CENTIFUGES AND CENTRIFUGATION

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- **Centrifuges, tubes etc – types and general use**
- **Types of centrifugation**
- **Centrifuges – safety of use**

Types of Centrifuges

1. Small Benchtop – with or without refrigeration
   - slow speed (eg up to 4000 RPM)
   - common in clinical labs (blood/plasma/serum separation)
   - can take approx (up to) 100 tubes, depending on diameter
2. Microcentrifuges ("microfuge", "Eppendorf")
   - take tubes of small vols (up to 2 mL)
   - very common in biochemistry/molecular biology/ biological labs
   - can generate forces up to ~15,000 x g
   - with or without refrigeration

Types of Centrifuges continued

3. High Speed centrifuges
   - 15,000 – 20,000 RPM
   - large sample capacity depending on rotor
   - normally refrigerated
   - research applications
4. Ultracentrifuges → 65,000 RPM (100,000's x g)
   - limited lifetime
   - expensive
   - require special rotors
   - care in use – balance critical!
   - research applications

Centrifuge Containers

- Bottles – large vols
- Screw-top / snap-top tubes
- Open tubes
- Flip-Tops ("Eppendorfs")
- May be reusable or disposable, depending on application

Cautions Regarding Containers

- ? solvent compatible
- ? sample compatible (eg binding of sample components to tube material)
- aluminium seals – check for corrosion
  - may need correct torsion to seal
  - susceptible to NaOH, $HClO_3$
- cleanliness (contamination) – disposble vs reusable
- aerosol formation with flip-tops

Care and Maintenance

- log book for centrifuge
- regular inspection by authorised personnel (metal fatigue)
- oiling, bearings, swivel points
- cleanliness
- seals of centrifuge and containers – aerosols
- ENSURE TUBES ARE BALANCED (esp. ultracentrifuges)

Specimen Recovery

- Recovery of pellet or supernatant most common
- Sealed ultracentrifuge tubes – collect wanted band:
  - with needle and syringe
  - by freezing and slicing
  - draining off fractions etc.

Special Application

Ultrafiltration – through molecular weight cut-off membranes

- results in protein- or lipid-free filtrates
- eg: drug to plasma protein binding studies:
  1. measure total drug in plasma sample
  2. spin plasma through ultrafiltration membrane to get protein-free supernatant
  3. measure drug in supernatant – "free" or unbound drug
  4. express result as % bound
- traditionally done with dialysis and radiolabelled drug
- may need very sensitive assay to measure drug in supernatant if drug is highly bound

Types of Centrifugation

1. Differential Pelleting (differential centrifugation)

- **Most common – separates according to size**
- **Material initially uniformly distributed in the solution**
- **During spin, particles move with varying velocities down tube**
- **After spin, pellet contains larger to smaller particles (ususlly mixture)**
- **Supernatant = liquid + most slowly sedimenting component**
- **Pellet can be washed and respun (reduced yield)**
- **If material not cleanly pelleted (smeared; fixed angle rotor), then: - acceleration too rapid**
  - sample too concentrated

Differential Pelleting - Diagrammatic

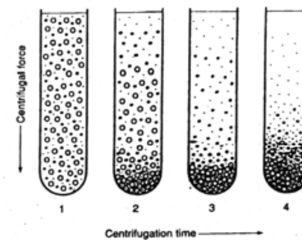
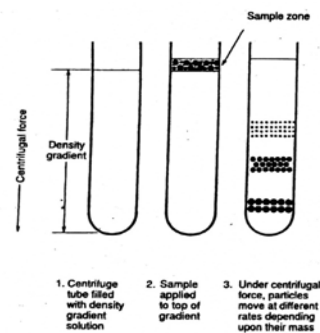


Figure 2. Diagrammatic representation of the fractionation of particles by differential pelleting.

