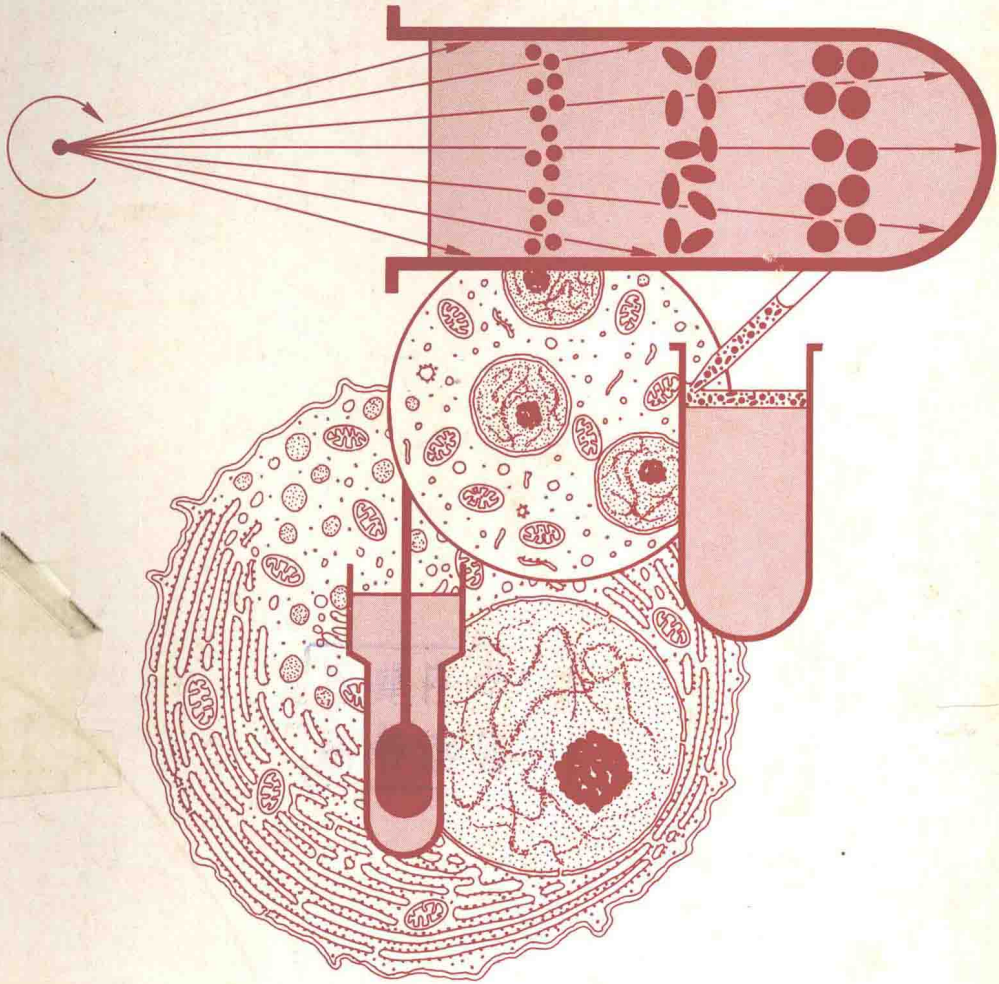
CENTRIFUGATION IN BIOLOGY AND MEDICAL SCIENCE



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Centrifugation in Biology and Medical Science

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Preface

It would be difficult to overstate the value of centrifugation as a tool of biomedical research, for much of what we know today about the composition, properties, and functions of specific macromolecules, supermolecular complexes, subcellular organelles, and even whole cells is the consequence of studies carried out following centrifugal purification, isolation, and/or characterization of these materials. In this book, I attempt to present a comprehensive account of the diverse forms that centrifugation takes in biological and medical science, noting particular applications, advantages, limitations, and other characteristics of each approach. The book was written with the view toward its usefulness not only to researchers but also to advanced students whose interests lie in physiology, cellular and molecular biology, biochemistry, and related fields. It is intended that the book's thorough consideration of criteria that underlie successful centrifugal fractionations can serve as a guide to the design of experimental protocols that meet specific tasks or goals. Special attention is paid to recent developments in the field and the appendices bring together a wealth of data of general usefulness.

