Centrifugal Separations in Molecular and Cell Biology

Edited by
G.D. BIRNIE *and* D. RICKWOOD

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BUTTERWORTHS
LONDON – BOSTON
Sydney – Wellington – Durban – Toronto

The Butterworth Group

United Kingdom
London
Butterworth & Co (Publishers) Ltd
88 Kingsway, WC2B 6AB

Australia
Sydney
Butterworths Pty Ltd
586 Pacific Highway, Chatswood, NSW 2067
Also at Melbourne, Brisbane, Adelaide and Perth

South Africa
Durban
Butterworth & Co (South Africa) (Pty) Ltd
152–154 Gale Street

New Zealand
Wellington
Butterworths of New Zealand Ltd
T & W Young Building, CPO Box 472
77–85 Customhouse Quay

Canada
Toronto
Butterworth & Co (Canada) Ltd
2265 Midland Avenue
Scarborough, Ontario, M1P 4S1

USA
Boston
Butterworth (Publishers) Inc
19 Cummings Park, Woburn, Massachusetts 01801

First published 1978

ISBN 0 408 70803 4

British Library Cataloguing in Publication Data

Centrifugal separations in molecular and cell biology
1. Centrifuges 2. Molecular biology – Technique
I. Birnie, George David II. Rickwood, David
574.8'8'028 QH324.9.C4 78-40042

ISBN 0-408-70803-4

Typeset by Scribe Design, Medway, Kent
Printed by W & J Mackay Ltd., Chatham

Preface

The centrifuge has played an essential, although not always acknowledged, role in almost every advance in molecular and cell biology, and it is now one of the most basic and valuable pieces of equipment available in biology laboratories of all kinds and sizes. Over the years the laboratory centrifuge, particularly the high-speed version, the ultracentrifuge, has undergone extensive development and modern machines are highly sophisticated and versatile instruments. However, despite daily use in many laboratories, the full potential of preparative centrifuges is rarely realized. This is because the proper application of many centrifugation techniques requires considerable expertise which is largely passed on by word of mouth, since many of the pertinent details are widely scattered in the literature. It was the realization that laboratory workers as well as students would find it beneficial to have these details drawn together in the one volume which prompted us to initiate the preparation of this book. The subject of the book is the application of modern centrifugation technology in molecular and cell biology. Its purpose is to present a detailed discussion of all aspects of the methodologies for the separation and fractionation of biological particles by centrifugation on both the preparative and analytical scales and, most important, to present this in such a form that readers with little or no previous experience of the techniques can easily make effective use of the most sophisticated of these methods.

The major emphasis of this book is on the practical aspects of the subject. Thus, a great deal of space has been devoted to describing how the various types of centrifugal separations are actually done, why they are best done in particular ways, the relative merits of different types of centrifugation techniques and how these vary according to circumstances, and the limitations of the methods and the ways in which these limitations may be minimized. Much space has also been given to the interpretation of the data obtained by the various centrifugation methods, and many examples of separations are described to illustrate the points made. In addition, the mathematics of basic sedimentation theory has been presented in a very simplified form, with many worked examples in illustration, to show non-mathematicians how they can easily apply the conclusions drawn from this theory to data from their own experiments and, thus, make more effective use of the equipment at their disposal. In many ways, therefore, the contents of this book complement those of its two predecessors, *Subcellular Components* (1972) and *Subnuclear Components* (1976), also published by Butterworths, since most of the methods described in these depend on one or more centrifugation techniques.

This book is multi-authored because centrifugation is such a wide field that it is difficult for any one author to give an equally authoritative, in-depth description of every one of its many aspects. We are indebted to the authors for having given so generously of their time and experience in preparing their chapters; we are particularly grateful to them for encouraging us to assemble this book, and for their patience and co-operation with our attempts to present a reasonably integrated overview of the whole field. We also thank the publishers for their help, and the personnel of the various centrifuge manufacturers for answering our questions promptly and in full detail.

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1 Introduction: Principles and Practices of Centrifugation

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This book is designed both as a complete guide for the novice and as an aid to the worker who has already had some experience of centrifugal techniques. This introductory chapter discusses the various techniques which are available to the researcher and describes the layout of the book, thus directing the reader to the relevant chapters which provide the information necessary to carry out each type of fractionation.

