12 Centrifugation and Ultracentrifugation

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12.1 INTRODUCTION

Biological centrifugation is a process that uses centrifugal force to separate and purify mixtures of biological particles in a liquid medium. The smaller the particles, the higher the g-forces (see next section) required for the separation. It is a key technique for isolating and analysing cells, subcellular fractions, supramolecular complexes and, with higher g-force instruments or 'ultra'-centrifuges (up to 60 000 revolutions per minute corresponding to ~200 000×g) isolated macromolecules such as proteins or nucleic acids. Such high-speed devices require a vacuum to avoid overheating of samples. The development of the first analytical ultracentrifuge – with a specially designed optical system for monitoring and recording the sedimentation process – by Svedberg in the late 1920s and the technical refinement of the preparative centrifugation technique by Claude and colleagues in the 1940s positioned centrifugation technology at the centre of biological and biomedical research for many decades. Today, centrifugation techniques represent a critical tool for modern biochemistry and are employed in almost all invasive subcellular studies. While analytical ultracentrifugation is mainly concerned with the study of purified macromolecules or isolated supramolecular assemblies, preparative centrifugation methodology is devoted to the actual separation of tissues, cells, subcellular structures, membrane vesicles and other particles of biochemical interest.

Most undergraduate students will be exposed to preparative centrifugation protocols during practical classes and might also experience a demonstration of analytical centrifugation techniques. This chapter is accordingly divided into a short introduction into the theoretical background of sedimentation, an overview of practical aspects of using centrifuges in the biochemical laboratory, an outline of preparative centrifugation and a description of the usefulness of ultracentrifugation techniques in the biochemical characterisation of macromolecules. To aid in the understanding of the basic principles

of centrifugation, the general designs of various rotors and separation processes are diagrammatically represented. Often, the learning process of undergraduate students is hampered by the lack of a proper linkage between theoretical knowledge and practical applications. To overcome this problem, the description of preparative centrifugation techniques is accompanied by an explanatory flow chart and the detailed discussion of the subcellular fractionation protocol for a specific tissue preparation. Taking the isolation of fractions from skeletal muscle homogenates as an example, the rationale behind individual preparative steps is explained. Since affinity isolation methods not only represent an extremely powerful tool in purifying biomolecules (see Chapter 5), but can also be utilised to separate intact organelles and membrane vesicles by centrifugation, lectin affinity agglutination of highly purified plasmalemmal vesicles from skeletal muscle is described. Traditionally, marker enzyme activities are used to determine the overall yield and enrichment of particular structures within subcellular fractions following centrifugation. As an example, the distribution of key enzyme activities in mitochondrial subfractions from liver is given. However, most modern fractionation procedures are evaluated by more convenient methods, such as protein gel analysis in conjunction with immunoblot analysis (Chapter 7). Miniature gel and blotting equipment can produce highly reliable results within a few hours, making it an ideal analytical tool for high-throughput testing. Since electrophoretic techniques are introduced in Chapter 6 and are used routinely in biochemical laboratories, the protein gel analysis of the distribution of typical marker proteins in affinity-isolated plasmalemma fractions is graphically represented and discussed.

Although monomeric peptides and proteins are capable of performing complex biochemical reactions, many physiologically important elements do not exist in isolation under native conditions. Therefore, if one considers individual proteins as the basic units of the proteome (see Chapter 21), protein complexes actually form the functional units of cell biology. This gives investigations into the supramolecular structure of protein complexes a central place in biochemical research. To illustrate this point, the sedimentation analysis of a high-molecular-mass membrane assembly, the dystrophin–glycoprotein complex of skeletal muscle, is shown and the use of sucrose gradient centrifugation explained.

Analytical ultracentrifugation – which unlike other analytical separation techniques does not require a separation medium, i.e. it is 'matrix-free' – has become a preferred or 'gold standard' technique for establishing the purity or homogeneity and state of aggregation of macromolecular or nanoparticle solutions, and to illustrate this point, we show how the purity of preparations of monoclonal antibodies can be routinely analysed with the modern ultracentrifuge, and how the inclusion of a density gradient, when appropriate, can enhance the resolution of the method even further.

12.2 BASIC PRINCIPLES OF SEDIMENTATION

From everyday experience, the effect of **sedimentation** due to the influence of the Earth's gravitational field (G = g = 9.81 m s^{-2}) versus the increased rate of sedimentation in a centrifugal field (G > 9.81 m s^{-2}) is apparent. To give a simple but illustrative example, crude sand particles added to a bucket of water travel slowly to the bottom of the bucket by gravitation, but sediment much faster when the bucket is swung around in a circle. Similarly, biological structures exhibit a drastic increase

in sedimentation when they undergo acceleration in a centrifugal field. The relative centrifugal field is usually expressed as a multiple of the acceleration due to gravity.

Below is a short description of equations used in practical centrifugation classes. When designing a centrifugation protocol, it is important to keep in mind that:

- the more dense a biological structure is, the faster it sediments in a centrifugal field
- the more massive a biological particle is, the faster it moves in a centrifugal field
- the denser the biological buffer system is, the slower the particle will move in a centrifugal field
- the greater the frictional coefficient is, the slower a particle will move
- the greater the centrifugal force is, the faster the particle sediments
- the sedimentation rate of a given particle will be zero when the density of the particle and the surrounding medium are equal.

Biological particles moving through a viscous medium experience a frictional drag, whereby the frictional force acts in the opposite direction to sedimentation and equals the velocity of the particle multiplied by the frictional coefficient. The frictional coefficient depends on the size and shape of the biological particle. As the sample moves towards the bottom of a centrifuge tube in swing-out or fixed-angle rotors (see Section 12.3.2), its velocity will increase due to the increase in radial distance. At the same time, the particles also encounter a frictional drag that is proportional to their velocity. The frictional force of a particle moving through a viscous fluid is the product of its velocity and its frictional coefficient, and acts in the opposite direction to sedimentation.

When the conditions for the centrifugal separation of a biological particle are described, a detailed listing of rotor speed and radial dimensions of centrifugation has to be provided. Essentially, the rate of sedimentation, v, is dependent upon the applied centrifugal field G (measured in cm s^{-2}). G is determined by the radial distance, r, of the particle from the axis of rotation (in cm) and the square of the angular velocity, ω, of the rotor (in radians per second):

$$G = \omega^2 \times r \qquad \text{(Eq 12.1)}$$

The average angular velocity of a rigid body that rotates around a fixed axis is defined as the ratio of the angular displacement in a given time interval. One radian, usually abbreviated as 1 rad, represents the angle subtended at the centre of a circle by an arc with length equal to the radius of the circle. Since 360° equals 2π radians (or rad), one revolution of the rotor can be expressed as 2π rad. Accordingly, the angular velocity of the rotor, given in rad s^{-1}. Note that rad is treated as a scalar and is related to the rotor speed in revolutions per minute (rpm = 1 min^{-1}) by

$$\omega = 2\pi \text{ rad} \times rpm \qquad \text{(Eq 12.2)}$$

and therefore the centrifugal field can be expressed as:

$$G = 4\pi^2 \text{rad}^2 \times rpm^2 \times r \qquad \text{(Eq 12.3)}$$

where the variable *rpm* is the rotor speed (measured in revolutions per minute, i.e. the non-italicised 'rpm' denotes the unit) and r is the radial distance from the centre of rotation. Note that 60 revolutions per minute is the same speed as one revolution per second, i.e. $rpm = 60 \text{ min}^{-1} = 1 \text{ s}^{-1}$.

Example 12.1 CALCULATION OF CENTRIFUGAL FIELD

Question 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}?

Answer The centrifugal field, G, at a point 5 cm from the centre of rotation may be calculated using Equation 12.1:

$$G = \omega^2 \times r = \left(3000 \frac{\text{rad}}{\text{s}}\right)^2 \times 5 \text{ cm} = 4.5 \times 10^7 \frac{\text{rad}^2 \text{ cm}}{\text{s}^2}$$

Example 12.2 CALCULATION OF ANGULAR VELOCITY

Question For the pelleting of the microsomal fraction from a liver homogenate, an ultracentrifuge is operated at a speed of 40 000 rpm. Calculate the angular velocity, ω, in radians per second.

Answer For a rotor speed of 40 000 rpm we obtain:

$$rpm = 40\,000 \text{ rpm} = \frac{40\,000}{1 \text{ min}} = \frac{40\,000}{60 \text{ s}}$$

The angular velocity may be calculated using Equation 12.2:

$$\omega = 2\pi \times s = 2\pi \times 40\,000 \text{ min}^{-1} = \frac{2\pi \times 40\,000}{1 \text{ min}} = \frac{2\pi \times 40\,000}{60 \text{ s}} = 4189 \text{ rad s}^{-1}$$

The centrifugal field is generally expressed in multiples of the earth's gravitational field, g (9.81 m s^{-2}). The **relative centrifugal field** RCF (or g-force) is the ratio of the centrifugal acceleration at a specified radius and the speed to the standard acceleration of gravity. The RCF can be calculated from the following equation:

$$\text{RCF} = \frac{G}{\text{g}} = \frac{4\pi^2 \text{ rad}^2 \times rpm^2 \times r}{\text{g}} \qquad \text{(Eq 12.4)}$$

RCF units are therefore dimensionless as they denote multiples of the gravitational constant g. Grouping numerical constants together leads to a more convenient form of Equation 12.4:

$$\text{RCF} = 1.12 \times 10^{-5} \times \frac{r}{1 \text{ cm}} \times \left(\frac{rpm}{1 \text{ min}^{-1}}\right)^2 \qquad \text{(Eq 12.5)}$$

with *r* given in cm. Although the relative centrifugal force can easily be calculated and can often be displayed on modern instruments, many centrifugation manuals contain a nomograph for the convenient conversion between relative centrifugal force and speed of the centrifuge at different radii of the centrifugation spindle to a point along