2. Rate-Zonal Density-Gradient Centrifugation

- Separates particles on basis of relative velocities
- Tube filled with inert liquid of varying density – pre-formed gradient within tube
- Sample layered onto top of liquid in tube (tradeoff: sample capacity greatly reduced)
- Gradient unchanged by centrifugation
- With centrifugation, faster-sedimenting particles in sample move ahead of slower ones
- i.e. sample separated as zones in the gradient
- Swing-out rotors preferred

Rate-Zonal Centrifugation - Diagrammatic



Application of Rate-Zonal (Density Gradient) Centrifugation: Separation of cellular organelles in continuous sucrose gradient

Organelle	P (g mL-1)
Nuclei	>130
Plasma membrane sheets	1.14 – 1.19
Mitochondria	1.17 – 1.21
Lysosomes	1.19 – 1.21
Peroxisomes	1.18 – 1.23
Vesicles	1.06 – 1.26

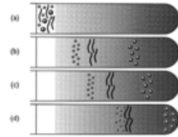


Figure 1.5

Fractionation of nuclei, plasma membrane and mitochondria in a continuous sucrose gradient. (a) Suspended nuclear pellet layered on top of gradient. During centrifugation at 6000 g for 40 min (b,c) nuclei (large circles), plasma membranes (lines) and mitochondria (small circles) sediment at rates proportional to their sizes. If centrifugation is continued for 2 h (d) the nuclei pellet and the mitochondria and plasma membranes band at the same buoyant density.

3. Isopycnic Density-Gradient Centrifugation

- Sample loaded into tube with gradient-forming solution (on top of orbelow pre-formed gradient, or mixed in with self-forming gradient)
- Particles move to point where their bouyand density equals that part of gradient and form bands
- "true" equilibrium procedure – depends on bouyant densities, not velocities
- eg: CsCl, NaI gradients for macromolecules and nucleotides – "self-forming" gradients under centrifugal force
- Density barrier centrifugation: if a single-step gradient is used to block the path of a particular particle in mixture

Isopycnic Centrifugation - Diagrammatic

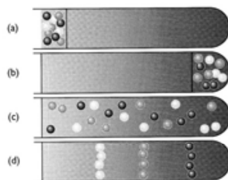


Figure 1.6

Buoyant density separation. Sample may be placed on top (a), beneath (b) or throughout density gradient (c). If centrifugation is carried out for a sufficient time, the particles will band at the same positions (d) irrespective of their starting point.

Choice of Material for Forming Gradients

1. Stable, soluble
2. Minimum osmotic effect, minimum change to ionic strength and pH
3. Totally inert toward biological materials (i.e. no affect on physical or chemiacl compositions)
4. Sterilizable
5. Not hydrated in aqeous solutions
6. No interference to assay
7. No interference to assay
8. Readily and completely separable from fractionated particles
9. Readily, obtainable, inexpensive or recoverable
10. Chemical, physical and thermodynamic properties known (esp. in quantitative work)

No one material which meets all criteria so need to choose carefully

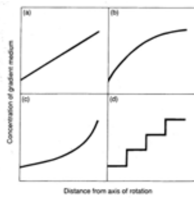
Examples of Gradient Materials

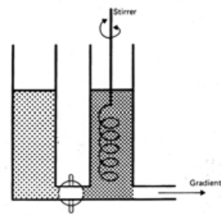
- Simple sugars – sucrose, sorbitol, glycerol
- Polysaccharides – Ficoll, dextran, glycogen
- Proteins – bovine serum albumin
- Deuterium oxide
- Inorganic salts – CsCl, Cs2So4
- Iodinated organic compounds – metrizamide, Renographin
- Colloidal silica – eg Percoll (15nm silica coated with polyvinylpyrrolidone)
- Solvents other than water – DMSO, formamide
- Mixtures of the above

