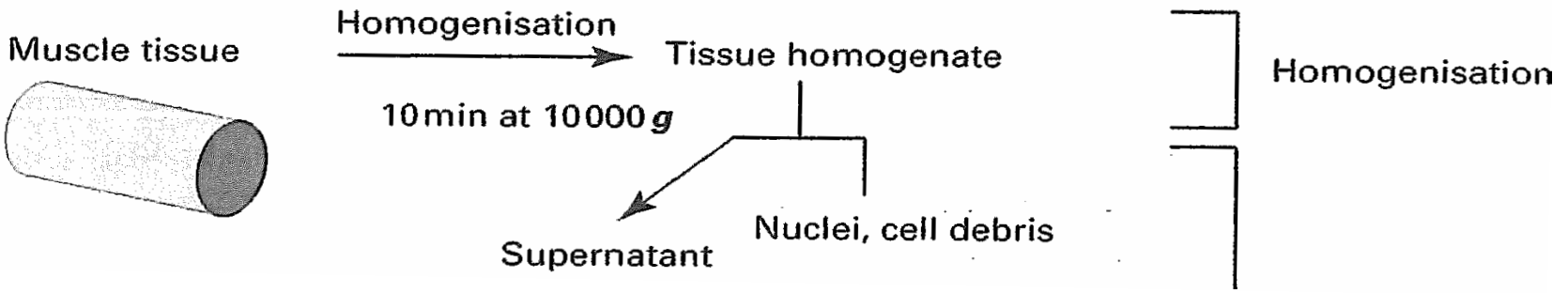
Organelle Separation

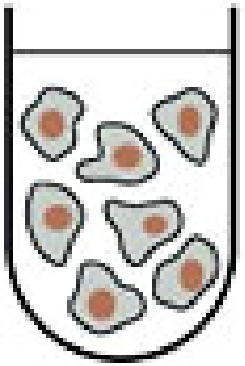


(Different centrifugation velocity)

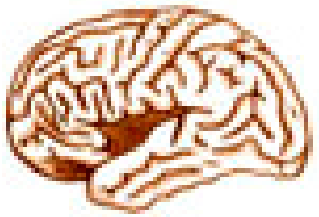
(Isopynic centrifugation)

Step 1- Cell homogenization



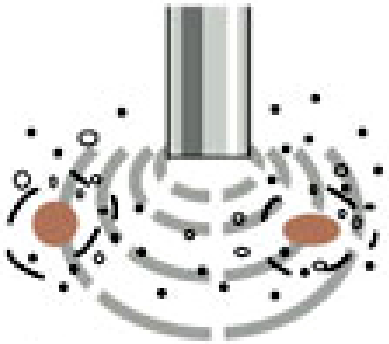


cell suspension or tissue

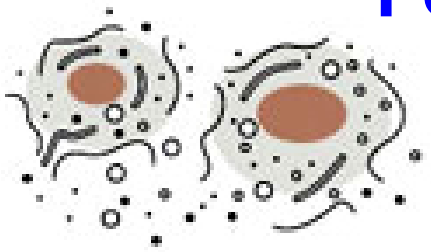


To obtain pure organelles, the cells must be ruptured, so that the cell membrane is broken, but the organelle to be studied is not. The process of rupturing a cell is known as **homogenization** of the cell and the subsequent isolation of organelles is **fractionation.**

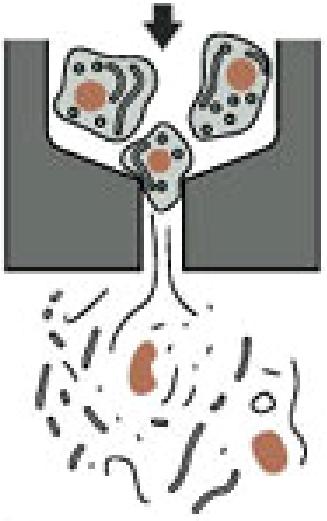
Four Common Methods



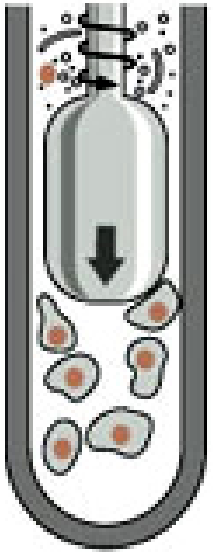
1. break cells with high frequency sound



2. use a mild detergent to make holes in the plasma membrane



3. force cells through a small hole using high pressure

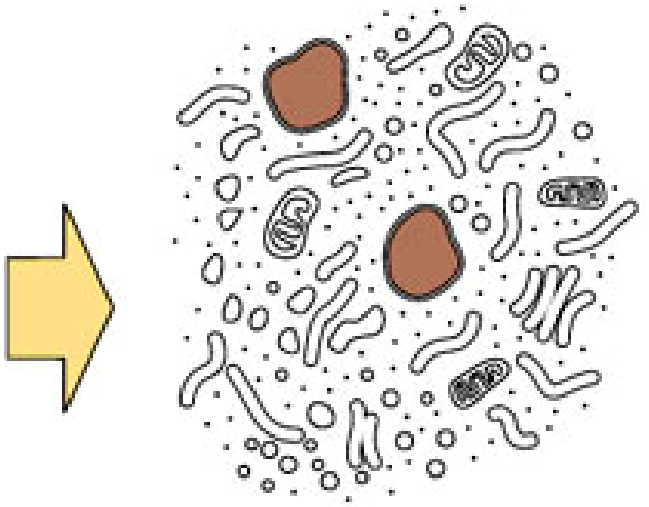


4. shear cells between a close-fitting rotating plunger and the thick walls of a glass vessel

