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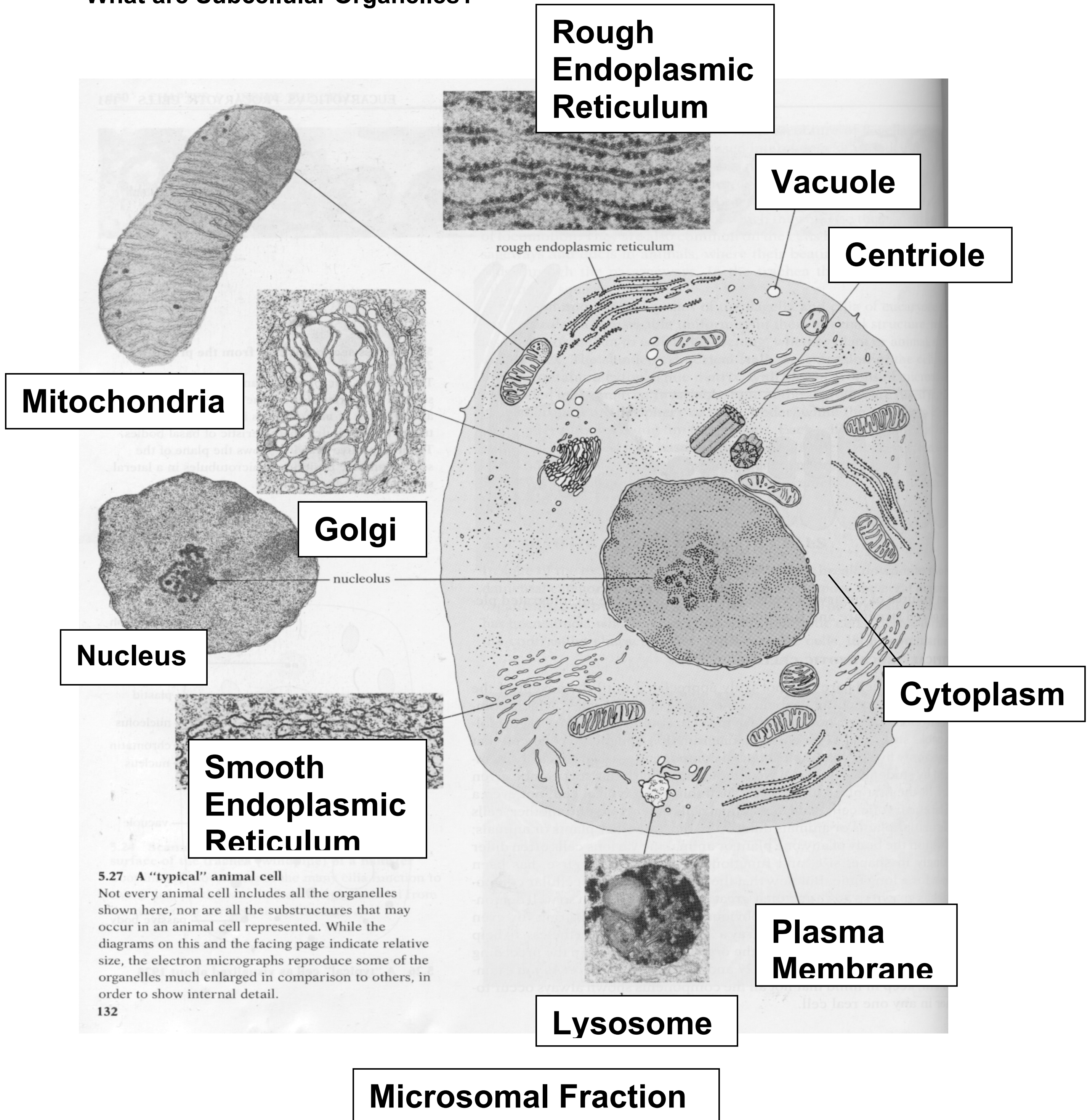
Subcellular Fractionation: Ultracentrifugation

<u>Subcellular Organelles</u> may be separated on the basis of differences in their SIZE, SHAPE and DENSITY using centrifugation procedures.

What are Subcellular Organelles?



Subcellular Fractionation:

Permits the isolation of specific cellular organelles

Step 1: Homogenization
Step 2: Fractionation by Centrifugation
Step 3: Marker Assays

Step 1: Homogenization

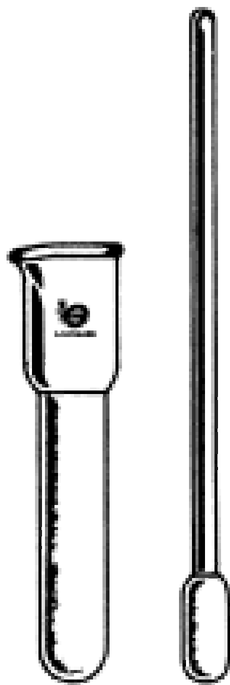
Shearing (disruption) of cells under conditions that prevent deteriorization for isolation of morphologically intact and functionally active organelles or microsomal fractions.

