3 Centrifugation

K. OHLENDIECK

3.1 INTRODUCTION

Biological **centrifugation** is a process that uses **centrifugal force** to separate and purify mixtures of biological particles in a liquid medium. It is a key technique for isolating and analysing cells, subcellular fractions, supramolecular complexes and isolated macromolecules such as proteins or nucleic acids. The development of the first **analytical ultracentrifuge** 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 centrifugation** 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 design of various rotors and separation processes is 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 of 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 11), but can also be utilised to separate intact organelles and membrane vesicles by centrifugation, lectin affinity agglutination of highly purified plasmalemma 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. 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 10 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 8), 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.

3.2 BASIC PRINCIPLES OF SEDIMENTATION

From everyday experience, the effect of **sedimentation** due to the influence of the Earth's gravitational field ($g = 981\ \mathrm{cm\,s^{-2}}$) versus the increased rate of sedimentation in a centrifugal field ($g > 981\ \mathrm{cm\,s^{-2}}$) 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, 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.

From the equation (3.1) for the calculation of the relative centrifugal field it becomes apparent that when the conditions for the centrifugal separation of a biological particle are described, a detailed listing of **rotor speed**, radial dimensions and duration of centrifugation has to be provided. Essentially, the rate of sedimentation is dependent upon the **applied centrifugal field** ($\mathrm{cm\,s^{-2}}$), G, that 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):

$$G = \omega^2 r \tag{3.1}$$

The average angular velocity of a rigid body that rotates about 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 a length equal to the radius of the circle. Since 360° equals 2π radians, one revolution of the rotor can be expressed as 2π rad. Accordingly, the angular velocity in rads per second of the rotor can be expressed in terms of rotor speed s as:

$$\omega = \frac{2\pi s}{60} \tag{3.2}$$

Example 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 $\mathrm{s^{-1}}$?

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

$G = \omega^2 r = (3000)^2 \times 5\ \mathrm{cm\,s^{-2}} = 4.5 \times 10^7\ \mathrm{cm\,s^{-2}}$

and therefore the centrifugal field can be expressed as:

$$G = \frac{4\pi^2(\text{rev min}^{-1})^2 r}{3600} = \frac{4\pi^2 s^2 r}{3600} \tag{3.3}$$

Example 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 r.p.m. Calculate the angular velocity, $\boldsymbol{\omega}$, in radians per second.

Answer The angular velocity, $\boldsymbol{\omega}$, may be calculated using the equation:

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

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

The centrifugal field is generally expressed in multiples of the gravitational field, g (981 cm s^{-2}). The **relative centrifugal field** (g), RCF, which is the ratio of the centrifugal acceleration at a specified radius and the speed to the standard acceleration of gravity, can be calculated from the following equation:

$$\text{RCF} = \frac{4\pi^2(\text{rev min}^{-1})^2 r}{3600 \times 981} = \frac{G}{g} \tag{3.4}$$

RCF units are therefore dimensionless (denoting multiples of g) and revolutions per minute are usually abbreviated as r.p.m.: RCF $= 1.12 \times 10^{-5}$ r.p.m.$^2 r$.

Although the relative centrifugal force can easily be calculated, centrifugation manuals usually 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 the centrifuge tube. A nomograph consists of three columns representing the radial distance (in mm), the relative centrifugal field and the rotor speed (in r.p.m.). For the conversion between relative centrifugal force and speed of the centrifuge spindle in r.p.m. 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 3.1 for an illustration of the usage of a nomograph.

In a suspension of biological particles, the rate of sedimentation is dependent not only upon the applied centrifugal field, but also on the nature of the particle, i.e. its density and radius, and also the viscosity of the surrounding medium. *Stokes' Law* describes these relationships for the sedimentation of a rigid spherical particle:

$$\nu = \frac{2}{9} \frac{r^2(\rho_p - \rho_m)}{\eta} \times g \tag{3.5}$$

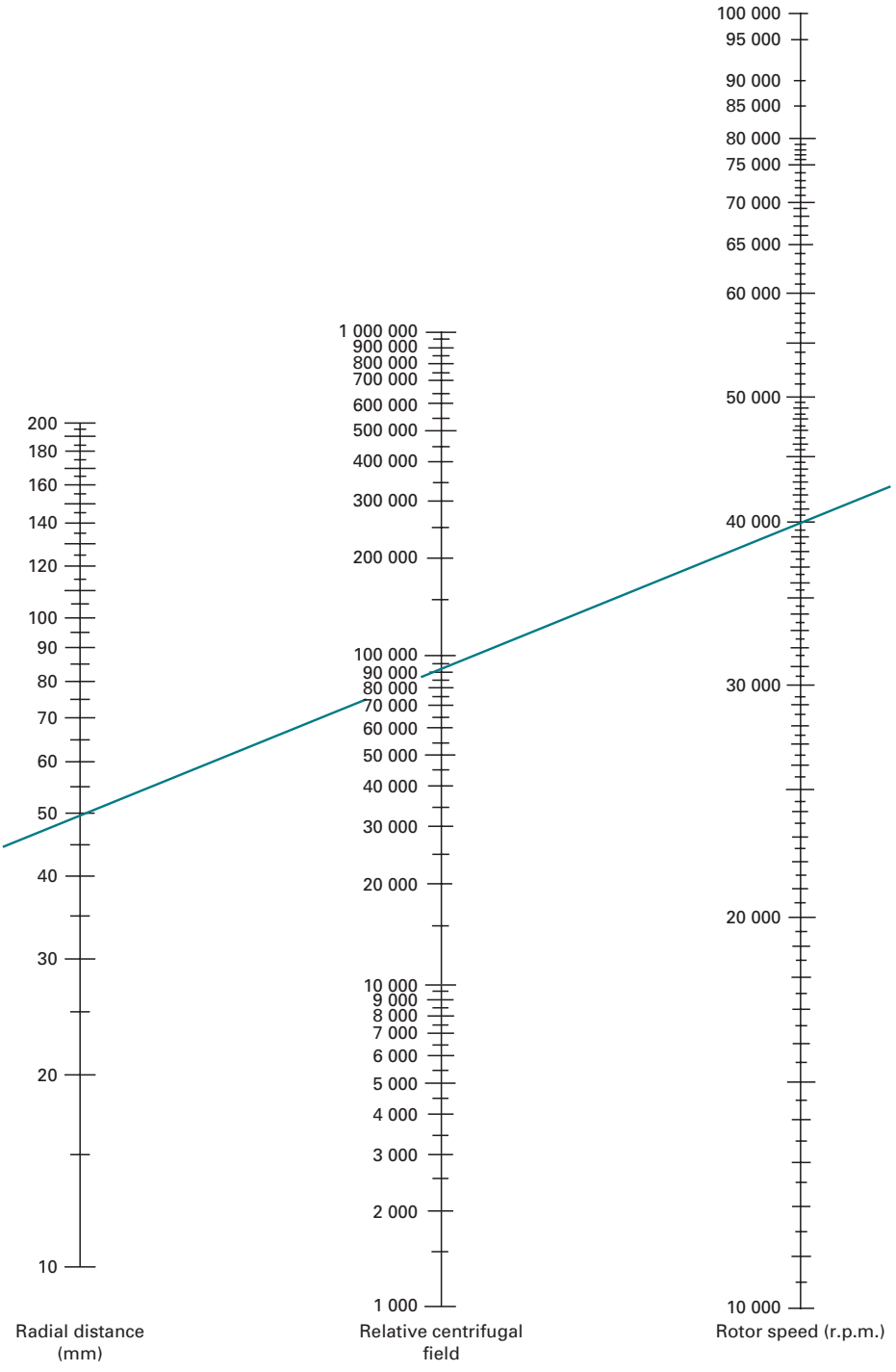


Fig. 3.1 Nomograph for the determination of the relative centrifugal field for a given rotor speed and radius. The three columns represent the radial distance (in mm), the relative centrifugal field and the rotor speed (in r.p.m.). For the conversion between relative centrifugal force and speed of the 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. (Courtesy of Beckman-Coulter.)

where ν is the sedimentation rate of the sphere, 2/9 is the shape factor constant for a sphere, r 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.

Example 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 Fig. 3.2a for a cross-sectional diagram of a fixed-angle rotor illustrating the position of the minimum and maximum radius. If the rotor is operated at a speed of 20 000 r.p.m., what is the relative centrifugal field, RCF, at the top and bottom of the centrifuge tube?

Answer The relative centrifugal field may be calculated using the equation:

$$RCF = 1{,}12 \times 10^{-5}\ \text{r.p.m.}^2 r$$

Top of centrifuge tube:

$$RCF = 1{,}12 \times 10^{-5} \times (20\,000)^2 \times 3.5 = 15\,680$$

Bottom of centrifuge tube:

$$RCF = 1{,}12 \times 10^{-5} \times (20\,000)^2 \times 7.0 = 31\,360$$

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 two-fold.

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:

$$t = \frac{9}{2} \frac{\eta}{\omega^2 r_p^2 (\rho_p - \rho_m)} \times \ln \frac{r_b}{r_t} \tag{3.6}$$

where t is the sedimentation time, η is the viscosity of medium, r_p is the radius of 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 rotor.

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

$$s = \frac{\nu}{\omega^2 r} \tag{3.7}$$

Since the sedimentation rate per unit centrifugal field can be determined at different temperatures and with various media, experimental values of the sedimentation coefficient are corrected to a sedimentation constant theoretically obtainable in water at 20 °C, yielding the $S_{20,W}$ value. The sedimentation coefficients of biological

macromolecules are relatively small, and are usually expressed (see Section 3.5), as Svedberg units, S. One Svedberg unit equals 10^{-13} s.

3.3 TYPES, CARE AND SAFETY ASPECTS OF CENTRIFUGES

3.3.1 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; and
- 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**;
- refrigerated high-speed preparative centrifuges;
- **analytical ultracentrifuges**;
- **preparative ultracentrifuges**;
- large-scale **clinical centrifuges**; and
- 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 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 3.4, 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. On the other hand, 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 3.3.2 and 3.3.4 for a more detailed discussion of safety and centrifugation). In contrast to preparative ultracentrifuges, analytical ultracentrifuges contain a solid rotor which in its simplest form incorporates one analytical cell and one counterbalancing cell. An optical system enables the sedimenting material to be observed throughout the duration of centrifugation. Using a light absorption system, a **Schlieren system** or a **Raleigh interferometric system**, concentration distributions in the biological sample are determined at any time during ultracentrifugation. The Raleigh and Schlieren optical systems detect changes in the refractive index of the solution caused by concentration changes and can thus be used for sedimentation equilibrium analysis. This makes analytical ultracentrifugation a relatively accurate tool for the determination of the molecular mass of an isolated macromolecule. It can also provide crucial information about the thermodynamic properties of a protein or other large biomolecules.

3.3.2 Types of rotors

To illustrate the difference in design of **fixed-angle rotors, vertical tube rotors** and **swinging-bucket rotors**, Fig. 3.2 outlines cross-sectional diagrams of these three main types of rotors. Companies usually name rotors according to their type of design, the maximum allowable speed and sometimes the material composition. Depending 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. 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 an electrochemically formed tough layer of aluminium oxide. In order to avoid damaging these protective layers, care should be taken during rotor handling.