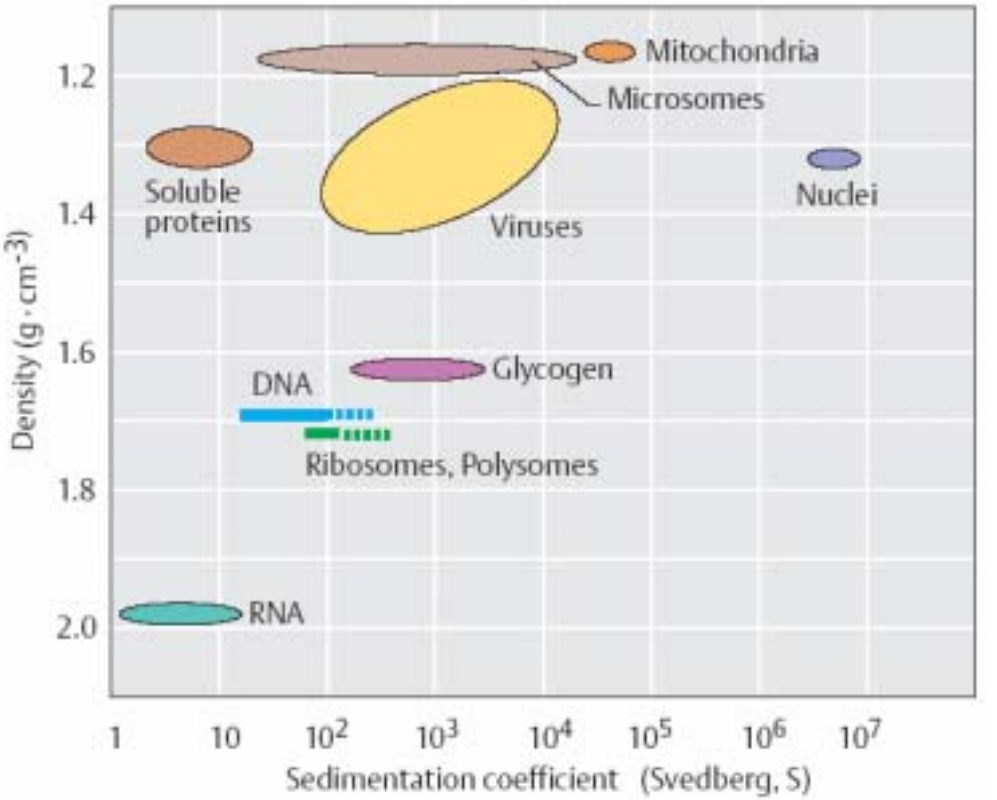
Using gentle mechanical procedures, called **homogenization**, the plasma membranes of cells can be ruptured so that the cell contents are released.

The resulting thick soup (called a homogenate or an extract) contains large and small molecules from the cytosol, such as enzymes, ribosomes, and metabolites, as well as all the membrane-bounded organelles.

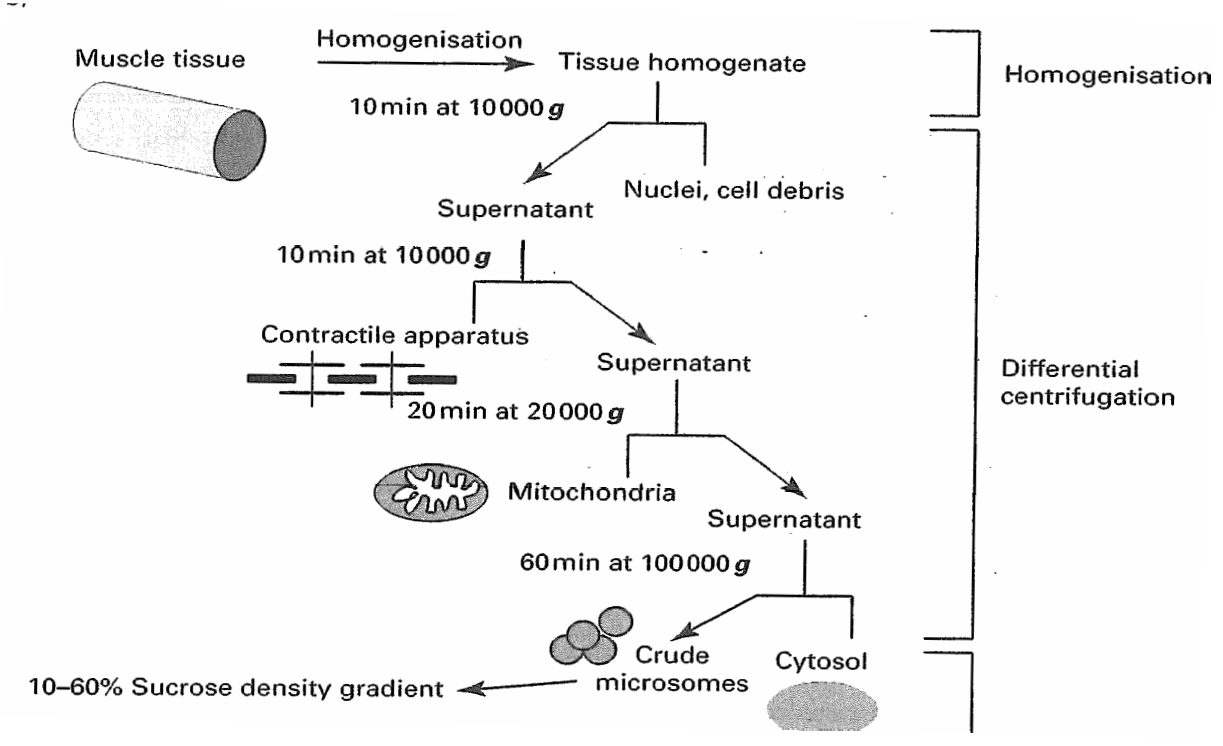
Ruptured cells producing a liquified cellular homogenate



When carefully applied, homogenization leaves most of the membrane-bounded organelles intact.