Methods

1. **Grinding: Potter-Elvehjem glass homogenizer (mortar and pestle)**
2. **Cutting: Waring Blender**
3. **Ultrasonic Vibration: Sonication**
4. **High Pressure: French Press**

Glass Homogenizer:

clearance of 0.004" to 0.006"



LG-10650

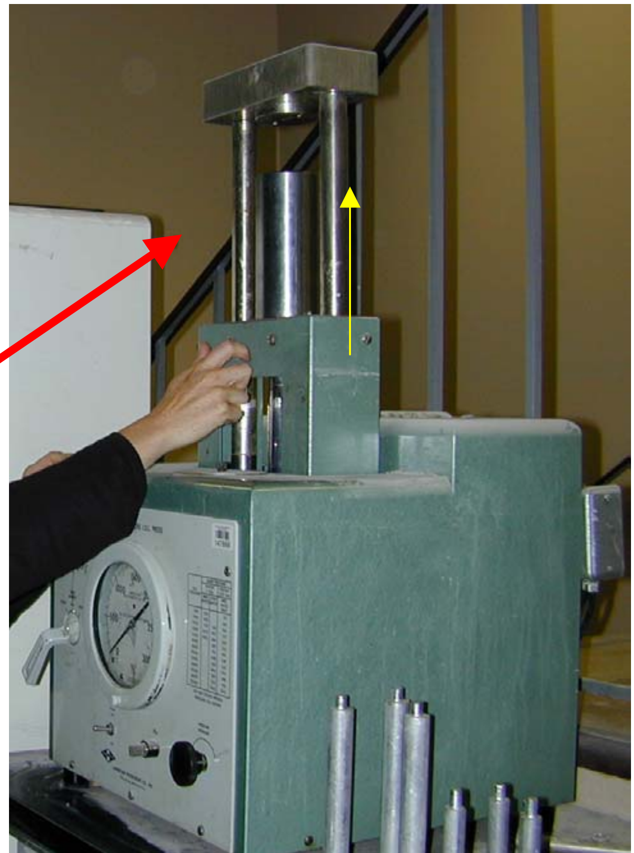
Warring Blender:

Ultra-Sonication:

French Press:

The Press

The Cell



Homogenization Conditions:

- **Low Temperature (4 degrees celcius)**

- **Buffer with pH ~7.0-7.4 and ionic concentration (e.g Tris-Cl pH 7.2, 50mM NaCl)**

In some instances:

- **High Osmolarity (up to 15% Sucrose) - isotonic**

- **Specific Ions ($CaCl_2$, $MgCl_2$) – stabilization**

- **Chelating Agent (EDTA) – protease inhibition**

- **Reducing Agent (mercaptoethanol) – prevents damaging oxidation reactions**

Step 2: Centrifugation

Separation based on differences in SIZE, SHAPE and DENSITY

- **Differential Centrifugation**
- **Density Gradient Centrifugation**

I) Differential Centrifugation:

- **Classical procedure used to isolate different particles by stepwise successive centrifugations at increasing RCF's (Relative Centrifugal Forces).**

- **Basic concept : Large heavy dense particles sediment faster than small light particles**

- **Crude resolution of subcellular fractions**

- **Usually performed prior to Density Gradient Centrifugation**

- **Carried out using Fixed Angle Rotors**

eg.) Crude Isolation of Plant Plasma Membrane by Differential Centrifugation

step i) **Filter homogenate through cheese cloth *or* centrifuge for 20 min. @ 1000g to pellet out nucleii and cell debris.**

step ii) **Centrifuge supernatant from 'a' for 20 min. @ 20,000g to pellet LARGE INTACT organelles including, mitochondria, peroxisome, lysosome, golgi, chloroplast.**

step iii) **Centrifuge supernatant from 'b' for 30 min. @ 80,000g to pellet 'microsomal fraction' including FREE membranes such as:**

plasma membrane,
mitochondrial membrane
golgi membrane,
endoplasmic reticulum membrane.

II) Density Gradient Centrifugation –

- **Used for higher resolution isolation of specific subcellular organelles by ultra-centrifugation through a density gradient**

- **Basic Concept: Particles move until density of medium equals density of particle, this is known as the isopycnic point.**

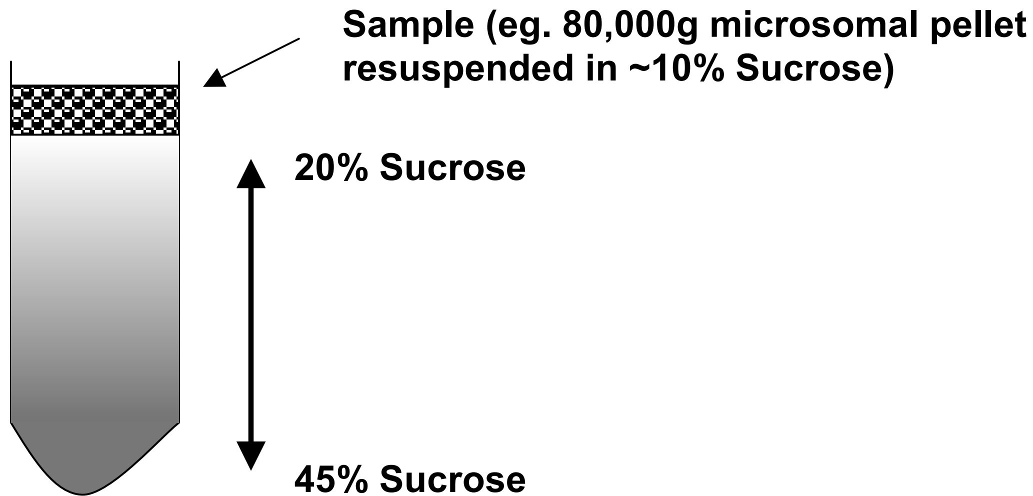
- **Most commonly use buffered sucrose gradients**