My interest in centrifugation began in 1965 when I had the good fortune to receive a postdoctoral fellowship to study at the Biology Division of the Oak Ridge National Laboratory under the guidance of Dr. Norman G. Anderson. At that time, Dr. Anderson's "Molecular Anatomy (MAN) Program" was devoted primarily to the development of zonal centrifuges and centrifugal fast analyzers. Although I had joined the laboratory to continue earlier studies on the mechanism by which iron is transferred across the plasma membrane of the developing red blood cell, I quickly became engrossed in the remarkable centrifugal developments being pioneered by Dr. Anderson and his associates. Fascinated by the seemingly unlimited applications of centrifugation in biomedical research and profoundly influenced by Dr. Anderson's inventive genius, my own research

interests and activities have been directed since that time to centrifugal methodology.

Norman Anderson's manifold contributions have helped turn the modern centrifuge into one of the most powerful and versatile tools in the research laboratory, and it is the focal point of many analytical and preparative studies. The effectiveness with which centrifugation is used depends to a large extent on the researcher's awareness of the spectrum of techniques that are available and appreciation of the specific advantages and limitations of each approach. By establishing the fundamental principles of centrifugation and surveying the field generally, this book provides a foundation on which such expertise can be developed.

This book could not have been prepared without the help and contributions of a number of individuals and companies. First, thanks to Dr. Norman G. Anderson of the Argonne National Laboratory (U.S.A.) for his permission to reproduce a number of photographs of early zonal rotors. My appreciation is also extended to Dr. W. Howard Evans of the National Institute for Medical Research (United Kingdom) for his contributions of photos of the A-XII rotor. I am grateful also to Mac Lawrence (Beckman Instruments, Inc.), Ronald Ostrom, Keelin Fry, and Rodger Nelson (DuPont/Sorvall Instruments), Patrick W. Goulding and V. N. Musmanno (Pennwalt Corporation, Sharples-Stokes Division), Ellen P. Aquilina (Bausch and Lomb) and Brian Collins (Buchler Instruments Division of Searle Diagnostics, Inc.) for their generous contributions of photographs and pertinent information.

Much of the original artwork appearing in the book was prepared by my good friend and colleague Mark H. Doolittle. Over the years, my own work in the field of centrifugation has been supported by the manifold talents of Harry R. White, to whom I owe special thanks. Finally, I should like to express my appreciation to Mary M. Conway, Life Sciences Editor, Denise Hillhouse, Sr. Production Supervisor, and Carole Schwager, Editorial Supervisor for Wiley-Interscience publications for their help and encouragement during the writing and production of this book.

PHILLIP SHEELER

Northridge, California
December, 1980

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Special thanks are extended to Mark H. Doolittle, who so masterfully prepared many of the illustrations in the book.

Contents

ONE

An Introduction

Centrifugation is one of the most important and widely applied research techniques in biochemistry, in cellular and molecular biology, and in medicine. In its various forms, centrifugation is regularly used to (1) remove cells or other suspended particles from their surrounding milieu on either a batch or a continuous-flow basis, (2) separate one cell type from another, (3) isolate viruses and macromolecules, including DNA, RNA, proteins, and lipids or establish physical parameters of these particles from their observed behavior during centrifugation, and (4) separate from dispersed tissue the various subcellular organelles including nuclei, mitochondria, chloroplasts, Golgi bodies, lysosomes, peroxisomes, glyoxysomes, plasma membranes, endoplasmic reticulum, polysomes, and ribosomal subunits. Indeed, much of our present knowledge concerning the morphology, chemical composition, and physiological functions of specific populations of cells in heterogeneous tissues and of the various subcellular organelles has been obtained either directly from analytical centrifugal techniques or by chemical or microscopic study following centrifugal isolation of these minute structures from disrupted tissues and cells.

It is important at the outset to clarify several terms regularly used either in connection with centrifugation as a technique or to classify different kinds of centrifuges; these are "ultracentrifuge," "analytical" ultracentrifuge, and "preparative" centrifuge (or ultracentrifuge). The term "ultracentrifuge" was introduced in 1924 by Theodor Svedberg to identify an instrument in which the sedimentation of amicroscopic colloidal particles could be directly observed and followed (Svedberg and Rinde, 1924). Svedberg's rationale was to make the identification of his centrifuge consonant with the recently developed "ultramicroscope"—a light microscope with special optics for rendering visible tiny particles not discernable by conventional light microscopy. It was not Svedberg's original

intent that the term "ultracentrifuge" suggest a centrifuge capable of producing very high rotational speeds; indeed, the rotors in Svedberg's 1924 ultracentrifuge reached only 10,000 rpm. However, in today's usage, the term "ultracentrifuge" is generally reserved for centrifuges (analytical *or* preparative) in which rotational speeds in excess of 25,000 rpm are routine, with the rotor spinning in an evacuated chamber. (Centrifuges operating at speeds below this are variously called "superspeed" or "high-speed" centrifuges.)