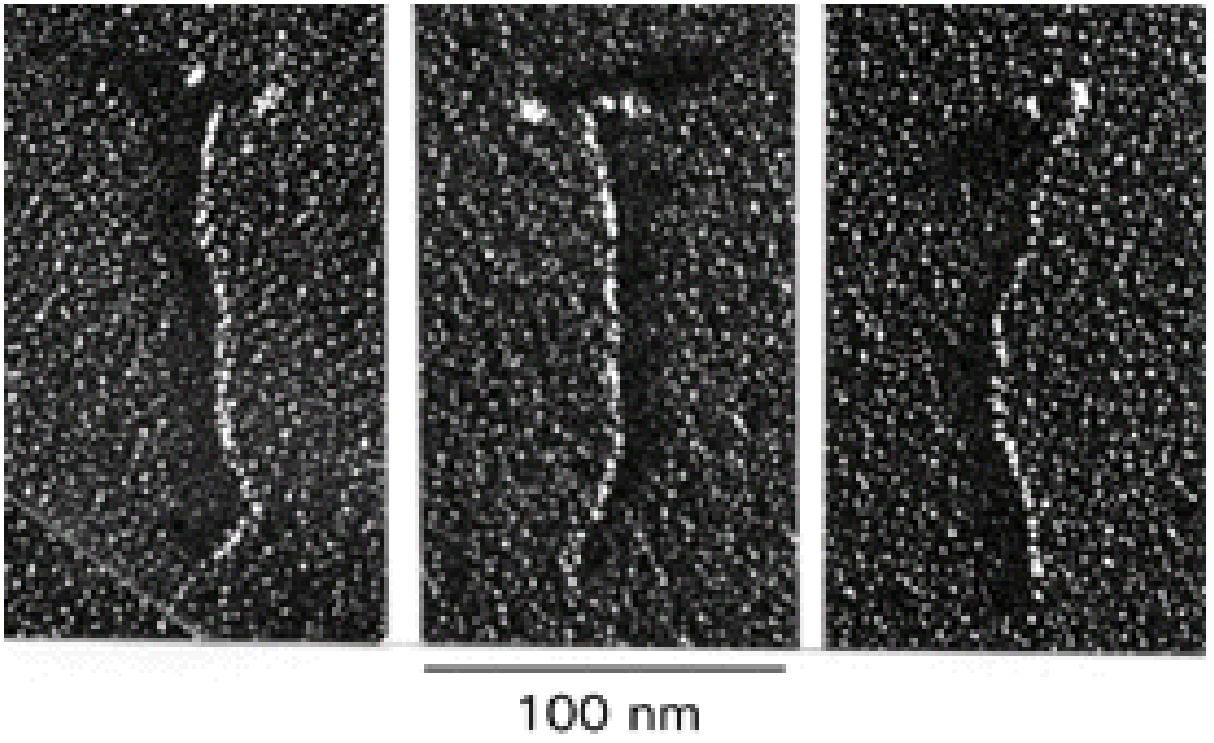


Step 2-Cell Fractionation by Centrifugation.



- Repeated centrifugation at progressively higher speeds will fractionate homogenates of cells into their components.
- In general, the smaller the subcellular component, the greater is the centrifugal force required to sediment it.

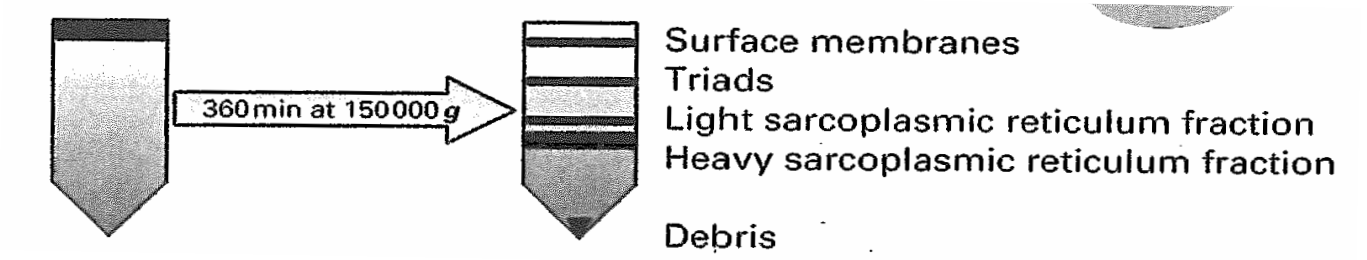
Contractile Apparatus of Muscle



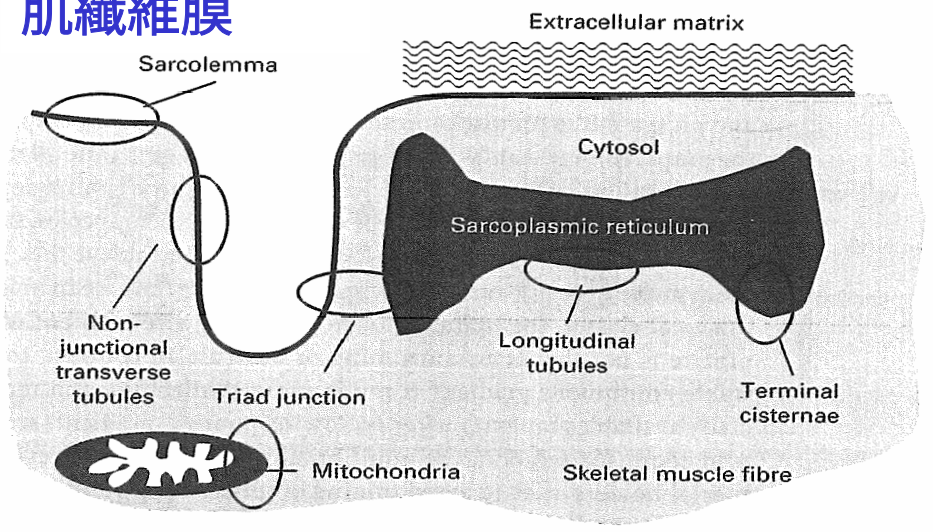
Electron micrographs of individual myosin protein molecules