- **Carried out in Swinging Bucket Rotors.**

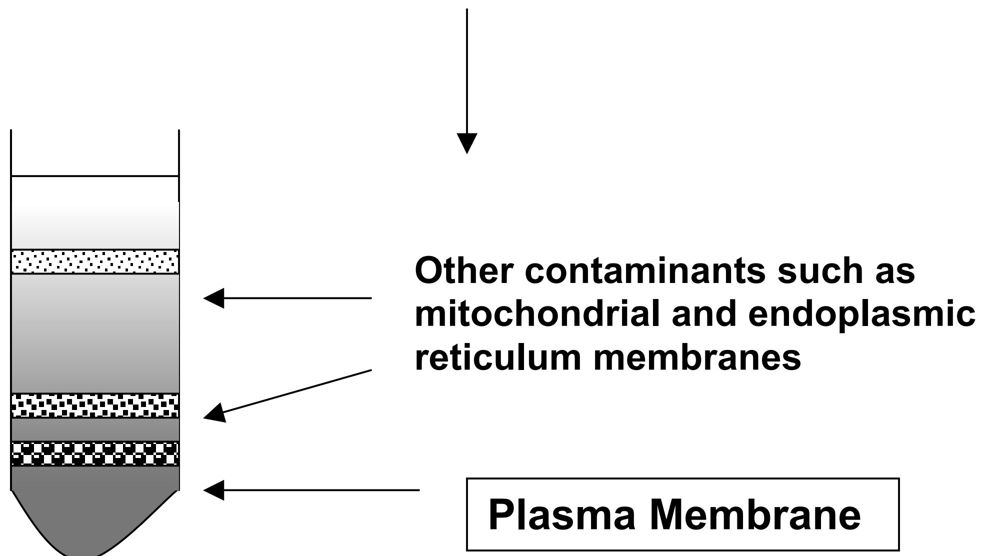
- **Two types of gradients: Continuous and Discontinuous**

A) Continous (Linear) Gradients

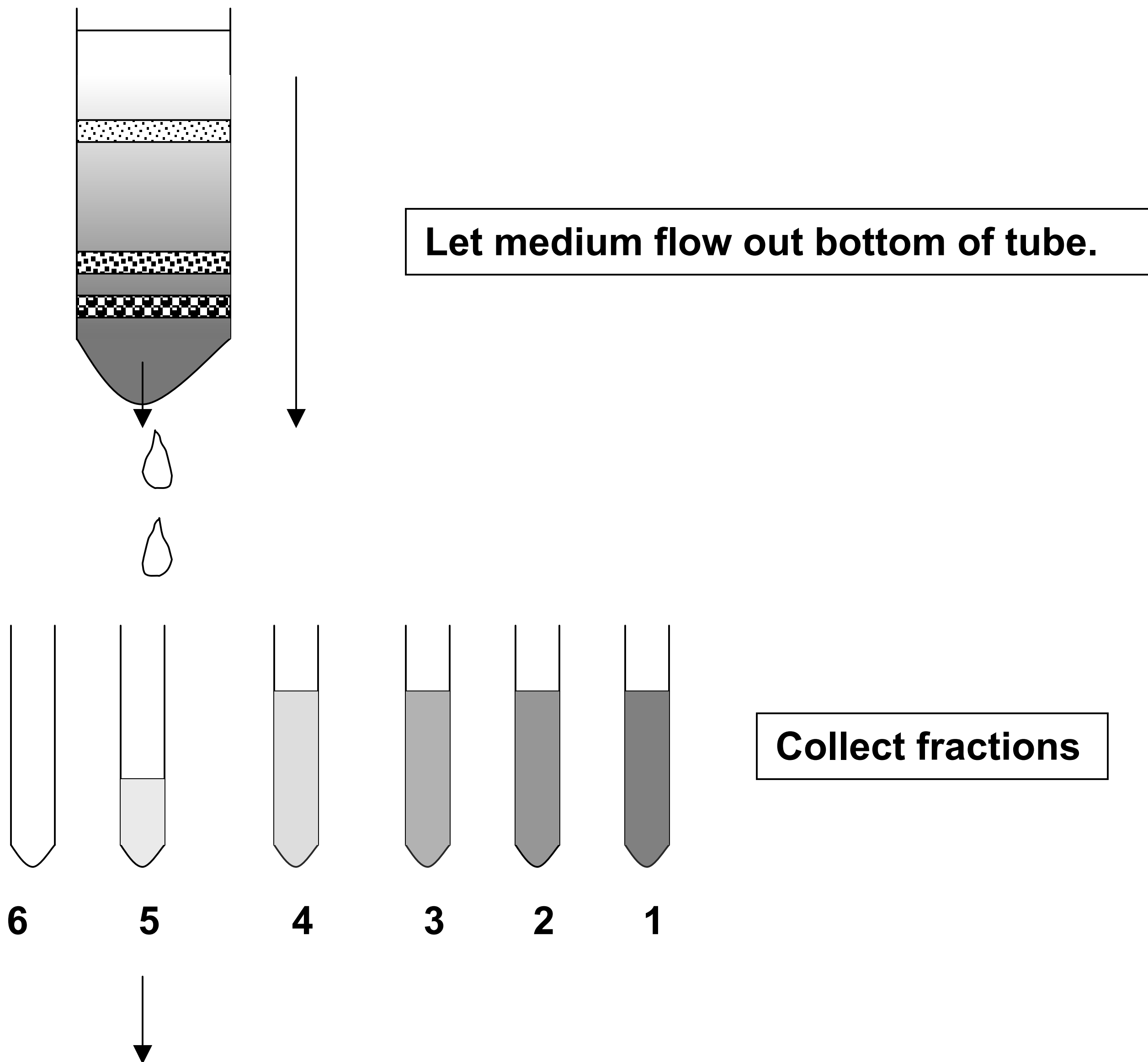
Preliminary determination of isopycnic points