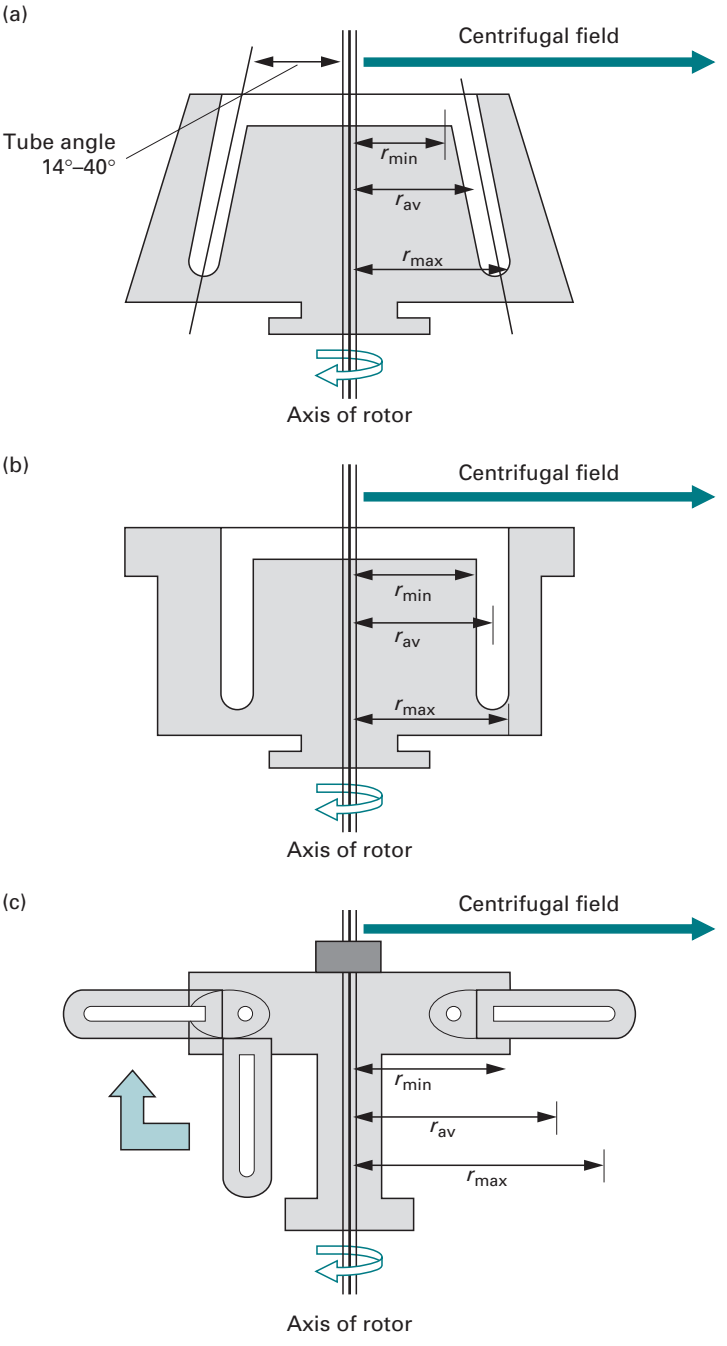


Fig. 3.2 Design of the three main types of rotors used in routine biochemical centrifugation techniques. Shown is a cross-sectional diagram of a fixed-angle rotor (a), a vertical tube rotor (b), and a swinging-bucket rotor (c). A fourth type of rotor is represented by the class of near-vertical rotors (not shown).

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 banding 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 rotors (Fig. 3.2a). 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 (Fig. 3.2b) 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 (Fig. 3.2c). They are loaded in a vertical position and during the initial acceleration phase, 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, Fig. 3.3 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 (Fig. 3.3a). 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 (Fig. 3.3b). 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 (Fig. 3.3c), such as in rate zonal studies based on the separation of biological particles as a function of sedimentation coefficient.

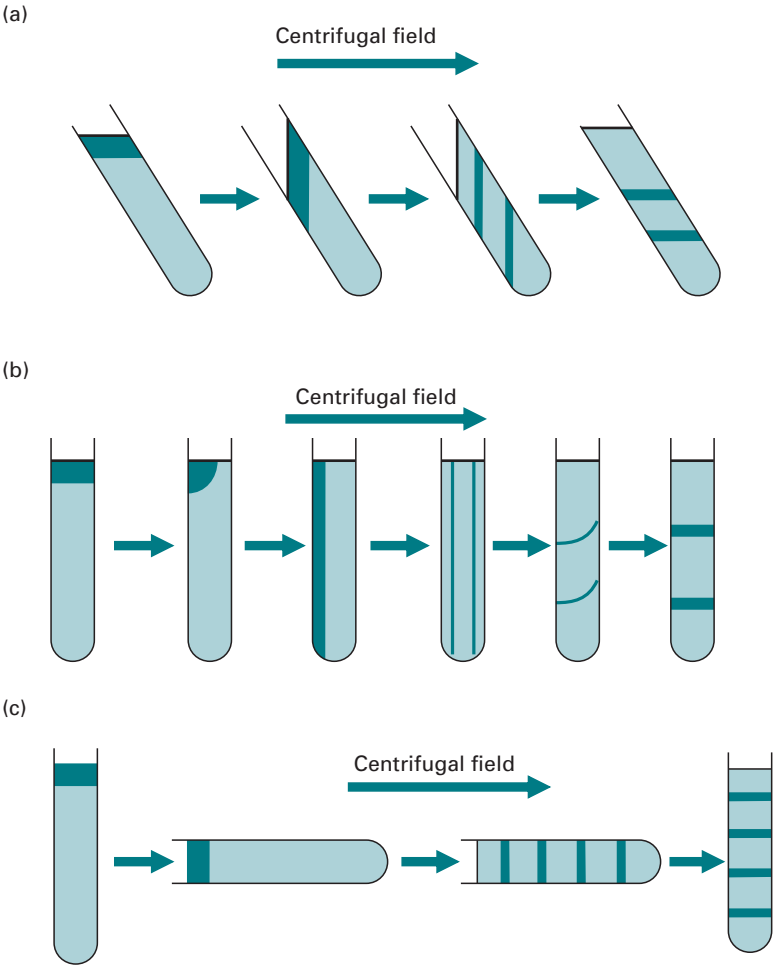


Fig. 3.3 Operation of the three main types of rotors used in routine biochemical centrifugation techniques. Shown is a cross-sectional diagram of a centrifuge tube positioned in a fixed-angle rotor (a), a vertical tube rotor (b), and a swinging-bucket rotor (c). The diagrams illustrate the movement of biological samples during the initial acceleration stage, the main centrifugal separation phase, de-acceleration 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.