Table 3. Physical properties and applications of density gradient materials

Material	Mol. wt	Max density (g/ml)	Ionic strength	Viscosity[a]	Osmolarity	Typical applications
Inorganic salts						
Caesium chloride	169	1.9	high	+	high	Nucleic acids and nucleoproteins
Caesium sulphate	362	2.0	high	+	high	
CsTFA[b]	246	2.6	high	+	high	Nucleic acids
Rubidium chloride	121	1.5	high	+	high	Proteins
Sodium bromide	103	1.5	high	+	high	Lipoproteins
Sodium iodide	150	1.9	high	+	high	Nucleic acids
Osmotically active compounds						
Sucrose	342	1.3	non-ionic	+ +	mod.	Very many
Sorbitol	182	1.26	non-ionic	+ +	mod.	Cells and membranes
Glycerol	92	1.26	non-ionic	+ + +	high	Membranes Proteins Nuclei
Iodinated compounds						
Urografin	614	1.45	mod.	+	mod.	Cells
Metrizamide	789	1.46	non-ionic	+ +	low	Many
Nycodenz	821	1.45	non-ionic	+ +	low	Many
Polymeric compounds						
Ficoll	400 000	1.23	non-ionic	+ + +	low	Membranes and cells
Dextran	50–500 000	1.05	non-ionic	+ + +	low	Membranes and cells
Bovine serum albumin	69 000	1.12	low	+ + +	low	Cells
Colloidal silica						
Ludox	–	1.2	low	+	low	Cells
Percoll	–	1.23	low	+	low	Cells and organelles

"Shapes" of Gradients





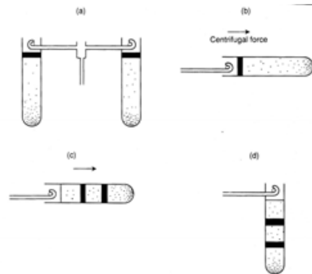
Simple Gradient Mixer

Types of Rotors

1. Swing-out (swinging bucket)

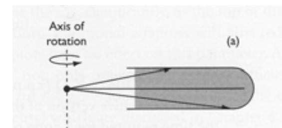
- Common in low speed centrifuges
- Also high speed, ultracentrifuges
- Tubes accommodated in a pivoted bucket which rotates from a vertical to a horizontal position during acceleration
- Bucket returns to vertical as centrifuge decelerates
- Meniscus of sample always remains at right angles to axis of tube
- Six-place rotor (6 buckets) most useful – can spin 2,3,4 or 6 samples (or sets of samples)

Swinging Bucket Rotor – cont'd



Pellet formation in Swinging Bucket Rotors

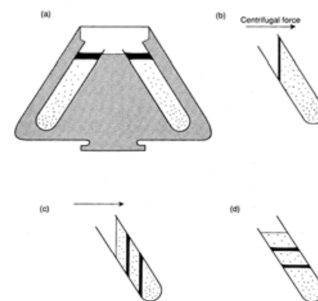
- Pelleted material symetrically distributed in a hemisperical section at bottom of tube
- Only particles in bottom of tube which move directly to bottom
- Other particles move first to wall of tube, then towards bottom



2. Fixed-Angle Rotors

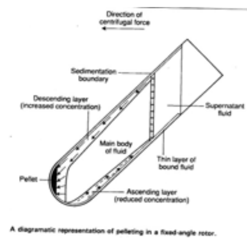
- Tubes in pocket at fixed angle in rotor
- Angle 10 to 50 degrees from vertical – at rest and during spin
- Use up to 600,000 x g

Fixed-angle rotors cont'd



Pellet formation in Fixed-Angle Rotors

- Particles migrate to wall before moving towards bottom
- Pellets always asymetrically distributed toward the outer aspect of the bottom of the tube

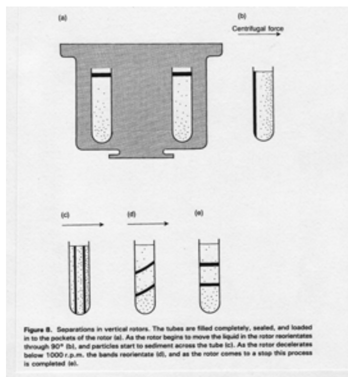


A diagramatic representation of pelleting in a fixed-angle rotor.