Centrifuge @ 115,000g for 8-15 hours to ensure fractions have reached their isopycnic points.



Determination of isopycnic points of the desired organelle



Determine Sucrose Concentration in each fraction using a refractometer

Assay each fraction for <u>Markers (Step 3)</u> to determine which fraction contains your desired organelle

Step 3: Molecular Marker Assays

Example: Plant Membrane Markers

a) Plasma Membrane Markers

- i) Potassium Stimulated ATPase
- ii) Vanadate-Sensitive ATPase
- iii) Glucan Synthase II
- iv) Cellulase
- v) Naphthylphthalmic Acid Binding

b) Mitochondrial Membrane Markers

- i) NADH cytochrome-C Oxidase
- ii) Azide-Sensitive ATPase

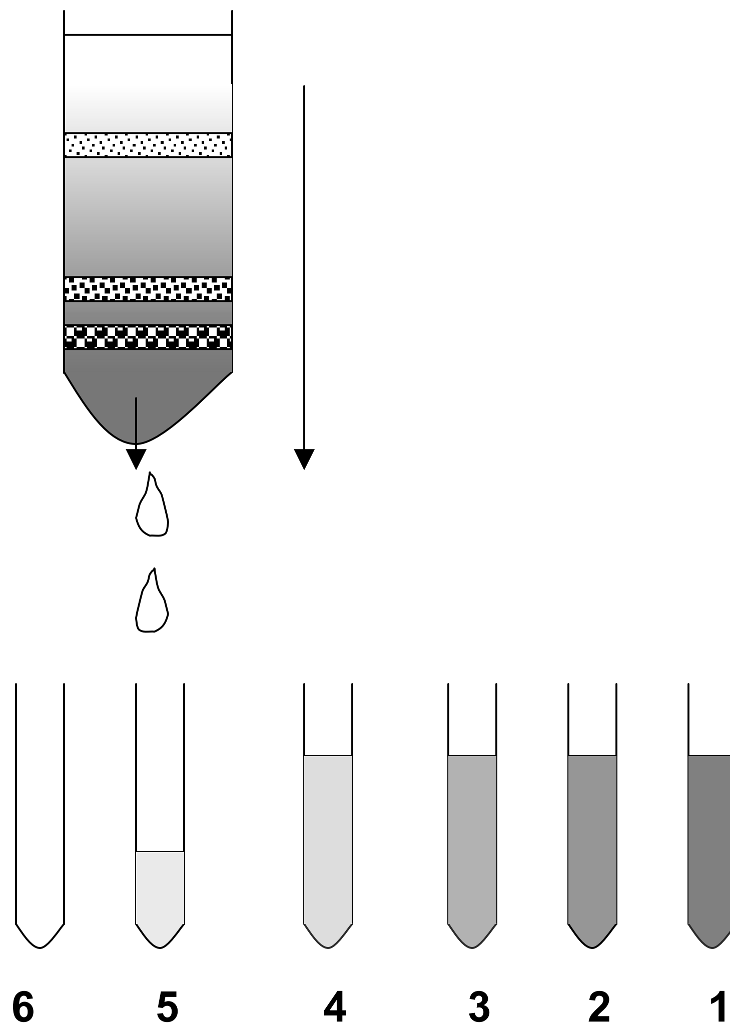
c) Endoplasmic Reticulum Markers

- i) NADH cytochrome-C Reductase

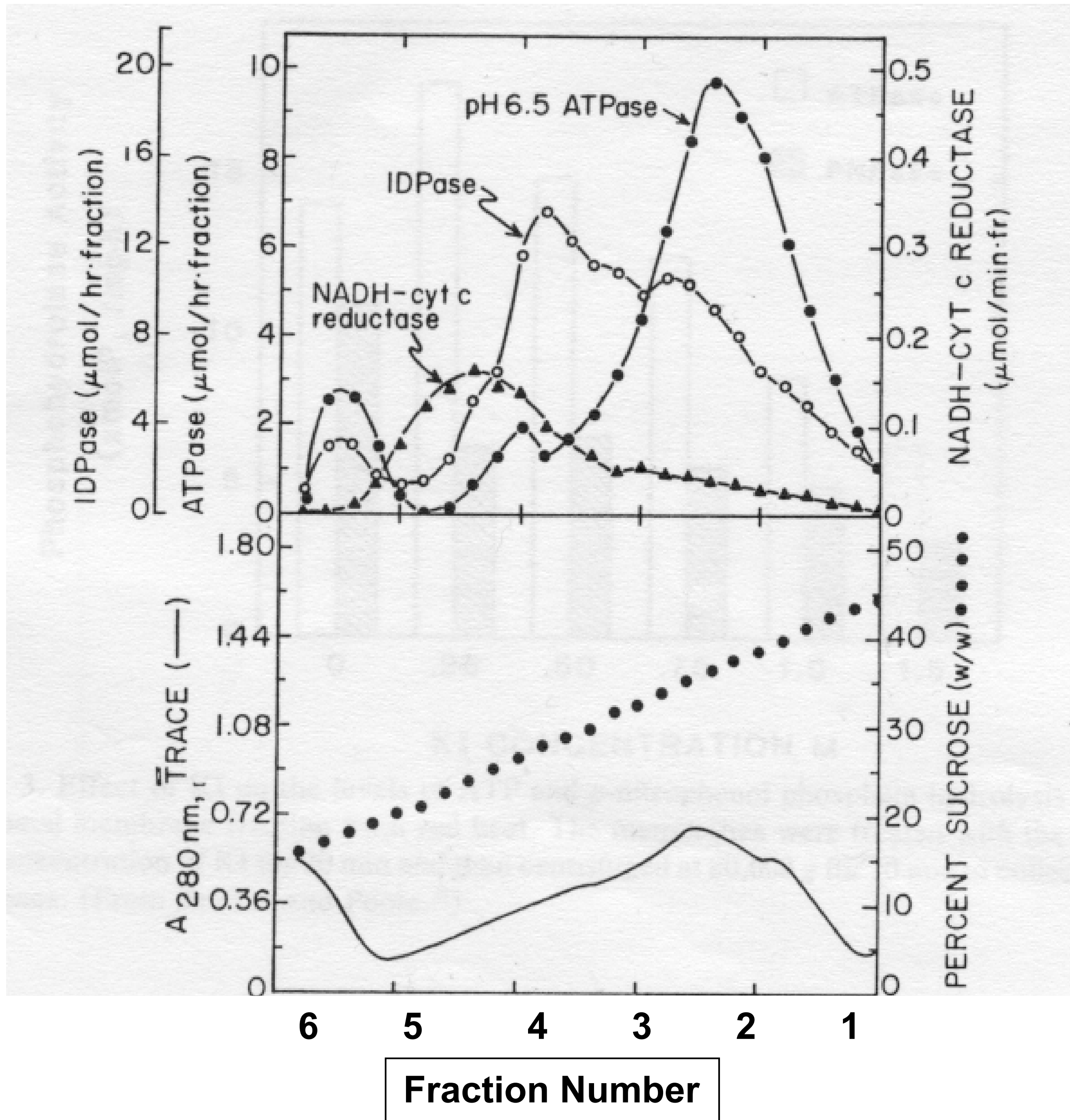
d) Golgi Membrane Markers

- i) Latent IDPase
- ii) Triton-Stimulated UDPase
- iii) Glucan Synthase I

See Hand Out for Details of Assay Conditions!!



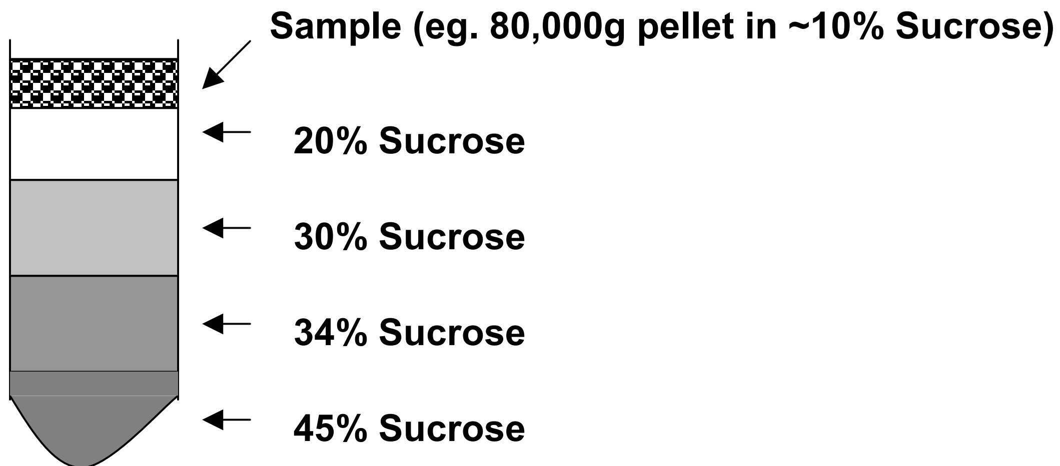
Plot Sucrose Concentrations and Marker Assay Activity for Each Tube:


pH 6.5 ATPase
IDPase
NADH-cyt c reductase
IDPase (µmol/hr·fraction)
ATPase (µmol/hr·fraction)
NADH-CYT c REDUCTASE (µmol/min·fr)
A 280 nm, TRACE (——)
PERCENT SUCROSE (w/w) ● ● ● ●
6
5
4
3
2
1
Fraction Number