Svedberg's ultracentrifuges were designed to characterize large molecules, especially proteins, on the basis of the behavior of these particles in centrifugal fields. The centrifuges accommodated only small quantities of sample and were equipped with an optical system that permitted the progress of particle sedimentation to be viewed and photographed. An analysis of their sedimentation behavior revealed certain physical properties of the particles even though individual particles were not actually isolated or collected. Accordingly, the Svedberg instruments were known as *analytical* ultracentrifuges in order to distinguish them from *preparative* instruments used to collect sediments from particle suspensions.

The practical distinction between analytical and preparative centrifuges became less clear as new procedures evolved for using progressively refined preparative instruments not only to quantitatively isolate particles, but to yield analytical data from the behavior of the particles during the isolation procedure. Even the optical system that for so many years uniquely characterized the Svedberg-type analytical ultracentrifuge has been modified and adapted to preparative instruments; moreover, special transparent rotors (i.e., zonal rotors; see Chapter 6) can be used with many preparative centrifuges, so that the investigator can directly observe the course of particle separations.

For the most part, the term "analytical untracentrifuge" is now restricted to a particular series of instruments characterized by an elaborate optical system and used either to establish physical parameters of particles on the basis of precise measurements of sedimentation made during centrifugation or to accurately assess the purity (or heterogeneity) of biological and chemical samples prepared by other techniques. In effect, these instruments are modern-day versions of the classical Svedberg concept. Other centrifuges are referred to as *preparative* instruments even though they may be used in either analytical experiments or in the quantitative isolation of particles.

Over the years, a number of books have been devoted to the subject of centrifugation, and these are listed at the end of this chapter. However, most of these books have focused either on classical analytical ultracentrifugation, dealing at length with mathematical models for the behavior

of sedimenting particles, or on specific applications of a given preparative approach. The present work is of a much more general character and encompasses methods that are not considered in any depth in other books dealing with the general subject of centrifugation. It is intended that this book serve several roles for the reader:

1 To provide a practical mathematical foundation in the physical principles on which centrifugal methodology is founded.
2 To provide an awareness of the remarkable variety of task-specific centrifugal equipment and accessories available today and an appreciation of the origin and the development of the major forms of centrifugation being used today.
3 To draw attention to the diversity of experimental approaches that can be taken in order to attack both general and specific biomedical problems.

Using the information presented here, it is hoped that the reader will be in a better position to make more prudent decisions concerning what centrifugal methods should (or should not) be tried in an effort to attain one's experimental objectives.

Following the cited references that appear at the end of each chapter are lists of additional articles dealing with specific problems that may bear more directly on the reader's own experimental objectives.

EARLY HISTORY OF CENTRIFUGATION

The practical application of centrifugal force dates back more than a thousand years to the extraction of "tung oil," a substance used in paints and varnishes, from the seeds of *Aleurites cordata* trees. However, the first recorded biological study using a "centrifuge" is that of T. A. Knight in 1806, who showed that the roots and stems of seedlings oriented themselves to centrifugal force when grown at the circumferential edge of a rotating water wheel. By the 1870s, small hand-operated centrifuges capable of spinning two small cylindrical test tubes at speeds up to about 3000 rpm were being used by chemists for collecting precipitates, whereas larger but slower devices were used by dairyworkers for removing the sediment present in fresh milk and for cream separation. Carl Gustaf de Laval, a Swedish inventor whose main efforts were directed toward the design of steam-driven turbine engines, introduced the *continuous-action* centrifugal cream separator in 1878 and completely revolutionized the dairy industry. This marked the beginning of continuous-flow centrifu-

gation and represented a major step forward in centrifuge technology. De Laval's basic approach to continuous centrifugal separations remains essentially unchanged even in modern instruments, and some of Svedberg's first analytical ultracentrifuges were modified versions of commercial de Laval machines. Although the electrical motor had been invented by Michael Faraday in 1822 and commercial electric motors were available by 1880, it was not until about 1910 that motor-driven centrifuges were built. Prior to 1910, centrifugal devices were either hand operated or driven by water or steam.

Small centrifuges were used in the 1880s by biologists to study properties of protoplasm and cellular inclusions. For example, the viscosity of protoplasm was investigated in 1880 by C. Dehnecke, who followed the movements of starch grains through the cytoplasm of plant cells subjected to small centrifugal forces. D. Mottier showed in 1899 that cellular inclusions visible with the light microscope formed strata within cells subjected to centrifugal force, suggesting that these inclusions possess different densities. Although several nineteenth century biologists effected the isolation of certain cellular inclusions, these isolations were achieved by noncentrifugal methods. Most notable, perhaps, was Friedrich Miescher's isolation of cell nuclei from leukocytes in 1868 and from sperm cells in 1874 by chemically digesting away the extranuclear cytoplasm.