BASIC CONCEPTS OF SEDIMENTATION THEORY

Essentially, a centrifuge is a device for separating particles from a solution. In biology, the particles are usually cells, subcellular organelles, or large molecules all of which are called 'particles' to simplify the terminology. The physical parameters which determine the extent of fractionation apply equally to such diverse particles as macromolecules and cells, although the nature of the particles (for example, their lability, sensitivity to osmotic pressure, etc.) may place restraints on the centrifugation conditions that can be used. The following is a simplified introduction to some of the basic parameters which govern the sedimentation and separation of particles in a centrifugal field. A detailed analysis of the theoretical aspects of centrifugal separations is given in Chapter 2.

Some of the basic principles of the sedimentation theory originate from Stokes's law. If the sedimentation of a sphere in a gravitational field is considered it can be shown that, as the velocity of a spherical particle reaches a constant value, the net force on the particle is equal to the force resisting its motion through the liquid. This resisting force is called frictional or drag force. From Stokes's law it can be calculated that the sedimentation rate, v, of a particle is given by

$$v = \frac{d^2(\rho_p - \rho_m)}{18\mu} \times g$$

From this equation, it can be seen that:

1. The sedimentation rate of a given particle is proportional to the square of the diameter, d, of the particle.

2. The sedimentation rate is proportional to the difference between the density of the particle and the density of the liquid medium, $(\rho_p - \rho_m)$.
3. The sedimentation rate is zero when the density of the particle is equal to the density of the liquid medium.
4. The sedimentation rate decreases as the viscosity, μ, of the liquid medium increases.
5. The sedimentation rate increases as the force field, g, increases.

The force field relative to the earth's gravitational field (RCF) exerted during centrifugation is defined by the equation

$$\text{RCF} = \frac{\omega^2 r}{980}$$

where r is the distance between the particle and the centre of rotation in cm; the rotor speed ω in rad/sec can be calculated from the equation

$$\omega = \text{rev/min} \times \frac{2\pi}{60} = \text{rev/min} \times 0.104\ 72$$

The sedimentation velocity per unit of centrifugal force is called the sedimentation coefficient, s:

$$s = \frac{1}{\omega^2 r} \times \frac{dr}{dt}$$

where dr/dt is the rate of movement of the particle in cm/sec. Sedimentation coefficients are usually expressed in svedbergs (S), equivalent to 10^{-13} sec. Thus, a particle whose sedimentation coefficient is measured at 10^{-12} sec, i.e. 10×10^{-13} sec, is said to have a sedimentation coefficient of 10 S.

The mathematical bases of sedimentation theory (both rate-zonal and isopycnic) have concerned many authors since the methods were first introduced, and a detailed analysis of this aspect of centrifugation is described in Chapter 2. Many biologists consider that the mathematics included in most discussions of sedimentation theory is esoteric in the extreme, and constitutes a considerable disincentive to their attempting to understand the basic parameters governing fractionations in a centrifugal field. To help counteract this feeling, Chapter 2 is so organized that the basic theories are presented in the text of the chapter with the main mathematical equations involved simply being stated, whereas the detailed derivations of the equations are grouped together in the Appendix to Chapter 2. The use of these equations for the interpretation of data from the analytical ultracentrifuge is explained in Chapter 8, which also gives some indication of the accuracy and reliability of the theory in practical situations. In fact, a considerable amount of valuable quantitative information can be obtained by applying some of these equations to data from experiments done in preparative ultracentrifuges, and the theme is extended in Chapters 5 and 6, which show how these quantitative data can easily be obtained by the use of no more than a little simple arithmetic.

CENTRIFUGATION METHODS

There are three main types of centrifugal fractionation, namely, (*i*) differential pelleting (differential centrifugation); (*ii*) rate-zonal density-gradient sedimentation; and (*iii*) isopycnic density-gradient sedimentation.