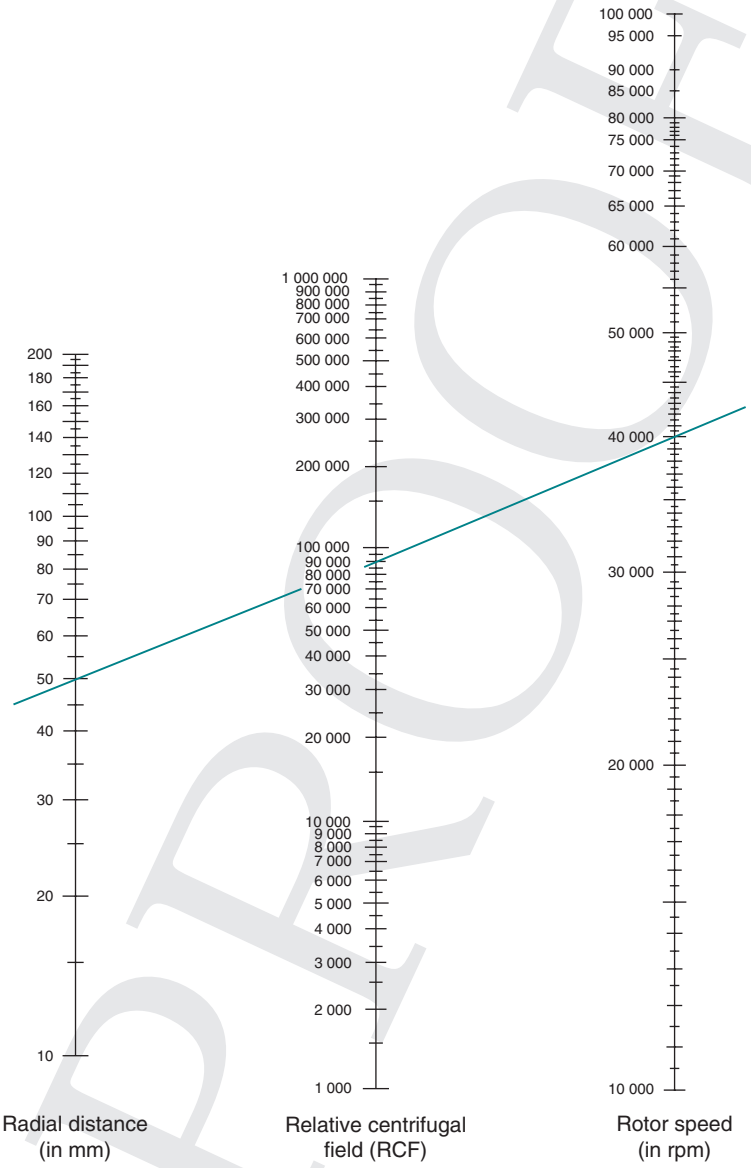


Figure 12.1 Nomograph for the determination of the relative centrifugal field for a given rotor speed and radius. The three columns represent the radial distance (in mm), the relative centrifugal field and the rotor speed *rpm* (in min^{-1}). For the conversion between relative centrifugal force and speed of the centrifuge spindle in revolutions per minute at different radii, draw a straight edge through known values in two columns. The desired figure can then be read where the straight edge intersects the third column. The example shown determines the relative centrifugal force for a 5 cm rotor operating at *rpm* = 40 000 min^{-1}, yielding an applied centrifugal field of about 90 000×g.

the centrifuge tube. A nomograph consists of three columns representing the radial distance (in mm), the relative centrifugal field and the rotor speed (in min^{-1}). For the conversion between relative centrifugal force RCF and speed of the centrifuge spindle (*rpm*, in min^{-1}) at different radii, a straight edge is aligned through known values in two columns, then the desired figure is read where the straight edge intersects the third column. See Figure 12.1 for an illustration of the usage of a nomograph.

In a suspension of biological particles, the rate of sedimentation (v) is dependent not only upon the applied centrifugal field, but also on the nature of the particle, i.e. its density ρ_p, its hydrodynamic radius R_{hydro}, (see Section 2.2.4) and also the density (ρ_m) and viscosity (η) of the surrounding medium. Stokes' law describes this relationship for the sedimentation of a rigid spherical particle:

$$v = \frac{2}{9} \times \frac{R_{hydro}^2 \times (\rho_p - \rho_m)}{\eta} \times g \qquad \text{(Eq 12.6)}$$

Example 12.3 CALCULATION OF RELATIVE CENTRIFUGAL FIELD

Question 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 Figure 12.2a 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 rpm, what is the relative centrifugal field (RCF) at the top and bottom of the centrifuge tube?

Answer The relative centrifugal field may be calculated using Equation 12.5:

$$RCF = 1.12 \times 10^{-5} \times \frac{r}{1\ cm} \times \left(\frac{rpm}{1\ min^{-1}} \right)^2$$

At the top of the centrifuge tube:

$$RCF_{top} = 1.12 \times 10^{-5} \times \frac{3.5\ cm}{1\ cm} \times \left(\frac{20\,000\ min^{-1}}{1\ min^{-1}} \right)^2 = 15\,680$$

At the bottom of the centrifuge tube:

$$RCF_{bottom} = 1.12 \times 10^{-5} \times \frac{7.0\ cm}{1\ cm} \times \left(\frac{20\,000\ min^{-1}}{1\ min^{-1}} \right)^2 = 31\,360$$

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 exactly twofold.

where v is the sedimentation rate of the sphere, R_{hydro} is the radius of particle, ρ_p is the density of particle, ρ_m is the density of medium, g is the gravitational acceleration and η is the viscosity of the medium. R_{hydro} will depend on the shape of the particle. Following from Equation 12.6, the sedimentation time can be simply calculated as the ratio of distance sedimented and v.

Accordingly, a mixture of biological particles exhibiting an approximately spherical shape can be separated in a centrifugal field based on their density and/or their size. The time of sedimentation (in seconds) for a spherical particle is:

$$t = \frac{2}{9} \times \frac{\eta}{\omega^2 \times R_{hydro}^2 \times (\rho_p - \rho_m)} \times \ln \frac{r_b}{r_t} \qquad \text{(Eq 12.7)}$$

where t is the sedimentation time, η is the viscosity of medium, R_{hydro} is the hydrodynamic radius of the particle, r_b is the radial distance from the centre of rotation to bottom of tube, r_t is the radial distance from the centre of rotation to liquid meniscus, ρ_p is the density of the particle, ρ_m is the density of the medium and ω is the angular velocity of the rotor.

The **sedimentation rate** or velocity of a biological particle can also be expressed as its **sedimentation coefficient** (s), whereby:

$$s = \frac{v}{\omega^2 \times r} \qquad \text{(Eq 12.8)}$$

measured in the units of time (i.e. seconds).

Since the sedimentation rate per unit centrifugal field can be determined at different temperatures and with various media of different densities and viscosities, experimental values of the sedimentation coefficient are often corrected or 'normalised' for comparison purposes to standard solvent conditions. These standard conditions are the density and viscosity of water at 20.0 °C and the symbol used for this normalised sedimentation coefficient is $s_{20,w}$. Importantly, $s_{20,w}$ will depend on the size and shape or conformation of the macromolecule. The sedimentation coefficients (corrected or non-corrected) of biological macromolecules are relatively small, and are usually expressed as svedberg units denoted as S (see Section 12.5); one svedberg units equals 10^{-13} s.

12.3 TYPES, CARE AND SAFETY ASPECTS OF CENTRIFUGES

12.3.1 Types of Centrifuges

Centrifugation techniques take a central position in modern biochemical, cellular and molecular biological studies. Depending on the particular application, centrifuges differ in their overall design and size. However, a common feature in all centrifuges is the central motor that spins a rotor containing the samples to be separated. Particles of biochemical interest are usually suspended in a liquid buffer system contained in specific tubes or separation chambers that are located in specialised rotors. The biological medium is chosen for the specific centrifugal application and may differ considerably between preparative and analytical approaches. As outlined below, the optimum pH value, salt concentration, stabilising cofactors and protective ingredients such as protease inhibitors have to be carefully evaluated in order to preserve biological function. The most obvious differences between centrifuges are:

- the maximum speed at which biological specimens are subjected to increased sedimentation
- the presence or absence of a vacuum
- the potential for refrigeration or general manipulation of the temperature during a centrifugation run
- the maximum volume of samples and capacity for individual centrifugation tubes.

Many different types of centrifuges are commercially available including:

- large-capacity low-speed preparative centrifuges
- preparative high-speed ultracentrifuges
- refrigerated preparative centrifuges/ultracentrifuges
- analytical ultracentrifuges
- large-scale clinical centrifuges
- small-scale laboratory microfuges.

Some large-volume centrifuge models are quite demanding on space and also generate considerable amounts of heat and noise, and are therefore often centrally positioned in special instrument rooms in biochemistry departments. However, the development of small-capacity **bench-top centrifuges** for biochemical applications, even in the case of ultracentrifuges, has led to the introduction of these models in many individual research laboratories.

The main types of centrifuge encountered by undergraduate students during introductory practicals may be divided into microfuges (so called because they centrifuge small volume samples in Eppendorf tubes), large-capacity preparative centrifuges, high-speed refrigerated centrifuges and ultracentrifuges. Simple bench-top centrifuges vary in design and are mainly used to collect small amounts of biological material, such as blood cells. To prevent denaturation of sensitive protein samples, refrigerated centrifuges should be employed. Modern refrigerated microfuges are equipped with adapters to accommodate standardised plastic tubes for the sedimentation of 0.5 to 1.5 cm^3 volumes. They can provide centrifugal fields of approximately 10 000×g and sediment biological samples in minutes, making microfuges an indispensable separation tool for many biochemical methods. Microfuges can also be used to concentrate protein samples. For example, the dilution of protein samples, eluted by column chromatography, can often represent a challenge for subsequent analyses. Accelerated **ultrafiltration** with the help of plastic tube-associated filter units, spun at low g-forces in a microfuge, can overcome this problem. Depending on the proteins of interest, the biological buffers used and the molecular mass cut-off point of the particular filters, a 10- to 20-fold concentration of samples can be achieved within minutes. Larger preparative bench-top centrifuges develop maximum centrifugal fields of 3000×g to 7000×g and can be used for the spinning of various types of containers. Depending on the range of available adapters, considerable quantities of 5 to 250 cm^3 plastic tubes or 96-well ELISA plates can be accommodated. This gives simple and relatively inexpensive bench centrifuges a central place in many high-throughput biochemical assays, where the quick and efficient separation of coarse precipitates or whole cells is of importance.

High-speed refrigerated centrifuges are absolutely essential for the sedimentation of protein precipitates, large intact organelles, cellular debris derived from tissue

homogenisation and microorganisms. As outlined in Section 12.4, the initial bulk separation of cellular elements prior to preparative ultracentrifugation is performed by these kinds of centrifuges. They operate at maximum centrifugal fields of approximately 100 000×g. Such centrifugal force is not sufficient to sediment smaller microsomal vesicles or ribosomes, but can be employed to differentially separate nuclei, mitochondria or chloroplasts. In addition, bulky protein aggregates can be sedimented using high-speed refrigerated centrifuges. An example is the contractile apparatus released from muscle fibres by homogenisation, mostly consisting of myosin and actin macromolecules aggregated in filaments. In order to harvest yeast cells or bacteria from large volumes of culture media, high-speed centrifugation may also be used in a **continuous flow mode** with **zonal rotors**. This approach does not therefore use centrifuge tubes, but a continuous flow of medium. As the medium enters the moving rotor, biological particles are sedimented against the rotor periphery and excess liquid removed through a special outlet port.