In medicine, S. G. Hedin and J. Daland showed in the early 1890s that they could more rapidly determine the ratio of plasma volume to packed cell volume by subjecting whole blood to centrifugal force rather than allowing the cells to settle through the plasma for several hours under the influence of gravity. These early "hematocrit centrifuges" were small, hand-operated instruments geared to provide speeds up to 10,000 rpm and capable of forming the packed blood cell sediment in about 3 min. In 1902, A. E. Wright, a pioneer in blood cytology, introduced the use of specially prepared glass centrifuge tubes in which the leukocyte-rich "buffy coat" could be conveniently separated from the bulk of erythrocytes for subsequent microscopic study. Wright's work probably represents the first successful fractionation of whole cells using centrifugal force.

The focal point of biochemistry at the turn of the century was the investigation of the chemical and physical properties of protein solutions and other colloids. The discovery by Emil Fischer in 1902 that proteins consisted of chains of amino acids linked together through peptide bonds turned out to be a major impetus for further development of centrifugal technology. Fischer and most other notable chemists of that period believed that individual protein species (hemoglobin, albumin, etc.) were *polydisperse;* that is, a given protein molecule occurred in various sizes

(i.e., polypeptide chain lengths). Reasoning that the polydispersity of colloidal suspensions might be amenable to study by measuring particle sedimentation rates, A. Dumanskii (Dumanskii, 1910; Dumanskii et al., 1913) attempted to correlate the results of ultramicroscopic studies of colloids with the behavior of the colloidal particles during centrifugation. Dumanskii's experiments, which were carried out with the use of a conventional centrifuge containing cylindrical tubes (and hence lacking "sector shape"), were unsuccessful because of the influence of convection on the sedimenting particles. The Svedberg had been concerned with colloid chemistry since 1905, and initially he shared the view that individual protein species were polydisperse. In 1923, Svedberg and J. B. Nichols built an "optical centrifuge"—the immediate forerunner of the analytical ultracentrifuge. The rotor of the optical centrifuge consisted of a metal tube encasing two cylindrical glass chambers. The tube was mounted directly on a small electric motor so that its long axis rotated in the horizontal plane. Narrow rectangular slots cut through the tube permitted the sedimentation of particles to be viewed and photographed during centrifugation. Rotor speed was limited to a few hundred revolutions per minute. Particle sedimentation rates could not be measured accurately in the optical centrifuge because of convective disturbances resulting from the lack of sector shape in the tube chambers and the absence of strict temperature control.

Later in 1923, Svedberg and H. Rinde built the first ultracentrifuge. The basic driving mechanism was constructed by using parts of a de Laval centrifugal cream separator. The cream separator bowl was replaced by the centrifuge rotor (constructed of brass) and the top of the separator modified to enclose the rotor in a hydrogen gas atmosphere that cooled the rotor during operation and reduced thermal convection in the samples subjected to analysis. The rotors used in this centrifuge were positioned on a vertical shaft, the lower portion of which formed a worm gear. The worm gear was rotated in the horizontal plane by a vertical gear wheel connected by a belt to an electric motor (Fig. 1-1). Svedberg soon replaced the original gear-driven ultracentrifuges by direct motor-driven machines.

To eliminate sedimentation artifacts resulting from convection currents within the sample compartments (called "cells"), the compartments were constructed with a sector shape; that is, the cell walls formed radii of the circle being swept out by the spinning rotor. The cells were fitted above and below with transparent windows permitting direct observation (and photography) during the course of sedimentation. Rotors attained speeds of 10,000 rpm (i.e., about 5000g) and were used to study the behavior of a number of colloids, including hemoglobin. Working with R. Fahraeus, Svedberg made the first estimations of the average molecular

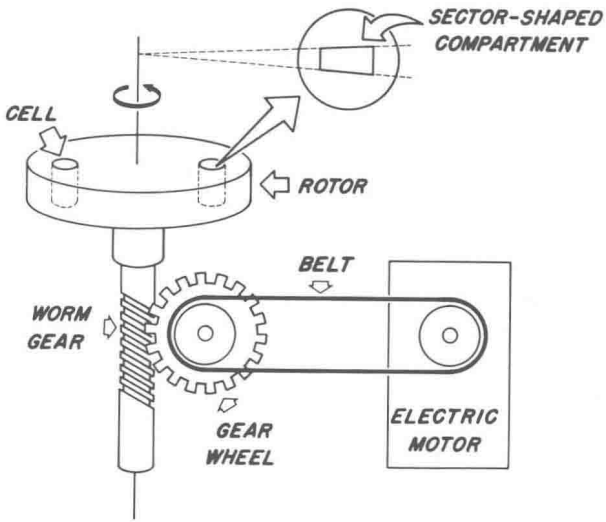

Fig. 1-1 Basic components of Theodor Svedberg's first analytical ultracentrifuge.

