Centrifugation (2nd Edition)

a practical approach

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Preface

The centrifuge has become one of the most basic laboratory instruments and as such it is used by a wide range of laboratory personnel. Instruction is usually freely available for the actual operation of all centrifuges from, for example, manufacturers' manuals. However, while there are a number of advanced treatises on centrifugation, these frequently only review various aspects of the subject and as such do not directly relate to the laboratory use of the various centrifugation techniques. This book is designed not only to detail the important criteria for optimising centrigual separations but also each section includes detailed protocols of experiments designed to illustrate the points made in each section.

While this book has been written primarily for novices, established research workers who already have some experience of centrifugation should find that the text, which emphasises the advantages of using newer types of rotors and gradient media, a useful reference source for their work. In addition, the general appendices at the end of the book provide a great deal of data which are extremely useful for everyone working in the field of centrifugation.

PREFACE TO THE SECOND EDITION

The enormous success of the first edition of this book emphasised the need for a book stressing the practical aspects of centrifugation. This second edition has a similar format to the first in providing extensive experimental details of protocols for all types of centrifugal separations from macromolecules to whole cells. It also describes the applications of centrifuges ranging from simple bench machines to analytical centrifuges. However, the opportunity has been taken to revise the text extensively not only to bring it up to date but also to expand its coverage to make it more comprehensive. The book has been revised and extended not only as a guide to novices but also as a reference source for experienced researchers. Finally, I would like to thank my many colleagues, both those involved in academic research and those associated with centrifuge manufacturers, for their helpful information which has enabled me to assemble such a detailed and comprehensive book.

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Abbreviations

BSA	bovine serum albumin
DAB	diaminobenzidine tetrahydrochloride
DFP	di-isopropylfluorophosphate
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycobis(β-aminoethyl)ether tetraacetic acid
GBSS	Gey's balanced salt solution
NP-40	Nonidet P-40
PBS	phosphate-buffered saline
PMSF	phenylmethylsulphonyl fluoride
POPOP	1,4-bis-(5-phenyloxazol-2-yl)benzene
PPO	2,5-diphenyloxazole
PVP	polyvinylpyrrolidone
RCF	relative centrifugal force
mRNP	messenger ribonucleoprotein
SDS	sodium dodecyl sulphate
SV40	simian virus 40
TCA	trichloroacetic acid

Contents

CHAPTER 1

The Theory and Practice of Centrifugation

D. RICKWOOD

1. INTRODUCTION

The aim of this chapter is to introduce the reader to some of the basic concepts in the area of centrifugation. The beginning of this chapter outlines the theoretical bases of centrifugation to introduce the reader to the most important parameters which are likely to be encountered. More rigorous and mathematically detailed treatments of centrifugation theory can be found elsewhere (1,2). The other parts of this chapter deal with the practical aspects of centrifugation in terms of descriptions of centrifuges and rotors, as well as describing the properties of the various gradient media used for centrifugal separations.

2. THEORY OF CENTRIFUGATION

2.1 Sedimentation Theory

In a suspension of particles, the rate at which the particles sediment depends not only on the nature of the particles but also on the nature of the medium in which the particles are suspended as well as the force applied to the particles. Intuitively, one would expect that larger particles should sediment more rapidly than smaller ones. In fact, although biological particles vary enormously in size from relatively small proteins to whole cells, the parameters affecting sedimentation are the same irrespective of size. One important factor affecting the sedimentation of particles is the viscosity of the medium. In 1856, Sir Gabriel Stokes proposed that the frictional force, F, acting on a rigid spherical particle of radius, r_p, was related to the viscosity, η, by the equation:

$$F = 6 \pi \eta . r_p . \frac{dr}{dt} \qquad \text{Equation 1}$$

where dr/dt is the velocity of the particle. As shown in *Figure 1* the actual force experienced by particles is determined not only by the gravitational force, g, but also the flotation effects which reflect the differences in the density of the medium (ϱ_m) and the particles (ϱ_p). Thus Equation 1 becomes:

$$(\varrho_p - \varrho_m) V . g = 6 \pi \eta r_p \frac{dr}{dt} \qquad \text{Equation 2}$$

Since the particle is assumed to be spherical, then the volume, V, can be ex-

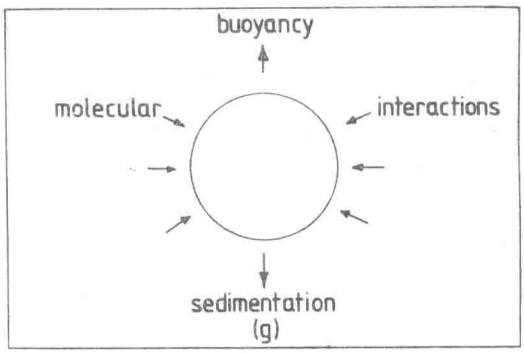

Figure 1. Forces acting on particles in solution.