Ultracentrifugation has decisively advanced the detailed biochemical analysis of subcellular structures and isolated biomolecules. Preparative ultracentrifugation can be operated at relative centrifugal fields of up to 900 000×g. In order to minimise excessive rotor temperatures generated by **frictional resistance** between the spinning rotor and air, the rotor chamber is sealed, evacuated and refrigerated. Depending on the type, age and condition of a particular ultracentrifuge, cooling to the required running temperature and the generation of a stable vacuum might take a considerable amount of time. To avoid delays during biochemical procedures involving ultracentrifugation, the cooling and evacuation system of older centrifuge models should be switched on at least an hour prior to the centrifugation run. In contrast, modern ultracentrifuges can be started even without a fully established vacuum and will proceed in the evacuation of the rotor chamber during the initial acceleration process. For safety reasons, heavy armour plating encapsulates the ultracentrifuge to prevent injury to the user in case of uncontrolled rotor movements or dangerous vibrations. A centrifugation run cannot be initiated without proper closing of the chamber system. To prevent unfavourable fluctuations in chamber temperature, excessive vibrations or operation of rotors above their maximum rated speed, newer models of ultracentrifuges contain sophisticated temperature regulation systems, **flexible drive shafts** and an **over-speed control** device. Although slight rotor imbalances can be absorbed by modern ultracentrifuges, a more severe misbalance of tubes will cause the centrifuge to switch off automatically. This is especially true for swinging-bucket rotors. The many safety features incorporated into modern ultracentrifuges make them a robust piece of equipment that tolerates a certain degree of misuse by an inexperienced operator (see Sections 12.3.2 and 12.3.4 for a more detailed discussion of safety and centrifugation). In contrast to preparative ultracentrifuges, analytical ultracentrifuges contain a solid rotor that incorporates one counterbalancing cell and typically either three or seven analytical cells. A specialised optical system enables the sedimenting material to be observed throughout the duration of a centrifuge run. Using either an **absorption optical system** (based on ultraviolet/visible light absorption; see also Section 13.2) or a **Rayleigh interference optical system** (based on light refraction; see also Section 12.5), or a combination of both, concentration distributions of macromolecules in solution can be

recorded at any time during ultracentrifugation. From these records, information about the purity/heterogeneity, sedimentation coefficient distribution, average molar mass and molar mass distributions, and ligand interaction information can be obtained.

12.3.2 Types of Rotor

To illustrate the difference in design of fixed-angle rotors, vertical tube rotors and swinging-bucket rotors, Figure 12.2 outlines cross-sectional diagrams of these three main types of rotor. Companies usually name rotors according to their design type, the maximum allowable speed and sometimes the material composition. Depending

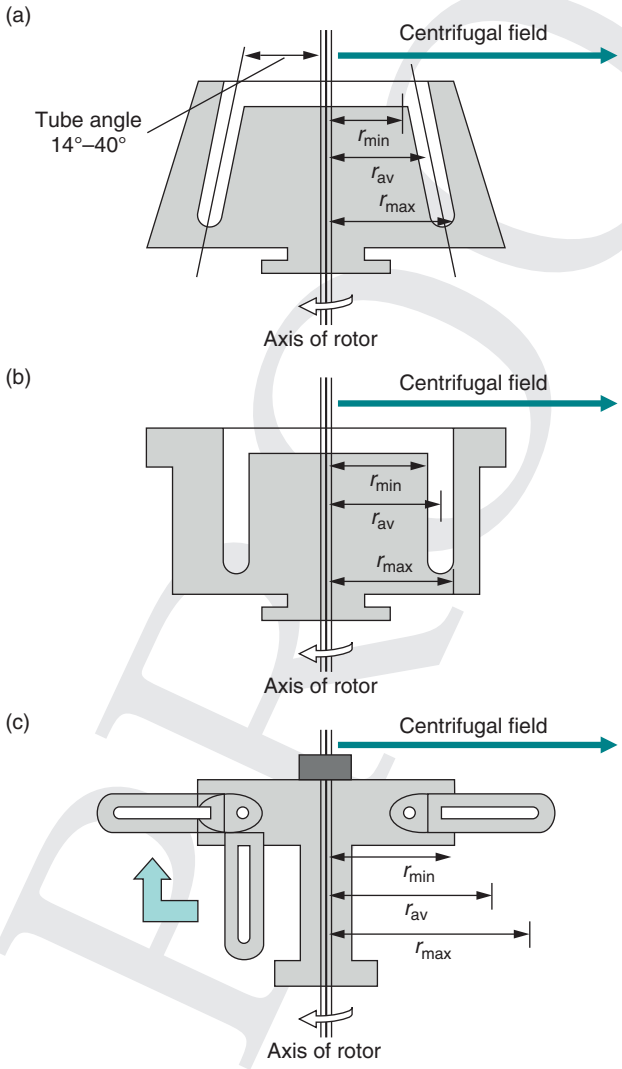


Figure 12.2 Design of the three main types of rotor used in routine biochemical centrifugation techniques. Shown is a cross-sectional diagram of a fixed-angle rotor (a), a vertical tube rotor (b) and a swinging-bucket rotor (c). A fourth type of rotor is represented by the class of near-vertical rotors (not shown).

on the use in a simple low-speed centrifuge, a high-speed centrifuge or an ultracentrifuge, different centrifugal forces are encountered by a spinning rotor (see also Example 12.3). Accordingly, different types of rotors are made from different materials. Low-speed rotors are usually made of steel or brass, while high-speed rotors consist of aluminium, titanium or fibre-reinforced composites. The exterior of specific rotors might be finished with protective paints. For example, rotors for ultracentrifugation made out of titanium alloy are covered with a polyurethane layer. Aluminium rotors are protected from corrosion by a tough, electrochemically formed layer of aluminium oxide. In order to avoid damaging these protective layers, care should be taken during rotor handling.

Fixed-angle rotors are an ideal tool for pelleting during the differential separation of biological particles where sedimentation rates differ significantly, for example when separating nuclei, mitochondria and microsomes. In addition, isopycnic ('matching density') banding – where the density of the substance matches that of the gradient at that radial position – may also be routinely performed with fixed-angle rotors. For isopycnic separation, centrifugation is continued until the biological particles of interest have reached their **isopycnic position** in a gradient. This means that the particle has reached a position where the sedimentation rate is zero because the density of the biological particle and the surrounding medium are equal. Centrifugation tubes are held at a fixed angle of between 14° and 40° to the vertical in this class of rotor (Figure 12.2a). Particles move radially outwards and since the centrifugal field is exerted at an angle, they only have to travel a short distance until they reach their isopycnic position in a gradient using an isodensity technique or before colliding with the outer wall of the centrifuge tube using a differential centrifugation method. **Vertical rotors** (Figure 12.2b) may be divided into true vertical rotors and **near-vertical rotors**. Sealed centrifuge tubes are held parallel to the axis of rotation in vertical rotors and are restrained in the rotor cavities by screws, special washers and plugs. Since samples are not separated down the length of the centrifuge tube, but across the diameter of the tube, isopycnic separation time is significantly shorter as compared to swinging-bucket rotors. In contrast to fixed-angle rotors, near-vertical rotors exhibit a reduced tube angle of 7° to 10° and also employ quick-seal tubes. The reduced angle results in much shorter run times as compared to fixed-angle rotors. Near-vertical rotors are useful for gradient centrifugation of biological elements that do not properly participate in conventional gradients. Hinge pins or a crossbar is used to attach rotor buckets in **swinging-bucket rotors** (Figure 12.2c). They are loaded in a vertical position and during the initial acceleration phase, the rotor buckets swing out horizontally and then position themselves at the rotor body for support.

To illustrate the separation of particles in the three main types of rotors, Figure 12.3 outlines the path of biological samples during the initial acceleration stage, the main centrifugal separation phase, de-acceleration and the final harvesting of separated particles in the rotor at rest. In the case of **isopycnic centrifugation** in a fixed-angle rotor, the centrifuge tubes are gradually filled with a suitable gradient, the sample carefully loaded on top of this solution and then the tubes placed at a specific fixed-angle into the rotor cavities. During rotor acceleration, the sample solution and the gradient undergo reorientation in the centrifugal field, followed by the

separation of particles with different sedimentation properties (Figure 12.3a). The gradient returns to its original position during the de-acceleration phase and separated particle bands can be taken from the tubes once the rotor is at rest. In analogy, similar reorientation of gradients and banding of particles occurs in a vertical rotor system (Figure 12.3b). Although run times are reduced and this kind of rotor can usually hold a large number of tubes, resolution of separated bands during isopycnic centrifugation is less when compared with swinging-bucket applications. Since a greater variety of gradients exhibiting different steepness can be used with swinging-bucket rotors, they are the method of choice when maximum resolution of banding zones is required (Figure 12.3c), such as in rate zonal studies based on the separation of biological particles as a function of sedimentation coefficient.

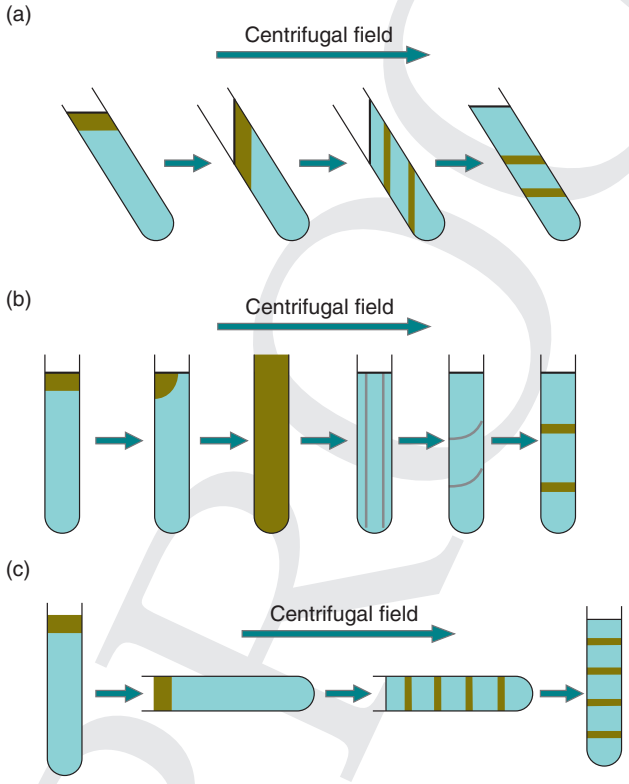


Figure 12.3 Operation of the three main types of rotor used in routine biochemical centrifugation techniques. Shown is a cross-sectional diagram of a centrifuge tube positioned in a fixed-angle rotor (a), a vertical tube rotor (b) and a swinging-bucket rotor (c). The diagrams illustrate the movement of biological samples during the initial acceleration stage, the main centrifugal separation phase, deceleration and the final harvesting of separated particles in the rotor at rest. Using a fixed-angle rotor, the tubes are filled with a gradient, the sample loaded on top of this solution and then the tubes placed at a specific fixed-angle into the rotor cavities. The sample and the gradient undergo reorientation in the centrifugal field during rotor acceleration, resulting in the separation of particles with different sedimentation properties. Similar reorientation of gradients and banding of particles occurs in a vertical rotor system. A great variety of gradients can be used with swinging-bucket rotors, making them the method of choice when maximum resolution of banding zones is required.