3.3.3 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 deacceleration of rotors may lead to metal fatigue.

3.3.4 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 recommendation 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.

3.4 PREPARATIVE CENTRIFUGATION

3.4.1 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

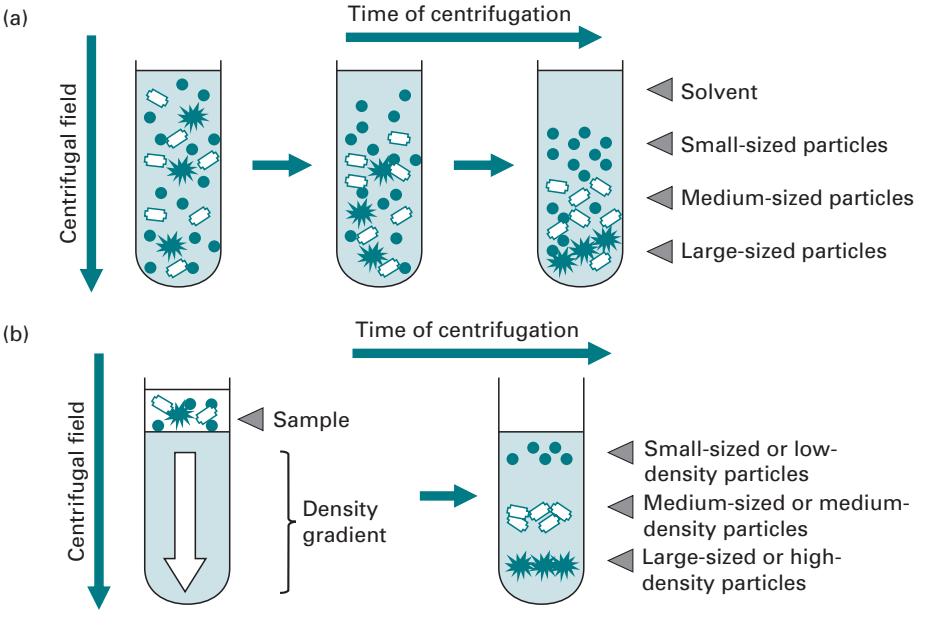


Fig. 3.4 Diagram of particle behaviour during differential and isopycnic separation. During differential sedimentation (a) of a particulate suspension in a centrifugal field, the movement of particles is dependent upon their density, shape and size. For separation of biological particles using a density 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.

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 recentrifuged. 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.

The differential sedimentation of a particulate suspension in a centrifugal field is diagrammatically shown in Fig. 3.4a. 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 recentrifugation 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.

3.4.2 Density-gradient centrifugation

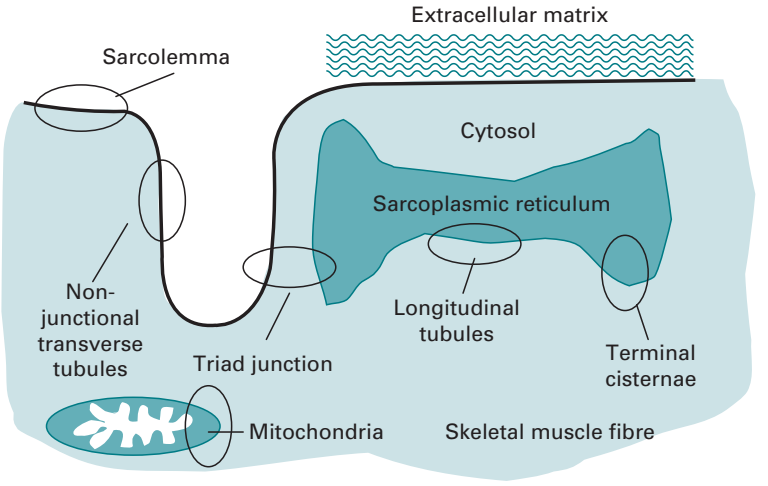
To further separate biological particles of similar size but differing density, ultracentrifugation with preformed or self-establishing **density gradients** is the method of choice. Both rate separation or equilibrium methods can be used. In Fig. 3.4b, the preparative ultracentrifugation of low- to 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 preformed 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 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.

3.4.3 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 (Fig. 3.5), the isolation of highly purified sarcolemma vesicles outlined (Fig. 3.6), and the subfractionation of liver mitochondrial membrane systems shown (Fig. 3.7). 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 Fig. 3.5a. 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 co-factors 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 2200 kDa, named titin. Commercially available protease inhibitor cocktails usually exhibit a broad specificity for the inhibition of cysteine-proteases, serine-proteases, aspartic-proteases, metallo-proteases and amino-peptidases. They are used in the micromolar concentration range and are best added 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