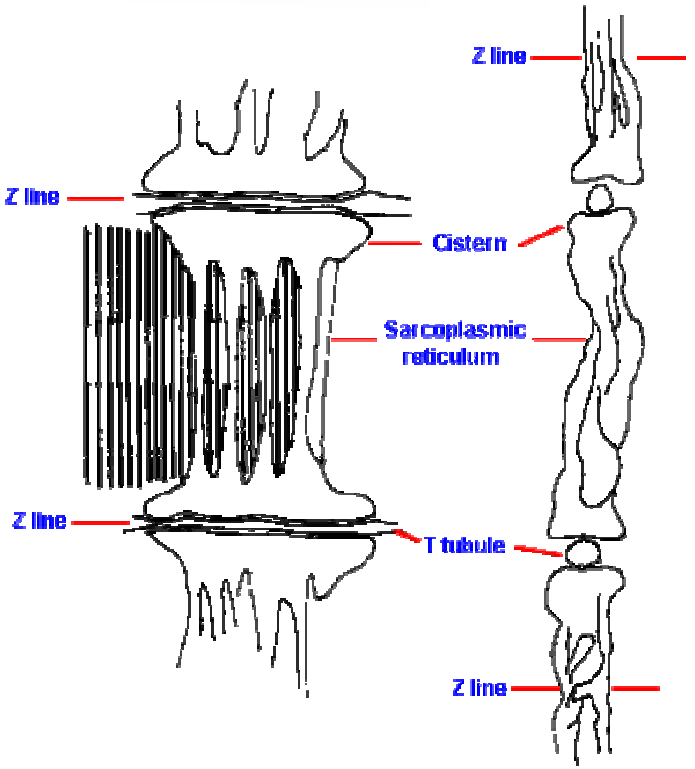
Myosin is a major component of the contractile apparatus of muscle. As shown here, it is composed of two globular head regions linked to a common rodlike tail.

Step 3- Density Gradient Centrifugation

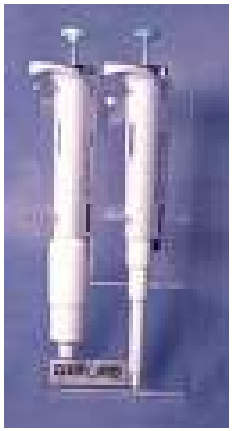


Sarcolemma
肌纖維膜

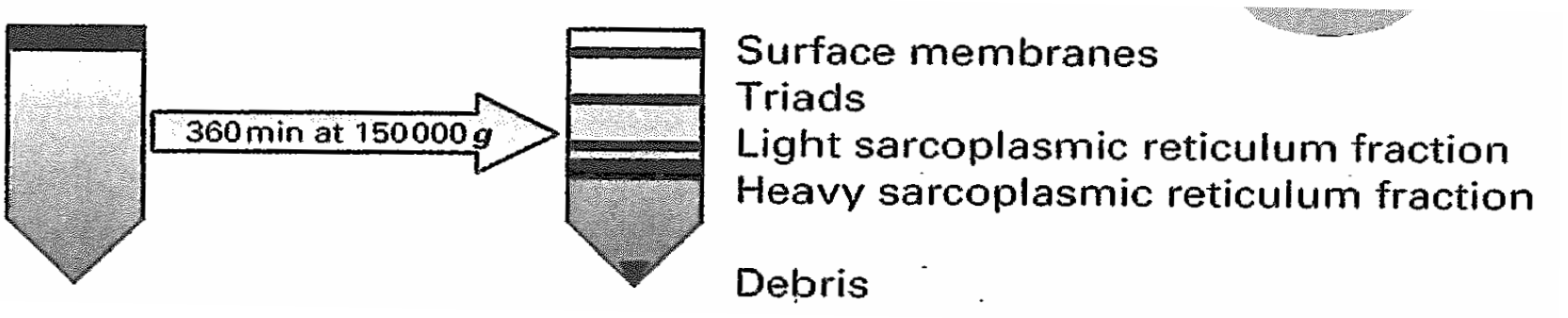




Step 4- Collection of Fractions

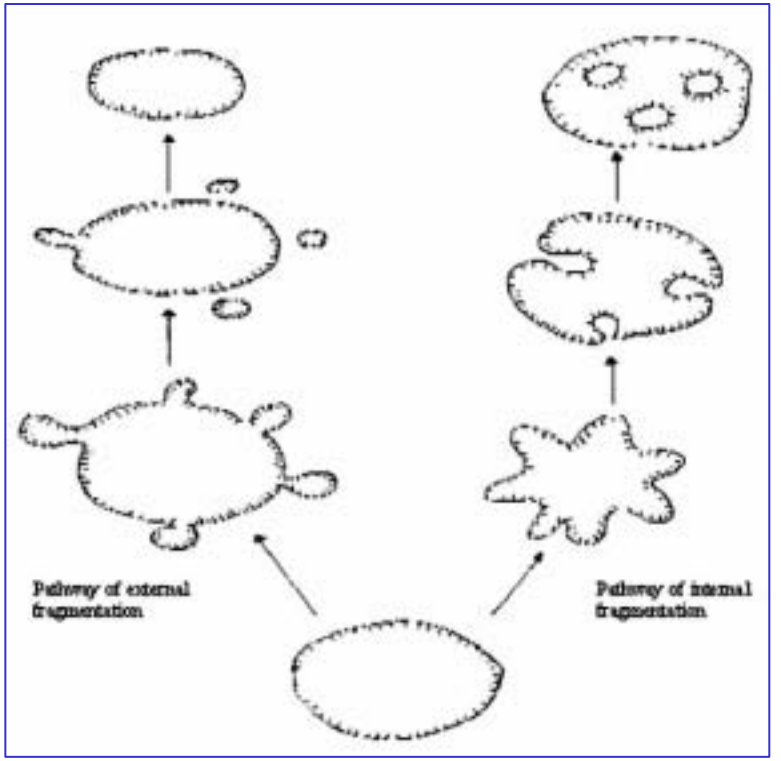


- Manual collection by pipette
- Automatic fraction collector for unstable gradient
- Freezing and slicing