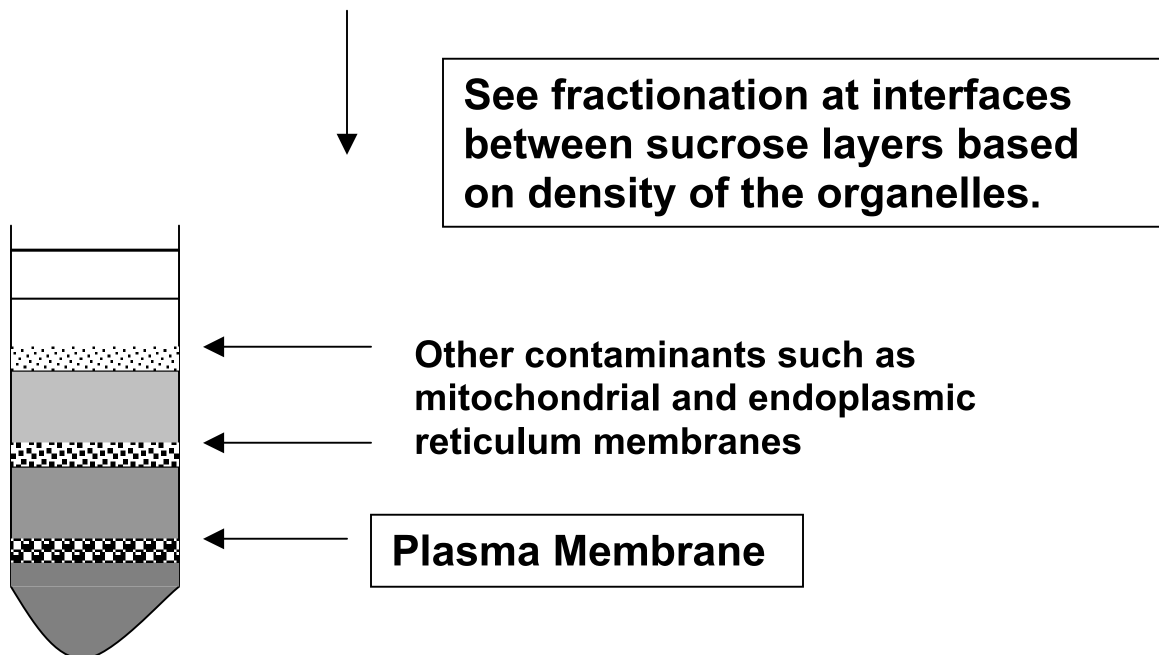
-

B) Discountinuous gradients:

- **For improved resolution of organelles**

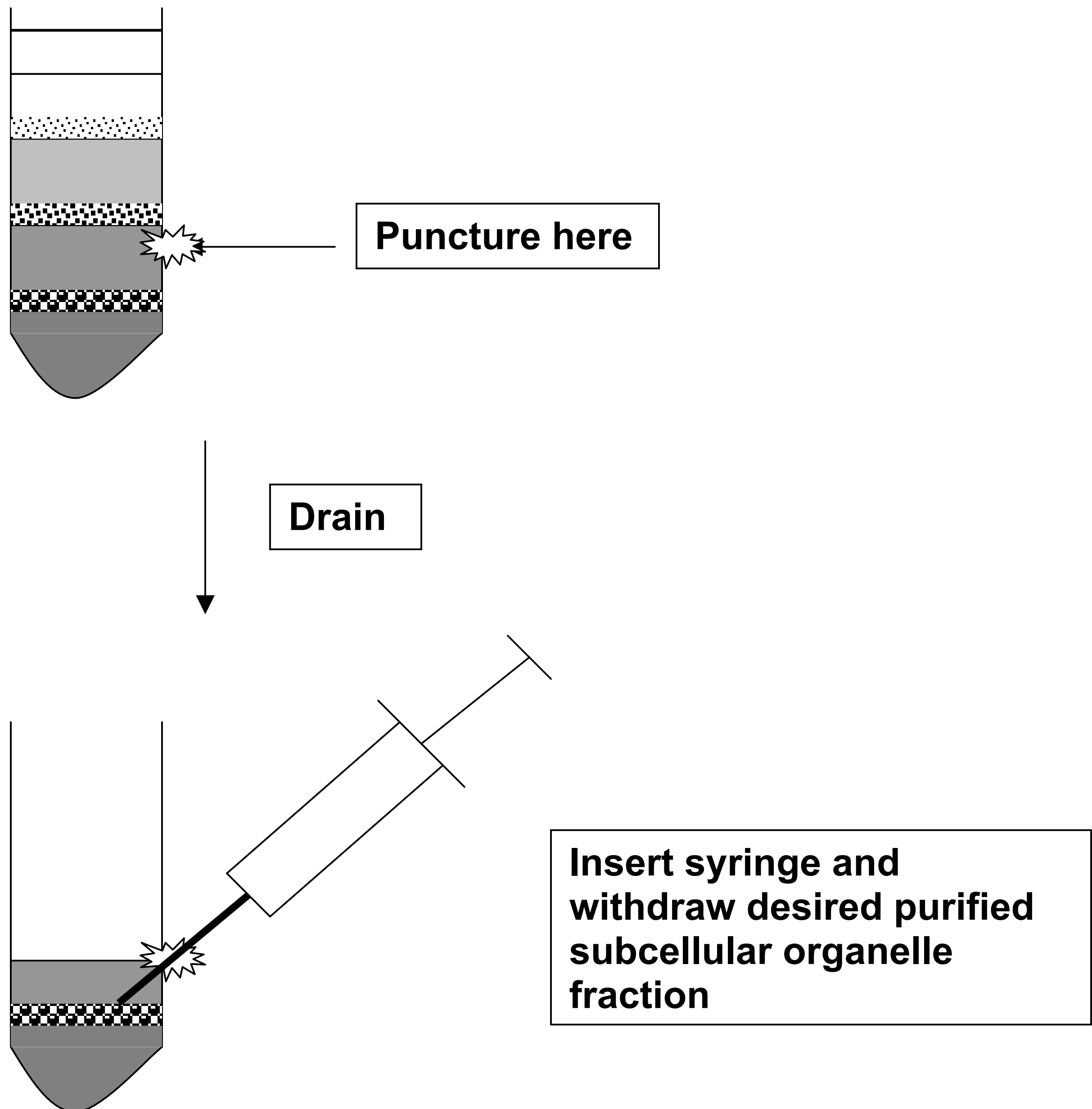


Centrifuge @ 115,000g for 2 hours in Swinging Bucket Rotor



Recovery of sample:

Use a needle to puncture tube 3-5 mm above your desired organelle, and let the medium flow out of the tube



The Physics of Ultra-Centrifugation:

Centrifugation separates particles in a suspension based on differences in size, shape and density that together define their sedimentation coefficient.

The tube containing the suspension of particles is rotated at a high speed, which exerts a centrifugal force directed from the center of the rotor towards the bottom of the tube.

This force acts on the suspended particles pushing them towards the bottom of the tube at a rate determined by the velocity of the spinning rotor (ie the size of the applied centrifugal force) and the particle's sedimentation coefficient. This rate is known as the 'sedimentation rate'.

Centrifugal Force:

Centrifugal Force = G

Angular velocity (radians/sec) = ω

Radius (distance from center of spinning) = r

$$G = \omega^2 r \quad (1)$$

$$\omega = \frac{[(2\pi(\text{rev/min})]}{60} \quad (2)$$

Substitute Formula (2) into Formula (1):

$$G = \frac{[4\pi^2(\text{rev/min})^2 \times r]}{3600} \quad (3)$$

Centrifugal Force 'G' is more commonly expressed as the Relative Centrifugal Force (RCF) in multiples of the earth's gravitational field 'g'.

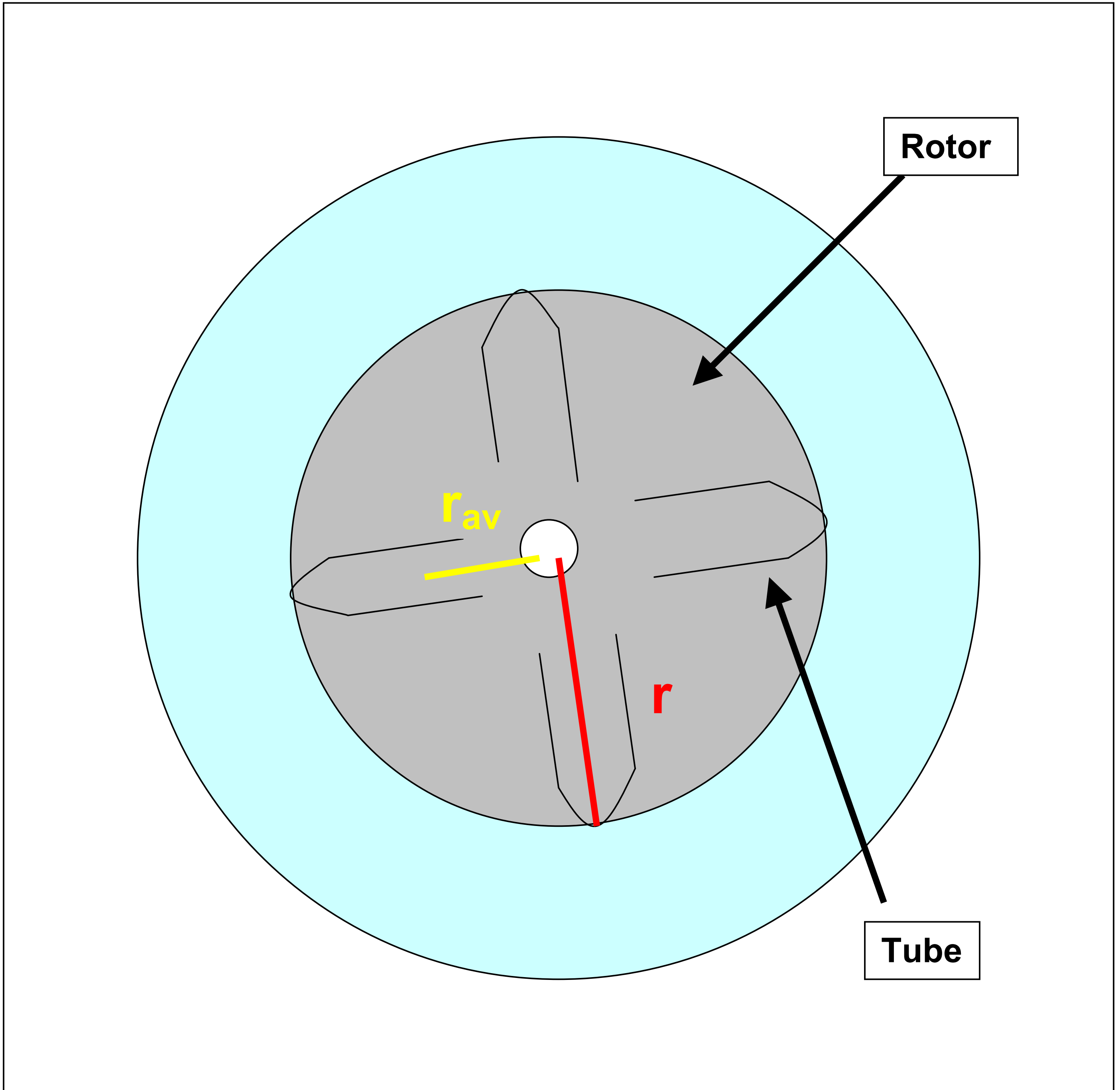
$$RCF = G/g \quad (4)$$

Earth's Gravitational Field = g = 981cm/sec^2
Substituting Formula (3) into (4):