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



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

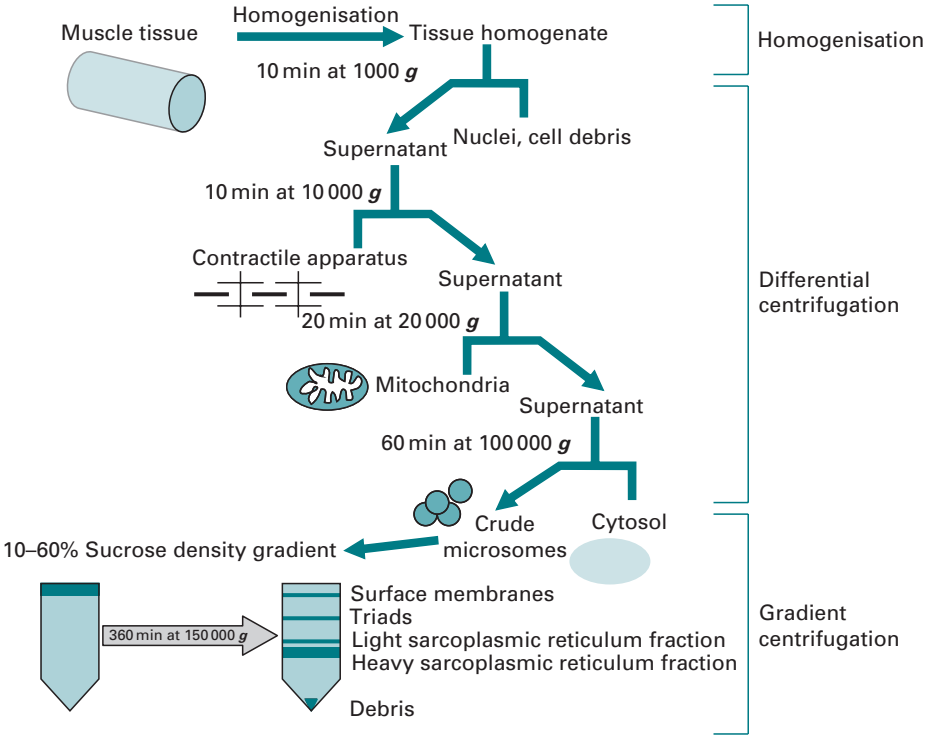


Fig. 3.5 Scheme of the fractionation of skeletal muscle homogenate into various subcellular fractions. Shown is a diagrammatic presentation of the subcellular membrane system from skeletal muscle fibres (a) and a flow chart of the fractionation protocol of these membranes from tissue homogenates using differential centrifugation and density gradient methodology (b).

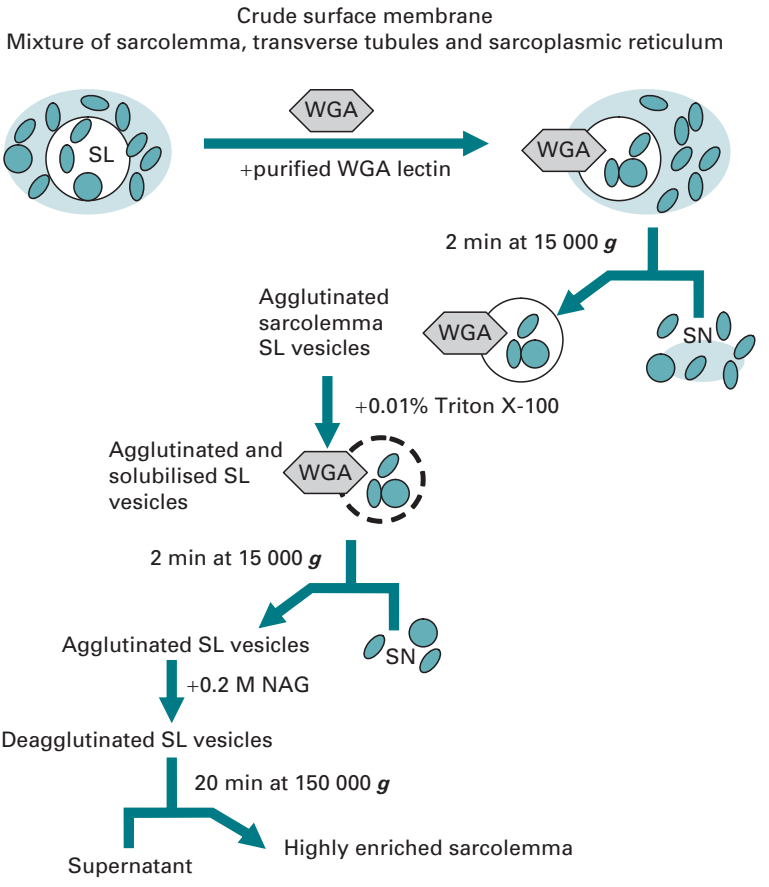
and consist of substances such as trypsin inhibitor, E-64, aminoethyl-benzenesulfonyl-fluoride, antipain, aprotinin, benzamidine, bestatin, chymostatin, ϵ-aminocaproic acid, *N*-ethylmaleimide, leupeptin, phosphoramidon and pepstatin. The most commonly used chelators of divalent cations for the inhibition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

3.4.4 Subcellular fractionation

A typical flow chart outlining a subcellular fractionation protocol is shown in Fig. 3.5b. Depending on the amount of starting material, which would usually range between 1 and 500 g in the case of skeletal muscle preparations, a particular type of rotor and size of centrifuge tubes 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 Fig. 3.6 illustrates both the preparative and analytical principles underlying such a biochemical approach. Modern preparative affinity techniques using centrifugation steps can be performed

(a) Scheme of subcellular fractionation of muscle sarcolemma



(b) Diagram of immunoblot analysis of subcellular fractionation procedures

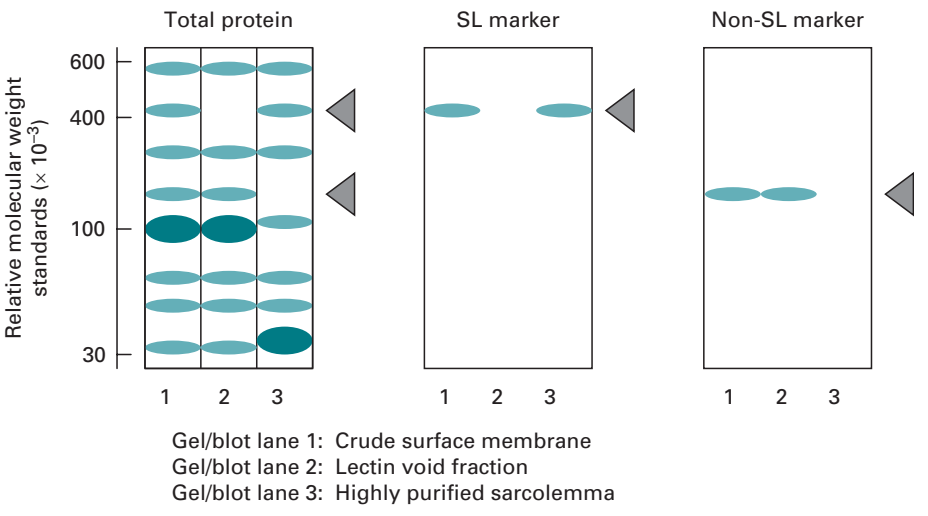


Fig. 3.6 Affinity separation method using centrifugation of lectin-agglutinated surface membrane vesicles from skeletal muscle. Shown is a flow chart of the various preparative steps in the isolation of highly purified sarcolemma vesicles (a) and a diagram of the immunoblot analysis of this subcellular fractionation procedure (b). The sarcolemma (SL) 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.