12.3.3 Care and Maintenance of Centrifuges

Corrosion and degradation due to biological buffer systems used within rotors or contamination of the interior or exterior of the centrifuge via spillage may seriously affect the lifetime of this equipment. Another important point is the proper balancing of centrifuge tubes. This is not only important with respect to safety, as outlined below, but might also cause vibration-induced damage to the rotor itself and the drive shaft of the centrifuge. Thus, proper handling and care, as well as regular maintenance of both centrifuges and rotors, is an important part of keeping this biochemical method available in the laboratory. In order to avoid damaging the protective layers of rotors, such as polyurethane paint or aluminium oxide, care should be taken in the cleaning of the rotor exterior. Coarse brushes that may scratch the finish should not be used and only non-corrosive detergents employed. Corrosion may be triggered by long-term exposure of rotors to alkaline solutions, acidic buffers, aggressive detergents or salt. Thus, rotors should be thoroughly washed with distilled or deionised water after every run. For overnight storage, rotors should be first left upside down to drain excess liquid and then positioned in a safe and dry place. To avoid damage to the hinge pins of swinging-bucket rotors, they should be dried with tissue paper following removal of biological buffers and washing with water. Centrifuge rotors are often not properly stored in a clean environment; this can quickly lead to the destruction of the protective rotor coating and should thus be avoided. It is advisable to keep rotors in a special clean room, physically separated from the actual centrifugation facility, with dedicated places for individual types of rotors. Some researchers might prefer to pre-cool their rotors prior to centrifugation by transferring them to a cold room. Although this is an acceptable practice and might keep proteolytic degradation to a minimum, rotors should not undergo long-term storage in a wet and cold environment. Regular maintenance of rotors and centrifuges by engineers is important for ensuring the safe operation of a centralised centrifugation facility. In order to judge properly the need for replacement of a rotor or parts of a centrifuge, it is essential that all users of core centrifuge equipment participate in proper book-keeping. Accurate record-keeping of run times and centrifugal speeds is important, since cyclic acceleration and deceleration of rotors may lead to metal fatigue.

12.3.4 Safety and Centrifugation

Modern centrifuges are not only highly sophisticated, but also relatively sturdy pieces of biochemical equipment that incorporate many safety features. Rotor chambers of high-speed and ultracentrifuges are always enclosed in heavy armour plating. Most centrifuges are designed to buffer a certain degree of imbalance and are usually equipped with an automatic switch-off mode. However, even in a well-balanced rotor, tube cracking during a centrifugation run might cause severe imbalance, resulting in dangerous vibrations. When the rotor can only be partially loaded, the order of tubes must be organised according to the manufacturer's instructions, so that the load is correctly distributed. This is important not only for ultracentrifugation with enormous centrifugal fields, but also for both small- and large-capacity

bench centrifuges, where the rotors are usually mounted on a more rigid suspension. When using swinging-bucket rotors, it is important always to load all buckets with their caps properly screwed on. Even if only two tubes are loaded with solutions, the empty swinging buckets also have to be assembled since they form an integral part of the overall balance of the rotor system. In some swinging-bucket rotors, individual rotor buckets are numbered and should not be interchanged between their designated positions on similarly numbered hinge pins. Centrifugation runs using swinging-bucket rotors are usually set up with low acceleration and deceleration rates, as to avoid any disturbance of delicate gradients, and reduce the risk of disturbing bucket attachment. This practice also avoids the occurrence of sudden imbalances due to tube deformation or cracking and thus eliminates potentially dangerous vibrations.

Generally, safety and good laboratory practice are important aspects of all research projects and the awareness of the exposure to potentially harmful substances should be a concern for every biochemist. If you use dangerous chemicals, potentially infectious material or radioactive substances during centrifugation protocols, refer to up-to-date safety manuals and the safety statement of your individual department. Perform mock runs of important experiments in order to avoid the loss of precious specimens or expensive chemicals. As with all other biochemical procedures, experiments should never be rushed and protective clothing should be worn at all times. Centrifuge tubes should be handled slowly and carefully so as not to disturb pellets, bands of separated particles or unstable gradients. To help you choose the right kind of centrifuge tube for a particular application, the manufacturers of rotors usually give detailed recommendations of suitable materials. For safety reasons and to guarantee experimental success, it is important to make sure that individual centrifuge tubes are chemically resistant to solvents used, have the right capacity for sample loading, can be used in the designated type of rotor and are able to withstand the maximum centrifugal forces and temperature range of a particular centrifuge. In fixed-angle rotors, large centrifugal forces tend to cause a collapse of centrifuge tubes, making thick-walled tubes the choice for these rotors. The volume of liquid and the sealing mechanisms of these tubes are very important for the integrity of the run and should be done according to manufacturer's instructions. In contrast, swinging-bucket rotor tubes are better protected from deformation and usually thin-walled polyallomer tubes are used. An important safety aspect is the proper handling of separated biological particles following centrifugation. In order to perform post-centrifugation analysis of individual fractions, centrifugation tubes often have to be punctured or sliced. For example, separated vesicle bands can be harvested from the pierced bottom of the centrifuge tube or can be collected by slicing of the tube following quick-freezing. If samples have been pre-incubated with radioactive markers or toxic ligands, contamination of the centrifugation chamber and rotor cavities or buckets should be avoided. If centrifugal separation processes have to be performed routinely with a potentially harmful substance, it makes sense to dedicate a particular centrifuge and accompanying rotors for this work and thereby eliminate the potential of cross-contamination.

12.4 PREPARATIVE CENTRIFUGATION

12.4.1 Differential Centrifugation

Cellular and subcellular fractionation techniques are indispensable methods used in biochemical research. Although the proper separation of many subcellular structures is absolutely dependent on preparative ultracentrifugation, the isolation of large cellular structures, the nuclear fraction, mitochondria, chloroplasts or large protein precipitates can be achieved by conventional high-speed refrigerated centrifugation. Differential centrifugation is based upon the differences in the sedimentation rate of biological particles of different size and density. Crude tissue homogenates containing organelles, membrane vesicles and other structural fragments are divided into different fractions by the stepwise increase of the applied centrifugal field. Following the initial sedimentation of the largest particles of a homogenate (such as cellular debris) by centrifugation, various biological structures or aggregates are separated into pellet and supernatant fractions, depending upon the speed and time of individual centrifugation steps and the density and relative size of the particles. To increase the yield of membrane structures and protein aggregates released, cellular debris pellets are often rehomogenised several times and then subjected to further centrifugation. This is especially important in the case of rigid biological structures such as muscular or connective tissues, or in the case of small tissue samples, as is the case with human biopsy material or primary cell cultures.

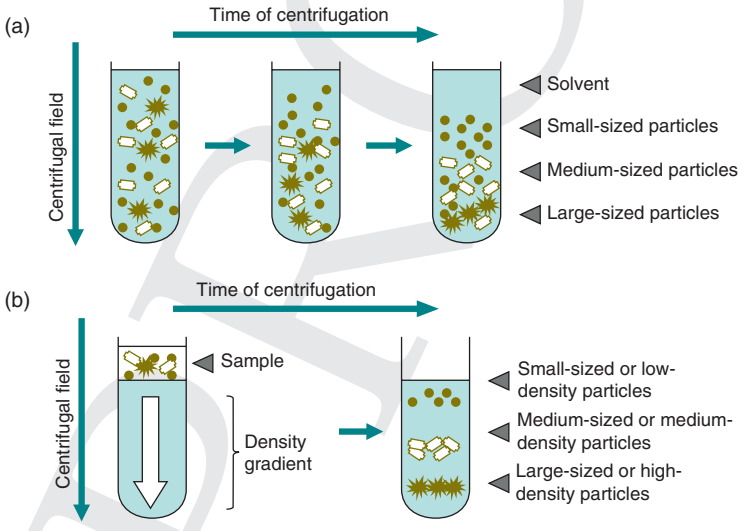


Figure 12.4 Diagram of particle behaviour during differential and isopycnic separation. During differential sedimentation (a) of a particulate suspension in a centrifugal field, the movement of particles is dependent upon their density, shape and size. For separation of biological particles using a density gradient (b), samples are carefully layered on top of a preformed density gradient prior to centrifugation. For isopycnic separation, centrifugation is continued until the desired particles have reached their isopycnic position in the liquid density gradient. In contrast, during rate separation, the required fraction does not reach its isopycnic position during the centrifugation run.

The differential sedimentation of a particulate suspension in a centrifugal field is diagrammatically shown in Figure 12.4a. Initially, all particles of a homogenate are evenly distributed throughout the centrifuge tube and then move down the tube at their respective sedimentation rate during centrifugation. The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant. However, during the initial centrifugation step, smaller particles also become entrapped in the pellet, causing a certain degree of contamination. At the end of each differential centrifugation step, the pellet and supernatant fraction are carefully separated from each other. To minimise cross-contamination, pellets are usually washed several times by resuspension in buffer and subsequent centrifugation under the same conditions. However, repeated washing steps may considerably reduce the yield of the final pellet fraction, and are therefore omitted in preparations with limiting starting material. Resulting supernatant fractions are centrifuged at a higher speed and for a longer time to separate medium-sized and small-sized particles. With respect to the separation of organelles and membrane vesicles, crude differential centrifugation techniques can be conveniently employed to isolate intact mitochondria and microsomes.