weight of hemoglobin (67,000) (Svedberg and Fahraeus, 1926). Because of the relatively low centrifugal forces produced in this centrifuge, the hemoglobin studies were necessarily *sedimentation equilibrium* experiments, in which particle sedimentation is balanced by diffusion, and therefore, could not provide information about molecular polydispersity or homogeneity. Such information could be provided only by *sedimentation velocity* methods, in which centrifugal force exceeds diffusion, so that a sedimenting boundary is formed. Sedimentation equilibrium and sedimentation velocity experiments are considered more fully later.

By 1926, Svedberg was also experimenting with centrifuges in which the rotor was driven by two small oil turbines positioned at either end of the rotor shaft. These rotors were spun about a horizontal axis at speeds up to 40,100 rpm (about 100,000*g*). In the same year, Svedberg received the Nobel prize in chemistry for his work on colloids. Using direct, motor-driven, and oil-turbine-driven ultracentrifuges, Svedberg continued his studies on colloids, and from a series of experiments conducted through 1931 drew two notable conclusions: (1) proteins may have molecular weights from thousands (e.g., egg albumin) to millions (e.g., snail hemocyanin); and (2) a given protein type (hemoglobin, albumin, hemocyanin, etc.) is not polydisperse but is homogeneous, with all molecules of that protein having the same molecular weight. The notion that proteins possessed well-defined molecular weights represented a departure from contemporary thinking and was received with considerable skepticism.

Between 1924 and 1940, Svedberg constructed and tested a number of rotors varying in size, shape, and the number and positions of their analytical cells. Some of these rotors were used at speeds in excess of 80,000 rpm. During the same period, E. Henriot and E. Huguenard pioneered the idea of using compressed air to spin centrifuge rotors. The air entered the centrifuge chamber from below and acted to support the rotor as well as to spin it; the rotors were small, solid devices that did not hold liquid samples for analysis (Henriot and Huguenard, 1925). By 1927, they reported attaining 660,000 rpm using the air-turbine approach (Henriot and Huguenard, 1927; Huguenard, 1927). Substituting compressed hydrogen gas for air and using a rotor of less than 1 cm in diameter, J. W. Beams and E. G. Pickels in 1935 achieved 1,560,000 rpm. The possibility of using the air-turbine approach for centrifuges and rotors suitable for biological and chemical studies was pursued in the late 1930s and early 1940s by E. G. Pickels and H. J. Bauer. Pickels and Bauer placed the air turbine in a separate compartment above the rotor chamber. A length of wire descended from the turbine into the rotor chamber and supported the rotor from above. Rotation of the turbine was translated through the wire to the rotor (Bauer and Pickels, 1936). The rotors used by Bauer and Pickels and the system for optical measurement of the sedimenting particles were similar to those developed by Svedberg.

Pickels, Bauer, J. L. Onceley, R. W. G. Wyckoff, and W. M. Stanley successfully employed the air-turbine analytical ultracentrifuge at speeds up to about 60,000 rpm in a series of studies on proteins and virus particles. In 1946, responding to the increasing interest in the analytical ultracentrifuge, Pickels formed the Special Instruments Company (SpInCo) for the commercial production of the instrument. Later this became the Spinco division of Beckman Instruments, Inc. For simplification and greater reliability, the air-turbine drive was replaced by an electric motor and gears, but in other respects the ultracentrifuge remained the same. Indeed, while the Spinco instrument (known as the "model E analytical ultracentrifuge") has undergone a number of modifications and improvements over the years, it still possesses much of the character of the original Pickels ultracentrifuge. Commercial analytical ultracentrifuges are also produced by other companies, including Measuring and Scientific Equipment, Ltd. (MSE) and Hereus-Christ, Inc.

The technological evolution of the analytical ultracentrifuge served as a catalyst for furthering the development of other kinds of centrifuges for chemical, biological, and medical study. The result is today's host of superspeed centrifuges, ultracentrifuges, and rotors for general- as well as special-purpose preparative, continuous-flow, and analytical applications. Many of the succeeding chapters of this book are devoted to a