with various biological or chemical ligands. In the case of immuno affinity purification, antibodies are used to specifically bind to their respective antigen.

3.4.5 Affinity purification of membrane vesicles

In Fig. 3.6a 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 sarcolemma 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 sarcolemma 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 sarcolemma 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 sarcolemma vesicles for the release of the much smaller sarcoplasmic reticulum vesicles. Low *g*-force centrifugation is then used to separate the agglutinated sarcolemma vesicles and the contaminants. To remove the lectin from the purified vesicles, the fraction is incubated with the competitive sugar *N*-acetylglucosamine that 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 sarcolemma 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 3.6b shows a diagram of the protein and antigen banding pattern of crude surface membranes, the lectin void fraction and the highly purified sarcolemma fraction. Using antibodies to markers of 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 3.7a 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

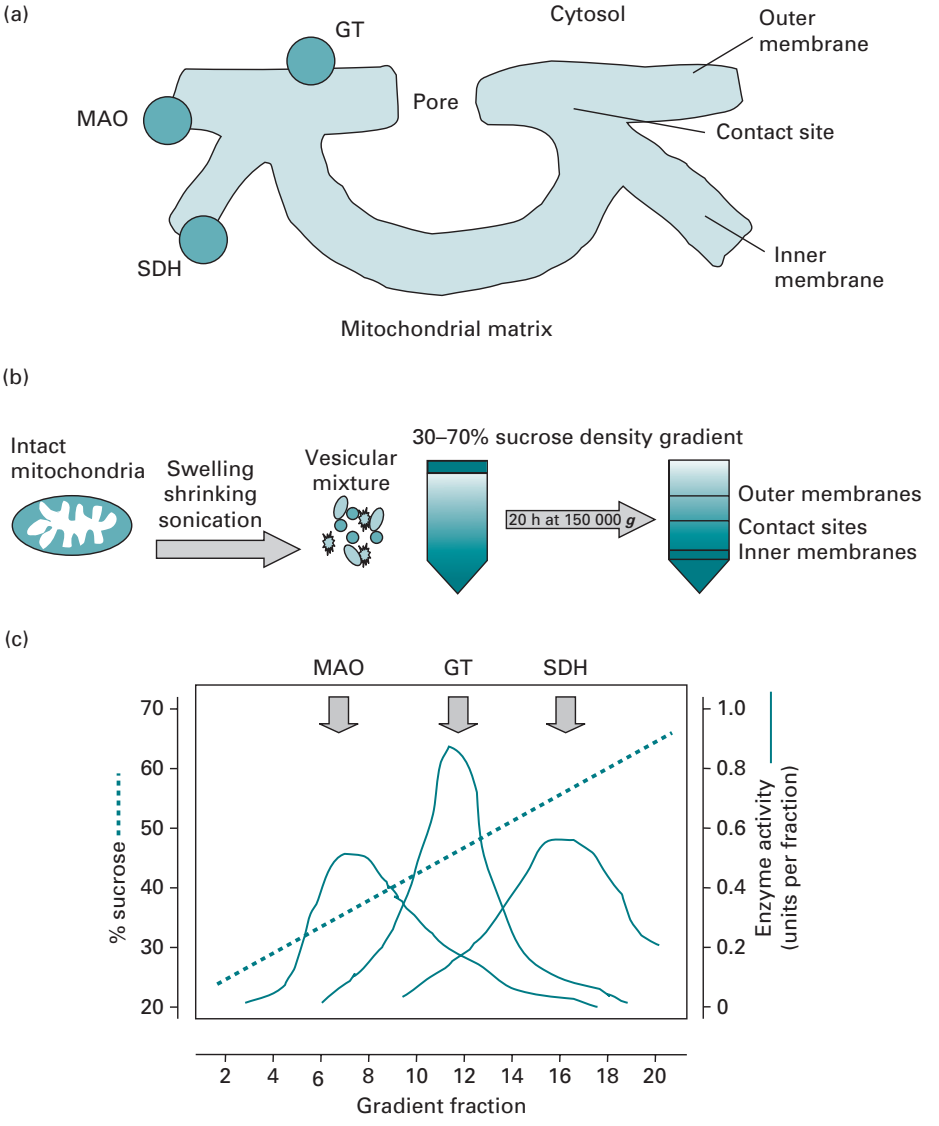


Fig. 3.7 Scheme of the fractionation of membranes derived from liver mitochondria. Shown is the distribution of marker enzymes in the micro compartments of liver mitochondria (MAO, monoamino oxidase; SDH, succinate dehydrogenase; GT, glutathione transferase) (a), the separation method to isolate fractions 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).

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 (Fig. 3.7b). 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 (Fig. 3.7c). Routinely used enzymes as subcellular markers would be the Na^+/K^+-ATPase for the plasmalemma, glucose-6-phosphatase for the endoplasmic reticulum, galactosyl transferase for the Golgi apparatus, succinate dehydrogenase for mitochondria, acid phosphatase for lysosomes, catalase for peroxisomes and lactate dehydrogenase for the cytosol.