12.4.2 Density-Gradient Centrifugation

To further separate biological particles of similar size but differing densities, ultracentrifugation with pre-formed or self-establishing **density gradients** is the method of choice. Both rate separation or equilibrium methods can be used. In Figure 12.4b, the preparative ultracentrifugation of low- to high-density particles is shown. A mixture of particles, such as is present in a heterogeneous microsomal membrane preparation, is layered on top of a pre-formed liquid density gradient. Depending on the particular biological application, a great variety of gradient materials are available. Caesium chloride is widely used for the banding of DNA and the isolation of plasmids, nucleoproteins and viruses. Sodium bromide and sodium iodide are employed for the fractionation of lipoproteins and the banding of DNA or RNA molecules, respectively. Various companies offer a range of gradient material for the separation of whole cells and subcellular particles, e.g. Percoll®, Ficoll®, dextran, metrizamide and Nycodenz®. For the separation of membrane vesicles derived from tissue homogenates, ultra-pure DNase-, RNase and protease-free sucrose represents a suitable and widely employed medium for the preparation of stable gradients. If one wants to separate all membrane species spanning the whole range of particle densities, the maximum density of the gradient must exceed the density of the most dense vesicle species. Both **step-gradient** and **continuous-gradient** systems are employed to achieve this. If automated gradient-makers are not available, which is probably the case in most undergraduate practical classes, the manual pouring of a stepwise gradient with the help of a pipette is not so time-consuming or difficult. In contrast, the formation of a stable continuous gradient is much more challenging and requires a commercially available gradient-maker. Following pouring, gradients are usually kept in a cold room for temperature equilibration and are moved extremely slowly in special holders so as to avoid mixing of different gradient layers. For rate separation (sedimentation velocity;

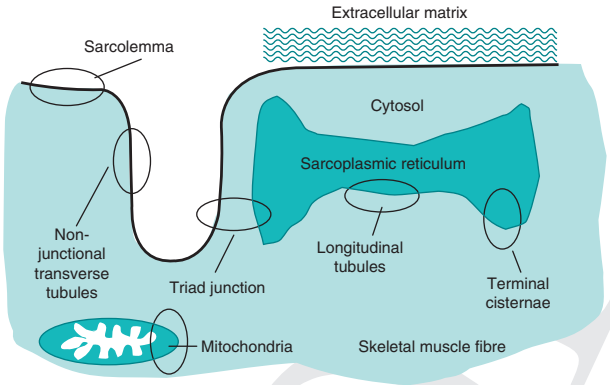
see Section 12.5.1) of subcellular particles, the required fraction does not reach its isopycnic position within the gradient. For **isopycnic separation**, density centrifugation is continued until the buoyant density of the particle of interest and the density of the gradient are equal.

12.4.3 Practical Applications of Preparative Centrifugation

To illustrate practical applications of differential centrifugation, density gradient ultracentrifugation and affinity methodology, the isolation of the microsomal fraction from muscle homogenates and subsequent separation of membrane vesicles with a differing density is described (Figure 12.5), the isolation of highly purified sarcolemmal vesicles outlined (Figure 12.6) and the subfractionation of liver mitochondrial membrane systems shown (Figure 12.7). Skeletal muscle fibres are highly specialised structures involved in contraction, and the membrane systems that maintain the regulation of excitation–contraction coupling, energy metabolism and the stabilisation of the cell periphery are diagrammatically shown in Figure 12.5a. The surface membrane consists of the sarcolemma and its invaginations, the transverse tubular membrane system. The transverse tubules may be subdivided into the non-junctional region and the triad part that forms contact zones with the terminal cisternae of the sarcoplasmic reticulum. Motor-neuron-induced depolarisation of the sarcolemma travels into the transverse tubules and activates a voltage-sensing receptor complex that directly initiates the transient opening of a junctional calcium-release channel. The membrane system that provides the luminal ion reservoir for the regulatory calcium cycling process is represented by the specialised endoplasmic reticulum. It forms membranous sheaths around the contractile apparatus whereby the longitudinal tubules are mainly involved in the uptake of calcium ions during muscle relaxation and the terminal cisternae provide the rapid calcium-release mechanism that initiates muscle contraction. Mitochondria are the site of oxidative phosphorylation and exhibit a complex system of inner and outer membranes involved in energy metabolism.

For the optimum homogenisation of tissue specimens, mincing of tissue has to be performed in the presence of a biological buffer system that exhibits the right pH value, salt concentration, stabilising cofactors and chelating agents. The optimum ratio between the wet weight of tissue and buffer volume, as well as the temperature (usually 4 °C) and presence of a protease inhibitor cocktail is also essential to minimise proteolytic degradation. Prior to the 1970s, researchers did not widely use protease inhibitors or chelating agents in their homogenisation buffers. This resulted in the degradation of many high-molecular-mass proteins. Since protective measures against endogenous enzymes have been routinely introduced into subcellular fractionation protocols, extremely large proteins have been isolated in their intact form, such as 427 kDa dystrophin, the 565 kDa ryanodine receptor, 800 kDa nebulin and the longest known polypeptide of 3800 kDa, named titin. Commercially available protease inhibitor cocktails usually exhibit a broad specificity for the inhibition of cysteine proteases, serine proteases, aspartic proteases, metalloproteases and aminopeptidases. They are used in the micromolar concentration range and are best added

(a) Subcellular membrane systems that can be isolated by differential centrifugation



(b) Scheme of subcellular fractionation of membranes from muscle homogenates

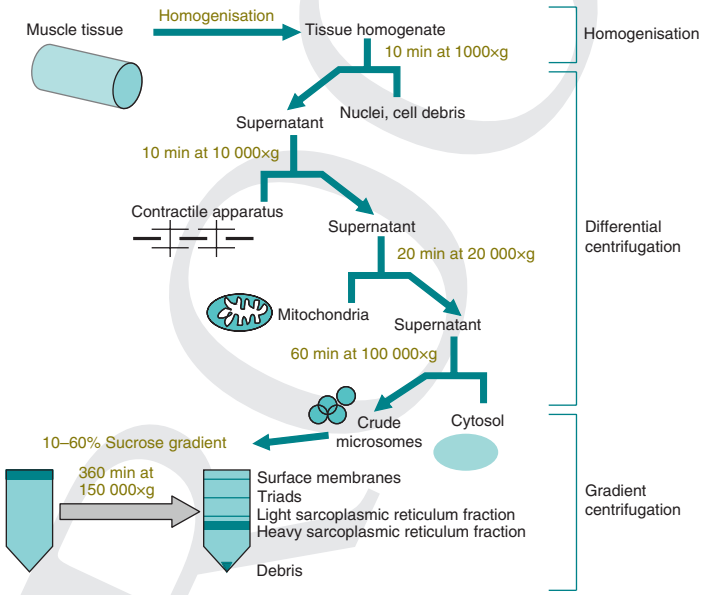


Figure 12.5 Scheme of the fractionation of skeletal muscle homogenate into various subcellular fractions. Shown is a diagrammatic presentation of the subcellular membrane system from skeletal muscle fibres (a) and a flow chart of the fractionation protocol of these membranes from tissue homogenates using differential centrifugation and density gradient methodology (b).

to buffer systems just prior to the tissue homogenisation process. Depending on the half-life of specific protease inhibitors, the length of a subcellular fractionation protocol and the amount of endogenous enzymes present in individual fractions, tissue suspensions might have to be replenished with a fresh aliquot of a protease inhibitor cocktail. Protease inhibitor kits for the creation of individualised cocktails are

also available and consist of substances such as trypsin inhibitor, E-64, aminoethyl-benzenesulfonyl-fluoride, antipain, aprotinin, benzamidine, bestatin, chymostatin, ε-aminocaproic acid, *N*-ethylmaleimide, leupeptin, phosphoramidon and pepstatin. The most commonly used chelators of divalent cations for the inhibition of degrading enzymes such as metalloproteases are EDTA and EGTA.

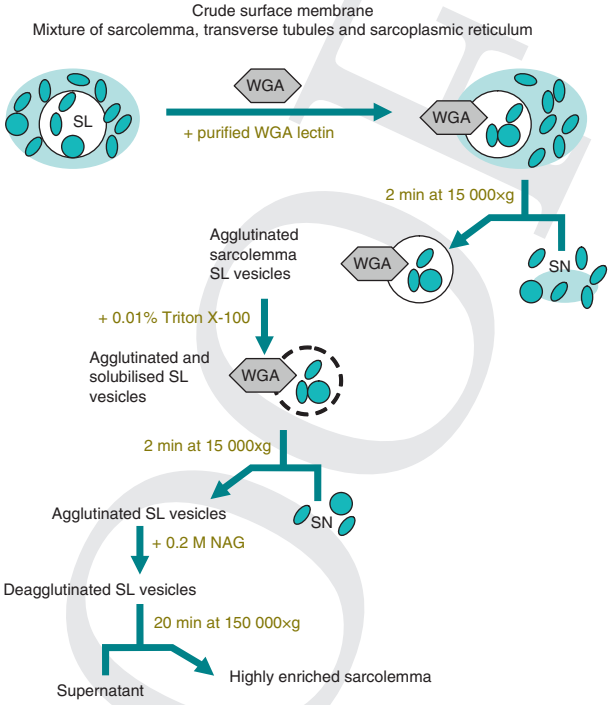
12.4.4 Subcellular Fractionation

A typical flow chart outlining a subcellular fractionation protocol is shown in Figure 12.5b. Depending on the amount of starting material, which would usually range between 1 g and 500 g in the case of skeletal muscle preparations, a particular type of rotor and size of centrifuge tube is chosen for individual stages of the isolation procedure. The repeated centrifugation at progressively higher speeds and longer centrifugation periods will divide the muscle homogenate into distinct fractions. Typical values for centrifugation steps are 10 min for 1000×g to pellet nuclei and cellular debris, 10 min for 10 000×g to pellet the contractile apparatus, 20 min at 20 000×g to pellet a fraction enriched in mitochondria, and 1 h at 100 000×g to separate the microsomal and cytosolic fractions. Mild salt washes can be carried out to remove myosin contamination of membrane preparations. Sucrose gradient centrifugation is then used to further separate microsomal subfractions derived from different muscle membranes. Using a vertical rotor or swinging-bucket rotor system at a sufficiently high g-force, the crude surface membrane fraction, triad junctions, longitudinal tubules and terminal cisternae membrane vesicles can be separated. To collect bands of fractions, the careful removal of fractions from the top can be achieved manually with a pipette. Alternatively, in the case of relatively unstable gradients or tight banding patterns, membrane vesicles can be harvested from the bottom by an automated fraction collector. In this case, the centrifuge tube is pierced and fractions collected by gravity or slowly forced out of the tube by a replacing liquid of higher density. Another method for collecting fractions from unstable gradients is the slicing of the centrifuge tube after freezing. Both latter methods destroy the centrifuge tubes and are routinely used in research laboratories.