3. Vertical Rotors

- First introduced in 1970’s – high-speed and ultracentrifuges
- Solution re-orientates below 800 rpm, no disruption to gradient
- Good for isopycnic and rate-zonal centrifugation
- Not used for pelleting – pellet would be along length of tube and would fall off as liquid decanted
- Also – “near-vertical” rotors – tube angle = 8 degrees

Vertical Rotors cont’d



Rotor Parameters

- Rotors generally described by their type, sample-volume capacity, number of tubes (of maximum volume) and maximum speed
- RCF: Relative Centrifugal Force
  - measured at 3 points along tube
- Can relate RCF to speed (RPM)

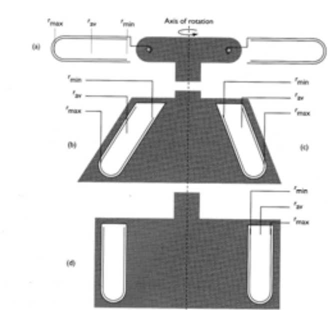


Figure 2.2
Comparison of τ values of rotors. Swinging-bucket (a), fixed-angle (b), near-vertical (c) and vertical (d) rotors.

Practical Applications of Centrifugation

- Purification of mammalian cells
- Fractionation of subcellular organelles (including membranes / membrane fractions)
- Fractionation of membrane vesicles

Membranes and Cellular Organelles

Why Purify?

- Provide reasonable amounts of membranes or organelles with known properties

eg:

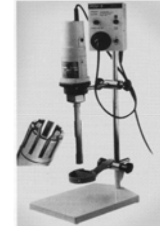
- – membranes: binding experiments – require pure preps
- – microsomes — eg CYP45O, drug metb’n
- – mitochondria
- – peroxisomes
- – bacterially grown proteins

Three Stages of Purification:
1. Cell disruption
2. Separation of subcellular components
3. Identification, yield assessment

(1) Cell Disruption:
- controlled, uniform breakage
- choose method → 90% disruption (confirm)
- physical (mechanical or liquid) force, compared with chemical methods which may damage membranes etc
- organelles intact, dispersed
- conversion of ER etc into sealed vesicles → "right-side-out"

Techniques for Cell Disruption:

(i) Potter-Elvehjem (teflon in glass), Dounce (glass in glass) Homogenisers
- liquid sheer, clearance 0.07 – 0.13 mm
- 5-10g soft tissue/100 mL medium
- cool on ice
- record number of strokes, speed etc eg 8-10 strokes @ 1500 rpm
- first chop tissue or use Ultraturrax etc

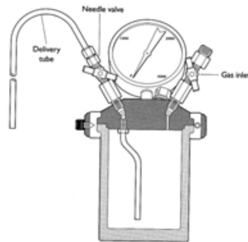


(ii) Polytron, Ultraturrax
- mechanical sheer, better for "hard" tissues
- 3000 – 4000 rpm for 60 sec, on ice
- micro tips also available (use in microcentrifuge tube)

Techniques for Cell Disruption continued:

(iii) Pressure cavitation – eg N_2 "bombs"
- used exclusively for single-cell suspensions
- gas dissolved in suspension at 20-100 atmospheres
- upon return to atmospheric pressure, cells disrupted by rapid expansion of dissolved nitrogen

Caution: although very efficient and highly reproducible, ribosomes and rough endoplasmic reticulum may be damaged



Techniques for Cell Disruption continued:

(iv) Sonicators
(v) Other mechanical – glass beads, ball bearings, force etc

After Cell Disruption:

- can check extent of disruption by staining homogeate with 0.1% toluidine blue in isotonic medium + microscopy at 400x

2. Separation of subcellular components: Centrifugal Separation

(i) Differential Pelleting – most common
- see example soon

(ii) Cation-catalysed pelleting:
- Precipitation of plasma membranes by addition of divalent cations (Mg++, Ca++)
- Cations bind to sialic groups on membranes