3.5 ANALYTICAL CENTRIFUGATION

3.5.1 Applications of analytical ultracentrifugation

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 250 000 *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 of Fig. 3.8 outlines the optical system of a modern analytical ultracentrifuge. The availability of high-intensity xenon flash lamps and the advance in instrumental sensitivity and wavelength range has made the accurate 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 relative molecular mass, purity and shape of these biomolecules. Analytical ultracentrifugation for the determination of the relative molecular mass of a macromolecule can be performed by a **sedimentation velocity approach** or **sedimentation equilibrium methodology**. The hydrodynamic properties of macromolecules are described by their sedimentation coefficients and can be determined from the rate that a concentration boundary of the particular biomolecules moves in the gravitational field. Such studies on the solution behaviour of macromolecules can give detailed insight into the properties of large aggregates and thereby confirm results from biochemical analyses on complex formation. The sedimentation coefficient can be used to characterise changes in the size and shape of macromolecules with changing experimental conditions. This allows for the detailed biophysical analysis of the effect of variations in the pH value, temperature or co-factors on molecular shape.

Analytical ultracentrifugation is most often employed in

- the determination of the purity of macromolecules;
- the determination of the relative molecular mass of solutes in their native state;
- the examination of changes in the molecular mass of supramolecular complexes;
- the detection of conformational changes; and in
- ligand-binding studies (Section 17.3.2).

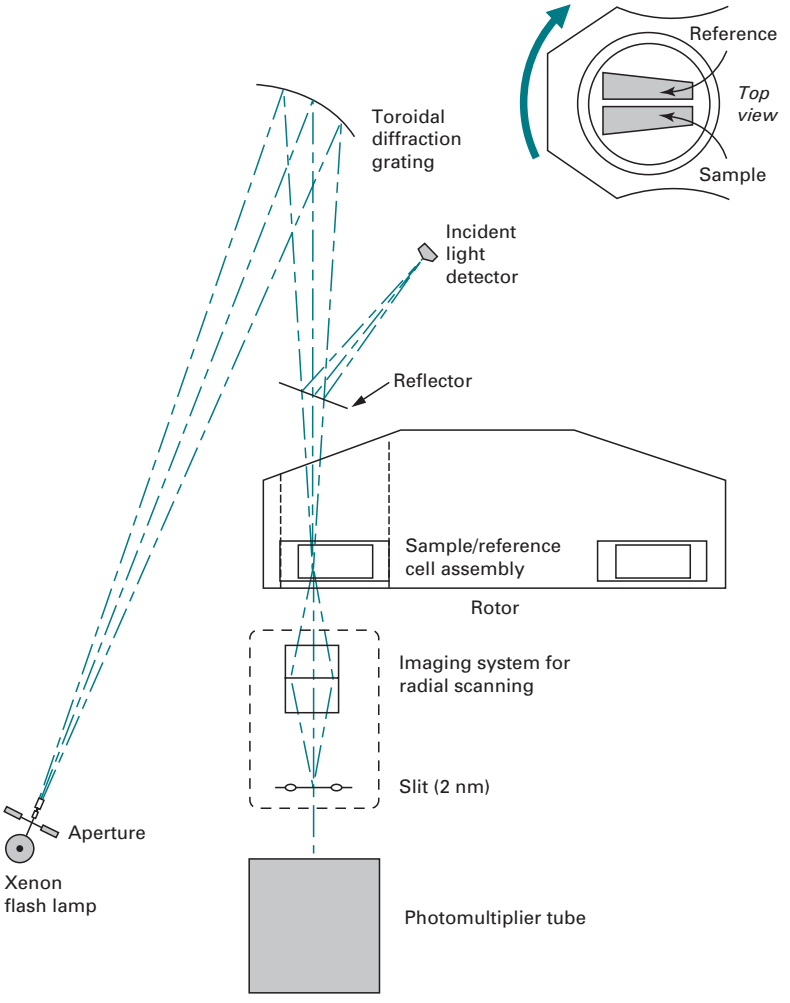


Fig. 3.8 Schematic diagram of the optical system of an analytical ultracentrifuge. The high-intensity xenon flash lamp of the Beckman Optima XL-A analytical ultracentrifuge shown here allows the use of wavelengths between 190 nm and 800 nm. The high sensitivity of the absorbance optics allows the measurement of highly dilute protein samples below 230 nm. (Courtesy of Beckman-Coulter.)

The sedimentation velocity method can be employed to estimate sample purity. Sedimentation patterns can be obtained using the Schlieren optical system. This method measures the refractive index gradient at each point in the ultracentrifugation cell at varying time intervals. During the entire duration of the sedimentation velocity analysis, a homogeneous preparation forms a single sharp symmetrical sedimenting boundary. Such a result demonstrates that the biological macromolecules analysed exhibit the same molecular mass, shape and size. However, one can not assume that the analysed particles exhibit an identical electrical charge or biological activity. Only additional biochemical studies using electrophoretic techniques and enzyme/bioassays can differentiate between these minor subtypes of macromolecules with similar molecular mass. The great advantage of the sedimentation velocity method is

that smaller or larger contaminants can be clearly recognised as shoulders on the main peak, asymmetry of the main peak and/or additional peaks. For a list of references outlining the applicability of ultracentrifugation to the characterisation of macromolecular behaviour in complex solution, please consult the review articles listed in Section 3.6. In addition, manufacturers of analytical ultracentrifuges make 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.