Cross-contamination of vesicular membrane populations is an inevitable problem during subcellular fractionation procedures. The technical reason for this is the lack of adequate control in the formation of various types of membrane species during tissue homogenisation. Membrane domains originally derived from a similar subcellular location might form a variety of structures, including inside-out vesicles, right-side-out vesicles, sealed structures, leaky vesicles and/or membrane sheets. In addition, smaller vesicles might become entrapped in larger vesicles. Different membrane systems might aggregate non-specifically or bind to or entrap abundant solubilised proteins. Hence, if highly purified membrane preparations are needed for sophisticated cell biological or biochemical studies, affinity separation methodology has to be employed. The flow chart and immunoblotting diagram in Figure 12.6 illustrates both the preparative and analytical principles underlying such a biochemical approach. Modern preparative affinity techniques using centrifugation steps can be performed with various biological or chemical ligands. In the case of immuno-affinity purification, antibodies are used to specifically bind to their respective antigen (see also Section 7.11). For a

list of references outlining the use of subcellular fractionation methods in routine biochemical and proteomic applications, please consult the articles listed in Section 12.6.

(a) Scheme of subcellular fractionation of muscle sarcolemma



(b) Diagram of immunoblot analysis of subcellular fractionation procedures

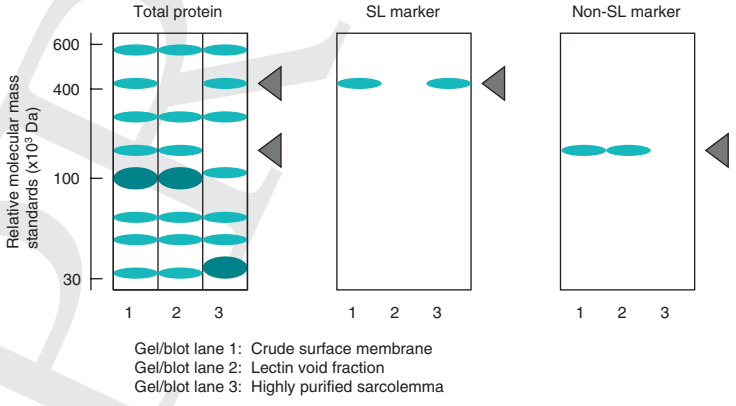


Figure 12.6 Affinity separation method using centrifugation of lectin-agglutinated surface membrane vesicles from skeletal muscle. Shown is a flow chart of the various preparative steps in the isolation of highly purified sarcolemma vesicles (a) (NAG, *N*-acetylglucosamine; SL, sarcolemma; SN, supernatant; WGA, wheat germ agglutinin) and a diagram of the immunoblot analysis of this subcellular fractionation procedure (b). The sarcolemma and non-SL markers are surface-associated dystrophin of 427 kDa and the transverse-tubular α_{1S}-subunit of the dihydropyridine receptor of 170 kDa, respectively.

12.4.5 Affinity Purification of Membrane Vesicles

In Figure 12.6a is shown a widely employed **lectin agglutination method**. Lectins are plant proteins that bind tightly to specific carbohydrate structures. The rationale behind using purified wheat germ agglutinin (WGA) lectin for the affinity purification of sarcolemmal vesicles is the fact that the muscle plasmalemma forms mostly right-side-out vesicles following homogenisation. By contrast, vesicles derived from the transverse tubules are mostly inside out and thus do not expose their carbohydrates. Glycoproteins from the abundant sarcoplasmic reticulum do not exhibit carbohydrate moieties that are recognised by this particular lectin species. Therefore, only sarcolemmal vesicles are agglutinated by the wheat germ lectin and the aggregate can be separated from the transverse tubular fraction by centrifugation for 2 min at 15 000×g. The electron microscopical characterisation of agglutinated surface membranes revealed large smooth sarcolemmal vesicles that had electron-dense entrapments. To remove these vesicular contaminants, originally derived from the sarcoplasmic reticulum, immobilised surface vesicles are treated with low concentrations of the non-ionic detergent Triton X-100. This procedure does not solubilise integral membrane proteins, but introduces openings in the sarcolemmal vesicles for the release of the much smaller sarcoplasmic reticulum vesicles. Low g-force centrifugation is then used to separate the agglutinated sarcolemmal vesicles and the contaminants. To remove the lectin from the purified vesicles, the fraction is incubated with the competitive sugar *N*-acetylglucosamine, which eliminates the bonds between the surface glycoproteins and the lectin. A final centrifugation step for 20 min at 150 000×g results in a pellet of highly purified sarcolemmal vesicles. A quick and convenient analytical method of confirming whether this subcellular fractionation procedure has resulted in the isolation of the muscle plasmalemma is immunoblotting with a mini electrophoresis unit. Figure 12.6b shows a diagram of the protein and antigen banding pattern of crude surface membranes, the lectin void fraction and the highly purified sarcolemmal fraction. Using antibodies to mark the transverse tubules and the sarcolemma, such as the α_{1S}-subunit of the dihydropyridine receptor of 170 kDa and dystrophin of 427 kDa, respectively, the separation of both membrane species can be monitored. This analytical method is especially useful for the characterisation of membrane vesicles, when no simple and fast assay systems for testing marker enzyme activities are available.

In the case of the separation of mitochondrial membranes, the distribution of enzyme activities rather than immunoblotting is routinely used for determining the distribution of the inner membrane, contact zones and the outer membrane in density gradients. Binding assays or enzyme testing represents the more traditional way of characterising subcellular fractions following centrifugation. Figure 12.7a outlines diagrammatically the micro compartments of liver mitochondria and the associated marker enzymes. While the monoamino oxidase (MAO) is enriched in the outer membrane, the enzyme succinate dehydrogenase (SDH) is associated with the inner membrane system and a representative marker of contact sites between both membranes is glutathione transferase (GT). Membrane vesicles from intact mitochondria

can be generated by consecutive swelling, shrinking and sonication of the suspended organelles. The vesicular mixture is then separated by sucrose density centrifugation into the three main types of mitochondrial membranes (Figure 12.7b). The distribution of marker enzyme activities in the various fractions demonstrates that the outer membrane has a lower density compared to the inner membrane. The glutathione transferase-containing contact zones are positioned in a band between the inner and outer mitochondrial membrane and contain enzyme activities characteristic for both systems (Figure 12.7c). Routinely used enzymes as subcellular markers would be Na^+/K^+-ATPase for the plasmalemma, glucose-6-phosphatase for the endoplasmic reticulum, galactosyl transferase for the Golgi apparatus, succinate dehydrogenase for

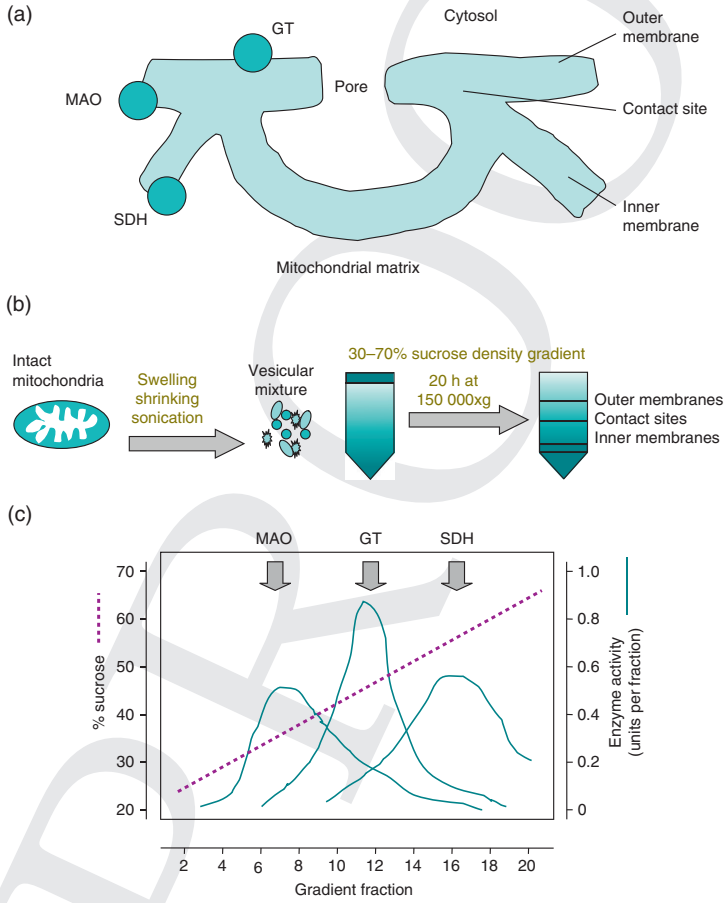


Figure 12.7 Scheme of the fractionation of membranes derived from liver mitochondria. Shown is the distribution of marker enzymes in the micro-compartments of liver mitochondria (MAO, monoamino oxidase; SDH, succinate dehydrogenase; GT, glutathione transferase) (a), the separation method to isolate fractions highly enriched in the inner cristae membrane, contact zones and the outer mitochondrial membrane (b), as well as the distribution of mitochondrial membranes after density-gradient centrifugation (c).

mitochondria, acid phosphatase for lysosomes, catalase for peroxisomes and lactate dehydrogenase for the cytosol.

12.4.6 Separation of DNA Components

A recent application of ultracentrifugation is in the genome-wide identification of gene regulatory regions, i.e. the open regions of DNA not protected by nucleosomes at that point in time. Intact nuclei are incubated with limiting amounts of DNase I, which is able to enter the nuclei and digest accessible DNA from chromatin. The digested DNA is then recovered and applied to a step gradient typically made with 10–40% sucrose (40% at the bottom of the tube, rising to 10% at the top), and subjected to 24 hours at 90 000×g (at 25 °C). Alternatively a 'sucrose cushion' can be used that is simply a fixed concentration of sucrose; typically 9%. The gentle separation of DNA fragments allows efficient molecular cloning of the DNA fragments that are then sequenced using massively parallel sequencing (Chapter 20).

12.5 ANALYTICAL ULTRACENTRIFUGATION

Analytical ultracentrifuges are high-speed ultracentrifuges with optical system(s) for recording the sedimentation process. As biological macromolecules exhibit random thermal motion, their relative uniform distribution in an aqueous environment is not significantly affected by the Earth's gravitational field. Isolated biomolecules in solution only exhibit distinguishable sedimentation when they undergo immense accelerations, e.g. in an ultracentrifugal field. A typical analytical ultracentrifuge can generate a centrifugal field of up to 200 000×g in its analytical cell. Within these extremely high gravitational fields, the ultracentrifuge cell has to allow light passage through the biological particles for proper measurement of the concentration distribution. The schematic diagram in Figure 12.8 outlines one of the two principal optical systems for a modern analytical ultracentrifuge. The availability of high-intensity xenon flash lamps and the advance in instrumental sensitivity and wavelength range has made the measurement of highly dilute protein samples below 230 nm possible. Analytical ultracentrifuges such as the Beckman Optima XL-A allow the use of wavelengths between 190 nm and 800 nm. Sedimentation of isolated proteins or nucleic acids can be useful in the determination of the molecular mass, purity and shape of these biomolecules. A second optical system (not shown) is the Rayleigh interference optical system, which detects the distribution of proteins or nucleic acids – or other macromolecules such as polysaccharides, glycoproteins and synthetic macromolecules – based on their different refractive index compared to the solvent they are dissolved in. The Beckman Optima XL-I, for example, has both optical systems. These two different optical systems can be applied individually, or simultaneously, which can be helpful for looking at interactions involving biomolecules with ligands. Other less common optical systems are based on fluorescence or refractive gradient "Schlieren" optics.