pressed in terms of the radius of the particle. Thus:

$$\frac{4}{3}\pi r_p^3(\varrho_p - \varrho_m)g = 6\pi\eta r_p\frac{dr}{dt} \qquad \text{Equation 3}$$

In practice, the centrifugal force which moves the particles away from the axis of rotation is very much greater than the Earth's gravitational field and so we can express centrifugal force relative to the Earth's gravitational field by the expression:

$$\text{centrifugal force} = \frac{\omega^2 r}{g} \qquad \text{Equation 4}$$

where r is the radial distance of the particle from the axis of rotation and ω is the angular velocity in terms of radians/sec. Substituting this relationship into Equation 4 and simplifying gives the expression in terms of the velocity of particles, namely:

$$\frac{dr}{dt} = \frac{2r_p^2(\varrho_p - \varrho_m)\omega^2 r}{9\eta} \qquad \text{Equation 5}$$

This expression is only true for spherical particles. Non-spherical particles have larger frictional coefficients. In the case of rod-like molecules the frictional coefficient of the molecule (f) can be as much as ten times that of the frictional coefficient of a sphere (f_o). To take this into account, Equation 5 can be modified to give the expression:

$$\frac{dr}{dt} = \frac{2r_p^2(\varrho_p - \varrho_m)\omega^2 r}{9\eta(f/f_o)} \qquad \text{Equation 6}$$

Besides the buoyancy and sedimentation forces, the particles are also subjected to molecular forces of the surrounding medium (see *Figure 1*). If the particles are small then considerable centrifugal force is necessary to counteract these forces and sediment particles.

2.2 Non-ideality of Biological Particles

One factor that can complicate sedimentation studies is that a number of biological particles have a dynamic nature. For example, proteins with a

subunit structure (e.g., haemoglobin) may undergo dissociation during centrifugation leading to the formation of multiple peaks. Another problem is that sedimentation down the gradient may alter the properties of the particle. As an example, the rate at which high molecular weight DNA migrates through a gradient depends on the relative centrifugal force, since higher centrifugal forces appear to alter the conformation and hence the sedimentation rate of the DNA (4).

The other feature of centrifugation is that the centrifugal force generates hydrostatic pressure within the solution. The hydrostatic pressure generated can be sufficient to permeabilise membranes to gradient solutes (5), to dissociate nucleoprotein complexes (6) and to disrupt protein complexes (3). Hence, in choosing centrifugation conditions or in interpreting sedimentation patterns, care must be taken to avoid conditions which may lead to the formation of artifacts.

2.3 Sedimentation Coefficients

From Equation 6, it can be seen that it is possible to define a particle in terms of its behaviour in a centrifugal field, that is in terms of its sedimentation coefficient, s, where:

$$s = \frac{dr/dt}{\omega^2 r} \qquad \text{Equation 7}$$

For most biological macromolecules the magnitude of s is about 10^{-13} sec and hence the unit of sedimentation, the Svedberg (S), has been defined as being equal to 10^{-13} sec. The definition of sedimentation coefficients is discussed in greater detail in Chapters 4 and 8. It is also important to realise that not only is the relationship between the sedimentation coefficient of a particle and its molecular weight not linear but also it varies from one type of particle to another.

2.4 Practical Calculations of Centrifugal Force and Centrifugation Times

As shown in Equation 4, the relative centrifugal force (RCF) can be calculated from the expression:

$$\text{RCF} = \frac{\omega^2 r}{g} \qquad \text{Equation 4}$$

It is inconvenient to measure the angular velocity, ω, and so it is more convenient to express the RCF in terms of revolutions per minute (r.p.m.), N, and this gives the expression:

$$\text{RCF} = 11.18 \times r\left(\frac{N}{1000}\right)^2 \qquad \text{Equation 8}$$

The centrifugal force is usually given in terms of 'g' and is written as such or as 'xg'.

From Equation 8, it can be seen that the centrifugal force acting on the particle is related to the square of the speed and hence doubling the speed in-

creases the centrifugal force by a factor of four. The centrifugal force also increases with the distance from the axis of rotation (r). Hence particles in a homogeneous medium will accelerate as the radial distance increases, although in sucrose gradients, where there is a viscosity gradient, increasing viscosity tends to minimise the effects of increasing the radial distance. The reader should note that centrifugal force can only be calculated if both the speed and radial dimensions of the rotor are known.