3.5.2 Relative molecular mass determination

For the accurate determination of the molecular mass of solutes in their native state, analytical ultracentrifugation represents an unrivalled technique. The method requires only small sample sizes (20–120 mm^3) and low particle concentrations (0.01–1 g dm^{-3}) and biological molecules with a wide range of molecular masses can be characterised. In conjunction with electrophoretic, chromatographic, crystallographic and sequencing data, the biochemical properties of a biological particle of interest can be determined in great detail. As long as the absorbance of the biomolecules to be investigated (such as proteins, carbohydrates or nucleic acids) is different from that of the surrounding solvent, analytical ultracentrifugation can be applied. At the start of an experiment using the **boundary sedimentation** method, the biological particles are uniformly distributed throughout the solution in the analytical cell. The application of a centrifugal field then causes a migration of the randomly distributed biomolecules through the solvent radially outwards from the centre of rotation. The solvent that has been cleared of particles and the solvent still containing the sedimenting material form a sharp boundary. The movement of the boundary with time is a measure of the rate of sedimentation of the biomolecules. The sedimentation coefficient depends directly on the mass of the biological particle. The concentration distribution is dependent on the buoyant molecular mass. The movement of biomolecules in a centrifugal field can be determined and a plot of the natural logarithm of the solute concentration versus the squared radial distance from the centre of rotation (ln c vs. r^2) yields a straight line with a slope proportional to the monomer molecular mass. Alternatively, the relative molecular mass of a biological macromolecule can be determined by the **band sedimentation** technique. In this case, the sample is layered on top of a denser solvent. During centrifugation, the solvent forms its own density gradient and the migration of the particle band is followed in the analytical cell. Molecular mass determination by analytical ultracentrifugation is applicable to values from a few hundred to several millions. It is therefore used for the analysis of small carbohydrates, proteins, nucleic acid macromolecules, viruses and subcellular particles such as mitochondria.

3.5.3 Sedimentation coefficient

Biochemical studies over the last few decades have clearly demonstrated that biological macromolecules do not perform their biochemical and physiological functions in isolation. Many proteins have been shown to be multifunctional and their activity

is regulated by complex interactions within homogeneous and heterogeneous complexes. Co-operative kinetics and the influence of micro-domains have been recognised to play a major role in the regulation of biochemical processes. Since conformational changes in biological macromolecules may cause differences in their sedimentation rates, analytical ultracentrifugation represents an ideal experimental tool for the determination of such structural modifications. For example, a macromolecule that changes its conformation into a more compact structure decreases its frictional resistance in the solvent. In contrast, the frictional resistance increases when a molecular assembly becomes more disorganised. The binding of ligands (such as inhibitors, activators or substrates) or a change in temperature or buffering conditions may induce conformational changes in subunits of biomolecules that in turn can result in major changes in the supramolecular structure of complexes. Such modifications can be determined by distinct differences in the sedimentation velocity of the molecular species. Sedimentation equilibrium experiments can be used to determine the relative size of individual subunits participating in complex formation, the stoichiometry and size of a complex assembly under different physiological conditions and the strength of interactions between subunits.

When a new protein species is identified that appears to exist under native conditions in a large complex, several biochemical techniques are available to evaluate the oligomeric status of such a macromolecule. Gel filtration analysis, blot overlay assays, affinity chromatography, differential immuno precipitation and chemical cross-linking are typical examples of such techniques. With respect to centrifugation, sedimentation analysis using a density gradient is an ideal method to support such biochemical data. For the initial determination of the size of a complex, the sedimentation of known marker proteins is compared to the novel protein complex. Biological particles with a different molecular mass, shape or size migrate with different velocities in a centrifugal field (Section 3.1). As can be seen in equation 3.7, the sedimentation coefficient has dimensions of seconds. The value of Svedberg units ($S = 10^{-13}$ s) 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. The prototype of a soluble protein, serum albumin of apparent 66 kDa, has a sedimentation coefficient of 4.5 S. Figure 3.9 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 (16S) and thyroglobulin (19 S). When the membrane cytoskeletal element dystrophin was first identified, it was shown to bind to a lectin column, although it does not exhibit any carbohydrate chains. This suggested that dystrophin might exist in a complex with surface glycoproteins. Sedimentation 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 its composition. Alkaline extraction, acid treatment or incubation with different types of detergent causes the differential disintegration 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

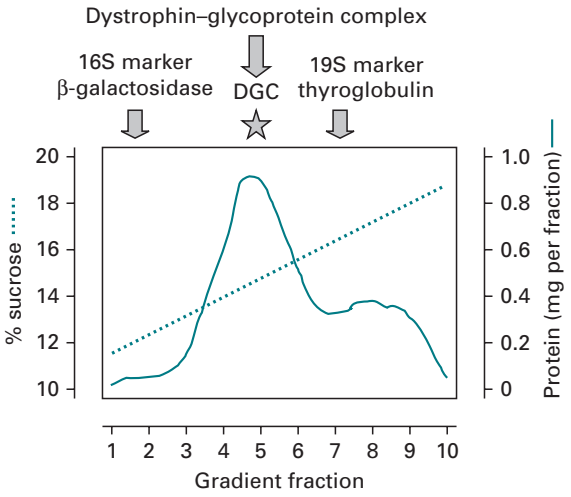


Fig. 3.9 Sedimentation analysis of a supramolecular protein complex. Shown is the sedimentation of the dystrophin–glycoprotein complex (DGC). Its size was estimated to be approximately 18 S by comparing its migration to that of the standards β-galactosidase (16 S) and thyroglobulin (19 S). Since the sedimentation coefficients of biological macromolecules are relatively small, they are expressed as Svedberg units, S, whereby 1 Svedberg unit equals 10^{-13} s.

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 gain biochemical knowledge of a newly discovered protein quickly.

3.6 SUGGESTIONS FOR FURTHER READING

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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. (Provides an excellent synopsis of the applicability of ultracentrifugation to the characterisation of macromolecular behaviour in complex solution.)

Cox, B. and Emili, A. (2006). Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics. *Nature Protocols*, **1**, 1872–1878. (Outlines differential centrifugation protocols for the isolation of the nuclear, cytosolic, mitochondrial and microsomal fraction.)

Girard, M., Allaire, P. D., Blondeau, F. and McPherson, P. S (2005). Isolation of clathrin-coated vesicles by differential and density gradient centrifugation. *Current Protocols in Cell Biology*, Chapter 3, Unit 3.13. (Describes a typical subcellular fractionation protocol used in modern biochemical applications.)

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. (Describes typical centrifugation protocol used for the isolation of DNA and RNA molecules.)