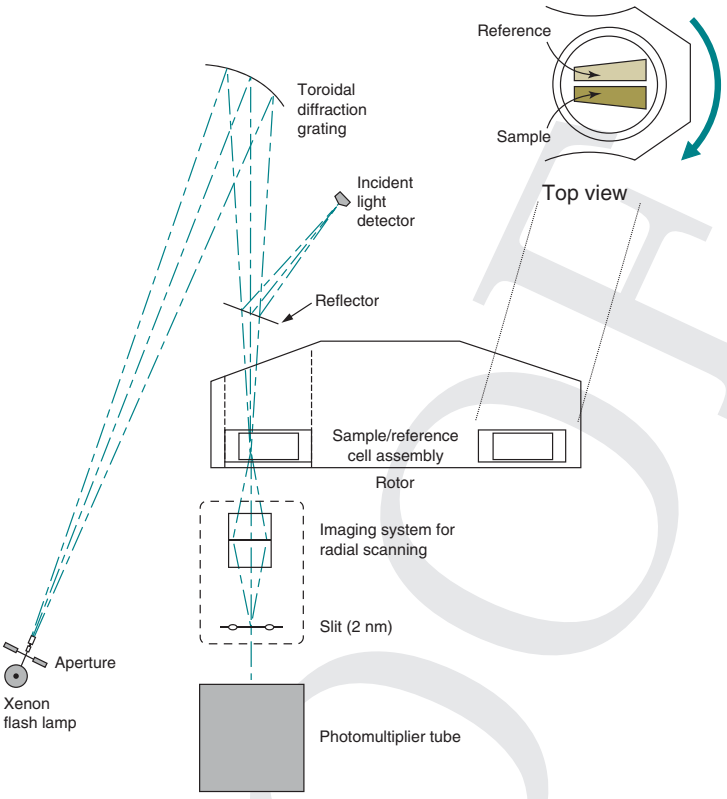


Figure 12.8 Schematic diagram of the absorption optical system of an analytical ultracentrifuge (courtesy of Beckman-Coulter). The high-intensity xenon flash lamp of the Beckman Optima XL-A analytical ultracentrifuge shown here allows the use of wavelengths between 190 nm and 800 nm. The high sensitivity of the absorbance optics allows the measurement of highly dilute protein samples below 230 nm. This is the simplest of the two main optical systems available on a modern analytical ultracentrifuge. An alternative optical system, based on refractive index properties, is known as the Rayleigh interference optical system.

12.5.1 Sedimentation Velocity, Sedimentation Equilibrium and Their Applications

There are two main types of analytical ultracentrifuge experiments: sedimentation velocity and sedimentation equilibrium experiments. In **sedimentation velocity** experiments, the change in concentration distribution in the ultracentrifuge cell at high rotor speeds is recorded. A boundary is formed between solution and clear solvent left behind and the rate of movement of the boundary per unit centrifugal field yields the sedimentation coefficient (Equation 12.8), which will be a function of the size (molecular mass) and shape (based on friction properties) of the system. For heterogeneous systems the method will give a **distribution of sedimentation coefficient**.

In contrast, in **sedimentation equilibrium** experiments an equilibrium or steady-state distribution of concentration is obtained. Here, lower rotor speeds are used and the centrifugal forces are countered by the back forces due to diffusion and no boundary is formed. Instead, after a period of time (usually at least several hours) an equilibrium is reached, and there will be no net movement of macromolecules, hence

no friction forces: the pattern only depends on molecular mass. The two methods – sedimentation velocity and sedimentation equilibrium – provide complementary information about a macromolecular system. Sedimentation velocity has a high resolution, which is excellent for monitoring heterogeneity and aggregation and also for evaluating molecular conformation in solution. Sedimentation equilibrium is an 'absolute' method (not requiring standards) for molecular mass and (in heterogeneous systems) average molecular mass determination. For systems containing non-covalent assemblies, both methods can provide valuable information about the stoichiometry and strength of self-association reactions (i.e. the quaternary structure: protein dimerisation, tetramerisation, etc.) or interactions with other molecules.

Analytical ultracentrifugation is most often employed for:

- the determination of the purity (including the presence of aggregates) and oligomeric state of macromolecules, from recording the distribution of sedimentation coefficients from sedimentation velocity
- the determination of the average molecular mass, or distribution of molecular mass of solutes in their native state, from sedimentation equilibrium
- the examination of changes in the molecular mass of supramolecular complexes, using either sedimentation velocity or sedimentation equilibrium (or both)
- the detection of conformation and conformational changes using sedimentation velocity
- ligand-binding studies (see also Section 23.5.3).

Since the mass of one molecule is extremely small, when researchers refer to the 'molecular mass' of a molecule, they really mean the **molar mass** M which describes the mass of 1 mol (= 6.023×10^{23} molecules) of macromolecules, in units of g mol^{-1}, or, equivalently, the **relative molecular mass** M_r, which is the mass of a macromolecule per one-twelfth of the mass of a carbon-12 atom (see also Section 2.3.1). Molar mass and relative molecular mass are numerically the same, but being a relative measure, M_r has no units.

For a list of references outlining the applicability of ultracentrifugation to the characterisation of macromolecular behaviour in solution, please consult the review articles listed in Section 12.6. In addition, manufacturers of analytical ultracentrifuges offer a large range of excellent brochures on the theoretical background of this method and its specific applications available. These introductory texts are usually written by research biochemists and are well worth reading to become familiar with this field.

12.5.2 Sedimentation Coefficient and Sedimentation Coefficient Distribution

The sedimentation coefficient, as defined by Equation 12.8 (after normalisation to standard conditions), will depend on the size (molecular mass) of a macromolecule. For particles of near-globular shape, $s_{20,w}$ is approximately proportional to $M^{2/3}$. The value of $s_{20,w}$ lies for many macromolecules of biochemical interest typically between 1 and 20, and for larger biological particles such as ribosomes, microsomes and mitochondria between 80 and several thousand. Figure 12.9 shows the optical records from a sedimentation velocity experiment on a tetanus toxoid protein used in the production of glycoconjugate vaccines against serious disease. The experiment was conducted to determine the sedimentation coefficient of the monomer species and any other components present, to establish the amount of monomer compared to other species. The presence of dimer can clearly be seen for a whole range of different concentrations used.

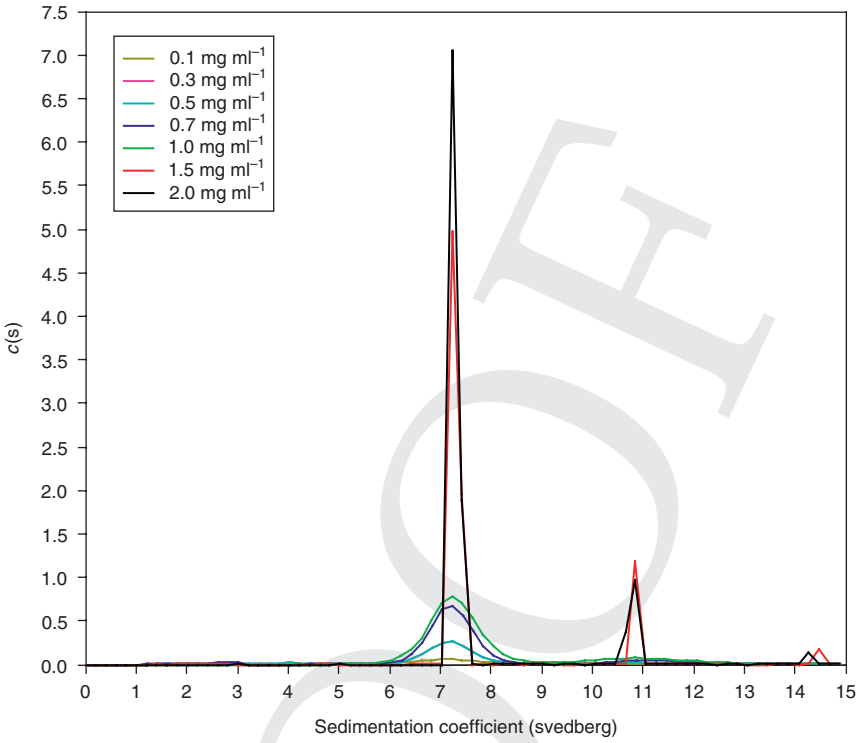


Figure 12.9 Sedimentation coefficient distribution $c(s)$ versus s for tetanus toxoid protein showing ~86% monomer with a sedimentation coefficient of 7.6 S and ~14% of dimer at higher s (11.6 S). A rotor speed of rpm = 45 000 min^{-1} was used at a temperature of 20.0 °C. Protein solutions were dissolved in a phosphate chloride buffer, pH 6.8, ionic strength 0.1 M. The relative proportions of monomer and dimer do not change with loading concentration, showing the dimerisation process is not reversible. If it was reversible then the proportion of dimer should increase with increasing concentration. Some evidence of a trace amount of a higher-molecular-weight species (with a sedimentation coefficient of approximately 14.5 S) is also seen.

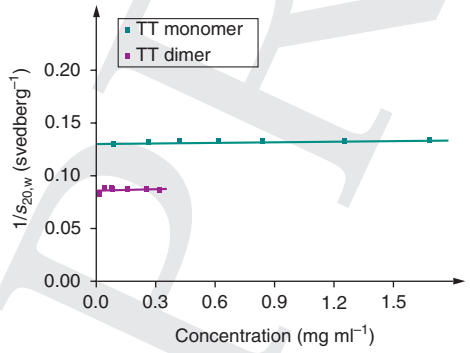


Figure 12.10 Extrapolation of the reciprocal sedimentation coefficients to zero concentration to correct for non-ideality effects of tetanus toxoid monomers and dimers.