Of these techniques, differential pelleting is the method most commonly used for fractionating material according to size. In this method, the material to be fractionated is initially distributed uniformly throughout the sample solution, which is the sole occupant of the centrifuge tube (*Figure 1.1a*). After centrifugation the pellet is enriched in the larger particles of the mixture (*Figure 1.1b*). However, the pellet obtained always consists of a mixture of the different species of particle, and it is only the most slowly sedimenting component of the mixture that remains in the supernatant liquid which can be purified by a single centrifugation. The amount of contamination in the pellet can be reduced by washing it (that is, by resuspending and recentrifuging), but this inevitably

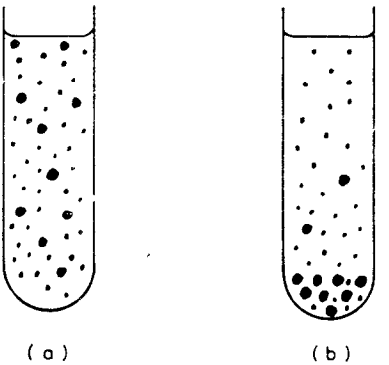
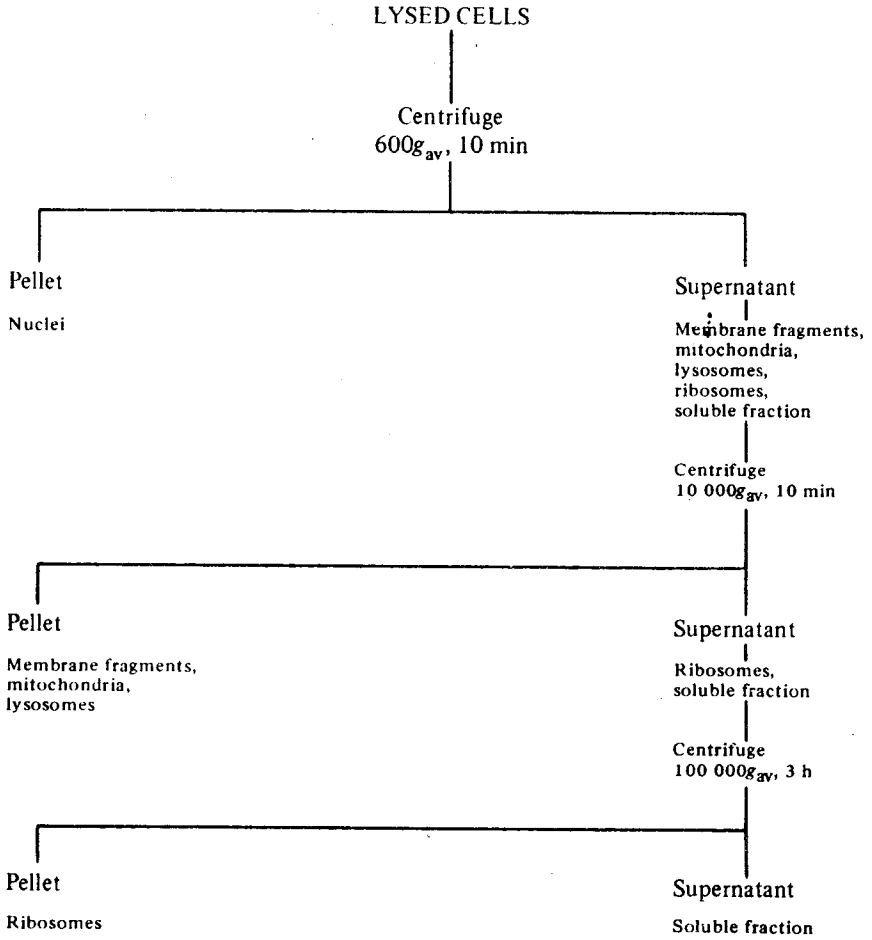


Figure 1.1 Effect of centrifuging a suspension of heterogeneous particles

reduces the yields obtained. Some improvement can be made by, for example, sedimenting the particles through a pad of dense sucrose, although such methods are not universally applicable. An example of a general scheme which is the basis of many used to fractionate cells into their components is outlined in *Chart 1.1*. However, it is not possible to discuss such general schemes in any detail. The actual method which should be used to isolate any particular species of particle depends on a host of independently variable factors, including the tissue from which the particles are to be isolated, the purpose for which they are required, the precise composition of the medium in which the cells are homogenized, and the interaction of other components of the homogenate with the particles of interest. No general scheme can make allowances for the variation in all of these factors, and it is, therefore, much more satisfactory to look at the problems involved from the point of view of each individual species of particle. This is beyond the scope of a book of this kind, and reference should be made to publications such as *Subcellular Components: Preparation and Fractionation* (2nd edn) and *Subnuclear Components: Preparation and Fractionation*, published by Butterworths in 1972 and 1976, respectively, which deal with the problems involved in isolating the various components of eukaryotic cells from a variety of tissues. The simplicity of differential pelleting methods makes them very attractive for a large number of fractionations on a preparative scale. Moreover, the methods have the advantage that they can be performed with fixed-angle rotors which generally have a higher capacity than the swing-out type.