$$RCF = \frac{[4\pi^2(rev/min)^2 \times r]}{3600 \times 981} \quad (5)$$

$$RCF = 1.119x10^{-5}(rev/min)^2 \times r_{av} \quad (6)$$

RCF is a ratio of two forces and has no units. However trditionally the numerical value of RCF is followed with the symbol 'g'.



In practice 'r' = r_{av}; half the distance from the center of the axis of rotation to the distal end of the centrifuge tube

Example Problem:

What is the relative centrifugal force experienced by a tube placed in a rotor with a radius of 25 centimeters spinning at 25,000 revolutions per minute?

Using Formula (6):

$$RCF = 1.119 \times 10^{-5} (rev/min)^2 \times r_{av}$$

$$RCF = 1.119 \times 10^{-5} (25{,}000)^2 \times 12.5$$

$$RCF = 87{,}344g$$

Rate of Sedimentation:

The rate of sedimentation in a centrifugal field is defined as follows:

$$\frac{dr}{dt} = \frac{M(1-\bar{\nu}\rho)}{N_A f}\,\omega^2 r \qquad (7)$$

r = radius at which the organelle is located
t = time
M = molecular weight
$\bar{\nu}$ = partial specific volume of the molecule; inverse of the density
ρ = density of the solvent
***f* = translational frictional coefficient**
ω = angular velocity
N_A = Avagadro's number

This equation simply states that the rate of sedimentation of a given particle is proportional to the molecular weight (M), the centrifugal force ($\omega^2 r$), and the density difference between the particle and the solvent ($1-\nu\rho$), and inversely proportional to the frictional coefficient.

When a particle has the same density as the solvent: $\bar{\nu} = 1/\rho$ the particle will not sediment (isopycnic point).

Example: particle density = 5

solvent density ρ = 5

therefore $\nu = \overline{1}/5$

$\bar{\nu}\rho = 1/5 \times 5 = 1$

$$\frac{dr}{dt} = \frac{M\,(1-\nu\rho)}{N_A f}\,\omega^2 r \qquad (7)$$

$$\frac{dr}{dt} = \frac{M\,(1-1)}{N_A f}\,\omega^2 r = 0$$

If a particle is <u>lighter</u> (less dense) than the solvent:

$\bar{\nu} > 1/\rho$ **the particle will rise.**

<u>Example</u>: **particle density = 2**

solvent density $\rho = 5$

therefore $\bar{\nu} = 1/2$

$\bar{\nu}\rho = 1/2 \times 5 = 5/2 = 2.5$

$$\frac{dr}{dt} = \frac{M\,(1-2.5)}{N_A f}\,\omega^2 r$$

$$\frac{dr}{dt} = \frac{M\,(-1.5)}{N_A f}\,\omega^2 r = \text{negative}$$

Rate of sedimentation to the bottom of the tube is negative.
The particle will accelerate up the tube.

If a particle is heavier (more dense) than the solvent:

$\bar{\nu} < 1/\rho$ **the particle will sink.**

Example:

particle density = 10

solvent density ρ = 5

therefore $\bar{\nu}$ = 1/10

$\bar{\nu}\rho$ **= 1/10 x 5 = 5/10 = 0.5**

$$\frac{dr}{dt} = \frac{M\,(1-0.5)}{N_A f}\,\omega^2 r$$

$$\frac{dr}{dt} = \frac{M\,(0.5)}{N_A f}\,\omega^2 r \quad = \text{positive}$$

Rate of sedimentation to the bottom of the tube is positive.
The particle will accelerate down the tube.

Sedimentation Coefficient (S):

The sedimentation coefficient is defined as follows:

$$S = \frac{dr}{dt} (1/ \omega^2 r) \qquad (8)$$

Rearrangement of Formula (7):

$$\frac{dr}{dt} (1/\omega^2 r) = \frac{M (1-\bar{\nu}\rho)}{N_A f} \qquad (9)$$

Substitution of Formula (8) into Formula (9):

$$S = \frac{M (1-\bar{\nu}\rho)}{N_A f} \qquad (10)$$

This is know as the <u>Svedberg equation</u> and is usually expressed in Svedberg units, S (= 10^{-13} second).

This equation indicates that 'S' is dependent upon the molecular weight, the density and the frictional coefficient.

Real Life Example Problem:

The bacterial ribosome is made up of two subunits:

50S and 30S

Which of these subunits will sediment faster in a linear density gradient experiment? Explain.

The 50S subunit will sediment faster.

'50S' and '30S' represent the subunits' sedimentation coefficients. According to the formula

$$S = \frac{dr}{dt}\,(1/\,\omega^2 r)$$

the sedimentation coefficient is directly proportional to the rate of sedimentation. So the greater the S value, the faster it will sediment.