If the molecular mass M is known, the sedimentation coefficient can be used to calculate the frictional coefficient f, which is a measure of the conformation of a macromolecule:

$$f = \frac{M \times \left(1 - 0.9982 \text{ g ml}^{-1} \times \overline{v}\right)}{\text{N}_\text{A} \times s_{20,\text{w}}} \qquad \text{(Eq 12.9)}$$

0.9982 g ml^{-1} is the density of water at 20.0 °C, N_A is the Avogadro constant and v is the partial specific volume (in ml g^{-1}) of the protein. For studies attempting accurate characterisation of conformation, another correction of the sedimentation coefficient is usually necessary. Because of the large size of macromolecules compared to the solvent they are dissolved in, they exhibit **non-ideality**: (i) they get in the way of each other, and (ii) if they are carrying charge, macromolecules can repel each other. The charge effect can be reduced by increasing the **ionic strength** (concentration of low-molecular-weight salt ions) of the solvent or, in the case of proteins, working near or at the isoelectric pH. Both effects can be reduced by working at low concentrations of macromolecules, or by taking measurements of $s_{20,w}$ at a series of concentrations, c and extrapolating to zero concentration to yield corrected ($s^0_{20,w}$) values. More accurate extrapolations are generally obtained if the reciprocal $1/s_{20,w}$ is plotted versus c. Figure 12.10 shows such an extrapolation for the tetanus toxoid data of Figure 12.9.

The conformation can be interpreted in terms of ellipsoid models for globular proteins (Figure 12.11a) or bead models (Figure 12.11b) for more complicated structures such as antibodies. It is usually necessary to combine f with other physical data, such as from viscosity or small-angle X-ray scattering measurements (see Section 14.3.5).

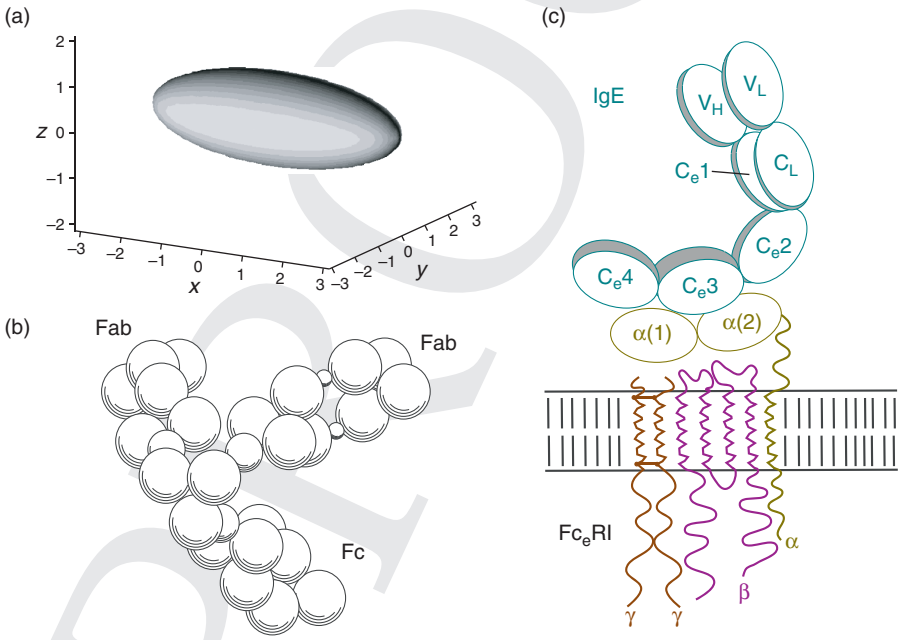


Figure 12.11 Models for the conformations of proteins from the sedimentation coefficient, molecular mass and other information. (a) Ellipsoid model of axial ratio (ratio of long axis to short axis) of 3:1 for tetanus toxoid protein, and was obtained by combining analytical ultracentrifugation data with intrinsic viscosity data. (b) Bead model for antibody IgE (note the individual beads do not represent atoms or domains). This was the first demonstration of the cusp shape of the IgE molecule and was obtained by combining analytical ultracentrifugation data with the radius of gyration from X-ray scattering, later confirmed by spectroscopic and crystallographic data. (c) Schematic representation showing how the cusp shape of IgE facilitates its binding via its $C_\varepsilon3$ domain to the α-chain of the high affinity membrane IgE receptor known as $Fc_\varepsilon RI$, shown with its constituent α, β, and two γ polypeptide chains.

Density Gradients

For very complex systems, the addition of a density gradient material can assist with the resolution of components. Figure 12.12 illustrates the sedimentation analysis of the dystrophin–glycoprotein complex (DGC) from skeletal muscle fibres. The size of this complex was estimated to be approximately 18 S by comparing its migration to that of the standards β-galactosidase (16 S) and thyroglobulin (19 S). When the membrane cytoskeletal protein dystrophin was first identified, it was shown to bind to a lectin matrix, although the protein does not possess any carbohydrate conjugation; this suggested that dystrophin might exist in a complex with surface glycoproteins. Sedimentation velocity analysis confirmed the existence of such a dystrophin–glycoprotein complex and centrifugation following various biochemical modifications of the protein assembly led to a detailed understanding of the composition of this complex. Alkaline extraction, acid treatment or incubation with different types of detergent causes the differential disassembly of the dystrophin–glycoprotein complex. It is now known that dystrophin is tightly associated with at least 10 different surface proteins that are involved in membrane stabilisation, receptor anchoring and signal transduction processes. The successful characterisation of the dystrophin–glycoprotein complex by sedimentation analysis is an excellent example of how centrifugation methodology can be exploited to quickly gain biochemical knowledge of a newly discovered protein.

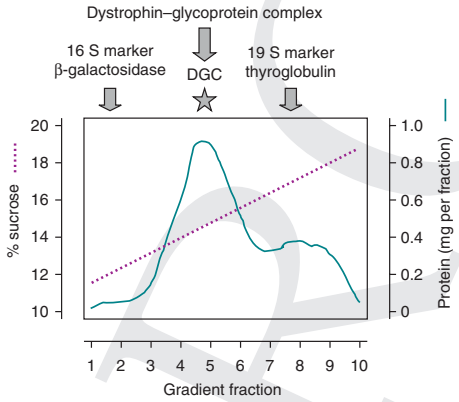


Figure 12.12 Sedimentation analysis of a supramolecular protein complex. Shown is the sedimentation of the dystrophin–glycoprotein complex (DGC). Its size was estimated to be approximately 18 S by comparing its migration to that of the standards β-galactosidase (16 S) and thyroglobulin (19 S).

12.5.3 Molecular Mass Determination

After equilibrium has been obtained in a sedimentation equilibrium experiment, the dataset of concentration in the ultracentrifuge cell $c(r)$ as a function of radial position r can be processed to yield the molar mass M (or the average molecular mass if the system is heterogeneous). For a single solute, a plot of ln $c(r)$ versus r^2 yields a straight line with a slope proportional to M (Figure 12.13). The proportionality constant depends on the rotor speed, temperature, density of the solvent, and the partial specific volume v. For a heterogeneous system, the plot will be curved upwards, and an average slope is defined, yielding what is known as the weight average molecular mass M_w. In addition, it is possible to see if M_w changes with concentration. If M_w increases with increasing

concentration this is symptomatic of an associating or interacting system – further analysis of this can yield the stoichiometry and strength of the interaction.

Molecular (molar) mass determination by analytical ultracentrifugation is applicable to values from a few hundred to several million Da. It is therefore used for the analysis of small carbohydrates, proteins, nucleic acid macromolecules, viruses and subcellular particles such as mitochondria.

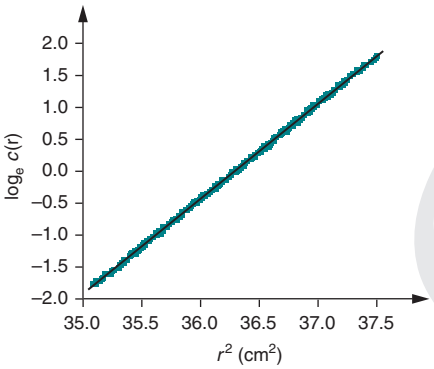


Figure 12.13 Sedimentation equilibrium experiment on IgG1 in phosphate-chloride buffer, pH 6.8, ionic strength 0.1 M, $\theta = 20.0°$; rotor speed $s = 13\,000$ rpm, loading concentration $\tilde{\rho}^{\circ} = 1$ mg ml^{-1}. Data points (blue squares), fitted line (black). M_w (from the slope) = (148±2) kDa.

12.6 SUGGESTIONS FOR FURTHER READING

12.6.1 Experimental Protocols

Abdelhameed A.S., Morris G.A., Adams G.G., *et al.* (2012) An asymmetric and slightly dimerized structure for the tetanus toxoid protein used in glycoconjugate vaccines. *Carbohydrate Polymers* **90**, 1831–1835.

Burgess N. K., Stanley A. M. and Fleming K. G. (2008). Determination of membrane protein molecular weights and association equilibrium constants using sedimentation equilibrium and sedimentation velocity. *Methods in Cell Biology* **84**, 181–211.

Carberry S., Zweyer M., Swandulla D. and Ohlendieck K. (2014) Comparative proteomic analysis of the contractile-protein-depleted fraction from normal versus dystrophic skeletal muscle. *Analytical Biochemistry* **446**, 108–115.

Davis K.G., Glennie M., Harding S.E. and Burton, D.R. (1990) A model for the solution conformation of rat IgE. *Biochemical Society Transactions* **18**, 935–936.

Klassen R., Fricke J., Pfeiffer A. and Meinhardt F. (2008) A modified DNA isolation protocol for obtaining pure RT-PCR grade RNA. *Biotechnology Letters* **30**, 1041–1044.

Lee Y.H., Tan H.T. and Chung M.C. (2010) Subcellular fractionation methods and strategies for proteomics. *Proteomics* **10**, 3935–3956.

Sutton B.J. and Gould H.J. (1993) The human IgE network. *Nature* **366**, 421–428.

12.6.2 General Texts

Schuck P., Zhao H., Brautigam C.A. and Ghirlando R. (2015) *Basic Principles of Analytical Ultracentrifugation*, CRC Press, Boca Raton, FL, USA.

Scott D., Harding S.E. and Rowe, A. (2005) *Analytical Ultracentrifugation: Techniques and Methods*, Royal Society of Chemistry, London, UK.

12.6.3 Review Articles

Cole J.L., Lary J.W., Moody T.P. and Laue T. M. (2008) Analytical ultracentrifugation: sedimentation velocity and sedimentation equilibrium, *Methods in Cell Biology* 84, 143–179.

12.6.4 Websites

SEDFIT Tutorials (Peter Schuck)
www.analyticalultracentrifugation.com/tutorials.htm (accessed May 2017)