Chart 1.1 FRACTIONATION OF CELL COMPONENTS



However, sometimes the material is not cleanly pelleted at the bottom of the tube in a fixed-angle rotor, but is smeared down the side of the tube. This is the result either of too rapid a rate of acceleration of the rotor (especially in the case of very large particles), or of too high a concentration of material in the tube.

The efficiency of the fractionation of particles according to size and shape can be improved markedly by using rate-zonal centrifugation through a density gradient, although this does mean that the sample capacity of each tube is greatly reduced. This technique involves layering the sample on to the top of a liquid column which is stabilized by a gradient of an inert solute, commonly sucrose. It is important that the maximum density of the gradient is less than the buoyant density of the particles and that the gradients are centrifuged in swing-out or zonal rotors to minimize wall effects (see p. 63).

When centrifuged, the particles move down the gradient at a rate which depends on the parameters given in Stokes's equation. Since one frequently only wants to separate the different particles rather than measure their sedimentation coefficients accurately, Chapter 3 discusses the general and practical aspects of such separations with the emphasis on the use of swing-out rotors. On the other hand, if very large amounts of sample are to be fractionated it is often more convenient to use a zonal rotor. Moreover, the resolution of a mixture into its components is significantly better in zonal rotors. Details of the design and use of these rotors are discussed in Chapter 4. Further, if care is taken these preparative techniques can be used analytically. Using the methods described in Chapter 5, the data obtained from rate-zonal sedimentation experiments in either tubes or zonal rotors will yield accurate estimates of the sedimentation coefficients of particles.

The third method for separating particles is isopycnic sedimentation in a gradient whose maximum density exceeds that of the particles. This is an equilibrium technique in which particles are separated on the basis of their buoyant densities, independently of the time of centrifugation and of the size and shape of the particles, although these parameters do determine the rate at which equilibrium is reached and the width of the bands formed at equilibrium. It is important to realize that the effective buoyant density of any particle is a function of the actual density of the particle (determined by its partial specific volume) and its degree of hydration. For example, the density of non-hydrated DNA is close to 2.0 g/cm^3, but its observed buoyant density can vary from 1.7 g/cm^3 to 1.1 g/cm^3, depending on the water activity of the gradient medium. For instance, the density of sodium DNA is 2.0 g/cm^3; in NaI the observed buoyant density of DNA is 1.52 g/cm^3, whereas in metrizamide it is 1.12 g/cm^3, corresponding to a hydration of 8 and 68 moles of water per mole of nucleotide, respectively. Isopycnic centrifugation in the salts of alkaline metals (for example, CsCl and NaI) has been widely used for the separation, fractionation and purification of macromolecules and nucleoproteins; this aspect is discussed in Chapter 6. On the other hand, isopycnic sucrose gradients have been used widely for the fractionation of such cell organelles as mitochondria and nuclei, whereas the more osmotically inert polysaccharides (for example, Ficoll) have been used for the separation of cells. Moreover, a number of other non-ionic density-gradient media have recently been introduced and comparisons between the established and new non-ionic gradient media are discussed in Chapter 7.

The techniques mentioned so far can be used for fractionating particles on either a preparative or an analytical scale. However, in order to obtain really accurate quantitative data it is necessary to use the purpose-built analytical ultracentrifuges. Using these centrifuges it is possible to make measurements on a number of different samples while they are actually being sedimented, the times required for such measurements usually being quite short. The use of the analytical centrifuge and its associated specialized techniques are discussed in detail in Chapter 8.