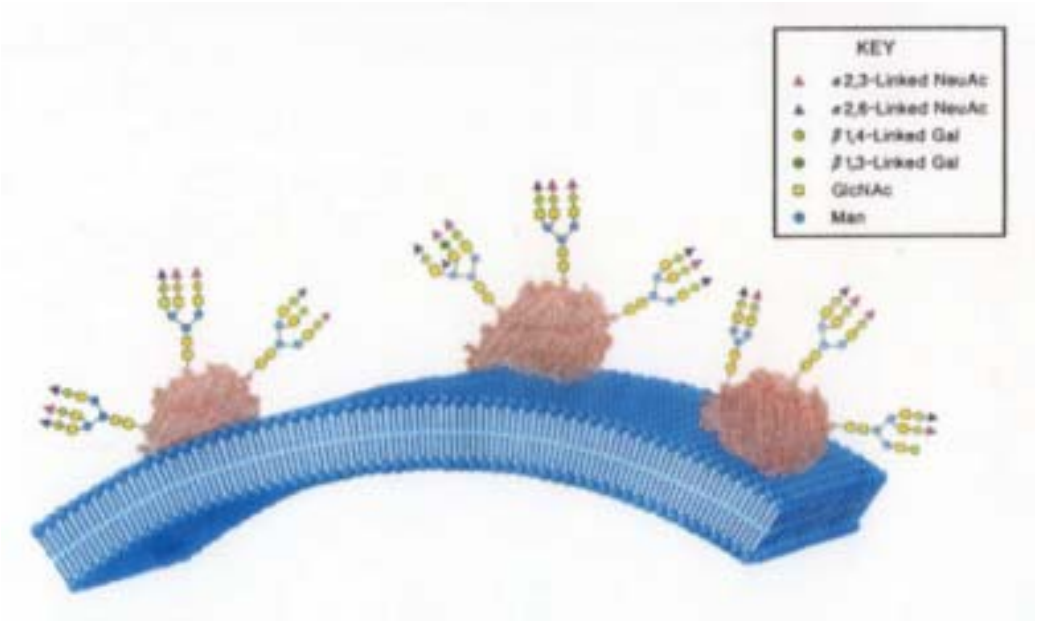
Affinity Purification of Membrane Vesicles (BMB 3.4.5)

- Cross-contamination of vesicular membrane protein
- Inside-out vesicles, right-side-out vesicle, membrane sheet, leaky vesicles
- Smaller vesicles are trapped in large vesicles



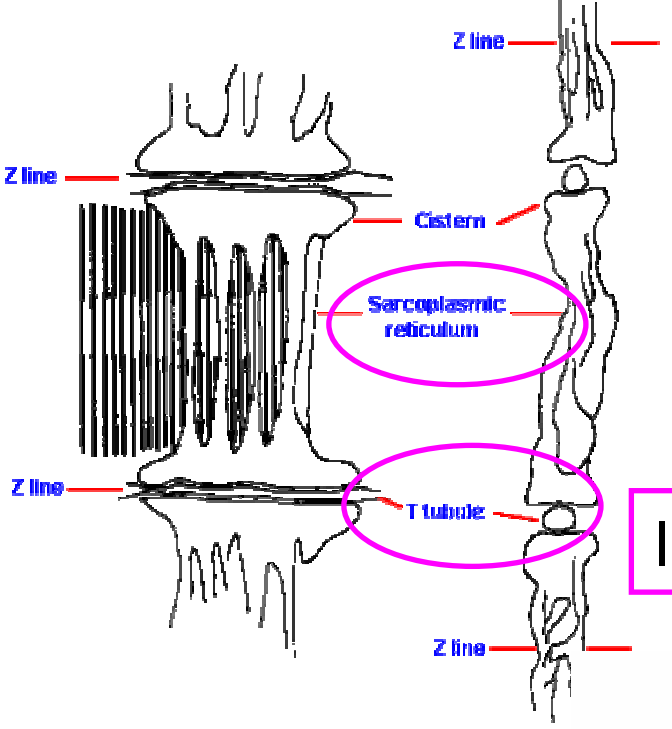
In-side-out (cytoplasmic side out)
Right-side-out (apoplastic side out) vesicles

Lectin Agglutination Method (by Lectin-carbohydrate Interaction)



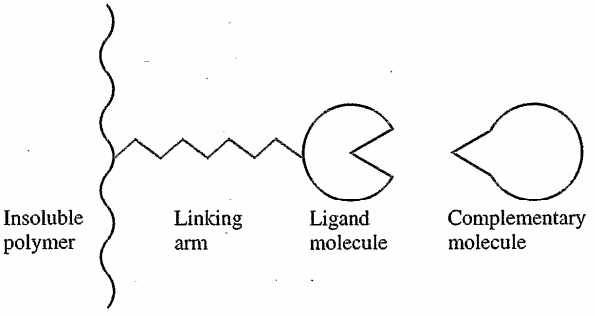
Lectin: protein that interact with carbohydrate