(iii) Density-gradient centrifugation:
- eg: sucrose, Percoll, metrizamide, Ficoll
- use different gradients to isolate mitochondria, peroxisomes, lysosomes, etc

Table 4. Organelle and membrane densities in Percoll gradients

Fraction	Source	Density (g/ml)*
Chromaffin granules	Bovine adrenal	1.067–1.081
Endocytic vesicles	K562 cells	1.03
	Rabbit alveolar macrophages	1.05
Glycosomes	*Trypanosoma brucei*	1.087
Golgi	Rat liver	1.028–1.057
Lysosomes	Rat liver	1.087
	Rat kidney cortex	1.15
	Human lymphoblasts	1.085
	Pig thyroid	1.14
Mitochondria	Rat liver	1.085–1.100
	Bovine skeletal muscle	1.035–1.070
Peroxisomes	Rat liver	1.075
Plasma membranes	Rat liver sinusoidal	1.02–1.04
	Rabbit kidney cortex basolateral	1.037
	Rabbit kidney cortex brush-border	1.042
Synaptosomes	Rat brain	1.04–1.05

*Density applies to Percoll in 0.25 M sucrose.

3. ID, Yield Assessment

- Use series of markers – assay isolated fraction for these
- Markers generally enzymic or immunological

Table 5. Markers for subcellular fractionation

Membrane type	Enzymic or chemical marker	Membrane subtype
Plasma membrane	Na^+K^+ ATPase	Basolateral
	Adenylate cyclase	Basolateral
	Specific cell surface receptors	Basolateral
	5′-nucleotidase	Apical
	Leucine aminopeptidase-glutamyltranspeptidase	Apical
	Alkaline phosphatase	Apical
Endoplasmic reticulum	Glucose-6-phosphatase	
	NADPH-cytochrome *c* reductase	
	Choline phosphotransferase	
	Cytochrome b_5	
Mitochondria	Succinate dehydrogenase	inner membrane
	Cytochrome oxidase	inner membrane
	Monoamine oxidase	outer membrane
	Kynurenine-3-hydrolase	outer membrane
Golgi apparatus	Galactosyltransferase	trans and middle regions
	Sialyltransferase	trans and middle regions
	NADP-phosphatase	trans and middle regions
	Mannosidase II	cis region
Lyosomes	Acid phosphatase	
	Glucuronidase	
	Aryl sulphatase	
Endosomes	Monensin-activated Mg^{2+}-ATPase in addition to un-degraded internalized ligands	
	Specific GTP binding proteins *rab* 7 'late' endosomes	
	Specific GTP binding proteins *rab* 4 and 5 'early' endosomes	
Peroxisomes	Catalase	
	Carnitine palmitoyl trans-ferase	
Cytosol	Lactate dehydrogenase	
Plant Cells (in addition to above)		
Plasma membrane	K^+ stimulated, Vanadate inhibited, ATPase, glucan synthetase II	
Tonoplast	C^1-stimulated, NO_3-inhibited ATPase	
Chloroplasts	Chlorophyll	
Amyloplasts	Monogalactosyldiglyceride synthetase	
Golgi apparatus	Inosine diphosphatase	
Bacterial membranes (specific)		
Inner membranes	D-lactate dehydrogenase	
Outer membranes (gram negative)	Phospholipase A1	