DESIGNING CENTRIFUGATION EXPERIMENTS

Most centrifugation experiments are generally adaptations of fractionation procedures published by other workers studying similar types of particles. The most

common difficulty is that they have used a different make or type of centrifuge or centrifuge rotor. It is not possible to give any sensible general advice as to the relative merits and demerits of different types and makes of centrifuge since the considerations which influence any one person's choice from among the models available embrace such diverse factors as the purposes for which the centrifuge is required and the skills of its users, and the location of the nearest centrifuge engineer, his competence, and the ready availability of spare parts. More serious and general problems arise when the other workers whose method is being followed have specified the type of rotor used and its speed, but not the relative centrifugal force (or other details such as tube angle) which is required to obtain the desired separation. In order to reproduce the experimental conditions when a different type or make of rotor is to be used, it is essential to know the precise dimensions and characteristics of each rotor, and for this purpose Chapter 9 provides tables of data from which direct comparisons of most commercially available rotors can be made. The other important factor to be considered is the choice of centrifuge tube, since some types of tube fail when exposed to particular solvents, extremes of pH, or large density differences. The suitability of each type of tube for a variety of situations is also discussed in Chapter 9.

CARE OF CENTRIFUGES AND ROTORS

The investment of buying an ultracentrifuge and rotors is similar to buying a Rolls-Royce car, and both should be held in the same high esteem and treated accordingly. Thus, for the complete novice with no experience of using a particular centrifuge or a particular rotor it is very important that he or she should read the manufacturers' instructions and, if possible, consult someone who can advise on practical details. For example, at first sight some rotors appear compatible with a particular model of centrifuge, but in reality they must not be used in that model because they are too heavy or, in the case of swing-out rotors, the bowl is too small! The second important factor is the balancing of tubes since, although the flexible drive of an ultracentrifuge is designed to withstand minor imbalances in the rotor, running under these conditions causes the centrifuge to deteriorate faster than normal. It is also important to contain the sample during centrifugation, particularly as the samples are often biohazardous or radioactive. Also, the rotors of all ultracentrifuges run in a high vacuum to minimize heating due to air resistance and so it is especially important to isolate the sample, to prevent evaporation, by capping the tubes securely. Another problem is that of spillage of sample material into the rotor buckets. Many solutions, particularly alkaline and salt solutions, rapidly corrode aluminium and, in some cases, even titanium rotors. Rotors *must always* be rinsed thoroughly with distilled water and left to drain after use. A more detailed account of rotor care is given in Chapter 9, whereas the special problems associated with the maintenance of zonal rotors are discussed in Chapter 4.

2 The Bases of Centrifugal Separations

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The origin of zonal centrifugation may be hidden in scientific literature but Brakke (1951) and Anderson (1955) are credited with being the first workers to demonstrate the practical advantages of the method. Since then, views have hardened and opinions divided between which of the many advocated procedures give the best results. Even terms have been disputed, but for this discussion 'zone' and 'band' are taken as being synonymous terms. In general, two separate types of zonal experiment have been applied to separating particles: rate-zonal and isopycnic equilibrium. In rate-zonal experiments the zone migrates with the field and its width increases with time, whereas in equilibrium experiments the zone settles at its isopycnic density (isodensity is considered to be synonymous with isopycnic): hence, its shape and position are invariant with time. Both methods differ from the widely applied differential procedure, in which a plateau of concentration is maintained throughout the experiment. In zonal experiments the concentrations both of the supporting gradient and the zonal constituents are never constant with respect to radius once the experiment has started. The introduction of the inert gradient into the experiment presents problems of resolution which are not normally encountered in differential experiments. In this chapter an outline is given of the theoretical treatments of some of the problems encountered in zonal experiments, and emphasis has been placed on conditions which affect resolution of zones when separating a mixture. Many of the factors which affected shape and migration of zones were understood by 1967, and Schumaker (1967) reviewed this work giving useful solutions to the transport equations for zones. Details of these solutions are not repeated here. Instead, an attempt is made to collect together those features which affect the shape and positioning of zones in both velocity and equilibrium experiments.

The major equations are given in the main text, whereas their derivation is given in the Appendix (pp. 22–30). A list of the symbols used and their definitions are given in *Table 2.1.*

COMPARISON OF ZONAL CENTRIFUGATION WITH COMPLEMENTARY TECHNIQUES

When Svedberg first introduced the use of the ultracentrifuge to biochemistry the major problem in physical biochemistry was defining and detecting heterogeneity of macromolecules. Since those early days, analytical techniques more powerful than centrifugation have been developed for detecting heterogeneity, leaving the analytical centrifuge for measuring absolute molecular weights and