There are many carbohydrates on the surface of cell

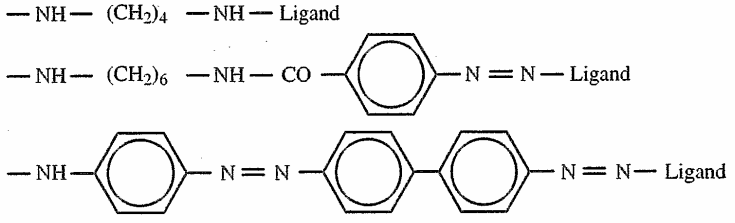


No carbohydrate

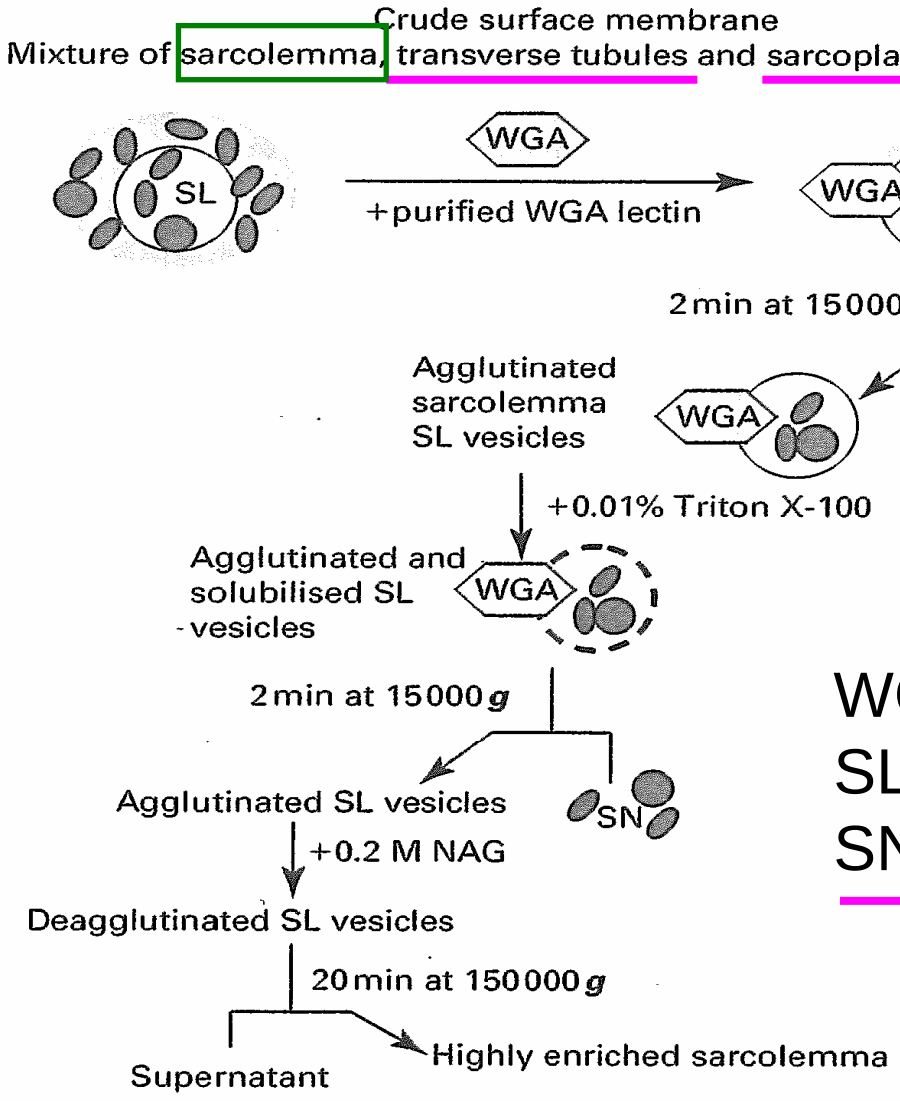
Inside-out: No carbohydrate



SOME LINKING GROUPS



Lectin Agglutination Method



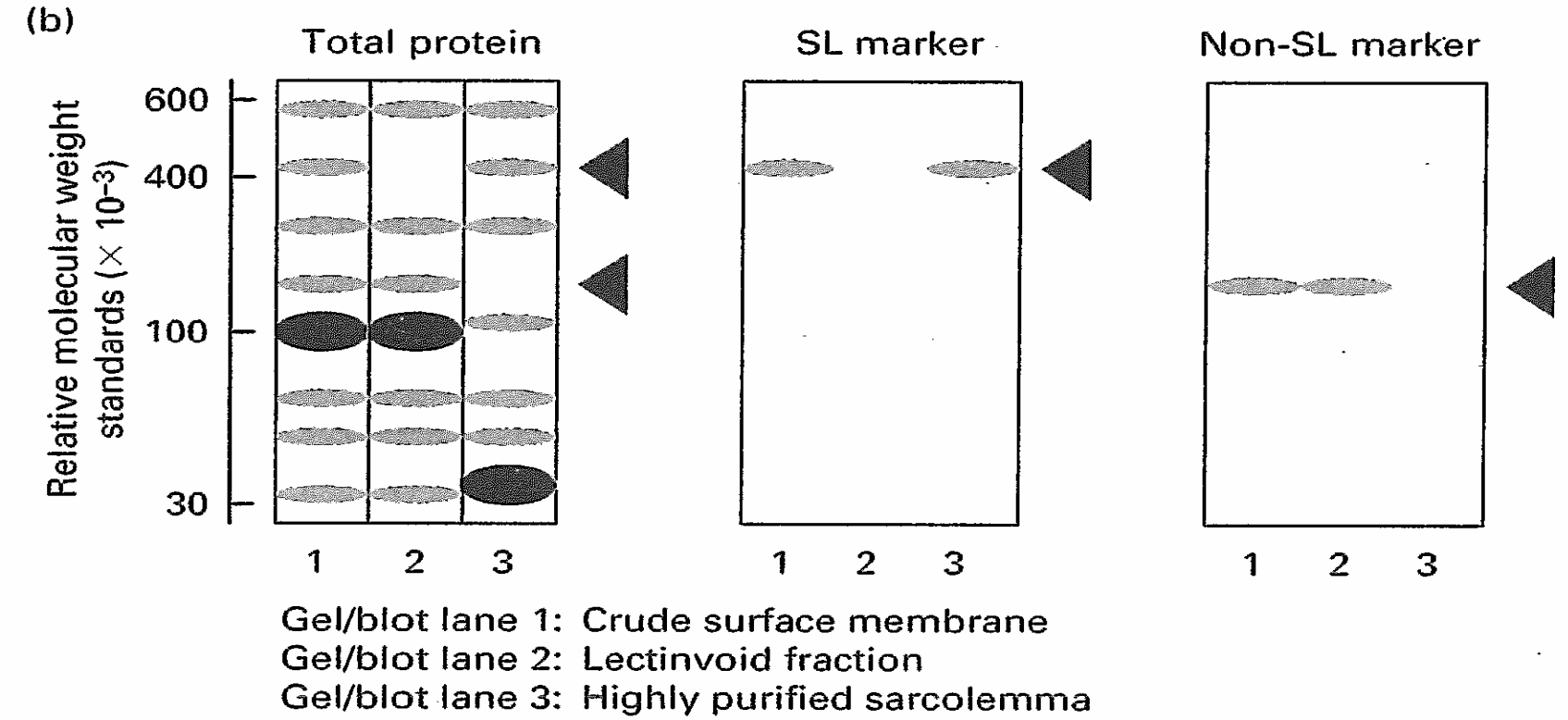
WGA: Wheat germ agglutinin

SL: Sarcolemma

SN: supernatant

No carbohydrate

Immunoblot Analysis for Verification of Different Subcellular Fractions



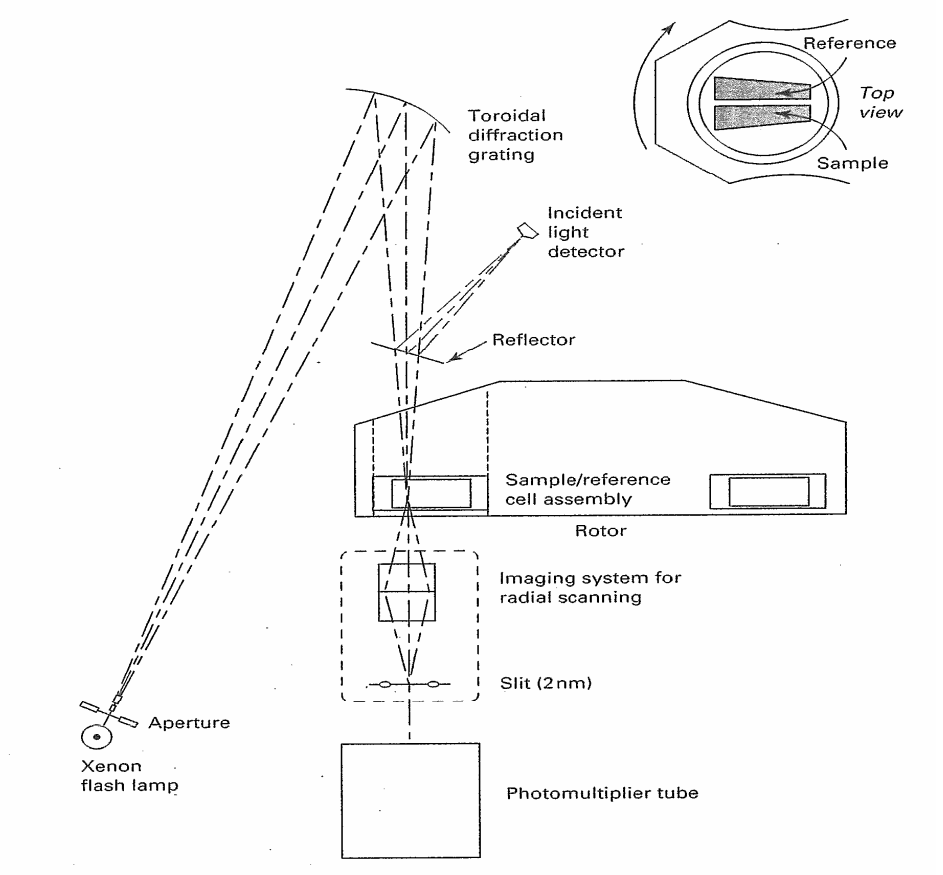
Analytical Ultracentrifugation MBM 3.5.1

An analytical ultracentrifuge spins a rotor at an accurately controlled speed and temperature. The concentration distribution of the sample is determined at known times using absorbance measurements. It can determine:

Continuously monitor the sedimentation process

- Purity of macromole
- Relative molecular mass of solute (within 5% SD)
- Change in relative molecular mass of supermolecular complexes
- Conformational change of protein structure
- Ligand-binding study

Optical System of an Analytical Ultracentrifugation

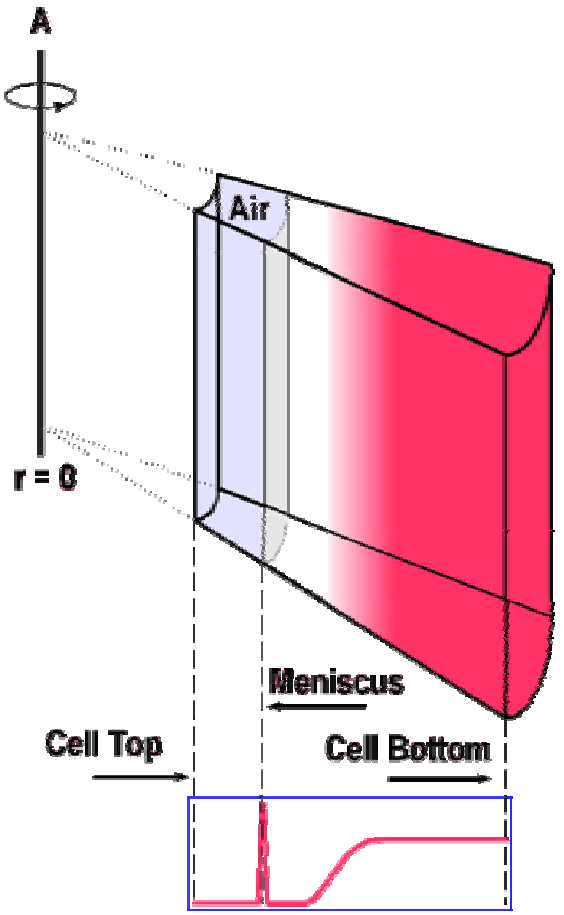


ck to top

·A):

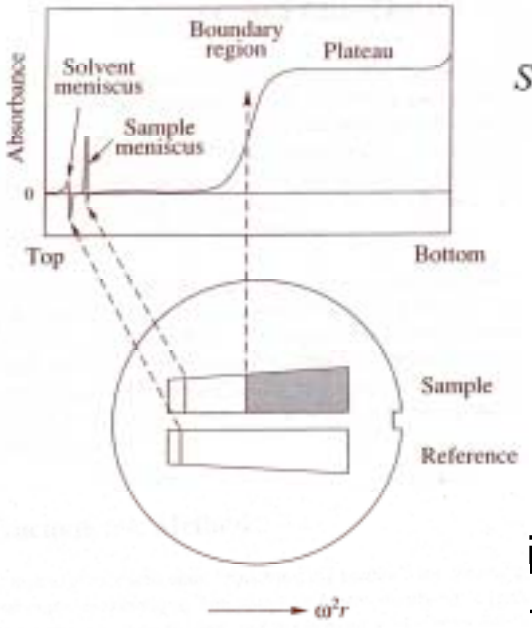
This figure displays a schematic diagram of the Beckman Optima XL-A absorbance system. A high intensity xenon flask lamp allows the use of wavelengths between 190 and 800nm. The lamp is fired briefly as a selected sector passes the detector.