Manufacturers usually give the dimensions of rotors in terms of the maximum and minimum radii, r_{max} and r_{min}, respectively (see Appendix III). The r_{max} quoted by manufacturers usually relates to the distance to the bottom of the bucket; especially where thick-walled tubes are being used the r_{max} and hence g_{max} may be significantly less. Throughout this book, unless otherwise stated, the centrifugal force will be given as that at the centre of the solution, that is, the average centrifugal force, g_{av}.

The other parameter of rotors that is usually quoted is the k-factor (see Appendix III). The smaller the k-factor the greater is the pelleting efficiency of the rotor. The k-factor can be calculated for rotors using the expression:

$$k = \frac{2.53 \times 10^{11}\left[\ln\left(\frac{r_{max}}{r_{min}}\right)\right]}{N^2} \qquad \text{Equation 9}$$

If the sedimentation coefficient (s) of particles is known, then the k-factor can be used to calculate the time in hours (t) required to pellet the particles using the relationship:

$$t = \frac{k}{s} \qquad \text{Equation 10}$$

It must be emphasised that the k-factor is related to the speed of the rotor. All k-factors relate to the maximum speed of the rotor; at lower speeds the k-factor is correspondingly increased according to the relationship:

$$k_{actual} = k\left(\frac{N_{max}}{N_{actual}}\right)^2 \qquad \text{Equation 11}$$

Also all k-factors quoted by manufacturers assume that the tubes are full; the k-factors of rotors are smaller if partially filled tubes are used because the shorter pathlength enables the particles to pellet more quickly. The other feature to be remembered is that the k-factor is calculated on the basis that the density and viscosity of the liquid medium in which the particles are suspended are not significantly different from the density and viscosity of water, increasing either of these effectively increases the k-factor.

In the case of sucrose gradients there is a viscosity gradient throughout the tube and hence the k-factor cannot be used. Instead a model system of the time needed to sediment particles to the bottom of a 5 – 20% (w/w) sucrose gradient at 5°C is used to define k′ and k* factors (7) which allow one to estimate the sedimentation pattern in a 5 – 20% (w/w) sucrose gradient. A series of k′-values are used depending on the density of the particle (7) while k* factors are calculated on the assumption that the density of the particle in sucrose is

1.3 g/cm^3; usually this assumption does not introduce large errors into the calculation (see Appendix III). However, for estimating the sedimentation of particles in sucrose gradients accurately it is often more useful to use computer simulation methods (see Section 4 of Chapter 4).

3. TYPES OF CENTRIFUGAL SEPARATIONS

3.1 **Differential Pelleting**

As might be predicted from Equation 5, centrifugation will first sediment those particles which are largest. In addition, as indicated in Equation 6, very asymmetrical molecules will sediment more slowly than spherical particles of the same mass and density. Increasing either the centrifugation speed or the time of centrifugation will cause smaller particles to pellet also (*Figure 2*). As might be expected from Equation 5, differential centrifugation separates particles not only according to size but also on the basis of density, since particles that are denser (e.g., nuclei) will pellet at a faster rate than less dense particles (e.g., membranes) of the same mass. Hence it is sometimes possible to obtain good separations of particles of similar sizes but different densities by differential pelleting. For particles of similar densities one usually requires about a 10-fold difference in mass to separate one particle from another efficiently by differential pelleting. The major problem with differential pelleting is that, as shown in *Figure 2*, the centrifugal force necessary to pellet the larger particles from the top of the solution is also often sufficient to pellet the smaller particles nearer the bottom of the tube. Hence in a single step it is only possible to obtain a pure preparation of the smallest particles since these will remain in solution after all the other larger particles have pelleted. The yield of such a procedure is, however, likely to be low. An alternative approach is to minimise

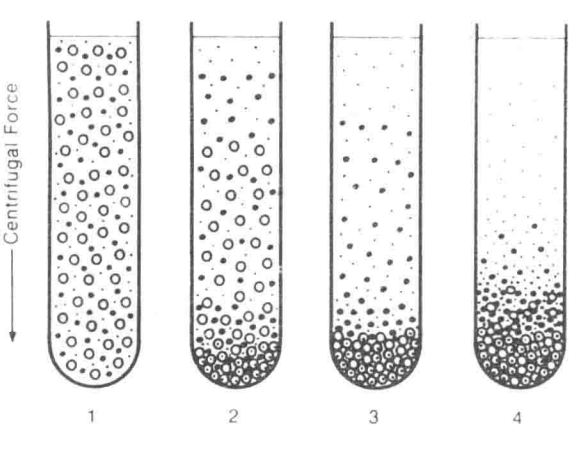

Figure 2. Fractionation of particles by differential pelleting. Reproduced from ref. 7 with permission.