Table 3. Properties of cell organelles and membranes

Organelle	% of homogenate protein	Particle dimension (μm)	Sedimentation force required to pellet	Density in sucrose (g/ml)
Plasma membrane[a]	0.4–2.5	3–20 (large sheets)[b]	1500*g*, 15 min	1.15–1.18
		0.05–3 (vesicles)	100 000*g*, 60 min	1.12–1.14
Nuclei	13	3–12	600*g*, 15 min	>1.30
Nuclear membrane	–	3–12	1500*g*, 15 min	1.18–1.22
Golgi apparatus[c]	1	1 (large)	2000*g*, 20 min	1.12–1.16
		0.05–0.5 (vesicles)	150 000*g*, 20 min	1.12–1.16
Mitochondria[d]	16	0.5–2	10 000*g*, 25 min	1.17–1.21
Lysosomes	2	0.5–0.8	10 000*g*, 25 min	1.19–1.22
Peroxisomes	3	0.5–0.8	10 000*g*, 25 min	1.18–1.23
Endoplasmic reticulum	24	0.05–0.30	150 000*g*, 40 min	1.06–1.23 (smooth)
				1.18–1.23 (rough)
Endocytic vesicles	1	0.1–0.5	150 000*g*, 40 min	1.10–1.13
Secretory vesicles[e]	–	0.1–0.5	150 000*g*, 40 min	

[a]Although in theory 5.4 mg/g liver should be recovered from rat liver, in practice values of 1 mg/litre (18–20%) are obtained. With cultured cells (e.g. RLT-28 hepatoma, 2.5 mg should be recovered from 10^8 cells, although in practice 0.3 mg (= 12%) is an acceptable value (43).
[b]Large sheets derive mainly from basolateral region and produced under low shear conditions. Vesicles derived from microvilli at apical or secretory aspect of the basolateral membrane. Note that under carefully defined conditions of tissue homogenization, large areas of the apical plasma membrane (e.g. bile canaliculi of liver parenchymal cells) when attached to lateral membranes sediment at low centrifugal forces. Tight-homogenization of these structures can then release the apical plasma membrane as vesicles (21).
[c]Golgi apparatus can be isolated as a single fraction in which the stacks or cisternae are intact or as those subfractions designated light, intermediate, and heavy (37–39). These are claimed to correspond roughly to the trans (light) and cis (heavy) aspects of the Golgi apparatus. Since Golgi fractions are cross-contaminated with endocytic vesicles, a method that isolates Golgi fractions and endocytic vesicles from the same homogenate offers advantages. (2, 42).
[d]Methods for the separation of mitochondria into inner and outer membranes exist (43). Note that the density of released outer membranes is 1.12–1.14 g/ml.
[e]Density of secretory vesicles and amount recovered varies according to the nature of the content. In liver, secretory vesicles are recovered mainly in Golgi-light fraction (see above).

Blood Tube Breakage in Centrifuge

- **Probably alerted by noise, imbalance etc**

What To Do:

- **Stop centrifuge**
- **Put up sign –“Do not open”, initial, date/time etc**
- **Leave 10 – 30 mins**

If tube is in enclosed capsule

- **Inspect capsule**
- **Remove from centrifuge and open in fume cupboard or biohazard hood**
- **Spray contents with hypochlorite / ethanol / commercial product**
- **Close capsule and leave 10 – 30 min - label with “do not open” etc**
- **Open capsule, carefully remove debris to sharps container**
- **Wash capsule in hot soapy water**
- **Sterilize / autoclave**
- **Inspect capsule and centrifuge for damage, rectify**
- **Organize recollection of sample**

If tube is NOT in enclosed capsule Caution: Aerosols!!

- **Clear other staffmembers away**
- **Wear personal protective equipment incl. mask**
- **Open centifuge, turn off power and spray with 80% ethanol - NOT HYPOCHLORITE**
- **Close lid, put up WARNING sign, leave ~30 mins**
- **Wear thick gloves – use thick absorbent paper towel to mop up liquid, place in biohazard waste**
- **Clean solid glass fragments with paper towel / tongs**
- **Spray again and leave 10 – 30 mins**
- **Wash with hot soapy water, spray again and let dry**

FOR ALL TUBE BREAKS:

- **Arrange sample recollection**
- **Conduct thorough inspection for damage**
- **Fill out log book / incident report form -**
  - **why did it happen?**
  - **could it have been prevented?**
  - **any suggestions for improvement / prevention of recurrence**