Sedimentation Velocity Method

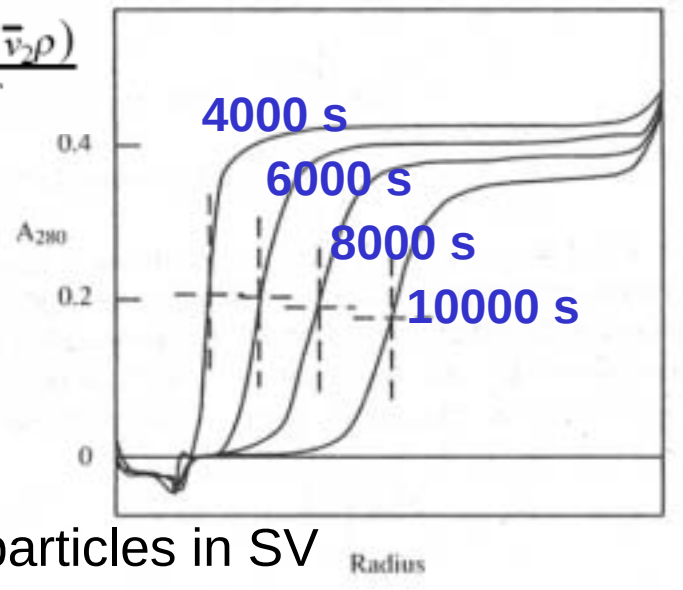


Sedimentation velocity experiments are performed at high speed to overcome the effect of diffusion. For a sedimentation velocity experiment, an initially uniform solution is placed in a cell and a sufficiently high angular velocity is applied to cause rapid sedimentation of solute towards the cell bottom. As a result, there is a depletion of solute near the meniscus, causing a characteristic spectrum as shown in the following figure. A sharp boundary occurs between the depleted region and the sedimenting solute (the plateau)

Determination of Sedimentation Coefficient (*s*)



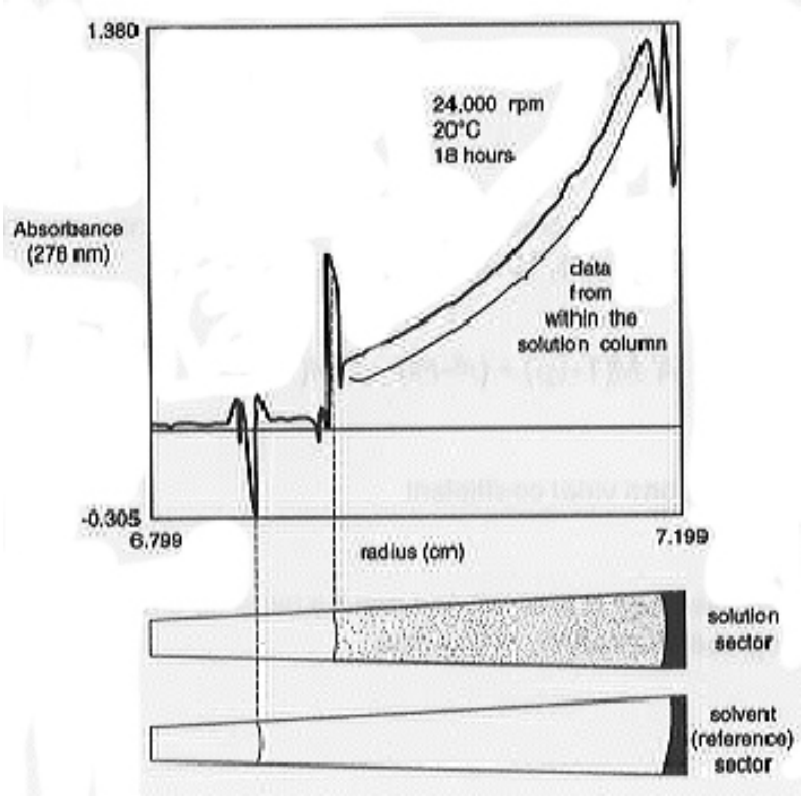
$$S = \frac{v_t}{\omega^2 x} = \frac{m(1-\bar{v}_2\rho)}{f}$$



individual particles in SV
t be resolved, but the rate of movement of the boundary region can be measured. From this, the **sedimentation coefficient (*s*)** can be determined. Remember, *s* depends directly on the **mass** of the solute particles and inversely on the **frictional coefficient**, which is a measure of **size** of the solute particles.

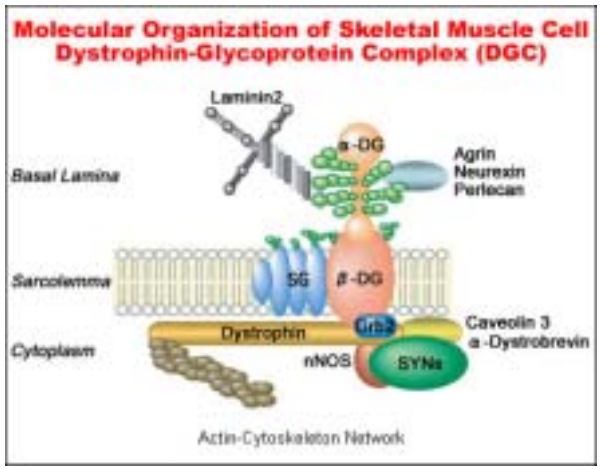
http://www.biochem.vt.edu/bios576/AU/AU_Page.html#au

Sedimentation Equilibrium Methods



- Sedimentation equilibrium experiments have a lower rotor speed than sedimentation velocity experiments. Solute particles do not pellet at the bottom of the cell, but instead the process of diffusion opposes the process of sedimentation until after a period of time, the two opposing forces reach equilibrium and the apparent concentration profile does not change. At equilibrium, the concentration of the solute increases exponentially towards the cell bottom. Each column displays a different absorbance profile, because the concentrations of sample are varied in each.

Sedimentation Analysis of Supramolecular Protein Complex



The binding of ligands may induce conformational changes in subunits of biomolecules, which changes the supramolecular structure of complex.